Assessing the Efficacy of Selected Wastewater Treatment Technologies to Reduce Dissemination of Environmental Antibiotic Resistance

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Abstract

The widespread occurrence and proliferation of antibiotic resistant bacteria (ARBs) continues to be a global concern. Wastewater treatment plants (WWTPs) are known to be hotspots for ARBs and antibiotic resistant genes (ARGs) because of high nutrient concentrations, presence of antibiotics that promote selective pressure, and a plethora of diverse bacteria populations, which can promote dissemination of ARG via horizontal gene transfer. WWTPs can potentially discharge ARBs and ARGs through aqueous and solid effluents, which raises concern about the role WWTPs play in disseminating ARBs and ARGs into the natural environment.

This dissertation assesses the behavior and fate of a model ARB, carbapenem-resistant *Enterobacterales*, and its corresponding ARG, bla_{KPC}, as they flow from a hospital via wastewater into a typical municipal WWTP and, from there, into the natural environment via its receiving waters. This research also examines the efficacy of selected conventional WWTP technologies in deactivating the model ARB and ARG via laboratory-scale experiments of chlorination and ultraviolet (UV) radiation using traditional methods used in previous literature studies. This dissertation then describes an effects-based assay to assess the potential of gene transfer from treated wastewater effluents to microbial communities in the downstream receiving water. The results of this dissertation contribute to the knowledge of the role of WWTPs in dissemination of antibiotic resistance. They also highlight the usefulness of microcosm experiments as a novel, effects-based assay for evaluating the potential for antimicrobial dissemination from WWTPs, which has not yet been well-described in existing literature.

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Definition of Acronyms

ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistant Gene
bla_{KPC}	*Klebsiella pneumoniae* carbapenemase gene
CFU	Colony Forming Units
CRE	Carbapenem-resistant *Enterobacterales*
HAI	Healthcare-associated infections
HGT	Horizontal gene transfer
KPC	*Klebsiella pneumoniae* carbapenemase
KPCO	*Klebsiella pneumoniae* carbapenemase-producing organisms
UV	Ultraviolet
WW	Wastewater
WWTP	Wastewater Treatment Plant

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PreDis_effluent = Pre-Disinfection Effluent, Upstream_Sed = Upstream Sediment, Downstream_Sed = Downstream Sediment.

Figure 3-5. Quantitative prevalence of bla_{KPC} and 16S RNA abundances for sampled locations along the hospital sewershed. The inset shows the solids stream. Samples with an asterisk(*) were only sampled on one occasion (Oct. 2020). The rest of the samples are averages from 3 sampling dates.

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Figure 4-1. Quantitative ARB and ARG at varying chlorination (left panel) and UV doses (right panel) taken from Stange et al., 2019. For both chlorination and UV radiation, the ARBs, *E. coli* (top left and right) and *E. faecium* (bottom left and right), were inactivated at lower doses than those needed to decrease the ARG copy numbers. There was negligible decrease in ARG copy numbers using UV radiation, but up to a 0.8-2.8 log reduction of ARG copy numbers for chlorination at 0.5 mg/L free chlorine dose. The doses in this study, 0-30 mg*min/L for chlorination and 0-60 mJ/cm^2 for UV, are relatively similar to the doses that will be used in our study, 0-60 mg*min/L free chlorine and 0-40 mJ/cm^2.

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Related Publications, Presentations, and Awards

Publications

1) **Loudermilk, E**., Kotay, S., Barry, K., Colosi, L., Mathers, A. Evaluating the Efficacy of Wastewater Disinfection on the Proliferation of Klebsiella pneumoniae carbapenemase (in preparation)
2) **Loudermilk, E.,** Kotay, S., Barry, K., Parikh, H., Colosi, L., Mathers, A. (2022). Tracking Klebsiella pneumoniae carbapenemase gene as an indicator of antimicrobial resistance dissemination from a hospital to surface water via a municipal wastewater treatment plant. *Water Research, 213*, 118151.
3) Singh, U., **Loudermilk, E.**, M., & Colosi, L. M. (2021). Accounting for the role of transport and storage infrastructure costs in carbon negative bioenergy deployment. *Greenhouse Gases: Science and Technology*, *11(1)*, 144-164.
4) Grimes, K.; Dunphy, L.; **Loudermilk, E.**; Melara, A.; Kolling, G.; Papin, J.; Colosi, L. (2019) Evaluating the efficacy of an algae-based treatment to mitigate elicitation of antibiotic resistance. *Chemosphere*, 124421.

Presentations

1) **Loudermilk, E**., Kotay, S., Barry, K., Parikh, H., Mathers, A., Colosi, L. *Fate of Nosocomial Resistant Klebsiella pneumoniae producing Enterobacterales in a Wastewater Treatment Plant and Receiving Waters.* Chesapeake Potomac Regional Chapter of the Society of Environmental Toxicology and Chemistry (CPRC-SETAC) Spring Conference. Virtual. April 2021.
2) **Loudermilk, E.;** Kotay, S.; Mathers, A.; Colosi, L. *Mitigating Antibiotic Resistance in Environmental Contexts.* Invited Speaker. Engineering Systems and Environment Department Seminar. Charlottesville, VA, February 2020.
3) **Loudermilk, E.**; Grimes, K.; Dunphy, L.; Melara, J.; Papin, J.; Colosi, L. *Using Adaptive Laboratory Evolution to Quantify Antibiotic Resistance Potential in Wastewater Treatment Plant Effluents.* CPRC-SETAC Spring Meeting. Caanan Valley, WV, April 2019.

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Chapter 1: Introduction and Background

1.1 Background and Motivation

1.1.1 Overview and background of Antibiotic Resistance

Antibiotic resistance has become a pressing human health concern over the last several decades with the continued widespread use of antibiotics, which leads to the evolution of antibiotic resistant bacteria (ARBs). ARBs are of serious concern because they can infect humans and animals with life-threatening infections that are very difficult to treat. It is estimated that over 35,000 deaths occur in the United States every year as a result of infections caused by ARBs (CDC, 2019). Antibiotic resistant genes (ARGs) can spread vertically through cell division and replication, transferring resistance from one organism to their offspring. ARGs can also spread horizontally via transfer of mobile genetic elements from one cell to another within the same generation (i.e., from a host cell to other cells that are not its direct offspring). The recipient cells can be of a different bacterial species, and can include pathogenic and non-pathogenic bacteria. This mechanism of ARG transmission is of particular concern insofar as it could contribute to very rapid, widespread resistance to clinically relevant drugs.

1.1.2 Wastewater treatment plants as a reservoir for antibiotic resistance

Antibiotics are essential for medical and veterinary purposes, but it is estimated that over 30% of antibiotics prescribed in the U.S. are unnecessary (Center for Disease Control and Prevention, 2018). Many antibiotics are not well metabolized by the human body and are becoming increasingly more prevalent in high concentrations in wastewater (WW) and in downstream receiving waters (Karkman et al., 2018; Noguera-Oviedo & Aga, 2016; Wang et al., 2020).

Wastewater treatment plants (WWTPs) have been identified as hotspots for ARBs and ARGs (Rizzo et al., 2013) and may even lead to increased concentrations of ARBs and ARGs through preferential selection and horizontal transfer of antibiotic resistance features (Czekalski et al., 2012; Luo et al., 2014). Thus, WWTPs are important reservoirs of antibiotics, ARBs, and ARGs that have the potential to spread antimicrobial resistance into the natural environment. Dissemination of ARBs and ARGs into the environment is of concern for several reasons, one of the largest being the potential spread of ARGs to a wide variety of bacterial hosts, both pathogenic and non-pathogenic, leading to large community resistance to classes of antibiotics. Many clinically relevant ARGs are thought to have originated or spread from non-pathogenic bacteria in environmental settings, such as rivers, raising the concern about environmental reservoirs as a pathway for widespread antibiotic resistance (Cantón & Coque, 2006; Finley et al., 2013; Poirel et al., 2005). Human exposure to antibiotic-resistant bacteria in environmental settings is also possible, resulting in serious infections, which has been documented previously (Europe, 2011; Laurens et al., 2018). WWTPs make use of multiple processes (biological, mechanical, chemical, physical, etc.) to treat wastewater, which have different impacts on ARBs and ARGs. Notably, WWTPs are primarily designed to reduce concentrations of nitrogen, phosphorus, dissolved organic carbon, and pathogenic bacteria, but they are not designed to remove antibiotics, ARBs, or ARGs in their aqueous discharge or dewatered biosolids, and these constituents are currently unregulated. The last step in WWTPs is typically disinfection, which aims to inactivate bacteria prior to discharge from the plant. Studies have shown that conventional WWTPs are ineffective in completely removing antibiotics (Noguera-Oviedo & Aga, 2016) as well as ARBs (Czekalski et al., 2014; Michael et al., 2013; Rizzo et al., 2013), leading to concern over their potential discharge into receiving waters and further dissemination

of antibiotic resistance in the environment. This also makes WWTPs an interesting node in a part of a larger pathway for antibiotics, ARBs, and ARGs to enter the environment (Figure 1-1). WWTPs are contained system boundaries that have the potential through engineering advancements and technologies to play a critical part in halting the spread of antibiotic resistance by removing these antibiotic resistance constituents prior to discharge.

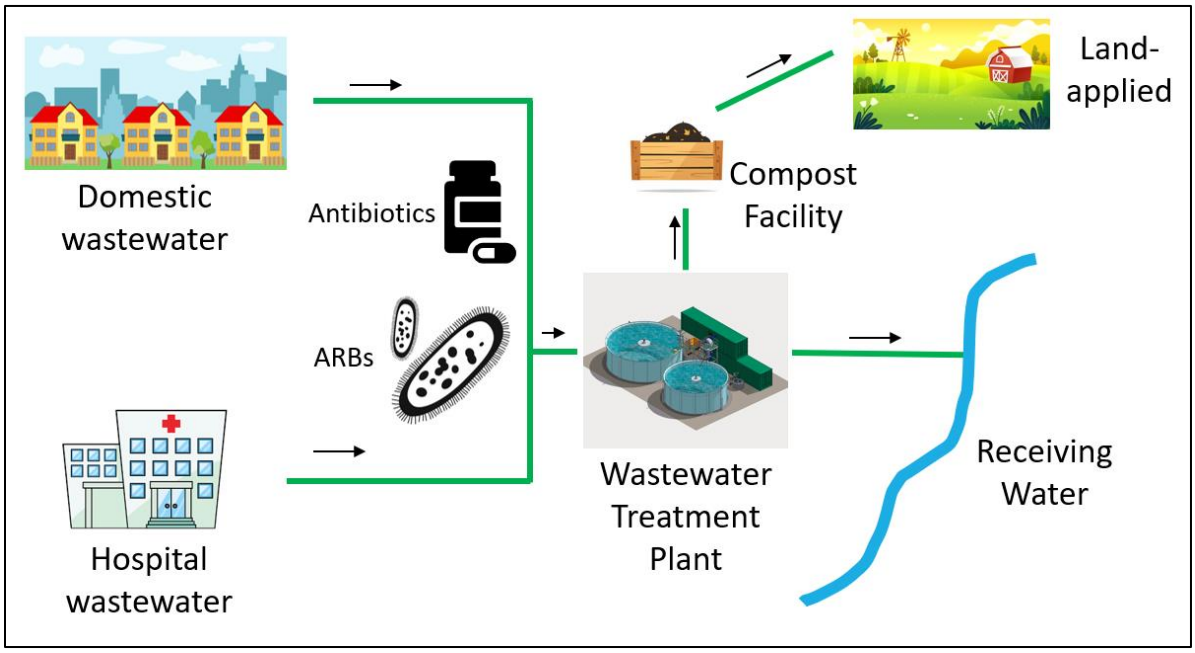

Figure 1-1. Flow pathway for antibiotic resistance to spread into various environmental communitities following discahrge from WWTPs. Antibiotic resistant bacteria and antibiotics enter WWTPs, and can then be possibly discharged through final effluent into a receiving body of water or land-applied through compost made from wastewater biosolids.

1.1.3 Carbapenem-resistant Enterobacterales as a model ARB?

In the last twenty years, there has been rising concern over the spread of Carbapenem-resistant *Enterobacterales* (CREs), a class of gram-negative bacteria that produce carbapenemases, which are β-lactamases that hydrolyze the carbapenem antibiotics. Carbapenems are highly effective antibiotics often reserved for use in serious multi-drug

resistant (MDR) bacterial infections caused by extended-spectrum beta-lactamase-producing *Enterobacterales* (Pitout & Laupland, 2008). The mortality rate is very high among patients acquiring infections with CREs (Bratu et al., 2005; Cassini et al., 2019; Patel et al., 2008). In 2017, the World Health Organization identified CREs as the most urgent priority for antibiotic resistance (WHO, 2017) and there were an estimated 13,100 hospital infections and 1,100 deaths due to CREs in the United States alone (CDC, 2019). Carbapenemase genes are almost exclusively carried on large conjugative plasmids that can be horizontally transferred to other bacterial cells. Carbapenemase genes are carried on plasmids, such as bla_{KPC} and bla_{NDM} (Bonomo et al., 2018).

The most concerning carbapenemase gene in the United State is the *Klebsiella pneumoniae* carbapenemase (bla_{KPC}), which was first discovered in 1996 on a *Klebsiella pneumoniae* strain in North Carolina and became endemic throughout hospitals in New York city in the early 2000s (Yigit et al., 2001). From 2000-2010, KPC-producing organizations (KPCO) spread throughout hospitals in the continental U.S. (Codjoe & Donkor, 2017; Munoz-Price et al., 2013). Fortunately, KPCO have been on the decline in the U.S. in the last several years and have not been found extensively in the U.S. outside of clinical settings (CDC, 2019). Their limited occurrence outside of clinical settings, in addition to the importance of limiting carbapenem resistance due to severe adverse health outcomes for contracting CRE infections, make it particularly advantageous from a research perspective as an ideal model ARB for characterizing wastewater-based dissemination of antibiotic resistance. In this dissertation, KPCO and bla_{KPC} were used as our model ARBs and ARG to better understand the impacts of WWTPs as an important reservoir for antibiotic resistance and as a location for engineering intervention methods to mediate the potential dissemination of antibiotic resistance to the larger community.

Chapter 2: Research Hypotheses and Objectives

The goal of my dissertation is to characterize the spread of antibiotic resistance spread from a hospital into the natural environment and to assess to what extent conventional wastewater treatment technologies are effective in mitigating the dissemination of ARBs and ARGs. The first step is to characterize the flow of ARBs and ARGS from a point source location, a hospital, through the different compartments of a WWTP, and into the natural environment through the aqueous effluent (downstream receiving water) and solids effluent (land-application of biosolids). The second step is to analyze how conventional WWTP technologies fair in removing and inactivating ARBs using the traditional method by calculating concentrations of ARB before and after disinfection. The final step is to develop and expand upon a potentially more informative method for determining the potential for antibiotic resistance dissemination through a novel effects-based assay of transconjugation microcosm experiments.

