THE CHARACTERIZATION, OCCURRENCE, AND MOBILITY OF ANTIBIOTIC RESISTANT BACTERIA, ANTIBIOTIC RESISTANCE GENES, AND PLASMIDS ISOLATED FROM URBAN WASTEWATER TREATMENT PLANTS

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Amir H. Tehrani

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The characterization, occurrence, and mobility of antibiotic resistant bacteria, antibiotic resistance genes, and plasmids isolated from urban wastewater treatment plants.
Amir H. Tehrani, Ph.D. 2020, Molecular Science, Ryerson University.

Abstract

Throughout the decades, there has been an increase in the prevalence and the spread of antibiotic resistance genes (ARGs) within the bacterial populations found in various environments. The continual use of antibiotics has contributed to a higher state of resistance in microorganisms and is recognized as a threat to global public health. The spread of ARGs within a bacterial population is still poorly understood, although, wastewater treatment plants (WWTPs) have been recognized as 'hotspots' for the dissemination and proliferation of ARGs. More research is required to further our understanding of the occurrence and mobility of antibiotic resistant bacteria (ARB), ARGs, and plasmids within densely populated microbial environments such as in WWTPs.

The first objective investigated the presence of tetracycline resistant and tetracycline sensitive bacteria and characterized them based on identity, morphology, and antibiotic resistance patterns. It was found that tetracycline resistant and sensitive populations differed greatly in composition. In addition, isolates that were resistant to tetracycline were more likely to carry resistances to other antibiotics, unlike sensitive ones. The knowledge acquired from this research will shed light upon resistance patterns and routes that can occur in a complex WWTP microbial population.

The second objective investigated the presence, host range, and characterization of plasmids found in both antibiotic resistant and sensitive isolates. The members belonging to the phylum Enterobacteriaceae were found to be the main carriers of plasmids. Numerous plasmids with conjugative properties, type secretion systems, antibiotic resistance genes, and virulence

factors were identified among the selected cultures. The genetic information obtained will contribute to the understanding of the role plasmids play in the mobility, host range, metabolic function, virulence, and the spread of ARGs.

The last objective monitored the conjugative transfer of two novel plasmids, pNT36-3, and pNT36-4, between two environmental strains of *Escherichia coli* NT36 and EB-G3. Furthermore, the effects of subinhibitory concentrations of antibiotics on plasmid transfer are also examined. In the presence of 1 μg/mL of carbenicillin, the plasmid transfer rate significantly increased while decreasing in the presence of other antibiotics. Understanding the environmental conditions, host requirements, and occurrence of plasmid transfer will bring about great insight in understanding gene transfer between environmental bacteria.

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List of Abbreviations

AB – Ashbridge's Bay WWTP

ABC – ATP-Binding Cassette

AMP – Ampicillin

ARB – Antibiotic Resistant Bacteria

ARGs – Antibiotic Resistance Genes

ARI – Antibiotic Resistance Index

BLAST – Basic Local Alignment Search Tool

CHL – Chloramphenicol

CIP – Ciprofloxacin

DNA – Deoxyribonucleic Acid

EHEC – Enterohemorrhagic *Escherichia coli*

ERY – Erythromycin

FACS – Fluorescence Activated Cell Sorting

GEN – Gentamicin

gfp – Green Fluorescence Protein (gene)

H – Humber WWTP

HGT – Horizontal Gene Transfer

I – Intermediate

IDT – Integrated DNA Technologies

KAN – Kanamycin

LB – Luria-Bertani

MATE – Multi-drug and Toxic compound Extrusion

MDR – Multidrug Resistance

MDR – Multidrug-Resistant

MEGA – Molecular Evolutionary Genetics Analysis

MFS – Major Facilitator Superfamily

MIC – Minimum Inhibitory Concentration

NCBI – National Center for Biotechnology Information

NT – North Toronto WWTP

oriT – Origin of Transfer Gene

PCR – Polymerase Chain Reaction

PMB – Polymyxin B

qPCR – Quantitative Polymerase Chain Reaction

R – Resistant

R2A – Reasoner's 2A agar

RND – Resistance-Nodulation-Division

rRNA – ribosomal Ribonucleic acid

S – Sensitive

SMR – Small Multi-drug Resistance

SPT – Spectinomycin

STR – Streptomycin

SXT – Sulfamethoxazole trimethoprim

TET – Tetracycline

Tet B – Tetracycline Resistance Gene B

Tet C – Tetracycline Resistance Gene C

Tet G – Tetracycline Resistance Gene G

Tet M – Tetracycline Resistance Gene M

Tet Q – Tetracycline Resistance Gene Q

Tet W – Tetracycline Resistance Gene W

Tet X – Tetracycline Resistance Gene X

TET^R – Tetracycline Resistant Bacteria

TET^S – Tetracycline Sensitive Bacteria

TIG – Tigecycline

VAN – Vancomycin

WWTPs – Wastewater Treatment Plants

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Chapter 1: Introduction

1.1 Background

1.1.1 Antibiotics

The term antibiotic is defined as any large group of organic compounds having the capacity to inhibit the growth or destroy microorganisms due to specific interactions with bacterial targets. Antibiotics are crucial for the treatment of bacterial infectious diseases; however, chronic exposure of antibiotics to microorganisms can promote the development of antibiotic resistance. Within the last few decades, there have been increasing concerns regarding the rapid emergence of resistant bacteria worldwide. This antibiotic resistance crisis has been attributed to the misuse of antibiotics alongside the lack of new drug development by pharmaceutical industries. In 2016, 40,752 kg of antimicrobials were purchased by various hospital sectors across Canada at a cost of ~$92 million

(CAD).[1] Due to the high importance of antimicrobials, the World Health Organization has published a selection of reserve group or "last resort" antibiotic products, which includes products that are intended to be used when all other alternatives have failed. The list of antimicrobials of last resort includes atrenozam, daptomycin, fosfomycin, 4th and 5th generation cephalosporins, colistin, polymixin B, and tigecycline.[2] These antibiotics are only prescribed by health professionals when they have exhausted all other alternatives. Despite this direction, the use of antibiotics has significantly increased in the Canadian community dispensaries between 2013 to 2016, while remaining relatively stable in hospital settings during this period.[1] In almost every case of developed/discovered antibiotic, resistance has eventually been seen in pathogenic microbes that were once previously susceptible.[3,4] The emergence of antibiotic resistant bacterial infections have left clinicians with no reliable alternatives to treat infected patients.

As a result of increased resistance, antibiotics in recent years have been characterized as an emerging environmental contaminant in aquatic environments because of their potential effects on public health and the ecosystem.[5–8] They are generally classified according to their spectrum of activity against bacterial species. The two main classes include broad-spectrum and narrow-spectrum antibiotics. Broad-spectrum antibiotics are active against both Gram-positive and Gram-negative organisms. Narrow spectrum antibiotics have a limited range of activity and are only useful for particular species. It is essential to note that the spectra of activity of a particular drug can change from broad to narrow-spectrum as microbes acquire or develop antibiotic resistant genes (ARGs). The three main origins of antibiotics include natural products, semi-synthetic variants, and antibacterial agents of purely synthetic origin. Throughout history, research and development in the pharmaceutical industry have gone to great lengths to discover new antibiotics. The process is extremely rigorous and requires brute-force screening efforts with a large resource

commitment. Furthermore, a newly found antibiotic must go through several clinical trials and stages, before becoming approved on a commercial level. This can be very time consuming and expensive. Despite all this, researchers and health officials still strive to discover new antibiotics to combat the ever-changing and resilient antibiotic resistant bacteria (ARB). Antibiotics of natural origins such as chlortetracycline and tetracycline are extracted from bacterial cultures belonging to phylum Actinobacteria.[9] The phylum Actinobacteria also contribute to the production of other classes of antibiotics including chloramphenicol, aminoglycosides, erythromycin A, lincomycin, vancomycin, rifamycin, novobiocin, spectinomycin, and streptogramins. After the discovery of naturally occurring antibiotics, researchers found ways of improving the antibiotic's bacterial affinity, decreasing human toxicity, optimizing the solubility, and stability by changing or moving functional groups present on the antibiotic. These chemical substances gave rise to semi-synthetic antibiotics such as minocycline. Although naturally occurring antibiotics played a major role in antibiotic discovery, there have been tremendous breakthroughs in discovering antibiotics originating in synthetic chemistry. Quinolones, a fully synthetic class of antibiotics, have played a major role in modern medicine giving rise to antibiotics such as nalidixic acid, ciprofloxacin, levofloxacin, and many more. Due to differences in the mode of action of each antibiotic, the effect experienced by microbes can either be bactericidal (cell death) and/or bacteriostatic (growth/replication inhibition).[10] Various antibiotics have different modes of action determined by the nature of their structure and affinity to unique target sites within a bacterial cell. There are five major biochemical and physiological targets for antibiotics including bacterial cell wall, cell membranes, protein synthesis, RNA and DNA synthesis, and metabolic processes.[11]

1.1.2 Antibiotic resistance

To appropriately understand the problem of antibiotic resistance, it is helpful to recognize some relevant concepts. Antibiotic resistance is an ancient phenomenon that is an expected result of the interaction of microbes with their environment. Most antibiotic compounds are naturally produced and some, as explained in section 1.1.1, are produced by bacteria to aid them to establish better environmental dominance in their community/environment. As a consequence, these organisms have evolved mechanisms to overcome the stress of these chemical compounds to survive. These bacteria are often considered to be intrinsically resistant to antibiotics, however, most researchers tend to rather focus on the movement of resistance genes. The main problem arises with the acquisition of resistance within a population that was originally susceptible to the same antibiotic compound. Horizontal gene transfer (HGT) is a well-recognized process of acquiring resistance from external genetic determinants, likely obtained from intrinsically resistant organisms present within the same environment. It is also important to recognize the concept of antibiotic susceptibility/resistance in bacteria has multiple layers of complexity within clinical settings. Identifying microbial clinical susceptibility breakpoints (resistant, intermediate, and susceptible) relies on *in vitro* activity of the chemical against a population of bacteria under appropriate parameters. Due to discrepancies when interpreting the susceptibility patterns of a microbe, the resistance observed may vary in clinical settings. In clinical environments, parameters such as infection site, pharmacological drug dose, type of infection, and inoculum population size all can influence the susceptibility breakpoints of the bacterium in question.

A microorganism that is referred to as resistant comes about when a change in susceptibility renders the antibiotic no longer effective at specific clinical dose concentrations. As discussed previously, the spread of ARGs through HGT also has significant implications in

reducing antibiotic activity.[12] Resistance to antibiotics occurs through four major mechanisms: (1) efflux pumps, (2) chemical alteration of the antibiotic, (3) destruction of the antibiotic, and (4) changes in the target site.

Efflux pumps are the major group of proteins that confer resistance to most classes of antibiotics. These complex bacterial proteins are capable of extruding toxic compounds out of the cell and minimize their interaction with their target sites. Since the discovery of the efflux system capable of pumping tetracycline out of *E.coli* cells in the 1980s, there have been great breakthroughs in characterizing other efflux systems in both gram-negative and gram-positive bacteria. These systems can be substrate-specific (such as TetA efflux pump specific to tetracycline) or have broad substrate specificity that is often found in multidrug-resistant (MDR) bacteria. To date there are five major classes of efflux systems that have been identified to cause resistance: (1) the ATP-binding cassette (ABC) family, (2) major facilitator superfamily (MFS), (3) resistance-nodulation-division (RND) family, (4) small multi-drug resistance (SMR) family, and (5) multi-drug and toxic compound extrusion (MATE) family.[13]

Another common strategy used by microbes to develop antibiotic resistance is to prevent the action of the antibiotic to its target site by interfering with its target site. Bacteria utilize multiple mechanisms to achieve this, including modification of the target site to reduce affinity and prevention of the antibiotic to reach its binding site. One of the classic examples of target protection mechanism is the tetracycline resistant determinant Tet(M). Tetracycline is a biostatic antibiotic that inhibits the ribosomal activities within the cell and prevents protein synthesis. Tet(M) interacts with the ribosome and displaces the tetracycline molecule from the ribosome.[14] Furthermore, Tet(M) alters the ribosomal structure to prevent the rebinding of the antibiotic.[14] Another example of target site modification is resistance developed as a result of genetic mutation.

Genetic changes as a result of mutation can alter the antibiotic's target site and reduce the binding affinity of the antibiotic. Resistance to the antibiotic, oxazolidinone, is an example of resistance development through genetic mutation. Oxazolidinones have a broad gram-positive activity that prevents protein synthesis by binding the A site of the bacterial ribosome. Methylation found in *E.coli* residue A2503 in the 23S rRNA subunit is a commonly characterized mutation that results in resistance to oxazolidinones by reducing antibiotic affinity to its target site. Another form of target site modification is an enzymatic alteration. Enzymes such as erythromycin ribosomal methylation proteins can result in macrolide resistance by mono- or dimethylating adenine residue in the 50S rRNA subunits. As a consequence, the binding of the antibiotic molecule to its target site will become impaired.[15,16]

1.1.3 Antibiotic resistance genes in wastewater treatment systems

When antibiotics enter the ecosystem, they can affect the evolution of microorganisms and the community structure.[17] As a result, the ecological function of that aquatic ecosystem, under environmentally relevant antibiotic concentrations, may exert a temporary selective pressure.[18] Numerous studies have been done to address rising concerns regarding the potential impact of antibiotics and antibiotic remnants in aquatic environments.[19–24] The presence of antibiotics within an ecosystem is not only recognized as a chemical/emerging contaminant but also can play a critical role in the development of ARB and ARGs.[8,19,25] A significant amount of antibiotics, ARGs, and ARB have been detected in the effluent of wastewater treatment and their downstream water bodies and are recognized to be a main anthropogenic point of contamination.[12,21,22,25–30] Due to incomplete metabolism during human or veterinary usage, or improper disposal of unused antibiotics, the accumulation of these compounds within water treatment systems has increased.[26] As a result, the wastewater treatment plants (WWTPs) are recognized as hotspots for ARGs and

ARB and their spread into the environment.[12] The biological treatment process serves as an environment that is suitable for resistance development and spread due to continuous exposure of subinhibitory concentrations of antibiotics within the microbial community.[22,30] However, there is still a lack of knowledge regarding the effects of subinhibitory concentrations of antibiotics on the proliferation of ARG in the microbial environment.

1.1.4 The fate of ARB and ARG in WWTPs

The WWTPs generally have three stages of treatment (primary, secondary, and tertiary). In the primary treatment, sewage flows into large tanks to allow for the settling of the sludge while large solids are separated and oil/grease layers are removed. In the secondary or the biological treatment, biological and chemical processes are used to degrade the biological/organic content of the waste via aerobic and anaerobic tanks. In tertiary treatment, flocks or suspended solids, additional nutrients, toxic materials, organics, and any additional components that failed to be removed by the previous treatments are separated from the water.

The microbial population in the WWTP is constantly changing as a result of numerous factors such as the flow rate, type of waste, and seasons. Whether the presence of subinhibitory concentrations of antibiotics increases the proliferation of ARG and favour ARB is still currently under discussion. Despite the vigorous treatment process, ARGs and ARB cannot be completely removed from the effluent.[30] As a consequence, higher levels of ARGs and ARB have constantly been detected in WWTP effluents than in any farm, urban, or hospital effluents.[25,30] The release of ARGs and ARB into downstream water bodies increases the prevalence of resistance in the environment. The presence of these antibiotic resistant determinants may increase the rate of HGT and contribute to the proliferation of ARGs and ARB in all downstream water bodies making the

WWTP potentially a major source of ARB and ARG dissemination. A study in Croatia found that the total abundance of ARGs was three times higher in the sludge of a WWTP that received wastewater from pharmaceutical production plants than from municipal sources.[31] In their study, it was concluded that environments with a higher concentration of antibiotic residues select and may favour taxonomic shifts towards resistant species.

Although the WWTP does remove some antibiotics through degradation and sorption during the treatment process, not all of the antibiotics are completely removed.[30-31] Scientists and engineers are constantly striving to find ways or processes to minimize ARGs and ARB in wastewater treatment plants as well as their release into receiving waters. One study found that coagulation may play a promising role in the removal of ARGs in wastewater treatment plants.[32] Coagulation is a chemical water treatment process that is applied before sedimentation and filtration to promote the removal of particles. Smaller particles bind together to form larger aggregates so that they can be easily trapped and separated from the water during the sedimentation/filtration step. They concluded that the coagulation process was successful in 0.5-3.1 log reduction of three tetracycline resistance genes, 2 sulfonamide resistance genes, and class 1 integrons.[32]

In Canada, there has been very little attention given to the distribution, transfer, and occurrence of ARGs and ARB in WWTPs and Canadian water systems. This information is vital for tracking and identifying potential hazards to public health and water quality. Identifying major habitats in which ARGs and ARB thrive is essential for learning their fate in various environments and perhaps environmental problems that can arise as a result. Currently, antibiotics, ARGs, and ARBs are not being reported in the annual reports released by Humber, Ashbridge's Bay, and North Toronto WWTPs in Toronto, Ontario.[33–35]

1.1.5 Horizontal Gene Transfer and Mobile Genetic Elements

It is well recognized that HGT plays an important role in bacterial evolution.[45] HGT is one of the main culprits that move ARGs from resistant to non-resistant bacteria and play an essential role in the development of multidrug resistance (MDR). The three well-recognized methods of transfer include transformation, transduction, and conjugation. Fragments of DNA, genes, or plasmids can be transferred from one donor bacteria to another recipient even if they are distantly related.[36] With the acquisition and concentration of different resistance genes and mechanisms, bacteria are capable of becoming MDR at an increased rate. In the case of tetracycline, there have been over 30 genes discovered that confer resistance to this antibiotic.[9] Ever since the wide use of tetracyclines in clinical settings and agriculture, numerous tetracycline resistance genes have been detected in the environment and have become prevalent in pathogenic bacteria.[21,37] Understanding the mechanisms and promoting factors of HGT between bacterial isolates would help in the development of new strategies to combat challenges caused by ARB.

One of the methods of DNA transfer recognized under HGT is known as transformation. Transformation is the natural process of cellular uptake of free DNA from their surrounding environment. It was first discovered in *Streptococcus pneumoniae* in 1928. Under certain conditions, bacteria are capable of becoming more physiologically susceptible to uptake exogenous DNA from their natural surroundings in a state known as competence. This ability is a genetically programmed process that is conserved in both Gram-positive and Gram-negative bacteria.[38-40] There are several factors including environmental signals, chemicals, condition, and expression of competence-induced proteins that can induce bacteria to more readily uptake natural DNA.[39] Several studies have found that antibiotic resistance can arise as a cause of natural transformation.[40–43] It has been found that natural transformation can also promote the transfer of

insertion sequence elements, transposons, and integrons along with ARGs.[43] During the final stages of WWTP before the water is released into the environment, the effluent undergoes various treatments to reduce the total number of bacterial cells released via ozonation, chlorination, and/or UV disinfection.[44] As a result of large scale cell death, a substantial amount of exogenous DNA is released into the effluent and downstream water bodies. Since the WWTP has been recognized to have higher than normal levels of ARGs and ARB[12,22,26,28,29,31], the microorganisms downstream of the WWTP may come in contact with higher concentrations of exogenous DNA and increase the likelihood of natural DNA transformation and ARG acquisition. The general mechanisms of DNA transformation are well described in multiple reviews or studies.[38,39,45]

Another method of DNA transfer as a result of HGT is known as transduction. Bacteriophages are viruses that are capable of infecting bacteria by inserting their DNA into their host's genome. Often when bacteriophages move from one bacterium to another, they can carry a small portion of DNA from the previous bacterium with them to their new host in a process known as transduction. Since the virus serves as the method of transfer, they do not require contact between the cell donating the DNA and the recipient cell. Bacteriophages can carry genes such as ARGs along with them to their new hosts.[46-47] Viruses have been detected at concentrations of 10^8-10^{10} virus-like particles per mL at various stages of WWTPs.[46] The amount of viruses found in WWTP is 10-1000 times higher than in natural aquatic environments thus further supporting the idea that WWTPs can serve as a reservoir for the transfer of ARGs using viruses as transfer vectors. Furthermore, a study performed in Singapore identified the virome of a WWTP using metagenomic sequencing and found that 5-20% of the viruses detected were phylogenetically assessed.[184] Furthermore, they also investigated the additional genes carried by the WWTP virome and found that the majority are involved in DNA metabolism rather than ARGs.[184] Other studies

performed under controlled lab environments have demonstrated that bacteriophage-mediated transduction of ARGs is certainly possible.[46, 47]

Bacterial conjugation is the last method of HGT that involves the transfer of genetic material through direct cell to cell contact in energy-driven transport. During this process, one bacterium serves as the donor while another bacterium serves as the recipient often transferring DNA as plasmid structures. Bacterial plasmids are extra-chromosomal DNA that replicate independently as a stable component of the cell's genome. They can vary in size ranging from 1-100+ kbp and can have various copy numbers from 1 to several hundred per cell. The copy number of plasmids is generally fixed under constant conditions which are controlled by plasmid-mediated systems. Plasmids often impose a fitness cost to their hosting cell as they take valuable resources for their maintenance and expression. As a consequence, the survivability of the plasmid in a cell depends on numerous factors including environmental stress, competition, fitness cost, plasmid-mediated regulatory elements and partitioning, and whether the plasmid offers any improvement to the host's survivability and metabolism.[48,49] Another factor which dictates the persistence of a plasmid in a cell is plasmid incompatibility. Plasmid incompatibility is defined as the failure of two or more plasmids to coexist in a cell due to sharing similar plasmids partitioning or replication systems (Rep proteins) and/or interference with the ability to maintain plasmid copy number.[49–52] Knowing which replicon types present in a plasmid can help predict plasmid copy number, host-range, and compatibility.[50] There are many plasmid incompatibility groups (Inc) known to date. Inc groups are categorized based on their genetic similarity and pilus structures summarized in Table 1 (adapted from Waters, 1999).[53]

Table 1. Incompatibility groups categorized based on genetic similarity and pilus structures

Main Groups	Sub-Incompatibility Groups
IncF	IncF, IncS, IncC, IncD
IncP	IncP, IncU, IncM, IncQ, IncW
IncP-Ti *	IncX, IncH, IncN, IncT
IncI	IncI, IncB, IncK

* = IncP plasmid group with a modified pilus structure

The conjugation process generally includes the following model: (1) contact between donor and recipient cells are made with a mating bridge, (2) single-stranded nick within the origin of transfer (*oriT*) initiates DNA relaxosomes, (3) conjugative replication of a single strand of DNA is transferred to the recipient, (4) DNA complementary strand synthesis and replication in the recipient and DNA recircularization.[53] This process is generally regulated by conjugative elements that are found on transferring plasmids that aid in the production of the protein machinery required for the process. The transfer of conjugative plasmids across the cell membrane of its host often relies on a large membrane-associated protein complex belonging to the type IV secretion system (T4SS) such as the Vir system.[53]

To this day, there have been many in vitro conjugational experiments performed to assess factors, conditions, and/or environments that promote the transfer of plasmids between bacteria. However, due to technological limitations and diversity of plasmids, it is extremely difficult to assess conjugation frequencies *in situ* environments such as the WWTP. Most studies can only predict the frequencies under *in vitro* settings using lab strains as donors or recipients. To our knowledge, there have been no studies to fully assess conjugation using native environmental cultures as both donor and recipient. There have been studies that attempt to characterize and identify antibiotic resistant plasmids from WWTP samples; however, not all identified plasmids carry conjugative elements.[54–56] Furthermore, these studies have found that plasmids are the main carriers of ARGs as opposed to other mobile genetic elements and can potentially disseminate

within bacterial populations. Other studies have tried to determine conjugation rates by using lab strain bacteria as a plasmid donor with a known broad host range plasmid (RP4 plasmid being the most popular) to conduct conjugation experiments in WWTP samples.[29,57–59] Although the majority of these studies have limitations in properly assessing the dangers of ARG-carrying-plasmids in WWTP bacterial populations, they all reach a similar conclusion that these plasmids are prevalent, have conjugative capabilities to proliferate, and they can contribute to increasing the overall resistance of the population.

Other mobile genetic elements outside of HGT that can contribute to increasing antibiotic resistance in bacterial populations are transposons and integrons. Transposons (transposable elements or "jumping genes") are a linear DNA sequence that is capable of changing their positions within a genome as well as move from the genome of one bacteria to another. The general structure of a bacterial transposon is composed of flanking insertion sequences, transposase, and resolvase gene that encodes for proteins involved in insertion and excision, and any structure or additional genes that they may carry such as ARGs. In addition to their ability to move from one genome to another, they are also capable of inserting themselves into plasmids and move between hosts via conjugation.[60] In addition, ARG and heavy metal resistance gene carrying transposons have been demonstrated to be present on plasmids.[56,60,61] A study performed by Cain and Hall (2012) in Australia, found that IncHI2 plasmids (pSRC26 and pSRC125) extracted from *Salmonella enterica* isolates recovered from cattle were carrying multiple ARG-carrying-transposons (Tn*10* and Tn*1696*-like).[60] Furthermore, another study performed by Stokes *et al.* (2007), demonstrated that certain transposons such as Tn*1403* isolated from clinical *Pseudomonas* strain were composed of three different transposons while carrying tetracycline resistance gene (TetC).[61] This demonstrates

the capabilities of transposons to modify their genetic sequence by integrating with one another to increase their propagation and persistence as well as contribute to the dissemination of ARGs.

Integrons are versatile sequences of DNA that specialize in gene acquisition and are commonly found in bacterial genomes. Integrons contribute greatly to genomic complexity, adaptive responses, and phenotypic diversity in microorganisms. One of their major genetic structures is known as a gene cassette that allows them to embed genes acquired mainly from the genome of their bacterial host. The general composition of an integron normally includes a gene encoding site-specific recombinase (*intI*), recombination site for gene insertion (*attI*), and a promoter that directs the transcription of all the genes in the gene cassette(s). Under most circumstances, integrons identified in clinical bacterial isolates carry less than 5 gene cassettes[62] but one clinical *Escherichia coli* MG-1 isolate was found to carry as many as 9.[63] Due to their minimalistic structure, integrons are not capable of transferring between different bacteria on their own as they require a mobile genetic vector such as a plasmid or a transposon to migrate between hosts. However, integrons play a critical role in gene acquisition and expression of multiple ARGs in clinical isolates.[63–66] Although most research involving integrons has been performed on clinical isolates, there have been numerous studies done to assess the prevalence of integrons in WWTPs.[67–70] Multiple ARGs carrying class 1 and 2 integrons were detected in all of the studies conducted at various stages of the treatment systems. The prevalence of ARG-carrying-integrons in both the environmental and clinical settings impose major concerns on public health and safety. While integrons may not be directly mobile, they certainly are prevalent in most environments and indirectly capable of disseminating ARGs within bacterial populations.

1.1.6 Tools for Assessing ARGs in WWTPs

The tools available for assessing bacterial populations leave scientists with two main approaches including molecular-based or culture-based. Each approach exhibits specific advantages or limitations that hinder one's ability to appropriately investigate large, complex, diverse, and ever-changing microbial populations. In recent times, molecular-based approaches have been valued to a greater extent for the detection of ARG and ARB by utilizing tools such as polymerase chain reaction (PCR), quantitative PCR, microscopy, and next-generation sequencing. Despite the technological advancements in molecular-based approaches, accurately demonstrating ARG transfer in WWTPs remains a difficult task. Given the absence of standardized methods to evaluate antibiotic resistance in environmental samples, the data obtained from different time points or regions of the world make it difficult to compare accurately. Nonetheless, scientists can look for and recognize trends involving certain bacterial groups or patterns. In this section, the advantages and disadvantages of molecular-based and culture-based approaches for characterizing ARGs, ARB, and gene transfer in environmental samples will be discussed.

Molecular methods have been utilized to a great extent for detecting, characterizing, and addressing the potential risks of gene transfer over the last decade.[21,55,56,58,71] One of the main advantages of molecular-based methods is that they can identify microorganisms that either grow at very slow rates or that are unculturable *in vitro*. It is suggested that only a small percentage of bacteria in a given microbial population can be grown in a lab.[72] Molecular-based techniques allow for the high-throughput detection of certain unculturable members of a population that may carry multiple ARGs that cannot be grown in a lab. The word "unculturable" does not signify that these bacteria can never be grown; instead, it means that scientists lack critical information on the conditions required to cultivate them. However, their presence and contribution to the overall

resistome and the transfer of mobile genetic elements in a bacterial community is of great importance. Another advantage of molecular-based techniques is that the majority of the available tools are high-throughput meaning that they can produce large quantities of data in a short amount of time such as next-generation sequencing. In addition, the majority of the techniques are far less laborious but can be much more expensive. The disadvantages of molecular-based methods are that the majority of the tools use some form of PCR-based technology. One of the main problems with PCR-based technologies is that several biases involving non-random distribution, error rates, and selection of the template DNA may favour specific fragments over others.[73,74] This favourtism may skew the data/results and may introduce false-positive or false-negative conclusions. Another disadvantage of molecular-based techniques for the detection of ARGs is that they provide no information regarding the functionality of the genes. Depending on their level of gene expression, some bacteria harbouring multiple ARGs may not express those genes and will contribute to false-positive data.

