WASTEWATER: A SURVEILLANCE SENTINEL FOR BACTERIAL PATHOGENS AND ANTIMICROBIAL RESISTANCE DISSEMINATION

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ABSTRACT

WASTEWATER: A SURVEILLANCE SENTINEL FOR BACTERIAL PATHOGENS AND ANTIMICROBIAL RESISTANCE DISSEMINATION

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Antimicrobial resistance (AMR) has emerged as one of the most significant global public health concerns, threatening the effective prevention and treatment of an ever-increasing range of infections caused by bacteria no longer susceptible to the common therapeutics used to treat them. The wide range of resistant infections, with decreasing treatment options, is leading to increased morbidity and mortality. Antimicrobial resistant bacteria can easily spread through the environment, and wastewater treatment plants (WWTPs) are recognized as a reservoir for AMR pathogens and genes. Although water reclamation is vital to the sustainability of water supplies, the highly diverse microbial environment in wastewater creates favorable conditions for the dissemination of antimicrobial resistance genes as well as the proliferation of resistant bacteria. Current wastewater testing methods fail to take into consideration AMR bacteria and AMR genes when evaluating the overall safety aspects of water reuse. In this study, AMR bacteria isolated from Southwestern United States (U.S.) wastewater were interrogated through meropenem antimicrobial susceptibility testing, 16S rRNA gene sequencing, multiplex species identification amplicon sequencing, and whole genome sequencing to identify the human pathogens present in the wastewater and determine if viable meropenem resistant bacteria persist in wastewater after treatment. Molecular assays, such as those developed in this study, represent a comprehensive and minimally invasive approach to provide community level surveillance of AMR pathogens as well as identify viable resistant bacteria present in wastewater. The application of AMR wastewater surveillance allows for better understanding of the risk of

transmission of harmful bacteria to humans as well as inform decisions on safe reclaimed effluent water reuse.

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DEDICATION

This Thesis is dedicated to my family and friends who have supported me throughout this entire process. Without everyone's love and support, I wouldn't be where I am today.

CHAPTER 1: INTRODUCTION

Wastewater Treatment Plants

Wastewater treatment plants (WWTPs) are designed to eliminate the harmful substances found in used water, including human waste, food scraps, oils, soaps, and chemicals (*Wastewater Treatment Water Use | U.S. Geological Survey*, n.d.). In homes, this includes water from sinks, showers, bathtubs, toilets, washing machines, and dishwashers. Businesses and industries also contribute a share of used water. The proper collection, treatment, and disposal of wastewater reduces potential negative impacts on the environment and is essential to protecting the health of the public. Influent water, the untreated or contaminated water flowing into the WWTP that is ready for processing, undergoes a combination of physical, chemical, and biological engineering treatments with the goal of removing as much of the suspended solids and harmful substances as possible before the remaining water, called effluent, is discharged back to the environment (*Wastewater Treatment Water Use | U.S. Geological Survey*, n.d.). Water generally undergoes a primary, secondary, and tertiary treatment at WWTPs. Primary treatment, the physical process of treatment, can include screens that remove large debris, grit removal systems, and clarifier settling tanks. Secondary treatments, the biological process, include activated sludge processes and secondary clarifier tanks. Tertiary treatment, the chemical process, includes membranes, sand filtration, and chlorine, ozone, or ultra-violet radiation disinfection. Once treated, effluent water can have several different uses in the community depending on the approval from a city's department of environmental control. In the Southwestern U.S., the focus area of this study, effluent water is approved to be used for irrigation, dust suppression, and toilet flushing (*Water Reclamation Plants | City of Flagstaff Official Website*, n.d.). As the population continues to grow and living standards improve, the production of domestic, industrial, and commercial wastewater has increased (Varela & Manaia, 2013). As a consequence of this, the chemical and biological complexity of wastewater has increased considerably over the last decades, with contamination from hygiene, pharmaceutical and personal care products as well as numerous natural, human, and animal derived microbes (Varela & Manaia, 2013).

The wastewater environment is an ideal medium for the concentration of a wide range of both pathogenic and nonpathogenic microorganisms, specifically bacteria, viruses, and protozoa (Abdel-Raouf et al., 2012). Bacteria are the most diverse group of human pathogens found in wastewater. Several types of bacteria colonize the human intestine and are shed in feces. While many bacteria found in the human microbiome are commensal and beneficial, some are pathogenic and are shed into wastewater. The presence of pathogenic microorganisms in wastewater highlights the need for treatment of used water. If left untreated, this water can act as a vehicle for microbial waterborne diseases as well as contribute to the dissemination of antimicrobial resistance (AMR).

Antimicrobial Resistance

Antimicrobial resistance is one of the biggest public health challenges of our time and is expected to kill more people than cancer by the year of 2050 (O'Neill, 2014). Progress in molecular biology has revealed new and significant challenges for AMR mitigation given the immense diversity of antimicrobial resistance genes (ARGs), the complexity of ARG transfer, and the broad range of omnipresent factors contributing to AMR (Nguyen et al., 2021). AMR has the potential to affect people at any stage of life, as well as the healthcare, veterinary, and agriculture industries, making it one of the world's most urgent public health problems (CDC, 2022). Each year in the United States, at least 2.8 million people get an antimicrobial-resistant infection, and more than 35,000 people die (CDC, 2022). AMR has become so severe that multidrug resistance and even pan-resistance is being seen in several different species of bacteria. Within 70 years of the clinical deployment of penicillin, there are increasing numbers of pathogens that are not only resistant to penicillin and its derivatives but also to all other available antibiotics (Mazel, 2004). Although there are a large number of antimicrobial agents, both natural and synthetic, to choose from for potential infection therapy, resistance has been documented for all of these, and resistance generally occurs quickly after the antimicrobial is approved for use (Reygaert, 2018).

Antimicrobial resistance is a natural phenomenon that predates the modern selective pressures of clinical antibiotic use. Resistance to antimicrobials is natural, ancient, and can be hard wired into some microbial pangenomes (Bhullar et al., 2012). Studies on ancient DNA from 4 million year old microbiome from the Lechuguilla Cave, New Mexico and 30,000-year-old Beringian permafrost sediments have discovered a vast number of genes that encode resistance to antimicrobials, including β-lactams, tetracyclines and glycopeptides (Bhullar et al., 2012) (D'Costa et al., 2011). Many antimicrobials are produced by bacterial species, considered self-producers. Self-producers have resistance mechanisms to protect themselves from the compounds they produce. Soil bacteria, including the Actinomycetes group, produce the antimicrobials streptomycin, actinomycin, erythromycin, vancomycin, chloramphenicol, and tetracycline (Clardy et al., 2009) (Clardy, 2005). Many of the antibiotics used today come from soil microbes, which employ them as weapons in the vast soil environment in order to compete for resources and survive (Society, n.d.). Natural resistance may be intrinsic, or always expressed in the species, or induced, meaning the genes are naturally occurring in the bacteria, but are only expressed when activated, such as after exposure to an antibiotic (Reygaert, 2018). However, the widespread administration of antimicrobials in the treatment and prevention of infectious disease provides a strong selective pressure that is driving the evolutionary response in the microbial world (Michael et al., 2014). This provoked evolutionary response in bacteria has caused an increase in frequency of antimicrobial resistance phenotypes among microbes (Michael et al., 2014). The development of resistance mechanisms in human pathogens causes antimicrobial therapeutics to be largely ineffective and makes treatment of bacterial infections increasingly complicated. Resistance is occurring much quicker than it would naturally in the environment, which has ultimately allowed for AMR infections to rapidly emerge in health care facilities (Davies & Davies, 2010).

Although resistance to antimicrobials occurs naturally in the environment, selective pressures from modern medicine and technological advancements are causing anthropogenic resistance to occur in pathogens. This is also known as acquired resistance, which stems from the acquisition of genetic material that confers resistance. ARGs are often located on plasmids or transposons, which are mobile genetic elements that can be transferred to other bacterial species through all the main routes by which bacteria acquire any genetic material: transformation, transposition, and conjugation. All these routes are termed horizontal gene transfer (HGT). The transfer of ARGs can cause initially susceptible populations of bacteria to become resistant to an antibacterial agent and proliferate and spread (Tenover, 2006). An example of this is the acquisition of genes that encode enzymes such as β-lactamases, which are being disseminated on mobile genetic elements and causing β-lactam antimicrobials to be ineffective in treatment (Tooke et al., 2019).

Antibiotics target many different essential cellular structures and functions in the bacterial cell, including inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of DNA or RNA synthesis, inhibition of folate synthesis, and membrane disruption

(Neu & Gootz, 1996). Cell wall synthesis is inhibited by intercepting one of the three phases of peptidoglycan assembly. Protein synthesis is inhibited by binding to the 30S or 50S bacterial ribosomal subunit. DNA and RNA synthesis is inhibited by targeting the DNA gyrase, topoisomerase IV, bacterial RNA polymerase, or interfering with DNA replication within the bacterial cell. Folate synthesis, which is essential for DNA/RNA synthesis, is disrupted by inhibiting bacterial synthesis of dihydrofolic acid or by binding to and inhibiting the enzyme dihydrofolate reductase. Membrane disruption is achieved by disrupting the integrity of the membrane, such as the creation of porins in the membrane resulting in the loss of the physiological function of the membrane. Because of this, bacteria have a wide range of resistance mechanisms that cause the antimicrobial to be ineffective that they develop through mutation, selection, or acquisition of genomic information that encodes resistance (Munita & Arias, 2016). One of the most effective resistance mechanisms is the efflux pump, which extrude the antibacterial agent from the cell before it can reach its target site and exert its effect (Soto, 2013). Understanding the resistance mechanisms that bacteria use is fundamental to monitoring and mitigating the spread of AMR. Unfortunately, many mechanisms are still unknown, which causes challenges when trying to advance this field of research.

Resistance can vary greatly across related bacterial groups, meaning that bacteria as a group or species are not necessarily uniformly susceptible or resistant to any particular antimicrobial agent. Bacteria can be classified as susceptible, intermediate, or resistant using a minimum inhibitory concentration (MIC), the minimum concentration of antimicrobial that will inhibit the growth of the bacteria. Classification as susceptible, intermediate, or resistant depends on the clinical breakpoints set for the specific organism. All bacterial species have different MIC breakpoints that are set for specific antimicrobials. The primary agencies responsible for determining MIC breakpoints include: the U.S. Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (*Updating Breakpoints in Antimicrobial Susceptibility Testing*, n.d.). Determining the optimal value at which a breakpoint should be set is multifactorial, requiring a multidisciplinary approach to incorporate data from both laboratory and clinical settings. An antimicrobial's *in vitro* activity against an organism is assessed as well as the pharmacokinetic and pharmacodynamic parameters, or the relationship between drug concentration and antimicrobial effect, are taken into consideration when determining breakpoints for antimicrobials. Outcomes from clinical data, such as treatment failures and success, are also used to determine breakpoints. Breakpoints may change when new data or resistance mechanisms emerge.

Meropenem Resistance

The growing burden of resistance has resulted in reliance on drugs of last resort, or reserve drugs. Reserve drugs are antimicrobials that retain activity against multidrug-resistant bacteria (Geneva: World Health Organization, 2022). These antimicrobials are to be used only for severe clinical infections for which the currently available treatment options are very limited (Geneva: World Health Organization, 2022). Ideally these antimicrobials are used for targeted treatment once a multidrug-resistant infection is confirmed. Multidrug-resistant phenotypes, such as carbapenem-resistance, have led to last resort drugs becoming the only treatment option for some infectious diseases (Bassetti et al., 2019). However, some of these last resort drugs can have substantial toxicity and are only used when the risk of death or permanent damage is high (Geneva: World Health Organization, 2022).

Meropenem, a last resort drug, is a β-lactam antimicrobial belonging to the carbapenem class. β-lactams are the most widely used class of antibiotics and they inhibit cell wall synthesis by interfering with the last step of peptidoglycan synthesis, transpeptidation. Specifically, they cause acylation of transpeptidase, an enzyme that catalyzes the cross-linking of peptidoglycan chains that form the rigid cell walls (Bush & Bradford, 2016). Meropenem is an important antimicrobial that has an ultra-broad spectrum of antimicrobial activity which encompasses Gram-positive and Gram-negative aerobes and anaerobes, including many strains resistant to other antimicrobials (Blumer, 1997). It proves particularly useful in severe and polymicrobial infections and those caused by organisms resistant to other agents (Blumer, 1997). It is listed as a reserve drug on the World Health Organization (WHO) AWaRe (Access, Watch, Reserve) antibiotic book published in 2019.

Resistance to meropenem is a serious public health concern as this drug is used to treat a variety of serious illnesses, including infections where the organism is resistant to the primary antimicrobial therapeutic. Bacteria achieve resistance to meropenem through overexpression of the MexAB-OprM, MexXY-OprM, or MexCD-OprJ efflux systems, the loss of porin OprD and the overproduction of extended-spectrum cephalosporinases (ESACs), the production of AmpC β-lactamases, and the production of metallo-β-lactamases (MBL) (Rodríguez-Martínez et al., 2009) (Sinha & Srinivasa, 2007) (Riera et al., 2011). Resistance to meropenem has been observed in *Pseudomonas aeruginosa, Acinetobacter* spp., *Escherichia coli, Bacillus cereus, Bacillus pumilus, Chryseobacterium indologenes* (Masuda & Ohya, 1992) (Gupta et al., 2006) (Sinha & Srinivasa, 2007) (Yasmin et al., 2022) (Beattie et al., 2020). It is worth noting that *Acinetobacter baumannii, P. aeruginosa,* and *E. coli* respectively are the top three bacteria in the WHO priority list of antibiotic resistant organisms. Meropenem resistant *Bacillus licheniformis*, *Pseudomonas geniculata*, and *Bacillus mageterium* have also been isolated from hospital effluent water in Seoul, Korea (Hwang & Kim, 2018). Co-resistance of colistin and meropenem had been documented in a *Stenotrophomonas* strain isolated from sewage water (Li et al., 2019).