Objective 1) Characterizing the occurrence and fate of a model ARB as it flows from hospital to natural environment

Objective 2) Evaluating the efficacy of selected conventional wastewater disinfection technologies for inactivation of a model ARB

Objective 3) Characterizing dissemination of wastewater-based antimicrobial resistance to representative downstream microbial communities

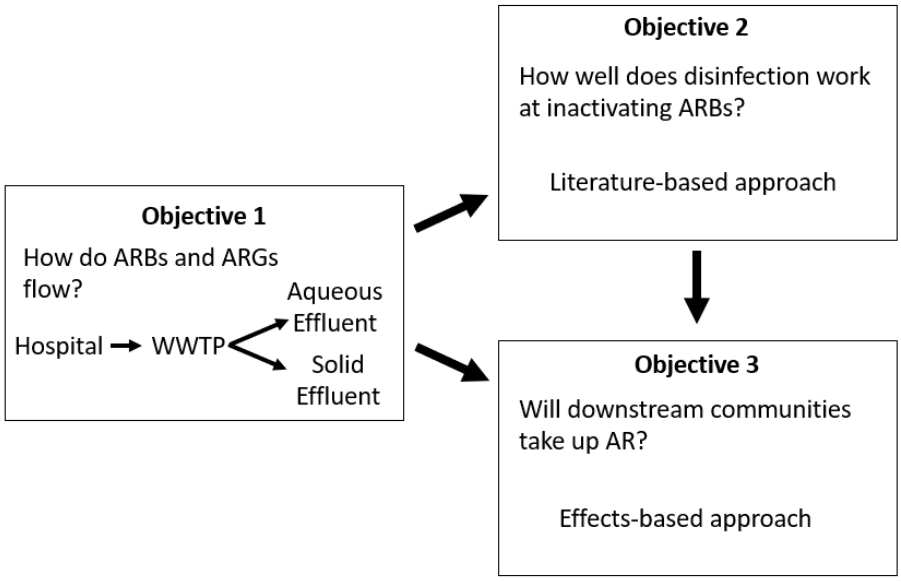

Figure 2-1. The overall framework for the dissertation with Objectives 1, 2 and 3 having interconnected goals.

Chapter 3: Objective 1 Characterizing the occurrence and fate of a model ARB as it flows from hospital to natural environment

The goal of Objective 1 was to characterize the flow and fate of ARBs and ARGs as they move throughout an integrated wastewater management system and, subsequently, into the natural environment. This was done by quantifying our model ARBs, Carbapenem-resistant *Enterobacterales* (CREs), in each section of the wastewater management system from the source, the UVA hospital, through each compartment of the WWTP, to the downstream receiving waters and sediments and the wastewater biosolids that are land-applied after composting. The work in Objective 1 was published in *Water Research* in April 2022 (Loudermilk et. al., 2022).

3.1 Background

Carbapenem-resistant *Enterobacterales* (CREs) are a subset of ARBs that are considered by the U.S. Center for Disease Control (CDC) to be one of the most urgent threats to human health (Lledo et al., 2009). The presence of CREs in community or environmental settings is of concern because it has been documented that exposure to CREs (e.g., via recreational contact or other means) can lead to life-threatening infections in humans (Laurens et al., 2018).

Hospitals are the main source of ARBs and other antibiotic resistance features entering WWTPs and this is especially true for CREs in the U.S., as carbapenem antibiotics are not typically used outside of hospital settings (CDC, 2019). UVA hospital, the source hospital for the model WWTP for Objective 1, has a documented history of CRE infections in patients, but also

CREs persisting in the biofilms of hospital plumbing (Mathers et al., 2018). Therefore, it is expected that our WWTP will be receiving influxes of CREs from the UVA hospital that may be proliferating in the WWTP itself.

It has been well-documented that WWTPs do not completely remove antibiotics, ARBs, or ARGs (Czekalski et al., 2012; McKinney & Pruden, 2012; Munir, Wong, & Xagoraraki, 2011; Noguera-Oviedo & Aga, 2016; Rizzo et al., 2013; Stange et al., 2019; Yuan, Guo, & Yang, 2015). Previous studies have even shown that WWTPs provide suitable environments, with large quantities and diversity of bacteria passing through daily with the elevated concentrations of antibiotics for selective pressure, for the growth and spread of antibiotic resistance through horizontal gene transfer and conjugation (Li et al., 2010). CREs have been extensively found in the receiving waters of other countries of the world (Caltagirone et al., 2017; Kittinger et al., 2016; Poirel et al., 2012; Yang et al., 2017). In the United States, Mathys et al. sampled 50 WWTPs and found that 15 of them, or 30%, yielded carbapenemase-producing bacteria (Mathys et al., 2019). They also reported that WWTPs utilizing ultraviolet (UV) radiation had lower rates of carbapenemase-producing bacteria (12%) compared to WWTPs utilizing chlorination (42%). The goal of Objective 1 was to characterize the flow and fate of KPCO and bla_{KPC} as they move throughout an integrated wastewater management system and, subsequently, into the natural environment in order to better understand the fate and behavior of ARBs and ARGs in the wastewater treatment process and identify potential areas for engineering intervention.

3.2 Methodology

3.2.1 Sampling Locations

Wastewater samples were collected from three different locations: 1) UVA hospital in Charlottesville, VA, 2) UVA residential dorm buildings and an academic building, together constituting our non-clinical "WW Control", 3) the Moores Creek Wastewater Treatment Plant operated by the Rivanna Water and Sewer Authority in Charlottesville, VA (Fig. 1). Within the WWTP, samples were taken from the following locations: i) raw influent, ii) secondary aeration basin, iii) digester influent, iv) digester effluent (prior to dewatering), v) pre-disinfection effluent, and vi) final effluent. Water and sediment samples were also collected on the same day from upstream and downstream locations in the WWTP's receiving water. Water samples were collected from five upstream locations (120-160m from the discharge point) and five downstream locations (30-70m from the discharge point). At each of the water sampling locations, sediment samples were also collected using a core sampler from the top 5 cm of the sediment layer. All samples were taken during three major sampling dates during September 2019, December 2019, and October 2020. Two smaller sampling dates during June 2019 and July 2020 only had a few of the samples taken. Two final product compost samples were collected from the McGill Composting Facility in Waverly, VA in December 2020. This facility accepts digested biosolids from the Moores Creek WWTP and other municipal WWTPs. One of the compost product samples was made using wastewater biosolids among its feedstocks. The other compost product sample was made from non-wastewater feedstocks. All samples were stored at 4°C for analysis within 24 hours of collection.

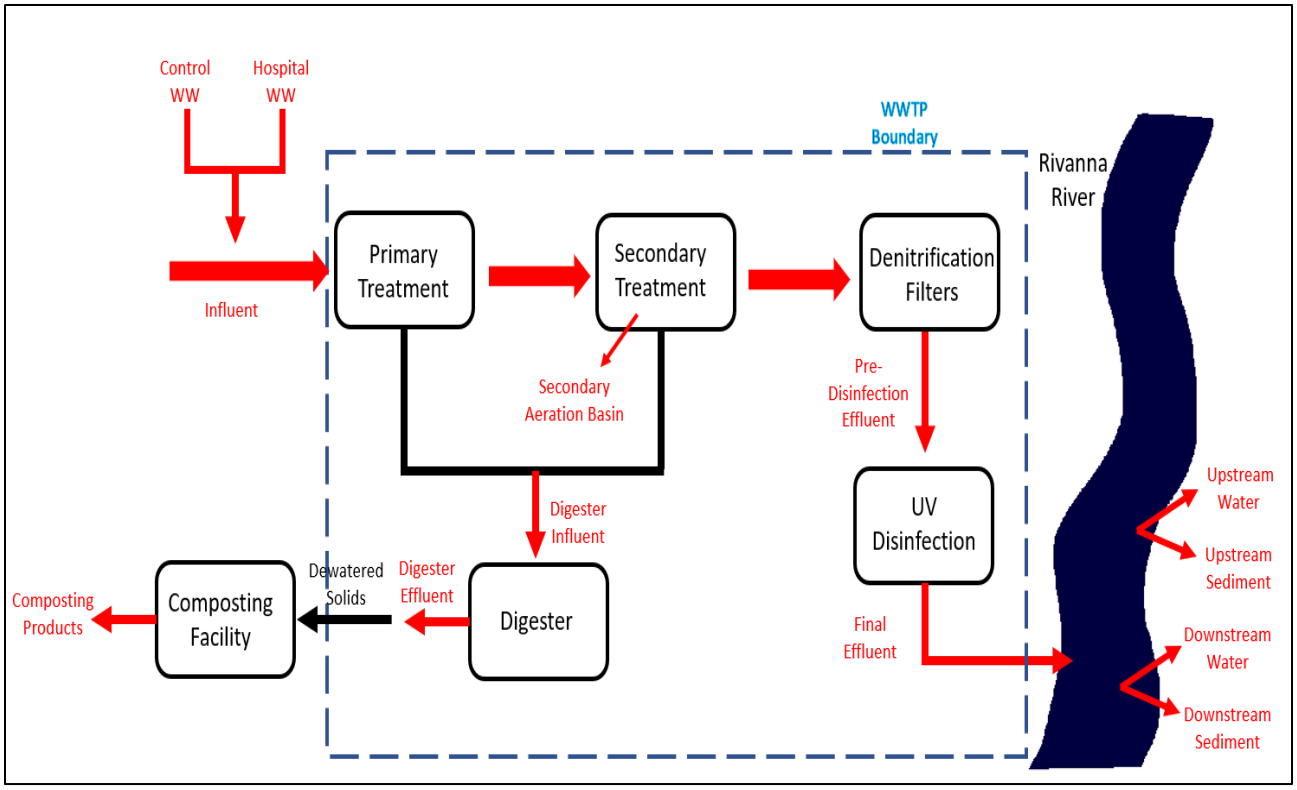

Figure 3-1. Schematic of sampling locations. Red arrows indicate locations that were sampled.

3.2.2 Microbial Analyses

Quantitative culturing was done by pipetting 1 mL of sample and serially diluting 1:10 six times with sterile water. Using a sterile pipette, 10 µL of each of the six dilutions for each sample were streaked on to both a MacConkey agar plate, which is selective from Gram-negative bacteria, and a ChromAgar plate, which is selective for carbapenem resistant bacteria and allows for *Enterobacterales* and *Aeromonas* to grow as pigmented colonies. Colonies were incubated for 24 hours at 37°C. After incubation, the mean number of colony forming units (CFUs) were recorded for each plate. For the enrichment analyses, samples were vacuum-filtered through 0.22 µm filters and the filters were added to a test tube containing 4.5 mL of tryptic soy broth and a 10-µg ertapenem disk. After the test tubes were incubated for 24 hours at 37°C, a 10 µL inoculating loop was used to streak each sample onto a ChromAgar plate and incubated for

another 24 hours at 37°C. Unique pigmented isolates for both the quantitative ChromAgar plates and the enrichment plates were subcultured on a sheep's blood agar plate for another 24 hours at 37°C and were saved for species identification via VITEK2 and PCR screening for carbapenemase genes.

3.2.3 PCR Analysis

The colonies isolated in 3.2.2 were screened for bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and $bla_{OXA\text{-}48\ like}$. A boil prep extraction was done by placing one colony into 100 µL wells and boiled for 10 minutes. From the boil prepped wells, 2 µL was pipetted into a new well plate containing 18 µL of mastermix containing reverse and forward primers, HotStart Taq, dNTPS, buffer, and nuclease-free water. For VIM, IMP, and KPC, a multiplex was done together and OXA and NDM were run separately. Samples were run on a Bio-Rad CFX96 Thermal Cycler with a positive and negative control and analyzed based on a C_t value of 35 cycles.

All wastewater samples were also processed using real-time quantitative PCR (qPCR) for 16S and KPC gene copy numbers. The samples first underwent DNA extraction using a Qiagen Dneasy PowerSoil HTP 96 Kit. 2 µL of the extracted sample were added to 18 µL of mastermix (PowerUp SYBR Green Mastermix, forward KPC/16S primer, reverse KPC/16S primer, and nuclease-free water). Samples were run in triplicate on a Bio-Rad CFX96 Thermal Cycler with a positive and negative control and analyzed based on a C_t value of 35 cycles. Standards for KPC and 16S were generated from 10^2 to 10^9 copy numbers to create a standard curve equation for each primer set. Three standards, along with a negative control, were run with each set of samples to ensure accuracy. The copy numbers for each sample were determined using the equation generated by the standard curve and averaged across the triplicate.

3.3 Results and Discussion

3.3.1 Detection of KPC-producing Enterobacterales

Table 3-1 summarizes the presence or absence of KPC-producing *Enterobacterales* in each sampled location by sampling date. KPC-producing *Enterobacterales* were consistently detected in the hospital wastewater and throughout most compartments of the WWTP; namely, in the raw influent, secondary aeration basin, digester influent, and digester effluent. Every sample from these locations yielded at least one KPC-producing *Enterobacterales* on every sampling date. In the final stages of the WWTP, that was not the case. KPC-producing *Enterobacterales* were detected in the pre-disinfection effluent on only one of three sampling dates. This suggests that the treatment processes prior to disinfection may be removing ARBs even before disinfection. KPC-producing *Enterobacterales* were not detected in the final effluent on any of the sampling dates. This appears to indicate that the WWTP is effective at removing ARBs prior to reaching the natural environment. KPC-producing *Enterobacterales* were not detected in the upstream water and sediment samples. They were also not detected in the downstream water and sediment samples, expect for one occurrence in the water column during September 2019. It may be of note that this is the same sampling date were KPC-producing *Enterobacterales* were present in the pre-disinfection effluent.

Table 3-1. Prevalence of KPCO at selected sampling locations. Green highlighted X's represent KPC-producing *Enterobacterales* present. Sampling locations with no X were not sampled on that particular sampling date.