DNA sequencing technologies and platforms are being updated at a tremendous pace to improve time efficiency, data throughput, cost efficiency, and accuracy. The term next-generation sequencing (NGS) is used when describing a massive parallel or deep DNA sequencing technology that has made great advancements in genomic research. NGS tools are constantly evolving and adapting to modern technology to improve their data quality from previous iterations. In the fields of antibiotic resistance and environmental research, sequencers are used in numerous strategies to identify phylogeny of microbial populations through 16S rRNA metagenomics[75], investigating the presence of mobile antibiotic resistomes in bacterial populations[29,55], characterization of ARG-carrying plasmids[55,76], and whole-genome sequencing of individual ARB isolates.[73] It should be noted that although NGS is capable of identifying microbes in a given population, the technology

only allows for their detection at a genus level. Furthermore, the sequencing process will also result in the death of all microorganisms in the sample, therefore, eliminating any culture-based isolation of individual strains for future work.

Quantitative PCR (qPCR) and qualitative PCR are useful approaches for detecting and quantifying ARGs, pathogens, mobile genetic elements, and certain bacteria in both unculturable and culturable populations.[12] The detection and quantification of ARGs and ARB using qPCR and PCR have been extensively used in environmental studies.[21,27,77,78] The advantages of this approach include cost and time effectiveness, easy to use with available primer sets, and it provides information on copy numbers and abundance of selected genes in a sample. A unique strategy developed by Bonot and Merlin (2010) used qPCR to monitor the dissemination of a conjugative plasmid (pB10) in a microbial community.[79] The dissemination of a plasmid in a population is a form of DNA replication and, as a result, the plasmid to donor DNA ratio increases when the plasmids move to other members of the population. This methodology is a useful tool for tracking plasmid conjugation in an environmental sample using a known donor bacterium with a plasmid of interest. The sensitivity of qPCR allows working with small sample inoculums and a greater sample size to appropriately detect plasmid movement. Additionally, novel conjugative plasmids lacking any form of fluorescent tags, markers, and/or ARGs can be investigated when otherwise not possible with other methodologies such as microscopy or culture-based techniques. One of the disadvantages of qPCR and PCR-approach is that you must know which genes you are looking for in a sample to develop appropriate primer sets. The primer sets used for the detection of genes of interest must also be designed to be very specific to their target to minimize any non-specific binding or primer dimers.

The fluorescence-based approach via microscopy is a commonly used method for tracking the conjugation of genetically modified/tagged plasmids in a bacterial population.[58] The plasmid of interest is normally genetically modified to express a green fluorescence protein (*gfp*) with the repressor gene inserted into the chromosome of its hosting cell. Furthermore, the hosting bacteria will also be genetically modified to contain another functional fluorescence gene with a different colour such as *mCherry* (red). Under normal circumstances, the donor bacteria will not express the *gfp* gene present on the plasmid but instead will fluoresce red due to the expression of the chromosomal *mCherry* gene. When the plasmid moves from the donor to a recipient, the *gfp* gene will start to express in its new host to exhibit a green colour when viewed under a fluorescence microscope. The detection of green fluorescent bacteria indicates the presence of the conjugative plasmid in a new host while the red fluorescent bacteria will represent the original donor. The transconjugant population can then be sorted from the remaining microbes using fluorescence activated cell sorting (FACS) to separate the population based on fluorescent labeling. Sorted transconjugant cells can then be collected, DNA extracted, and subjected to 16S rRNA gene sequencing to identify all possible recipients. The fluorescence-based approach is quite successful at displaying a broad-host-range of both the bacteria and the plasmid in a time-efficient manner as well as identifying all possible recipients at the genus level. The disadvantage of this technique is that it requires a known genetically modified plasmid and lab-strain bacteria as the donor. Lab strain bacteria and plasmids often do not well represent the environmental population as they are accustomed to laboratory conditions. As a result, it is difficult to replicate the environmental conditions required for conjugation and may result in false conclusions or assessments. Lastly, the fluorescence-based approach is extremely expensive and requires a fair amount of resources.

Despite the technological advancements for molecular-based research; the culture-based approach remains to be a useful tool for investigating the spread of ARGs and the presence of ARB. One of the main advantages of this approach is that it provides information on the gene functionality and phenotype of ARB through techniques such as antibiotic disk diffusion testing. Furthermore, it provides useful information on the degree of resistance microbes have to specific antibiotics through minimum inhibitory concentration (MIC) tests. The molecular-based approach can often be successful in predicting the resistance phenotype of a microbe; however, scientists do not have the bioinformatics tools to determine their degree of resistance and MIC. The culture-based approach is especially useful when targeting certain groups of microorganisms in a population such as coliform bacteria. The total coliform group is a diverse set of microbes with faecal and nonfaecal origins and is conventionally used as indicators for disease-causing organisms in water systems.[80] Furthermore, the culture-based techniques coupled with molecular tests allow for the identification of isolates at a species level through a complete 16S rRNA gene sequencing or PCR detection of species-specific genes. Although the molecular-based approaches are useful for the detection and characterization of ARGs, plasmids, and/or mobile genetic elements, they fail to determine which microbe is carrying them. The culture-based approach helps bridge that gap of information by identifying the individual capabilities of each isolate or strain. Monitoring plasmid conjugational transfer between microbes through a liquid or solid interface mating provides useful information on transfer efficiency, frequency, functionality, and host-range of plasmids and bacteria. However, culture-based conjugation often requires the donor and recipient to be compatible and have antibiotic resistance or phenotypic selective markers for transconjugant identification and selection. The two main disadvantages of a culture-based approach are that the techniques are often laborious and can only be used on culturable populations while ignoring the

unculturable bacteria. As a consequence, these techniques often fail to properly represent the entire environmental population and only looks at certain selected members or conditions. Due to the advantages and disadvantages of both culture-based and molecular-based methods, it is vital when designing experiments to utilize both approaches to provide unique and valuable insights into the complex field of antibiotic resistance. To solely base conclusions on one method will not paint a complete image and will fail to adequately represent what occurs in the environment.

1.2 Research Gaps and Thesis Objectives

There are critical knowledge gaps that need to be addressed to overcome the global challenge of antibiotic resistance. The ubiquity of ARB and ARGs in humans, animals, the environment, and the ability to move between all three ecosystems has contributed immensely to the increased resistance patterns observed in microorganisms. The role of the environment as a transmission route for bacterial pathogens has been well detailed in the previous section. The common understanding that most resistance genes identified in ARB and pathogens have originated from bacteria that normally thrive in the environment. Hence, densely populated microbial environments like the WWTP act as a reservoir and dispersal route for ARGs and ARB. Additional research is required to understand the occurrence and mobility of ARB and ARGs in the environment. In this section, the current critical knowledge gaps and the contribution of this thesis to address the missing knowledge will be discussed below.

One of the major gaps in this field is identifying the relative contributions of different sources of antibiotics and ARB into the environment. By better quantifying the degree of contribution from major sources, exposure routes, and propagation pathways on environmental bacteria will help in assessing the risk of ARB. The selective pressure imposed on environmental bacteria depends on numerous factors such as concentration, type, co-exposure, and time of exposure of the chemical agent(s) and the hostility of the environment to promote bacterial growth. The lack of information on the interaction between chemical agents, such as antibiotics, on environmental bacteria, brings forth uncertainties in understanding the extent that they contribute to promoting resistance development. To better understand the role of different sources of antibiotics, ARGs, and ARB in the environment, knowledge of the characterization, occurrence, natural variability, and mobility of ARGs, mobile genetic elements, as well as their

mobilization/transfer frequencies in the environment is required. This data is valuable when assessing the risk and the degree of intervention required to remediate potential dangers. One of the difficulties in analyzing microbial communities and assessing their potential risk is that they are highly complex and vary over time. Furthermore, the lack of standardized surveillance methods for tracking mobile genetic elements, ARGs, ARB, contaminants, and their mobility complicates the data analysis and risk assessment process.

Another major gap is to understand the effects of anthropogenic inputs on the environment and their role in the evolution of antibiotic resistance. Antibiotics, metals, biocides, and other non-antibiotic chemical agents introduce additional challenges in understanding their impact and mechanism on microbial communities within the environment. It is difficult to address this challenge as most techniques for investigating the effects of contaminants are laborious and time consuming. In addition, the variability and complex nature of contaminants and their many interactions leave scientists with more questions than answers about their impact. Evaluating the resistome of a microbial population brings about great insight into the potential range of ARGs by investigating their occurrence and mobility (intrinsic vs mobile resistance). Sufficient methods and research to address the evolution, selection, mobility, and persistence of ARB and ARGs in microbial communities are urgently needed. Furthermore, additional research is required to determine the effects of selective pressure on the dissemination of ARGs and ARB. Growth conditions and selective pressures that favour the growth of ARB and the transfer of mobile genetic elements must be taken into consideration when evaluating environmental risks. The development of methods with improved sensitivity to establish a link between ARGs and ARB without the need for cultivation has not yet been established. As a consequence, culture-based techniques are still valuable when investigating the relationship between ARGs and ARB.

Most research done in the field of antibiotic resistance generally investigates the transfer between lab strains or between lab strains and environmental isolates which may misrepresent what can occur in nature. Furthermore, environmental isolates are often selectively picked for their resistance phenotypes and the rest of the population is often ignored. Other members within the population may play a role in the mobility of ARGs between different members of the population. Although most microbial members may be sensitive to an antibiotic, they may still be a carrier for unexpressed ARGs that may become functional upon transferring to a different host through HGT. The information gained on this topic could enhance our understanding of the fate of ARGs and their potential effect on bacterial communities. The research performed in this dissertation aims to shed light on several knowledge gaps to more efficiently manage the spread and emergence of ARGs and ARBs in the environment. **The primary goal of this research is to characterize and investigate the occurrence and mobility of ARB and ARGs in three urban WWTPs.** To achieve this overall goal the following objectives will be carried out:

A) The first objective (Chapter 3) was to devise a characterization strategy to investigate the prevalence and routes of ARGs in WWTPs and demonstrate their interaction between antibiotic sensitive and resistant populations. This objective was accomplished by:
    - Determining whether the selection of tetracycline resistance increased the likelihood of having resistances to 7 other antibiotics in environmental bacteria,
    - Determining whether antibiotic resistance is correlated with microbial genera.

- And Identifying the presence of tetracycline resistance genes in WWTP and determining whether the same determinants can be found in connected water bodies and other nearby WWTPs.

This objective investigated the occurrence of tetracycline resistant and sensitive populations provided insight into not just the susceptibility phenotypes but community composition as well. The uniqueness of this methodology helps to investigate the antibiotic resistance problem from a different perspective by looking at the population as a whole rather than specific sought after members. The knowledge acquired from this research will shed light upon resistance patterns and routes that can occur in a complex WWTP microbial population. We suspected that the tetracycline resistant and sensitive populations would have a high diversity and be distinct from one another. Furthermore, the results of this objective will lay the foundation for identifying mobile genetic elements and their role in the dissemination of ARGs in subsequent chapters.

B) The aim of objective 2 was to identify plasmid carrying isolates from the tetracycline resistant and sensitive populations and determine the role of the plasmid in their host. This objective goal was accomplished by:
    - Determining if the annotated plasmids carry ARGs and whether the isolate's antibiotic resistance profiles are linked to the found plasmids.
    - Determining the role and transfer capabilities of each plasmid isolated from environmental bacterial isolates.
    - Identifying the host range of plasmids that were sequenced from *Providencia, Acinetobacter, Klebsiella,* and *Escherichia* isolates via computational methods and the BLAST database.

This objective will elucidate the presence of plasmids in antibiotic resistant and sensitive environmental bacterial isolates. The genetic information obtained in this research will contribute to the understanding of the role plasmids play in the mobility, host range, metabolic function, virulence, and the spread of ARGs. The uniqueness of this research is that we attempt to identify plasmids from both antibiotic resistant and sensitive environmental populations rather than selecting for specific strains or plasmids. This methodology will shed light upon the commonality of plasmids in bacterial populations since ARB are often expected to be carrying plasmids with ARGs.

C) The aim of objective 3 is to utilize molecular and culture-based techniques to monitor the conjugation of plasmids, pNT36-3, and pNT36-4, between two environmental *Escherichia coli* isolates while exposed to a subinhibitory concentration of antibiotics. This objective was accomplished by:

   - Determining the plasmid host range, stability, and maintenance of pNT36-3 via culture-based conjugative mating and growth experiments.
   - Using molecular-based techniques, determine whether subinhibitory concentrations of carbenicillin, tetracycline, ciprofloxacin, and gentamicin affect the rate of conjugation of both pNT36-3 and pNT36-4 between two environmental *E. coli* strains.

This research will contribute to the knowledge gap by demonstrating the impact of subinhibitory concentrations of antibiotics on the mobility and stability of conjugative plasmids between environmental bacterial strains. What makes this research unique is that it investigates conjugation by tracking the transfer of two novel plasmids between two environmental *E. coli* strains using the molecular-based techniques. To our knowledge, the methodology in selecting the

antibiotic pressures for investigating conjugation via qPCR has not yet been performed. In literature, most conjugation experiments often involve the use of lab strains as either plasmid recipient or donor. We hypothesize that the presence of subinhibitory concentrations of antibiotic pressure will increase the rate of plasmid transfer between the donor and recipient. Understanding the environmental conditions, host requirements, and occurrence of plasmid transfer will aid in the risk assessment process and bring about great insight into the potential dangers of gene transfer between environmental bacteria.

2

Chapter 2: Materials and Methods

2.1 WWTP sample sites and collection

Wastewater samples were collected from the secondary treatment of three major plants (North Toronto, Ashbridge's Bay, and Humber) all located in Toronto, Ontario (Figure 1). Individual plant capabilities are outlined in Table 2 and obtained from each respective plant's annual report for 2017.[33-35] Each plant's aerated tanks and activated sludge were sampled and mixed on multiple occasions throughout the year. All samples were transferred into the laboratory in 1 L bottles and processed on the same day.

Table 2. The 2017 annual report summary of the three sampled wastewater treatment plants

	North Toronto	Ashbridge's Bay	Humber
Total Suspended Solids (mg/L)	276	279.5	301
Biological Oxygen Demand (mg/L)	192	201.9	255
Total Phosphorus (mg/L)	5.2	6.4	5.3
Influent Flow Rate (ML/day)	15.7	659.8	331.7
Capacity (ML/day)	45.5	818	473
Population Served	55,000	1.5 million	685,000
Effluent Discharge	Don River	Lake Ontario	Lake Ontario

2.2 Bacterial Isolation and Identification

Diluted wastewater samples were plated on Reasoner's 2A agar (R2A) with and without tetracycline (16 µg/mL) and were incubated at room temperature for up to 3 days. Cultures were selected at random and grown as pure cultures and DNA extracted using DNeasy PowerSoil Kit (Qiagen, Toronto, ON). The culture's genus was identified using previously described techniques by Sanger sequencing the V3-V4 region of the 16S rRNA.

2.3 Whole Genome DNA Extraction

The DNA was extracted from both pure isolates and aerated tanks wastewater/activated sludge samples of each WWTP using the MoBio UltraClean Soil DNA Extraction Kit (MoBio Laboratories Inc., Carlsbad, CA, USA), following the manufacturer's protocol. Certain steps in the protocol were modified to obtain better DNA yield which included increasing the bead-lysis step for certain cultures from 10 minutes to up to 20 minutes. Furthermore, the elution step incubation period was increased by an additional 5 minutes. The concentration and purity of the DNA were determined via a nanophotometer and gel-electrophoresis. DNA was stored at -20 °C until needed for PCR amplification and sequencing.

2.4 Antibiotic Resistance Profiles

The resistance profiles of each culture were determined using standard Kirby-Bauer disk diffusion method following the manufacturer's protocol except that the cultures were tested on R2A media instead of Mueller-Hinton. Laboratory strains of *Escherichia coli* DH5α, *Pseudomonas putida* BBC443, and ATCC 12633 were used as controls to determine whether the change in media affected the inhibition zones of the antibiotics. The profiles were classified into three categories (resistant, intermediate, and susceptible) depending on the size of the inhibition rings observed. The tested antibiotics include ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), kanamycin (30 µg), streptomycin (10 µg), sulfamethoxazole/trimethoprim (23.75/1.25 µg), tetracycline (30 µg), and tigecycline (8 µg).

2.5 Plasmid Extraction and Gel Electrophoresis

Cultures were selected at random from both tetracycline sensitive and resistant populations to undergo plasmid extraction using the NucleoBond Xtra Midi extraction kit (Macherey-Nagel, Germany). The procedure was followed as stated in the manufacturer's protocol. The cell pellet size of each bacteria was adjusted to the same size as that of an *E. coli* MM294 grown under the conditions stated by the manufacturer's protocol since the growth of each bacteria will differ. The presence of the plasmids was confirmed by running the plasmid extracts on a 0.7 % agarose gel stained with SYBR safe for ~1 hrs at 50 V and visualized with GelDoc EZ system (BIO-RAD, ON, Canada).

2.6 Plasmid Sequencing, Assembly, and Annotation

The extractions that yielded plasmids were sent away to the University of Regina and sequenced by Dr. Christopher Yost's lab group via Illumina MiSeq. The sequencing reads were assembled using SPAdes (St. Petersburg Genome Assembler) v3.8.0[81] and the scaffolds were

categorized and assessed as plasmid/chromosomal fragments using PlasScope's Centrifuge V1.0.3 tool via the default database.[82] The obtained scaffolds from both chromosomal, plasmid, and unclassified were annotated using Rapid Annotation using Subsystem Technology V2.0 (RAST).[83] All the downstream analysis used in the annotation process were limited to contigs and scaffolds that had a minimum coverage of 20x.

2.7 Whole Plasmid Assembly

Larger scaffolds obtained from the sequencing analysis were closed and confirmed by designing primers to stretch between the ends of each scaffold. If the expected PCR product is obtained then we can safely assume that the two scaffolds are connected. The primers were specifically designed to target each scaffold using Primer3 V4.1.0 online tool (http://bioinfo.ut.ee/primer3/). The PCR reaction was performed in 25 μL volumes with 100 ng of template, 200 mM of dNTPs, 200 nM of each forward and reverse primer, Taq buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl^2$) with 1.25 U Taq polymerase (New England BioLabs, MA, USA). The thermocycler protocol used for the primer sets are as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 1 min denaturation, variable annealing temperatures (45-60 °C) for 1 min, an extension at 72 °C for 1 min, and lastly 10 min of final extension for 72 °C. The PCR products were confirmed on a 1 % agarose gel stained with SYBR Safe at 100 V for 30 min and visualized with the GelDoc EZ system.

2.8 Scaffold Alignment to BLAST Plasmid as Reference

Large scaffolds that could not be assembled into whole plasmids were entered into the Basic Local Alignment Search Tool (BLAST). The matches/accession numbers that were provided by each scaffold were recorded and scaffolds that contained common similarities to a certain match were grouped as one. The grouped scaffolds were then aligned together to reconstruct the plasmid

by using plasmids in the BLAST database as a reference. All complete and partial plasmids were graphically generated using SnapGene Viewer V4.3.11.

2.9 Statistical Analysis of Antibiotic Resistance Profiles

The percentage of multiple antibiotic resistant bacteria at each location was determined. An isolate was considered to be multiple antibiotic resistant (MAR) if it was found to be resistant to three or more antibiotics [84]. The antibacterial resistance index (ARI) is used for analyzing the prevalence of bacterial resistant determinants in a population at a specific location. The following formula was used to calculate the ARI:

$$ARI = A/NY$$

where *A* is the total number of resistant determinates recorded in the population, *N* is the number of isolates in the population, and *Y* is the total number of antibiotics tested [85].

2.10 Determination of tetracycline resistance determinant

A PCR assay was performed to determine which of the seven tetracycline resistance determinants (Tet B Tet C, Tet G, Tet M, Tet Q Tet W, and/or Tet X) were present in the WWTPs community extracts. The genes and primers are shown in Table 3. Each reaction was tested alongside an appropriate positive and negative control to ensure the validity of the PCR protocol. Positive controls were plasmids obtained from M.C. Roberts (University of Washington) containing the appropriate gene to Tet B, Tet C, Tet G, Tet M, Tet Q and Tet W and DNA from strains containing Tet X provided by G. Vora (Naval Research Base, Washington) as shown in Table 3. Genomic DNA extract of an *E.coli* DH5α lab strain with no tetracycline resistance was used as the negative control for this assay.

Each PCR reaction was performed in 25 µL reactions containing 50 ng of template DNA, 0.5 uM of forward and reverse primers, 3.44 µg BSA, 200 µM dNTPs, Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) with 1.25 U Taq (New England BioLabs, Pickering, ON, Canada). Touch Down-PCR (TD-PCR) was performed on Tet B, Tet C, Tet G, Tet M, and Tet W due to the high efficacy. The first step involved sample denaturation at 96 °C for 5 min followed by thermocycler at 94 °C for 1 min. An initial annealing temperature of 65 °C was decreased by 1 ° C for every cycle for a total of 10 cycles with an elongation time of 3 min at 72 °C. The annealing temperature of 55 °C was used for an additional 20 cycles. The reaction composition of Tet Q and Tet X were similar to the previously listed reaction except 0.7 µM forward and reverse primers were used. The thermocycler settings for Tet Q included: initial denaturation at 94 °C for 5 min, 94 °C denaturation for 30 s, annealing temperature at 50 °C for 30 s, and elongation temperature at 72 °C for 1.5 min for a total of 30 cycles. Lastly, the following thermocycler settings were used for Tet X: initial denaturation at 94 °C for 2.5 min, 94 °C denaturation for 15 s, annealing temperature at 55 °C for 30 s, and elongation temperature at 72 °C for 30 s for a total of 35 cycles. Four microliters of the PCR products were run on a 1 % agarose gel (stained with SYBR Safe) at 100 V for 25-30 minutes via gel electrophoresis (Invitrogen, Burlington, ON, Canada).

2.11 16S rRNA sequencing and phylogenetic analysis

Isolates for sequencing were selected from each WWTP to provide the same representation in the isolated pool as seen in the community pool as determined by morphology and antibiotic profiles. Of the 173 original isolates, DNA was extracted from 90 isolates (45 TET^S and 45 TET^R). The 16S rRNA gene was then amplified by PCR using the forward primer U341 F and reverse primer U758 R (Table 3). The reaction composition and thermocycler setting that was used to

carry out the 16S rRNA gene amplification was identical to that of the TD-PCR mentioned above. The DNA sequencing of the 16S rDNA PCR products was performed at the ACGT Corp. (Toronto, ON, Canada) with a Sanger sequencing system. A single consensus sequence was generated and edited from the forward and the reverse nucleotide sequences using Sequence Scanner v1.0 (Applied Biosystems, 2005).

The sequences obtained were imported into the NCBI Nucleotide-BLAST database to determine the identity of each isolate. Once the species were identified, appropriate type strains were selected from the NCBI database and both were imported into Molecular Evolutionary Genetic Analysis (MEGA 7.0) software. By using the Clustal W alignment tool, the sequences were aligned with each other and the fragment lengths were accommodated to the shortest sequence (>400bp). Both dendrograms were constructed separately to address the tetracycline resistant and tetracycline sensitive isolates. Dendrograms were created using the neighbour-joining statistical method and bootstrap values were generated from 500 replications.

The Shannon-Weaver Diversity Index[183] was used to test the evenness and diversity in each of the populations and the Dice-Coefficient[182] was used to determine the similarity of the two populations. To identify distinct patterns of resistance among the isolates, hierarchical cluster analysis was performed. The inhibition zones obtained from the antimicrobial disc susceptibility test were categorized into nominal values based on their phenotypes (resistant, susceptible, and intermediate). The nominal values were then imported into IBM SPSS statistics program version 23.0 to generate the clusters using Square Euclidean distance and the Ward method. The patterns of resistance observed in each cluster were organized based on the number of isolates demonstrating the same type of resistance for a given antibiotic. If 75 % or more of the isolates had an identical phenotype; they were categorized accordingly as S (susceptible) or R (resistant).

If less than 75 % of the isolates in a given cluster demonstrated a particular phenotype, they were categorized as Variable Resistance (Vx-%, where x is phenotype demonstrated by the majority of the cultures followed by the percentage).

2.12 Plasmid Donor Verification

From the NGS sequencing data obtained from the previous section (2.6), *Escherichia coli* NT36 and *Klebsiella pneumonia* H11A containing conjugative plasmids were selected to undergo solid and liquid mating with a recipient *E. coli* MM294-pKan lab strain. The solid mating was performed by diluting an overnight culture of both donors and recipients in Luria-Bertani (LB) Broth and inoculated together in a ratio of 1:3:3 (donor: recipient: sterile saline). The mixture was then spot plated numerous times (100 µl) on R2A agar plates containing kanamycin (50 µg/mL)/carbenicillin (100 µg/mL) and incubated overnight at 37 °C. The plates were examined for growth of transconjugants the following day and were re-cultured on kanamycin/carbenicillin R2A agar plates for verification. The liquid mating was performed by diluting an overnight culture of both parents similarly as mentioned above but instead in LB media rather than saline. The donor-recipient mixture was incubated overnight at 37 °C. On the following day, the mixture was diluted in sterile saline 0-100 folds, spread plated (100 µl) on R2A agar plate containing kanamycin (50 µg/mL)/carbenicillin (100 µg/mL), and incubate overnight once more at 37 °C. The plates were examined for the presence of transconjugants and were re-cultured on kanamycin/carbenicillin R2A agar plates for verification. These experiments verified whether each culture is suitable as a potential plasmid donor for the transfer of carbenicillin resistance.

2.13 Recipient Collection and Solid/Liquid Conjugative Screening

To screen for suitable environmental recipients, samples collected from WWTP were cultured in a similar fashion as previously described in section 2.2 but instead plated on MacConkey agar

containing chloramphenicol (25 µg/mL), gentamicin (10 µg/mL), kanamycin (50 µg/mL), ciprofloxacin (10 µg/mL), erythromycin (10 µg/mL), and tetracycline (16 µg/mL). Single colonies were isolated and grown as a pure culture on a correct corresponding antibiotic plate. Antibiotic disk diffusion method was used to identify the antibiotic resistance profiles of each bacteria as previously described in section 2.4. Cultures that had antibiotic resistance towards any of the tested antibiotics while being sensitive to carbenicillin/ampicillin were selected for conjugative mating experiments (Table 10). Solid/liquid mating was performed using the same method as earlier described in this section. All transconjugants were re-cultured on their respective double antibiotic MacConkey agar plate and their morphologies were cross-examined to determine which of the two parents are the donor and recipient. MacConkey agar was used to help differentiate between lactose fermenting isolates that share similar phenotypes to the donor. Since the donor bacteria, *E. coli* NT36 is lac negative and cannot utilize lactose, MacConkey agar will aid in differentiating between possible lac positive recipients. The obtained transconjugants from the donor/recipient mixture can be tested on MacConkey to determine which of the two parents was the correct donor.

2.14 Quantitative-PCR Primer Design for Tracking Parent Bacteria and Plasmids

To measure the copy number of the two plasmids, IncI1 and IncF, in relativity to the donor's *E. coli* NT36 chromosomal copy number is critical for evaluating plasmid mobility via qPCR. Quantitative-PCR primers were designed from *E. coli* NT36 plasmid sequences using IDT PrimerQuest Tool to track IncI1 and IncF genes of donor plasmids (Table 4). The primer set YaiO obtained from (Table 4) amplifies an *E. coli* specific gene[86] that is used to track both *E. coli* parents (*E. coli* NT36 and *E. coli* EB-G3) for qPCR analysis and referencing. The reaction mix and thermocycler settings for all three primer sets were performed in the same manner as the Touch Down-PCR.

It was suspected that the recipient *E. coli* EB-G3 would carry point mutations in its gyrase gene as a result of its ciprofloxacin resistance. To track the donor *E. coli* NT36 copy number and measure plasmid mobility, the primer set (*gyrA*-F and *gyrA*-R) obtained from literature was used to amplify the gyrase gene (*gyrA*) to identify any possible point mutations between the two parents.[87] The PCR protocol was followed as outlined[87] and the PCR products were sent to ACGT Corp. (Toronto, ON, Canada) for DNA sequencing. The single consensus sequence was edited using Sequence Scanner v1.0 (Applied Biosystems, 2005) and point mutations between *E. coli* NT36 and EB-G3 strains were identified by using nBLAST sequence alignment. Quantitative-PCR primer sets (gyrNT36) (Table 4) were designed based on the differences between the two sequences and with high specificity for *E. coli* NT36. As such, the primer sets (gyrNT36) were designed using IDT PrimerQuest and New England BioLabs (NEB) Tm calculator tools and were best optimized for specificity to amplify *E. coli* NT36 *gyrA* gene. The goal here is to create a qPCR primer set that specifically amplifies the donor NT36 and not the recipient EB-G3. PCR was carried out to verify primer specificity using the reaction mix outlined in 2.10 with the following thermocycler settings: initial denaturation of 95 °C for 5 min, 30 cycles of denaturation 95 °C (1 min), annealing 55 °C (30 s), and elongation 72 °C (30 s).

2.15 Growth Curve and Subinhibitory Concentration

Maximum subinhibitory concentrations of *E. coli* NT36 and EB-G3 were determined for the antibiotics gentamicin, ciprofloxacin, tetracycline, and carbenicillin using Epoch 2 microplate spectrophotometer (BiotTek, USA). The parameters set for the 96-well microplate reader include incubation at 37 °C at constant orbital shaking. All absorbances were read at 600 nm and measurements were taken at 30 min intervals for a total of 5.5 hrs. An overnight culture of each parent was prepared separately with the corresponding antibiotic pressure (*E. coli* NT36:

carbenicillin and *E. coli* EB-G3: gentamicin), saline washed twice, resuspended and diluted in LB to a final OD of ~0.05. This was placed into the 96-well plate in replicates of four at various increasing antibiotic concentrations (Table 5). The growth curves were generated and analyzed using Gen5 (BioTek, USA) microplate data collection and analysis software.