The Importance of Wastewater Surveillance

Reclaimed water has become an incredibly valuable source for irrigation, industrial, and other purposes, with the goal of conserving potable water sources for human consumption and domestic uses. Although water reclamation is vital to the sustainability of water supplies, wastewater habitats contain a rich and diversified bacterial community. Several bacteria, such as *Nitrospira* species that oxidize nitrite to less toxic nitrate, perform a series of biochemical and metabolic transformations, which are fundamental in the wastewater treatment processes (Varela & Manaia, 2013) (*How Microbes Help Us Reclaim Our Wastewater*, n.d.). However, a non-negligible number of bacteria persists in treated wastewater, and these habitats may favor the evolution and dissemination of new resistance and virulence genes and the emergence of new pathogens (Varela & Manaia, 2013). Safe and sustainable wastewater reuse requires a better understanding of the risk of transmission of harmful bacteria to humans, specifically taking into consideration antimicrobial resistant bacteria and antimicrobial resistance genes when evaluating the overall safety aspects of water reuse (Hong et al., 2018).

Using molecular tools and assays to monitor wastewater is one way to better understand the microbial community in wastewater and it serves as an extremely powerful tool. One wastewater sample can provide data on the average infection rate of thousands of people. This means that it is also a low-cost testing method, because instead of individually testing thousands of people, collecting and testing a small number of wastewater samples is much more cost effective for obtaining population wide data. It also allows for the establishment of trends in current outbreaks, identification of new outbreaks, and the determination of the prevalence of the infection in a population (Bivins & Bibby, 2021) (Randazzo et al., 2020) (*Public Health Interpretation and Use of Wastewater Surveillance Data | National Wastewater Surveillance System | CDC*, 2023). The use of wastewater surveillance for the detection of viable AMR bacteria, including known human pathogens, can inform decisions on reclaimed effluent reuse applications. Most importantly, wastewater testing is comprehensive, unbiased, and it captures asymptomatic infections.

Wastewater Surveillance History

The analysis of wastewater to identify the presence of biologicals or chemicals for the purpose of monitoring public health is not a new concept. It started more than 80 years ago with epidemiological studies that used cell-culture methods to detect and monitor poliovirus in sewage and water (Trask & Paul, 1942). In the 1980s, hybridized single-stranded RNA probes were designed to detect Hepatitis A in water (Jiang et al., 1987). By the late 1990s, PCR had proven to be a rapid, highly sensitive and accurate method to detect pathogenic viruses, bacteria and protozoa in water and wastewaters (Toze, 1999).

Today, the SARS-CoV-2 pandemic has opened the door for a massive amount of wastewater research. The SARS-CoV-2 virus is shed in feces, even if the infected persons don’t have symptoms. The virus can then be detected in wastewater, enabling wastewater surveillance to capture the presence of SARS-CoV-2 shed by people with and without symptoms. This prompted vast wastewater studies all over the globe, even resuling in the Centers for Disease Control and Prevention’s (CDC) National Wastewater Surveillance System (NWSS). These wastewater studies allowed for collaboration with local health departments to track SARS-CoV-

2 levels in wastewater so communities could act quickly to prevent the spread of the virus (CDC, 2023).

There has been a significant increase in wastewater research due to its success with SARS-CoV-2, including studies using the already functioning SARS-CoV-2 wastewater surveillance methods to also detect the influenza virus and mpox virus (Mercier et al., 2022) (Wolfe et al., 2023). The CDC is also extending their National Wastewater Surveillance System developed for SARS-CoV-2 to include research of antimicrobial resistant bacteria in wastewater, incorporating a One Health approach to understanding AMR (*Understanding Antibiotic Resistance in Water*, 2022). Even with the increase in wastewater surveillance, it is important to continue the development of molecular assays and techniques to detect the spread of pathogens in wastewater. Wastewater treatment can play a crucial role in controlling AMR, but research lacks in how wastewater and its treatment is related to the spread of human pathogens and multi-drug resistant bacteria. Furthermore, as resistance continues to rise globally it is imperative that we are actively looking for resistance to last resort drugs, such as meropenem, with the goal of maintaining the therapeutic efficacy of these antimicrobial agents.

While there are guidelines set for WWTPs that require monitoring the quality of the water prior to reuse, they are outdated and not informative. WWTPs generally look for the number of fecal coliforms, which indicate the presence of fecal contamination, in the water to determine if it is safe. Fecal coliforms were used as early as 1914 by the U.S. Public health Service to test drinking water for fecal contamination (Bitton, 1999). Fecal coliforms are indicators of contamination, but this method in no way informs as to what is contaminating the water. Coliform counting was developed prior to the massive rise in AMR, and it does not take into consideration ARGs and multidrug resistant strains of bacteria that pose a significant threat to public health. Until recently, direct detection of pathogenic bacteria, viruses, and parasites present in wastewater was seen as a costly and time-consuming procedure. However, through molecular assays, antimicrobial susceptibility testing, and genomic sequencing, such as those developed and used in this study, this can be accomplished quickly and cost-effectively. Recent studies have shown that municipal, hospital, and abattoir wastewater create favorable conditions for the transfer of ARGs and proliferation of antibiotic resistant bacteria (Nguyen et al., 2021). Numerous ARGs including types and subtypes of almost all common antibiotics have been detected in wastewater influent, effluent and biosolids or sludge. A study by Mathys et al., 2019 found that WWTP effluent and nearby surface waters in the US are routinely contaminated with carbapenemase-producing bacteria with clinically important genotypes including those producing *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo-beta-lactamase (NDM). Another study by Oliveira et al., 2021 found the presence of high concentrations of clinically relevant and globally distributed carbapenem (*bla*) and fluoroquinolone (*qnr*) genes in the wastewater influents, discharged effluents, as well as reused effluents. These studies indicate that current wastewater treatments are inefficient in the removal of antibiotic resistant bacteria and genes. It is apparent that WWTPs harbor antimicrobial resistant bacteria and their genes, but assessing the viability of these bacterial species through culturing techniques is vital in determining if WWTPs are producing effluent water that contains viable pathogenic bacteria that pose a risk to human health. Additional research needs to be done that will allow for informed decisions on the use of effluent water in communities and educate on the risks of potential infection from viable human pathogens present in the reclaimed water.

The purpose of this thesis was to address these gaps in research concerning the persistence of viable bacterial pathogens and the presence of meropenem resistance in Southwestern U.S. wastewater. There were four primary research objectives for this thesis:

1. To develop molecular assays to screen wastewater samples collected in the Southwestern U.S. for known AMR bacteria associated with human infection and determine if AMR bacteria are widely circulating in the community.
2. Analyze influent and effluent wastewater samples to determine if meropenem resistance persisted in viable bacteria following treatment.
3. To look at the changes in microbial diversity in the wastewater as it is exposed to different concentrations of meropenem and select for highly resistant organisms.
4. To provide a comprehensive and minimally invasive approach to community level surveillance of AMR pathogens present in wastewater.

These research objectives were accomplished by interrogating AMR bacteria isolated from Southwestern U.S. wastewater through antimicrobial susceptibility testing, 16S rRNA gene sequencing, multiplex species identification amplicon sequencing, and whole genome sequencing. These objectives are addressed in the below two chapters. Chapter 2, titled “The Use of Molecular Assays and Amplicon Sequencing to Detect gDNA and Identify Relevant Human Pathogens in Wastewater”, discusses the development and application of the molecular assays used to screen wastewater samples for multiple relevant AMR and human pathogen bacterial species. Chapter 3, titled “The Investigation and Identification of Viable Meropenem Resistant Bacteria in Wastewater”, addresses all research that pertains to the investigation of viable meropenem resistant bacteria present in Southwestern U.S. wastewater and sludge.

CHAPTER 2: THE USE OF MOLECULAR ASSAYS AND AMPLICON SEQUENCING TO DETECT gDNA AND IDENTIFY RELEVANT HUMAN PATHOGENS IN WASTEWATER

Wastewater surveillance is an extremely cost-effective and efficient way to monitor wastewater for multiple harmful bacterial and viral pathogens. This chapter will discuss the use of primer and assay design, multiplex optimization, extraction methods, PCR, and amplicon sequencing to examine Southwestern U.S. wastewater and sludge for relevant human pathogens.

Materials and Methods

Water Sample Organization and Preliminary Testing

Wastewater samples were collected from wastewater treatment plants and manholes outside congregate living areas across the Southwestern U.S. Wastewater samples had to be inventoried and organized to determine the quantity of samples available for processing. A total of 359 wastewater samples (165 influent, 23 effluent, 6 sludge, and 165 congregate living) collected across the Southwestern U.S. were analyzed in this project.

Prior to processing all wastewater and sludge samples, an appropriate extraction method had to be selected and tested. The Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Valencia, CA, USA) was chosen due to its use in the isolation of microbial genomic DNA and efficient lysis of bacterial cells. The purpose of this preliminary testing was to confirm that pelleting and extracting with the PowerSoil Pro Kit would work for this type of sample. Five wastewater samples were pelleted by centrifugation and processed following the PowerSoil Pro protocol. Two polymerase chain reaction (PCR) assays were used to confirm the success of the genomic DNA extraction: the BactQuant 16S rRNA assay (Liu et al., 2012) as well as an unpublished

139F/248R *E. coli* species assay designed through comparative genomics analysis by including cryptic *Escherichia* species. The amplification of these assays confirmed that the extraction was successful. Based on these results, the DNeasy PowerSoil Pro 96-well plate extraction kit was used to process the remaining wastewater samples.

Primer Design/Pan-assay Optimization

Two different multiplex assays were designed and optimized to detect clinically and/or environmentally relevant pathogens in the wastewater. These two assays consist of a multiplex containing AMR bacterial pathogen targets as well as a multiplex containing sexually transmitted infection (STI) targets. The bacterial pathogen panel includes the $bla_{\text{OXA-51}}$ gene (an intrinsic carbapenemase gene found in all *Acinetobacter* species), *Klebsiella pneumoniae*, *Clostridium difficile*, and the *mecA* gene found in methicillin-resistant *Staphylococcus aureus* (MRSA). The Shiga toxin 2 gene (*stx2a*) was to be included in the bacterial pathogen multiplex as well, but due to negative interactions with the other primers in the multiplex the wastewater DNA was screened with Shiga toxin 2 primers in singleplex. The STI panel includes molecular targets for *Neisseria gonorrhoeae* (gonorrhea), *Treponema pallidum* (syphilis), *Chlamydia trachomatis* (chlamydia), and human alphaherpesvirus 2 (herpes simplex virus type 2, genital herpes). The PCR primers for *K. pneumoniae, C. difficile*, Shiga toxin 2, human alpha herpes virus 2, and *N. gonorrhoeae* were designed with Dr. Jason Sahl's primer design pipeline; this pipeline is described in detail below for the design of *K. pneumoniae* primers. The $bla_{\text{OXA-51}}$, *mecA, T. pallidum,* and *C. trachomatis* primers were found in literature from previous studies (Börjesson et al., 2009) (Turton et al., 2006). Primers were ordered with universal tails to facilitate Illumina index ligation. All primer pairs were validated for specificity in singleplex with a positive control, a non-target species negative control, and water prior to multiplex optimization. All multiplexes were validated with a single positive control that contained equal concentrations of each target, a non-target species negative control, and water.

gBlocks were ordered from IDT for *C. trachomatis,* human alphaherpesvirus 2, *T. pallidum,* and *N. gonorrhoeae* and were used as positive controls for validating these assays. gBlocks were designed by first creating a BLAST database of one reference genome. The primer amplicon, which had previously been determined through *in silico* PCR, was then aligned against the genomes with BLASTn. The BLAST output was used to determine where the amplicon aligned in the genome. If this region was shorter than 125 bases, the region was lengthened to meet IDT's gBlock length requirement. This region was then informatically extracted from the genome and complexity was checked through IDT's website before ordering.

The lack of previously described primers available to detect the bacterial pathogen *Klebsiella pneumoniae* in traditional PCR reactions prompted the need to identify a region uniquely conserved within *K. pneumoniae* to be used to design primers that will detect this pathogen in wastewater samples. Due to the large amount of gene conservation among bacterial species, it can be difficult to avoid amplifying non-target species. Genome plasticity, or the ability for the genome to easily change and evolve, complicates assay design as well. Very specific regions within the genome must be identified to design primers around. To accomplish this, I used a primer design pipeline that compares primers between a large set of target and non-target genomes.

All the of the *Klebsiella* genomes from NCBI's GenBank assembly database were downloaded using the NCBI genome download tool available on Github (https://github.com/kblin/ncbi-genome-download). This tool allows for the specification of organism type, genus and species, and assembly level. For this design, the specifications made were organism type “bacteria”, genus *“Klebsiella”*, and assembly level “complete, contig, chromosome, scaffold”. With these parameters, approximately 17,000 genomes were downloaded. All genomes that were downloaded were copied into a new directory, unzipped, and renamed from FNA to FASTA files. The genomes were renamed with a more informative name using a script written by Dr. Jason Sahl as the genomes are downloaded with accession number only. For example, GCA 000219945.2 ASM21994v2 was renamed to GCA 000219945.2 Klebsiella pneumoniae JH1 Contig.

A dendrogram was generated using the 17,000 *Klebsiella* genomes using pairwise genomic distances with Mash (Ondov et al., 2016); Mash calculates pairwise similarity between two genomes in nucleotide content using k-mers. The generated tree was then visualized using FigTree. This tree was then used to define where the *Klebsiella pneumoniae* species started and ended. This was done to ensure that there were no incorrectly speciated genomes in the sample set. The genomes were then separated into *“Klebsiella pneumoniae”* and “non-*pneumoniae*” directories for downstream analyses. This resulted in ~15,000 *Klebsiella pneumoniae* genomes and ~2,000 non-*pneumoniae* genomes.

Prokka is a command line software tool used to annotate bacterial, archaeal, and viral genomes (Seemann, 2014). Prokka was run on one complete genome of *K. pneumoniae* (GenBank accession number GCA_904863175.1) to get a file of annotated genes. The Large Scale Blast Score Ratio (LS-BSR), a tool to quickly compare the genetic content between a large number of bacterial genomes, was then used to query the set of *K. pneumoniae* genes against both the *K. pneumoniae* and non-*pneumoniae* genomes to identify gene distribution and conservation (Sahl et al., 2014). BLAT was used for alignment because it quickly finds similarity in DNA within a large sample set. The output of LS-BSR is a pairwise matrix of blast score ratios ranging from zero to one. Count BSR, another tool included in this software that counts the number of genomes each gene hits, was then used on the generated matrix at a specified BSR value of greater than or equal to 0.8 in target *K. pneumoniae* genomes and less than or equal to 0.4 in non-target genomes. These values are equivalent to ~80% or greater identity across ~100% of the alignment in *K. pneumoniae* genomes and ~40% or less identity across ~100% of the alignment in non-*pneumoniae* genomes. Strict BSR values were set to identify genes that hit all the *K. pneumoniae* genomes and none of the non-*pneumoniae* genomes, indicating that this is a unique region found only in *K. pneumoniae* and should be focused on for primer design.