Sample Location	June 2019	Sept 2019	Dec 2019	July 2020	Oct 2020
Hospital WW		X	X	X	X
Non-Hospital WW Control (different locations)			X	X	X
Raw Influent	X	X	X	X	X
Primary Solids					X
Secondary Aeration Basin	X	X	X	X	X
Digester Influent		X	X		X
Digester Effluent		X	X		X
Pre-Disinfection Effluent		X	X		X
Final Effluent	X	X	X		X
Upstream Water (different locations)		X	X		X
Upstream Sediment (different locations)		X	X		X
Downstream Water (different locations)		X	X		X
Downstream Sediment (different locations)		X	X		X
Composting Products					X

Figure 3-2 shows the different strains of CREs detected from various sampling locations by date. Many of the bacteria detected in the raw hospital wastewater can be traced throughout the WWTP, reaffirming the hypothesis that the WWTP is the source of KPC-producing *Enterobacterales* into the WWTP. This is also supported by the absence of KPC-producing *Enterobacterales* in the wastewater control samples from other non-clinical UVA buildings.

Most of the CREs detected in the upstream and downstream water and sediment samples were *Aeromonas* species, which did not test positive for KPC. *Aeromonas* are intrinsically resistant to carbapenemases and they are unlikely to produce human infections(Queenan & Bush, 2007). However, of huge concern was a *Klebsiella oxytoca* isolate recovered from one of the downstream water locations on the September 2019 sampling date. *K. oxytoca* is a clinical strain

that is not naturally found in environmental contexts. Genetic matching was done on this isolate to compare it with a database of previously sequenced isolates collected from patients, but also sink drains, toilets, waste hoppers, and other locations within UVA hospital. It was found that this *K. oxytoca* strain was almost identical to a strain previously isolated from UVA hospital, exhibiting genetic differences of less than 10 single nucleotide polymorphisms (SNPs). This is very significant, as it almost assuredly indicates that the *K. oxytoca* strain found 37m downstream of the WWTP originated from UVA hospital.

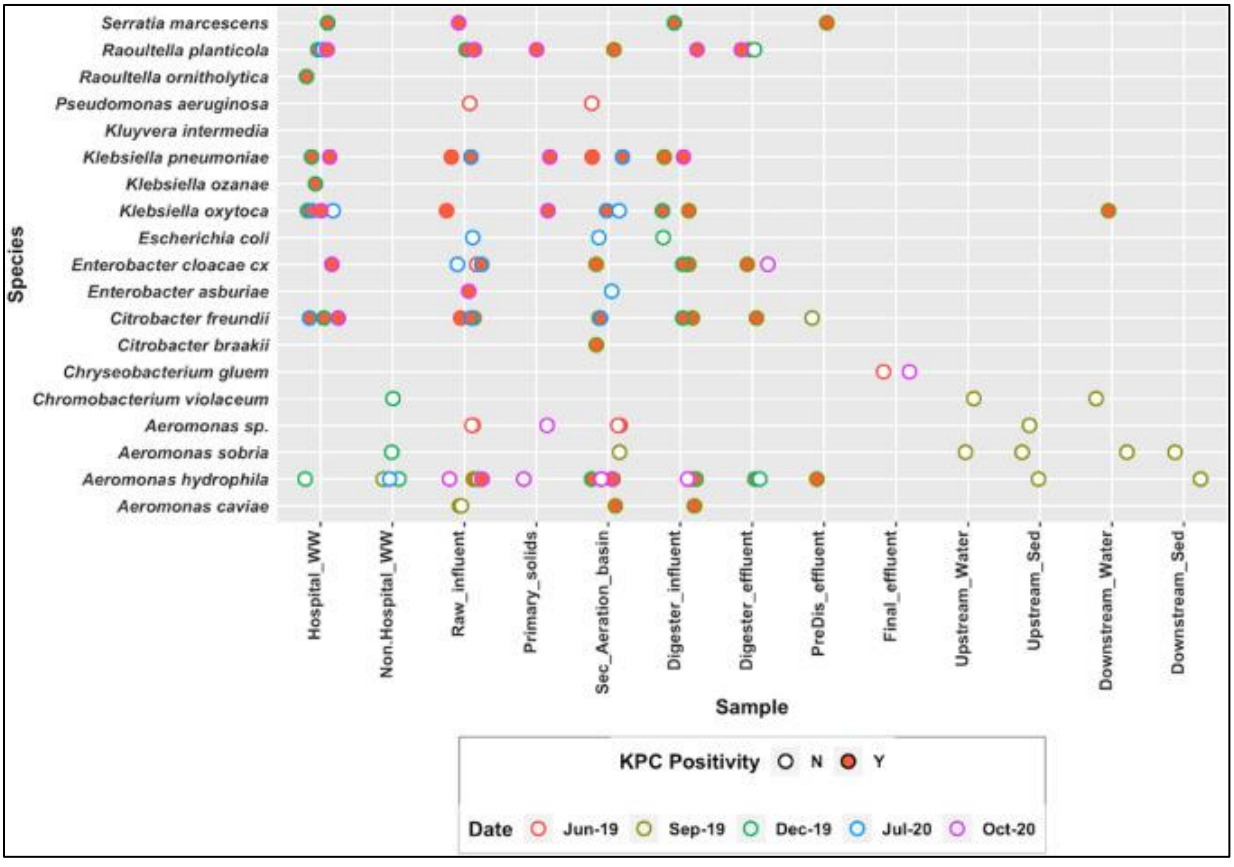

Figure 3-2. Prevalence of KPC-producing *Enterobacterales* across each stage of the treatment chain (hospital and municipal WW, WWTP compartments, and upstream/downstream water and sediment locations) across each of the sampling dates. Abbreviations: Hospital_WW = Hospital Wastewater, Non-Hospital_WW = Non-Hospital Wastewater, Sec_Aeration_Basin = Secondary

Aeration Basin, PreDis_effluent = Pre-Disinfection Effluent, Upstream_Sed = Upstream Sediment, Downstream_Sed = Downstream Sediment. The Non-Hospital WW, Upstream Water, Upstream Sediment, Downstream Water, and Downstream Sediment locations were aggregates of the different locations sampled.

The quantitative measurements of KPCO across the treatment chain (Figure 3-3) show that the hospital WW had the highest concentrations of KPCO, at least a log-fold higher than the next-highest concentration, the raw influent. KPCO abundance decreased throughout the WWTP process, with a 2-log reduction to the post-digester effluent. This decreasing trend seems to suggest that the model WWTP is not actively amplifying KPCO, but is steadily decreasing KPCO throughout the WWTP process. No KPCO were subcultured in the pre-disinfection and final effluent sampling locations.

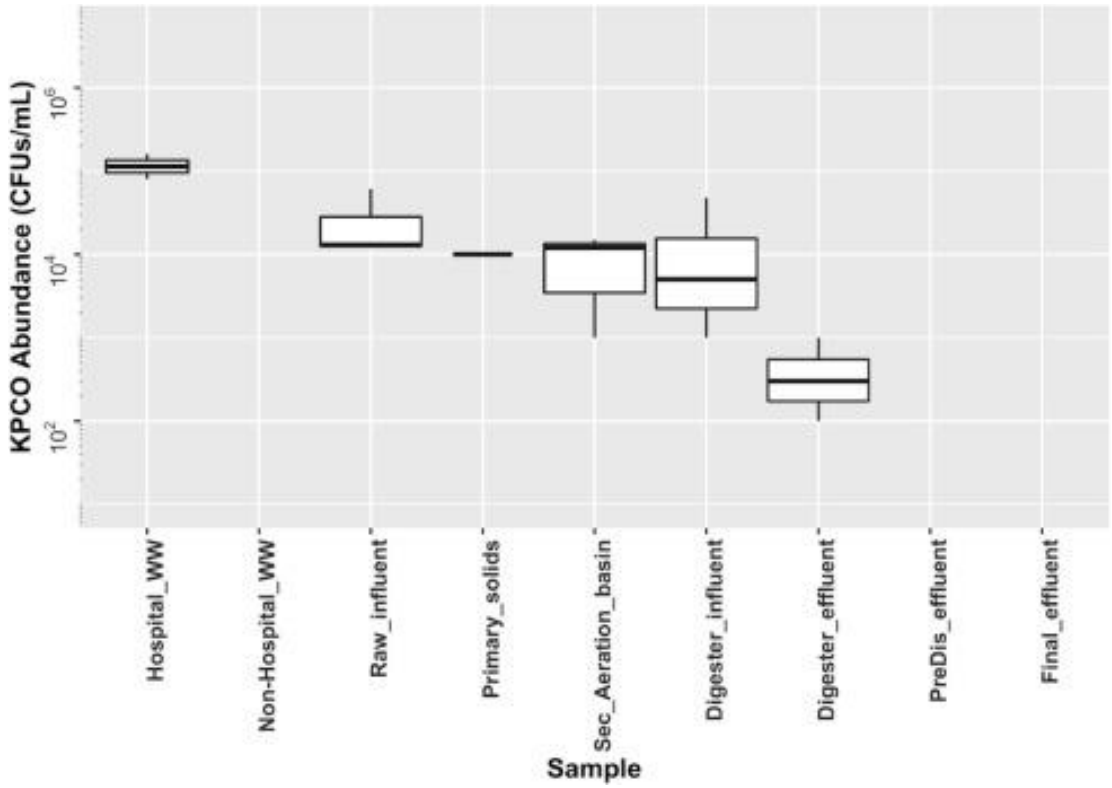

Figure 3-3. Abundance of KPCO across hospital and municipal wastewaters and different compartments of the WWTP. Abbreviations: Hospital_WW = Hospital Wastewater, Non-Hospital_WW = Non-hospital WW, Sec_Aeration_basin = Secondary Aeration Basin, PreDis_effluent = Pre-disinfection Effluent.

The abundance of Gram-negative bacteria (GNR) was realtively similar from hospital watewater and non-hospital wastewater (Figure 3-4). Lower GNR bacterial populations were observed in the pre-disinfection effluent (3-log reduction), final effluent, and upstream/downstream water and sediment.

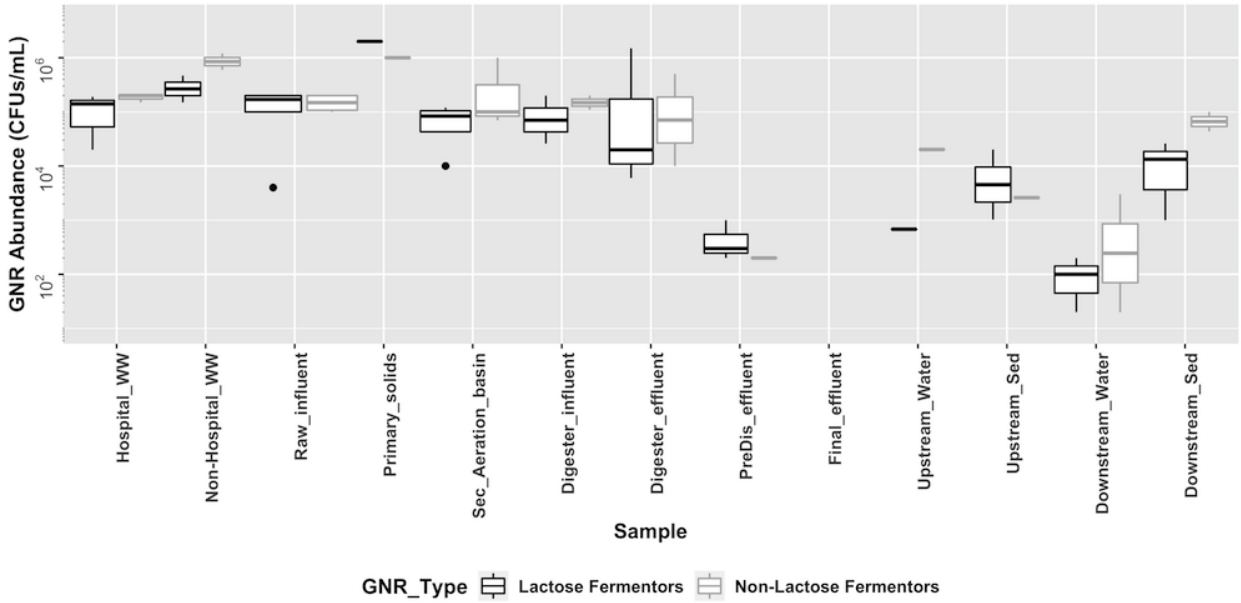

Figure 3-4. Gram-negative bacterial abundance throughout the hospital and municipal wastewaters, the WWTP compartments, and the upstream/downstream water and sediment sampling locations across all sampling dates. Lactose and on-lactose fermenters were quantified by culturing on MacConkey agar. Abbreviations: Hospital_WW = Hospital Wastewater, Non-Hospital_WW = Non-Hospital Wastewater, Sec_Aeration_Basin = Secondary Aeration Basin, PreDis_effluent = Pre-Disinfection Effluent, Upstream_Sed = Upstream Sediment, Downstream_Sed = Downstream Sediment.

3.3.2 Quantitative Prevalence of bla$_{KPC}$ and 16S genes

Figure 3-5 shows the quantitative prevalence of 16S and *bla*$_{KPC}$ throughout the WWTP. As expected, the hospital WW showed the highest ratio of *bla*$_{KPC}$ to 16S genes and the highest magnitude of *bla*$_{KPC}$ compared to the rest of the treatment chain. Similar to the microbiological results, *bla*$_{KPC}$ was present throughout the WWTP, but below the detectable limit for the pre-disinfection effluent and final effluent, as well as downstream in the receiving water. The *bla*$_{KPC}$ gene does not appear to be significantly amplified by the WWTP process.

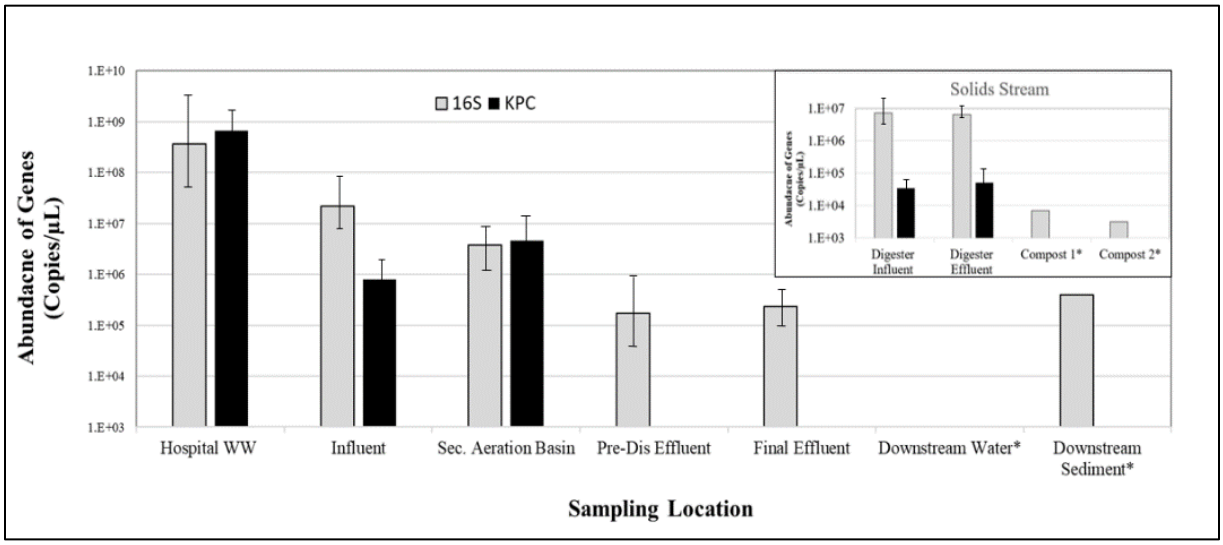

Figure 3-5. Quantitative prevalence of *bla*$_{KPC}$ and 16S RNA abundances for sampled locations along the hospital sewershed. The inset shows the solids stream. Samples with an asterisk(*) were only sampled on one occasion (Oct. 2020). The rest of the samples are averages from 3 sampling dates.