2.16 Conjugation Reactor Assembly

Overnight cultures of *E. coli* NT36 and EB-G3 were prepared in LB media and incubated at 37 °C while shaking. The cultures were washed twice with 0.9 % saline and resuspended in 10 mL of sterile LB media. The culture suspensions were then diluted 100 folds and their OD measurements were taken and adjusted to ~0.05. Once adjusted, the reactors were assembled with 150 mL LB media containing 1:3 donor to recipient ratio with the appropriate final OD of ~0.05. The selected donor to recipient ratio will increase the likelihood of developing transconjugants since there are more available cells to receive the plasmids per donating bacteria. The reactors with the antibiotic pressure were assembled in triplicates alongside a single reactor with no antibiotic pressure as reference. The antibiotic pressures used for this experiment include carbenicillin (1 µg/mL), tetracycline (64 µg/mL), gentamicin (6 µg/mL), and ciprofloxacin (0.25 µg/mL). The concentration of the antibiotic pressures was determined from the subinhibitory concentrations of each parent obtained from the microplate growth curves (section 2.15). The reactors were incubated at 37 °C for 3.5 hrs indicative by the initial stages of their stationary growth phase. After 3.5 hrs of growth, the reactors were plasmid extracted using the NucleoBond Xtra Midi extraction kit (Macherey-Nagel, Germany) with a slight modification to the protocol by extending the lysis step to 10 min to extract chromosomal DNA. Lastly, the purity and concentration of the genomic extract of each reactor were measured using a nanophotometer™ Pearl (Implen, Germany).

2.17 Tracking Plasmid Transfer via Quantitative-PCR

Four primer sets were used to measure the migration of two plasmids hosted by *E. coli* NT36 to *E. coli* EB-G3 (gyrNT36, YaiO, IncI1-TraW, and IncF-TraN) (Table 4). Standard curves for each primer set were established to measure and verify qPCR efficiency at various template concentrations (100 ng to 0.001 ng) (Table 4). The qPCR assay was performed in triplicates on *E. coli* NT36 genomic DNA as a reference, no-antibiotic reactor DNA extract as calibrator sample, three antibiotic-containing reactor DNA extracts, and Milli-Q water as contamination control. SYBR green qPCR supermix (Bio-Rad, ON, Canada) and 100 ng of appropriate template DNA were used for each reaction by following the manufacturer's protocol. The thermocycler settings for all reaction and primer sets are as follows: initial denaturation at 95 °C for 2min, 40 cycles of denaturation at 95 °C for 10 s, and annealing at 55 °C for 30 s. A melting curve was also performed at 95 °C for 10 s and 65 °C to 95 °C at increments of 0.5 °C for 5 s. The fold difference in the relative abundance of YaiO, IncI1-TraW, and IncF-TraN to gyrNT36 was calculated using the Livak method. In this scenario, the reference primer set is gyrNT36, the calibrator sample is the no-antibiotic containing reactors, and the treatment groups are the antibiotic-containing reactors. After the qPCR run, the samples were run on a 1 % gel electrophoresis to ensure that there is no primer dimers or non-specific binding.

Table 3. A list of PCR primers used for tetracycline resistance determinants and the 16S rRNA gene.

Gene	Primer	Sequence (5'to 3')	Amplicon size (bp)	Annealing Temperature (°C)	Resistance mechanism	Positive Control	Reference
Tet B	Tet B F Tet B R	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	659	TD* (65-55)	Efflux pump	E. coli HB101 (pRT11)	[87]
Tet C	Tet C F Tet C R	CTTGAGAGCCTTCAACCCAG ATGGTCGTCATCTACCTGCC	418	TD (65-55)	Efflux pump	*E.coli* DO-7 (pBR322)	[87]
Tet G	Tet G F Tet G R	GCTCGGTGGTATCTCTGCTC AGCAACAGAATCGGGAACAC	468	TD (65-55)	Efflux pump	TOPO10	[87]
Tet M	Tet M F Tet M R	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	406	TD (65-55)	Ribosomal protection protein	*E.coli* DH1 (pACYC177)	[87]
Tet Q	Tet Q F Tet Q R	ATCGGTATCAATGAGTTGTT GACTGATTCTGGAGGAAGTA	40	50	Ribosomal protection protein	pNFD 13.2	[88]
Tet W	Tet W F Tet W R	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	168	TD (65-55)	Ribosomal protection protein	pIE1120 (pGEM-TW)	[89]
Tet X	Tet X F Tet X R	TTAGCCTTACCAATGGGTGT CAAATCTGCTGTTTCACTCG	223	55	Degradation enzyme	DNA	[88]
16S rRNA	U341 F U758 R	CCTACGGGAGGCAGCAG CTACCAGGGTATCTAATCC	500	55	NA**	Any bacteria	NA

*TD = touchdown PCR (initial temperature = 65°C and final temperature = 55°C). **NA – not applicable

Table 4. List of primer sets used for the PCR and qPCR detection of *E. coli* NT36, EB-G3, pNT36-3, and pNT36-4.

Primer Set	Sequence (5' to 3')	Amplicon size (bp)	Target	Tm (°C)	qPCR Efficiency (%)	Reference
IncI1-TraY	F: AGC GAT ACT CCA GCC ATT TC R: CCG CCT CTT CAT TAC CTC TTA C	716	pNT36-3	63.2	-	This study
IncI1-TraG	F: GGC CAG GTA AAT AGC CTC ATA G R: GGT AGG AAC TGA CCA CGA TTA C	984	pNT36-3	63.1	-	This study
IncI1-TraB	F: GAG TCT GCC CGT CTT ATC TTT C R: CAG ACG GTG TCC CAG TTA TTT	474	pNT36-3	63.4	-	This study
IncI1-PilM	F: GTT AAT GGC TGA GTG GAG GTA G R: ACA AGG GAT GGT CGC TAA TG	962	pNT36-3	63.2	-	This study
IncI1-TraW	F: CGA CGA CGG TGA CTG AAT AA R: CCC GAG CAG GAG ACA ATA AA	108	pNT36-3	63.0	110.0	This study
IncI1-TrbB	F: TAC TGC TTC AGG CGT TGT ATC R: GCG TTG TGC TGT TCG TAA TG	92	pNT36-3	63.4	-	This study
IncI1-PilQ	F: GTT GGA CCT GAC AGG ACT ATT T R: GAT ACC CGA TGA CGG AGA TAA AG	123	pNT36-3	63.1	-	This study
IncF-TraN	F: CAG GTT CCC TCA TCG GAA TAA A R: CAG GAT GAA GGT CCG TGA TAA A	291	pNT36-4	63.2	104.6	This study
IncF-TraQ	F: GTA TCC ATC CGC GCC ATA AA R: CCT GGG TGT CTG GTT TCA TAT C	715	pNT36-4	63.6	-	This study
IncF-TraG	F: GGT ACG CTC TCC ATT CCT TTA C R: CGA CCA GTA CAC GAC GAA TAT G	294	pNT36-4	63.4	-	This study
gyrNT36	F: GGC TCG GCG GTC TAT G R: GGG ACT TTT TGC CGT G	225	*E. coli* NT36	55.0	99.3	This study
YaiO	F: TGA TTT CCG TGC GTC TGA ATG R: ATG CTG CCG TAG CGT GTT TC	115	*E. coli* EB-G3	58.0	108.8	[86]

Table 5. The concentration of antibiotics used to determine the maximum subinhibitory concentration of *E. coli* NT36 and EB-G3

Gentamicin (µg/mL)	0.5	1	2	4	6	8	12	16	20	30
Carbenicillin (µg/mL)	1	10	20	40	64	80	100			
Tetracycline (µg/mL)	1	2	4	8	16	32	64	100		
Ciprofloxacin (µg/mL)	1	2	4	8	10	15	20			
Ciprofloxacin (µg/mL)	0.004	0.015	0.0625	0.125	0.25	0.5	1			

3

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Chapter 3: Characterization of Antibiotic Resistant and Sensitive Bacteria Found in Three Urban WWTPs

3.1 Introduction

The use of antibiotics in the treatment of infectious diseases is crucial for the protection of public health. However, the significant increase in the use and misuse of antimicrobials drugs, both in clinical and agricultural settings, has contributed to a corresponding increase in the concentration of compounds found in waste streams and the environment in general.[90] In urban settings, humans contribute to most of the pharmaceutical waste that ends up in domestic sewers that eventually are transported to the wastewater treatment plant (WWTP). Wastewater treatment includes the removal of particulate matter and the degradation of organic and inorganic contaminants, however,

conventional wastewater treatment facilities have not been designed to remove emerging contaminants during treatment.[91] Furthermore, removal rates depend heavily on the operating conditions of the plant.[92,93] Concurrently, the daily use of pharmaceuticals for medical treatment and agricultural uses results in pseudo-persistent concentrations of these drugs in the secondary process of the WWTPs. Surveys of Canadian WWTPs have shown that antibiotics can frequently be detected in effluents[93,94] and can enter the environment through discharges from the wastewater treatment process.

The increase in antibiotic waste released into municipal wastewater has coincided with an increase in the prevalence of resistance genes in wastewater treatment processes. Both antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have been found in wastewater samples from China, Japan, Germany, Portugal, and the US (Arkansas, Colorado, Louisiana, Michigan) among others.[95–103] Limited data is available on the abundance and identification of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in WWTPs in Canada [104] and it is not clear what role the WWTPs play in discharging ARGs into the natural environment along with treated effluent.[99] Concerns about WWTPs acting as sites for the transfer and evolution of antibiotic resistance genes prompts the following questions: What is the prevalence of antibiotic resistance genes in WWTPs in a large Canadian urban metropolis? Are ARGs escaping from WWTPs and contaminating downstream water bodies? And what are the ARBs in the WWTP that carry the resistance genes? Are ARGs in wastewater carried as single entities or they are part of multiple gene mobility factors and how are they inherited?

Although antibiotic resistance and corresponding gene determinants are ubiquitous in the environment, WWTPs are considered ‘hotspots’ for horizontal gene transfer between bacteria due to their high nutrient and high density load.[105] Furthermore, the subinhibitory concentrations of

antibiotics, like those observed in WWTPs, have been shown to increase the frequency of transfer of resistance genes.[106] The ability of WWTPs to act as an ideal environment to promote gene transfer between bacteria endorses the potential for the increased occurrence of ARGs within the bacterial population and the potential for resistance genes to be transferred from indigenous populations to pathogens or from one pathogen to another.[107] Overall, the WWTP may accelerate the evolutionary timeline of ARGs by enhancing the mobilization of environmental resistance genes into clinical isolates.[108] Studies examining the relative abundance of ARGs in water and biofilms samples collected downstream of WWTP sites suggest that effluent discharges could be a source of ARGs in the environment.[108,109]

Tetracycline is an antibiotic that has been used extensively in human and veterinary medicine for decades. Although its usage in human treatment has decreased in recent years its consumption in agricultural and animal husbandry settings is still widespread. Resistance to tetracycline is due to numerous genes that code for one of three mechanisms: efflux pumps, ribosomal protection proteins or enzyme degradation. Many of these genes are found on mobile genetic elements that carry resistance to other antibiotics and/or metals.[110,111] With more than 40 determinants identified that code for resistance to tetracycline[112] and the genetic basis of the resistance well established, molecular methods can be used to track the identification and location of the determinants in populations.[113]

This chapter aims to devise a characterization strategy to permit the investigation of the prevalence and fate of ARGs in WWTPs and demonstrate the interaction between antibiotic sensitive and resistant populations. We hypothesize that by using both molecular and culture-based techniques, we can identify suitable donors and recipients from each respective population that can serve as ideal candidates to understand the fate of ARGs in wastewater communities. Since culture-

dependent methods may isolate only a small percentage of the overall bacterial population and culture-independent methods are limited in their ability to identify the antibiotic resistance isolates or determine multiple antibiotic resistance in the population, both culture-dependent and culture-independent methods were employed in this study to minimize the limitations of either approach. The objectives were to isolate tetracycline sensitive and tetracycline resistant bacteria from multiple urban WWTPs to determine the frequency of resistance and whether selection for a single resistance increased the likelihood that isolates carried multiple resistance; to identity isolates from the two populations to determine whether genus identity was correlated with antibiotic resistance phenotypes profiles; to track tetracycline resistance genes to determine if WWTP is seeding natural environments and to perform hierarchical cluster analysis on the resistance profiles as a way to detect gene dissemination. Although culture-independent techniques such as next-generation sequencing can provide more information with regards to the entire population, they cannot identify which of the members carries individual genes. Our combined methods of characterization enable downstream population investigations that monitor the proliferation and transfer of mobile genetic elements amongst native members in a given environment and the fate of those elements after transfer.

3.2 Results and Discussion

Both tetracycline resistant and sensitive cultures were isolated from wastewater samples from three urban WWTPs in Toronto (Figure 1). The pure culture isolates were characterized using culture-dependent/independent methods and analyzed statistically. The advantage of combining these methods of characterization allows the collection of information about the community as a whole with respect to the array of resistance determinants that are contained within that community.

3.2.1 Antibiotic Resistance

Tetracycline resistance genes have been shown to be widespread in the microbial community in hospital and urban WWTPs. Furthermore, the percent of bacteria isolates exhibiting antibiotic resistance within WWTPs was found to be greater than that found in the natural environment.[105,114] In 2007, Auerbach *et al.*[21] used culture-independent methods to show that tetracycline resistance genes were more abundant in WWTPs than in natural lake samples and that Tet Q was found to be highest in influent and Tet G to be highest in activated sludge. Moreover, ARBs and ARGS have been found to be released from WWTP in the effluent and biosolids generated during the treatment process.[103]

In this study, bacterial isolates were collected from three WWTPs in a large urban area by spread plating activated sludge samples on R2A plates with and without selective antibiotic. Sixty-four tetracycline sensitive isolates representing different morphotypes were selected for further analysis, 13 were from the North York plant, 22 were from the Humber plant and 29 were from the Ashbridges Bay plant. It was found that when these isolates were tested for their resistance to eight antibiotics, many were found to have resistance to one or more of the antibiotics (Figure 2). Antibiotic resistance to each of the 8 tested antibiotics was found in all the plants albeit at varying levels of resistance. Overall, it was found that 33 - 37 % of the isolates were resistant to ampicillin,

5 - 18.5 % were resistant to chloramphenicol, 0 - 7.6 % resistant to ciprofloxacin, 26 - 29 % were resistant to gentamicin, 0 - 14.8 % resistant to kanamycin, 5.2 - 7.7 % resistant to streptomycin and 5 - 44 % were resistant to sulfamethoxazole /trimethoprim. Although 30 % percent of the isolates were not resistant to any of the antibiotics tested, 13.6 % percent were found to be resistant to three or more antibiotics and, therefore, considered to have multiple antibiotic resistance (MAR).

Regardless of the WWTP sampled the resistance to ampicillin appeared to be quite consistent with approximately 1/3 of the bacteria carrying resistance to the β-lactam antibiotics. Other studies have found the ampicillin resistance varies from 3.3 - 42%[105] and is among the most common resistance found. Ampicillin resistance is mediated by the *bla* (TEM-1) gene that can be carried on a transposon or plasmid that contributes substantially to the spread of the antibiotic resistance determinant among bacterial populations.[115]

Resistance to sulfamethoxazole/trimethoprim was also found to be high and bacteria have been found to remain resistant to this drug even in the absence of selective pressure.[116] Resistance to kanamycin was only observed in the Ashbridges Bay WWTP isolates and resistance to ciprofloxacin was only observed in the Humber and Ashbridges Bay plant isolates although several additional isolates displayed intermediate resistance to these antibiotics. Overall, the isolates that were collected without antibiotic selection carried a wide diversity of resistance (Figure 2).

The percent of tetracycline resistant culturable bacteria in the WWTPs was determined by plating samples on R2A plates supplemented with tetracycline (16 μg/mL) and expressing the number of isolates that grew on tetracycline plates as a percentage of the total number of bacteria that grew on plates with no antibiotic. Each WWTP was sampled three times and the percentage tetracycline resistant isolates varied from as little as 0.13 % to as high as 7.18 % of the total culturable population. Several of the isolates that grew on the tetracycline selective plates later

showed only intermediate resistance when tested in the antibiotic disc test probably since the discs contained 30 µg while the selective plates had only contained 16 µg/ml tetracycline. Overall, it was found that 0.94% of the culturable bacterial population from Ashbridges Bay, 1.84% from Humber, and 3.66 % from North Toronto were resistant to tetracycline.

One hundred and nine of these isolates were then tested for their resistance to the additional seven antibiotics, 68 from the North Toronto, 25 from Ashbridges Bay, and 16 from the Humber WWTP. Overall, it was found that 75 - 94% of the tetracycline resistant isolates were also resistant to ampicillin, 52 -81% were resistant to chloramphenicol, 32 - 75% resistant to ciprofloxacin, 37 - 94% were resistant to gentamicin, 31 - 63% resistant to kanamycin, 32 - 81% resistant to streptomycin and 40 - 75 % were resistant to sulfamethoxazole /trimethoprim (Figure 2). In total 78% were considered to be MAR (Table 6).

Comparison of the levels of resistance between isolates selected as tetracycline sensitive and tetracycline resistant suggests that selection for a single resistant determinant makes it more likely the isolates have additional resistances probably because resistance genes are often found clustered on mobile genetic elements that can be transferred to other bacteria.[117]

The ARI scores (Table 6) of the tetracycline sensitive and tetracycline resistant isolates were calculated. An ARI value above 0.2 indicates that isolates are exposed to selectivity due to the presence of contaminants such as antibiotics.[85] Since selective pressure can promote the dissemination of the resistance determinants, a population with a high ARI score would have more members carrying resistance genes that were likely to proliferate or transferring resistance genes to other organisms. In our case, the ARI scores for the tetracycline resistance population in all three WWTP (0.60, 0.47, 0.83) were up to 8 times higher than those in the tetracycline sensitive population (0.11, 0.21, 0.10) suggesting that dissemination of antibiotic resistance genes had

occurred and that mobile genetic elements carrying multiple gene resistances were likely present (corresponds with high MAR).

Both the Humber and North Toronto plants had higher ARI values than Ashbridges Bay possibly because Ashbridges Bay collects a much greater volume of water including stormwater that may dilute the antibiotic concentrations from municipal sources thereby lowering the selection pressure.

3.2.2 Diversity and abundance of species

Forty-five tetracycline sensitive and forty-five tetracycline resistance isolates were sequenced and identified (Table 7). All of the isolates presented 16S rRNA gene sequence similarity values higher than 95% with the type strain of a validly named species and were, therefore, considered members of that genus. Most (67%) of the tetracycline sensitive isolates were identified as *Acidovorax, Acinetobacter, Aeromonas, Flavobacterium,* and *Pseudomonas* while most (56%) of the tetracycline resistant isolates were found to be *Chryseobacterium, Microbacterium, Stenotrophomonas,* and *Variovorax,* showing that the composition of the populations was dominated by different genera. The Shannon-Weaver Index calculation confirmed that both the tetracycline sensitive population (0.64) and the tetracycline resistant population (0.66) contained a large amount of diversity and a Dice-Coefficient calculation (0.20) indicated that the populations did not have a significant overlap in composition suggesting that they were distinctly different from one another.

In terms of dissemination of tetracycline resistance determinants, we were able to find both sensitive and resistant variants of some genera, however, seven genera (*Enterobacter, Exiguobacterium, Flavobacterium, Herminiimonas, Riemerella, Sinorhizobium, and Yersinia*), representing 14 isolates (31%), were only found among the tetracycline sensitive strains (Table 7). Interestingly, many of the possible pathogenic genera (*Escherichia* and *Serratia*) were only found

in the tetracycline resistant population, confirming the potential concern of antibiotic resistance dissemination among pathogens in wastewater treatment communities. Although some pathogens were found in the tetracycline sensitive population, none were only represented in that population. This observation confirms that antibiotic resistance in potential pathogens is quite widespread in WWTPs.

The uniqueness in the composition of the two populations possibly reflects the limitation of antibiotic gene dissemination among some bacterial genera. Although the culturable population represents only a fraction of the total community it may indicate that not all bacteria are capable of carrying or expressing every antibiotic resistance determinant. Furthermore, it appears that horizontal gene transfer may be restricted to certain members of the overall bacterial community. Further characterization of these isolates will determine if they share common genetic elements that can be used to carry antibiotic genes.

3.2.3 Distribution of antibiotic resistance determinants

Antibiotic resistance was analyzed in conjunction with the phylogenetic data using cluster analysis to compare the antibiotic resistance determinant patterns within each genera cluster and throughout each population (Figures 3 and4). Differences in antibiotic resistance patterns can result from the ecology and physiology of the bacteria and may suggest distinct modes and mechanisms of resistance acquisition. In one case, there were no distinct resistance patterns associated with any of the genera clusters. For example, within the tetracycline sensitive population, the *Acinetobacter* cluster contained 7 different antibiotic profiles, none of which were more dominant than the other or more prevalent in any one of the WWTPs. This possibly suggests that individual isolates had acquired their resistance genes independently of others. Since the identification process did not

identify specific species within each cluster, it may be possible that antibiotic resistance patterns may emerge at the species level.[118]

In the tetracycline resistance population, the *Stenotrophomonas* cluster (Figure 4) contained isolates from both the North Toronto and Humber plant that presented the same pattern (resistant to all eight antibiotics) but also contained isolates with different patterns within and between the different WWTPs. Again, this suggests that individual isolates may have acquired resistance genes independently of each other or such that these determinants are not actively expressed despite their presence. Nevertheless, on some occasions, different isolates of the same genera, isolated from either the same or different WWTPs, yielded the same antibiotic resistance pattern. In general, it was observed that members of the same genera did not necessarily share common antibiotic resistance profiles. Moreover, it was not possible to establish a relationship between the resistance phenotype and the site of isolation. However, the absence of any patterns across any of the parameters - genera, location, or antibiotic resistance, suggests the relevance of population dynamics for the hypothetical dissemination of resistance. To evaluate whether vertical and horizontal gene transfer is the major process for dissemination of antibiotic resistance within WWTPs requires a deeper analysis to include multiple isolations of the same species from different time points.

3.2.4 Dissemination of antibiotic genes into the environment

Antibiotic resistance bacteria and genes not removed during the wastewater treatment process could potentially be disseminated into the environment downstream of the discharge pipe.[104] Although ARB are seldom released from the WWT process, ARGs can escape removal.[95] If ARGs are present on small genetic elements they may be able to pass through the discharge process and be available for uptake (via transformation) by bacteria downstream of the plants. ARGs themselves have been recognized as emerging contaminants, independent of their bacterial carriers.[109]

Therefore, identifying the contribution source of the ARGs in the downstream water sources can help to determine how much the WWTPs contribute to urban water ARG contamination.

Two wastewater flow pathways were investigated. The North Toronto Plant discharges its effluent by gravity to the Ashbridges Bay plant which after treatment releases the effluent into Lake Ontario on the east side of the city (LOS1). The Humber plant discharges its final effluent directly into Lake Ontario on the west side of the city (LOS2). Identification of the tetracycline determinants may assist in monitoring the dissemination of tetracycline resistance and the evolution of gene exchange. The previous sampling showed that tetracycline resistant bacteria could be isolated from all 5 locations (data not shown). However, whether the observed tetracycline resistant bacteria in the lake was due to intrinsic resistance or due to the acquisition of ARGs from the WWTP discharge was unknown.

Overall, the percentage of tetracycline resistant bacteria in the lake was lower than in the WWTP (data not shown) which was expected since a large dilution effect must be taken into account when the discharge is released into the lake body. To determine if the antibiotic genes in the WWTP are indeed escaping to the lake, seven of the tetracycline resistance determinants were monitored using PCR primers to the seven genes to create ARG gene profiles of the bacterial populations within the three urban WWTPs and the receiving waters. The seven genes used were Tet B, Tet C, Tet G, Tet M, Tet Q, Tet W, and Tet X.

Table 8 shows the detection of each of the genes in the 5 locations. Tet C, Tet Q, and Tet X were found in all locations suggesting that these gene determinants are ubiquitous in Toronto water, whereas, Tet B was not detected in any of the locations. Tet G was found in the North Toronto plant and Ashbridges Bay plant but not in the Humber plant, however, this determinant was absent from all lake samples at both locations. Likewise, Tet M was found in all three WWTPs although not in

all samples from the Ashbridges Bay plant and was not in the downstream water body. This evidence suggests that the WWTP process may effectively remove some ARGs from the effluent before their release. Interestingly, however, Tet W was found in all three WWTPs and the downstream lake water but only in the water column and not in the sediment. The detection of Tet W in the water column and not the sediment perhaps represents a transient location for the ARG where the determinant has not been deposited into the lake in high enough concentration or over enough time to allow the determinant to be deposited into the sediment or be picked up by a bacterium that eventually settles into the sediment. Moreover, it could not be determined whether the determinant was indigenous to the lake water or had come from being released from the WWTP although the same result was observed in both wastewater pathways. Further investigation of the presence of this determinant in water sources upstream of the plants could help to determine if the WWTP contributed to the presence of Tet W in the lake water or whether this determinant is indigenous to the lake.

3.2.5 Hierarchical cluster analysis of antibiotic profiles

The antibiotic profile patterns of 160 isolates were compared using hierarchical cluster analysis to determine if common patterns could be distinguished. After analysis, the 160 profiles were clustered into 10 major patterns (Table 9). There are two ways of interpreting the data obtained through the cluster analysis. The first is to recognize R (resistance) and S (susceptibility) patterns in each cluster that are over the 75% cut-off. Possible mobile genetic elements could be present within the cluster-populations that confer resistance to certain groups of antibiotics within the populations. In other words, one or more mobile elements may be responsible for conferring similar patterns of resistance in each cluster. Because of this phenomenon, the proliferation of multiple-antibiotic resistance carrying elements may have spread throughout various members of the population thus

giving them a similar resistance pattern. For example, by observing clusters 9 and 10, there are 25 isolates in cluster 10 with various morphologies but an identical resistance phenotype. Similarly, cluster 9 also has a similar pattern of resistance except for a high number of intermediate levels of resistance to gentamicin and kanamycin. The resistance patterns observed for both clusters 9 and 10 may be due to a similar mobile element that lacks the gentamicin and kanamycin resistance genes in cluster 9; alternatively, isolates in cluster 10 have an additional element with resistance genes to these two antibiotics.

The second way of interpreting the data is to pay attention to the variable resistance (V^R) phenotypes across the clusters. Resistances to certain antibiotics across most clusters show various degrees of susceptibility (resistant, susceptible, and intermediate). As a result, only the prominent phenotype is indicated in Table 9 (e.g. V^S-58% indicates that 58% of the isolates in this cluster were susceptible to the antibiotic). The variable phenotypes in a given cluster introduce discrepancies across isolates in a given cluster making it difficult to categorize the susceptibility of the cultures. Meanwhile, these variable phenotypes could indicate the possibility of mobile-mediated-resistance genes being present in some cases and absent in others. For example, cluster 8 contains a population where the majority are resistant to ampicillin, chloramphenicol, and sulfamethoxazole-trimethoprim yet remain variable for tetracycline, gentamicin, kanamycin, and streptomycin. This pattern of resistance could be the result of a mobile genetic element carrying resistance to aminoglycosides and/or tetracycline in some of the isolates within this cluster while absent in others. Cluster 7 also shares a similar concept for resistance. The majority of the isolates in cluster 7 are resistant to tetracycline, ampicillin, and gentamicin, yet remain variable for chloramphenicol, ciprofloxacin, kanamycin, and streptomycin. It is possible for a mobile genetic element carrying resistance for the

natural aminoglycosides to be present among some of these isolates with a possibility of also carrying resistance to chloramphenicol and ciprofloxacin.

After examining the collected data, it is difficult to differentiate the root cause of these patterns and whether they are caused by a single mobile genetic (with insertions or deletions) or by numerous/combinations of genetic elements (plasmid, transposons, or chromosomal). However, these patterns do provide the incentive for investigating wastewater cultures to determine which genetic elements are responsible for resistance to the targeted antibiotics and to determine whether they can horizontally be transferred and particularly to pathogenic microbes.

3.3 Conclusion

Overall, bacterial isolates were collected from the three urban WWTPs and some found to have multiple resistances to eight antibiotics. Bacteria that carried a single resistance to tetracycline were found to be more likely to have resistance to three or more antibiotics than those isolates that were not tetracycline resistant. This suggests that resistance could be acquired as a cassette containing several determinants or that a single determinant could code for a mechanism that can offer resistance to several different antibiotics simultaneously. A more diverse tetracycline determinant library was seen in the WWTP than in the receiving waters indicating that ARGs may be removed during the treatment process (Table 8). However, sampling of receiving waters at a later date will determine if determinants only seen in the WWTP may potentially appear in the receiving waters. Identification of isolates showed that there was a large diversity of species in both the tetracycline resistant and tetracycline sensitive populations and that the two groups had unique compositions suggesting that antibiotic resistance determinants may be more likely to be present in some strains than in others (Table 7). Furthermore, a large diversity of antibiotic resistance patterns existed within genera of each population suggesting that transmission of ARG within the WWTP process may happen by several different mechanisms. Lastly, in future studies, it would be valuable to identify which mobile genetic elements are carried by these bacterial cultures. Not only would it provide insight into how mobile genetic elements may proliferate in a population but which members are involved in their transfer. By characterizing the population using our combination of methods, we were able to link genotypes to specific communities and phenotypes to specific community members. It allowed us to gain a deeper understanding of how gene transfer may or may not occur in highly dense populations and who the possible donors and recipients may be.

Figure 1. Map of the three WWTPs (North Toronto, Humber, and Ashbridge's Bay) located in Toronto, Ontario, Canada. The wastewater of all three plants was sampled for analysis. This image was generated through Google Maps.