No genes were found that had zero non-*pneumoniae* genome hits using the count BSR hits tool at a BSR threshold of 0.8. However, two genes were found that hit only two of the non-*pneumoniae* genomes. One of these genes was annotated as a LysR family transcriptional regulator (locus tag HJW71_18195) and the other was a nuclear transport factor 2 family protein (locus tag PF051_13275). The non-*pneumoniae* genomes that these two genes hit were investigated. One genome was found to be contaminated as it was 11 Mb in size, which is more than double the size of a typical *K. pneumoniae* genome (approximately 5Mb). The other genome that these genes hit was identified as *pneumoniae*, but it clustered into a group in the phylogenetic tree containing mainly *Klebsiella variicola* genomes. One hypothesis is that this genome was incorrectly classified when uploaded to NCBI, possibly due to incorrect taxonomic classification stemming from shared phenotypes. The decision was made to move forward with these genes as both the genomes they were hitting were likely mis-classified. Both genes were considered for primer design.

The two genes, HJW71_18195 and PF051_13275, were put into Primer3, which is integrated into the primer design pipeline (PDP), and primers were designed (*Primer3—New*

Capabilities and Interfaces - PMC, n.d.). The PDP generates a “best primers” output file that contains three possible primer pairs for each gene. The output of the PDP includes important information, such as number of mis alignments within target genomes, number of alignments in non-target genomes, number of degeneracies, thermalcycling temperatures, and amplicon length. Using the output information, HJW71_18195, a LysR family transcriptional regulator gene, was chosen and all primer options were tested with *in silico* PCR to ensure the chosen primers worked effectively and did not amplify non-targets.

Multiplex Targets

The $bla_{\text{OXA-51}}$ gene was included in the AMR multiplex because it is an intrinsic resistance gene found in *Acinetobacter* species. There is a growing global concern of AMR in *Acinetobacter baumannii*, specifically Carbapenem-resistant *A. baumannii* (CRAB). CRAB are resistant to nearly all antimicrobials, including broad-spectrum carbapenem drugs: meropenem, imipenem, and doripenem (*Carbapenem-Resistant Acinetobacter Baumannii (CRAB): An Urgent Public Health Threat in United States Healthcare Facilities | A.R. & Patient Safety Portal*, n.d.). *Acinetobacter* can cause human infections in the blood, urinary tract, and lungs (pneumonia), or in wounds in other parts of the body (*Acinetobacter in Healthcare Settings | HAI | CDC*, 2019). These carbapenemase genes can be transferred to other bacteria and cause the spread of multidrug resistance (MDR).

Klebsiella pneumoniae is a pathogen included in the Carbapenem-resistant Enterobacterales (CRE) order of bacteria, which commonly cause infections in health care settings. CRE are difficult to treat because they do not respond to commonly used antimicrobials and are sometimes resistant to all antimicrobial therapies, known as pan resistance. *K. pneumoniae* carbapenemase (KPC)-producing bacteria are a group of highly drug-resistant

Gram-negative bacilli causing infections associated with significant morbidity and mortality (Arnold et al., 2011). KPCs are an important mechanism of resistance for an increasingly wide range of Gram-negative bacteria and are no longer limited to *K pneumoniae*.

Clostridium difficile is a spore forming Gram-positive bacterium. *C. difficile* is shed in feces and will end up in wastewater. Bacteria form spores to withstand very harsh environmental conditions as a survival mechanism, leading to the thought that *C. difficile* spores may be persisting in wastewater treatment plants/sludge.

The *mecA* gene found in methicillin-resistant *Staphylococcus aureus* is one of the causes of very difficult to treat Staph infections due to its antimicrobial resistance. Staph infections can spread in hospitals, other healthcare facilities, and in the community (*MRSA | CDC*, 2019). They can cause serious infections that can lead to sepsis or death.

Shiga toxin 2, a very potent toxin produced by bacteria, was included in the screening of wastewater and sludge samples because Shiga toxin-producing *E. coli* are found in the intestine of healthy cattle. Shiga toxin-producing *E. coli* (STEC) infections can be a major source of infection for humans. *E. coli* O157:H7 is one of the most commonly identified type of STEC in the United States. Diarrheal STEC infections can cause complications leading to hemolytic uremic syndrome (HUS). HUS affects the kidneys and blood clotting functions of infected people, leading to the destruction of red blood cells and possible kidney failure (*Hemolytic-Uremic Syndrome*, n.d.). If storm water runoff from agricultural areas with cattle operations ends up in the wastewater, the *stx2a* gene could be detected.

Genomic DNA Extraction from Wastewater Samples

In preliminary testing, it was determined that the Qiagen DNeasy PowerSoil Pro 96-well plate extraction kit would be the most efficient way to extract DNA from wastewater. The 353 previously collected wastewater samples were stored in 50 ml conical tubes (conicals). The six sludge samples were stored in 15ml conicals. The conicals were thawed on ice, then 5ml of water were transferred from the 15 or 50ml conicals to 5ml conicals and centrifuged at 3220 rcf for 45 minutes. Once centrifuged, pellets were formed in the bottom of the conicals. The majority of the supernatant was poured off without disturbing the pellet, leaving a small amount of water behind. The pellet was mixed with the small remaining amount of water and half of the pellet was pipetted into its corresponding well of the 96-well PowerBead Pro plates included in the extraction kit. The other half of the pellet was pipetted into Mueller Hinton II cation adjusted broth to be cultured for later antimicrobial resistance testing discussed in Chapter 3. The samples were then processed following the Qiagen DNeasy 96 PowerSoil Pro Kit protocol. A total of four 96 well plates were processed in this manner. Once the DNA was extracted, it was run with the BactQuant 16S rRNA assay to confirm the success of the extraction. DNA was stored in foil sealed 96 well half skirted plates at -20°C for downstream use.

Molecular Testing: Sample Screening

All extracted DNA were screened with the BactQuant16S rRNA assay, the bacterial pathogen multiplex, the STI multiplex, Shiga Toxin 2 singleplex, and *A. baumannii* singleplex. gDNA was amplified with the bacterial pathogen multiplex using the following protocol: 1X Promega PCR Master Mix (Promega, Fitchburg, WI), 2.5 µl gDNA template, and multiplexed primer concentrations listed in Table 1. Specific PCR parameters were as follows: initial denaturation at 95°C for 2 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 5 minutes. gDNA was amplified with the STI multiplex using the following protocol: 1X Promega PCR Master Mix, 1 µl gDNA template, and multiplexed primer concentrations listed in

Table 1. Specific PCR parameters were as follows: initial denaturation at 95°C for 2 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 5 minutes.

Following the assay screenings, the PCR product was visualized on a 2% lithium borate agarose gel through gel electrophoresis. Gels were run at 250 volts for approximately 35 minutes using the GeneRuler 100bp ladder, then viewed with the Bio-Rad Gel Doc XR+ Gel Documentation system (Bio-Rad, Hercules, CA).

Table 1. PCR primer sequences, concentrations, and amplicon size

Target	Amplicon Target Size (bp)	Forward primer (5'-3')	Reverse primer (5'-3')	Final Concentration (uM)	Multiplex
Acinetobacter Species - blaOXA-51-like	353	TAATGCTTTGATCGGCCTTG	TGGATTGCACTTCATCTTGG	0.3	Bacterial Pathogen
Clostridium difficle	239	CTCCATATGGTAGGTACTCC	CCTACAACACGRTGACATGG	0.3	Bacterial Pathogen
Klebsiella pneumoniae - putative HTH-type transcriptional regulator YbdO)	398	GAGCACCATAACCTGACCAC	AAGCTGCATAGTGGGTGAGA	0.3	Bacterial Pathogen
MecA gene (MRSA)	108	TGCTCAATATAAAATTAAAACAAACTACG	GAAGTATGACGCTATGATCCCAATCTAACTTC	0.3	Bacterial Pathogen
Neisseria gonorrhoeae (gonorrhea)	248	AGTCTGTTTTCAAATCGGGA	TATTCAATAAACGCCCGTCA	0.3	STI
Treponema pallidum (syphilis, *polA gene)*	105	GGTAGAAGGGAGGGCTAGTA	CTAAGATCTCTATTTTCTATAGGTATGG	0.3	STI
Chlamydia trachomatis (chlamydia, gyrA gene)	463	TGATGCTAGGGACGGATTAAAACC	TTCCCCTAAATTATGCGGTGGAA	0.45	STI
Herpes Simplex 2 Virus	311	AAAACACGTATCTGCTGCCC	GTCGTACAGGGGATTTTCCG	0.3	STI
Shiga Toxin 2	248	AACGGGTAAATAAAGGAGTT	ACAGGTACTGGATTTGATTG	0.5	n/a
E.coli Species	146	GAGCGTATCTCTGAACAAGG	GAAGCTTCRYTGGTCAGTTC	0.4	n/a
16S rRNA - 8F/1492R	1484	AGAGTTTGATCMTGGCTCAG	TACGGYTACCTTGTTACGACTT	0.4	n/a
16S rRNA - Earth Microbime Project	390	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	0.4	n/a
16S rRNA - BactQuant	467	CCTACGGGDGGCWGCA	GGACTACHVGGGTMTCTAATC	0.4	n/a

Amplicon Sequencing (AmpSeq)

Thirty-four paired influent and effluent wastewater samples that were collected on the same day from the same location were chosen to undergo amplicon sequencing. All six sludge samples were also sequenced. Three additional samples were included due to amplification with the *K. pneumoniae*, the *bla*$_{\mathrm{OXA\text{-}51}}$ gene, and the *mecA* gene targets when screened with the bacterial pathogen multiplex. These three additional samples were unique as majority of the samples did not amplify with all three targets within one sample. A total of 43 samples underwent amplicon sequencing.

After screening and visualization of the bacterial multiplex, a 1.5X Agencourt AMPure bead (Beckman Coulter, Brea, CA) clean-up was performed on the 46 samples according to manufacturer's instructions. This was followed by an indexing PCR with the following final concentrations: 1X HiFi HotStart ReadyMix (Kapa Biosystems Inc., Wilmington, MA), 0.4 µM of each indexing primer, and 2 µl of PCR product. The indexing PCR parameters were as follows: initial denaturation at 98°C for 2 minutes, 6 cycles of denaturation at 98°C for 30 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. A 1X Agencourt AMPure bead clean-up was preformed after the indexing PCR. All amplicons were normalized with SequalPrep (Thermo Fisher Scientific, Applied Biosystems), pooled, and sequenced with the Illumina MiSeq Reagent nano Kit v2 (300 cycles) (Illumina Inc., San Diego, CA).

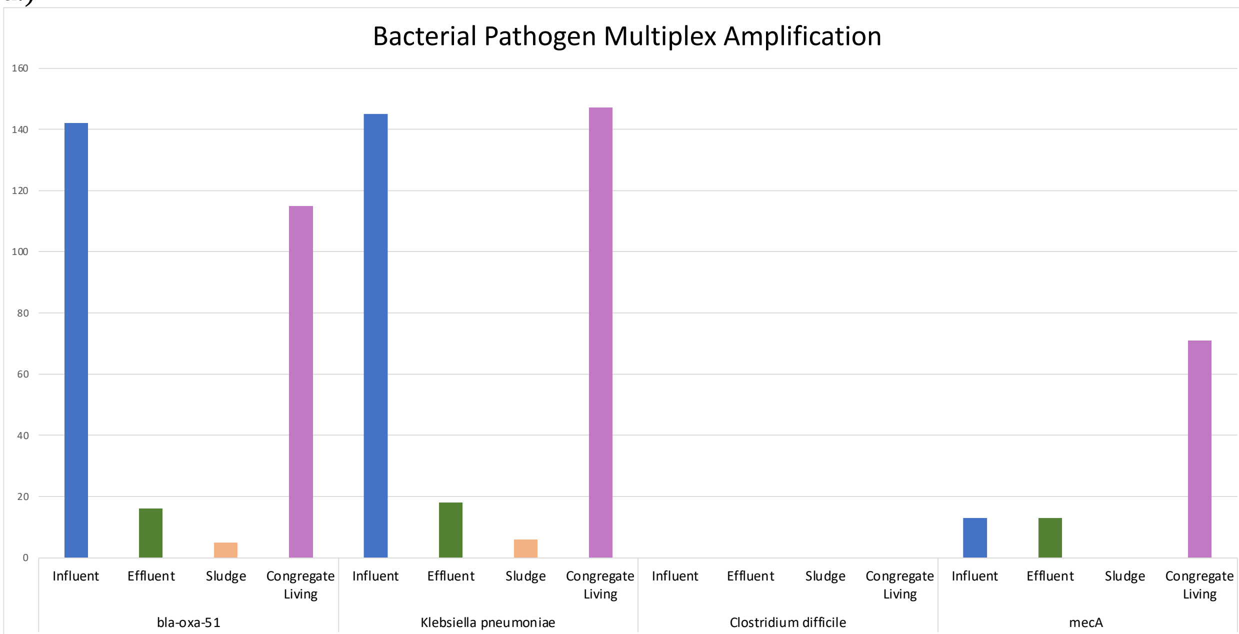
Amplicon sequencing results were analyzed on Northern Arizona University's high performance computing cluster Monsoon. A coverage matrix and breadth matrix were generated to evaluate the number of reads that mapped to each target within each sample. The breadth is the number of bases in the reference genome that are covered by the sample reads at a certain coverage depth. The coverage depth for the generation of the breadth matrix was 3X. The *bla*$_{\mathrm{OXA-51}}$ amplicon was analyzed further to investigate the presence of variants and diversity. Diversity within the target was analyzed using Qiime2 (Bolyen et al., 2019) and LS-BSR. Sequencing adapters and PCR primers were trimmed off the reads using the qiime cutadapt command. Then the qiime demux summarize command was used to generate a .qzv file to determine truncation parameters, which were then used to run DADA2. The qiime feature-table tabulate-seqs command was used to get the representative sequences, followed by the qiime phylogeny align-to-tree-mafft-fasttree command, generated unrooted trees to look at diversity among the amplicons. A LS-BSR matrix was then generated using a directory of representative *Acinetobacter* spp. genomes (n=731) and the amplicon sequencing representative sequences generated with Qiime2. Representative *Acinetobacter* spp. genomes were generated through Mash based clustering, which filtered out highly similar sequences and keeping only a single representative.

