ABSTRACT

Antibiotic Resistant Bacteria in an Urban Freshwater Ecosystem in Central Texas

Annie Kwok

Director: Sanghoon Kang, Ph.D.

Antibiotic resistance is a growing concern for the human population due to an increasing occurrence of antibiotic resistance genes in aquatic ecosystems and the risk of pathogenic microorganisms acquiring those resistance genes (ARGs). It is desired to more closely examine the relationship between antibiotic resistant bacteria (ARB) and antibiotic residues in an urban freshwater environment. Thus, the main objective of this study is to investigate the presence of antibiotic resistance in wastewater treatment plant (WWTP) effluent leading into the Brazos River. The study additionally explores possible mechanisms of resistance gene emergence among different antibiotics using classical techniques such as replica plating, Luria-Delbrück's Fluctuation Test, Newcombe Test, and 16S rRNA gene sequencing. According to the Luria- Delbrück Fluctuation Test and the Newcombe Test, different antibiotics appear to be associated with different tendencies of resistance emergence – raising questions about the roles of random mutation and induction. This study provides a baseline understanding of the urban freshwater ecosystem status in central Texas and quantitatively examines the degree of resistance emergences.

APPROVED BY DIRECTOR OF HONORS THESIS:

_____

Dr. Sanghoon Kang, Department of Biology

APPROVED BY THE HONORS PROGRAM:

_____

Dr. Elizabeth Corey, Director

DATE: _____

ANTIBIOTIC RESISTANT BACTERIA IN AN URBAN FRESHWATER ECOSYSTEM IN CENTRAL TEXAS

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

Annie Kwok

Waco, Texas

May 2017

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

The studies and work conducted in this paper were supported by Sanghoon Kang, Michael Davis, Erick LeBrun, Swastika Raut, and Abigail Antrich in the Kang Microbial Ecology Laboratory at Baylor University. Funding was provided by the Jack G. and Norma Jean Folmar Research Grant, the Undergraduate Research and Scholarly Achievement (URSA) initiative at Baylor University, the Baylor University Honors Program, and the Texas Branch of the American Society for Microbiology. This research is in compliance with institutional policies relating to infectious agents.

CHAPTER ONE

Introduction

Since the discovery of penicillin in 1928 (Fleming, 1929; Geddes, 2008), antibiotics have played a crucial role in the fight against pathogens and infections, as well as increasing livestock growth and health (Teuber, 2001). However, recent concerns have become more pressing due to the increasing number of antibiotic resistant pathogens in medical settings across the world – resulting in the loss of viable treatment methods. In an attempt to stifle the alarming rate at which antibiotic resistance has been occurring, researchers have begun to investigate the role of the environment in the spread of antibiotic resistance to both human and animal populations (Kümmerer, 2004). Modern wastewater treatment plants (WWTPs) have been found to serve as an important source of antibiotic resistant bacteria (ARB) and antibiotics (Czekalski, Diez, & Bürgmann, 2014; Kim and Aga, 2007). The primary goals of WWTPs are to remove solids, reduce numbers of pathogens, and sequester nutrients such as organic carbon, nitrogen, phosphorous, and fatty acids; and are not designed to remove antibiotics and other pharmaceuticals that ultimately pollute receiving bodies of water (Golet et al., 2001; Kümmerer, 2004; Zuccato, Calamari, & Natangelo, 2000). Some of these remaining antibiotics are minimally biodegradable in freshwater ecosystems (Al-Ahmad, Daschner, & Kümmerer, 1999), which can result in antibiotic residues of variable concentrations with unknown selective consequences for environmental bacteria (Hirsch et al., 1999).

The presence of antibiotics can select for bacteria already carrying antibiotic resistant genes (ARGs), allowing for preferential growth and propagation of their genome through horizontal gene transfer (HGT) and asexual reproduction (Aminov, 2009; Martinez, 2009). There have been numerous studies within the last several decades which present data on the exacerbation of antibiotic resistance in microbial communities in various sources of freshwater as a result of improperly treated run-offs from large facilities such as hospitals, pharmaceutical production factories, and livestock farms (Anderson and Sobsey, 2006; Chee-Sanford et al., 2001; Parveen et al., 2006; Sapktoa et al., 2007; Sayah et al., 2005). In addition to the problem of antibiotic and pharmaceutical compounds, many treatment measures used in WWTPs may not be effective in removing resistant microbes themselves from sewage influent (Huang et al., 2011; Li et al., 2013; Martins da Costa, Vaz-Pires, & Bernardo, 2006). This allows bacterial contaminants to be flushed into receiving bodies of freshwater (Jury et al., 2011; Michael et al., 2013; Teuber, 2001; Zurfluh, Hachler, & Nuesch-Inderbinen, 2013). These bacterial contaminants enhance the likelihood of spreading ARGs by residing in environments with potential for frequent HGT to downstream aquatic microbiota.

This cycle produces an accumulation of ARGs and ARB, allowing urban water sources to serve as both reservoirs and breeding grounds of resistance (Allen et al., 2010). Urban aquatic ecosystems in Brazil have demonstrated communities capable of tolerating antibiotic concentrations up to 600 times higher than levels in clinical usage (Coutinho et al., 2014). ARGs in aquatic microbes may be relatively harmless to humans when found in non–pathogens, but they can become transferred to pathogens or human and animal commensals (Cantas et al., 2013; Czekalsi, Diez, & Bürgmann, 2014). When resistance

occurs, especially in opportunistic pathogens, it can serve as a risk to human health when affected freshwater sources are used for human consumption and recreation. Infections become increasingly dangerous as readily available and widely used treatments may no longer be effective (Bush et al., 2011) with the accumulation and spread of antibiotic resistance.