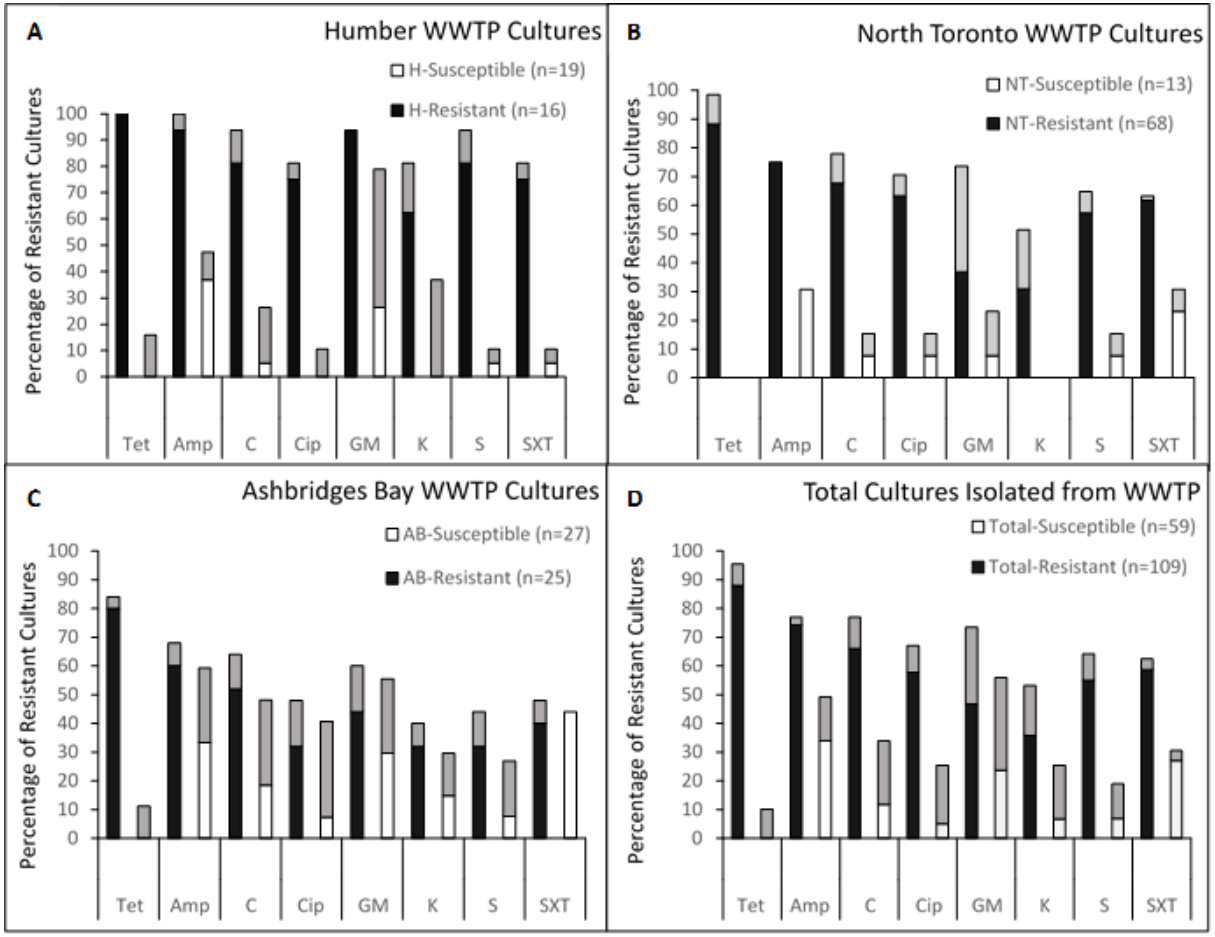
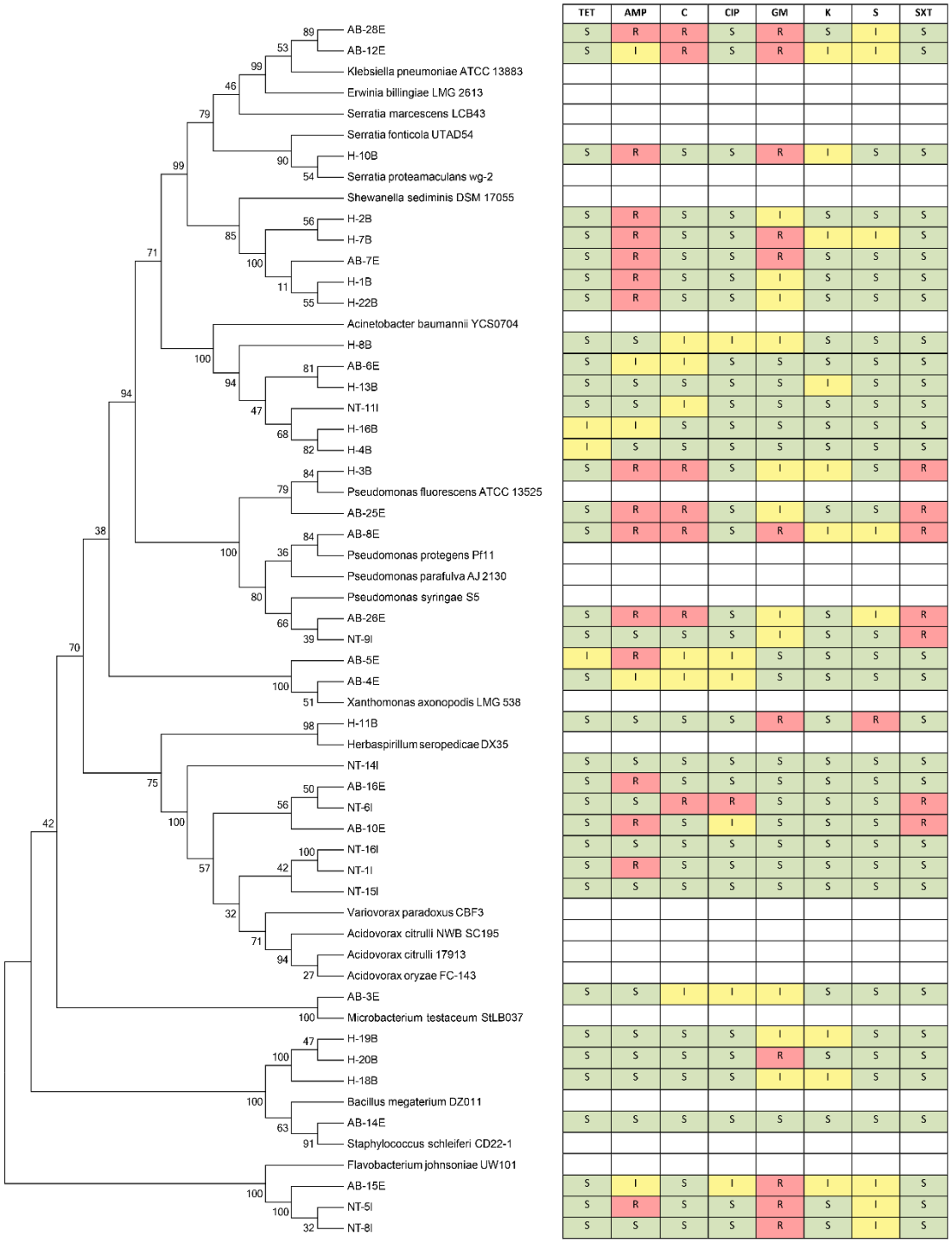
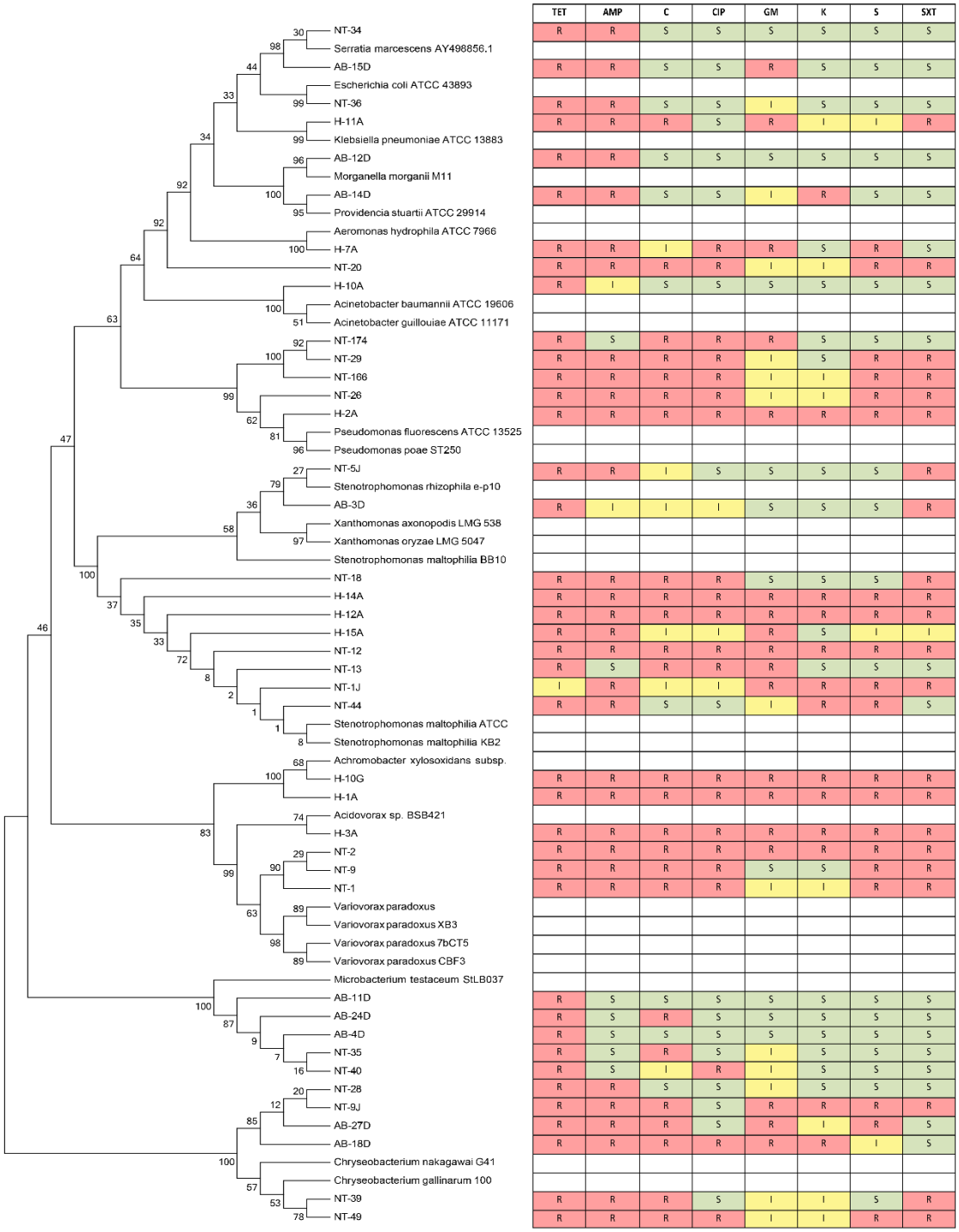


Figure 2. Antibiotic resistance profiles of tetracycline sensitive and tetracycline resistant isolates from three urban wastewater treatment plants; A) Isolate profiles from North Toronto WWTP B) Isolate profiles from Humber WWTP, C) Isolate profiles from Ashbridges Bay WWTP and D) cumulative total from all three locations. The black bars show the percentage of resistant isolates and the white bars represent the percentage of sensitive isolates. The grey bars present the percentage of isolates exhibiting intermediate resistance. Tet = tetracycline, Amp =ampicillin, C = chloramphenicol, Cip = ciprofloxacin, GM = gentamicin, K = kanamycin, S = streptomycin, SxT = sulfamethoxazole/trimethoprim.



TET	AMP	C	CIP	GM	K	S	SXT
S	R	R	S	R	S	I	S
S	I	R	S	R	I	I	S
S	R	S	S	R	I	S	S
S	R	S	S	I	S	S	S
S	R	S	S	R	I	I	S
S	R	S	S	R	S	S	S
S	R	S	S	I	S	S	S
S	R	S	S	I	S	S	S
S	S	I	I	I	S	S	S
S	I	I	S	S	S	S	S
S	S	S	S	S	I	S	S
S	S	I	S	S	S	S	S
I	I	S	S	S	S	S	S
I	S	S	S	S	S	S	S
S	R	R	S	I	I	S	R
S	R	R	S	I	S	S	R
S	R	R	S	R	I	I	R
S	R	R	S	I	S	I	R
S	S	S	S	I	S	S	R
I	R	I	I	S	S	S	S
S	I	I	I	S	S	S	S
S	S	S	S	R	S	R	S
S	S	S	S	S	S	S	S
S	R	S	S	S	S	S	S
S	S	R	R	S	S	S	R
S	R	S	I	S	S	S	R
S	S	S	S	S	S	S	S
S	R	S	S	S	S	S	S
S	S	S	S	S	S	S	S
S	S	I	I	I	S	S	S
S	S	S	S	I	I	S	S
S	S	S	S	R	S	S	S
S	S	S	S	I	I	S	S
S	S	S	S	S	S	S	S
S	I	S	I	R	I	I	S
S	R	S	S	R	S	I	S
S	S	S	S	R	S	I	S

Figure 3. Dendrogram of the alignment of the 16S rRNA partial sequences of various tetracycline susceptible isolates to known type strains with their corresponding antibiotic resistance profiles. Antibiotics used: TET: tetracycline (30 µg), AMP: ampicillin (10 µg), C: chloramphenicol (30 µg), CIP: ciprofloxacin (5 µg), GM: gentamicin (10 µg), K: kanamycin (30 µg), S: streptomycin (10 µg), and SXT: sulfamethoxazole-trimethoprim (23.75/1.25 µg). R = resistance, I = intermediate, S = sensitive.



TET	AMP	C	CIP	GM	K	S	SXT
R	R	S	S	S	S	S	S
R	R	S	S	R	S	S	S
R	R	S	S	I	S	S	S
R	R	R	S	R	I	I	R
R	R	S	S	S	S	S	S
R	R	S	S	I	R	S	S
R	R	I	R	R	S	R	S
R	R	R	R	I	I	R	R
R	I	S	S	S	S	S	S
R	S	R	R	R	S	S	S
R	R	R	R	I	S	R	R
R	R	R	R	I	I	R	R
R	R	R	R	I	I	R	R
R	R	R	R	R	R	R	R
R	R	I	S	S	S	S	R
R	I	I	I	S	S	S	R
R	R	R	R	S	S	S	R
R	R	R	R	R	R	R	R
R	R	R	R	R	R	R	R
R	R	I	I	R	S	I	I
R	R	R	R	R	R	R	R
R	S	R	R	R	S	S	S
I	R	I	I	R	R	R	R
R	R	S	S	I	R	R	S
R	R	R	R	R	R	R	R
R	R	R	R	R	R	R	R
R	R	R	R	R	R	R	R
R	R	R	R	R	R	R	R
R	R	R	R	S	S	R	R
R	R	R	R	I	I	R	R
R	S	S	S	S	S	S	S
R	S	R	S	S	S	S	S
R	S	S	S	S	S	S	S
R	S	R	S	I	S	S	S
R	S	I	R	I	S	S	S
R	R	S	S	I	S	S	S
R	R	R	S	R	R	R	R
R	R	R	S	R	I	R	S
R	R	R	R	R	R	I	S
R	R	R	S	I	I	S	R
R	R	R	R	I	I	R	R

Figure 4. Dendrogram of the alignment of the 16S rRNA partial sequences of various tetracycline resistant isolates to the known type strains with their corresponding antibiotic resistance profiles. Antibiotics used: TET: tetracycline (30 µg), AMP: ampicillin (10 µg), C: chloramphenicol (30 µg), CIP: ciprofloxacin (5 µg), GM: gentamicin (10 µg), K: kanamycin (30 µg), S: streptomycin (10 µg), and SXT: sulfamethoxazole-trimethoprim (23.75/1.25 µg). R = resistance, I = intermediate, S = sensitive.

Table 6. The MAR and ARI scores for the tetracycline sensitive and tetracycline resistant isolates from each of the urban WWTPs.

WWTP	Tet profile (n)	MAR	ARI
North Toronto	Tet sensitive (13)	7.7	0.11
	Tet-resistant (68)	80.9	0.60
Ashbridges Bay	Tet sensitive (29)	22.2	0.21
	Tet-resistant (25)	56.0	0.47
Humber	Tet sensitive (22)	5.3	0.10
	Tet-resistant (16)	100	0.83
Total	Tet sensitive (64)	13.6	0.14
	Tet-resistant (109)	78.0	0.60

MAR = Multiple Antibiotic Resistance
ARI = Antibiotic Resistance Index

Table 7. Identities of the tetracycline resistant and tetracycline sensitive WWTP isolates.

Genus	**Tet resistant – n (%)**	**Tet sensitive – n (%)**
Achromobacter	2 (4.4)	0
Acidovorax	1 (2.2)	6 (13.3)
Acinetobacter	3 (6.7)	10 (22.2)
Aeromonas	1 (2.2)	5 (11.1)
Chryseobacterium	6 (13.3)	0
Enterobacter	0	2 (4.4)
Escherichia	2 (4.4)	0
Exiguobacterium	0	3 (6.7)
Flavobacterium	0	4 (8.9)
Herminiimonas	0	1 (2.2)
Klebsiella	1 (2.2)	0
Microbacterium	6 (13.3)	1 (2.2)
Morganella	1 (2.2)	0
Providencia	1 (2.2)	0
Pseudomonas	2 (4.4)	5 (11.1)
Riemerella	0	2 (4.4)
Serratia	3 (6.7)	0
Sinorhizobium	0	1 (2.2)
Staphylococcus	1 (2.2)	1 (2.2)
Stenotrophomonas	9 (20.0)	1 (2.2)
Variovorax	4 (8.9)	1 (2.2)
Xanthomonas	2 (4.4)	1 (2.2)
Yersinia	0	1 (2.2)
Total (n)	**45**	**45**

Table 8. The detection of tetracycline gene determinants in metagenomics DNA in the two wastewater flow pathways.

Pathway 1	Gene Determinant						
	Tet B	**Tet C**	**Tet G**	**Tet M**	**Tet Q**	**Tet W**	**Tet X**
North Toronto ↓	-	+	+	+	+	+	+
Ashbridges Bay ↓	-	+	+	+/-	+	+	+
Lake Ontario (east) water column	-	+	-	-	+	+	+
or **Lake Ontario (east) sediment**	-	+	-	-	+	-	+
Pathway 2							
Humber ↓	-	+	-	+	+	+	+
Lake Ontario (west) water column	-	+	-	-	+	+	+
or **Lake Ontario (west) sediment**	-	+	-	-	+	-	+

Table 9. Distinct patterns of resistance of the isolates obtained through hierarchical cluster analysis

Cluster	TET	AMP	C	CIP	GM	K	S	SXT	Number of isolates in each cluster
1	S	S	V^{S}-68%	S	V^{S}-64%	S	S	S	28
2	$V^{S/R}$-46%	R	S	S	$V^{I/R}$-46%	S	S	S	13
3	S	$V^{S/R}$-42%	S	S	R	V^{S}-58%	V^{I}-50%	S	12
4	R	S	V^{S}-50%	V^{S}-71%	V^{S}-64%	S	S	S	14
5	S	V^{S}-58%	V^{S}-58%	V^{I}-58%	S	S	S	R	12
6	R	R	V^{R}-54%	V^{R}-45%	S	S	S	V^{R}-63%	11
7	R	R	$V^{S/R}$ -36%	V^{S}-54%	R	V^{R}-73%	V^{R}-73%	S	11
8	V^{R}-58%	R	R	S	V^{R}-58%	V^{R}-42%	V^{I}-42%	R	12
9	R	R	R	R	V^{I}-50%	V^{I}-50%	R	R	22
10	R	R	R	R	R	R	R	R	25

Tet=tetracycline, Amp=ampicillin, C=chloramphenicol, Cip=ciprofloxacin, GM=gentamicin, K=kanamycin, S=streptomycin, SXT= sulfamethoxazole/trimethoprim, S-(≥75% Susceptible), R-(≥75% Resistant), V^{S}-% (<75% susceptible as majority), V^{R}-% (<75% resistant as majority), V^{I}-% (<75% Intermediate as majority), $V^{S/R}$-% (<75% susceptible and resistant in equal distribution), $V^{I/R}$-% (<75% intermediate and resistant in equal distribution)

4

Chapter 4: Annotation and Surveillance of Novel Plasmids found in Environmental Bacterial Cultures Isolated from the WWTPs

4.1 Introduction

Within the last few decades, there have been increasing concerns regarding the rapid emergence of resistant bacteria worldwide. This antibiotic resistance crisis has been attributed to the misuse of antimicrobials as well as the lack of new drug development by pharmaceutical industries. In 2016, 40,752 kg of antimicrobials were purchased by various hospital sectors across Canada at a cost of ~$92 million (CAD).[1] In almost every case of developed/discovered antibiotic, resistance has eventually been seen in pathogenic microbes that were once previously susceptible. Due to the emergence of antibiotic resistant infections, it leaves clinicians with no reliable alternatives to treat infected patients.

One of the main sources for the dissemination of ARGs in bacteria has been attributed to various aquatic systems affected by anthropogenic activities. When antibiotics enter the ecosystem, they can affect the evolution of microorganisms and community structure.[119] As a result, the ecological function of an exposed aquatic ecosystem, under environmentally relevant antibiotic concentrations, may exert a temporary selective pressure.[19] Numerous studies have been done to address rising concerns regarding the potential impact of antibiotics and antibiotic residues in aquatic environments.[20,21,23,24,121] The presence of antibiotics within an ecosystem is not only recognized as a chemical/emerging contaminant but they also play a critical role in the development of ARB and ARGs.[8,19,25] A significant amount of antibiotics, ARGs, and ARB have been detected in the effluent of wastewater treatment systems and their downstream water bodies and are recognized to be a main anthropogenic source of contamination.[12,27,29,121–123] As a result, the wastewater treatment plants (WWTPs) are recognized as hotspots for ARGs and ARB and their spread into the environment.[12] The biological treatment process serves as an environment that is suitable for resistance development and spread due to continuous exposure of subinhibitory concentrations of antibiotics within the microbial community.[30,121] However, there is still a lack of knowledge regarding the mechanisms used by microorganisms to transfer ARGs between the members within their population.

In Canada, there has been very little attention given to the distribution, transfer, and occurrence of ARGs and ARB in WWTPs and Canadian water systems. This information is vital for tracking and identifying potential hazards to public health and water quality. Identifying major habitats in which ARGs and ARB thrive is essential for learning their fate in the environments and perhaps future problems that may arise as a result. Currently, antibiotics, ARGs, and ARB concentrations and frequencies are not being reported in Canadian WWTPs annual reports.[33–35]

One of the main vectors that are used by microorganisms to transfer ARGs is plasmids through the process of bacterial conjugation. Bacterial plasmids are extra-chromosomal DNA that replicates independently as a stable component of the cell's genome. They can vary in size ranging from 1-100+ kbp and can have various copy numbers from 1 to several hundred per cell. The copy number of plasmids are generally fixed under constant conditions and are controlled by plasmid-mediated systems. Plasmids often impose a fitness cost to their hosting cell as they take valuable resources for their maintenance and expression. The survivability of the plasmid in a cell depends on numerous factors including environmental stress, competition, fitness cost, plasmid-mediated regulatory elements and partitioning systems, and whether if the plasmid offers any improvement to the host's survivability and metabolism.[124,125] As an example, a plasmid can be beneficial when its bacterial host is exposed to an environmental stressor. In the absence of the stressor, the same plasmid can be detrimental due to the fitness cost imposed by it. As a consequence, plasmid-free bacterial strains will have a competitive advantage over plasmid carrying strains in the absence of the stressor, therefore costly plasmids are expected to be lost from a population as a result of competition. Several possible explanations have been proposed to explain the underlying cost of hosting plasmids including altering host gene expression, the metabolic load imposed by plasmid replication, the introduction of efflux pumps that may extrude essential biomolecules, and the disruption of essential host genes by the integration of plasmid genes.[181] Another factor that dictates the stability of a plasmid in a cell is plasmid incompatibility. Plasmid incompatibility is defined as the failure of two or more plasmids coexisting in a cell by sharing similar plasmids partitioning or replication systems (Rep proteins) and/or interference with the ability to maintain plasmid copy number.[51,125–127] The copy number, host-range, and compatibility of a plasmid can be predicted by identifying the presence of its replicon type.[51] Larger plasmids can slowly be lost from a population as a result of segregational loss. Segregational instability is described as plasmid loss that occurs

during cell division whereby the plasmid fails to separate into each daughter cell. Segregational instability is often associated with larger plasmids as a result of their low copy number nature. If the rate of plasmid loss exceeds the rate of vertical and horizontal gene transfer, it is expected that the plasmid will eventually be lost from a population.[181] The metabolic burden (fitness cost) imposed by plasmid carriage can divert resources such as essential biomolecules, energy-rich compounds, or machinery away from its bacterial host, which may stress the cell and reduce overall fitness. [181]

In literature, it has been documented that antibiotic resistant plasmids are predominant and available within environmental populations.[55–57] However, most studies neglect to investigate the non-resistant members of a population while focusing on specific members or genetic elements through selective sampling. In this study, we attempted to approach the issue differently by identifying members in culturable populations in three WWTPs and investigating their plasmidome (entire plasmid contents) of individual isolates. The advantages of this method include (1) knowing the identity of the isolate hosting the plasmid, (2) investigating a wider diversity of bacteria, (3) distinguishing plasmid content between antibiotic sensitive and resistant populations, (4) obtain a better insight on plasmid host range, and (5) establish a link between antibiotic resistance phenotype/genotype of individual bacteria.

4.2 Results and Discussion

4.2.1 Bacterial selection, resistance profiles, and plasmid detection

A total of 46 isolates from the WWTP system were selected for plasmid extraction. Their resistance profiles were examined for eight different antibiotics (Table 10) and categorized based on their initial selection. Among the selected isolates, 63% (n=29) were tetracycline resistant, 37% (n=17) tetracycline sensitive, 39% (n=18) carried 6 or more resistances to antibiotics, 24% (n=11) carried less than 3 resistances to the tested antibiotics, and 20% (n=9) were resistant to all tested antibiotics. As previously reported, selection for resistance to one antibiotic (tetracycline) was associated with resistance to other antibiotics (Chapter 3). Regardless of their resistance patterns, only 30% (n=14) of the isolates carried any plasmid(s). Surprisingly, it was found that nearly all of the highly resistant bacteria (≥ 6 resistances) did not carry any plasmid(s) except for two isolates, *Stenotrophomonas* NT1J and *Chryseobacterium* AB27D. Previous studies have indicated that plasmids and mobile gene elements play a critical role in the dissemination of ARGs and we expected that multi-resistant isolates would be more likely to carry plasmids with ARGs. A possible explanation for the high resistance patterns observed in these isolates could be as a result of a combination of numerous chromosomal-based genetic elements such as transposon, integrons, chromosomal resistance genes, and/or multidrug efflux systems. Transposons and integrons have been recognized in the past as carriers of multiple ARGs such as Tn*10* and Tn*1696*-like as well as class 1-2 integrons.[61,65,67,128] In addition, the overexpression of multidrug efflux systems such as the AdeABC system, belonging to the resistance-nodulation-division (RND) family found in *Acinetobacter baumannii*, has been demonstrated to induce resistance to aminoglycosides, fluoroquinolones, tetracycline, tigecycline, chloramphenicol, erythromycin, trimethoprim, netilmicin, and meropenem.[129] The observed high resistance patterns may be a result of the overexpression of an uncharacterized multidrug efflux system. Additionally, extraction of plasmid

DNA from some isolates may have been compromised due to numerous factors including inadequate lysis procedures for that strain or large plasmid sizes that cause co-isolation of the plasmid with the chromosomal fraction. In this case, whole-genome sequencing may be required to confirm the presence of plasmids in hard-to-extract isolates for increased sensitivity. Overall, nine plasmids from four isolates representing the following genera, *Providencia, Escherichia, Klebsiella*, and *Acinetobacter*, were sequenced and annotated. Four of the plasmids were successfully closed by performing PCR on the ends of their respective sequences as outlined in methods and materials section 2.7.