Given the presence of KPC-producing *Enterobacterales* in the digester effluent on all three sampling dates, it was of particular interest to investigate the fate of those organisms after they leave the WWTP. The wastewater biosolids are transported to a local composting facility and the compost (Class A biosolids) made from that facility are eventually land-applied as soil amendment. We collected samples of the final composting products from that facility and found

that neither of the composting samples contained KPC-producing *Enterobacterales* or *bla*$_{KPC}$. This is reassuring that KPC-producing *Enterobacterales* are being effectively removed for both the aqueous effluent and the solids effluent.

3.3.3 Genomic Relatedness of K. oxytoca Isolates

The discovery of the *K. oxytoca* isolate 37 meters downstream in the receiving water prompts the question of the origin of the isolate. We took the *K. oxytoca* isolates found in this study (4 from the hospital WW, 8 from the WWTP and the 1 from the receiving river) and analyzed their genetic relatedness to historic *K. oxytoca* isolates from the UVA hospital (16 from the hospital environment and 4 from hospital patients) found in Figure 3-6. The *K. oxytoca* (CAV8493) isolated from the downstream receiving water was a clonal isolate (less than 100 SNPs) to isolates found historically in UVA hospital, in both patients (CAV1374, CAV1755, CAVP131 and CAVP26) and the hospital environment (CAV3083, CAV3248, CAV3258, CAV6574, CAV6582), as well as an isolate found in the WWTP digester effluent (CAV8586). This genetic relatedness between these isolates indicates this *K. oxytoca* in the receiving water almost assuredly originated in UVA hospital and most likely spread to the receiving water via the WWTP, despite the fact no KPCO were found in the final effluent of the WWTP. This raises the concern that although the WWTP has strict disinfection procedures and appears to be removing KPCO and other ARBs, there is still the concern of some spillover, which can lead to the spread of ARBs downstream in the environment.

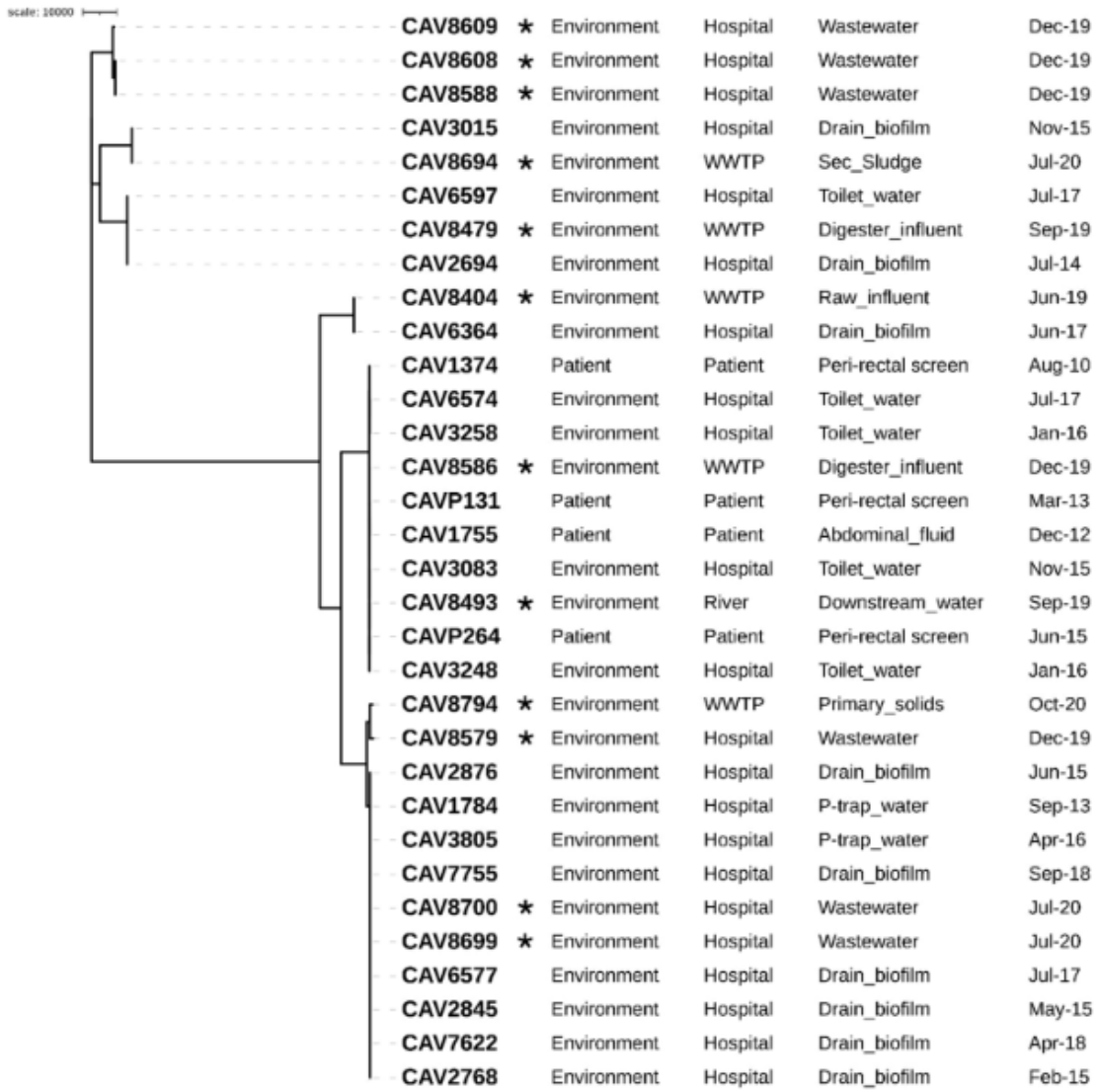

Figure 3-6. Phylogenetic tree of the relatedness of *K. oxytoca* isolates between environmental and clinical isolates. Asterisks (*) denote isolates cultured from this current study and the other isolates are historical isolates from UVA hospital either from patients or the hospital environment (drain biofilm, p-trap water, or toilet water). The first column is the CAV# of the isolate; the second and third columns are the type of location where the isolate was found; the fourth column is the compartment of isolation; the fifth column is the month and year of isolation.

3.4 Conclusions

KPC-producing *Enterobacterales* and *bla*$_{KPC}$ were found in high concentrations in hospital wastewater and were present throughout the sampled WWTP. These findings were expected given the long-standing documentation of KPC-producing *Enterobacterales* in UVA hospital's plumbing over the last decade (Mathers et al., 2018). KPC-producing *Enterobacterales* were not detected in the final effluent of the WWTP and although KPC-producing *Enterobacterales* and *bla*$_{KPC}$ were found in the digester effluent, they were not detected in the final compost products made using wastewater biosolids. By using KPC-producing *Enterobacterales* as a model for ARBs, it was shown that the integrated wastewater management system (WWTP and biosolids management) appears to be generally effective at removing ARBs prior to discharge for both the aqueous and solid phases.

While KPC-producing *Enterobacterales* were not detected in the discharge from the WWTP, it was of significant concern that a *K. oxytoca* isolate was recovered 37m downstream of the WWTP that was almost genetically identical to strains found in the UVA hospital and the WWTP. Given that no isolates were recovered upstream, it seems likely that this strain was transported to the environment from the hospital via the WWTP.

Results from Objective 1 provide valuable insights regarding how KPC, our model ARG, persists in the wastewater treatment process. However, there are still some concerns about the potential for spillover of ARBs and/or ARGs into the outflow via the effluent and the potential for spread of antibiotic resistance downstream to native microbial assemblages, especially given the *K. oxytoca* strain found in the receiving waters that likely came through the WWTP. Therefore, it is of interest to investigate the efficacy of different wastewater disinfection

treatments on the removal of ARBs within the WWTP to prevent possible spillover of already-resistant bacteria to the natural environment, which is presented in Objective 2 (Chapter 4). But is also raises the concern of "spillover" ARBs that are released in the outflow of the WWTP and their potential to transfer resistance features to other bacterial hosts downstream of the WWTP, which forms the basis for the microcosm transconjugation experiments presented in Objective 3 (Chapter 5).

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Chapter 4: Objective 2 Evaluating the efficacy of selected conventional wastewater disinfection technologies for inactivation of a model ARB

4.1 Background on Wastewater Disinfection Treatment

Disinfection is used in WWTPs to inactivate pathogenic bacteria in treated effluents prior to discharge. In the United States, the Environmental Protection Agency (EPA) enforces wastewater effluent guidelines through the National Pollutant Discharge Elimination System (NPDES) ("U.S. Environmental Protection Agency)," 2021). Disinfection techniques most commonly used are chlorination and ultraviolet radiation, but other options are also used, including ozonation, peracetic acid, nanofiltration, copper ionization, and others. In this study, chlorination and UV radiation were the two disinfection technologies considered for several reasons. First, they are widely used in the U.S. with chlorination and/or UV radiation accounting for disinfection in 96% of all WWTPs (Leong, Kuo, & Tang, 2008). Second, they utilize different mechanisms to inactivate bacteria (see Sections 4.1.1 and 4.1.2), such that examination of both together is expected to yield insights into the mechanisms by which ARBs and ARGs are inactivated.

4.1.1 Chlorination

Table 4-1. Literature overview of chlorination disinfection efficacy on ARBs and ARGs.

Source	ARB	ARG	Dose (mg*min)/L	Results
Stange et al., 2019	*E. coli* and *E. faecium*	*tet*A, *amp*C	15	3.8-5.6 log reduction of ARBs, 0.8-2.8 reduction of ARGs
Yoon et al., 2017	*E. coli*	amp^R and kan^R	33-72	4-log reduction of ARGs
Huang et al., 2013	*E. coli*	*tet*A	10	5-log reduction of ARBs
Pang et al., 2016	*E. coli*	bla_{TEM1}	10	3-log reduction of ARBs; no apparent reduction of ARGs
Destiani & Templeton, 2019	*E. coli* and *Pseudomonas aeruginosa*	*tet*A, bla_{TEM1}, *sul*1, *mph*A	30	1.7-log reduction of ARGs

Chlorine is the most commonly used wastewater disinfection technology in the United States. Chlorine compounds used for wastewater disinfection can include chlorine gas, sodium hypochlorite, calcium hypochlorite, and chlorine dioxide, however, many facilities have switched from chlorine gas to sodium hypochlorite for safety concerns related to liquid-gaseous chlorine (Stover et al., 2012; Tchobanoglus, Burton, & Stensel, 2003). Free available chlorine (FAC) inactivates bacteria by breaking down bacterial cell walls via reactions with amino acid side-chains and peptidoglycan (Dodd, 2012). The effectiveness of chlorination on inactivating bacteria depends on the type and age of microorganisms. Older bacteria cultures require higher chlorine doses, likely due to the increased development of the polysaccharide sheath (Tchobanoglus et al., 2003). Chlorination is often followed by dechlorination because chlorine

residuals and byproducts are toxic to aquatic and human life. Sulfur dioxide is the most common dechlorinating agent used, but sodium sulfite compounds are also used (Tchobanoglus et al., 2003).

4.1.2 Ultraviolet Radiation

Table 4-2. Literature overview of UV disinfection efficacy on ARBs and ARGs.

Source	ARB	ARG	Dose (mJ* cm^{-2})	Results
Stange et al., 2019	*E. coli* and *E. faecium*	*tet*A, *amp*C	60	4.8-5.5 log reduction of ARBs, negligible reduction (0-1.0 log) of ARGs
Yoon et al., 2017	*E. coli*	amp^R and kan^R	50-130	4- log reduction of ARGs
Huang et al., 2013	*E. coli*	*tet*A	10	4-log reduction of ARBs
Pang et al., 2016	*E. coli*	bla_{TEM1}	40; 80	40 mJ*cm^{-2} for 5.5-reduction of ARBs; 80 mJ*cm^{-2} for 1.2-log reduction of ARGs
Destiani & Templeton, 2019	*E. coli* and *Pseudomonas aeruginosa*	*tet*A, bla_{TEM1}, *sul*1, *mph*A	200	1.2-log reduction of ARGs
McKinney & Pruden, 2012	MRSA, VRE, *E. coli*, *Pseudomonas aeruginosa*	*mec*A, *van*A, *tet*A, *amp*C	10-20; 200-400	10-20 mJ*cm^{-2} for 4- to 5-log reduction of ARBs; 200-400 mJ*cm^{-2} for 3- to 4- log reduction of ARGs

Ultraviolet (UV) radiation utilizes electromagnetic radiation (200-300 nm wavelength) to penetrate through bacteria cell walls and cytoplasm to inactivate bacteria through bacterial cell walls. UV light results in the formation of structural lesions on DNA, which inhibits cell replication. The most significant lesion produced by UV light in the is the formation of pyrimidine primers. Some microorganisms have shown the ability to repair DNA damage from UV radiation by removing pyrimidine primers via visible light reactions (photoreactivation) or

light-independent reactions (dark repair) (Friedberg et al, 2005; Harm, 1980; Lindenauer & Darby, 1994).