Results

The Qiagen DNeasy PowerSoil Pro 96-well plate kit was a reliable extraction method that generated gDNA from pelleted wastewater samples. Amplification with the BactQuant 16S rRNA assay in 342 out of 359 samples confirmed the success of this extraction method. The samples that did not amplify with the BactQuant assay may not have had any bacterial species present, experienced an error in the extraction, or had PCR inhibitors present.

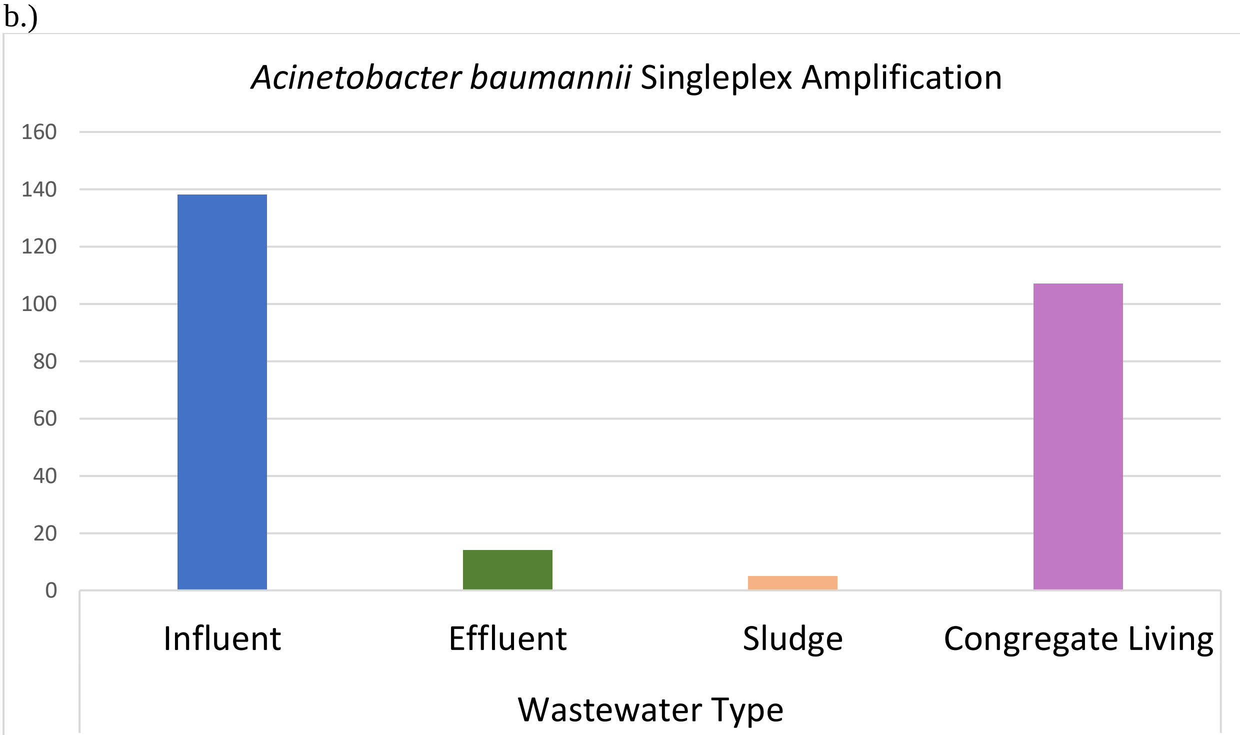
The bacterial pathogen multiplex proved to be effective at amplifying the *K. pneumoniae*, *bla*$_{\mathrm{OXA\text{-}51}}$ gene, and *mecA* gene targets in gDNA extracted from wastewater. The *K. pneumoniae* primers that were designed for the bacterial pathogen multiplex were highly effective in amplifying only *K. pneumoniae.* There was not any unspecific amplification, negative interactions with other primers in the multiplex, or cross reactivity. There was no visible amplification with the *C. difficile* assay, however the assay did amplify *C. difficile* in the positive control, indicating that the assay was working properly. The amplification of STI gDNA using the STI multiplex was unsuccessful as none of the wastewater or sludge samples amplified with any of the targets in the STI multiplex. There was also no amplification in the Shiga toxin 2 singleplex. A total of 264 samples amplified with the *A. baumannii* singleplex. The number of samples that amplified in each assay, separated by wastewater type, is shown in Figure 1.

a.)



Bacterial Pathogen Multiplex Amplification
160
140
120
100
80
60
40
20
0
Influent
Effluent
Sludge
Congregate Living
bla-oxa-51
Klebsiella pneumoniae
Clostridium difficile
mecA

b.)



Acinetobacter baumannii Singleplex Amplification
160
140
120
100
80
60
40
20
0
Influent
Effluent
Sludge
Congregate Living
Wastewater Type

c.)

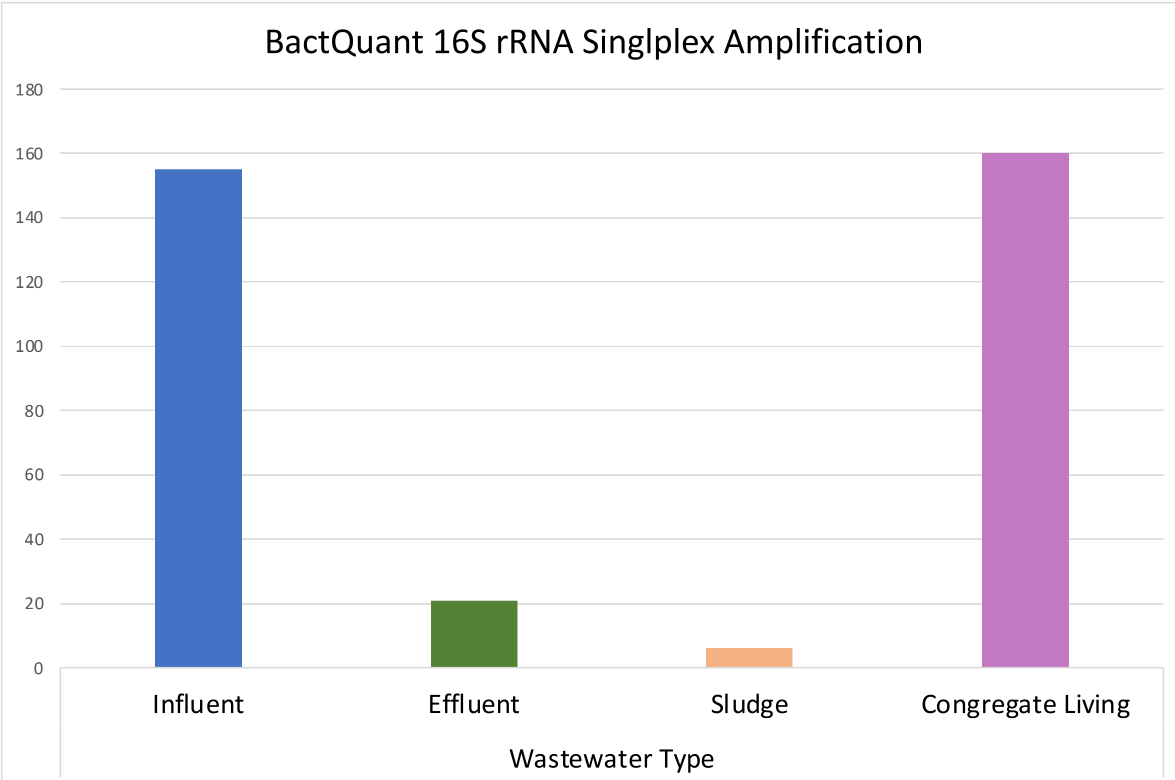


Figure 1. Assay Amplification Bar Charts
The number of samples that amplified in each assay, organized by wastewater type: influent (n=165), effluent (n=23), sludge (n=6), and congregate living (n=165). 1a.) Amplification for the bacterial pathogen multiplex, including the *bla*$_{\text{OXA-51}}$ gene, *K. pneumoniae, C. difficile,* and *mecA* gene targets. 1b.) Amplification with the *A. baumannii* singleplex. 1c.) Amplification with the BactQuant 16S rRNA assay.

All putative amplicons visualized on the gel were confirmed with amplicon sequencing. The targets that amplified in the multiplex were also present in the amplicon sequencing results. In addition to this result, the *C. difficile* target that did not appear to amplify in the multiplex aligned to the amplicon sequencing reads in 15 samples, including WWTP influent, effluent, and sludge as well as congregate living water samples. Five effluent samples had reads align to all four amplicons. Four of the six sludge samples also had reads that aligned to all four amplicons.

The read counts for the positive control included in the AmpSeq data were analyzed to determine how evenly the amplicons sequenced. Read counts were not evenly distributed in the positive control, with *C. difficile* receiving the least amount of reads, followed by *K. pneumoniae*, then *bla*$_{\text{OXA-51}}$, and *mecA* receiving the most reads. This was used to examine relative abundance of the amplicon within each sample and determine which amplicons were enriched in a sample when compared to the positive control reads. *K. pneumoniae* was found in the greatest relative abundance in all 46 samples that underwent amplicon sequencing. *C. difficile* was found in the lowest abundance when compared to the positive control reads.

The amplicon sequencing data was also analyzed to identify variants within the *bla*$_{\text{OXA-51}}$ gene, which could be representative of different *Acinetobacter* species. Using QIIME2, 79 variants were found for the *bla*$_{\text{OXA-51}}$ amplicon. These variants can be separated by a single SNP. After screening the 79 identified variants against the representative *Acinetobacter* spp. genomes, 16 of the bla$_{\text{OXA-51}}$ variants had a BSR value of 0.9 or greater. The genomes that each of the 16 sequences hit were further investigated. All but two of the representative sequences had a mixture of *Acinetobacter* species within them, including *A. lwoffii, A. pittii, A. johnsonii, A. gyllenbergii, A. ursingii, A. seifertii, A. oleivorans, A. junii,* and *A. baumannii.* The two ASVs that only hit one genome were classified as *A. bouvetii and Acinetobacter* sp. VT 511.

Discussion

The standard molecular-based method for wastewater surveillance is the quantitative polymerase chain reaction (qPCR) technique on the basis of nucleic acid amplification (Hiller et al., 2019). The advantage of using qPCR for wastewater testing is the high specificity, the rapid examination, and low measurement error of about 10% (Kitchen et al., 2010). However, qPCR only provides cycle threshold (Ct) values, which allows for determination of presence or absence of the target within the sample. qPCR detection alone does not identify the bacteria carrying the specific resistance genes and their relevance to human health. qPCR multiplexes are also limited to three or four gene targets within a reaction (*What Is Multiplexing?*, n.d.). The innovation of using the highly targeted approach of amplicon sequencing for wastewater is that it not only provides presence and absence information, but it allows for numerous downstream analyses that give insight into the genetic variation in specific genomic regions. Unlike qPCR, amplicon sequencing uses a highly multiplexed PCR approach to expedite research by assessing multiple genes simultaneously and allowing sequence targets ranging from a few to hundreds of genes in a single run (*Library Prep and Array Kit Selector*, n.d.).

The methods described in this chapter serve as a proof of concept that amplicon sequencing multiplex PCR assays can act as a quick, cost-effective, and noninvasive way to monitor the pathogens circulating in the community and assess diversity through wastewater and sludge testing. The amplification of *K. pneumoniae,* the *bla*$_{\mathrm{OXA\text{-}51}}$ gene found in *Acinetobacter* spp., and the *mecA* gene found in Methicillin-resistant *S. aureus* indicates that the gDNA of these bacterial species were present in the wastewater. However, downstream experiments and culturing were required to determine if this DNA came from viable bacteria in the wastewater or if it was extracellular DNA. The identification of these targets was not a surprising finding, as *K. pneumoniae,* the *bla*$_{\mathrm{OXA\text{-}51}}$ gene, and the *mecA* gene have all been identified in wastewater prior to this study (Surleac et al., 2020) (Ferreira et al., 2011) (Börjesson et al., 2009).

Amplicon sequencing was performed on 46 wastewater samples screened with the bacterial pathogen multiplex. This confirmed that the targets with observed amplification were truly present in the water sample, but furthermore allowed for the comparison of paired influent and effluent samples. Using the read counts from the positive control and observing relative abundance, it was discovered that bacterial pathogens persist in treated effluent water. This analysis does not confirm absolute abundance of pathogens in the wastewater samples; therefore, the efficacy of treatment cannot be commented on. However, the persistence of human pathogens in effluent water highlights the need to move away from fecal coliform detection and implement water standards that include the detection of human pathogens prior to reuse. Also using the amplicon sequencing read counts, five samples were observed that did not have *C. difficile* reads in the influent sample but had reads in the paired effluent sample. It is hypothesized that these samples may be picking up *C. difficile* from the WWTP, indicating that WWTPs may possibly be a source for *C. difficile.* This could be due to the spore forming ability of *C. difficile*, which allows it to survive water treatment and persist in the treatment plant. Another factor that may contribute to this is the use of anaerobic digesters at WWTPs, which creates an ideal environment for *C. difficile* as it is a strict anaerobe.

After visualization of the bacterial pathogen multiplex amplicons on a gel, it did not appear that there was any amplification with the *C. difficile* primers. This was an unexpected result, as *C. difficile* is shed in feces and studies have identified *C. difficile* in wastewater (Romano et al., 2012) (Chisholm et al., 2022). However, the amplicon sequencing results did indicate a percentage of reads that aligned to *C. difficile. C. difficile* could have been present in the wastewater and sludge in very low concentrations that could not be detected with traditional PCR and gel electrophoresis. Because *C. difficile* is a spore forming organism, extraction of DNA can be challenging and require a more intense lysis and bead beating steps than used in the chosen extraction method for these samples.

Although it was expected that STI targets would be present in wastewater, the 359 gDNA samples isolated from wastewater and sludge were negative for all targets in the STI multiplex. It was thought that the bacteria or virus would be able to be detected once it was shed into the wastewater. However, after observing no amplification with this multiplex, the conclusion was made that a small amount of people in the community experiencing a flare up or infection with a STI would likely not result in a detectable quantity of bacteria or virus in the wastewater. It is unknown if STI DNA persists in Southwestern U.S. wastewater and further investigation would provide more insight. However, based on our validations, we would expect to amplify this DNA if it was present in sufficient quantities.