Many of the aforementioned studies in freshwater ARB and ARG presence have been conducted in Europe and Asia, however, there have been no such studies for urban aquatic ecosystems in central Texas. This research gap highlights the lack of information on antibiotic presence and resistance in important Texas watersheds, and as a result, the severity of ARB proliferation needs to be studied to gain a grasp on the current situation. The research objective was to investigate the current status of ARB in a central Texas urban aquatic ecosystem, and the possibility of antibiotics in WWTP influent and effluent genetically influencing and selecting for ARB in an urban freshwater environment.

CHAPTER TWO

Materials and Methods

Sampling

Water samples were collected from the Waco Metropolitan Area Regional Sewage System (WMARSS). WMARSS is a joint wastewater treatment plant that serves eight urban cities with an average flow of 37.8 million gallons per day. The effluent from the treatment plant leads into the Brazos River. In addition to the WMARSS, other local watersheds entering the Brazos River near Waco include several cattle pastures, other smaller treatment subsets of WMARRS, and an artificial wetland ecosystem constructed by the city of Waco north of the Brazos River.

Four 500 mL samples of influent and effluent were collected in Nalgene™ Lab Quality Amber HDPE Wide Mouth bottles. The bottles were set in ice immediately after collection and transported to the laboratory to be placed in refrigeration, and were processed within 48 hours. Sampling was carried out over four weeks, taken once a week in the afternoon.

Isolation of Antibiotic Resistant Bacteria

Two types of media were used: Trypticase Soy Agar (TSA) to enumerate general growth and Eosin Methylene Blue (EMB) to enumerate coliform growth. Each influent sample was processed into 10^{-4} and 10^{-6} dilutions; 100 μL of these dilutions were plated onto one TSA and one EMB plate, respectively. 250 mL of each effluent sample was filtered on Pall GN-6 Metricel® MCE Membrane Disc Filters to concentrate the bacteria.

The filter was then vortexed for 5 minutes at full speed in conical polypropylene tubes with 10 mL of the respective effluent sample for five minutes to release the bacteria into the solution, with 10 μL of this solution plated onto one TSA and one EMB media plate. These four plates comprised the master plates for each sampling date. All EMB plates were incubated for 24 hours at 37 ^{o}C and all TSA plates for 24 hours at room temperature.

Seven classes of antibiotics were chosen based on common use in medicine and agriculture, and were used to create antibiotic infused TSA and EMB media culturing plates. Concentration values for each antibiotic (Table 1) were referenced from minimum bactericidal concentrations (MBCs) determined by an article that has established MBCs for a common gut microbe (Ingham et al., 1968). For each type of sample master plate – influent-TSA, influent-EMB, effluent-TSA, effluent-EMB – seven antibiotics were placed on sterile TSA and EMB media culturing plates and left for several hours to sit and absorb into the agar. Using velvet, the master plates were replica plated onto the antibiotic infused plates. A total of 28 antibiotic plates were incubated for 24 hours, at 37 ^{o}C for EMB plates and room temperature for TSA plates. Bacterial colony growth on each antibiotic plate were deemed resistant to the specific antibiotic and recorded.

Antibiotic Class	Antibiotic Used	Media Concentration (μg/mL)
Beta-Lactam	Penicillin	128
Fluoroquinolone	Ciprofloxacin	1.0
Macrolide	Erythromycin	128
Anisoles	Trimethoprim	0.298
Tetracycline	Tetracycline	128
Sulfonamide	Sulfamethoxazole	7.15
Aminoglycoside	Gentamicin	6250

Table 1: Antibiotic Concentrations Used in Media.

The Luria-Delbrück Fluctuation Test

During the isolation process, each set of antibiotic infused plates were compared to their respective master plates and a colony that was observed to be susceptible to all seven antibiotics was chosen from each master plate. A first round of pure culture was inoculated on a single plate of respective media using this colony, incubated for 24 hours as before on EMB and TSA plates. Using a random colony from the first round, a second round of pure culture was inoculated on a single plate of respective media and incubated again with the same conditions. This process was repeated, using the previous round to inoculate the next round, until the fifth round. The fifth round was used to inoculate seven antibiotic infused media plates and then incubated. This last procedure was replicated five times to produce 35 plates, and the number of antibiotic resistant colonies were observed and recorded.

The Luria–Delbrück experiment intends to test two possibilities: induced or spontaneous mutation (Luria and Delbrück, 1943; Smith et al., 2015). The repeated culturing between each generation provides time for mutation to occur during cell division. If the mechanism for antibiotic resistance in bacteria is induction by antibiotics, the number of colonies between the five sets of antibiotic infused plates from the final step should not vary greatly. However, if antibiotic resistance in bacteria is due to spontaneous mutation, then a mutation can occur at any point in the culturing process – either in earlier generations or later generations. This should produce a large amount of variance in colony number between each set of antibiotic infused plates. Levene's test of equality of variances was used to test the significance of results from the Luria-Delbrück test.

The Newcombe Test

The fifth round from each respective sample and plate was used to additionally inoculate seven respective antibiotic infused plates. The plates were incubated at their respective temperatures for 24 hours and then re-spread before being incubated for another additional 24 hours. If the colonies and bacterial cells had spontaneously mutated prior to exposure to the antibiotics, then the re-spread plate should have a higher number of bacteria present due to the moved bacterial cells forming new colonies of their own (Newcombe, 1949). Analysis of variance (ANOVA) was used to test the significance of results from the Newcombe test.

16S rRNA Gene Sequencing

Using the Qiagen DNeasy® Blood and Tissue Kit, DNA was extracted from resistant isolates of the final sets of antibiotic infused plates from the Luria-Delbrück's experiment cultured in BD Difco™ Nutrient Broth. The extracted DNA was run on 1% agarose gel at 85V at 115mA for 45 minutes in order to ensure bacterial DNA was intact. Using the isolated DNA, a 25 µL PCR reaction mixture consisting of 200 µM primers (universal primers set 27F-1492R), 1 µL of template DNA (~1 ug), 9.5 µL of DNA safe water, and 12.5 µL of Amaresco® Hot Start PCR-toGel TAQ PCR Master Mix 2X was made for each sample.