4.1.3 Effects of Wastewater Disinfection on ARBs and ARGs

Tables 4-1 and 4-2 summarize the literature data regarding the inactivation and reduction of several ARBs and ARGs disinfected via chlorination and UV radiation, respectively. Overall, chlorination and UV disinfection reduce total concentrations ARBs of ARGs as shown in numerous studies, but the effectiveness varies (Huang et al., 2013; McKinney & Pruden, 2012; Stange et al., 2019; Yoon et al., 2017). Previous studies have shown that UV treatment has wide variability in the effectiveness against ARBs- it can select for certain strains of ARBs in effluent, while also being more effective against other types of ARBs (Di Cesare et al., 2016; Guo et al., 2013). Guo et al., 2015 showed that low chlorine doses (less than 40 mg Cl*min/L) can even promote horizontal gene transfer of ARGs by 2-5-fold via chloramines improving cell permeability, followed by increased pili on cell surfaces that act as pathways for ARG transfer. Even if ARBs are removed prior to discharge, ARGs remain a concern as several studies have documented that intracellular activity from deactivated ARB cells can still retain transforming activity (Roller, Olivieri, & Kawata, 1980; Shih & Lederberg, 1976; Zhang et al., 2019). Several studies have investigated the impacts of different disinfection technologies on ARGs, but these have had mixed results. Previous studies have documented that UV radiation results in incomplete degradation for several different ARGs (McKinney & Pruden, 2012; Stange et al., 2019), including *tet*(A) (Stange et al., 2019), *amp*(C) (Stange et al., 2019), *van*(A) (Stange et al., 2019), *erm*(B) (Stange et al., 2019), and bla_{TEM1}(Pang et al., 2016). McKinney and Pruden (2012) and Pang et al (2015) found that UV inactivation of ARGs tested required doses at least one order of magnitude higher than for the inactivation of host bacterial cells (i.e., ARBs)

(McKinney & Pruden, 2012; Pang et al., 2016). Figure 4-1 shows the results from Stange et al (2019) for deactivation of *E. coli*, *E. faecium*, *tet*(A), and *amp*C, *erm*B, and *van*A, using various doses of chlorine and UV (Stange et al., 2019). However, to our knowledge, there is not yet any study documenting disinfection removal efficiencies for KPC-producing bacteria or bla_{KPC}, which may behave slightly differently under disinfection than other classes of ARBs and ARGs.

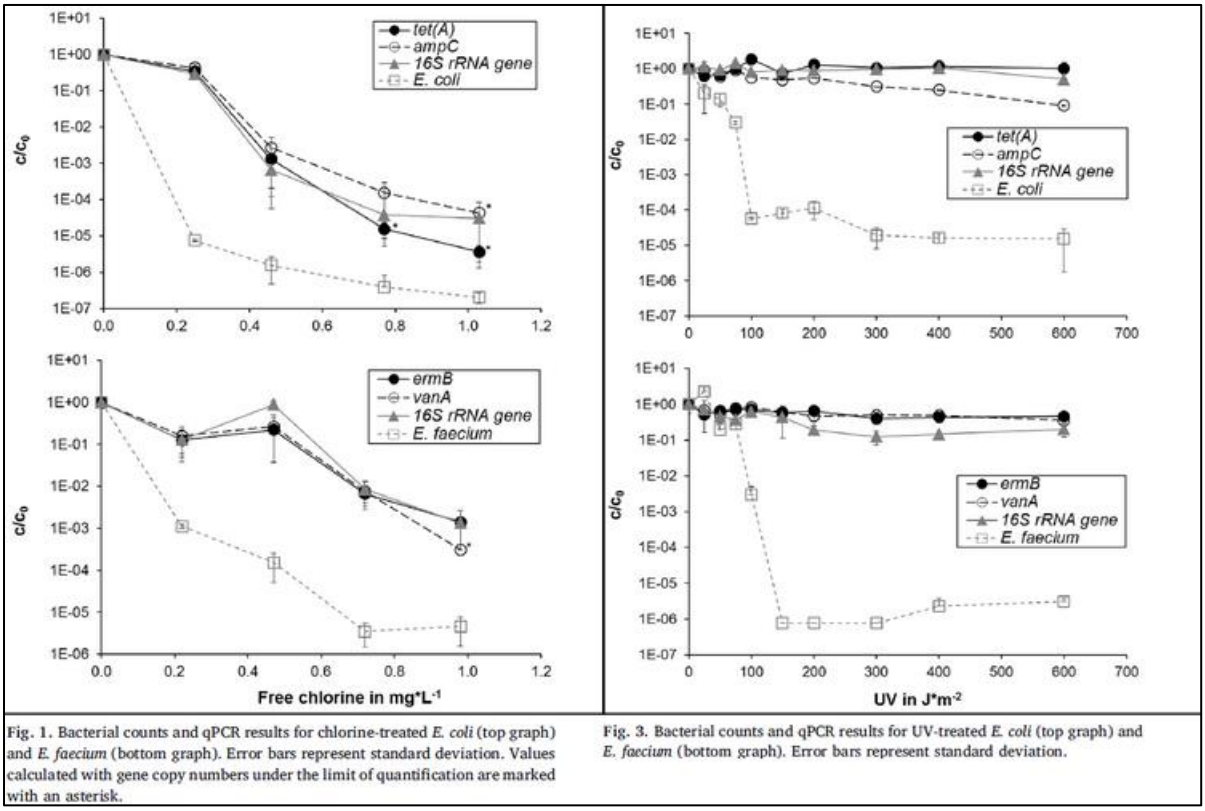

Fig. 1. Bacterial counts and qPCR results for chlorine-treated *E. coli* (top graph) and *E. faecium* (bottom graph). Error bars represent standard deviation. Values calculated with gene copy numbers under the limit of quantification are marked with an asterisk.

Fig. 3. Bacterial counts and qPCR results for UV-treated *E. coli* (top graph) and *E. faecium* (bottom graph). Error bars represent standard deviation.

Figure 4-1. Quantitative ARB and ARG at varying chlorination (left panel) and UV doses (right panel) taken from Stange et al., 2019. For both chlorination and UV radiation, the ARBs, *E. coli* (top left and right) and *E. faecium* (bottom left and right), were inactivated at lower doses than those needed to decrease the ARG copy numbers. There was negligible decrease in post-disinfection ARG copy numbers using UV radiation, but up to a 0.8-2.8 log reduction of ARG copy numbers for chlorination at 0.5 mg/L free chlorine dose. The doses in this study, 0-30 mg*min/L for chlorination and 0-60 mJ/cm^2 for UV, are comparable to the doses used in this

study, 0-60 mg*min/L free chlorine and 0-40 mJ/cm^2. Figure from Stange et al., 2019 [used without permission]

4.1.4 Photoreactivation and growth following UV-disinfection

There are generally three explanations that account for the increase in bacterial counts post-disinfection: 1) the growth and spread of bacteria that were uninjured during disinfection; 2) the reactivation of injured bacteria through light- or dark-repair; 3) the growth and spread of re-activated bacteria. The growth of bacteria after disinfection is highly dependent on the number of bacteria eliminated during disinfection and the amount of nutrients present in the WW post-disinfection, among other potential factors. All three of these processes may be present in this study. Lower doses of disinfection would be expected to have more growth due to spread of uninjured bacteria, while higher doses would be expected to have more growth and repair of injured and re-activated bacteria.

It has been well-documented that many microorganisms, including *E. coli* and other pathogenic bacteria, can repair the damage done by UV, which weakens the effectiveness of UV disinfection and raises concerns over the potential of ARBs to pass through the UV disinfection process (Hijnen et al., 2006; Locas et al., 2008; MeiTing et al., 2011; Zhou et al., 2017). This process is called photoreactivation or light-repair. Bacteria can also repair themselves through dark-repair mechanisms, which are light-independent (Britt, 1996; Friedberg et al., 2005; Harm, 1980). Considering that exposure to sunlight is practically inevitable for most treated WW effluents, it remains important to consider the potential for photoreactivation to occur.

The largest class of structural lesions induced by UV irradiation are cyclobutane-primidine dimers (CPD), which account for 75% of UV-induced DNA products (Sinha & Häder, 2002). Photoreactivation is a process in which a DNA photolyase enzyme binds to the CPDs and

reverses the UV damage through light reactions (310-480 nm). Photolyases have chromophores that absorb blue-light photons and transfers the excitation energy to a catalytic cofactor, which can then donate an electron to a CPD and split the cyclobutene ring of the CPD with very high efficiency, close to one dimer split for every blue-light photon absorbed (Britt, 1996). *E. coli* have a *phr* gene, likely adapted from other bacteria, that codes for deoxyribodipyrimidine photolyase, which can bind to a CPD with a folic acid cofactor. When that bond is exposed to light, the folic acid cofactor absorbs a light photon and the energy can be used to break the cyclobutane ring (Sinha & Häder, 2002). Hoyer 1998 found that a minimum dose of 30 mJ/cm^2 was required to achieve a 4-log reduction of ATCC 11229 *E. coli*.

Dark-repair mechanisms are more complex than light-repair as they do not directly reverse UV-induced damage, but instead replace sections of damaged DNA with new nucleotides (Britt, 1996; Seeberg et al., 1995). There are several different mechanisms for dark-repair. One method is through base excision repair (BER), where DNA glycosylases recognizes abnormal nucleotide bases, and then cleave cross-links in the DNA between the glycosidic bond and the deoxyribose sugar, leaving an AP site. An AP nuclease removes the sugar-phosphate group and then DNA polymerase is able replicate the missing nucleotide before the DNA ligase seals it (Seeberg et al., 1995; Sinha & Häder, 2002). Another method is nucleotide excision repair (NER), where large UV-induced lesions are removed by excinucleases. Then, DNA polymerase is able to fill the gap of nucleotides using the opposite side of DNA as a template with DNA ligase following up to seal it (Lehmann, 1995).

4.2 Methodology

4.2.1 Antibiotic Resistant Bacteria

A laboratory-strain of *Escherichia coli* (*E. coli* $J53_{rif}$) was selected to serve as a representative strain of gram-negative bacteria for the disinfection and subsequent microcosm experiments, which are described in Chapter 5. $J53_{rif}$ *E. coli* was chosen for several reasons: 1) *E. coli* is capable of repairing itself after UV disinfection (Locas et al., 2008; MeiTing et al., 2011; Zhou et al., 2017); 2) 1) *E. coli* is a gram-negative lactose-fermenting bacteria, similar to CREs, so it may behave similarly under disinfection to other clinically relevant ARBs; 3) it is a competent cell that is suitable for the transconjugation experiments described in Chapter 5. Two versions of $J53_{rif}$ *E. coli* were used in separate experiences: one non-KPC isolate, and another that had been previously mated with a CAV1016 bla_{KPC} plasmid (KPC isolate). These two strains were used together in the disinfection processes, to evaluate whether the presence of bla_{KPC} would impact the effectiveness of the selected disinfection.

4.2.2 Preparation of Bacteria

Protocols for ARB disinfection experiments were adapted from the studies summarized in Tables 4-1 and 4-2. Colonies were grown from frozen bacterial stocks (40% glycerol, -80°C) and streaked onto sheep's blood agar plates and incubated for 24 hours at 37°C. Single colonies were chosen using an inoculating loop and grown in LB broth for 12 hours (to reach mid-exponential phase), then pelleted via centrifugation and resuspended in Phosphate Buffer Solution (PBS) to concentrations of ~10^8 colony forming units (CFUS) per mL. The optical density (OD) was measured using a nanophotometer adjusted to 1. Test samples were made in duplicate and underwent chlorination and ultraviolet disinfection.

4.2.3 Quantitative Analysis

1-mL of sample was taken pre-disinfection and post-disinfection. These were analyzed for quantitative counts. These samples were serially diluted 1:10 six times onto LB plates (w/ 1 µg/mL meropenem for the bla_{KPC} positive *E. coli*) and incubated for 24 hours at 37°C. The average number of CFUs per mL were recorded for each plate and CFUS per mL were averaged based on the replicates.

Rate constants, *k*, for disinfection were calculated using a first-order reaction according to Chick's Law:

$$k * t = -log \frac{[bacteria]_{time=n}}{[bacteria]_{time=0}}$$

4.2.4 Chlorination

The chlorinating agent was a stock solution of 5 mg/L sodium hypochlorite solution that was added to test tubes containing 10mL of the simulated effluent (*E. coli* in PBS) with varying exposure times to simulate different free chlorine concentrations (0, 0.5, 1.0, 1.5, 2, and 4 mg/L free chlorine) in the test tubes. Free chlorine concentrations were confirmed using Hach method 10241. Samples were gently shaken for their respective contact times. The chlorination process was terminated by adding 1.5% sodium thiosulfate as a dechlorinating agent. 1-mL of pre- and post- chlorination samples were collected and analyzed for quantitative counts as described in Section 4.2.3.

927 *4.2.5 Ultraviolet Radiation*

928

929 A laboratory-scale, benchtop collimated beam
930 UV apparatus was adapted from Bolton and Linden
931 (2003). It consisted of a germicidal UV sterilizing lamp
932 (Fischer Scientific) emitting monochromatic light at a
933 wavelength of 254 nm. UV intensity was 0.7 mW/cm^2,
934 which was confirmed using a UVC radiometer. 30 mL of
935 test sample were added to transparent, plastic petri dishes
936 (9 cm diameter) and fluence doses were applied (0, 5, 10,
937 20, 40, 80, and 100 mJ/cm^2) to the samples by UV light
938 at a constant intensity of 0.7 mW/cm^2, but altering the
939 exposure time to achieve the desired fluence using the
940 following equation:

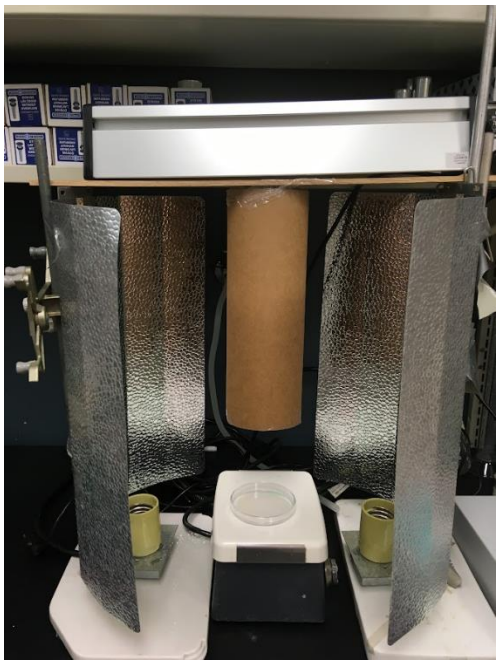

Figure 4-2. UV apparatus used in the disinfection experiments.

941 $$\text{Dosage (mJ/cm}^2\text{)} = \text{UV Intensity (mW/cm}^2\text{)} \times \text{Exposure Time (s)}$$

942 Samples were gently mixed using a magnetic stirrer plate during irradiation. 1-mL of pre-
943 and post- irradiation samples were collected and analyzed for quantitative counts as described in
944 Section 4.2.3.

