Abundance of Antibiotic Resistance Genes in Two Municipal Wastewater Treatment Plants and Receiving Water in Atlantic Canada

by

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Abstract

Antibiotic resistance genes (ARGs) in pathogenic bacteria confer resistance to many clinically important antibiotics and will, if found in treated wastewater and the environment, threaten public health. Using quantitative (qPCR), ARGs were assessed throughout two municipal wastewater treatment plants (WWTPs) that use different types of biological treatment (aerated lagoons (AL) vs. biological nutrient removal (BNR)). Furthermore, ARG presence was assessed in the receiving river of the AL plant upstream and downstream of the effluent discharge location. Both WWTPs reduced ARG levels, however ARGs persisted through the treatment. Relative abundance of ARGs (per 16s rRNA gene) was slightly decreased at the BNR plant, suggesting this treatment type improved removal of resistant bacterial populations. ARGs were detected both upstream and downstream of the AL WWTP, however higher levels were detected downstream. Overall results suggested that these WWTPs cannot remove total ARGs and are impacting ARG levels in the receiving environment.

List of Abbreviations Used

AB	Antibiotic
AL	Aerated Lagoon
ANOVA	Analysis of Variance
AR	Antibiotic Resistance
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistance Gene
BNR	Biological Nutrient Removal
BOD	Biochemical Oxygen Demand
$CBOD_5$	Five-Day Carbonaceous Biochemical Oxygen Demand
CI	Chromosomal Integron
COD	Chemical Oxygen Demand
CT	Cycle Threshold
DI	Deionized
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
eDNA	Exogenous DNA
E_{fi}	Efficiency from Fluorescence Increase
GC	Gene Copy
gDNA	Genomic DNA
HE	Hospital Effluent
HGT	Horizontal Gene Transfer
HRT	Hydraulic Retention Time

iDNA	Intracellular DNA
IQR	Interquartile Range
LOD	Limit of Detection
LOQ	Limit of Quantification
MGD	Millions of Gallons per Day
MGE	Mobile Genetic Element
MDR	Multidrug Resistance
MIC	Minimum Inhibitory Concentration
MPN	Most Probable Number
OPC	One Point Calibration
PAO	Phosphorus Accumulating Organisms
PCA	Principal Component Analysis
qPCR	Quantitative Polymerase Chain Reaction
RI	Resistance Integron
rRNA	Ribosomal Ribonucleic Acid
SRT	Solids Retention Time
SPE	Solid Phase Extraction
TN	Total Nitrogen
TP	Total Phosphorous
TSS	Total Suspended Solids
UV	Ultraviolet
VSS	Volatile Suspended Solids
WHO	World Health Organization

WWTP	Wastewater Treatment Plant

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First and foremost, I would like to thank my supervisors and mentors Dr. Lisbeth Truelstrup Hansen and Dr. Rob Jamieson for this amazing opportunity. It has truly been an enlightening and fulfilling experience. Thank you for your continued support, encouragement, and guidance throughout these past few years. Also thank you to my committee member Dr. Chris Yost for your wise input and direction throughout the project. Thank you to all of lab mates who offered your help both in the lab and by giving valuable advice and support. Audrey Hiscock, Robbie Johnson, Amy Jackson, Joanna Poltarowicz, Jenny Hayward, Dr. Colin Ragush, Yannan Huang, Amit Ross, Dr. Marta Piercey, Michael McDonald and everyone else, you all made it a great and fun experience. A special thanks to Dr. Kara Neudorf for your mentorship and continued support throughout the project. All of your patience and our many exchanges of cat stories and laughs made the project even more enjoyable. Last but not least, thank you to my family and friends for your unconditional support, understanding, and hilarity over the years that have gotten me to where I am today.