The PCR was run on the Applied Biosystems® Veriti ® 96-Well Thermal Cycler, beginning at 96°C for five minutes, then 30 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for one minute, and extension at 72°C for two minutes. The PCR was finished with a seven-minute final extension at 72°C. Products were run on a 1% agarose gel at 85V at 115mA for 45 minutes to ensure presence, with quantity confirmed using

NanoDrop 2000 (Thermo Scientific). PCR products were sent for sequencing to Macrogen USA (Rockville, MD), and the sequence results were analyzed through the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) and the Ribosomal Database Project's (RDP) SeqMatch tool.

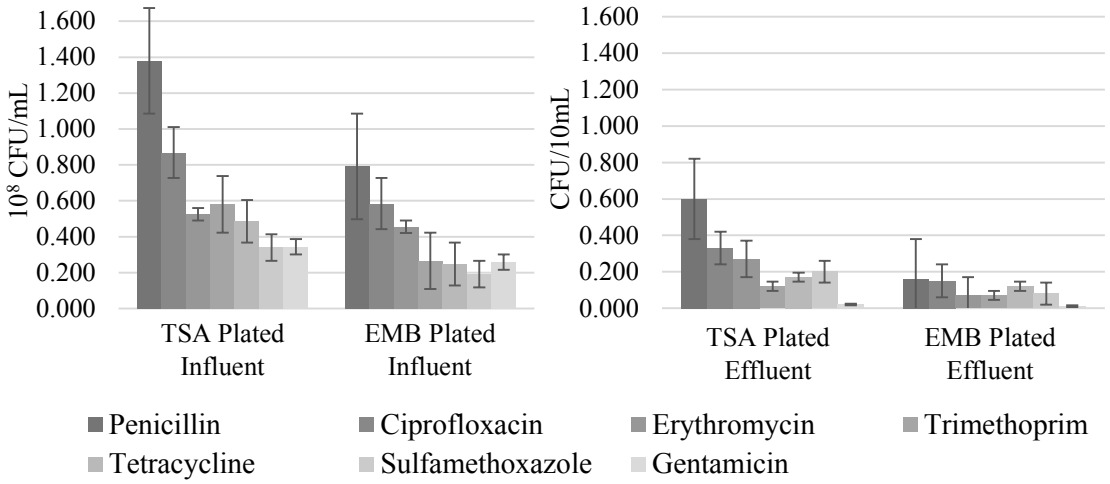
CHAPTER THREE

Results

Isolation of Antibiotic Resistant Bacteria

In the replicate-plate antibiotic resistance assay, total culturable count of ARB drastically decreased post-treatment in effluent in comparison to influent (Figure 1.a). However, while overall culturable count of the samples had decreased in the effluent by 10^7 CFU/mL, percentages of resistant bacterial colonies in comparison to total bacterial colonies on the master plate were comparable (Figure 1.b). The penicillin-infused media agar plates had the highest percentage of surviving bacteria grown across all four types of samples when compared to the master plates. Resistance to ciprofloxacin produced the second highest percentages among the samples, with the exception of influent-EMB. Erythromycin-infused media agar plates generally had the third highest percentage of surviving bacteria followed loosely by trimethoprim, tetracycline, and sulfamethoxazole. Gentamicin-infused media agar plates had the lowest percentages of resistance.

a



b

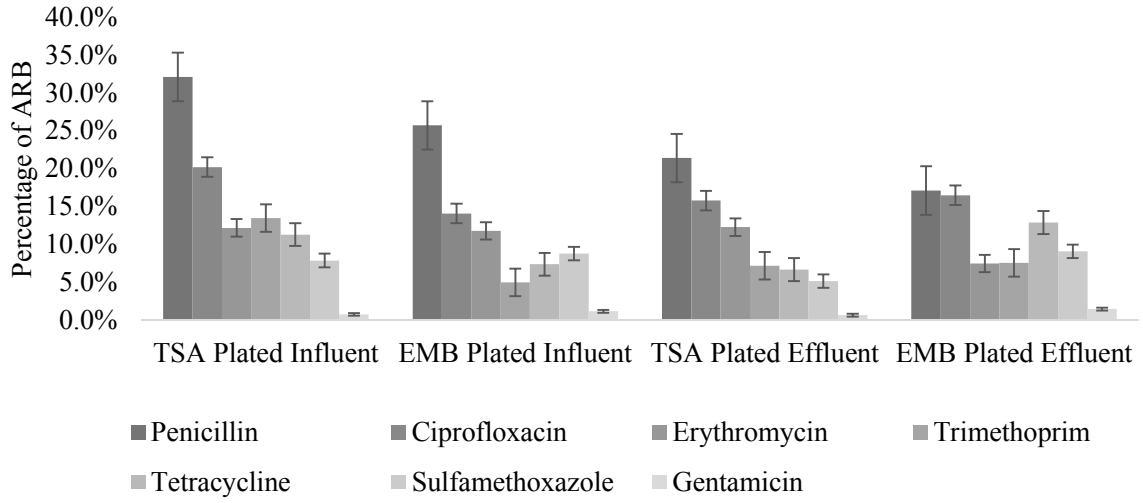


Figure 1: Results of the Replica-Plate Antibiotic Resistance Assay. Error bars indicate standard error of the means. (a) Total culturable count (colony forming unit per unit volume) of surviving bacteria from the replicate-plate antibiotic resistance assay. (b) Percentage of surviving bacteria in comparison to the master plates from the replicate plate antibiotic resistance assay.