945 *4.2.6 Light- and Dark-repair Experiments*

946 After exposure to UV radiation treatments (as described in 2.1.2) of 5, 40, and 80 mJ/cm^2
947 doses (low, medium, and high UV doses, respectively), the test samples for both the non-KPC *E.*
948 *coli* and the KPC-positive *E. coli* were exposed to three different light treatments: ambient light,

artificial light, and no light. Ambient light samples were placed on the benchtop with exposure to natural sunlight through the windows, which provided approximately 10 hours of daylight over the 24-hour period (completed in December in the Northern Hemisphere). Artificial light samples were exposed to an artificial light source, a fluorescent growth lamp (125W, 30 $\mu W/cm^2$), which provided 12 hours of light exposure followed by 12 hours of no light exposure. No light samples were placed on the benchtop with a box placed over the samples to prevent light from reaching the samples.

Samples under all three conditions were evaluated in triplicate and left for 24 hours following UV disinfection of each of the three UV doses: 5, 40, and 80 mJ/cm^2. All samples were gently stirred using a stirring plate at room temperature (approximately 20°C). At 3 and 24 hours, the samples were analyzed for CFU/mL concentrations as described in Section 4.2.3

Percent repair was calculated using the following equation,

$$\%\ Repair = \frac{[bacteria]_{t=time} - [bacteria]_{t=post-disinfection}}{[bacteria]_{t=pre-disinfection} - [bacteria]_{t=post-disinfection}}$$

4.3 Results and Discussion

4.3.1 Chlorination Results

The resistant and non-resistant *E. coli* were inactivated appreciably via chlorination, as seen in Figure 4-3, with a 4.7-log removal and 5.1-log removal, respectively, for the maximum dose of 4 mg/L free chlorine. As expected, greater log-removal was seen as concentrations of free chlorine increased. The removal efficiencies at higher doses of chlorination of 2 and 4 mg/L Chl were comparable with ARBs in previous literature (Table 4-1), with our study exhibiting 4.1- and 4.7-log removal for the resistant *E. coli.* Interestingly, our results showed lower *E. coli*

removal for both the resistant and non-resistant *E. coli* at lower chlorine doses (0.5 mg/L free chlorine), with only 1.7- and 2.7-log removal, respectively, compared to Stange et al., 2019 exhibiting over 5-log removal at the same free chlorine concentration. The resistant and non-resistant *E. coli* removal efficiencies were comparable, so it does not appear that the presence of bla_{KPC} impacts the efficacy of chlorination.

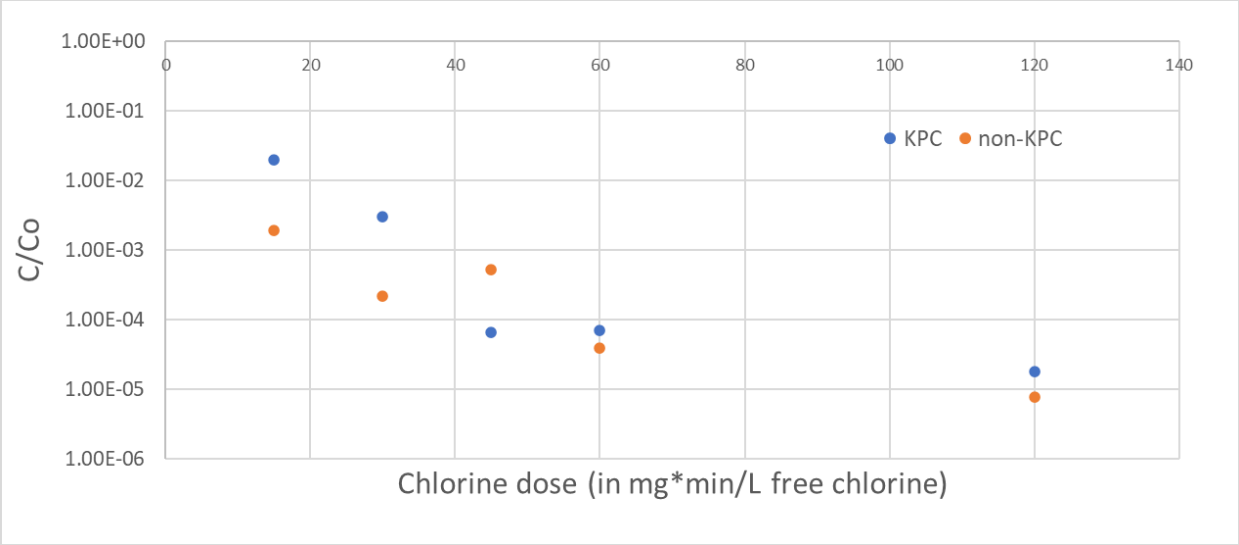

Figure 4-3. Quantitative ARB reduction for varying doses of chlorination. KPC (blue) = *E. coli* with the bla_{KPC} plasmid. Non-KPC (orange) = *E. coli* with no KPC resistance.

4.3.2 UV Results

The *E. coli* samples were also disinfected via UV irradiation with UV doses from 0 to 100 mJ/cm^2 (Figure 4-4). There was appreciable bacterial removal, especially at higher UV doses, with up to 6.1-log removal for the resistant *E. coli* and 4.8-log removal for the non-resistant *E. coli*. The removal efficiencies were comparable to the results found in literature

(Table 4-2) (Huang et al., 2013; McKinney & Pruden, 2012; Pang et al., 2016; Stange et al., 2019).

The resistant and non-resistant *E. coli* were comparable at lower UV doses, but exhibited a larger spread in removal rates at higher UV doses of 80 and 100 mJ/cm^2, with the resistant *E. coli* averaging 5.8- and 6.1-log removal and the non-resistant *E. coli* achieving 3.9- and 4.8-log removal, respectively. While this may be due to experimental variability, this result is promising as UV disinfection does not appear to be less effective due to KPC-resistance, which has been seen in previous studies for other types of ARBs (Destiani & Templeton, 2019).

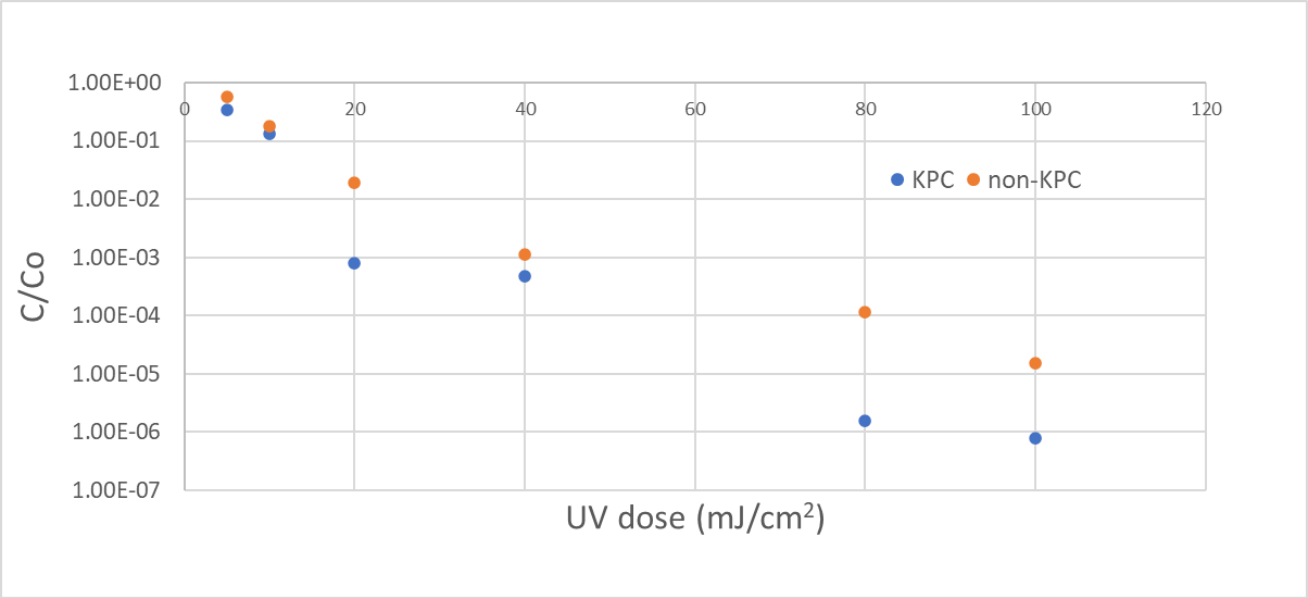

Figure 4-4. Quantitative ARB reduction for varying doses of UV radiation. KPC (blue) = *E. coli* with the *bla*$_{KPC}$ plasmid. Non-KPC (orange) = *E. coli* with no KPC resistance.

From the results, there does not appear to be an obvious answer to whether chlorination or UV disinfection is superior at inactivating and removing ARBs, as both disinfections at higher concentrations exhibited up to about 5-log removal (or 99.999% reduction) of *E. coli*. Neither

disinfection appeared to be less effective at removing the KPC-resistant *E. coli* compared to the non-resistant *E. coli.* Mathys et al., 2019 had surveyed 50 WWTPs across the U.S. and found that WWTPs utilizing ultraviolet (UV) radiation had lower rates of carbapenemase-producing bacteria (12%) compared to WWTPs utilizing chlorination (42%), so we had expected that perhaps UV irradiation would be more effective against ARBs. While both chlorination and UV disinfection appear to be effective at inactivating the vast majority of bacteria and ARBs exiting the WWTP, these results fall short of fully illustrating whether chlorination or UV disinfection truly stop the dissemination of wastewater-based antibiotic resistance downstream.

4.3.3 Potential regrowth and repair following UV disinfection

With previous studies showing the potential for bacterial regrowth and repair after UV disinfection (Destiani & Templeton, 2019; Friedberg et al., 2005; Harm, 1980; Lindenauer & Darby, 1994), it was of interest to evaluate the ability of ARBs to repair themselves in the hours post-UV disinfection. Figure 4-5 and Figure 4-6 illustrate the regrowth of the resistant and the non-resistant *E. coli*, respectively. The two light treatments, both simulated and natural light, exhibited higher repair percentages than the dark repair simulation for 5 and 40 mJ/cm^2, which was statistically significant. This difference emerges by the 3-hour mark, which is consistent with previous studies that have shown that *E. coli* observes the highest photoreactivation in the first 2-3 hours post UV-disinfection before leveling off (Zimmer & Slawson, 2002). Dark-repair, while exhibiting less repair than the two light treatments, did exhibit substantial repair percentages with 10.5% repair for the resistant *E. coli* and 15.4% for the non-resistant *E. coli* at 40 mJ/cm^2, a UV fluence in the range of what would be expected at a typical WWTP.

There is a decreasing trend of *E. coli* repair for increasing UV disinfection doses with the highest observed repair being 31.5% at 24 hours for 5 mJ/cm^2 for the resistant *E. coli,* but only

0.9% repair for 80 mJ/cm^2. The 5 mJ/cm^2 is a relatively low dose of UV fluence that only saw 0.5-log removal, so it is perhaps unsurprising that there was a large amount of regrowth following UV-disinfection. At the highest UV disinfection dose of 80 mJ/cm^2, there was very little *E. coli* repair for any of the three light treatments with less than 1.0% repair for any light treatment, which is consistent with findings in other literature (MeiTing et al., 2011). This was not surprising as 80 mJ/cm^2 is a very aggressive UV dose, likely above what would be used in an average WWTP. At a fluence of 40 mJ/cm^2, in the range of UV dosage used in WWTPs, there was *E. coli* repair up to 22.7% for the resistant *E. coli* and 25.7% for the non-resistant *E. coli*.

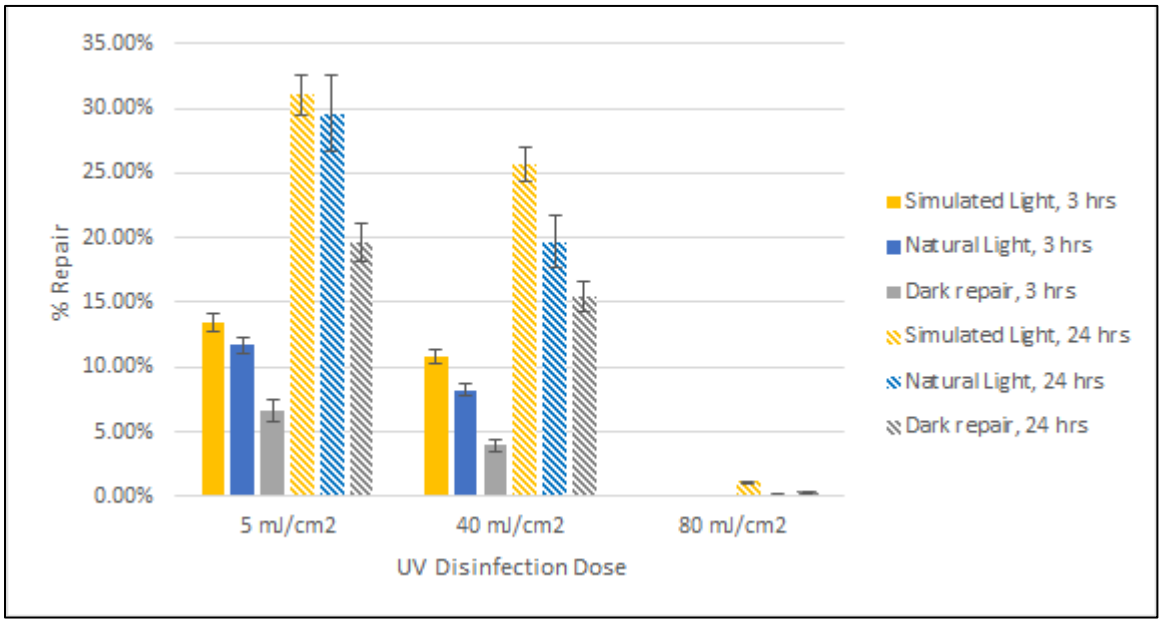

Figure 4-5. Percent repair of for the resistant *E. coli* with the *bla*$_{KPC}$ plasmid under three conditions: simulated light, natural light, and dark repair, at 3 and 24 hours, for varying doses of UV radiation (5, 40, and 80 mJ/cm^2).