Chapter 1: Introduction

1.1 Project Context

The World Health Organization (WHO) released a report in 2000 focusing on antibiotic resistance (AR) as one of the most critical human health challenges of the next century, announcing the need for "a global strategy to contain resistance" (WHO, 2000). It has been suggested that some resistant pathogens found in clinical environments have obtained their resistant genes from environmental reservoirs (Wright, 2010). Therefore, the link between the environment and clinical resistance has become of increased concern, and finding and managing resistant reservoirs is of interest. Wastewater treatment plants (WWTPs) have been previously described as “hotspots” for high levels of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Rizzo et al., 2013). The Government of Canada has reported in a news release that “wastewater treatment plants represent an important control point in the many steps taken to reduce the spread of antibiotic resistance” (Government of Canada, 2015). Little attention has been given to the occurrence, distribution, and transfer of AR and resistance genes between bacterial populations within municipal wastewater treatment plant (WWTP) systems. This information is essential for identification of public health risks and treatment options. Whether wastewater treatment processes are eliminating resistance elements, or potentially enriching them is still debated (Guardabassi et al., 2002; Huang et al., 2011). By examining the prevalence of ARB and ARGs throughout different WWTP systems, and examining the effects of different treatment steps, we can advance our understanding of their role in the dissemination of resistance elements into the

environment. This may help highlight the severity of treatment plants' impact on the rise of AR as well as highlight potential ways to decrease their contribution to the problem.

After treatment, WWTPs discharge their effluent into receiving environments such as rivers, lakes, or oceans. Currently, there are no regulations or guidelines for the permitted or desirable maximum levels of ARB, ARGs, or antibiotics (ABs) that can be released into the receiving environment. Therefore, AR determinants that are still present after treatment ultimately end up in the environment and are not monitored. Once present in the environment, genetic elements such as ARGs can spread among bacteria and be subsequently obtained by dangerous pathogens (Allen et al., 2010). Antibiotics can also exert a selective force for the maintenance of and acquisition of ARGs, thus further increasing AR in receiving environments (Davies and Davies, 2010). Along with WWTP effluents, other anthropogenic activities can also increase ARGs in the environment as higher levels have been associated with areas closer to urbanization and agriculture (Berglund et al., 2015; Ouyang et al., 2015). Runoff from these areas, as well as the discharge of WWTP effluents into watersheds allows increased transport of AR determinants, permitting their spread throughout the environment (Allen et al., 2010).

As mentioned above, currently there are no regulations on what is considered high for levels of ARGs in wastewater or environmental areas such as surface waters (Proia et al., 2016). Therefore, investigating a combination of ARG levels in wastewater, effluent receiving environments, and other surface waters, can allow for a starting point to begin to define what levels are actually high as well as assess the influence of different sources (e.g. WWTP effluents, proximity to urbanization, agriculture, etc.) on the rising AR problem. The purpose of this study was to determine the levels entering and leaving

WWTPs with a focus on the impact of individual treatment steps on ARG abundances in the municipal wastewater being treated. Furthermore, levels both downstream and upstream of a WWTP discharge point were assessed to determine that WWTPs impact on the receiving water. The upstream watershed was also assessed to determine ARG background levels in both more urbanized and remote locations along that watershed and its headwaters.

1.2 Research Objectives

The research objectives of this study were divided into two main sections 1) Presence and abundance of ARGs after the main treatment steps of two WWTPs that use different biological treatment (aerated lagoons (ALs) vs. biological nutrient removal (BNR) reactors), and 2) Presence and abundance of ARGs in the receiving river both downstream and upstream from a WWTP outlet, as well as further upstream to determine background ARG levels in the watershed. The first objective aimed at determining how each step along the wastewater treatment train impacts ARG abundances, as well as comparing WWTPs that use different treatment trains in order to assess differences caused by different designs. More specifically, different biological treatments were examined at the two plants to compare how they perform in terms of ARG removal. The specific objectives of this section were as follows:

1. Use quantitative PCR (qPCR) to determine both absolute abundance (copies/mL) and relative abundance (copies/16S rRNA copies) of nine different ARGs after each main treatment step in two different WWTPs to determine quantitatively which steps increase/reduce ARG abundance.

2. Use quantitative PCR (qPCR) to assess *int1* genetic marker as a proxy to measure ARG cassette presence and potential for ARG mobility in the wastewater.
3. Assess potential relationships between water quality indicators and ARG levels at both plants
4. Assess potential relationships between antibiotic levels and ARG levels at both plants
5. Assess seasonal, and temporal consistencies in ARG abundances at one WWTP

The second objective aimed to determine if one WWTP (based on an AL treatment and which has very strict effluent regulations as it discharges its effluent into a small river) impacts ARG levels downstream of the plant. ARG levels were also assessed at various locations upstream from the plant in order to determine baseline levels and to see if proximity to anthropogenic activities impacted these levels. The specific objectives of this section were as follows:

1. Use quantitative PCR (qPCR) to determine both absolute abundance (copies/mL) and relative abundance (copies/16S rRNA copies) of nine different ARGs both upstream and downstream of the WWTP outlet in order to determine if the WWTP is significantly impacting ARG levels in the river.
2. Use quantitative PCR (qPCR) to determine absolute abundance (copies/mL) of ARGs in the upstream watershed of WWTP outlet to determine background levels of ARGs both closer to and further away from urbanization.
3. Use quantitative PCR (qPCR) to assess *int1* genetic marker as a proxy to measure ARG cassette presence and potential for ARG mobility in the wastewater.
4. Assess potential relationships between water quality indicators and ARG levels

5. Assess potential relationships between AB levels and ARG levels
6. Assess seasonal differences and consistency of ARG levels in the river

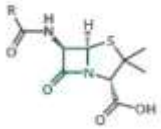
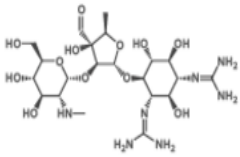
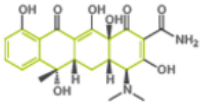
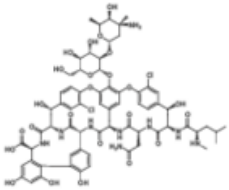
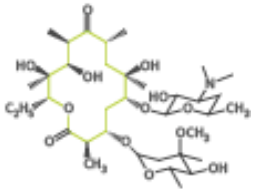
Chapter 2: Literature Review

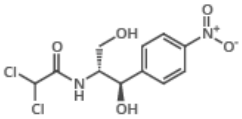
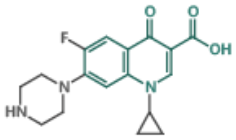
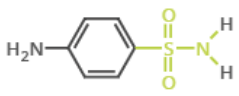
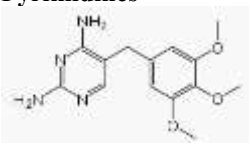
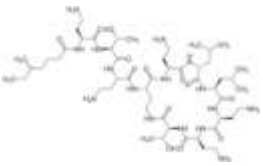
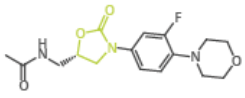
2.1 Background on Antibiotic Resistance

2.1.1 Defining Antibiotics and Antibiotic Resistance

Antibiotics are organic molecules that interact with cellular targets and cause microbial cell death or growth inhibition. Antibiotics are essential for treating bacterial infections in human and animals, thus preserving their efficacy should be a top priority (Allen et al., 2010). Five major AB targets exist in bacteria: the bacterial cell wall, the cell membrane, protein synthesis, DNA and RNA synthesis, and folic acid (vitamin B9) metabolism (Wright, 2010). Different ABs can be divided into classes based on their chemical structures. Antibiotics within a similar structural class will generally show similar patterns of effectiveness, toxicity, and mechanism of action. Antibiotics can also be classified as broad-spectrum, acting against a wide range of Gram-positive and Gram-negative bacteria; or narrow spectrum, which are only effective against specific families of bacteria (Wright, 2010). Table 1 lists some commonly used AB classes along with examples of ABs, their bacterial targets, as well as resistance mechanisms used against the ABs. Some examples of ARGs that encode resistance to the ABs are also listed in the table.

Table 1: Modes of action and resistance mechanisms of commonly used antibiotics (adapted from Morar and Wright, 2010 and Brunning, 2015).

Antibiotic Class	Known for	Examples of antibiotics	Bacterial Target	Mode(s) of resistance*
Beta-Lactams 	All contain a beta-lactam ring and are the most widely used antibiotics	Penicillins [shown] (ampicillin, methicillin), cephalosporins, (cephamycin), penems (meropenem), monobactams (aztreonam)	Cell wall synthesis	Hydrolysis (*bla*$_{TEM-1}$, *bla*$_{CTX-M}$), efflux (*acrB*), altered target (*mecA*)
Aminoglycosides 	All contain aminosugar substructures and include a family of over 20 antibiotics	Gentamicin, Streptomycin (shown), kanomycin	30S ribosomal subunit (protein synthesis)	Phosphorylation (*aph33ia*), acetylation (*aac(2'')-I)*, nucleotidylation (*aadD*), efflux, altered target
Tetracyclines 	All contain 4 adjacent cyclic hydrocarbon rings and are becoming less popular due to a rise in resistance	Tetracycline (shown), Minocycline, Tigecycline	30S ribosomal subunit (protein synthesis)	Monooxygenation (*tetX*), efflux (*tetA*), altered target, immunity (*tetO*)
Glycopeptides 	Consist of a carbohydrate linked to a peptide and are a common drug of last resort	Vancomycin (shown), Teicoplanin	Cell wall synthesis	Reprogramming of peptidoglycan biosynthesis (*vanA*)
Macrolides 	All contain a macrolide ring and are the second most prescribed antibiotic	Erythromycin (shown), Azithromycin	50S ribosomal subunit (protein synthesis)	Hydrolysis, Glycosylation, Phosphorylation (*mphA*), Efflux (*mefA*), Altered target (*ermB*)