The Luria-Delbrück Fluctuation Test

Bacterial colony forming units (CFUs) were observed and recorded across all five sets of plates within each sampling group. CFUs of respective antibiotic-infused plates from each set were averaged and the deviation of each plate from the mean was calculated. To display the variability in resistant colonies for each antibiotic, the range of bacterial colony count deviation from the mean was used and the significance was tested using Levene's test (Figure 2). The range in bacterial CFU among the antibiotic-infused plates did not have a clear trend, with ranges for each antibiotic sometimes varying widely. However, trimethoprim, tetracycline and sulfamethoxazole produced consistently low range values in comparison to other antibiotics. Levene's test showed significant differences in variances between the lower variance (trimethroprim, tetracycline and sulfamethoxazole) and the higher variance group (rest of antibiotics); in which only influent samples on TSA media had marginal significance ($P = 0.070$) while other set of samples showed much stronger significance ($P < 0.001$).

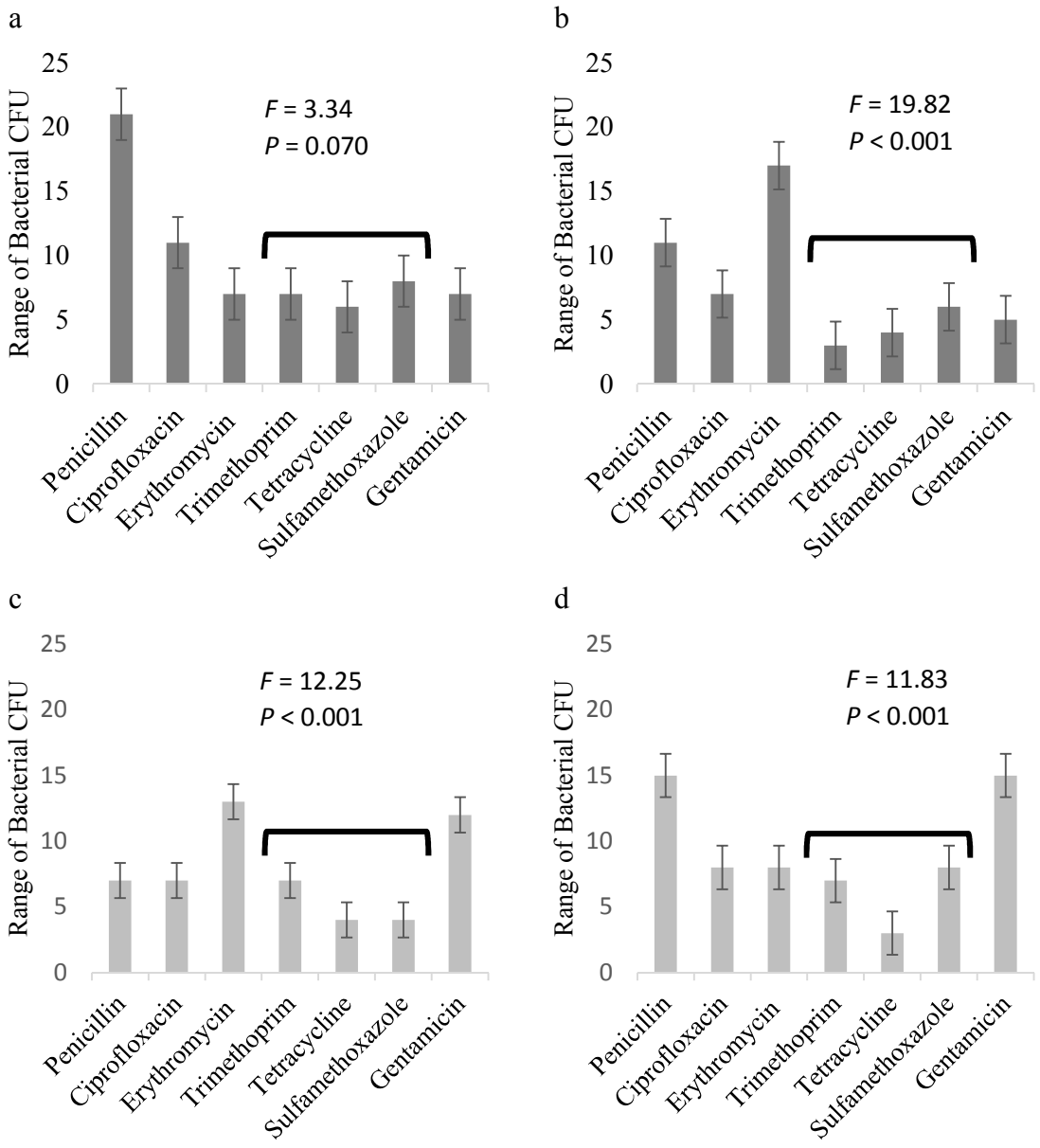


Figure 2: Range in deviation of bacterial colonies from mean colony count across respective antibiotic-infused plates. Accompanied statistical results are by Levene's test of equality of variances between lower and higher variance group. Error bars indicate standard error of the mean. (a) TSA Plated Influent (b) EMB Plated Influent (c) TSA Plated Effluent (d) EMB Plated Effluent

The Newcombe Test

In order to compare the number of CFUs on plates before and after the re-spreading process, the difference in the number of colonies formed before spreading and after spreading was calculated (Figure 3). Overall tetracycline, trimethoprim and sulfamethoxazole are among the lowest in CFU difference, but there was no statistical significance between this group from rest of antibiotics. An ANOVA was not able to reject the null hypothesis for the global test of significance as well as the pairwise comparisons (Tukey's method) at $\alpha = 0.05$.