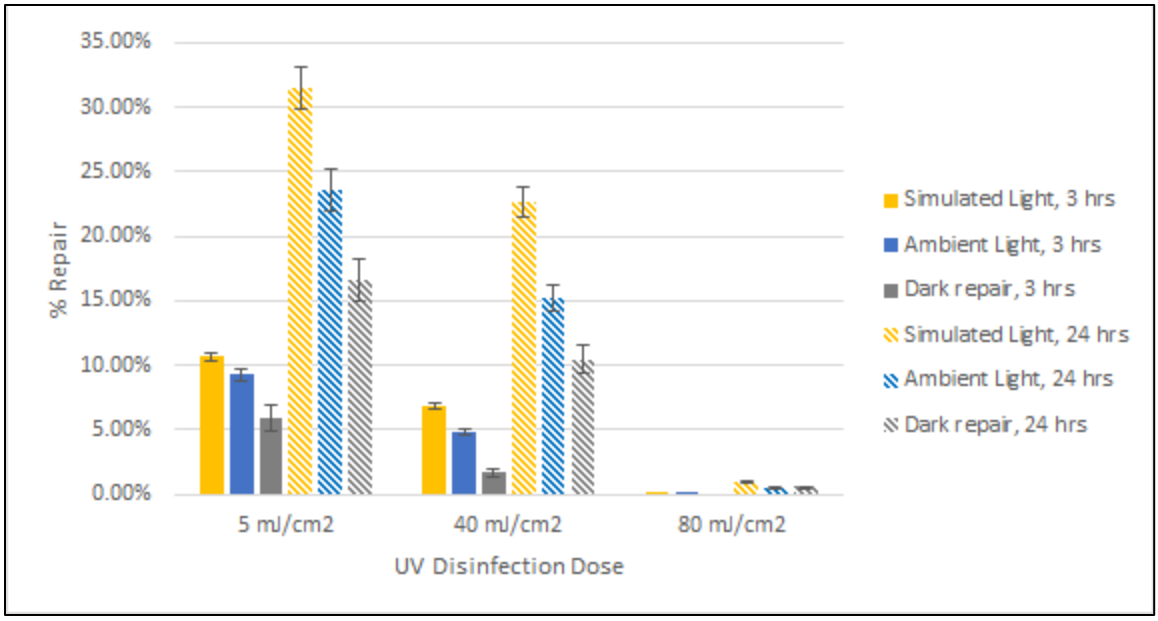

Figure 4-6. Percent repair of for the non-resistant *E. coli* without the bla_{KPC} plasmid under three conditions: simulated light, natural light, and dark repair, at 3 and 24 hours, for varying doses of UV radiation (5, 40, and 80 mJ/cm^2).

4.4 Conclusions

Chlorination and UV irradiation were shown to be effective disinfection treatments against inactivating both the resistant and non-resistant *E. coli*, up to 5.1-log removal for chlorination and 6.1-log removal for UV irradiation. The photoreactivation and dark-repair experiments post-UV disinfection show the potential for *E. coli* and other ARBs to repair and regrow substantially in the hours post-disinfection, up to 32% repair at the lowest UV fluence. However, aggressive UV doses greatly reduced repair potential, with less than 1% for any of the light- or dark- repair experiments at the highest fluence of 80 mJ/cm^2. Of concern was the significant repair of *E. coli* at the 40 mJ/cm^2, a fluence in the range that would be used at WWTPs, with repairs up to 22.7% for the resistant *E. coli* and 25.7% for the non-resistant *E. coli*.

While the efficacy of ARB removal for different disinfections is enlightening, this and the other previous studies fall short of fully illustrating whether application of the selected treatments truly mitigates the risk of wastewater-based antibiotic resistance dissemination to the receiving water and downstream environment. Additional research is needed to address this limitation, which will be addressed in Chapter 5.

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Chapter 5: Objective 3. Characterizing dissemination of wastewater-based antimicrobial resistance to representative downstream microbial communities

5.1 Motivation and Background

As described in Chapter 4, there are limitations in using current qualitative and quantitative methodologies for truly determining the efficacy of WWTPs in reducing antibiotic resistance dissemination since it remains unclear whether ARB and/or ARG in the treated effluents can disseminate resistance to microbial communities to the downstream receiving waters. The experiments summarized in Chapter 4 were relevant to discharge and propagation of ARB that were already resistant before release from the WWTP. This chapter focuses on the extent to which the selected treatments mitigate possible transmission of antimicrobial resistance to other organisms, not present in the initial effluent, including those that exist in sediment communities in the downstream receiving waters. This secondary transmission is of significant concern because it could result in rapid proliferation of multiple resistant lineages within a single environmental compartment via horizontal gene transfer (Finley et al., 2013; Rizzo et al., 2013).

In this objective, we apply a "microcosm" approach to analyze whether ARBs and/or ARGs in wastewater-treated effluents are able to transfer resistance to select microbial assemblages. This approach simulates environmental conditions of treated effluents mixing with native bacteria, whose ability to uptake antimicrobial resistance features is not previously well-documented. This microcosm transformation assay used in this study extends the methodological

framework that has been used most widely in existing literature, which has focused mostly on the kinds of methodologies used in Chapter 4.

Transconjugation is the process of transferring genetic material from bacterial cell to bacterial cell through direct contact. In a previous study, Luo et al. (2014) used a microcosm experiment to document the transfer of the NDM-1 gene, the predominant ARG for dissemination of carbapenemase resistance in Asia (Luo et al., 2014). An NDM-1-positive *Achromobacter* sp. strain was isolated from the aeration tank of a WWTP in China and inoculated with native river sediment bacteria through a 9-day microcosm. An indigenous bacterium, which was determined to be phylogenetically close to *Comamonas* sp., was isolated after 9 days, which contained the NDM-1 gene that was determined to be 100% identical to the donor *Achromobacter* sp. strain. This precedent shows the potential for transformation of CRE genes to indigenous bacteria in receiving waters. To our knowledge, the work by Luo et al (2013) is the only existing study in which a microcosm approach has been used to evaluate the extent to which selected WWTP treatments truly reduce the likelihood of antibiotic resistance transmission to downstream microbial assemblages.

In this study, a laboratory strain of $J53_{rif}$ *E. coli* was used as a recipient in some of the iterations of the microcosm experiments used in Objective 3, the same used in the disinfection experiments in Objective 2. The *E. coli* cells used were competent cells, meaning that the membranes of the cell were genetically modified to make them more receptive to taking up foreign DNA. Competent cells are more easily be able to take up the bla_{KPC}-carrying plasmid than normal bacterial cells because of their weakened cell structure. The goal of Objective 3 is to determine the extent to which ARBs, such as ones that spillover from WWTPs, disseminate antimicrobial resistance to other organisms that exist in sediment communities in the

downstream receiving waters. This was done through transconjugation microcosms to see if transconjugation would occur from the KPC-positive donor isolates to our KPC-negative recipient cells.

5.2 Methodology

5.2.1 Microcosm Transconjugation Experiments

Microcosm experiments were loosely adapted from the protocol from Luo et al. (2014). Three iterations of the experiment were performed, as described in Table 5-1. Iteration 0 was the initial experiment performed trying to transfer *bla*$_{KPC}$ from a KPC-positive *E. coli* isolate to a KPC-negative *E. coli* isolate. Iteration I made use of seven individual KPC-producing bacteria, which had all been identified in the WWTP in Objective 1 (see Figure 3-2 in Section 3.3.1) plus a competent J53 *E. coli* laboratory strain as a recipient. Iterations II and III made use of a single KPC-positive donor from previous WWTP sampling plus the lab-grown competent J53 plus sediment bacteria collected from downstream from a municipal WWTP.

Table 5-1. Iterations 0, I, II, and III of the microcosm experiments with the donors and recipients listed for each iteration.

Iteration	Donor	Recipient
0	J53$_{rif}$ *E. coli* w/ *bla*$_{KPC}$	Sediment Bacteria
I	KPCO from WWTP (*K. oxytoca, K. pneumoniae, A. hydrophilia, R. planticola, S. marcescens, C. freundii, Enterobacter cloacae)*	J53 *E. coli*
II	KPCO from WWTP (*R. planticola)*	Sediment Bacteria + J53 *E. coli*
III	KPCO from WWTP (*K. pnuemonia)*	Sediment Bacteria + J53 *E. coli*

In Iteration 0, the donor was the J53$_{rif}$ *E. coli* mated with *bla*$_{KPC}$ and the donor were sediment samples collected from the top 5 cm of the sediment layer from Moores Creek at a

location approximately 150m downstream of the Moores Creek WWTP discharge point. In this initial experiment, *E. coli* stocks were grown overnight in LB broth, then pelleted and resuspended in R2A and then inoculated in triplicate with the sediment for 72 hours at 20°C and mixed at 150 rpm. At 72 hours, 1-mL of sample was taken and processed via quantitative analysis by pipetting 100 µL of sample and serially diluting 1:10 six times with sterile DI water. 10 µL of each dilution was streaked on to a ChromAgar plate, plates that are selective for carbapenem-resistant bacteria, and incubated for 24 hours at 37°C. Then, a random selection of 4 pink pigmented colonies (presumed to be *E. coli*) and 8 non-pigmented colonies (presumed to be sediment bacteria) were taken for each triplicate and analyzed for bla_{KPC} using the methods described in Section 5.2.3.

In Iteration I, the seven bla_{KPC}-positive donor isolates were *Klebsiella pneumoniae, Klebsiella oxytoca, Citrobacter freundii, Raoutella planticola, Serratia marcescens, Enterobacter cloacae,* and *Aeromonas hydrophila*. The donor isolates were all isolated from either patients or the environment in the UVA hospital and have been previously screened and sequenced. Individual stocks of these isolates and the *E. coli* were grown overnight in LB broth (with 1 µg/mL meropenem for the donor isolates). Each of the donor isolates were pelleted and resuspended in R2A media in triplicate and combined with the $J53_{rif}$ *E. coli* recipient at a ratio of 1:7 donor to recipient ratio and inoculated for 12 hours at 20°C and mixed at 150 rpm. Samples were then analyzed using selective plating (as described in Section 2.4). Samples were also plated on LB agar plates at hour 0 (pre-inoculation) to determine the transformation efficiency, which was calculated using the following equation,

$$Transformation\ efficiency = \frac{\#\ of\ transconjugants}{\frac{CFU}{mL} of\ donor\ isolate} * volume\ of\ donor\ isolate$$

In Iteration II, sediment samples were collected from the top 5 cm of the sediment layer from Moores Creek at a location approximately 600m upstream of the Moores Creek WWTP discharge point. Upstream samples were collected to ensure that the samples were initially KPC free before co-inoculation with the ARG donor. The sediment samples were processed via quantitative analysis by pipetting 100 µL of sample and serially diluting 1:10 six times with sterile DI water. 10 µL of each dilution was streaked on to a ChromAgar plate and incubated for 24 hours at 37°C. Sediment samples were also tested via enrichment analyses by vacuum-filtering samples through a 0.22-µm filter and adding that filter to a test tube with 4.5 mL tryptic soy broth (TSB) and a 10-µg ertapenem disk. After the test tubes were incubated for 24 hours at 37°C, 10 µL was streaked onto a ChromAgar plate using an inoculating loop and incubated for another 24 hours at 37°C. Sediment samples were also tested for *bla*$_{KPC}$ using PCR Analysis as described in Section 5.2.3. No pigmented colonies grew on the ChromAgar plates from either the quantitative or enriched plates and the *bla*$_{KPC}$ was below the detectable limit, indicating that the sediment was KPC-negative prior to the sediment microcosms. Sediment samples were also tested on MacConkey plates to identify the number of gram-negative and gram-positive bacteria.

Based on the success of producing J53$_{rif}$ *E. coli* transconjugants from Iteration I, *bla*$_{KPC}$-positive *Raoultella planticola* (CAV 2118) was chosen as the donor for the sediment microcosm experiments in Iteration II and *Klebsiella pnuemoniae* (CAV1016) was chosen for Iteration III. *R. planticola* and *K. pnumoniae* were grown overnight in LB (with 1µg/mL meropenem) and then pelleted and resuspended in R2A with the KPC-negative sediment samples in duplicate. Microcosms were inoculated for 12 hours at 20°C and mixed at 150 rpm and then serially diluted 1:10 six times and streaked on ChromAgar for 24 hours at 37°C. Any pigmented colonies were

subcultured onto sheep's blood agar and analyzed for KPC via PCR. Non-pigmented colonies were subcultured and analyzed using the mCIM method (as described in Section 2.4).

In Iterations II and III, there were three sample conditions run in triplicate: positive controls, consisting of the donor isolate, the sediment, and the $J53_{rif}$ *E. coli*; experimental control, consisting of the donor isolate and the sediment; and a negative control, consisting of the sediment.

5.2.2 Selective Testing

In Iteration I, after 12 hours, microcosm samples were serially diluted six times (1:10) in sterile DI water and plated on selective LB plates (0.5 µg/mL meropenem and 250 µg/mL rifampicin or 600 µg/mL for the *K. pneumoniae* microcosm) and incubated for 24 hours at 37°C. Any colonies that grew on those selective plates were subcultured on sheep's blood agar plates and tested for KPC using PCR. This process was used because only *E. coli* transconjugants should grow on the double selective plates because the addition of 0.5 µg/mL meropenem should select for bacteria that produce carbapenemases, and the 250 µg/mL rifampicin should select only *E. coli* and not any of the donor organisms. This was confirmed prior to the experiment through a wide testing of all recipients, donors, and a known *E. coli* transconjugant that served as a positive control on different concentrations of meropenem plates, rifampicin plates, and meropenem plus rifampicin plates (Figure 5-1). An increased dose of 600 µg/mL rifampicin was used for the microcosms containing the *K. pneumoniae* donor because *K. pneumoniae* grew on plates containing only 250 µg/mL rifampicin.

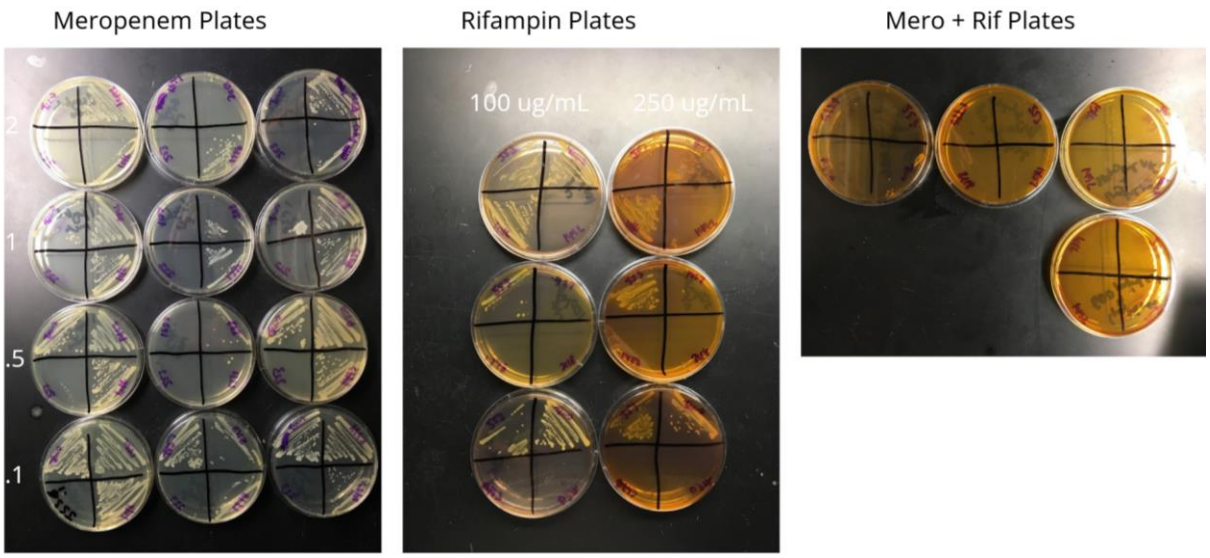

Figure 5-1. Widescale testing of selective plates to ensure that only *E .coli* transconjugants would grow on the LB plates containing meropenem and rifampin.