Antibiotic Class	Known for	Examples of antibiotics	Bacterial Target	Mode(s) of resistance*
Phenicols 	Distinct compound and commonly used in low income countries. No longer first line drug in developed countries due to increased resistance and safety concerns	Chloramphenicol (shown)	50S ribosomal subunit (protein synthesis)	Acetylation (*catA1*), Efflux (*adeB*), Altered target
Quinolones 	All contain fused aromatic rings with carboxylic acid group attached. Resistance evolves rapidly.	Ciprofloxacin (shown), levofloxacin	DNA gyrase (replication)	Acetylation, Efflux (*smeD*), Altered target (*qnrS*)
Sulfonamides 	All contain sulfonamide group and were the first commercial antibiotics	Sulfamethoxazole ,Sulfanilamide (shown)	Folate synthesis	Efflux, Altered target (*sul1, sul2*), immunity and bypass
Pyrimidines 	Resistance is increasing, but it is still a first line antibiotic in many countries	Trimethoprim (shown)	Folate synthesis	Efflux, Altered target, immunity and bypass (*dfrA1*)
Polymyxins 	Consist of a cyclic peptide with a long hydrophobic tail and are often only used as a last resort antibiotic	Colistin (shown)	Cell membrane	Efflux, Altered target (MCR-1)
Oxazolidinones 	All contain 2-oxazolidone in their structure and are commonly used as last resort antibiotics	Linezolid (shown), cycloserine	50S ribosomal subunit (protein synthesis)	Altered target

*() indicates ARG example (Liu et al., 2009)

Resistance to ABs occurs through four main mechanisms: target modification; efflux; immunity and bypass; and enzyme-catalyzed destruction (Wright, 2010). Target modification can occur through mutation of the targets themselves or by the production of enzymes that modify AB targets. An example of target modification is the *mecA* gene, which causes methicillin resistance. *MecA* codes for a modified penicillin binding protein that has a low affinity for β-lactams, therefore promoting resistance to methicillin (Liu et al., 2009). Efflux occurs through protein pumps that eject ABs from the inside of the cell, disallowing them to attack their target. An example of efflux is the *tetA* gene, which causes tetracycline resistance. *TetA* encodes a tetracycline efflux protein that functions as a tetracycline antiporter, eliminating the AB from the cell (Liu et al., 2009). Immunity consists of ABs or their targets being bound by proteins that prevent the AB binding to its target, therefore rendering them useless. An example of immunity is the *tetO* gene, which causes tetracycline resistance. *TetO* encodes a ribosomal protection protein, which protects the ribosome from the translation inhibition caused by tetracycline (Liu et al., 2009). Enzyme-catalyzed destruction recognizes antibiotics and modifies them in such a way as to destroy their functions, eliminating their ability to interact with their targets. For example, β-lactamase enzymes, encoded by such genes as bla_{TEM}, recognize and hydrolytically cleave the β-lactam ring of β-lactam antibiotics, which destroys their function (Wright, 2010). Figure 1 (adapted from Wright, 2010) illustrates some common mechanisms of action of AB groups and the resistance mechanisms that exist to counteract them.