The lack of amplification in the Shiga toxin 2 singleplex could indicate that storm water runoff from agricultural areas with cattle that shed STEC is not a significant problem in Southwestern U.S. wastewater treatment plants. The Southwest does not have a substantial amount of cattle farming when compared to other parts of the country, which could explain the absence of this target in the wastewater. It is also possible that the Shiga toxin gene could have been present at extremely low levels in the water and sludge that caused it to be undetectable in the testing methods used. A study by Bibbal et al. 2018 isolated STEC strains of *E. coli* with varying Shiga toxin subtypes associated with serious human disease in city wastewater. This suggests that Shiga toxin 2 could be present in wastewater, but it may require different detection methods than used in this study.

All samples were screened with an *A. baumannii* specific singleplex to further investigate which *Acinetobacter* species were causing amplification of the $bla_{\text{OXA-51}}$ gene in the bacterial pathogen multiplex. A total of 264 samples amplified with this singleplex, suggesting that *A. baumannii* is present in Southwestern U.S. wastewater. Although the samples amplified with the *A. baumannii* assay, it is possible that all samples could contain a mixture of *Acinetobacter* species. Eleven samples that amplified with the $bla_{\text{OXA-51}}$ gene did not amplify in the *A. baumannii* assay, which implied that there are other species of *Acinetobacter* present in the wastewater.

To explore the diversity of *Acinetobacter* species in the wastewater samples, the amplicon sequencing results were analyzed using QIIME2 and LS-BSR to gain insight into the variation within the *bla*$_{OXA-51}$ amplicon. Based on this analysis, the majority of the wastewater samples contained a wide variety of *Acinetobacter* species. Although carbapenem resistant *A. baumannii* is considered one of the largest public health threats, being classified as a Priority 1 pathogen by the WHO, other species of *Acinetobacter* have the potential to cause human infection. A study by Turton et al. 2010 found that significant numbers of other *Acinetobacter* species, particularly *A. lwoffii*, *A. ursingii*, and *A. johnsonii*, were often associated with bacteremia. All of these species were also identified with the *bla*$_{OXA-51}$ amplicon variant analysis. Not only is there the potential to cause infection, but these species can harbor and transfer resistance genes. *A. johnsonii,* one of the species identified through this analysis, has been found to actively acquire exogenous DNA from other bacterial species and become a reservoir of resistance genes (Montaña et al., 2016). The *Acinetobacter* species identified in the wastewater have the potential to spread antibiotic resistance genes, such as *bla*$_{OXA-51}$, to many of the other bacterial species present in the wastewater. As more bacterial species acquire carbapenem resistance genes, controlling AMR becomes even more challenging.

CHAPTER 3: THE INVESTIGATION AND IDENTIFICATION OF VIABLE MEROPENEM RESISTANT BACTERIA IN WASTEWATER

The ability for bacterial pathogens to persist even after treatment at WWTPs is a potential public health threat. AMR and multidrug resistance can rapidly spread within bacterial populations of related and unrelated species, which makes WWTPs hot spots for the dissemination of AMR through both the horizontal gene transfer of mobile genetic elements and the selective pressures of antimicrobials present in wastewater. Looking at antimicrobial resistance from a One Health perspective is essential to understanding how the aquatic environment of the urban water cycle is contributing to the growing threat of AMR. In this chapter, the antimicrobial susceptibility testing methods used to investigate viable meropenem resistant bacteria in both influent and effluent Southwestern U.S. wastewater are explained, with the goal of identifying highly meropenem resistant bacterial species circulating in the community.

Materials and Methods

Meropenem Antimicrobial Susceptibility Plate Testing (2μg/ml and 4μg/ml)

To determine if meropenem resistance persisted in viable bacteria cultured from wastewater, antimicrobial susceptibility plate testing was done on all 359 wastewater and sludge samples. The second half of the pellet formed during the gDNA extraction mentioned above in chapter 2 was placed in a deep 96-well plate with 1ml of Mueller Hinton II cation adjusted broth and placed in an incubator at 37°C overnight for at least 12 hours. After incubation, 100μl of overnight culture was placed into a clear flat bottom costar plate and was read at 620nm using the Fisher Scientific accuSkan FC to obtain an OD_{600} reading verifying there was adequate growth (OD_{600} of greater than or equal to 0.2). After confirming growth, 10μl of overnight culture was then subbed into a new deep 96 well plate with 1ml of fresh Mueller Hinton II cation adjusted broth, followed by pipette mixing. Immediately after mixing, 100μl of the fresh sub was placed in a new clear flat bottom costar plate and was read with the accuSkan FC to obtain an initial OD_{600} reading. The purpose of this reading was to establish a baseline OD_{600} of the subbed culture prior to incubation to ensure adequate growth. The deep 96 well plate with overnight culture subbed into fresh broth was then placed in the incubator at 37°C for approximately two hours to allow the bacterial cells to reach log growth before exposure to meropenem.

During the two-hour incubation, a 2X working stock of meropenem was made and filter sterilized with a 0.22μm filter. The concentration of the 2X working stock for the 2μg/ml experiment was 4μg/ml. The concentration of the 2X working stock for the 4μg/ml experiment was 8μg/ml. The meropenem working stock had to be made right before use as it is not stable for extended periods of time. As each sample needed to be grown in the presence of meropenem and in fresh MHII broth to determine if there was inhibition of growth, the antimicrobial and fresh broth were aliquoted into clear flat bottom plates in a sterile BSC. These plates also included a growth control (50μl MHII broth and 50μl BSL1 *E. coli* culture), a broth control (100μl of fresh MHII broth), and an antimicrobial control (50μl 2X working stock of meropenem and 50μl BSL1 *E. coli* culture). The layout of these plates can be seen in Table 2.

Table 2. Meropenem Susceptibility Testing Plate Map

		1	2	3	4	5	6	7	8	9	10	11	12
A	Sample not in presence of drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
B	Sample in presence of drug	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
C	Sample not in presence of drug	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
D	Sample in presence of drug	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
E	Sample not in presence of drug	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36
F	Sample in presence of drug	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36
G	Sample not in presence of drug	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43	Sample 44	Sample 45	Sample 46	Broth Control	ABX control
H	Sample in presence of drug	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43	Sample 44	Sample 45	Sample 46	Broth Control	Growth Control

After the two-hour incubation, 100μl of the culture was transferred to a new clear flat bottom plate and an OD_{600} reading was taken. The OD_{600} needed to be close to 0.02, which is approximately 10^7 bacterial cells. Placing too many cells in meropenem will produce false resistance readings as the antimicrobial will be used up before inhibiting all the cells present. Ensuring the proper number of cells is used will prevent overloading the antimicrobial with too many cells. If the OD_{600} was not at the appropriate reading of 0.02, the plate was placed back in the incubator and reread after 30 minutes to an hour depending on the first reading. Once the OD_{600} was at the desired value, the meropenem plates were inoculated by transferring 50μl of culture into the plates following the plate map in Table 2. Immediately after inoculation, the meropenem plates were read in the accuSkan FC to get initial OD_{600} and then placed in the incubator. Again, this initial reading was taken to compare to the OD_{600} taken after incubation to ensure the cultures were growing. Plates were incubated for 16.5 hours.

After the 16.5-hour incubation, all meropenem plates were read in the accuSkan FC plate reader. The OD_{600} data were exported and the doubling times for each sample were calculated using the following equation: ((incubation duration) x (log(2))) / (log(final concentration) – log(initial concentration)). If the sample doubled roughly the same number of times not in the presence of the drug and in the presence of the drug, then there was no inhibition of growth due to the antimicrobial agent, deeming that sample resistant.

These steps were completed for a concentration of first 2μg/ml and then 4μg/ml of meropenem. 2μg/ml of meropenem is an intermediate MIC, which was used first to cast a wider net as MICs can vary greatly for every organism and wastewater samples are highly complex. All 359 wastewater and sludge samples were tested at a 2μg/ml concentration. Samples that exhibited resistance to meropenem at a 2μg/ml concentration were then tested at a higher concentration of 4μg/ml. Samples that still showed resistance at 4μg/ml were moved forward to meropenem susceptibility testing using Etest strips. 20% glycerol + LB stocks were made from the overnight pellet culture, 2μg/ml culture, and 4μg/ml culture.

Meropenem Susceptibility Testing using Etest Strips

To further interrogate the viable meropenem resistant bacteria in Southwestern U.S. wastewater samples, the samples in which resistance persisted when grown in the presence of 4μg/ml of meropenem underwent additional antimicrobial susceptibility testing. Etest strips were chosen to look at the MICs of the wastewater samples as well as to assist in sample selection for downstream analyses. The MIC range on the meropenem Etest strips is 0.002-32μg/ml. The methods used for this experiment come directly from bioMérieux's Etest antimicrobial susceptibility testing protocol.

Media was chosen using the Etest recommended media, inoculum and incubation table following the aerobe organism group recommendations. Mueller Hinton II cation adjusted agar was prepared following the manufacturer's instructions. After allowing the media to cool, a serological pipette was used to place exactly 25ml of agar into each plate, which resulted in a depth of 4.0 ± 0.5 mm of agar in the plate. Mueller Hinton II cation adjusted broth was also made following manufactures instructions to be used for overnight and sub-cultures. Lastly, a 0.85% saline solution was prepared by placing 0.85g of NaCl into 100ml of DI water. The solution was then filter sterilized with a 0.22µm filter and vacuum apparatus.

Day one of this experiment consisted of inoculating overnight cultures. Inside a Biosafety cabinet (BSC), 5ml of Mueller Hinton II cation adjusted broth was placed into a 15ml conical tube and inoculated with the overnight glycerol stocks created during the antimicrobial susceptibility plate testing. Glycerol stocks were thawed slightly and a 1µl inoculation loop was used to inoculate the broth. The conical tubes were placed in a shaking incubator at 37°C for at least 12 hours overnight.

After overnight incubation, 50µl of overnight culture was sub-cultured into 5ml of fresh Mueller Hinton II cation adjusted broth and an OD_{600} reading was taken using the accuSkan FC. The sub was placed into the shaking incubator at 37°C for approximately 2 hours or until the cultures reached an OD_{600} of 0.20 – 0.50, indicating log phase of the bacteria. Once the culture was at the appropriate OD_{600}, the volume of culture that needed be added to 3ml of saline to bring the OD_{600} to 0.08 – 0.1 was calculated using $M_1C_1=M_2C_2$. Following the Etest inoculum recommendations, the saline and culture mixture should have a McFarland Standard of 0.5, which correlates to a 0.08 – 0.1 OD_{600}. The OD_{600} of the inoculum was read to confirm that the M_1C_1 calculation was correct and the OD_{600} fell between 0.08 and 0.1. A sterile cotton swab was then dipped into the inoculum and streaked onto the previously made MHII cation adjusted agar plate 3 times to evenly distribute the inoculum. The plates were dried for 15 minutes to allow any excess moisture to absorb into the agar. An Etest Mini Grip-It tool was then used to place the meropenem Etest strip in the center of each agar plate, taking careful consideration to avoid creating bubbles under the strip. Plates were incubated at 37°C for 16-20 hours.

After the 16–20-hour incubation, MICs were recorded for each sample based on growth and zones of inhibition around the Etest strip and recorded in Table 3. Photos were taken of all plates as well and can be seen in Figure 2. Culture was taken from each plate and stored in 20% glycerol + LB at -80°C.

Table 3. Meropenem Etest MICs

Sample	Etest MIC	Resistant/Intermediate/Susceptible
Inf 5/21/2020	>32	R
Inf 7/12-13/2021	0.38, >32	S/R
Inf 7/16/2020	>32	R
Inf 7/30/2020	>32	R
Inf 9/1/2020	4, >32	R
Inf 9/14-15/2020	1.5, >32	I/R
Inf 10/5-6/2020	2, >32	I/R
Inf 1/11-12/2021	>32	R
Inf 8/23-24/2021	>32	R
Eff 6/25/2020	8, >32	R
Inf 7/16/2020	.023, .25, >32	S/R
Inf 7/23/2020	.016, 0.25, >32	S/R
Inf 9/15/2020	>32	R
Inf 10/6/2020	4, >32	R
Eff 4/20/2021	4, >32	R
Eff 4/26/2021	3, >32	I/R
Eff 4/27/2021	.25, 1.5, >32	S/I/R
Inf 5/11/2021	.19, 1.5, >32	S/I/R
Eff 5/14/2021	4, >32	R
Inf 5/18/2021	0.016, 0.25, >32	S/R
Inf 5/25/2021	1.00, >32	S/R
Inf 6/1/2021	>32	R
Inf 7/3/2020	>32	R
Inf 7/18/2020	.064, >32	S/R
Inf 8/1/2020	0.25, >32	S/R
Inf 8/14/2020	1.5, >32	I/R
Inf 4/9/2020	>32	R
Inf 6/18/2020	16, >32	R
Inf 7/31/2020	6-8, >32	R
Inf 7/2/2020	8, >32	R
Inf 7/9/2020	8, >32	R
Inf 7/16/2020	8, >32	R
Inf 7/23/2020	6, >32	R
Inf 11/6/2020	>32	R
Inf 6/16/2021	2, >32	I/R
Inf 7/1/2021	>32	R
Inf 7/14/2021	8, >32	R
Inf 9/15/2021	>32	R
Inf 9/16/2021	2, >32	I/R
Congregate Living 8/17/21	.032, 2, >32	S/I/R
Congregate Living 8/26/21	0.032, .50, >32	S/R
Congregate Living 8/30/21	.064-0.094, >32	S/R
Congregate Living 8/30/21	.047, 1.0, >32	S/R
Congregate Living 9/7/21	.047, >32	S/R
Congregate Living 9/13/21	0.094, >32	S/R
Congregate Living 9/16/21	.032, >32	S/R
Congregate Living 9/20/21	.023, 0.25, >32	S/R
Congregate Living 9/23/21	.032, >32	S/R
Control - BSL1 E. coli	0.094, .032	S

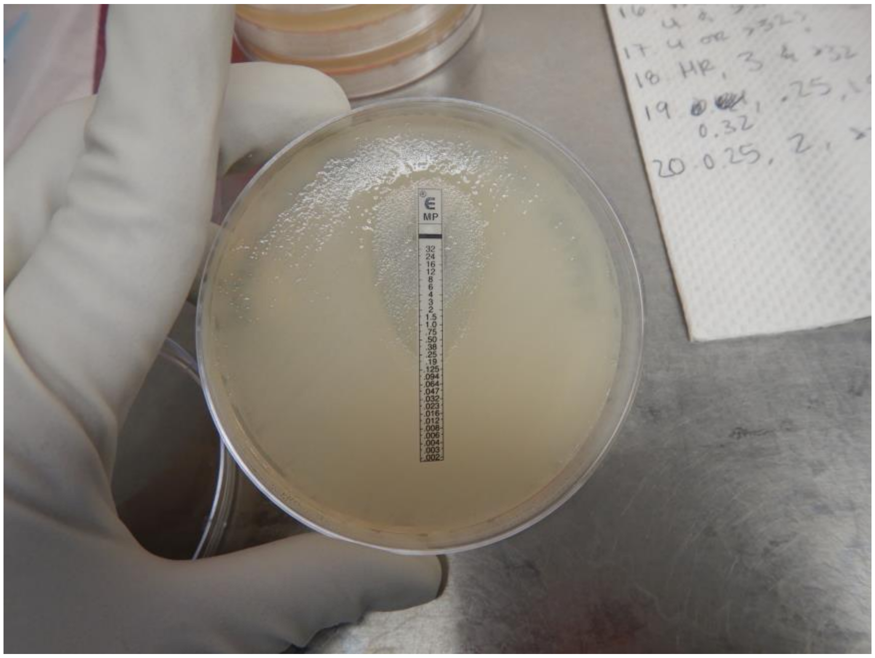


Figure 2. Etest Photo – Multiple Zones of Inhibition
Picture taken of a sample grown on MHII cation adjusted broth with a meropenem Etest strip with three zones of inhibition. MICs of 0.19, 2, and >32, which are classified as susceptible, intermediate, and resistant.