In Iterations II and III, colonies that grew on ChromAgar were tested using the modified carabapenemase inactivation method (mCIM). 1 µL of each test isolate was added to 2 µL of TSB broth and a 10-µg meropenem disk and incubated for 4 hours at 37°C. A lawn of 0.5 MacFarland solution of Escherichia coli ATCC 25922 was streaked onto a Mueller Hinton agar plate. After 4 hours, the meropenem disks were removed from the tube using a 10-µL inoculating loop and placed onto the Mueller Hinton agar plate and incubated for 24 hours at 37°C and then analyzed for presence or absence of a zone of inhibition around the meropenem disk as follows: cabapenemase-positive if the test isolate inactivates the meropenem in the disk and grows up to the disk; carbapenamase-negative if a zone of inhibition of >20 mm appears around the meropenem disk. Carbapenemase-positive colonies were then also confirmed using PCR.

5.2.3 PCR Analysis

Isolates from the microcosm experiments that grew on either the selective LB plates (Iteration I) or were carbapenemase-producing from the Mueller Hinton plates (Iteration II) were screened for bla_{KPC} via PCR to confirm the presence of the KPC gene. A boil prep extraction was done by placing one colony into 100 µL wells and boiled for 10 minutes. 2 µL of the boiled prep sample was added to a well plate containing 18 µL of a mastermix consisting of reverse and forward KPC primers, PowerUp SYBR Green Mastermix, and nuclease-free water. Samples were run on a Bio-Rad CFX96 Thermal Cycler with a method adapted from a previous study (Dallene et al., 2010)[19] with a positive and negative control and analyzed based on a C_t value of 35 cycles.

Quantitative PCR (qPCR) was done on the sediment samples prior to the microcosm experiment to ensure it was KPC-negative. The samples were extracted using a Qiagen DNEasy PowerSoil HTP 96 Kit. 2 µL of the extracted sample was added to the same mastermix mentioned above. Samples were run in triplicate on the Bio-Rad CFX96 Thermal Cycler against three standards for KPC and 16S (10^4, 10^6, 10^8 copy numbers), which had previously been used to make a standard curve, ranging from 10^2 to 10^9 copy numbers. Copy numbers for each sample were calculated using the equation generated from the standard curve and averaged across the triplicate.

In Iteration I, isolates that grew on the selective LB plates and tested positive for bla_{KPC} were confirmed as *Escheriela coli* transconjugants through species identification via the VITEK2 (Biomerieux, Durham, NC).

5.3 Results and Discussion

5.3.1 Microcosm Iteration 0

My initial hypothesis for Iteration 0 of the microcosm experiment, based on the Luo et al (2014) study involving the NDM-1 gene (Luo et al., 2014), was that bla_{KPC} would be transferred from the donor *E. coli* strain to one or more of the indigenous bacteria in the river sediment. However, this transfer was not observed (Table 5-2). Twelve *E. coli* isolates (the donor strain) were randomly selected from plates inoculated with pre- and post- treatment test solutions. All of these isolates tested positive for KPC at 0 hours. This analysis was repeated at 72 hours and yielded the same result. None of the indigenous sediment bacteria isolates taken from the control, pre-chlorination, or post-chlorination samples tested positive for KPC at either the 0- or 72-hour mark. Species identification was not done for either of the experiments because none of the indigenous bacteria isolates tested positive for KPC.

Table 5-2. Results from Iteration 0 showing the detection of bla_{KPC} in *E. coli* and the native bacteria. All E. coli isolates tested positive for KPC and all of the native bacteria tested negative for KPC.

		Time	
		0 hrs	72 hrs
Control	*E. coli*	N/A	N/A
	Native Bacteria	KPC-	KPC-
Pre-chlorination	*E. coli*	KPC+	KPC+
	Native Bacteria	KPC-	KPC-
Post-chlorination	*E. coli*	KPC+	KPC+
	Native Bacteria	KPC-	KPC-

5.3.2 Microcosm Iteration I

Following the initial Iteration 0 experiment, the methodology was refined prior to the next three iterations to better selectively test for experimental isolates (selective testing for Iteration I and mCIM testing for Iterations II and III).

In Iteration I, seven donors from KPCO observed in the WWTP in Objective 1, were used to see if any transconjugation would occur to our positive control donors, the $J53_{rif}$ *E. coli.* The only microcosms to yield KPC-positive *E. coli* transconjuagnts, (i.e., successful transfer of the plasmid with bla_{KPC} from a donor KPCO to *E. coli)* were the microcosms containing the donor *Raoultella planticola* (Table 5-3). This transformation was consistent across duplicate microcosms on two separate runs of Iteration I. All other microcosms did not exhibit transconjugation (no growth on the selective plates). Successful transfer of bla_{KPC} indicates the potential for the spread of KPC resistance within and downstream of the WWTP if ARBs are able to spill over, as we saw with the *K. oxytoca* strain in Objective 1.

Table 5-3. Results of the transconjugation experiment for Iteration I. Trancosnjugation was observed for the microcosms with one of the donors, *Raoultella planticola.*

Donor	Recipient	Did Transconjugation Occur?
Aeromonas hydrophilia (CAVP338)	$J53_{rif}$ *E. coli*	No
Citrobacter freundii (CAV1857)	$J53_{rif}$ *E. coli*	No
Enterobacter cloacae (CAV1778)	$J53_{rif}$ *E. coli*	No
Klebsiella oxytoca (CAV8493)	$J53_{rif}$ *E. coli*	No
Klebsiella Pneumoniae (CAV1016)	$J53_{rif}$ *E. coli*	No
Raoultella planticola (CAV2118)	$J53_{rif}$ *E. coli*	Yes
Serratia marcescens (CAV1492)	$J53_{rif}$ *E. coli*	No

5.3.3 Microcosm Iteration II

In Iteration II, we used the successful donor from Iteration I, *Raoultella planticola*, as the donor. As we saw in Iteration I, there was successful transfer of the bla_{KPC} from the donor, *R. planticola*, to *E. coli* in the positive control samples. However, there was no transconjugation seen from the *R. planticola* to the native sediment bacteria recipients. However, the number of potential recipients were limited by the growth of the *R. planticola*, which limited the number of native sediment colonies (n = 10), we were able to isolate. Therefore, we repeated this iteration with a decrease in the amount of starting *R. planticola* donors from ~10^8 to 10^6 CFUs/mL in order to provide more ample opportunity for observing transconjugation to occur to the native sediment bacteria colonies, which we will call Iteration IIb.

In Iteration IIb, we were able to isolate more of the native sediment bacteria colonies (n=21). However, all of these colonies were KPC-negative, meaning that once again transconjugation was not seen from the donor to the native sediment bacteria colonies. Similar to Iteration IIa, transconjugation did still occur in the positive control between the *R. planticola* and the *E. coli*.

5.3.4 Microcosm Iteration III

Having not seen transconjugation occur between the donor and the native sediment bacteria in either Iteration IIa or IIb using the *R. planticola* donor, the experiment was repeated using a different potential donor, *Klebsiella pneumoniae*, one of the donors used in the Iteration I experiment. In Iteration III, there was once again no observable transonjugation that occurred between the donor *K. pneumoniae* and the native sediment bacteria as none of the sediment isolates (n =22) picked up the bla_{KPC} gene. However, unlike Iteration IIa and IIb, there was no

transconjugation between the donor *K. pneumoniae* and the *E. coli* in the positive control sample, which is consistent with the findings from Iteration I.

Table 5-4. Results of the Iterations. Far right column lists the result if any transconjugation happened in that iteration.

Iteration	Donor	Recipient	Results
Iteration 0	J53 *E. coli* w/ bla_{KPC}	Sediment bacteria	No transconjugants
Iteration I (selective plating)	7 donors from WWTP	J53 *E. coli*	Transconjugants (*Raoultella -> E. coli*)
Iteration IIa (mCIM)	CAV2118 *Raoultella planticola*	Sediment bacteria, J53 *E. coli*	Transconjugants (*Raoultella -> E. coli*) No sediment transconjugants
Iteration IIb (mCIM)- less donors	CAV2118 *Raoultella planticola*	Sediment bacteria, J53 *E. coli*	Transconjugants (*Raoultella -> E. coli*) No sediment transconjugants
Iteration III (mCIM)	CAV1016 *Klebsiella pneumoniae*	Sediment bacteria, J53 *E. coli*	No transconjugants

5.4 Conclusions

Successful transfer of the KPC plasmid from a *R. planticola* to a competent J53 *E. coli* strain in Iterations I, IIa, and IIb illustrates the potential for concern for regarding dissemination antimicrobial resistance from KPC-producing bacteria to other bacterial recipients in receiving waters of WWTPs. However, ultimately, we did not see the transconjugation of any

environmental sediment bacteria when exposed to the KPCO donors in the microcosm experiments. While transconjugation could potentially still occur naturally downstream of the WWTP, it seems unlikely given the highly inflated concentrations of KPCO donors in the microcosm experiments, whereas we only observed one instance of a KPCO existing in the downstream receiving waters and sediments in all sampling dates in Objective 1. While this is good news from a human health perspective, there is a strong risk of KPCO and other ARB spillover into natural environments, especially at WWTPs where WW disinfection practices may not be as stringent or reliable. Further research is needed to look at other WWTPs than the one described in this dissertation and the use of the microcosm transconjugation study described in this chapter is an excellent tool for realizing the potential risks of ARB dissemination to different microbiological communities of downstream environments.

Chapter 6: Overall Conclusions and Future Study

6.1 Research Conclusions

This dissertation evaluated the risk and potential of the antibiotic resistance spread in the natural environment and the role wastewater treatment plants play, as laid out in the three objectives discussed in this proposal. Objective 1 used KPC-producing *Enterobacterales* as a model for antibiotic resistance fate and behavior through a WWTP. We were able to identify the model WWTP as a potential reservoir for KPC-producing organisms and $bla_{\text{KPC.}}$ KPCO were present throughout all compartments of the WWTP, except for the final effluent, and appear to be largely linked to the hospital WW influent. Although no KPCO were identified in the final effluent of the WWTP, a *K. oxytoca* strain was isolated from downstream receiving waters that was almost identical to *K. oxytoca* strains historically found in the UVA hospital, raising concern that KPCO may be passing through the WWTP. Objective 2 replicated previous literature in analyzing conventional wastewater disinfection technologies in activating ARBs and adapts it to analyzing the removal of KPC-producing bacteria. Chlorination exhibited up to 4.7-log removal of the ARB, while UV irradiation exhibited up to 6.1-log removal. The *E. coli* were able to repair themselves post-UV disinfection, which was aided by exposure to light, either artificial or natural sunlight. Objective 3 took a novel effects-based approach at evaluating the potential for transformation of indigenous bacteria through microcosm transconjugation experiments. We were successful in transferring the KPC plasmid from donor KPCO to J53 *E. coli*, demonstrating potential concern for transconjugation in downstream environments if KPCO are able to escape the WWTP. However, we did not see any transconjugation from donor KPCO to bacteria natively found in the WWTP receiving waters and sediment, making it seemingly unlikely for

transconjugation to occur in this natural environment, however the possibility remains for other WWTPs and their receiving environments.

6.2 Recommendations for Future Study

This dissertation utilized one single WWTP and focuses on KPC-producing bacteria, but has larger implications for our scientific community's collective knowledge on environmental antibiotic resistance spread and the effectiveness of current wastewater technologies are in reducing spread. However, there is more research than can and should be done in future works to better understand the risks of the dissemination of ARBs and ARGs from WWTPs into the native environment. Therefore, I would recommend the following:

1. A more widescale, comprehensive study of KPC-producing bacteria and bla_{KPC} in WWTPs and receiving waters and sediments throughout the United States. Since we were able to identify a *K. oxytoca* isolate in the receiving waters of our model WWTP and significant concentrations of KPC-producing bacteria within the WWTP compartments, it seems very likely other WWTPs in the United States are receiving similar inputs of KPCO, which are potentially being discharged into the receiving environment. A comprehensive study of WWTPs across the U.S. would shed light on the extent to which KPC-producing bacteria are entering natural waters and surrounding environments and their potential risk to cause HAIs in humans coming into contact with these KPCO in the environment.

2. More research on the effectiveness of different disinfection technologies. While there is extensive research on the effectiveness of chlorination and UV disinfection in inactivating different ARBs and ARGs, there is little to no research on KPCO and bla_{KPC}

aside from the experiments done in this dissertation. Additionally, the development of more advanced water treatment technologies provides more potential treatment options for WWTPs, which may be more effective at removing and inactivating ARBs and ARGs than the traditional WWTP disinfection technologies. So, more research needs to be done on the emerging advanced water treatment technologies and their effectiveness in stopping dissemination of ARBs and ARGs as possible alternatives to current WWTP treatments.

3. More future research studies to utilize the effects-based approach of transconjugation experiments to determine the likelihood of risk of KPC dissemination in the receiving environment. Transconjugation experiments offer a definitive approach to seeing if recipient bacteria will pick up resistance features from ARBs or ARGs. Expanding upon the work in this dissertation, I would recommend using multiple locations of WWTP receiving waters and sediments to increase the diversity of the microbiological community. Diversity of microorganisms could lead to more potential recipients of the bla_{KPC} gene. I would also recommend using more potential donors and varying conditions, such as nutrient availability, temperature, sunlight, etc.

This proposed future work would build upon the groundwork laid by this dissertation in providing essential knowledge to the scientific community on the potential for spread of antibiotic resistance to natural environments and waterways through WWTP effluents, which can then be used to better optimize and improve existing and new WWTP technology to minimize the risks of WWTPs as agents in dissemination of antibiotic resistance.

Chapter 7: References

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