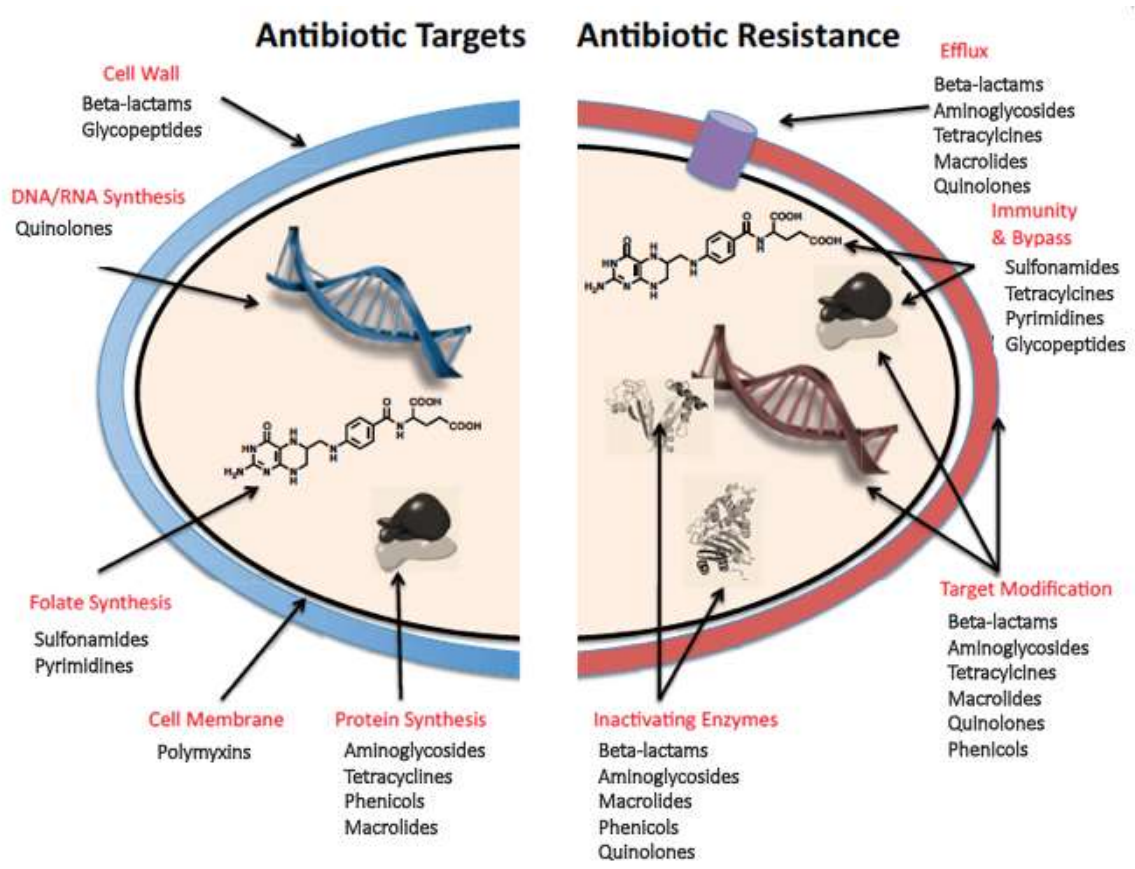


Figure 1: Antibiotic targets and mechanisms of resistance examples (adapted from Wright, 2010). See text for details

2.2.2 Why is Antibiotic Resistance an Important Issue and What Caused its Emergence?

Resistance to ABs was recorded as early as the 1940's, even before the first clinical use of penicillin (Wright, 2010). Resistance to sulfonamide in the 1930's and penicillin in the 1940's made treating infections in hospitals difficult and has been an increasing problem since (Levy and Marshall, 2004). Currently, resistance to all classes of ABs has developed, leaving no AB for which a resistance mechanism does not exist

(Rizzo et al., 2013). Multidrug resistance (MDR) was first detected among enteric bacteria in the late 1950s, and such strains cost many lives especially in developing countries (Levy and Marshall, 2004). There has even been a re-emergence of tuberculosis since the 1980's caused by MDR *Mycobacterium* spp., which often requires several different drugs to treat. Treating MDR bacteria is costly and more dangerous, sometimes resulting in treatment failure and even death (Levy and Marshall, 2004). The frequency of drug resistance has extended beyond hospitals and is present in the environmental reservoirs as well. The increasing rates of resistance around the world cause human health risks and undermine a physician's ability to treat infections, which increases health costs and mortality rates globally.