16S rRNA gene Sequencing

Using the results from the meropenem Etest strips, 19 samples from all collection sites that exhibited resistance to meropenem were chosen to undergo 16S rRNA gene sequencing. All samples chosen had MICs greater than or equal to 4μg/ml, but there was a specific focus on samples that had MICs of greater than or equal to 32μg/ml. All 19 samples had glycerol stocks from raw wastewater, culture at 2μg/ml of meropenem, culture at 4μg/ml of meropenem, and culture from Etest analyses. This presented the opportunity to watch the microbial diversity of the samples change as the selection pressure of meropenem was added in increasing concentrations. A total of 82 samples underwent 16S rRNA gene sequencing: 19 raw, 19 at 2μg/ml, 19 at 4μg/ml, and 25 from Etest analyses.

gDNA was already extracted for the raw wastewater samples from the genomic DNA extraction described in Chapter 2. Heat soaks were done on the 2μg/ml, 4μg/ml, and Etest glycerols. A 1μl inoculation loop was dipped into the slightly thawed glycerol stocks and then swirled in 15μl of molecular grade water in a 96 well plate. The plate was then heated at 95°C for 10 minutes followed by centrifugation at 2204 rcf for one minute. The raw gDNA and heat soak product were processed with the 16S rRNA Earth Microbiome Project assay, which amplifies the 515F–806R region (V4) of the 16S small subunit rRNA gene, using the primer set listed in Table 1 (*16S Illumina Amplicon Protocol*, n.d.). The assay was run using the following protocol: 1X PCR Buffer, 2.5mM $MgCl_2$, 0.2 nM dNTPs, 0.08 U/μl Platinum Taq, 2.5 μl gDNA template, and primer concentrations listed in Table 1. Specific PCR parameters were as follows: initial denaturation at 95°C for 10 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. The reverse primers in this assay have unique barcodes on them to make sequencing preparation simpler.

The PCR product was visualized through gel electrophoresis on a 2% lithium borate agarose gel at 250 volts for approximately 35 minutes using the GeneRuler 100bp ladder and viewed with the Bio-rad Gel Doc. A 1.5X Agencourt AMPure bead clean-up was performed on the PCR product. The amplicons were quantified with Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher/Invitrogen) following manufacturer's instructions. Using the Quant-iT results, amplicons were pooled at equal amounts into a single sterile tube. The final pool was quantified with the Qubit 4 Fluorometer (ThermoFisher/Invitrogen). The samples were then sequenced with the Illumina MiSeq Reagent Kit v3 (600 cycles).

Sequencing results were analyzed on Northern Arizona University's high performance computing cluster Monsoon using the bioinformatic tool Qiime2 following the "Moving Pictures" tutorial. A tab separated value metadata text file was created with the following information for each sample: sample ID, barcode number, barcode sequence, antimicrobial concentration, and whether the sample was influent or effluent. The paired end sequences were then downloaded to the working directory on Monsoon using the qiime tools import command. To demultiplex the sequences, the barcode sequence associated with each sample was needed. The sequences were demultiplexed using the metadata file containing the barcode sequences and the qiime demux command. A summary of the demultiplexing results was generated with the qiime demux summarize command and the .qzv output file was visualized using Qiime2 view to determine how many sequences were obtained per sample and to get a summary of the distribution of sequence qualities at each position in the sequence data. The DADA2 Qiime plugin was used with the command qiime dada2 denoise-paired to correct and remove low quality regions of the sequences for quality control. This command requires truncation parameters which were determined by viewing the interactive quality plot generated in the demux summarize command preformed. The forward truncation parameter was set to zero bases and the reverse parameter was set to 240 bases. These parameters were chosen because the quality of the initial bases was high, meaning bases did not need to be trimmed from the beginning of the sequences. Using the plot, the quality dropped off around position 240, so sequences were truncated at 240 bases. A feature table as well as the representative sequences were generated through the qiime feature-table summarize and the qiime feature-table tabulate-seqs commands, respectively. This gave information on how many sequences are associated with each sample. Both rooted and unrooted trees were generated to look at phylogenetic diversity using qiime phylogeny align-to-tree-mafft-fasttree. Using the rooted tree, the qiime diversity core-metrics-phylogenetic command was used to compute alpha and beta diversity metrics. Shannon's diversity index was generated for the alpha diversity metric using the equation $H = (-\Sigma p_i * \ln(p_i))$, where p_i is the proportion of the entire community made up of species *i* (Shannon, 1948). The higher the value of H, the higher the diversity of species in a particular community. The lower the value of H, the lower the diversity. A value of H = 0 indicates a community that only has one species. The calculated Shannon diversity indices can be seen in Table 4. The unweighted UniFrac distances were generated for the beta diversity metric (Lozupone & Knight, 2005). The PCoA of the unweighted UniFrac distances can be seen in Figure 3. The sampling depth parameter had to be determined to run this command, which was done by viewing the feature table. The sampling depth was set to 4,166x. Lastly, the taxonomy of the samples was explored by generating an interactive bar plot using the following commands: qiime feature-classifier classify-sklearn, qiime metadata tabulate, qiime taxa collapse, and qiime taxa barplot. The taxonomy bar plot generated can be seen in Figure 4.

Table 4. Shannon Diversity Indices for 16S rRNA Gene Sequencing

Sample	Shannon Diversity Index
Raw-Congregate-Living-20210817	5.627515689
Raw-Congregate-Living-20210916	5.498029393
Raw-Eff-20210426	6.447743399
Raw-Eff-20210427	6.05525813
Raw-Eff-20210514	5.610560009
Raw-Inf-20210511	6.102367545
Raw-Inf-20200730	5.780047544
Raw-Inf-20201005-06	5.851389963
Raw-Inf-20210712-13	5.82467409
Raw-Inf-20210823-24	5.442595331
Raw-Inf-20200703	4.747060388
Raw-Inf-20200718	5.337048956
Raw-Inf-20200814	4.73330732
Raw-Inf-20200409	4.509671111
Raw-Inf-20200618	5.447477767
Raw-Inf-20200731	5.573481146
Raw-Inf-20200702	5.463520108
Raw-Inf-20210616	4.909583938
Raw-Inf-20210915	5.174357489
2ug-ml-Congregate-Living-20210817	2.116417565
2ug-ml-Congregate-Living-20210916	1.146613954
2ug-ml-Eff-20210426	3.025710065
2ug-ml-Eff-20210427	3.074828391
2ug-ml-Eff-20210514	3.476730623
2ug-ml-Inf-20210511	3.994924002
2ug-ml-Inf-20200730	3.214328799
2ug-ml-Inf-20201005-06	3.773577723
2ug-ml-Inf-20210712-13	4.165728019
2ug-ml-Inf-20210823-24	3.309593158
2ug-ml-Inf-20200703	3.734374384
2ug-ml-Inf-20200718	3.504862112
2ug-ml-Inf-20200814	3.325129419
2ug-ml-Inf-20200409	3.765883311
2ug-ml-Inf-20200618	3.224753568
2ug-ml-Inf-20200731	3.225761415
2ug-ml-Inf-20200702	3.678336261
2ug-ml-Inf-20210616	3.825401957
2ug-ml-Inf-20210915	4.059955076
4ug-ml-Congregate-Living-20210817	1.836167862
4ug-ml-Congregate-Living-20210916	0.008556164
4ug-ml-Eff-20210426	2.441354057
4ug-ml-Eff-20210427	1.960055739
4ug-ml-Eff-20210514	2.466561567
4ug-ml-Inf-20210511	2.972060776
4ug-ml-Inf-20200730	0.729298979
4ug-ml-Inf-20201005-06	1.39413438
4ug-ml-Inf-20210712-13	1.260554917
4ug-ml-Inf-20210823-24	1.6015563
4ug-ml-Inf-20200703	0
4ug-ml-Inf-20200718	1.235315708
4ug-ml-Inf-20200814	0.355826163
4ug-ml-Inf-20200409	1.253892814
4ug-ml-Inf-20200618	1.377906196
4ug-ml-Inf-20200731	0.633195029
4ug-ml-Inf-20200702	1.461026676
4ug-ml-Inf-20210616	2.035592129
4ug-ml-Inf-20210915	0.941037621
Control-Ecoli-Type-VIII	0

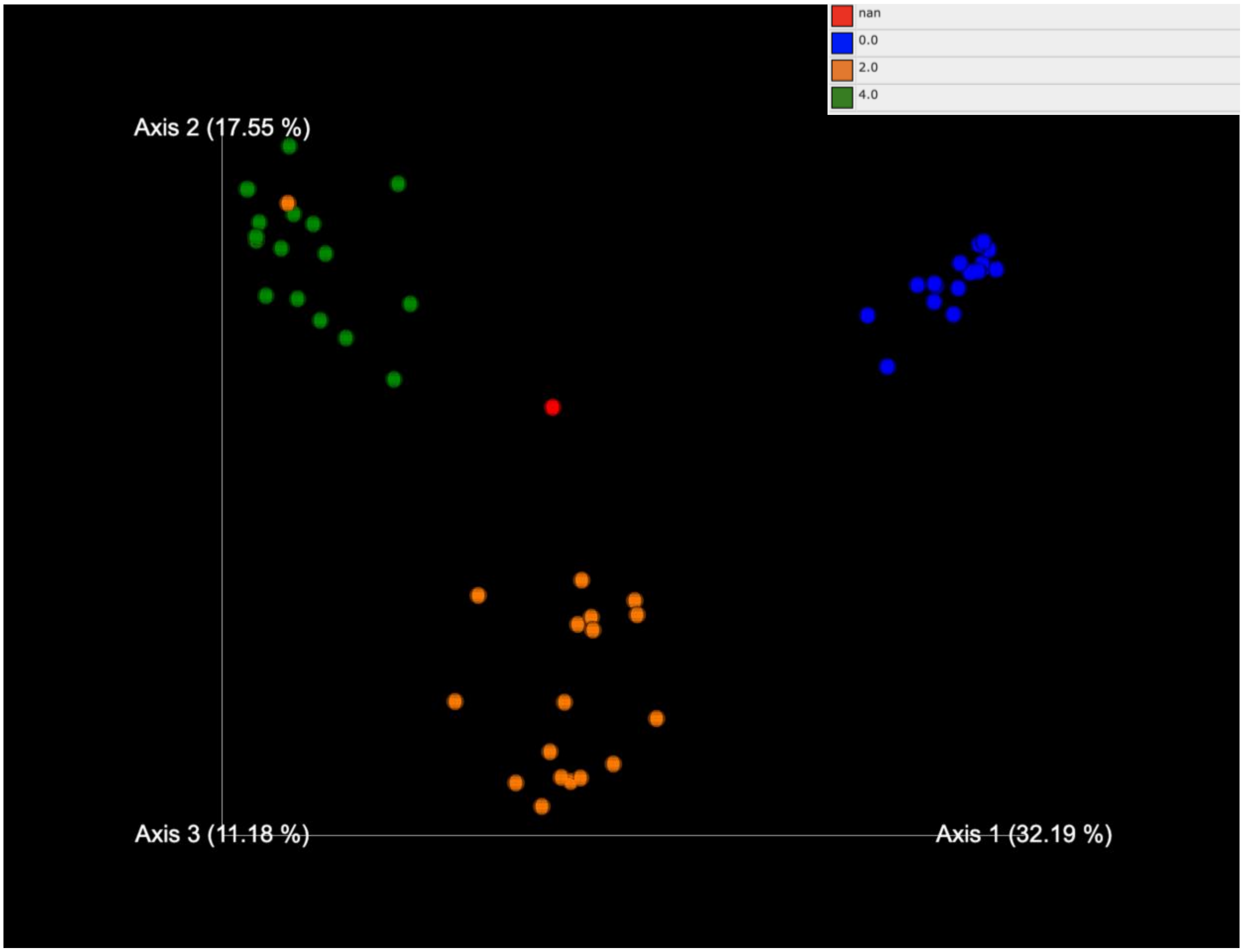


Figure 3. Unweighted UniFrac Distances PCoA – 16S rRNA Gene Sequencing
Unweighted Unifrac PCoA comparing microbial diversity between samples generated from 16S rRNA gene sequencing using Qiime2. Raw wastewater (blue, 0.0), wastewater grown at 2μg/ml meropenem (orange, 2.0), and wastewater grown at 4μg/ml (green, 4.0) all cluster together with their respective group, meaning the bacterial communities within these samples are highly similar. Microbial diversity changed as samples were actively selected for highly resistant species through the exposure to meropenem. The red point (nan) is the BSL1 *E. coli* positive control included in sequencing.