Table 10. The identity and antibiotic resistance profiles of all 46 cultures that were isolated and plasmid extracted from each WWTP

Genus	Strain	TET	AMP	C	CIP	G	K	S	SXT	TG	Plasmid
Achromobacter	H1A	R	R	R	R	R	R	R	R	R	-
Acinetobacter	AB6E	S	I	R	S	S	S	S	S	S	+
Acinetobacter	AB14C	R	R	I	I	R	S	S	S	R	-
Acinetobacter	H16B	I	I	S	S	S	S	S	S	S	-
Acinetobacter	H2F	R	R	I	R	R	S	I	S	R	-
Acinetobacter	NT10K	R	R	I	I	R	I	I	S	R	-
Acinetobacter	H10A	R	I	S	S	S	S	S	S	R	-
Acinetobacter	H4B	I	S	S	S	S	S	S	S	S	+
Acinetobacter	H13B	S	S	S	S	S	I	S	S	S	+
Acinetobacter	H15B*	S	S	S	S	R	S	S	S	S	+
Acinetobacter	NT2K	R	R	S	S	I	S	S	R	R	+
Acinetobacter	NT12K	R	I	R	S	R	S	S	I	R	-
Aeromonas	H7A	R	R	I	R	R	S	R	S	S	+
Aeromonas	NT13K	R	R	S	S	S	S	S	S	R	-
Aeromonas	AB9C	R	R	S	R	R	R	S	R	R	-
Chryseobacterium	AB18D	R	R	R	R	R	R	I	S	I	-
Chryseobacterium	AB27D	R	R	R	S	R	I	R	S	R	+
Enterobacter	AB12E	S	I	R	S	R	I	I	S	R	+
Escherichia	NT36*	R	R	S	S	I	S	S	S	S	+
Klebsiella	H11A*	R	R	S	S	R	I	I	R	S	+
Neisseria	NT15K	S	S	S	S	I	S	S	S	R	-
Providencia	AB14D*	R	R	S	S	I	R	S	S	R	+
Pseudomonas	AB8E	S	R	R	S	R	I	I	R	I	+
Pseudomonas	AB25E	S	R	R	S	I	S	S	R	R	+
Pseudomonas	H4F	I	R	R	R	I	S	S	R	R	-
Pseudomonas	NT9K	S	R	R	S	R	I	S	R	R	-
Serratia	AB15D	R	R	S	S	R	S	S	S	R	-
Serratia	NT29	R	R	R	R	I	S	R	R	R	-
Shewanella	H5F	I	R	S	S	S	S	S	S	R	-
Stenotrophomonas	NT1J	I	R	I	I	R	R	R	R	R	+
Stenotrophomonas	NT7K	I	R	S	S	S	S	S	S	R	-
Stenotrophomonas	NT8K	I	R	S	S	S	R	S	R	R	-
Variovorax	NT9	R	R	R	R	S	S	R	R	R	-
Variovorax	NT6	R	R	R	R	S	R	I	R	R	-
Varivorax	NT16F	R	R	R	R	R	R	R	R	R	-
Xanthomonas	H12F	I	R	R	S	S	S	S	R	R	-
Xanthomonas	NT5J	R	R	I	S	S	S	S	R	R	-

Genus	Strain	TET	AMP	C	CIP	G	K	STP	SXT	TG	Plasmid
N/A	AB3C	R	R	R	R	R	R	R	R	R	-
N/A	AB6C	R	R	R	R	R	I	R	R	R	-
N/A	AB11C	R	R	R	R	R	R	R	R	R	-
N/A	AB12C	R	R	R	R	R	R	R	R	R	-
N/A	AB13D	S	S	I	I	S	S	S	I	R	-
N/A	AB21D	R	S	S	S	S	S	S	S	R	-
N/A	AB28D	S	I	S	I	S	S	S	R	R	-
N/A	H3A	R	R	R	R	R	R	R	R	R	-
N/A	H6A	R	R	R	R	R	R	R	R	R	-
N/A	H8A	R	R	R	R	R	R	R	R	R	-

WWTP-wastewater treatment plant, AMP-ampicillin (10 µg), C-chloramphenicol (30 µg), CIP-ciprofloxacin (5 µg), G-gentamicin (10 µg), K-kanamycin (30 µg), STP-streptomycin (10 µg), SXT-sulfamethoxazole/trimethoprim (23.75/1.25 µg), TET-tetracycline (30 µg), and TG-tigecycline (8 µg), S-sensitive, I-intermediate, R-Resistant, "+"- plasmid(s) present, N/A-data not available. Strain isolation location "H"-Humber WWTP, "AB"- Ashbridge's Bay WWTP, and "NT"-North Toronto WWTP, *denotes the isolates that had plasmids extracted and sequenced.

4.2.2 *Providencia* sp. plasmid sequencing analysis

The genus *Providencia* is a gram-negative bacillus belonging to the Enterobacteriaceae family. The most studied *Providencia* species include *Providencia alcalifaciens, P. heimbachae, P. stuartii,* and *P. rettgeri*. They are recognized for their urease-producing capabilities and can cause urinary tract infections as an opportunistic pathogen. *Providencia* sp. are commonly found in soil, water systems, animal reservoirs and are known to be multi-antibiotic resistant.[130,131] Furthermore, *P. stuartii* has been documented for carrying conjugative plasmids capable of transferring to *Escherichia coli*.[130,132,133] In clinical studies, *Providencia* sp. is of great interest due to its ability to cause nosocomial infections, spread antibiotic resistance, and/or virulence factors through conjugative plasmids. In this section, two large plasmids, belonging to an isolate *Providencia* sp. AB14D will be discussed in terms of the implications that they may have in a bacterial community.

The first plasmid, pAB14D-1, is a 108,963 bp plasmid containing conjugative elements that are part of the IncF family, therefore, suggesting that this plasmid is capable of conjugating between compatible recipients (Figure 10). Furthermore, pAB14D-1 contains a type III secretion system that provides gram-negative bacteria a unique virulence mechanism enabling them to infect eukaryotic cells by secreting effector proteins.[134] Type III secretion systems (T3SS) have been characterized in plasmids found in various *Providencia* sp.[135] In the case of pAB14D-1, numerous translocator and effector proteins were found including pathogenicity island (SPI-1), MxiG, and SipC-D. Many of these translocator and effector proteins have been associated with T3SS of various microorganisms including *Shigella dysenteriae*, *Salmonella enterica,* and *Yersinia* sp.[134] Since this plasmid also carries IncF conjugative elements, it may be possible for this plasmid to transfer horizontally to other microorganisms within the same family and increase the pathogenicity of its population. The dendrogram generated through nBLAST demonstrates the commonality of this plasmid to other

plasmids/hosts found within the BLAST database (Figure 6). The pAB14D-1 plasmid shares a slight query coverage to plasmids found within *Pectobacterium carotovorum* (27%), *P. rettgeri* (27%), and *P. stuartii* (24%) with all similarities falling partially within the IncF-plasmid transfer regions. As a result, this plasmid appears to be novel, therefore making it difficult to predict its compatibility with other closely related bacteria. Another key feature of pAB14D-1 is that it contains a transposon that is similar to the transposase IS3/IS911 family. BLAST results of this transposon indicate that its sequence is commonly found in plasmids and chromosomes of *P. alcalifaciens, P. stuartii, P. rettgeri,* and *Proteus mirabilis* while partially found in *Morganella, Klebsiella,* and *Yersinia* genera. The sequences of this transposon flanks an 8616 bp hypothetical protein with an unrecognizable protein sequence. In its current unaltered state, pAB14D-1 may not contribute to the spread of ARGs, however, it may pose a different threat by increasing the pathogenicity of other members in its population and perhaps acquire ARG through genetic recombination.

The second plasmid identified in *Providencia* sp. AB14D is a smaller 42,469 bp plasmid designated as pAB14D-2 (Figure 11). Unlike pAB14D-1, pAB14D-2 carries the type IV secretion system (T4SS)-VirB1-11/D4. T4SS-VirB/D4 is homologous to conjugative machinery of incompatibility groups found in bacteria, therefore, they can mediate the conjugative transfer of plasmids to a wide range of bacterial species.[136,137] Furthermore, T4SSs have a wide variety of functions including facilitating the release of effector/virulence proteins and the release and uptake of DNA in the extracellular environment [136]. The plasmid pAB14D-2 carries a major structural gene (VirB2) that is essential for the secretion and biogenesis of the pilus structure.[137] The complete T4SS complex can produce the structural scaffold required for connecting the inner and outer membrane between the donor and recipient and allow the horizontal transfer of plasmid DNA.[138] Additionally, pAB14D-2 also contains a 9681bp gene encoding for an immunosuppressive bacterial effector-like

protein, lymphostatin *Efa1*/*LifA*. This gene shares the closest homology (62% DNA identity) to the lymphostatin found in Enterohemorrhagic *Escherichia coli* (EHEC).[139] Lymphostatins are recognized as a large toxin with a key role in bacterial pathogenesis in gram-negative bacteria and is strongly associated with the development of hemolytic uremic syndrome and infectious diarrhea.[139–141] Numerous pathogens have been described in the past to encode lymphostatins including *Chlamydia psittaci*, *Chlamydia muridarum*, EHEC, enteropathogenic *Escherichia coli*, and *Citrobacter rodentium.*[142–144] To our knowledge, this is the first instance that T4SS-VirB/D and lymphostatin *Efa1*/*LifA* genes have been detected on plasmids for any species of *Providencia*. The dendrogram generated from the closest BLAST alignments indicates that plasmids isolated from three *Proteus mirabilis* and one *P. rettgeri* have the closest similarity (91%) with a maximum DNA coverage of 57% (Figure 7). According to this data, pAB14D-2 is not a commonly found plasmid and it shares very little alignment outside of the entries shown in Figure 7. Lastly, pAB14D-2 encodes for a nucleoid-associated (H-NS) like-protein that shares a high identity (78%) to plasmid and chromosomal H-NS that are primarily found in *Providencia* and *Proteus* species. Plasmid-mediated H-NS like-proteins have been shown to play important roles in transcriptional regulation between the host's chromosome and plasmids while improving the overall plasmid stability and maintenance.[145] Chromosomal H-NS proteins are recognized for their ability to bind to regions of DNA that are rich in A/T content and can act as a global transcriptional repressor.[146] The plasmid pAB14D-2 has an A/T content of 64.3% and, as a consequence, can become susceptible to H-NS proteins encoded from its hosting bacteria. In its defense, pAB14D-2 encodes for a protein that has high homology to H-NS and may serve as a substitute for H-NS binding regions. Similar mechanisms have been found in the literature such as the H-NS homolog, Sfh, encoded by the plasmid pSfR27.[145,147–149] The *Sfh* gene was suggested to be a "stealth gene " that allows A/T-rich

plasmids to horizontally enter new hosts with minimal re-precautions on fitness and gene expression thus ensuring plasmid survivability and compatibility.[146]

To further add to plasmid persistence and survivability, both pAB14D-1 and pAB14D-2 carry a toxin-antitoxin stabilization system similar to RelE/RelB system. Homologs of RelBE systems have been recognized across prokaryotes as well as in plasmids.[150] The gene *relE* encodes for a cytotoxin that inhibits protein synthesis while *relB* encodes for an antitoxin to prevent RelE activity. The RelBE system ensures the survival of both plasmids within the next generation of daughter cells by killing off plasmid-free cells since they lack the antitoxin producing gene (*relB*).[151] As a result, this system serves a similar function to that of ARGs/antibiotic pressure to ensure plasmid persistence following cell division. In addition to the RelBE toxin-antitoxin system, the plasmid pAB14D-2 also encodes for another toxin-antitoxin system known as VapBC. Similar to RelBE, VapB serves as the antitoxin and neutralizes the translational inhibitor, VapC.[152] Both plasmids (pAB14D-1/2) help facilitate pathogenicity in *Providencia* sp. and have built-in mechanisms to promote their transfer, survival, and persistence within their host and in the bacterial population. Although no ARGs were identified on pAB14D-1 and pAB14D-2, the remaining partial sequencing data indicated the presence of tetracycline efflux protein (*tetA*), chloramphenicol acetyltransferase, beta-lactamase, fosfomycin resistance (*FosA*), and numerous multidrug-efflux transporter genes (data not shown). The antibiotic resistance profile of *Providencia* sp. *AB14D* indicates resistances to tetracycline, ampicillin, kanamycin, and tigecycline (Table 10). The discrepancies between the genotype and phenotype could be the result of unidentified genes, in-active genes, and/or mis-represented annotated sequences.

4.2.3 *Klebsiella* sp. plasmid sequencing analysis

Klebsiella sp. is a genus of gram-negative rod-shaped bacteria belonging to the Enterobacteriaceae family. *Klebsiella pneumoniae* is a well-studied bacteria due to its importance as a pathogen in both the community and hospital-acquired infections. There has been an everlasting struggle for the management and treatment of this bacteria due to its remarkable ability to acquire resistance to numerous classes of antibiotics including carbapenems, aminoglycosides, quinolones, and last resort antibiotics tigecycline and colistin.[153] Hypervirulent variants of *Klebsiella pneumoniae* have been recognized to cause serious life-threatening infections in healthy individuals including liver abscess, meningitis, pneumonia, and endophthalmitis.[154] *K. pneumonia* is ubiquitous in the environment and is commonly found in water, sewage, soil, humans, and plant surfaces.[155,156] It has been documented in the literature that pathogenic potential of environmental *K. pneumonia* isolates was found to be the same as those isolated from clinical environments.[157,158] However, environmental strains of *K. pneumonia* had a 90% or greater susceptibility to all antibiotics than those found within clinical settings with exception to ampicillin and carbenicillin resistance.[158] These findings suggest that selective pressure may be responsible for the resistance phenotypes observed in clinical isolates. In this section, one complete large-plasmid and one partially sequenced plasmid belonging to *Klebsiella* sp. H11A strain will be closely examined. *Klebsiella* sp. H11A was found to have resistance to tetracycline, ampicillin, gentamicin, and sulfamethoxazole/trimethoprim and moderately resistance to kanamycin and streptomycin (Table 10). As a result, the strain H11A can be considered as a multi-antibiotic resistant environmental isolate.

One of the plasmids that were successfully sequenced and closed is the large 110,448bp plasmid pH11A-1 (Figures 5 and 12). Unfortunately, this plasmid's annotation data primarily resulted in uncharacterized hypothetical proteins, with a few metabolic-associated genes and

unidentified phage tail proteins (Figure 5 and 12). Although numerous open reading frames of varying sizes are present on this plasmid, there is still a lack of research done in identifying these proteins. However, after observing the BLAST generated dendrogram (Figure 9), it was apparent that numerous plasmids share a strong coverage (88-93%) and identity (~99%) to pH11A-1. The pH11A-1 homologs have been found all over the world including China, USA, Hong Kong, and England. Only one of the many plasmid entries (pPMK1-B; CP008931.1) was published and, similar to our findings, they were unsuccessful in appropriately annotating their plasmid other than identifying a tellurite resistance gene.[159] Following BLAST results, pH11A-1 and its close relatives appear to be highly specific to *K. pneumoniae* bacteria. As for now, this plasmid's function and its role in *K. pneumoniae* isolates remain a mystery.

The second plasmid, denoted as pH11A-2, is partially sequenced/assembled ~240,771bp plasmid that shares high similarity to its reference, *K. quasivariicola* pKPN1705-1 (CP022824.1) (Figures 5 and 18). Both the reference and pH11A-2 plasmids encode for the complete IncF conjugative plasmid transfer genes. *K. pneumoniae* has been a well-recognized bacteria for hosting numerous plasmids including IncF-like plasmids.[132,160] Furthermore, pH11A-2 and pKPN1705.1 encode for a similar IS1-like transposon carrying numerous metal resistance/transport genes including copper, zinc, cadmium, cobalt, lead, mercury, and arsenic. Although heavy metal resistance directly causes no harm, they often can be accompanied by ARGs.[161] The presence and long-term accumulation of heavy metals in the environment can select for such antibiotics/metal co-resistance plasmids, therefore, contributing to the persistence of ARGs within the bacterial population. Iron transport system (*Fec*) and Arsenic resistance proteins (*ArsH*) were also present on both plasmids. The last unique feature of pH11A-2 is that it carries another transposable element belonging to the IS3/IS911 family. Numerous membrane transport genes were also found

accompanied with this transposon such as ABC transporters, urea carboxylase aminomethyltransferase, ABC-type amino acid transporter, and arsenic resistance genes.

4.2.4 *Acinetobacter* sp. plasmid sequencing analysis

Acinetobacter sp. is primarily round/rod-shaped gram-negative bacteria that are ubiquitous in the environment. They can commonly be found in the soil and water-bound environments. Certain species of *Acinetobacter* are a key source of hospital-acquired infection, especially when infecting immunocompromised patients. One of the most well-studied species of *Acinetobacter* is *A. baumannii* due to its disease-causing capabilities. *A. baumannii* is generally considered to have a high rate of antibiotic resistance including having resistance to major last-resort antibiotics. However, similarly to *K. pneumoniae*, most of the environmental cultures appear to be more susceptible to major antibiotics. Common infections caused by *A. baumannii* include pneumonia, meningitis, bloodstream infection, urinary tract infection, and necrotizing fasciitis. In this study, 11 unique *Acinetobacter* isolates were successfully characterized and tested for their plasmid content (Table 10). Only 5 out of 11 isolates were shown to be carrying plasmid(s). Unfortunately, out of the 5 sequenced *Acinetobacter* sp., only one of them was successfully sequenced as the rest were heavily contaminated with chromosomal reads.

The only plasmid that was successfully sequenced and closed was isolated from *Acinetobacter* H15B that resulted in a 163,876bp plasmid (pH15B-1) (Figures 5 and 13). *Acinetobacter* H15B is sensitive to all of the tested antibiotics with exception to gentamicin. Interestingly, although this plasmid was extracted from *Acinetobacter* sp., it appears that pH15B-1 only shares similarities to plasmids found in gram-positive *Bacillus cereus* and *Bacillus cytotoxicus* strains (Figure 8). The plasmid pH15B-1 was likely acquired by *Acinetobacter* H15B through the means of HGT from *Bacillus cereus* cultures within the same environment. The highest BLAST

match contained a poor coverage of 53% with a sequence identity of 100% (CP016361.1) while the rest of the matches were anywhere from 28% and below in coverage. There were no secretion systems or incompatibility groups identified on pH15B-1, therefore, suggesting that it had to be mobilized through other means of transfer from *Bacillus*. Previous studies have documented plasmid transfer between gram-positive *Enterococcus faecalis* and gram-negative *E. coli.*[162] Although the transfer between gram-positive to gram-negative may be rare, it is still possible for it occurs especially in a highly dense microbial population such as in WWTPs. Unique genes were found carried by pH15B-1 such as various sporulating genes (*SpoVAE1*, *SpoVAD*, and *SpoVAC*) common to *Bacillus* species. Furthermore, transposon (Tn552) genes were found accompanied by the plasmid along with various DNA integration/recombination/inversion like-genes. Unfortunately, similar to other plasmids, the majority of the open reading frames annotated as hypothetical proteins and their function/purpose on the plasmid remains a mystery. However, for this plasmid to remain stable in a non-native host, it may be possible that it provides *Acinetobacter* sp. H15B with an evolutionary advantage. *Acinetobacter* sp. H15B was tested to only be resistant to gentamicin (Table 10), however, after examining the remaining sequencing data, it was found that it carries ARGs for tetracycline, ampicillin, fluoroquinolones, aminoglycoside, and fosfomycin, and various multidrug resistance proteins. The discrepancies between antibiotic phenotype and genotype patterns could be explained by the lack of genetic expression. Although the genes are all present, they may no longer be functional to provide the correct resistance phenotype to the bacteria.

4.2.5 *Escherichia* sp. plasmid sequencing analysis

The genus *Escherichia* is a gram-negative, rod-shaped, facultative anaerobic bacteria that are ubiquitous in most environments including gastrointestinal tracts of animals, soil, and aquatic systems. The most characterized species belonging to this genus is *Escherichia coli* because of its beneficial or pathogenic relationship to humans. Most commensal *E. coli* strains rarely cause disease in healthy individuals; however, certain virulent strains are well-adapted to cause a variety of diseases including gastroenteritis, urinary tract infection, Crohn's disease, and more.[163] Mobile genetic elements have been identified for influencing *E. coli* pathogenicity through virulence factors and ARGs via transposons, bacteriophages, plasmids, and pathogenicity islands.[163] Even plasmids lacking conjugative and/or type secretion systems can move between different hosts via accompanying another plasmid with transfer capabilities. As a consequence, mobile genetic elements have contributed greatly to the evolution of highly resistant pathogenic *E. coli*. Although pathogenic *E. coli* have been shown to be more prominent in clinical settings, environmental strains have also been demonstrated to carry virulence genes/elements.[164] In addition, another study found that both clinical and environmental isolates had similar multiple antibiotic resistance indices and both groups carried resistances to major classes of antibiotics.[165] In this study, one *Escherichia* strain isolated from the WWTP was identified to carry four large plasmids with resistances to tetracycline and ampicillin. Unfortunately, the sequence reads of these plasmids were not successfully closed and instead were assembled/grouped with similar matching plasmids found in the BLAST database as a reference.

The largest matching plasmid found in the BLAST database to resemble *E. coli* NT36 sequences is the plasmid p0.1229-2 isolated from *E. coli* O18H1 by the Food and Drug Administration (FDA) in the USA (CP028322.1, Figure 15). *E. coli* NT36 plasmid sequences

(pNT36-4) share a 61% coverage to p0.1229-2 including genes such as the complete IncF-plasmid transfer genes, tetracycline resistance gene (TetA), and various unidentified hypothetical proteins. The differences between the two plasmids are that p0.1229-2 also carries ARGs and metal transport genes for aminoglycosides, quaternary ammonium compounds, sulfonamides, macrolides, and mercury transport proteins. It may be possible that the two plasmids share a similar evolutionary pathway and the introduction of the additional genes could have been made possible through mobile genetic elements such as transposons. The plasmid p0.1229-2 antibiotic and metal resistance genes are also accompanied by scattered remnants of the transposon (Tn21) genes. Tn21 has been characterized in the past to encode for mercury, sulfonamide, and aminoglycoside resistances hosted by conjugative plasmids.[166] The high similarity in the observed resistance patterns between p0.1229-2 and the literature suggests that Tn21 may be responsible for the additional resistance genes that are found in p0.1229-2 and not in pNT36-4.

The second plasmid that was found to share high similarity to *E. coli* NT36 (pNT36-2) sequence belongs to reference plasmid isolated from *Salmonella enterica*, pSH146_87 (JX445149.1, Figure 14). The plasmid pSH146_87 is a large 86 586bp conjugative plasmid originally sequenced in the USA. The similarities between pSH146_87 and pNT36-2 are relatively high with a coverage of 90%. The only notable feature between the two plasmids is that they both carry the complete IncI1-plasmid transfer genes. The majority of the remaining sequences were annotated as hypothetical proteins with unknown functions. Although the purpose of this plasmid within its host remains to be undetermined, it may still aid in the movement of other mobile plasmids that lack conjugative properties to move from one host to another. Both pSH146_87 and pNT36-4 may have a wider host range since they are found in *E. coli* and *S. enterica*, therefore, complementing the plasmid's conjugative ability to move across different genera.

The third plasmid pNT36-3 shared high similarity to a plasmid (p86) found in another *E. coli* strain 1190 isolated from veterinary clinical care in Great Britain (CP023387, Figure 16). There is a 88% coverage in the similarity between the two plasmids with little notable differences. One of the unique properties of this bacteria, similar to pNT36-2, is that it contains the complete IncI1-plasmid transfer genes. Although it is unlikely for a bacteria to host two plasmids of the same incompatibility group, pNT36-2 and pNT36-3 may encode for different plasmid recognition or partitioning proteins that may enable them to co-exist in *E. coli* NT36. Further sequencing analysis also indicates that *E. coli* NT36 carries two sets of IncI1-transfer genes with minor differences in their gene order (data not shown). To our knowledge, there have not been any cases where two plasmids of the same incompatibility co-exist under stable conditions within one bacteria. Other unique features that are shared between both plasmids are the presence of a beta-lactamase ARG followed by a partial transposase-like gene. The beta-lactamase resistance gene may have been introduced into the plasmid as a part of an unknown transposon that may no longer be functional due to the loss of important functional genes.

The final plasmid hosted by *E. coli* NT36 is interesting in the sense that it encodes partial IncI1-pilus structural genes (*PilV, PilT, PilP,* and *PilM*) and partial type IV secretion system genes (*VirB1*, *VirB2*, *VirB4*, *VirB8*, *VirB10*, *VirB11*, and *VirD4*). The closest plasmid found on BLAST to resemble pNT36-1 with coverage of 95% was a 59 599bp plasmid p75-02_2 isolated from *Shigella sonnei* strain p75-02 (CP019690.1, Figure 17). The only interesting genes found in both plasmids are the IncI1 pilus structural, Type IV secretion system, and remnants of phage protein genes (EaA). The plasmid pNT36-1 may once have had a functional type IV secretion system and/or IncI1-transfer capabilities that allow it to transfer between different genera within the same family. As a result of genetic modification, pNT36-1 has lost those essential genes and may no longer be able to

transfer on its own. The role of these plasmids within the bacteria remains a mystery and as to why its host chooses to maintain it despite fitness/maintenance costs imposed by them. Until there are better tools and strategies that scientists can employ to identify the function and roles of the uncharacterized hypothetical proteins, it will prove difficult to associate the role and function of certain plasmids within their hosts.

Overall, the environmental isolate *E. coli* NT36 contains numerous large conjugative plasmids that are suggested to be able to transfer between multiple hosts within closely related genera. This can be problematic as it will contribute to the overall spread of ARGs and/or virulence factors across numerous closely-related pathogenic species. More research is required to fully understand the stability, persistence, and the transfer of large conjugative/mobile plasmids within a highly diverse population such as those found in the WWTP. It remains difficult to fully assess the risks of these plasmids within a population as not all conjugative genetic elements are directly involved in encoding for ARGs and virulence factors.

4.3 Conclusion

It has been shown that ARGs and ARB are found in Toronto WWTPs as well as downstream water bodies (Chapter 3). The pseudo-persistence of antibiotic residues in wastewater has been demonstrated to support plasmid maintenance and possible transfer between microbes.[3] Possible pathogenic bacteria may employ various strategies to help maintain plasmids through toxin/antitoxin systems. Numerous plasmids were found to carry conjugative transfer systems including (IncF) pAB14D-1, pH11A-2, pNT36-4 and (IncI1) pNT36-2 and pNT36-3. Although not all conjugative plasmids carry ARGs, different virulence factors, type IV, and type III secretion systems were found prevalent amongst them. The host range and migration of these plasmids were found to be mainly inclusive to closely related genera, however, one plasmid (pH15B-1) was predicted to have originated from gram-positive *Bacillus cereus* and *Bacillus cytotoxicus* strains and transferred into a gram-negative *Acinetobacter* strain. This demonstrates that perhaps the transfer of plasmids is possible across different family groups, nevertheless, bacteria share a preference for closely related genera.

To our surprise, nearly all of the highly antibiotic resistant bacterial isolates appeared to not carry any plasmids (Table 10). Instead, plasmids were found more frequently among the antibiotic sensitive isolates, therefore suggesting that most multi-antibiotic resistant environmental bacteria must carry chromosome-bound resistance mechanisms. ARG-carrying elements may have integrated within the chromosome of these plasmid-lacking multi-resistant bacteria at some point in evolution. Furthermore, two out of the nine plasmids were found to carry a single ARG while type secretion systems and conjugative transfer genes were more frequently found several plasmids. This study suggests that although ARB are common in WWTPs, most observed resistance patterns are not directly related to ARG carrying plasmids.

However, plasmid transfer genes are far more common than ARGs amongst the plasmids found in such environments. The mobility of plasmids in microbial populations still poses a great threat even from plasmids that lack ARGs. The transfer of virulence factors, effector proteins, and the potential of acquiring ARGs through genetic recombination and transposons can lead to public health concerns, potential outbreaks, or environmental contamination. The ability for plasmids to carry virulence factors, ARGs, transfer genes, and move to closely-related and distantly-related microbes all can contribute to the development of pathogenic ARB. These findings support the need for more research, tools, and policies for better management of emerging contaminants and ARB in WWTPs.

Despite modern advancements made in the field of sequencing, better sequencing libraries are required to adequately annotate hypothetical protein sequences and to understand the role of plasmids within their hosts. Additionally, both culture-dependent and independent techniques must be utilized to identify plasmid hosts since plasmid sequencing cannot associate them with the identity of their host in a mixed population. When conducting a study to assess environmental risks, it is imperative to analyze the entire microbial population rather than selecting for specific plasmids, ARB, or ARGs. Otherwise, overestimated conclusions may be inferred from incomplete data that may result in poor policymaking and actions. Future studies are still warranted to understand the stability, transfer, and role of plasmids in a large/mixed environmental bacterial populations and their fate upon release into the surrounding ecosystems.