Antibiotic resistance is a natural phenomenon as ABs have been produced naturally for over 500 million years, thus creating selective forces for bacteria to obtain or maintain resistance (Baltz, 2008). Antibiotic resistance genes have been suggested to have alternative cellular functions other than resistance in some environmental bacteria, therefore these genes can also be found in areas without typical selective pressures from ABs (Allen et al., 2010). However, human activities have greatly contributed to the generation and spread of AR environmental reservoirs. Humans have been manufacturing and clinically using increased amounts of ABs since the 1940's (Davies and Davies, 2010). This has caused a widely disseminated release of ABs into the environment, which provides constant selection and maintenance pressure for populations of resistant strains in the environment (Davies and Davies, 2010). Due to the fact that the only available evidence suggests that a very small proportion of ABs is contributed by naturally occurring AB-producing strains in their native environments (Gottlieb, 1976), it

is assumed that commercial production by humans provides the majority of ABs found in the biosphere (Davies and Davies, 2010). Therefore, although natural ABs and AR does exist, anthropogenic activities have contributed to most of the increased development of AR. Some other uses of antimicrobial agents in addition to human therapeutics include growth promotion and therapeutic uses in animals, pest control in agriculture, biocides in cleaning products, as well as uses in research labs, which can ultimately end up in the environment and promote AR development. Some factors thought to contribute to dissemination of AR determinants from the above sources throughout the environment include biological forces such as human and animals, physical forces such as wind and watersheds, as well as runoff from areas of urbanization/agriculture (Allen et al., 2010). Therefore, even areas that are not under direct influence of anthropogenic activities have been shown to contain resistance genes as spread from anthropogenic impacted areas increases (Allen et al., 2010; Ouyang et al., 2015). Antibiotic resistance genes can persist even in the absence of typical selective pressures and after its host has died, making them extremely difficult to get rid of (Martinez, 2009; Salyers and Amábile-Cuevas, 1997). Antibiotic resistance genes have therefore been suggested as emerging environmental pollutants (Pruden et al., 2006). It is important to note that human therapeutic uses accounts for less than half of all AB applications (Davies and Davies, 2010), therefore these other sources are of major concern as well. Studies have also suggested correlations between water quality and ARG levels, indicating that wastewater effluents and waters polluted by urbanization and agriculture, which are known to have decreased water quality, may be hotspots for AR (Al-badaii and Shuhaimi-Othman, 2015).

2.3.3 Mechanisms for Acquiring Antibiotic Resistance

Both vertical and horizontal gene transfer are responsible for the evolution of resistance in bacteria. Vertical gene transfer involves the mutation of existing genes being passed to future generations. Horizontal gene transfer (HGT) involves mobile genetic elements (MGEs) containing resistance genes being transferred between bacteria (Martinez and Baquero, 2000). Antibiotic resistance genes present on these mobile elements encode specific mechanisms which challenge an AB's ability to harm a microorganism. Bacteria participate in HGT via uptake of foreign DNA by means of conjugation, transduction or transformation (Schlüter et al., 2007). Conjugation involves physical contact between a donor and a recipient cell via a conjugation pilus, through which genetic material is transferred, therefore involving live bacteria. Transformation is the uptake of exogenous DNA (eDNA) by bacteria, and transduction is the delivery of genetic material through phage predation (Soucy et al., 2015).

Mobile genetic elements include plasmids, integrons, and transposons, which are responsible for transferring ARGs between bacteria (Bennett, 2008). Plasmids can encode conjugative transfer genes that enable gene movement between bacteria of the same or different species, therefore they are important vectors for accumulating and spreading ARGs among bacteria (Bennett, 2008). Resistance genes are often found on plasmids within integrons, which are genetic elements able to acquire, exchange and express ARGs embedded in gene cassettes, and/or within transposons, which are genetic sequences capable of moving within genomes (Rizzo et al., 2013). Therefore, these plasmids are able to code for various resistance mechanisms to ABs, heavy metals, and quaternary ammonium compounds (Davies and Davies, 2010). This enables bacteria

receiving a resistance plasmid to become resistant to multiple ABs as well as heavy metals, which may co-select for each other (Levy and Marshall, 2004). Mobile elements are of great concern not only for the spread of ARGs among environmental bacteria, but also due the risk of transfer of ARGs to opportunistic pathogens present in these environmental reservoirs (Wright, 2010). In fact, many ARGs and proteins responsible for resistance in environmental bacteria are homologous to those found in pathogens, strongly suggesting HGT of these mobile elements and that the environment is a large reservoir of resistant genes (Wright, 2010). Due to vast numbers of bacteria on the planet and selection pressure which is provided by ABs, the movement of resistance genes into previously susceptible pathogens from resistant, but benign bacteria is of major concern (Wright, 2010). Therefore, determining hotspots for resistance is becoming more important in the research community.