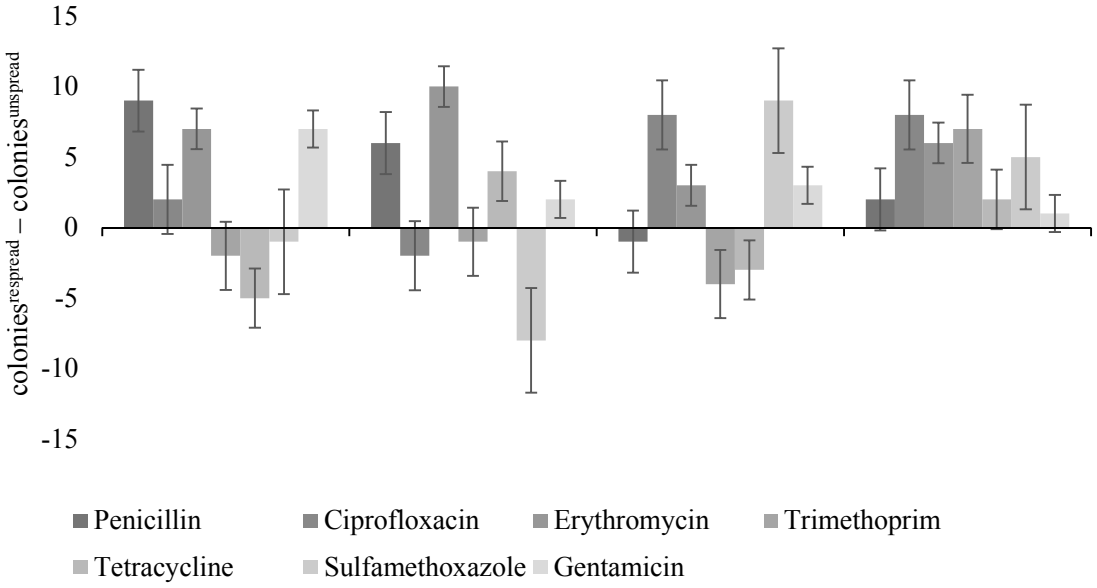


Figure 3: Difference between number of colonies grown on re-spread plates and un-spread plates. Error bars indicate standard error of the means.

16S rRNA Gene Sequencing

Using NCBI's BLAST and RDP's SeqMatch tool, 16S rRNA gene sequencing results from the final round of the Luria–Delbrück Fluctuation Test were analyzed. Gentamicin infused plates did not produce the growth of any resistant isolates.

All identified bacteria fell under the phylum γ-*Proteobacteria,* and included the genera *Klebsiella, Enterobacter,* and *Aeromonas* (Figure 4). Several bacterial species were identified (Table 2).

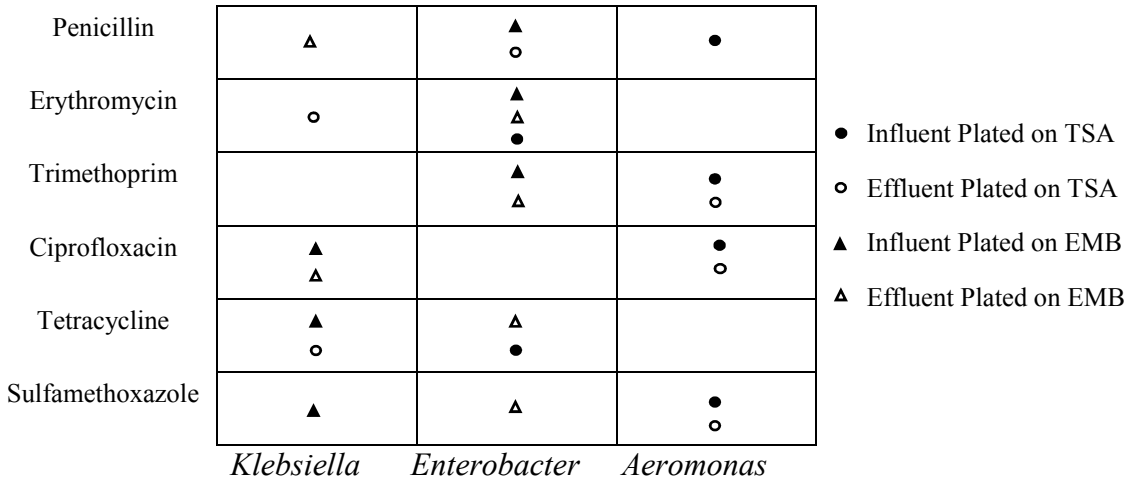


Figure 4: Distribution of different cultured samples and media among detected genera from 16S rRNA gene sequencing.

Type of Sample	Type of Media	Resistance to
Klebsiella pneumoniae and Klebsiella variicola		
Influent	EMB	trimethoprim, ciprofloxacin, tetracycline, sulfamethoxazole
Effluent	TSA	erythromycin
	EMB	penicillin and sulfamethoxazole
Enterobacter cancerogenus		
Influent	TSA	erythromycin, tetracycline
Effluent	TSA	penicillin
Enterobacter cloacae		
Influent	EMB	penicillin
Aeromonas jandaei		
Influent	TSA	penicillin, trimethoprim, ciprofloxacin
Effluent	TSA	sulfamethoxazole
Aeromonas ve onii and *Aeromonas hydrophila*		
Influent	TSA	sulfamethoxazole
Effluent	TSA	ciprofloxacin

Table 2: Bacterial Species Identified in Resistant Isolates. Isolates were taken from the Luria–Delbrück Fluctuation Test.

CHAPTER FOUR

Discussion

We carried out these experiments to investigate the current situation of antibiotic resistance in a central Texas urban freshwater ecosystem, particularly in an area of the Brazos River where the WMARRS operation releases the effluent. Minimal bactericidal concentrations (MBCs) of antibiotics in media were used to produce a selective environment in which resistant bacteria would be able to survive and grow, allowing for the observation of active ARB. Results demonstrated that antibiotic resistance was clearly exhibited in influent sewage leading into the treatment plant as well as treated effluent water leading into the Brazos River, in both generally grown and coliform-selective bacteria (Figure 1). The most notable resistance was to common antibiotics, including penicillin, fluoroquinolone, and macrolide. It is notable that the third most common type of antibiotic resistant bacteria in TSA-plated effluent are resistant to an antibiotic that has been (repeatedly) detected in WMARRS effluent – erythromycin (Bryan Brooks, personal communication). Fluoroquinolone and macrolide were not detected in WMARRS effluent, although bacteria resistant to them were among the most abundant. It is possible that there may be temporal variations in antibiotic concentration, and the particular sampling might have missed the overall trend which may be responsible for the high resistant bacteria abundance. Further studies including that possibility are being planned. Treatment provided by the WWTP was still effective in reducing overall total culturable ARB between influent and released effluent.