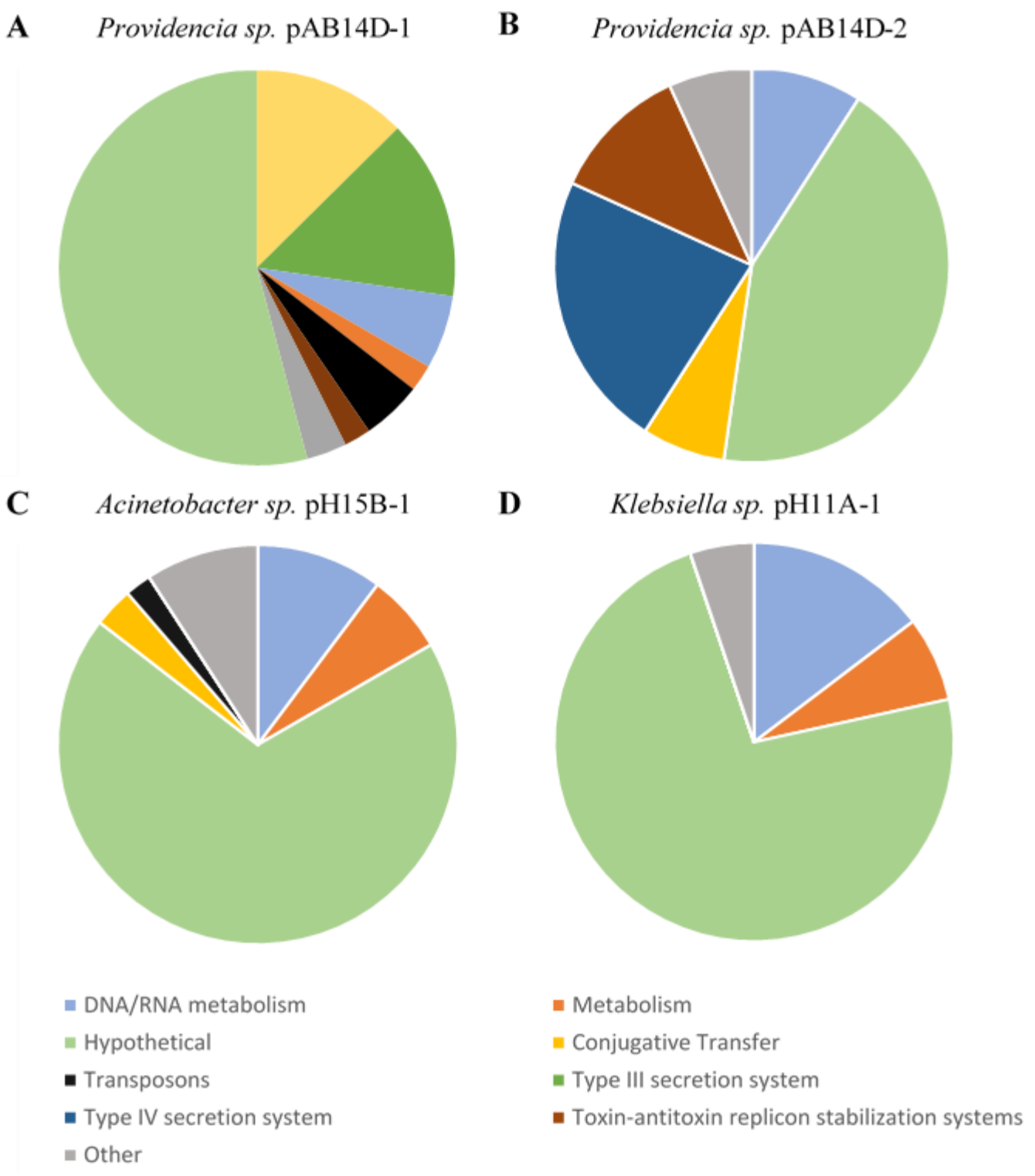


Figure 5. The distribution of the gene annotations of all 4 plasmids that were successfully closed. (A) and (B) are two top plasmids that belong to a single strain of *Providencia* sp. while the bottom two belong to *Acinetobacter* sp. (C) and *Klebsiella* sp. (D) isolates.

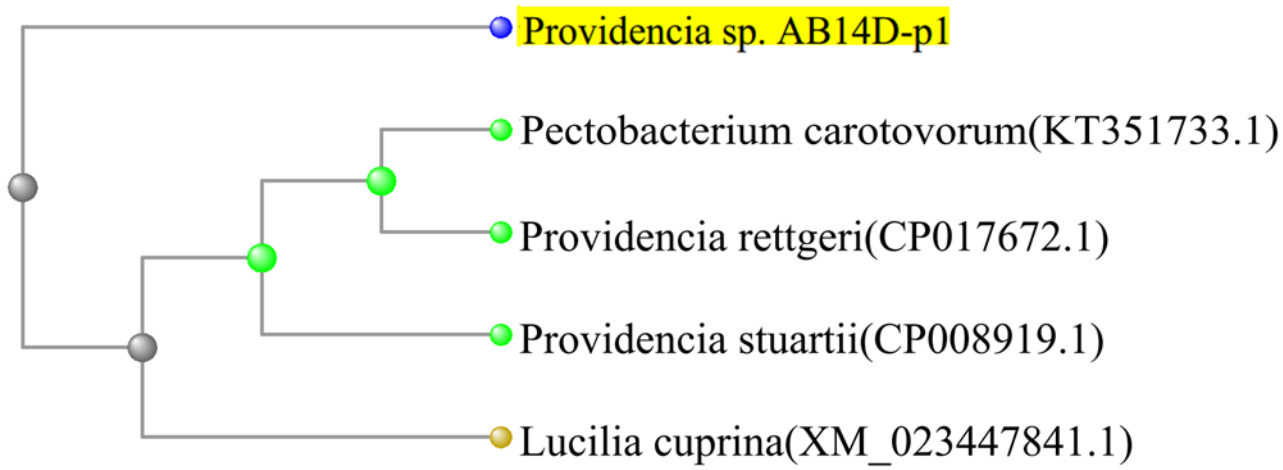


Figure 6. Dendrogram of the plasmid AB14D-p1 matched with bacterial hosts carrying the most similar plasmid found in BLAST database. The dendrogram was constructed using the distance tree tool on BLAST via the fast minimum evolution tree method.

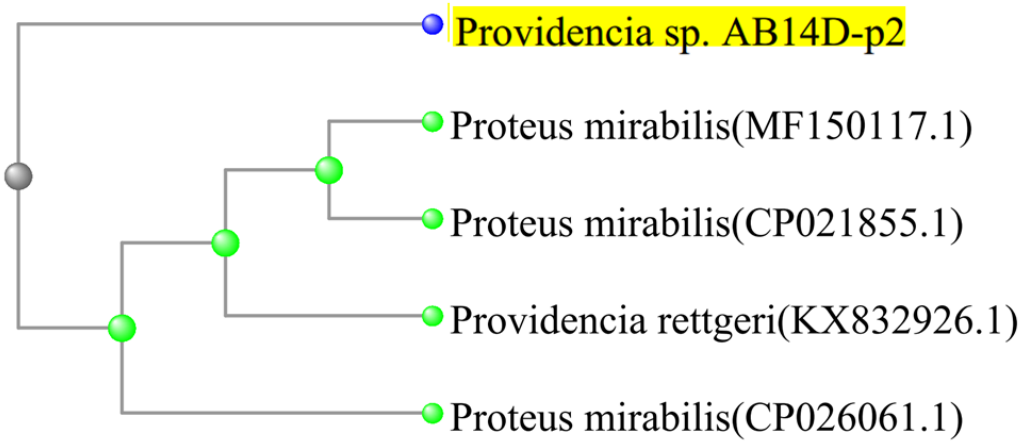


Figure 7. Dendrogram of the plasmid AB14D-p2 matched with bacterial hosts carrying the most similar plasmid found in BLAST database. The dendrogram was constructed using the distance tree tool on BLAST via the fast minimum evolution tree method.

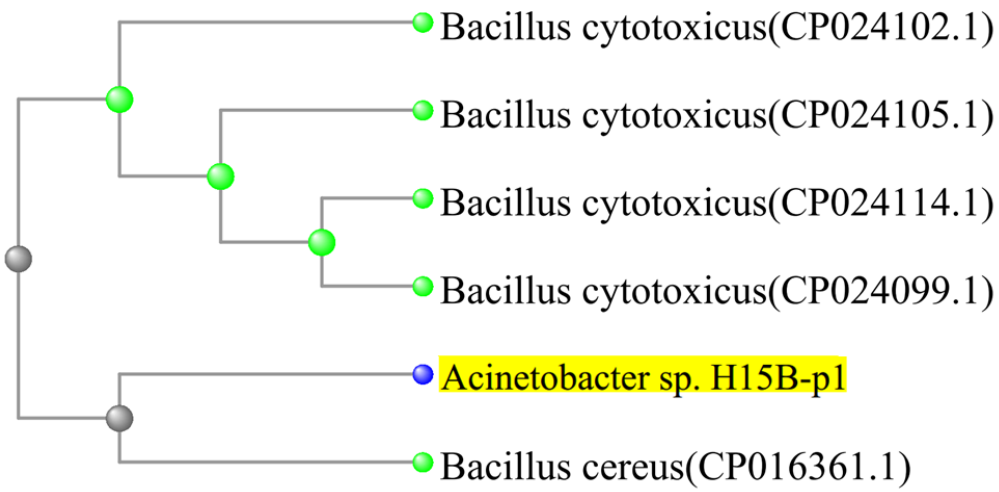


Figure 8 Dendrogram of the plasmid H15B-p1 matched with bacterial hosts carrying the most similar plasmid found in BLAST database. The dendrogram was constructed using the distance tree tool on BLAST via the fast minimum evolution tree method.

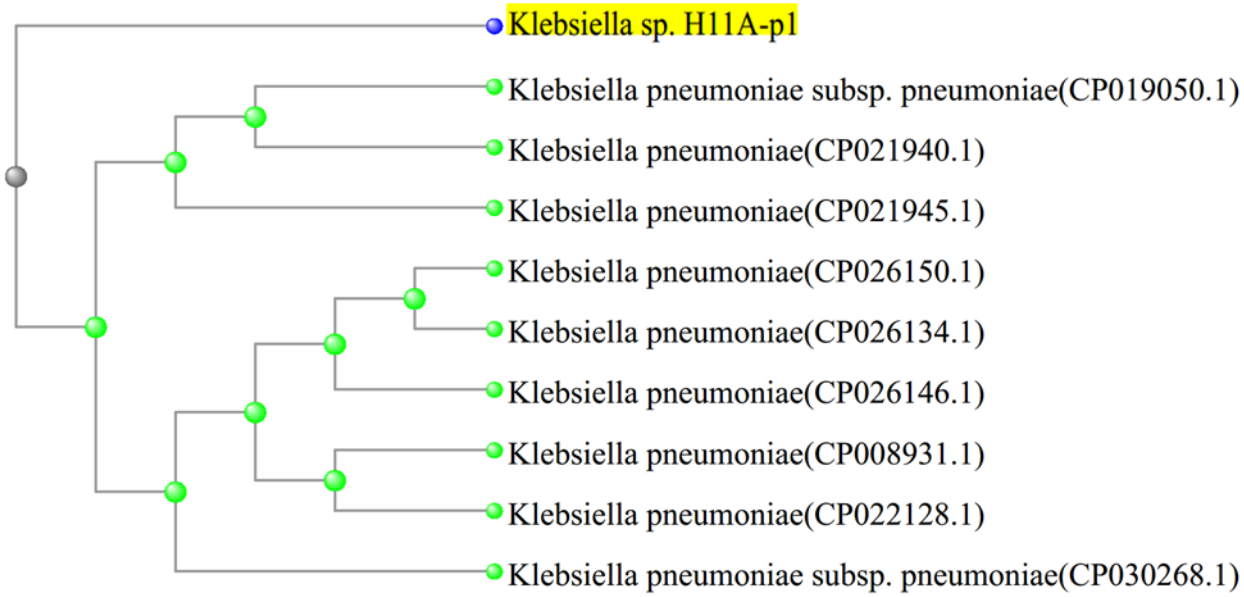


Figure 9. Dendrogram of the plasmid H11A-p1 matched with bacterial hosts carrying the most similar plasmid found in BLAST database. The dendrogram was constructed using the distance tree tool on BLAST via the fast minimum evolution tree method.

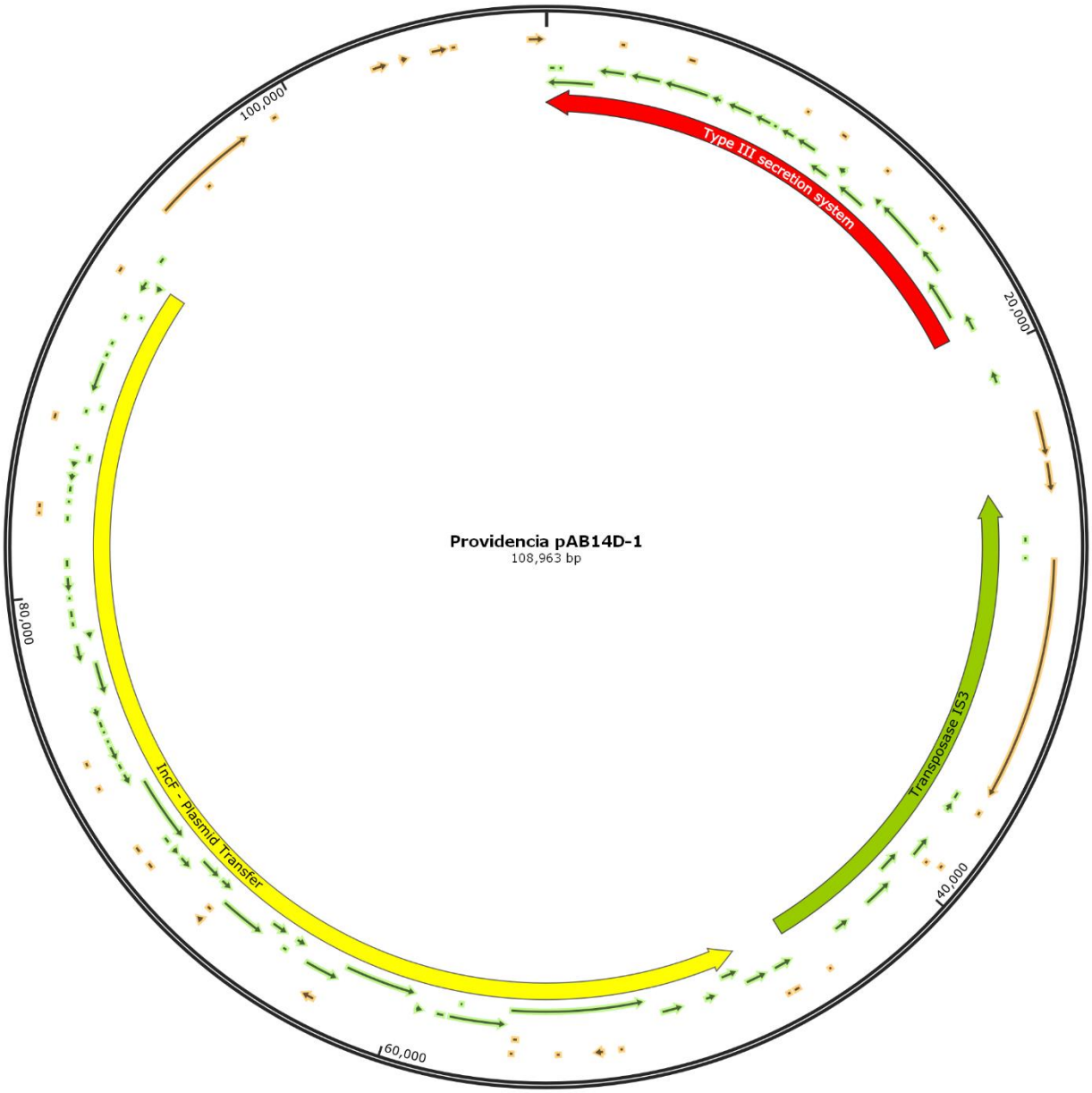


Figure 10. Complete encircled plasmid isolated from *Providencia* sp. AB14D-p1. The total size of the plasmid is 108 963 bp. The large coloured arrows indicate the type of genes and the direction of their open reading frame. The empty regions are non-open reading frames or hypothetical proteins. The image was constructed using SnapGene Viewer V4.3.10.

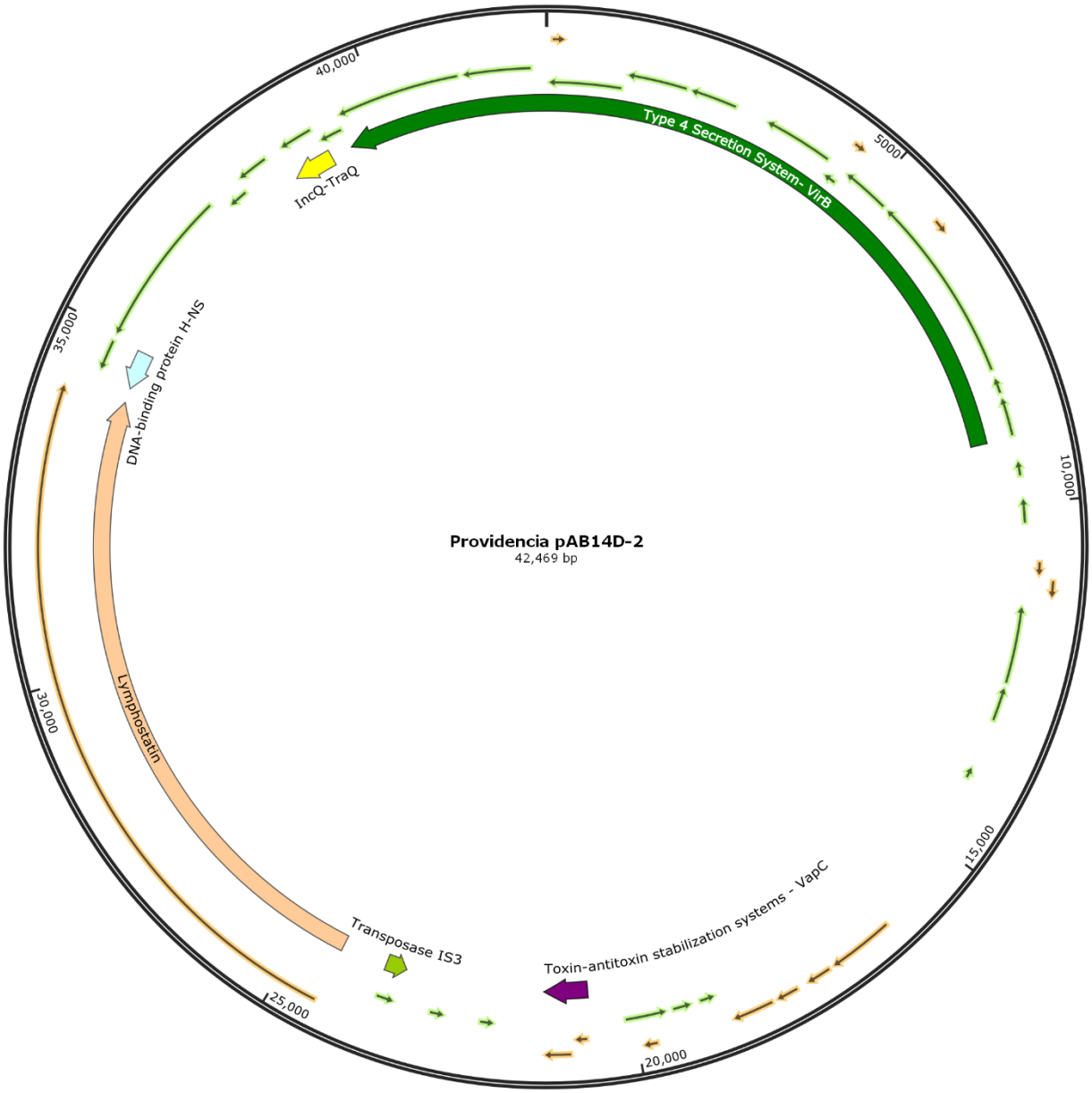


Figure 11. Complete encircled plasmid isolated from *Providencia* sp. AB14D-p2. The total size of the plasmid is 42 469 bp. The large coloured arrows indicate the type of genes and the direction of their open reading frame. The empty regions are non-open reading frames or hypothetical proteins. The image was constructed using SnapGene Viewer V4.3.10.

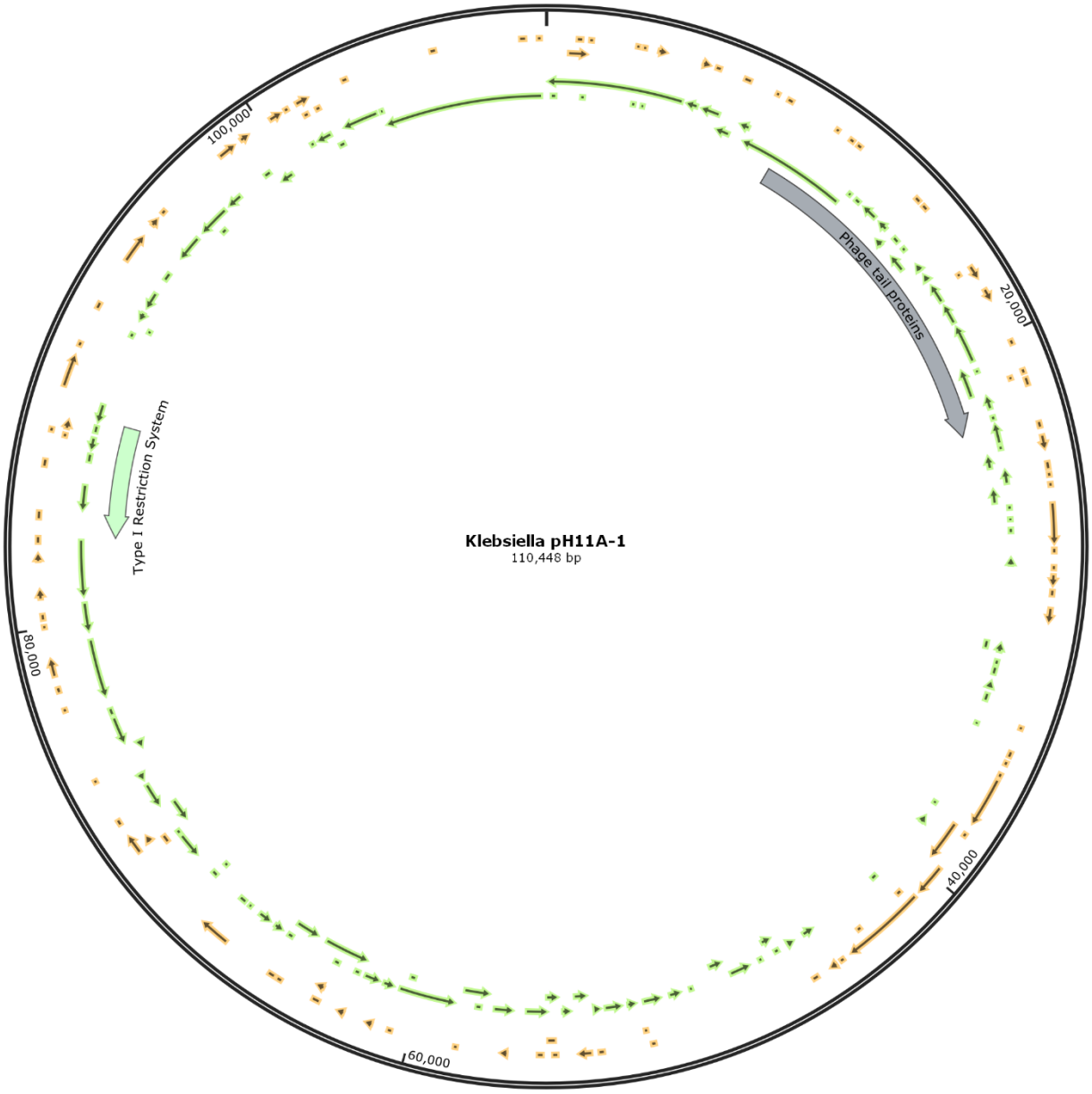


Figure 12. Complete encircled plasmid isolated from *Klebsiella* sp. H11A-p1. The total size of the plasmid is 110 448 bp. The large coloured arrows indicate the type of genes and the direction of their open reading frame. The empty regions are non-open reading frames or hypothetical proteins. The image was constructed using SnapGene Viewer V4.3.10.

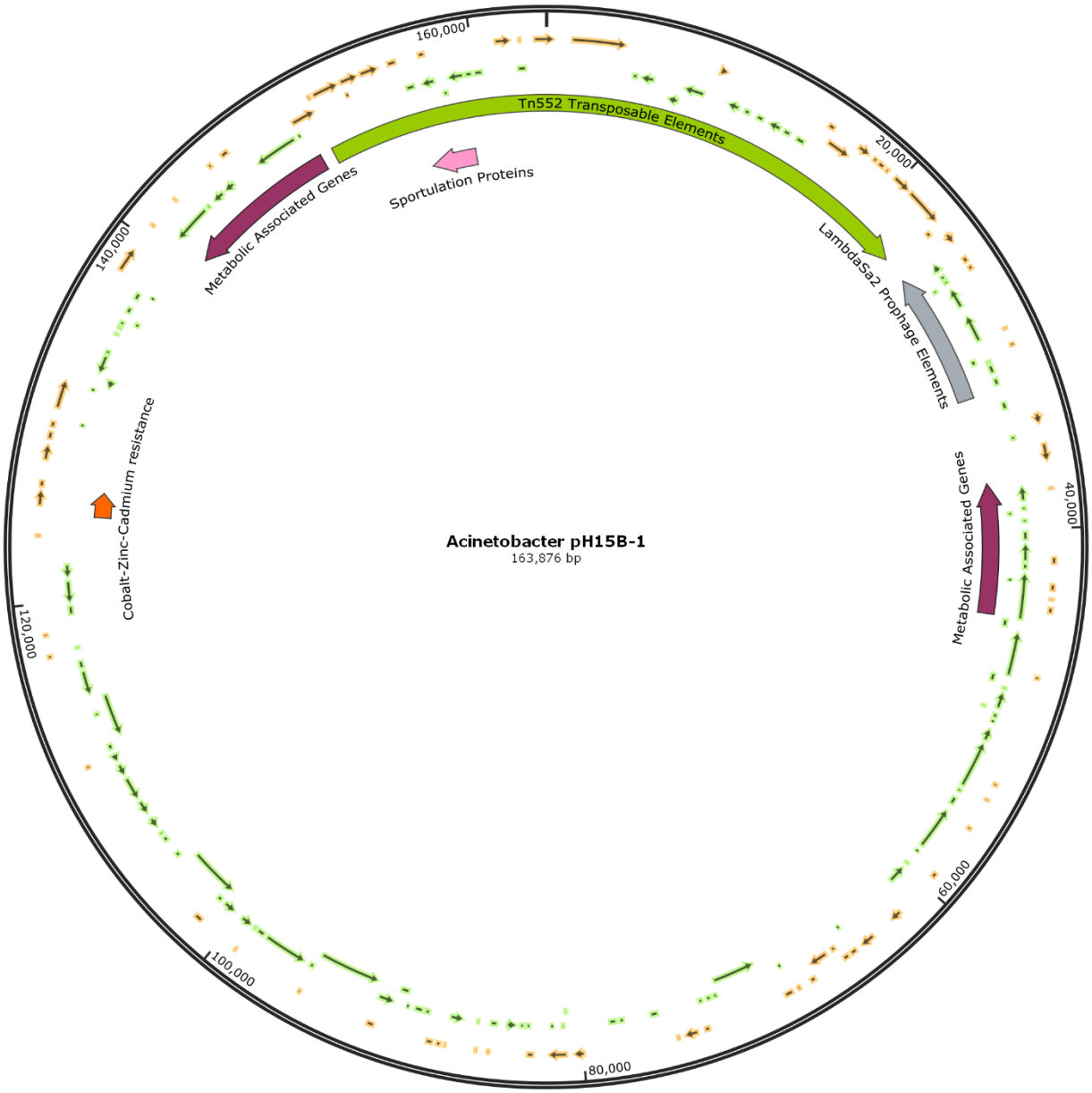


Figure 13. Complete encircled plasmid isolated from *Acinetobacter* sp. H15B-p1. The total size of the plasmid is 163 876 bp. The large coloured arrows indicate the type of genes and the direction of their open reading frame. The empty regions are non-open reading frames or hypothetical proteins. The image was constructed using SnapGene Viewer V4.3.10.

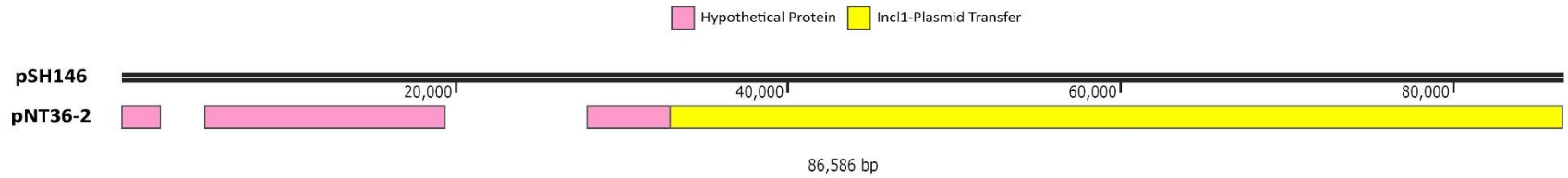


Figure 14. Partially constructed plasmid belonging to *Escherichia coli* pNT36-2 matched to its closest counterpart as a reference, *Salmonella enterica* pSH146_87 (JX445149.1). The black bar represents the reference match and the colour bars represent the regions of similarities between the sequences of the two plasmids. The image was constructed using SnapGene Viewer V4.3.10.

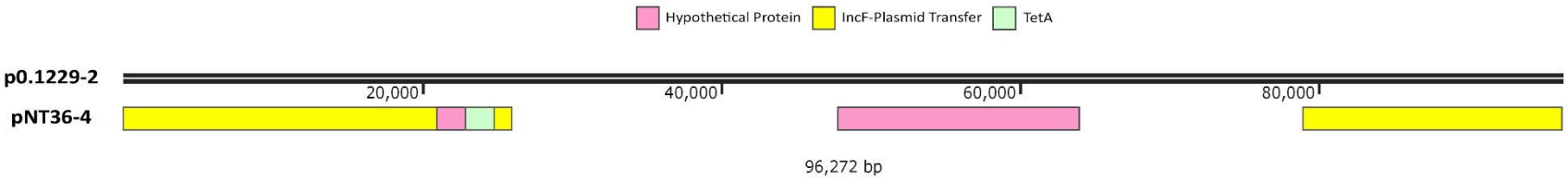


Figure 15. Partially constructed plasmid belonging to *Escherichia coli* pNT36-4 matched to its closest counterpart as a reference, *Escherichia coli* O18H1-p0.1229-2 (CP028322.1). The black bar represents the reference match and the colour bars represent the regions of similarities between the sequences of the two plasmids. The image was constructed using SnapGene Viewer V4.3.10.