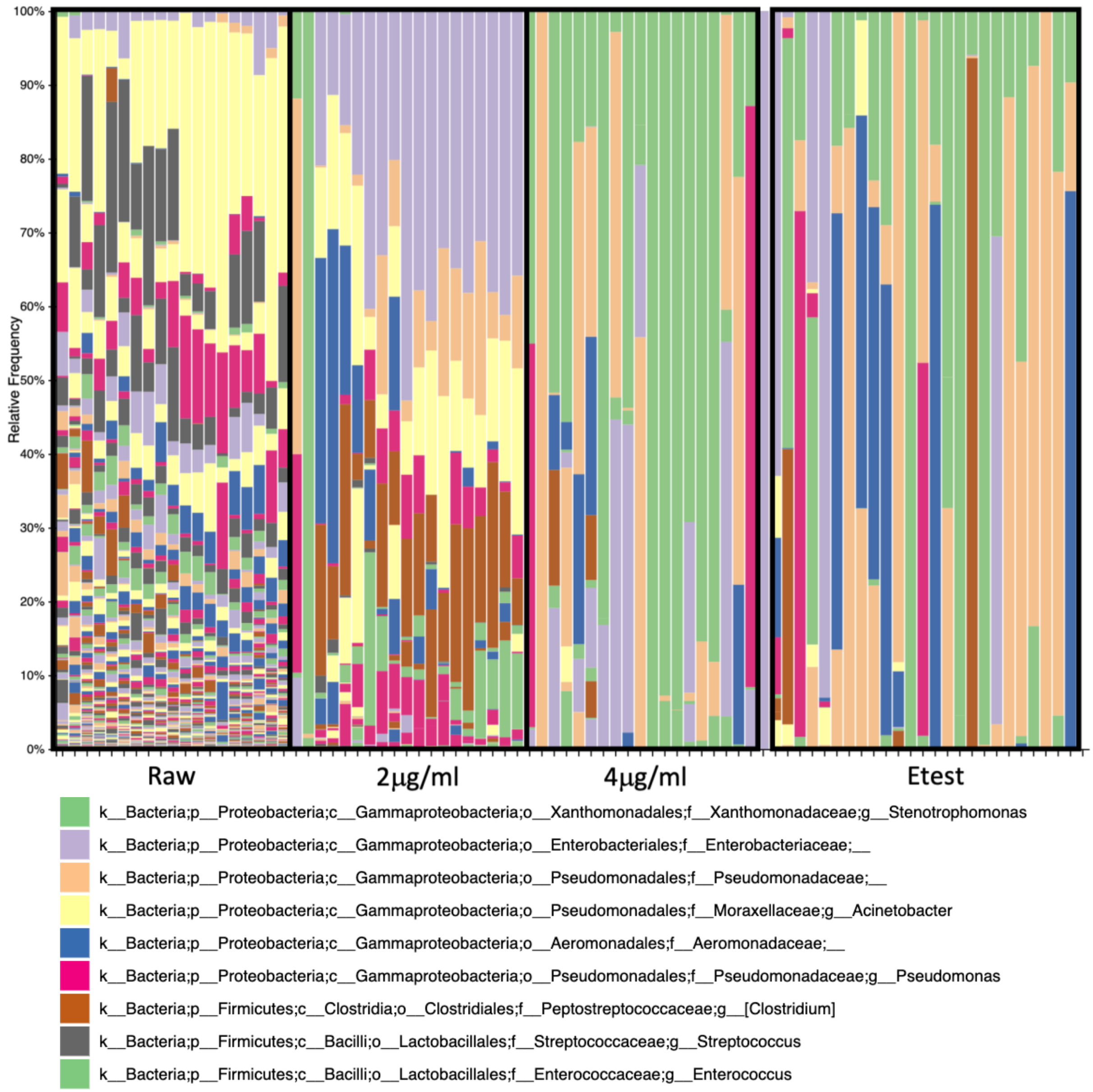


Figure 4. 16S rRNA Gene Sequencing Taxonomy Bar Chart
A taxonomy bar chart generated from 16S rRNA sequencing data using Qiime2. Each color within the bars represents a different genus identified within a single sample. The legend below identifies the most prominent genera identified. Microbial diversity decreases as the concentration of meropenem increases.

Restriction Fragment Length Polymorphism (RFLP) Single Colony Whole Genome Sequencing

Six different wastewater samples, five influent and one effluent, that had MICs of greater than or equal to 32μg/ml and had data from the previously done 16S rRNA gene sequencing were chosen for single colony restriction fragment length polymorphism enzymatic reactions.

This technique allowed for easy detection of diversity among the samples. The restriction enzymes cut the genomic DNA of the sample and produce a unique fingerprint that can be visualized through banding patterns on a gel.

Luria Broth (LB) agar plates containing 32µg/ml meropenem were made. The $M_1C_1 = M_2C_2$ calculation was used to make a 1ml meropenem working stock. LB agar was made following the manufacturer's instructions and allowed to cool to at least 55°C before adding the 1ml meropenem working stock to prevent degradation of the antimicrobial. The media and meropenem were stirred with a stir bar on a magnetic stir plate to create a homogenous mixture prior to pouring. A total of 25ml of agar was added to petri dishes and allowed to dry on the benchtop. Plates were made the same day of use to ensure proper antimicrobial activity.

Isolation streaks were made from Etest glycerol stocks on the 32µg/ml meropenem LB agar plates for the six wastewater samples. A BSL1 *E. coli* isolation streak as well as a blank agar plate were also included to ensure the antimicrobial was functioning properly and there was no contamination of the media. The isolation streaks were stored inverted and incubated at 37°C overnight. After incubation, 10 single colonies were picked using an inoculation needle for each sample (60 single colonies total) and swirled into 15µl of molecular grade water. Quarter lawns were made for each single colony as well by streaking the single colony on ¼ of a LB agar plate prior to swirling it in the water and incubating the plates at 37°C. The single colonies underwent a heat soak at 95°C for 10 minutes, followed by centrifugation at 2204rcf for 1 minute. The heat soak product was then used in the 16S rRNA 8F/1492R assay with the following protocol: 1X Promega PCR Master Mix, 1.5µl gDNA template, and multiplexed primer concentrations listed in Table 1. Specific PCR parameters were as follows: initial denaturation at 95°C for 2 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 5 minutes.

The 60 single colony 16S rRNA PCR products then underwent restriction fragment length polymorphism enzymatic reactions using the restriction enzymes HinP1I (New England BioLabs) and MspI (New England BioLabs). HinP1I has a cut site of G/CGC and MspI has a cut site of C/CGG. The enzymatic reaction protocol from New England BioLabs was as follows: 5µl 10X rCutSmart Buffer, 2µl DNA, 1µl HinP1I, 1µl MspI, and 41µl of molecular grade water for a total reaction volume of 50µl. Enzymes were kept on a cold block and added to the reaction last. The reactions were incubated at 37°C for 15 minutes.

RFLP single colony enzyme reactions were then run on a 2% lithium borate agarose gel at 250 volts for approximately 35 minutes using the GeneRuler 100bp ladder, then viewed with the Bio-rad Gel Doc. Banding patterns were analyzed to assess diversity within and among each sample. Single colonies that had unique DNA banding pattern fingerprints were chosen and gDNA was extracted from the quarter lawns using the MilliporeSigma GenElute™ Bacterial Genomic DNA Kit following the Gram-positive bacterial preparation protocol.

After extraction, the single colony gDNA samples were quantified using the Qubit 4.0 as well as through a 0.7% lithium borate quantification gel. The samples were then combined with Tris-EDTA to reach a concentration of 1ng per 100µl. The samples were whole genome sequenced using the Illumina MiSeq Reagent Kit v2 (500 cycles). Genomes were assembled with SPAdes v3.15.5 (PMID:22506599). 16S rRNA gene sequences were extracted from genome assemblies with barrnap v0.9 (https://github.com/tseemann/barrnap) and BLASTn was used to determine complete identity.

Results

Antimicrobial Susceptibility Plate Testing

Viable bacteria were successfully cultured from the wastewater and sludge samples, allowing for antimicrobial susceptibility testing to be performed. Of the 359 wastewater and sludge samples, 95 of those samples were deemed resistant to meropenem at 2μg/ml: 40 were influent municipal wastewater, 5 were effluent municipal wastewater, and 50 were collected at congregate living sites. No sludge samples exhibited resistance to meropenem at 2μg/ml and therefore were not tested at higher concentrations of meropenem. The 95 samples were moved forward and tested at 4μg/ml of meropenem; 48 samples (34 influent, 5 effluent, 9 congregate living) still showed resistance at 4μg/ml. These samples were moved forward to meropenem susceptibility testing with meropenem Etest strips.

Meropenem Susceptibility Testing using Etest Strips

24 of the 48 wastewater samples that were tested for meropenem susceptibility with Etest strips had MICs of greater than or equal to 4μg/ml, which was the threshold set for resistance in this project. Three of the 24 resistant samples were effluent and were all collected from the same municipal WWTP in the Southwestern U.S.. The remaining 24 samples exhibited mixed MICs, meaning that there were several zones of inhibition observed on the agar plate. Fifteen samples had susceptible and resistant MICs within one sample. Six samples had both intermediate and resistant MICs. Three samples had susceptible, intermediate, and resistant MICs recorded within a single sample. Figure 2 shows images of the multiple zones of inhibition observed. All recorded MICs can be viewed in Table 3.

16S rRNA gene Sequencing

The 16S rRNA gene sequencing provided family and genus level resolution of the bacteria present in both the raw wastewater samples and the wastewater samples that were exposed to 2µg/ml, 4µg/ml, and Etest strips of meropenem. The V4 region of the 16S rRNA gene is slow changing and highly conserved across bacterial species, therefore sequences could only be classified as much as the sequence allows. Some samples could not be classified down to genus and resulted in only family classification. A feature table and taxonomy bar plot were generated through Qiime2 and used to assess the microbial diversity in the wastewater (Figure5). The Shannon diversity index as well as the unweighted UniFrac distances were also generated from the 16S rRNA gene sequencing data (Table 4, Figure 3). These data show that as the concentration of meropenem increased, microbial diversity within the sample decreased. Vast microbial diversity was observed in all raw wastewater samples, with the raw samples containing over 100 genera within each sample identified through the 16S rRNA gene sequencing. Raw wastewater samples also had the highest Shannon diversity indices, meaning that these samples had the highest species diversity. In contrast, the samples grown at 4µg/ml meropenem had a range of 1-13 genera identified within them and the lowest Shannon diversity indices, indicating less species diversity within the sample. Notable genera that were identified though 16S rRNA gene sequencing include *Stenotrophomonas, Pseudomonas, Acinetobacter, Clostridium, Dysgonomonas,* and *Enterococcus.* The *Enterobacteriaceae* family was also prevalent in the samples exposed to 2µg/ml, 4µg/ml, and Etest strips. *Stenotrophomonas* was one of the most dominant genera in the samples grown in 4µg/ml of meropenem, with 10 of the 19 samples containing 50% or greater *Stenotrophomonas. Stenotrophomonas* did not become the dominant genus until it was present in higher levels of meropenem, as it was only present in 5 samples gown at 2µg/ml.

RFLP Single Colony Whole Genome Sequencing

Adequate growth was seen on all six isolation streaks grown on 32µg/ml meropenem LB agar plates. No growth was observed on the BSL1 *E. coli* plate or on the blank plate. Ten single colonies were successfully isolated from each sample. Two distinct colony morphologies were observed while selecting single colonies. The morphologies consisted of small clear colonies and small cream-colored mucoid colonies. These differences in morphologies were also seen in the single colony quarter lawns. This can be observed in Figure 5.

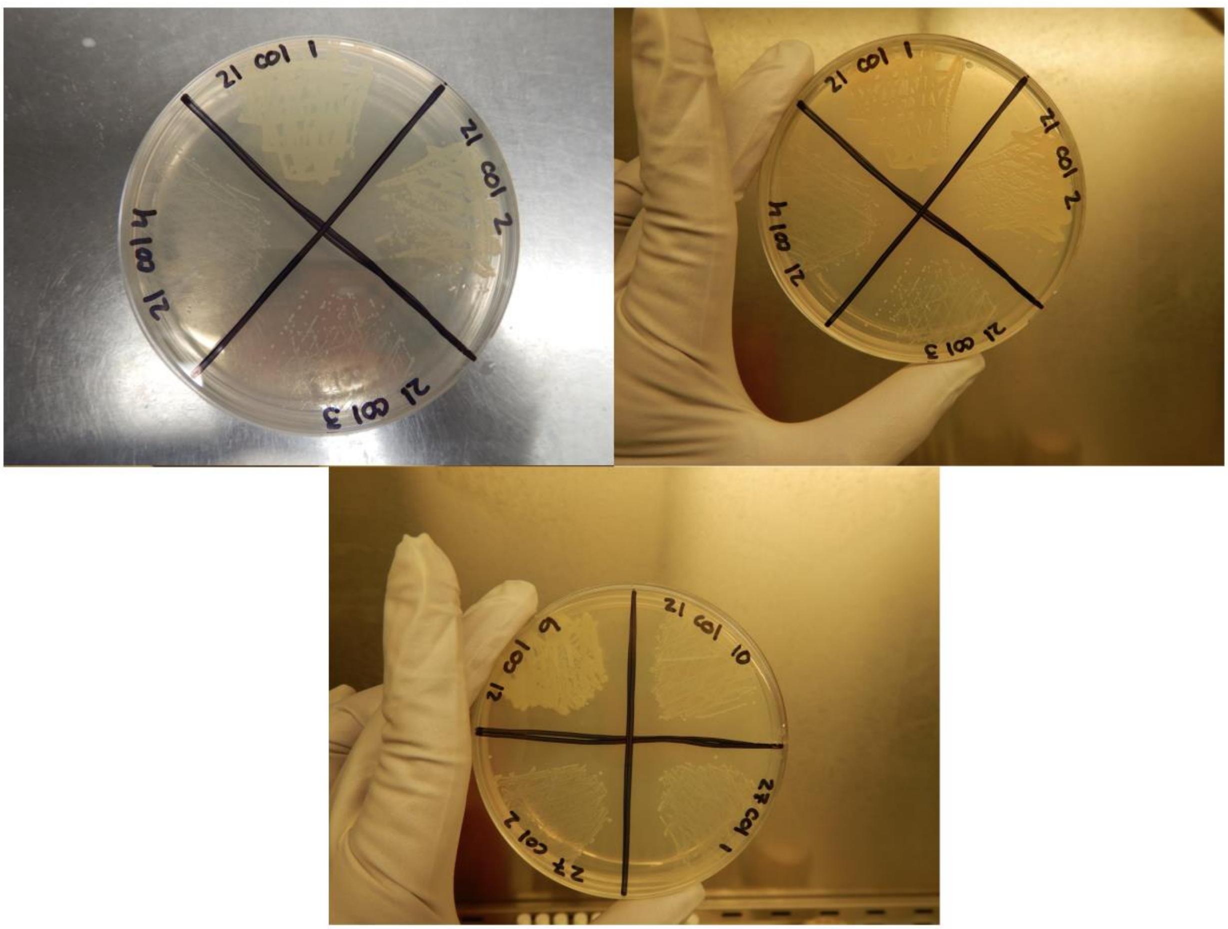


Figure 5. RFLP Single Colony Quarter Lawns
Images of the two different colony morphologies observed from RFLP single colony lawns.