Despite being an effective plant to reduce solid waste, nutrients, and pathogenic microbes, the WMARRS, as with most WWTPs (Huang et al., 2011; Li et al., 2013; Martins da Coasta, Vaz-Pires, & Bernardo, 2006), was ineffective in removing the presence of ARB in the effluent samples. In fact, a higher percentage of ARB were found in effluent in some cases, which has also been reported by several previous studies (Huang et al., 2011; Li et al., 2013; Martins da Coasta, Vaz-Pires, & Bernardo, 2006) possibly due to the increased opportunity of horizontal gene transfer within the WWTP. This presents an alarming issue, as accumulation of resistant bacteria in this freshwater ecosystem can create a significant reservoir for the spread and persistence of antibiotic resistance in the environment. As most WWTPs are not designed to remove antibiotics, pharmaceuticals, and other personal care products, the ineffectiveness of reducing antibiotics have been noted in other areas in the world (Batt and Aga, 2005; Carballa et al., 2004; Costanzo, Murby, & Bates; 2005; Miao et al., 2004).

The detection of antibiotic residues creates the possibility for an induction of mutation mechanisms, in addition to selective pressure, increasing the prevalence of antibiotic resistance in the environment. Mutation tests, the Luria–Delbrück Fluctuation Test and the Newcombe Test, were used to test this possibility and to serve as a premise for any future experiments to come. In the Luria–Delbrück Fluctuation Test, a lower range in deviation from mean colony count would indicate an induction mechanism due to antibiotic selective pressures, whereas a larger range in deviation would indicate spontaneous mutation occurring through replication and cell division. Although there was no significant trend for the majority of antibiotic residues tested, some antibiotics such as trimethoprim, tetracycline and sulfamethoxazole may be more pre-disposed to being

induced as they were consistently similar in colony count (Figure 2). Additionally, the fact that antibiotic resistance emerged from antibiotics at MBCs with five rounds of incubation indicates realistic possibilities in the aquatic environment in which conditions are not far from what was used in the Luria–Delbrück Fluctuation Test. Results from the Newcombe Test were not conclusive (Figure 3). After taking the difference between number of colonies on the un-spread plates and the re-spread plates, a positive number indicated that the re-spread plate had a greater number of colony forming units. A higher difference would be indicative of spontaneous mutation, whereas a lower difference would be indicative of induced mutation. Again, the lowest numbers in CFU differences were found with trimethoprim, tetracycline and sulfamethoxazole. There was no clear trend between samples and media, which may have been due to the inadequate number of replicates, which points to the need for follow-up experimentation to determine whether the presence of antibiotics and other factors could be altering the mutation rate leading to these resistance genotypes.

In the final part of the study, 16S rRNA gene sequencing was used to provide a potential list of ARB that have emerged quickly under strong antibiotic concentration pressures. The presence of γ-*Proteobacteria* was expected, as it is one of the main bacterial phyla present in the human gut microbiome (Khanna and Tosh, 2014; The Human Microbiome Project Consortium, 2012). However, the types of agar media used may have been more selective than was originally anticipated, resulting in little diversity in the isolated cultures. Among the identified species, many were opportunistic and commensal pathogenic microbes, and multidrug resistant. Influent plated on penicillin and ciprofloxacin –infused TSA media, and effluent plated on sulfamethoxazole –infused EMB

media were identified as *Aeromonas jandaei* strain ASH05 (GenBank accession number KU725738), a multi-drug resistant pathogenic strain isolated post-flood in Chennai (unpublished). A species of *Aeromonas* identified in influent cultured on trimethoprim and ciprofloxacin –infused TSA plates, and effluent cultured on sulfamethoxazole – infused TSA plates (GenBank accession number EU260204), has been referenced in a study examining the antimicrobial resistance in gram-negative bacteria in a lake under distinct anthropogenic influence (Pontes et al., 2009). In effluent cultured on penicillin-infused TSA, an environmental *Enterobacter* species (GenBank accession number EU420931) has been cited in a study exploring the incidence of extended spectrum beta-lactamases (ESBL), and plasmid-mediated AmpC betalactamase genes and integrons in a eutrophic bay (unpublished). Sequence matches for *Klebsiella pneumoniae* involving multi-drug resistance and nosocomial infections (GenBank accession numbers CP019772, CP017985, CP015392) were found from influent bacteria isolated on tetracycline and sulfamethoxazole –infused EMB media (Ruppé et al., 2017). Effluent cultured on ciprofloxacin-infused EMB media produced other identified strains of *Klebsiella pneumoniae* that exhibit antibiotic resistance in the environment (GenBank accession numbers KJ806466 and KP297443, unpublished).

A wide variety of identified strains isolated in this experiment had resistance profiles which matched some of the antibiotics detected at WMARRS; and with strains known for antibiotic resistance, presence in WWTPs, pathogenic potential, and nosocomial infections. All of the identified bacteria are in the clinically relevant genera *Klebsiella, Enterobacter,* and *Aeromonas*, with specific species known for lethal virulence factors (Grim, 2014; Sayah et al., 2005). This supports the idea that the natural environment and

WWTPs can be a significant reservoir for the exchange and maintenance of antibiotic resistance, either through horizontal gene transfer or possible selective pressures (Luria and Delbrück, 1943; Zhang, Zhang, & Ye, 2011). Despite antibiotic concentrations used in this study being minimum bactericidal concentrations for common gut microbes, numerous microbes were still cultured in effluent samples leading into a freshwater ecosystem. If freshwater sources containing multi-drug resistant pathogens are intended for anthropogenic use, it can serve as an alarming issue. Recently, a study has examined drinking water in six states in the United States and identified the presence of CTXM (an extended-spectrum β-lactamase) and OXA-48 (a carbapenemase) genes (Tanner et al., 2017).