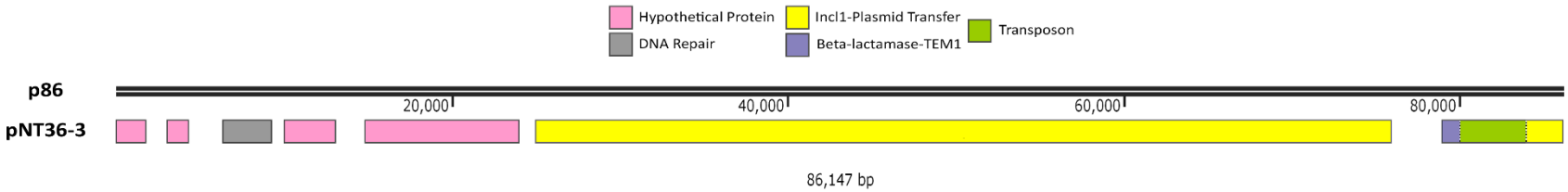


Figure 16. Partially constructed plasmid belonging to *Escherichia coli* pNT36-3 matched to its closest counterpart as a reference, *Escherichia coli* 1190 p86 (CP023387). The black bar represents the reference match and the colour bars represent the regions of similarities between the sequences of the two plasmids. The image was constructed using SnapGene Viewer V4.3.10.

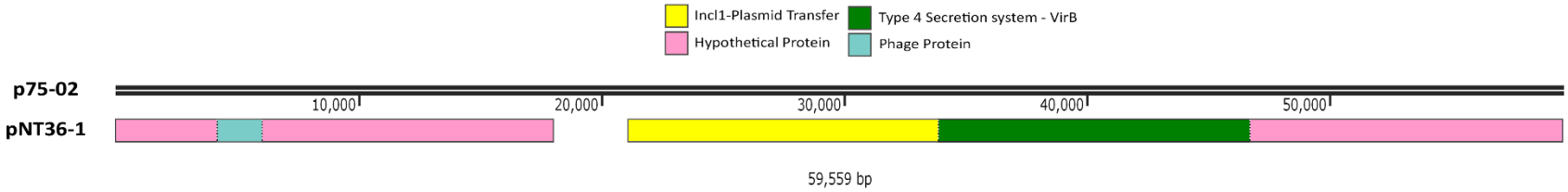


Figure 17. Partially constructed plasmid belonging to *Escherichia coli* pNT36-1 matched to its closest counterpart as a reference, *Shigella sonnei* 75/02 p75-02_2 (CP019690.1). The black bar represents the reference match and the colour bars represent the regions of similarities between the sequences of the two plasmids. The image was constructed using SnapGene Viewer V4.3.10.

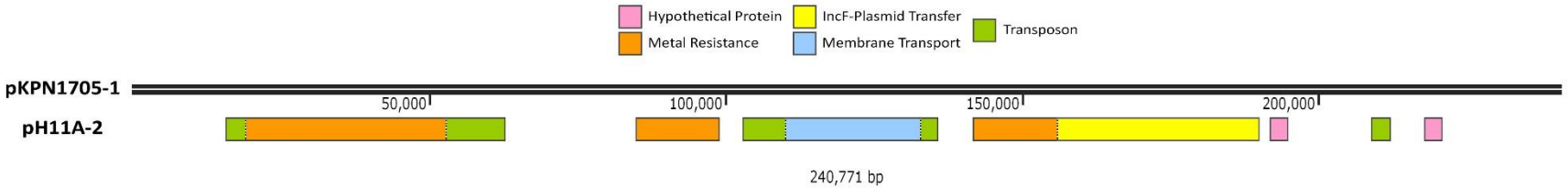


Figure 18. Partially constructed plasmid belonging to *Klebsiella* sp. pH11A-2 matched to its closest counterpart as a reference, *Klebsiella quasivariicola* KPN1705 pKPN1705-1 (CP022824.1). The black bar represents the reference match and the colour bars represent the regions of similarities between the sequences of the two plasmids. The image was constructed using SnapGene Viewer V4.3.10.

5

Chapter 5: The Impact of Antibiotics on Conjugational Transfer of pNT36-3 and pNT36-4 Between Two Environmental *Escherichia coli* Cultures Isolated from WWTP

5.1 Introduction

Studying the transfer of mobile genetic elements between members of a bacterial population is difficult due to the lack of suitable molecular tools and laborious culture-dependent techniques. Both culture-dependent and independent techniques often use lab strain bacteria as either the donor or recipient when transferring mobile genetic elements such as plasmids. The use of lab strains can often misrepresent what occurs in the environment as most lab strain microbes are not as robust as those found in the wild. The stability of plasmids in lab strain bacteria can become comprised because of the higher energy demand imposed by their maintenance. Despite the progress made in understanding the basic mechanisms involved in HGT, the major causes and

behaviours that are imposed on microbes within the natural environment remain a mystery. The presence of antimicrobial compounds and residues in the environment has attracted great attention due to their potential implications on microbial activity.[167] Antibiotics, hormones, and pharmaceuticals have been previously detected in WWTPs influent, effluent, and downstream water bodies at various concentrations.[26,31,103,168,169] The pseudo-persistence of antimicrobial compounds in the environment can result in increased antibiotic resistance levels .[20–24] Transfer of ARGs carrying plasmids (pB10) in pure and activated sludge culture has been previously described using lab strain *E. coli* DH5α as the plasmid donor.[170] It was found that increased plasmid transfer was detected in the presence of ppb levels of tetracycline and sulfamethoxazole. However, not in all cases of antibiotic pressure does the rate of plasmid transfer increase. In another study, it was found that transfer rates from foodborne *E. coli* strain to lab strain *E. coli* MG1655 were highest in the absence of antibiotic pressure.[171] Based on conflicting results it appears that the rate of plasmid transfer can be influenced by multiple factors including (1) plasmid type, (2) donor bacteria, (3) recipient bacteria, (4) growth conditions, and (5) the presence of stressors. Due to the vast diversity of microbes, environmental conditions, and plasmids, the parameters that affect plasmid transfer may differ between each study/scenario. Since there are no standardized methods to track the rate of plasmid transfer, it becomes difficult to properly address which parameters have an impact on plasmid transfer. Furthermore, the variability in the responsiveness of different bacterial strains and plasmids also adds to the difficulty of comparing other studies when identifying transfer parameters.

In this chapter, a series of experiments were conducted to monitor the dissemination of two large conjugative plasmids (IncI1-pNT36-3 and IncF-pNT36-4) between two environmental bacteria strains (donor: *E. coli* NT36, recipient: *E. coli* EB-G3). Furthermore, various subinhibitory

concentrations of antibiotic pressures will also be imposed to determine whether they affect the rate of transfer between the two microbes. The antibiotics selected for this study is the highest subinhibitory concentration of the most sensitive parent which includes carbenicillin (1 µg/mL), tetracycline (64 µg/mL), ciprofloxacin (0.25 µg/mL), and gentamicin (6 µg/mL). Carbenicillin was selected for this study since the resistance gene is carried by the donor's conjugative plasmid pNT36-3, tetracycline was selected since both parents are resistant to it, while gentamicin and ciprofloxacin were selected since only the recipient is resistant. These antibiotics were chosen to determine whether subinhibitory concentrations of antibiotic stress on the donor, recipient, or both influences the rate of transfer of each plasmid. Both molecular-based (quantitative PCR) and culture-based approaches will be utilized to monitor the dissemination of each plasmid within their population. Quantitative PCR has been demonstrated in the past to monitor the dissemination of broad-host-range plasmids pB10 in microcosms.[80,172] Similarly, this approach utilizes highly specific PCR primers to track the DNA copy numbers of each plasmid and total bacteria (donor and recipient) and compares them in a relative ratio to donor bacteria (*E. coli* NT36). If the rate of plasmid transfer is expected to increase, then the ratio between plasmid:donor would increase since there will be more plasmid copy numbers present relative to the donor's copy number. Overall, this strategy can serve as one of the best methods for tracking plasmid transfer in mix environmental populations as long as the design of the primers is highly specific for the donor and plasmid(s) being investigated.

5.2 Results and Discussion

5.2.1 Plasmid conjugation, maintenance, and stability

A total of fifty-five environmental isolates were selected to undergo conjugative mating in liquid and solid media (Table 11). Amongst the selected isolates, nine bacteria were successful in producing transconjugants on a corresponding double antibiotic R2A plate including two isolates of *Microbacterium testaceum*, two *Acinetobacter johnsonii*, one *Pseudomonas stutzeri*, and four *E. coli*. The identified transconjugants are only capable of demonstrating the conjugative properties of one of the two plasmids (pNT36-3) carried by *E. coli* NT36. Through culture-dependent techniques, one can only select for pNT36-3 as it is the only plasmid carrying the antibiotic resistance gene, *TEM-1* (demonstrated in chapter 2). As a result, the fate of the other conjugative plasmid, pNT36-4, is unclear whether it can successfully conjugate between the mating pairs since there is no way to select for it without genetic manipulation. The identified transconjugants primarily belong to the phylum Proteobacteria with exception to *M. testaceum* which belongs to the phylum Actinobacteria. Although successful conjugation was observed with the 9 tested isolates, only three of the *E. coli* were capable of maintaining pNT36-3 after numerous growth attempts on their corresponding selective media. According to literature, most of the successful cases of plasmid conjugation involving *E. coli* transfer from or to other bacteria occurs within the same phylum or family.[30,58,60,170,171,173] However, temporary conjugation between *E. coli* and *M.testaceum* has never been documented. A few of the reasons why pNT36-3 was lost after multiple generations of growth could be a result of the fitness cost imposed by the plasmid, slower growth rates, plasmid incompatibility, reduced survivability in the stationary phase, and possible loss of resistance from the plasmid itself.[49] In either case, the only isolates that were capable of maintaining pNT36-3 after numerous growth periods were *E. coli* EB-G3, 51, and 55 strains (Table

11). The reason for the survival of the plasmid remains unknown but it appears to have a high preference for other *E. coli* strains concurring with the BLAST sequencing data observed in chapter 4.

5.2.2 The importance of primer specificity

To properly monitor the dissemination of plasmid pNT36-3 and pNT36-4, four vital primer sets were utilized (YaiO, gyrNT36, IncI1-TraW, and IncF-TraN) for their detection via qPCR (Table 4). The primer set YaiO amplifies an orphan gene (*yaiO*) that specifically is used for the identification of *E. coli* species.[174] Since the *yaiO* gene is present in both donor and recipient, it serves as a detection tool for identifying the total amount of bacteria between the mating pair. As shown in Figure 19, both NT36 and EB-G3 are positive for the amplification of this gene. The primer set gyrNT36 is used to specifically amplify the *gyrA* gene of *E. coli* NT36. This primer set was designed for this study based on the point mutations identified between NT36 and EB-G3 gyrase gene to maximize its specificity for the donor (NT36). It is crucial that gyrNT36 only targets the donor bacteria as all the other primer sets will be compared with gyrNT36 as a relative ratio to determine plasmid transfer, as demonstrated in Figure 22 (adapted).[12] As shown in Figure 19, gyrNT36 is only positive when amplifying *E. coli* NT36 template DNA while negative for *E. coli* EB-G3. A series of highly specific primer sets were designed based on the plasmid sequencing data obtained from chapter two (Table 4) including IncI1 genes (TraY, PilQ, TraW, PilM, TrbB, TraG, TraB; Figure 20) and IncF genes (TraQ, TraN, and TraG; Figure 21). Amongst these primer sets, IncI1-TraW (belonging to pNT36-3) and IncF-TraN (belonging to pNT36-4) were selected to monitor the dissemination of the plasmids within the population.

5.2.3 Determination of antibiotic concentrations and growth curves

One of the main objectives of this study was to determine whether subinhibitory concentrations of antibiotics impact plasmid transfer. It was hypothesized that sub-lethal antibiotic concentrations can promote horizontal gene transfer between the donor and recipient. To achieve this goal, the maximum antibiotic concentration that each parent could withstand was determined via growth curves. Four classes of antibiotics were selected based on the resistance patterns observed from each parent and plasmid (Table 5). The highest concentration that both parents can withstand without major hindrance to their growth was selected for the reactors (tetracycline 64 µg/mL, gentamicin 6 µg/mL, carbenicillin 1 µg/mL, and ciprofloxacin 0.25 µg/mL) (Figures 23-24). Due to an undetermined reason, each parent can grow individually well under 0.25 µg/mL of ciprofloxacin but as a mixture, *E. coli* NT36 growth becomes completely inhibited (data not shown). The interaction between the two parents in the presence of 0.25 µg/mL of ciprofloxacin appears to affect NT36 growth but not EB-G3.

5.2.4 Monitoring conjugation via culture-based approach

The plate counts observed from each antibiotic treatment group are represented as a percentage in Figure 25. The total percentage of donor and transconjugant counts were relatively stable at approximately 86-87% of the population for three of the reactors with exception to the gentamicin reactor (Figure 25). Since the *E. coli* NT36 is not resistant to gentamicin, unlike EB-G3, it may have imposed a slight hindrance to its growth as observed in both Figures 23 and 25. However, the total percentage of transconjugants was not as consistent across each treatment group (Figure 25). The antibiotic-treated reactors, tetracycline, and gentamicin, both had a significant reduction in transconjugant formation while the carbenicillin reactor had a ~3 fold increase. It is apparent that transconjugants form at a greater number in the presence of 1 µg/mL carbenicillin

while at greatly reduced in the presence of 64 µg/mL tetracycline, 6 µg/mL gentamicin, and no growth at all in the presence of 0.25 µg/mL ciprofloxacin. The information provided from this culture-dependent mating experiment only demonstrates the transfer of pNT36-3 due to its ARG, TEM-1. Unfortunately, there is no way to track the transfer of the conjugative plasmid, pNT36-4, using culture-dependent techniques since it carries no selective properties.

5.2.5 Monitoring conjugation via molecular-based approach

The molecular-based qPCR approach was also employed in tandem to track the spread of both target plasmids within the same reactors. As demonstrated in Figure 26A, the YaiO primer set tracks the total amount of bacterial copy numbers within the treatment group. The tetracycline, gentamicin, and carbenicillin treatment groups respectively had a 12.9, 12.4, and 1.09 times fold increase relative to the donor's tracking gene (gyrNT36). The high numbers observed in the tetracycline and gentamicin treatment groups indicate that the EB-G3 population may have thrived within the mixture. The relative fold difference observed in Figure 26B indicates that there is a decrease in plasmid abundance under the presence of tetracycline (0.93) and gentamicin (0.76) while increasing in the presence of carbenicillin (1.29). As a consequence, the plasmid pNT36-4 may have been lost and/or cured of its host under the influence of tetracycline and gentamicin, while on the other hand, the plasmid proliferated in the population when exposed to carbenicillin. Slightly similar results are also observed when examining the relative abundance of IncI1-TraW (Figure 26C). The plasmid, pNT36-3, increased in the population under the influence of tetracycline (1.19) and carbenicillin (1.33) while still decreasing when exposed to gentamicin (0.71). In other studies, the relative plasmid transfer frequencies were observed to increase in *E. coli* and *P. aeruginosa* cultures when exposed to low concentrations (10-1000 µg/L) of tetracycline and sulfamethoxazole.[170] Similar to our findings, pNT36-3 experienced an increase in relative

abundance while pNT36-4 experienced a slight loss in the presence of tetracycline. The reason for using a much higher concentration of tetracycline (64 µg/mL) was due to the fact that both parents are resistant to the antibiotic and we wanted to maximize the pressure without hindering their growth. However, the direct relationship between tetracycline and plasmid transfer remains unknown but it is evident that the antibiotic can potentially have an impact on plasmid migration, possibly through SOS responses or global regulators. The transfer of the plasmids pwG613 and pSK41 has been reported to increase 3-10 folds in *Staphylococcus aureus* after being exposed to 500 µg/L and 100 µg/L of gentamicin, respectively.[175,176] To our knowledge, the effects of gentamicin on plasmid transfer frequencies have not been fully investigated in gram-negative bacteria. In this study, it is apparent that both pNT36-3 and pNT36-4 saw a decrease in the relative plasmid abundance when exposed to 6 µg/mL gentamicin. Other closely related antibiotics, kanamycin, and streptomycin have been shown to promote conjugation of pRK2013 from *E. coli* DH5α to HB101.[177] It was found that the activity of two antibiotic-induced genes, oligopeptide-binding protein (OppA) and ribose-binding protein (RbsB), were positively correlated and possibly responsible for the increased development of transconjugants.[177]

The only antibiotic pressure that caused an increase in the relative abundance of both plasmids was in the presence of 1 µg/mL carbenicillin. Carbenicillin and its closely related counterpart, ampicillin, have been reported in the past to significantly increase plasmid transfer of pTF2 in numerous strains *E. coli*.[178] Furthermore, it was observed that ampicillin and ciprofloxacin exposure significantly upregulates *tra* gene expression. It was suggested that the presence of ampicillin may activate SOS response and, as a result, increase the upregulation of numerous plasmid associated genes.[178] The activity of plasmid transfer may be affected depending on the certain response released by the bacteria. However, not in all cases of ampicillin exposure does the

plasmid transfer frequency increase as certain strains of *E. coli* was shown to be unaffected.[178] Another study showed that amoxicillin, ampicillin, and chloramphenicol exposure significantly reduced the transfer of an incI1 β-lactam resistance plasmid, pESBL-283, from foodborne *E. coli* strains to *E. coli* MG1655.[171] In their findings, it was suggested that the transfer rates were highest in the absence of antibiotics while raising the antibiotic concentrations above MIC resulted in reduced transfer rates.

Although the *E. coli* mixture was not able to grow in the presence of ciprofloxacin, adequate quantities of DNA were salvaged from the reactors and examined via qPCR (Figure 26). The high fold difference observed for the primer set YaiO (23.3) is the result of the EB-G3 population over-growing within the mixture. Since EB-G3 is already ciprofloxacin resistant, it would not have problems growing in the presence of the antibiotic while NT36 appears to be nearly inhibited. However, a very small population of NT36 did manage to survive. Among the surviving members, the plasmid pNT36-4 (0.99) had nearly no change in its relative abundance while pNT36-3 was significantly reduced (0.48). It is apparent that in the presence of ciprofloxacin and EB-G3, the growth of NT36 is significantly hindered with an increased loss of its plasmid pNT36-3 while no change was observed in the relative abundance of pNT36-4. According to literature, antibiotics such as ciprofloxacin may have plasmid curing properties at low concentrations when exposed to *E. coli* and *S. sonnei* cultures.[179–181] These findings also support the results of this study since ciprofloxacin also had a major impact on the loss of pNT36-3 from the population. Ciprofloxacin interferes with DNA gyrase activity and as a consequence can impact plasmid maintenance and stability.[180] However, plasmid loss was not observed in all cases of quinolone exposure as certain plasmids such as R16 and R386 experienced minimal loss.[179,181] While plasmids such as pST7, pST3, and pST16 experienced an increase in plasmid transfer.[177] The

plasmid, pNT36-4, also experienced a similar fate as the relative plasmid abundance experienced nearly no change. In summary, it appears that ciprofloxacin curing activity is selective for certain plasmids and strains of bacteria, while in other cases, it may induce plasmid transfer or have no effect.

One of the limitations of this study is that the experimental design is based on the assumption that pNT36-3 and pNT36-4 are both low and stable copy number plasmids. If, however, the plasmid copy numbers are inconsistent and/or increase in number within the cell rather than through conjugation, then it can lead to a false-positive conclusion. To ensure that this is not the case, culture-dependent plate counts had to be done to cross-reference the data within each experiment and verify that transconjugants are being produced (Figure 25). However culture-dependent plate counts can often be very laborious and inconsistent. In this experiment, both culture and molecular-based techniques appear to show similar trends in results. The only treatment group that did not follow a similar trend between each approach was the tetracycline reactor. The plate counts of this reactor indicated a significant reduction in transconjugant formation (Figure 25B) while the qPCR assay demonstrated an increase (Figure 26C). It is critical to ensure that the plasmids being investigated are consistent in their copy numbers so that the values obtained are not over or underestimated. The plasmid sequencing data obtained from chapter 2 helped validate that pNT36-3 and pNT36-4 are large plasmids (~86-96 kbp) that are often identified as low copy number. Furthermore, the qPCR assays had very little to no discrepancies between reactor controls and replicates with less than 0.5 Ct values (data not shown).

5.3 Conclusion

This work was initiated to utilize molecular and culture-based approaches to investigate the conditions that two environmental *E. coli* isolates require to promote the conjugative transfer of two large plasmids. This methodology was successful in tracking the transfer of IncI1 plasmid (pNT36-3) and IncF plasmid (pNT36-4) between two *E. coli* strains NT36 and EB-G3. It was found that pNT36-3 has the highest rate of transfer when *E. coli* NT36 and EB-G3 mixture was exposed to 64 µg/mL tetracycline and 1 µg/mL carbenicillin and the lowest in the presence of 0.25 µg/mL ciprofloxacin and 6 µg/mL gentamicin. However, the IncF plasmid (pNT36-4) had the highest rate of transfer in the presence of 1 µg/mL carbenicillin while having reduced rates in the presence of tetracycline, gentamicin, and ciprofloxacin. Furthermore, it was found that *E. coli* NT36 is capable of conjugating with numerous strains and species of bacteria including *Microbacterium testaceum*, *Acinetobacter johnsonii*, *Pseudomonas stutzeri*, and other *E. coli* strains but only in a select few *E. coli* isolates was pNT36-3 maintained with adequate stability. The temporary transfer of pNT36-3 was shown to be relatively common among other bacterial species and members of the WWTP. This temporary transfer of pNT36-3 can lead to possible genetic integration or recombination of essential genes within its new host even though the plasmid itself is eventually lost from the population. Regardless of whether the plasmid temporary transfer or remains stable in its host, it can contribute to the development of ARB and the spread of ARGs. The findings of this study further validate that plasmid transfers are highly dependent on numerous factors including stressor type, stressor concentration, growth media, growth period, donor bacteria, recipient bacteria, and the plasmid of choice. The variability within each category and the lack of standardization may give contradictory results as often seen in the literature, therefore, making it difficult to form risk assessment conclusions. In this case, the presence of subinhibitory

concentrations of carbenicillin greatly increased the mobility of both plasmids while decreased mobility was observed in the presence of other antibiotics. Although 1 µg/mL of carbenicillin far exceeds environmentally relevant concentration, it is possible that at lower concentrations similar effects on plasmid mobility can be expected. Unfortunately, it remains unclear whether the presence of antibiotics poses a great threat to the environment since, under certain circumstances, they can promote plasmid loss while promoting plasmid transfer in other cases.

Table 11. The result of the mating experiment (host range) between various recipients and *E. coli* NT36

Recipient Identity	Isolate #	Antibiotic Resistance Phenotype	Conjugation Occurrence	Maintenance of plasmids
Chloramphenicol Antibiotic Group				
Acinetobacter johnsonii	27	CHL, TET	(-)	(-)
Acinetobacter johnsonii	37	CHL	(-)	(-)
Arcobacter sp.	11E	CHL, CIP, GEN, KAN, STR, SXT	(-)	(-)
Microbacterium testaceum	24D	CHL, TET	(+)	(-)
Microbacterium testaceum	3E	CHL, CIP, GEN	(-)	(-)
Stenotrophomonas maltophilia	13	CHL, TET, CIP, GEN	(-)	(-)
Gentamicin Antibiotic Group				
Acinetobacter baumannii	15B	GEN	(-)	(-)
Arcobacter sp.	11E	GEN, CHL, CIP, KAN, STR, SXT	(-)	(-)
Escherichia coli	EB-G3	GEN, TET, CIP, SXT, ERY, VAN	(+)	(+)
Exiquobacterium sibiricum	20B	GEN	(-)	(-)
Flavobacterium hydatis	15E	GEN	(-)	(-)
Herminiimonas arsenicoxydans	11B	GEN, STR	(-)	(-)
Klebsiella pneumoniae	G1	GEN	(-)	(-)
Microbacterium testaceum	3E	GEN, CHL, CIP	(+)	(-)
Riemerella sp.	2E	GEN, SXT	(-)	(-)
Stenotrophomonas maltophilia	13	GEN, TET, CHL, CIP	(-)	(-)
Ciprofloxacin Antibiotic Group				
Acinetobacter haemolyticus	F9	CIP, SXT	(-)	(-)
Acinetobacter johnsonii	EB-CIP 11	CIP, TET, GEN, TIG, ERY, VAN	(+)	(-)
Acinetobacter johnsonii	EB-CIP 9	CIP, TET, GEN, TIG, ERY, VAN	(+)	(-)
Acinetobacter schinderi	Tig 6	CIP, TET, TIG, ERY, VAN	(-)	(-)
Aeromonas sp.	U12	CIP, GEN, SXT, PMB	(-)	(-)
Arcobacter sp.	11E	CIP, CHL, GEN, KAN, STR, SXT	(-)	(-)
Arthrobacter aurescens	B7	CIP, GEN, PMB	(-)	(-)

Arthrobacter sp.	J3	CIP, GEN, PMB	(-)	(-)
Arthrobacter sp.	J8	CIP, GEN, PMB	(-)	(-)
Brevundimonas naejangsanensis	F15	CIP, CHL, GEN, SXT, TIG, VAN, PMB	(-)	(-)
Brevundimonas sp.	U15	CIP, SXT, PMB	(-)	(-)
Escherichia coli	23	CIP, GEN, ERY, VAN	(+)	(-)
Escherichia coli	51	CIP, GEN, ERY, VAN	(+)	(+)
Escherichia coli	55	CIP, TET, GEN	(+)	(+)
Exiguobacterium antarticum	I4	CIP, GEN, STR, PMB	(-)	(-)
Flavobacterium commune	F11	CIP, GEN, KAN, STR, ERY, VAN, PMB	(-)	(-)
Flavobacterium commune	F18	CIP, GEN, KAN, STR, ERY, SPT, PMB	(-)	(-)
Flavobacterium commune	F21	CIP, GEN, KAN, ERY, VAN, PMB	(-)	(-)
Flavobacterium crassostreae	F2	CIP, GEN, KAN, STR, ERY, VAN, PMB	(-)	(-)
Flavobacterium crassostreae	F7	CIP, GEN, KAN, ERY, VAN, PMB	(-)	(-)
Hymenobacter swuensis	J6	CIP, GEN, PMB	(-)	(-)
Microbacterium aurum	J9	CIP, GEN, PMB	(-)	(-)
Microbacterium chocolatum	J10	CIP, GEN, PMB	(-)	(-)
Microbacterium chocolatum	U13	CIP, GEN, SXT, PMB	(-)	(-)
Microbacterium sp.	J2	CIP, GEN, PMB	(-)	(-)
Microbacterium sp.	J12	CIP, GEN, SXT	(-)	(-)
Microbacterium sp.	BHSC8	CIP, GEN, PMB	(-)	(-)
Microbacterium sp.	BHSC10	CIP, GEN	(-)	(-)
Microbacterium testaceum	3E	CIP, CHL, GEN	(-)	(-)
Novosphingobium aromaticivorans	U2	CIP, GEN, STR, PMB	(-)	(-)
Pedobacter cryoconitis	H1	CIP, GEN, KAN, STR, VAN, PMB	(-)	(-)
Pseudomonas stutzeri	8	CIP	(+)	(-)
Sphingobacteriaceae bacterium	B1	CIP, GEN, KAN, PMB	(-)	(-)
Stenotrophomonas maltophilia	13	CIP, TET, CHL, GEN	(-)	(-)

Erythromycin Antibiotic Group				
Acinetobacter baumannii	EB-CIP 10	ERY, CIP, VAN	(-)	(-)
Acinetobacter bouveii	Tig6	ERY, TET, CIP, TIG, VAN	(-)	(-)
Acinetobacter bouveii	14C	ERY, TET, CIP, TIG, VAN	(-)	(-)
Acinetobacter johnsonii	27	ERY, CHL, TIG, VAN	(-)	(-)
Acinetobacter johnsonii	5B	ERY, VAN	(-)	(-)
Acinetobacter johnsonii	4B	ERY, VAN	(-)	(-)
Acinetobacter johnsonii	9B	ERY	(-)	(-)
Acinetobacter johnsonii	EB-CIP 9	ERY, TET, CIP, GEN, TIG, VAN	(-)	(-)
Acinetobacter johnsonii	EB-CIP 11	ERY, TET, CIP, GEN, TIG, VAN	(-)	(-)
Acinetobacter johnsonii	16B	ERY	(-)	(-)
Enterobacter asburiae	EB-9	ERY, VAN	(-)	(-)
Enterobacter cloacae	EB-1	ERY, VAN	(-)	(-)
Escherichia coli	EB-7	ERY, SXT, VAN	(-)	(-)
Escherichia coli	EU8-121	ERY,VAN	(-)	(-)
Escherichia coli	EB-G3	ERY, TET, CIP, GEN, TIG, VAN	(-)	(-)
Escherichia coli	EB-CIP 8	ERY, CHL, CIP, TIG, VAN	(-)	(-)
Total Number of Isolates:		55		

CHL=Chloramphenicol, CIP=Ciprofloxacin, ERY=Erythromycin, GEN=Gentamicin, TET=Tetracycline, VAN=Vancomycin, SXT=Sulfamethoxazole Trimethoprim, TIG=Tigecycline, PMB=Polymyxin B, KAN=Kanamycin, SPT=Spectinomycin, and STR=Streptomycin

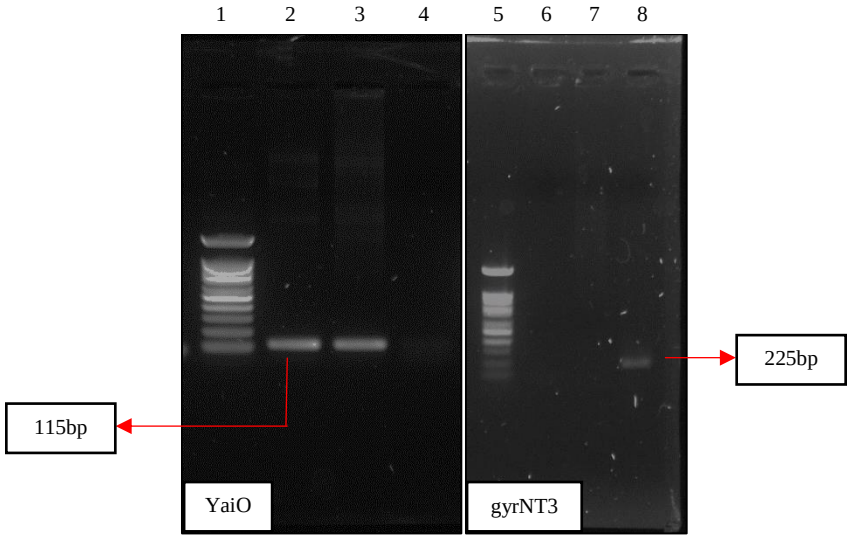


Figure 19. PCR amplification of YaiO (Left) and gyrNT36 (right) genes. Lanes 2 and 6 amplify E. coli EB-G3 template DNA while lanes 3 and 8 amplify E. coli NT36 template DNA. Lanes 1 and 5 are 1 kb ladder and lanes 4 and 7 are no template control.