All 60 single colony heat soaks amplified in the 16S rRNA 8F/1492R assay. After the 16S PCR product underwent the RFLP enzymatic reaction and was visualized, four samples had the same banding pattern across all 10 single colonies. Two samples had two different banding patterns observed across the 10 single colonies which can be seen in Figure 6. For the six samples that did not have more than one banding pattern, one colony was chosen and extracted. For the two samples that had unique banding patterns within the 10 colonies, two colonies that produced different banding patterns were chosen for each sample and extracted as well. A total of eight colonies were extracted.

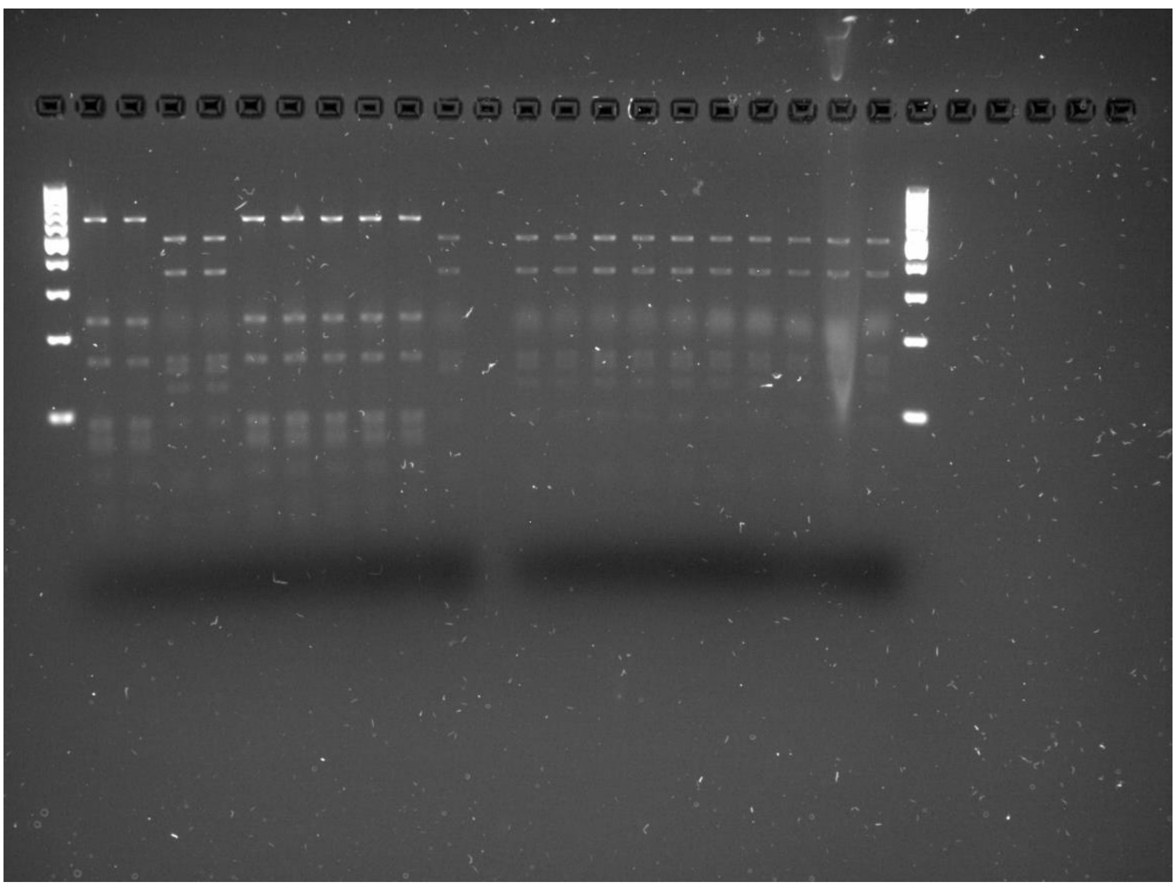


Figure 6. RFLP Banding Patterns – Gel Picture
Two different banding patterns within a single sample after performing single colony RFLP enzymatic reactions.

All eight colonies were whole genome sequenced, and the analysis of these sequences resulted in a diverse mixture of species within each colony. All colonies contained three to six different species that were identified after the extracted 16S rRNA gene sequences were aligned with BLASTn. In the first sample that had two banding patterns observed across the 10 single colonies, *S. maltophilia* was identified in both colony 1 and colony 3. In the second sample that had two banding patterns, both colonies contained *S. maltophilia* These observations can be viewed in Table 5.

Table 5. RFLP WGS Organisms

Sample	RFLP WGS Identification
Inf-7-30-2020-Colony-3	*Enterococcus faecium, Stenotrophomonas maltophilia, Staphylococcus petrasii, Staphylococcus epidermidis, Staphylococcus aureus*
Inf-8-23-24-2021-Colony-5	*Stenotrophomonas maltophilia, Enterococcus faecium, Escherichia coli, Staphylococcus epidermidis, Staphylococcus aureus*
Eff-5-14-2021-Colony-1	*Stenotrophomonas maltophilia, Staphylococcus epidermidis, Enterococcus faecium, Escherichia coli*
Eff-5-14-2021-Colony-3	*Stenotrophomonas maltophilia, Staphylococcus epidermidis, Enterococcus faecium*
Inf-7-3-2020-Colony-3	*Enterococcus faecium, Escherichia coli, Staphylococcus epidermidis*
Inf-4-9-2020-Colony-7	*Stenotrophomonas maltophilia, Escherichia coli, Staphylococcus epidermidis, Enterococcus faecium, Shigella flexneri*
Inf-9-15-2021-Colony-6	*Stenotrophomonas maltophilia, Enterococcus faecalis, Staphylococcus epidermidis, Escherichia coli*
Inf-9-15-2021-Colony-7	*Stenotrophomonas maltophilia, Staphylococcus epidermidis, Escherichia coli, Enterococcus gallinarum*

A single *Stenotrophomonas maltophilia* genome isolated from effluent sample Eff-5-14-2021 from the whole genome sequencing was annotated with Bakta and multiple genes associated with carbapenem resistance were identified (Schwengers et al., 2021). These include multidrug efflux ABC transporter SmrA, multidrug efflux system transcriptional regulator EmrR, multidrug efflux transporter outer membrane subunit EmrC, multidrug efflux MFS transporter periplasmic adaptor subunit EmrA, multidrug efflux MFS transporter permease subunit EmrB, Multidrug efflux pump subunit AcrA, ABC-type multidrug transport system, multidrug efflux RND transporter permease subunit, Multidrug efflux pump subunit AcrA (membrane-fusion protein), multidrug ABC transporter ATP-binding protein, multidrug transporter AcrB, beta-lactamases, and metallo-beta-lactamases.

Discussion

Antimicrobial resistance is a complex and significant threat that requires greater innovation and investment to detect, respond, and prevent resistance. The global spread of multi- and pan-resistant bacteria continues to threaten our ability to treat infections. In the United States, The National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) monitors antimicrobial resistance among enteric bacteria from humans, retail meats, and food animals through collaborations with the Centers for Disease Control and Prevention, US Food and Drug Administration, and the US Department of Agriculture (*National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) | NARMS | CDC*, 2023). Comparable environmental monitoring of AMR is lacking, but the recent establishment of wastewater surveillance for the SARS-CoV-2 virus provides an impetus for analogous AMR monitoring (Pruden et al., 2021). The CDC is now planning to use the NWSS developed for the SARS-CoV-2 virus as a valuable tool to also track antibiotic resistance genes across the country, giving insight to how the environment, people, and animals are exposed to and contributing to their emergence and spread (*Understanding Antibiotic Resistance in Water*, 2022). However, unlike the SARS-CoV-2 virus, there are many species of antimicrobial resistant bacteria of concern, making tracking the dissemination of AMR daunting because there is no single agent responsible.

In these experiments, both influent and treated effluent Southwestern U.S. wastewater along with congregate living wastewater were tested for resistance to the last resort drug meropenem. It was discovered that viable meropenem resistant bacteria were present in both influent and treated effluent wastewater. An increasing number of studies have also identified meropenem and other carbapenem resistant bacteria in water sources (Oliveira et al., 2021) (Mathys et al., 2019) (Oliveira et al., 2020) (Beattie et al., 2020) (Hwang & Kim, 2018) (Masuda & Ohya, 1992) (Yasmin et al., 2022). The viable resistant bacteria discovered in the experiments discussed in this chapter as well as other published studies highlights the need to incorporate a One Health approach to antimicrobial resistance monitoring and surveillance, specifically looking at the role water plays as a reservoir and source for the dissemination of antimicrobial resistance into the environment.

16S rRNA gene sequencing analysis of the wastewater samples cultured at increasing concentrations of meropenem allowed for the observation of active selection of AMR bacteria. The difference in microbial diversity from raw wastewater, wastewater cultured at 2μg/ml of meropenem, and wastewater cultured at 4μg/ml of meropenem was very apparent when looking at the 16S rRNA gene sequencing taxonomy bar chart and the Shannon diversity indices (Figure 4, Table 4). This was to be expected, as selection for AMR can occur when the bacteria are exposed to a single antimicrobial agent, even at very low concentrations (Gullberg et al., 2011).

Across the six samples that had both 16S rRNA and single colony whole genome sequencing data, the results were almost completely in agreement for genus identification. A sample was identified in the 16S data that was 100% *Stenotrophomonas* at 4μg/ml and a mixture of 4.39% *Enterococcus*/95.48% *Stenotrophomonas* grown with the Etest strip. When this sample's WGS data was analyzed, it was 78% dominant for *Enterococcus faecium*. These slight differences in percent identity are likely due to the difference in media used in each experiment. Cultures for the 16S rRNA sequencing and Etesting were grown with Mueller Hinton II cation adjusted media. WGS single colonies were grown on LB agar plates with 32μg/ml meropenem. The single colony work performed for WGS could also have allowed for isolation of mixtures within the sample.

Using both 16S rRNA gene sequencing and RFLP single colony whole genome sequencing, the identity of the meropenem resistant bacteria were identified to be relevant human pathogens. Despite the attempt to isolate single colonies for the RFLP experiment, mixtures of several species were identified through single colony WGS. Many of the same species were identified across all colonies sequenced, including *Stenotrophomonas maltophilia, Enterococcus faecium, Staphylococcus epidermidis,* and *Escherichia coli.* One possibility for why several species were identified in what was thought to be an isolated single colony is that these resistant bacterial species co-colonize together in wastewater. Co-colonization processes appear in many diverse ecological communities, from plant and marine ecosystems to infectious diseases (Madec & Gjini, 2020). Additional isolation and sequencing would be needed to further explore co-colonization of meropenem resistant bacteria in wastewater,

Stenotrophomonas maltophilia, one of the most abundant AMR species identified in these wastewater samples, was of great interest. *S. maltophilia* is an emerging multidrug-resistant global opportunistic pathogen (Brooke, 2012). *S. maltophilia* is an environmental bacterium found in aqueous habitats that is causing an increasing incidence of nosocomial and community-acquired infections, most commonly associated with respiratory infections in humans (Brooke, 2012). The presence of highly meropenem resistant (greater than or equal to 32μg/ml) *Stenotrophomonas* in effluent water is a concerning finding as it is an emerging opportunistic pathogen and has the potential to cause infection.

Annotation of a *S. maltophilia* genome that was identified from an effluent wastewater sample revealed several genes that are associated with carbapenem resistance. *S. maltophilia* has been classified as intrinsically resistant, containing numerous antibiotic resistance mechanisms. The best characterized mechanisms include several multidrug efflux pumps, beta-lactamases and aminoglycoside-inactivating enzymes (Sanchez et al., 2009). The identification of the SmrA efflux transporter, EmrR efflux pump, EmrB efflux pump, AcrA efflux pump, AcrB efflux pump, multidrug ABC transporter, RND efflux pump, beta-lactamases, and metallo-beta-lactamases expressed in the genome explains the resistance observed to high concentrations of meropenem.

Etest analyses resulted in wastewater samples that did not have uniform MICs within them. 21 samples had two zones of inhibition and 3 samples had three zones of inhibition. This variation in resistance within one sample cannot necessarily be classified as heteroresistance, because it was unknown if the varying MICs came from a subpopulation of a single species or if it was multiple bacterial species. The multiple MICs within a single sample was expected because the samples were not passaged to isolate single species from the diverse mixture within the wastewater prior to Etest analyses. Due to the large number of genera identified in the wastewater samples through 16S rRNA sequencing, the samples could not be isolated to a single species for Etest analyses as time and resources for this project were limited. The observed multiple zones of inhibition in the Etest results suggest that there was a mixture of species with different MICs within a single sample. The mixtures observed from Etest analyses were supported with the 16S rRNA data, as there were multiple genera identified within the Etest samples. The recorded MICs were used to determine which samples were highly resistant to meropenem and should be further interrogated through RFLP singly colony WGS.

CONCLUSION

Wastewater treatment and effluent reuse has become an incredibly important resource for supplementing dwindling freshwater supplies. Drought in the Southwestern United States has led to unprecedented water shortages, threatening both drinking and agricultural water supplies. To combat this drought, wastewater reclamation will have to be addressed. Continued wastewater testing is required to ensure optimal treatment for managing the risk from pathogens in wastewater. The current fecal coliform counting methods for wastewater effluent standards are outdated and do not provide enough information to ensure safe water reuse. Through the development of molecular assays and amplicon sequencing, significant human pathogens were detected in both influent and effluent Southwestern U.S. wastewater samples. Culturing and antimicrobial susceptibility testing allowed for the identification of viable meropenem resistant bacterial pathogens in both the influent and effluent wastewater samples. Viable human pathogens in effluent wastewater that are also resistant to the last resort drug meropenem is a significant public health concern.

Future studies that could come from the work conducted in this thesis include further isolation of viable bacterial species to determine co-colonization and the role this might play in AMR. Determining the resistance mechanisms that these species are using to be highly resistant to meropenem would allow for the understanding of possible transfer of ARGs that confer resistance to meropenem. Lastly, designing additional assays to screen for other targets of interest in wastewater would expand the knowledge of human pathogens circulating in the community.

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