Using culturing techniques, antibiotic resistant bacteria have been characterized at a WWTP in central Texas and the freshwater ecosystem receiving its effluent. There are substantial percentages of bacteria discovered to be resistant to most antibiotics, penicillin being the most abundant and gentamicin being the last abundant. The Luria–Delbrück Fluctuation and Newcombe Tests indicate certain antibiotics having particular mechanisms of mutation and evolution, or that resistance may arise through a mixture of induction and random mutation. The identified resistant isolates that rapidly emerged from the experiment have presented findings that include known pathogens with multi-drug resistance, which imposes this WWTP and urban aquatic ecosystem in central Texas as a potential risk. Future work in this area includes more detailed examinations of mutation emergence through more extensive mutation tests, general gene sequencing of samples taken from WMARRS, looking at other freshwater ecosystems affected by anthropogenic

activity in central Texas, and examining other commonly used antibiotics as selective pressures in resistance reservoirs.

BIBLIOGRAPHY

Al-Ahmad, A., Daschner, F., & Kümmerer, K. 1999. Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G, and sulfamethoxazole and inhibition of waste water bacteria. *Archives of Environmental Contamination and Toxicology* 37(2):158-63.

Allen, H., Donato, J., Wang, H., Cloud-Hansen, K., Advies, J., & Handelsman, J. 2010. Call of the wild: antibiotic resistance genes in natural environments. *Natural Reviews Microbiology* 8:251-9.

Aminov, R. 2009. The role of antibiotics and antibiotic resistance in nature. *Environmental Microbiology* 11(12):2970-2988.

Anderson, M., & Sobsey, M. 2006. Detection and occurrence of antimicrobially resistant E. coli in groundwater on or near swine farms in eastern North Carolina. *Water Science & Technology* 54(3):211-8.

Batt, A. L., Aga, D. S. 2005. Simultaneous analysis of multiple classes of antibiotics by ion trap LC/MS/MS for assessing surface water and groundwater contamination. *Analytical Chemistry* 77:2940-2947.

Bush, K., Courvalin, P., Dantas, G., Davis, J., Eisenstein, B., Huovinen, P. 2011. Tackling antibiotic resistance. *Nature Reviews Microbiology* 9(12):894-6.

Cantas, L., Shah, S., Cavaco, L., Manaia, C., Walsh, F., & Popowska, M. 2013. A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota. *Frontiers in Microbiology* 4:96.

Carballa, M., Omil, F., Lema, J. M., Llompart, M., Garcia-Jares, C., Rodriguez, I., Gomez, M., Ternes, T. 2004. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Resesarch* 38:2918-2926.

Chee-Sanford, J., Aminov, R., Krapac, I., Garriques-Jeanjean, N., & Mackie, R. 2001. Occurence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Applied and Environmental Microbiology* 67(4):1494-502.

Costanzo, S. D., Murby, J., Bates, J. 2005. Ecosystem response to antibiotics entering the aquatic environment. *Marine Pollution Bulletin* 51:218-223.

Coutinho, F., Silveira, C., Pinto, L., Salloto, G., Cardoso, A., Marins, O., Clementino, M. 2014. Antibiotic resistance is widespread in urban aquatic environments of Rio de Janeiro, Brazil. *Microbial Ecology* 68(3):441-52.

Czekalski, N., Diez, E. G., & Burgmann, H. 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. *International Society for Microbial Ecology* 8:1381-1390.

Fleming, A. 1929. On the antibacterial action of cultures of a Pencillium, with special reference to their use in the isolation of B. influenzae. *British Journal of Experimental Pathology* 10:226-236.

Geddes, A. 2008. 80th Anniversary of the discovery of penicillin: An appreciation of Sir Alexander Fleming. *International Journal of Antimicrobial Agents* 32(5):373.

Golet, E., Alder, A., & Hartmann, A. 2001. Trace determination of fluoroquinolone antibacterial agents in urban wastewater by solid-phase extraction and liquid chromatography with fluorescence detection. *Analytical Chemistry* 73(15):3632-8.

Grim, C. J., Kozlova, E. V., Ponnusamy, D., Fitts, E. C., Sha, J., Kirtley, M. L., van Lier, C. J., Tiner, B. L., Erova, T. E., Joseph, S. D., Read, T. D., Shak, J.R., Joseph, S. W., Singletary, E., Felland, T., Blaze, W. B., Horneman, A. J., Chopra, A. K. 2014. Functional Genomic Characterization of Virulence Factors from Necrotizing Fasciitis-Causing Strains of Aeromonas hydrophila. *Applied and Environmental Microbiology* 80(14): 4162-4183.

Hirsch, R., Thomas, T., Haberer, K., & Kratz, K. L. 1999. Occurrence of antibiotics in the aquatic environment. *The Science of Total Environment* 12;225(1-2):109-118.

Huang, J. J., Hu, H. Y., Tang, F., Li, Y., Lu, S. Q., Lu, Y. 2011. Inactivation and reactivation of antibiotic-resistant bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant. *Water Research* 45(9):2775-2781.

Ingham, H. R., Selkon, J. B., Codd, A. A., & Hale, J. H. 1968. A study in vitro of the sensitivity to antibiotics of Bacteroides fragilis. *Journal of Clinical Pathology* 21:432-436.

Jury, K., Khan, S., Vancov, T., Stuetz, R., & Ashbolt, N. 2011. Are sewage treatment plants promoting antibiotic resistance? *Critical Reviews in Environmental Science and Technology* 41:243-270.