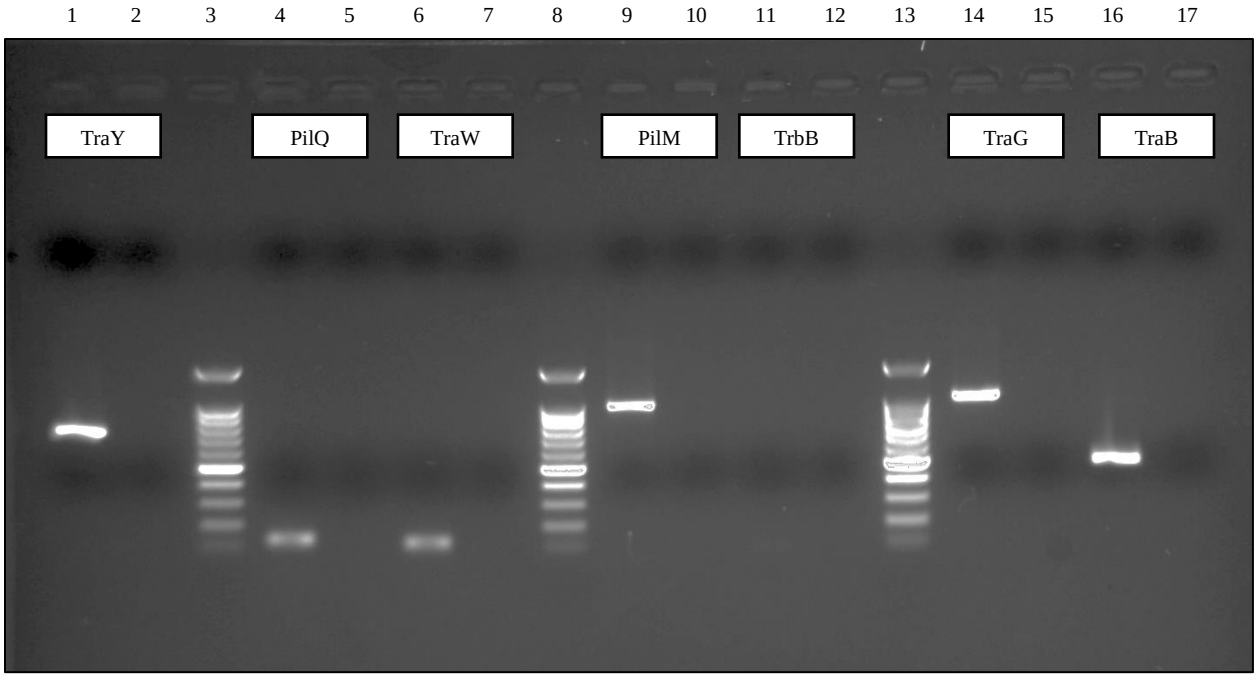


Figure 20. Specific primer sets designed for the PCR amplification of various IncI1 genes carried by pNT36-3 isolated from E. coli NT36. Lanes 1, 4, 6, 9, 11, 14, and 16 use E. coli NT36 template DNA while lanes 2, 5, 7, 10, 12, 15, and 17 use E. coli EB-G3 template DNA. Lanes 3, 8, and 13 contain a 1 kb ladder.

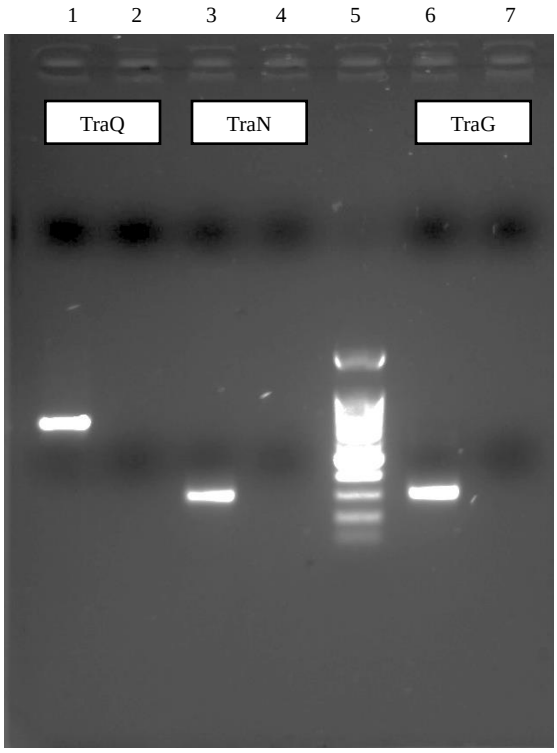


Figure 21. Specific primer sets designed for the PCR amplification of various IncF genes carried by pNT36-4 isolated from E. coli NT36. Lanes 1, 3, and 6 use E. coli NT36 template DNA while lanes 2, 4, and 7 use E. coli EB-G3 template DNA. Lanes 5 contains a 1 kb ladder.

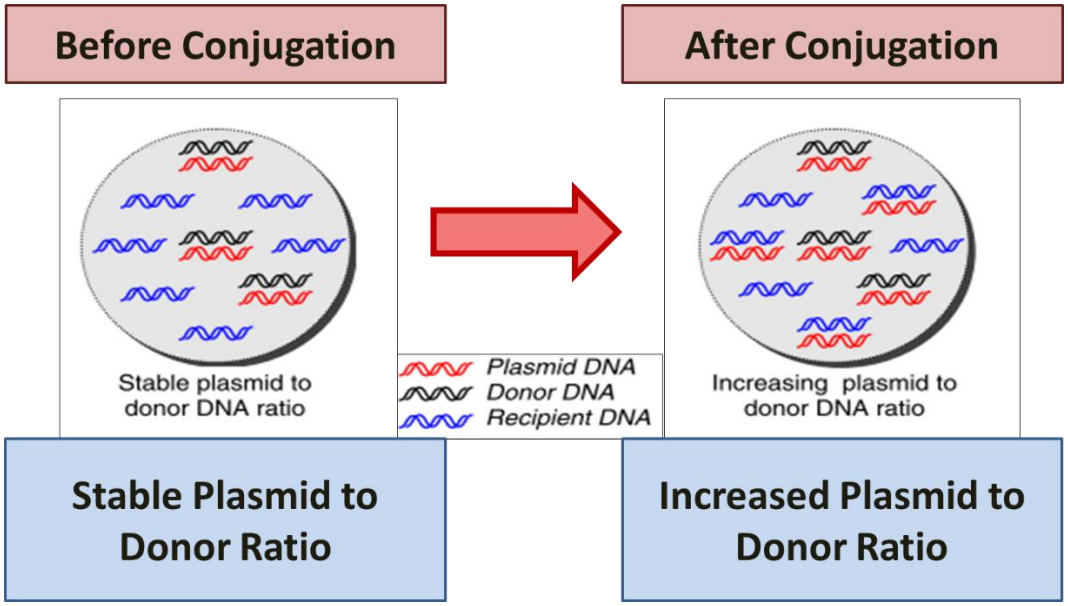


Figure 22. Schematic for monitoring plasmid transfer between donor and recipient using a molecular-based approach. This method relies on the idea that as the plasmid moves from the donor to the recipient, the copy numbers of the plasmid increase in relativity to the donor copy number.

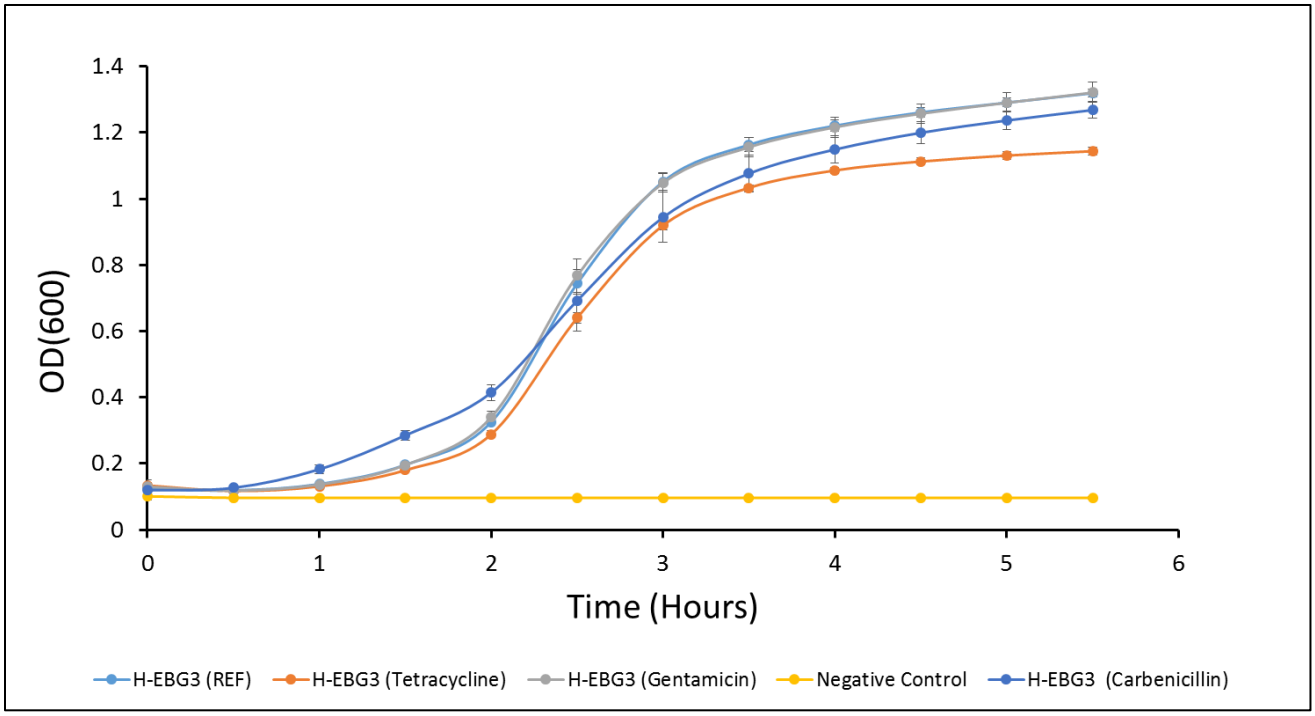


Figure 23. E. coli EB-G3 growth under various subinhibitory concentrations of antibiotics over a period of 5.5 hrs. The antibiotic concentrations include tetracycline (64 µg/mL), gentamicin (6 µg/mL), and carbenicillin (1 µg/mL). Sterile LB media was used as the negative control.

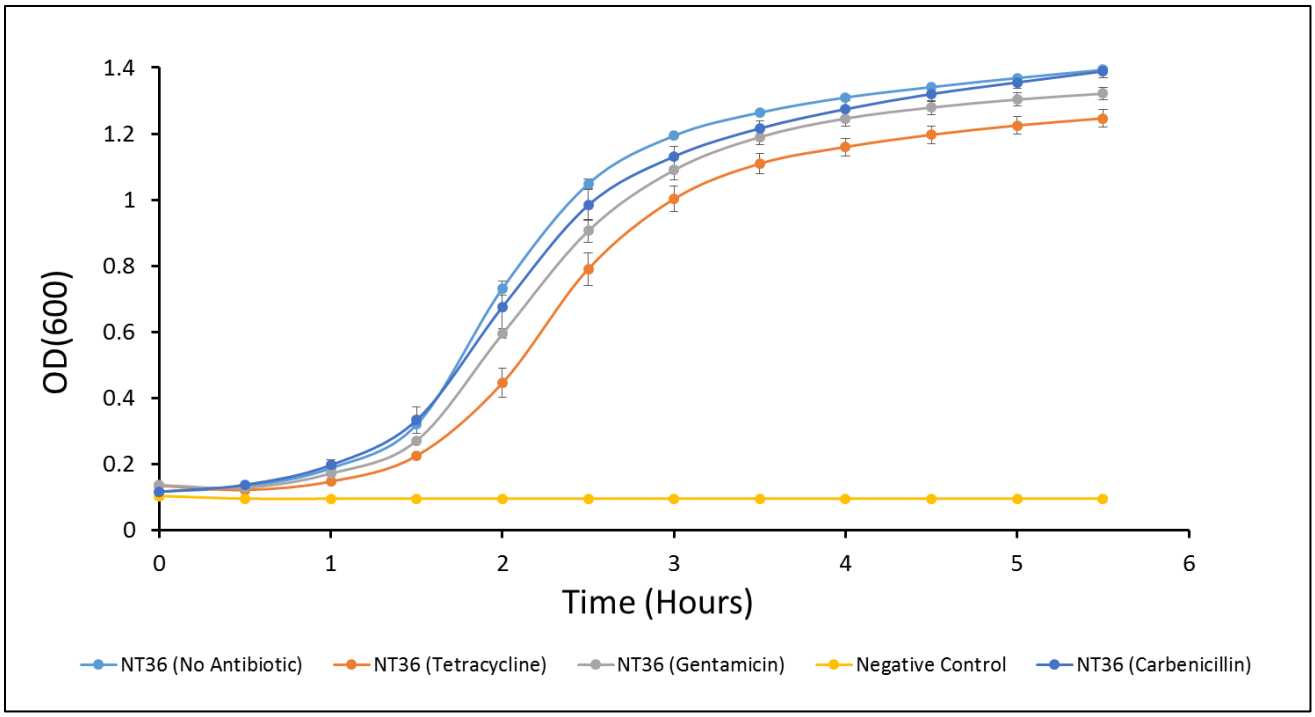


Figure 24. E. coli NT36 growth under various subinhibitory concentrations of antibiotics over a period of 5.5 hrs. The antibiotic concentrations include tetracycline (64 µg/mL), gentamicin (6 µg/mL), and carbenicillin (1 µg/mL). Sterile LB media was used as the negative control.

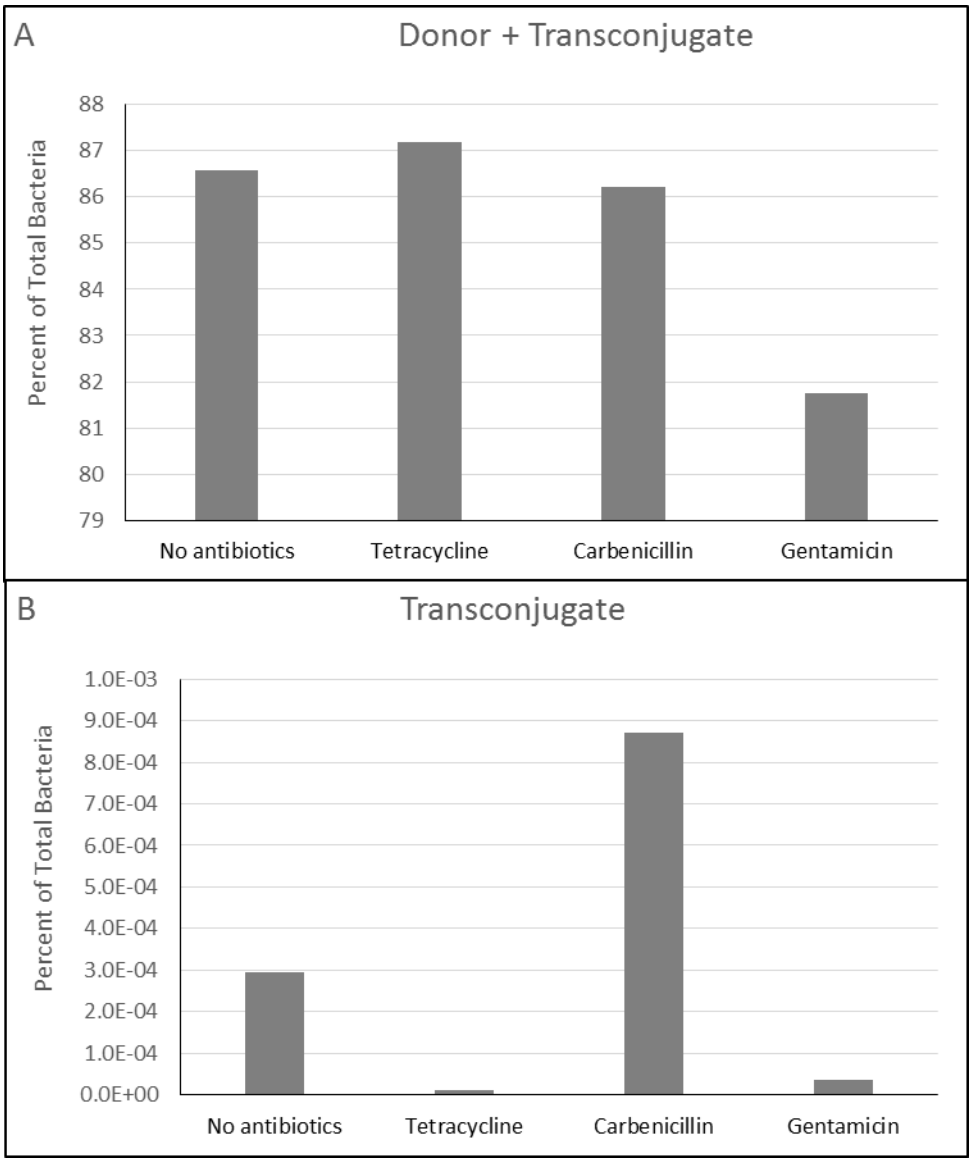


Figure 25. The percentage of colony-forming units of (A) donor bacteria (*E. coli* NT36) + transconjugants and (B) transconjugants cultured from each reactor under different antibiotic stress after 3.5 hrs of incubation.

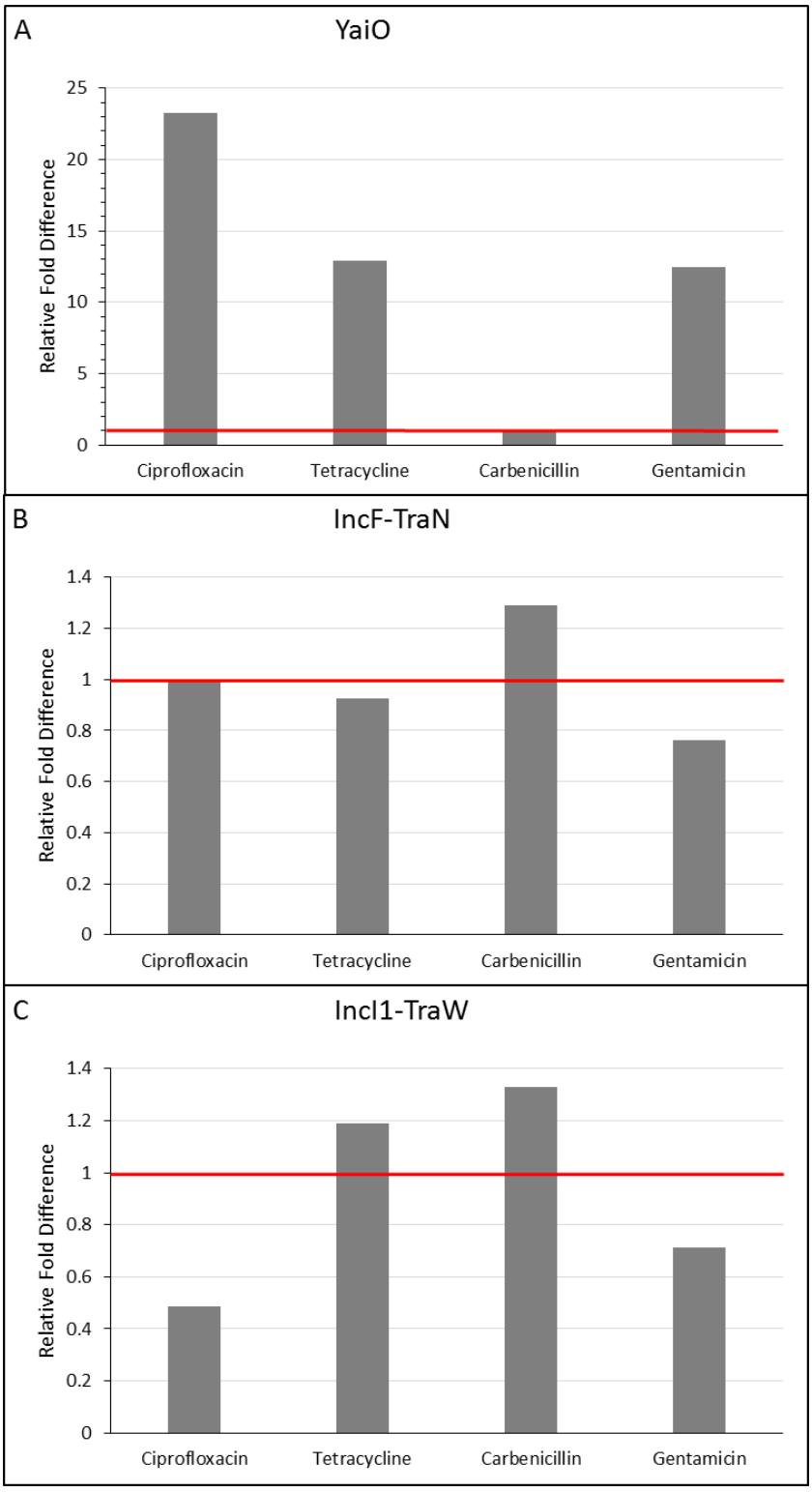


Figure 26. The fold difference in copy numbers of (A) YaiO, (B) IncF-TraN, and (C) IncI1-TraW genes relative to donor E. coli NT36 copy number (gyrNT36). The red line represents the baseline for an equivalent ratio. Values above the red line indicate an increased fold difference while values below the line indicate a decrease.

6

Chapter 6: Thesis Conclusions & Recommendations

6.1 Overall Summary & Contributions

Additional research is required to understand the occurrence, mobility, and fate of ARB and ARGs in the environment. This area of research brings forward essential information in understanding environmental sources and the development of ARB to better assess the risks imposed on public health and identify better treatment options in clinical settings. There are still on-going concerns regarding the presence of ARB and ARGs in the environment. As previously demonstrated, WWTPs serves as a hotspot for the occurrence, proliferation, and persistence of ARB and ARGs. The primary goal of this research was to characterize and investigate the occurrence and mobility of ARB and ARGs in three urban WWTPs (Humber, Ashbridge's Bay,

and North Toronto). To achieve this primary goal, three supporting goals and objectives were carried out to investigate this phenomenon, as outlined in Section 1.2.

The first objective was to devise a characterization strategy to investigate the prevalence and fate of ARGs in WWTPs and demonstrate the occurrence and composition of antibiotic sensitive and resistant bacteria in the WWTP population. This research validated the importance of culture-based techniques as opposed to the widely used molecular-based technologies, such as NGS. The differences in population composition between tetracycline resistant and sensitive members indicate that the populations are separate from each other and antibiotic sensitivity patterns are shared among members within the same genus. This type of research will assist in future investigations on ARB by identifying commonalities between members that are suspected of carrying multiple ARGs and those that are not. Furthermore, this method of examining the bacterial population as a whole, rather than through specific selection, will bring greater insight into population dynamics and their potential dangers in developing ARB. Lastly, this objective laid the foundation for subsequent studies by providing unique bacterial strains for investigating the presence and mobility of plasmids.

Objective two utilized NGS to identify conjugative plasmids carried by environmental bacterial strains and individually evaluated the potential risk each plasmid or bacteria can impose in the development of pathogenic ARB. The disadvantage of this method of culture collection and characterization is that it is laborious and can often result in the collections of novel uncharacterized genes or bacteria, with little to no information about their role in their host. As database, sequencing technology, and annotation tools improve over time, bacterial and genomic characterization will become more available and reliable. Utilizing both culture-based and molecular-based approaches will provide the necessary data to appropriately investigate mobile

genetic elements and ARB in WWTPs. This study demonstrated that ARGs are not as common on plasmids as originally expected and that plasmids are not always associated with ARB. However, transfer genes and mechanisms were found on nearly all sequenced plasmids, therefore playing a critical role in plasmid mobility. The ability of plasmids to proliferate between different members of the population contributes to bacterial evolution and eventual acquisition of ARGs. Furthermore, plasmids were found to carry toxin/antitoxin systems to improve plasmid persistence and survivability in their host, population, and sequentially the environment. The spread of plasmids that carry genes that encoding virulence factors and effector proteins will enhance microbial pathogenicity and consequently endanger public health and safety. After considering all the factors mentioned above, it is clear that even though the majority of the microbes do not host ARG-carrying plasmids, they still are instrumental in the development of pathogenic ARB.

The last objective aimed to utilize molecular-based techniques to monitor the conjugation/transfer of plasmids, pNT36-3, and pNT36-4, between two environmental *Escherichia coli* isolates exposed to a subinhibitory concentration of antibiotics. This experiment uses an experimental model, rather than a predictive model, to evaluate the plasmid's host range, stability, and transfer rates under antibiotic pressure. This section helps bridge the gap between what is predicted from characterizing each plasmid to what they are capable of accomplishing when given the opportunity. Furthermore, this study utilizes a unique methodology to monitor plasmid transfer in a cost and time-effective manner as long as correct/specific primer sets are available. Microbes are capable of transferring plasmid to closely or distantly-related members within their population. However, conjugated plasmids are often not maintained in their new host with exception to certain strains that belong to the same species. The presence of subinhibitory concentrations of carbenicillin increased plasmid transfer rates of pNT36-3 and pNT36-4 between

two *E. coli* strains. Although, the same effects were not observed in the presence of other antibiotics as they did not affect plasmid transfer and possibly induced plasmid loss. The findings of this study further validate that plasmid transfers are highly dependent on numerous factors including stressor type, stressor concentration, growth media, growth period, donor bacteria, recipient bacteria, and the plasmid of interest. By gaining insight into plasmid persistence, transfer rates, and the effects of antibiotic pressure gives us a better understanding of the potential hazards plasmids, ARGs, ARB, and antibiotics that can inflict on the environment.

6.2 Thesis Recommendations

Several technical recommendations can be proposed to improve the quality of this thesis. First, it would be ideal to perform 16S rRNA metagenomics on the wastewater community to get a higher profile comparison between each WWTP, as well as be able to identify non-culturable members of the population. The sequencing data can then be compared to the culturable profiles to obtain a better comparison between the molecular and culture-based populations. Second, it would be beneficial to also perform quantitative PCR to detect the concentration of tetracycline resistance genes found in each WWTP and downstream water body. Although traditional PCR will detect gene presence, it will not put into perspective the quantity and severity of their presence. Lastly, I would recommend using other nutrient media to enrich for Gram-positive bacteria and to culture a broader portion of the population. Although R2A media is a non-selective media, Gram-negative bacteria were found to only grow on it. It would be interesting to see what other community compositions may result when cultivating samples on different nutrient media and how it may affect antibiotic resistance patterns.

The plasmid extraction procedure must be better optimized for each bacteria to address poor DNA and sequencing qualities. I would recommend increasing the isolate count within each genus or species to obtain a cohesive story with regards to their plasmid profiles/content. Furthermore, I would recommend performing whole genome sequencing on isolates that were highly resistant to all of the tested antibiotics but appeared to not carry any plasmids such as *Variovorax* sp. NT16F. Other pathways, ARGs, or mechanisms may be responsible for their highly resistant phenotype and WGS may provide insight into their highly resistant nature.

Future research into the significance of health impacts resulting from environmental exposure to ARB would be essential for assessing risks for human health. Identifying exposure

routes from food consumption, communities, or nosocomial transmission will bring great assessments on potential health problems that can arise from ARB. Without additional knowledge on the severity of the impacts caused by ARB, it will be difficult to derive long-term goals or place essential policies on minimizing the risks to human health and the environment. Current information on how ARGs, ARB, and emerging contaminants are released into the environment does not provide adequate evidence to conclude on the importance of environmental transmission routes and their effects on human health. Additional information on the transfer of ARGs and ARB will help us identify what are the critical exposure routes and the risks that are associated with them. Having standardized methodologies on environmental sampling, time points, assessments, and analysis would provide better quality data that can be compared across different regions and achieve a greater understanding of the potential dangers caused by ARB, ARGs, and mobile genetic elements.

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