Khanna, S., Tosh, P. K. 2014. A Clinician's Primer on the Role of the Microbiome in Human Health and Disease. *Mayo Clinic Proceedings* 89(1):107-114.

Kim, S., & Aga, D. S. 2007. Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *Journal of Toxicology and Environmental Health* 10(8):559-573.

Kümmerer, K. 2004. Resistance in the environment. *Journal of Antimicrobial Chemotherapy* 54(2):311-320.

Kümmerer, K. 2009. The presence of pharmaceuticals in the environment due to human use - present knowledge and future challenges. *Journal of Environmental Management* 90(8):2354-2366.

Li, D., Zeng, S., Gu, A. Z., Miao, H., Shi, H. 2013. Inactivation, reactivation and regrowth of indigenous bacteria in reclaimed water after chlorine disinfection of a municipal wastewater treatment plant. *Journal of Environmental Sciences* 25(7):1319-1325.

Luria, S. E., Delbrück, M. 1943. Mutations of Bacteria from Virus Sensitivity to Virus Resistance. *Genetics* 28(6): 491–511.

Ma, L., Zhang, X. X., Zhao, F., Wu, B., Cheng, S., et al. 2013. Sewage treatment plant serves as a hot-spot reservoir of integrons and gene cassettes. *Journal of Environmental Biology* 34(2 Spec No):391-9.

Martinez, J. 2009. The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proceedings of the Royal Society of London B: Biological Sciences* 276(1667):2521-2530.

Martins da Costa, P., Paulo, V. P., Bernardo, F. 2006. Antimicrobial resistance in Enterococcus spp. isolated in inflow, effluent, and sludge from municipal sewage water treatment plants. *Water Research* 40(8):1735-1740.

Miao, X. S., Bishay, F., Chen, M., Metcalfe, C. D. 2004. Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. *Environmental Science and Technology* 38:3533-3541.

Michael, I., Rizzo, L., McArdell, C., Manaia, C., Merlin, C., Schwartz, T., & Fatta-Kassinos, D. 2013. Urban freshwater treatment plants as hotspots for the release of antibiotics in the environment: A review. *Water Research* 47(3):957-955.

Newcombe, H. B. 1949. Origin of Bacterial Variants. *Nature* 164:150-151.

Parveen, S., Lukasik, J., Scott, T., Tamplin, M., Portier, K., Sheperd, S., Braun, K., Farrah, S. 2006. Geographical variation in antibiotic resistance profiles of Escherichia coli isolated from swine, poultry, beef and dairy cattle farm water retention ponds in Florida. *Journal of Applied Microbiology* 100(1):50-57.

Pontes D. S., Pinheiro F. A., Lima-Bittencourt C. I., Guedes R. L., Cursino L., Barbosa F., Santos F. R., Chartone-Souza E., Nascimento A.M. 2009. Multiple antimicrobial resistance of gram-negative bacteria from natural oligotrophic lakes under distinct anthropogenic influence in a tropical region. *Microbial Ecology* 58(4):762-772.

Ruppe, E., Olearo, F., Pires, D., Baud, D., Renzi, G., Cherkaoui, A., Goldenberger, D., Huttner, A., Francois, P., Harbarth, S. and Schrenzel, J. 2017. Clonal or not clonal? Investigating hospital outbreaks of KPC-producing Klebsiella pneumoniae with whole-genome sequencing. *Clinical Microbiology and Infection* S1198-743X(17)30048-4.

Sapktoa, A. R., Curriero, F. C., Gibson, K. E., & Schwab, K. J. 2007. Antibiotic-Resistant Enterococci and Fecal Indicators in Surface Water and Groundwater Impacted by a Concentrated Swine Feeding Operation. *Environmental Health Perspectives* 115(7):1040-1045.

Sayah, R., Kaneen, J., Johnson, Y., & Miller, R. 2005. Patterns of antimicrobial resistance observed in Escherichia coli isolates obtained from domestic-and wild-animal fecal samples, human septage, and surface water. *Applied and Environmental Microbiology* 71(3):1394-1404.

Sha, J., Rosenzweig, J. A., Kozlova, E. V., Wang, S., Erova, T. E., Kirtley, M. L., van Lier, C. J., Chopra, A. K. 2013. Evaluation of the roles played by Hcp and VgrG type 6 secretion system effectors in Aeromonas hydrophila SSU pathogenesis. *Microbiology* 159:1120-1135.

Smith, G.P., Golomb, M., Billstein, S.K., Smith, S.M. 2015. The Luria-Delbrück Fluctuation Test as a Classroom Investigation in Darwinian Evolution. *The American Biology Teacher* 77:614-619.

Tanner, W. D., VanDerslice, J. A., Goel, R. K., Gundlapalli, A. V. 2017. CTX-M and OXA-48 Genes in U.S. Drinking Water. *ASM Conference on Innovative Microbial Ecology for Mitigation of Antibiotic Resistance and Bacterial Diseases*, 3/22-25, 2017, Crystal City, VA.

Teuber, M. 2001. Veterinary use and antibiotic resistance. *Current Opinion in Microbiology* 4(5):493-499.

The Human Microbiome Project Consortium. 2012. Structure, function and diversity of the healthy human microbiome. *Nature* 486:207-214.

Zhang, T., Zhang, X. X., Ye, Lin. 2011. Plasmid Metagenome Reveals High Levels of Antibiotic Resistance Genes and Mobile Genetic Elements in Activated Sludge. *PloS One* http://dx.doi.org/10.1371/journal.pone.0026041

Zuccato, E., Calamari, D., & Natangelo, M. 2000. Presence of therapeutic drugs in the environment. *Lancet* 355(9217):1789-90.

Zurfluh, K., Hachler, H., Nuesch-Inderbinen, M., & Stephan, R. 2013. Characteristics of extended-spectrum b-Lactamase and Carbapenemase-producing Enterobacteriaceae isolates from rivers and lakes in Switzerland. *Applied Environmental Microbiology* 79(9):3021-3026.