2.4 Potential Hotspots and Reservoirs of AR in the Environment

2.4.1 Municipal Wastewater Treatment Plants as Potential Hotspots for Antibiotic Resistance

The potential role of wastewater treatment facilities in the dissemination of AR is of increasing concern, as they may harbour favorable conditions for both selection and transfer of ARB and ARGs (Rizzo et al., 2013). Millions of cubic metres of sanitary sewage are flushed from homes, businesses, institutions and industries through sink drains and toilets into city sewer systems every day (Government of Canada, 2010). Municipal wastewater contains sanitary sewage and is sometimes combined with stormwater from rain or melting snow. The sewer system either takes the wastewater to a

municipal WWTP or releases it directly into a lake, river or ocean. However, despite treatment in municipal WWTPs, pollutants remain in treated wastewater and are discharged into surface waters. Treated wastewater may contain grit, debris, biological wastes, disease-causing and resistant bacteria, nutrients, and hundreds of chemicals such as those present in pharmaceuticals (e.g. ABs) and in personal care products (Government of Canada, 2010). The biological treatment process during wastewater treatment creates especially suitable conditions for selection and HGT due to bacteria being continuously mixed with sub-inhibitory AB concentrations in nutrient-rich, high cell density activated sludge (Auerbach et al., 2007). Treatment plants may also receive hospital effluents, which may have higher abundances of antibiotics and ARB/ARGs (Schluter et al., 2007). Hospitals effluents commonly contain high amounts of ABs in their effluents and are an important input source of resistant bacteria due to the use of ABs in medical treatment (Kümmerer, 2004). Wastewater treatment plants include different processes such as mechanical, biological, and physical treatments that may affect the fate of ARB and ARGs in different ways (Rizzo et al., 2013). The enumeration and detection of ARB and ARGs within various stages of municipal wastewater treatment systems and their receiving waters could provide a better understanding of the baseline levels and fate of AR throughout a WWTP as well as their dissemination into the environment.

2.4.2 Aquatic Environments as Reservoirs for Antibiotic Resistance

Aquatic environments such as rivers, lakes, and oceans are often impacted by anthropogenic activities, allowing the acquisition and spread of AR determinants (Marti

et al., 2014). As previously mentioned, a mixture of ABs and other pollutants, ARB, and ARGs reach the aquatic environment through such sources as treated and untreated sewage, hospital waste, aquaculture discharges, as well as agricultural/urban runoff (Marti et al., 2014). These aquatic environments, which include both water and sediment, may have a significant role in driving AR.

Water environments that receive effluents from WWTPs have been found to contain higher levels of AR determinants such as ARGs and ABs than other surface waters that do not receive wastewater (LaPara et al., 2011). The aquatic environment also allows for the transport of these AR elements downstream and their subsequent spread around the environment. Studies have also found higher levels of ARGs in both biofilm and sediment samples downstream from wastewater outlets (Knapp et al., 2012; Proia et al., 2016), demonstrating the ability of ARGs to be transferred from the water column to more stable environments. Studies have suggested that biofilms may contribute to AR due to high cell density, close proximity, and accumulation of MGEs, as well as serving as long-term reservoirs for ARGs (Engemann et al., 2008). Sediments have also been suggested as reservoirs for the accumulations and transfer of ARGs (Marti et al., 2014). Studies have shown increased ARG abundance in sediments more impacted by urbanization as well as downstream from wastewater outlets (Knapp et al., 2012; Marti et al., 2013). Studies have also suggested that ARGs once transferred to sediment can persist for months, thus contributing to long term AR reservoirs (Mao et al., 2014). Antibiotic resistance genes can also be transferred from sediment back into the water column during times of high flows, thus allowing the continual spread of ARGs (Knapp et al., 2012). Therefore, due to the increased abundance of AR determinants entering

aquatic environments, their increased spread and transport through watersheds, as well as the ability of ARGs to be transferred from the water column to more long-term reservoirs, aquatic environments may represent significant environmental reservoirs of AR.

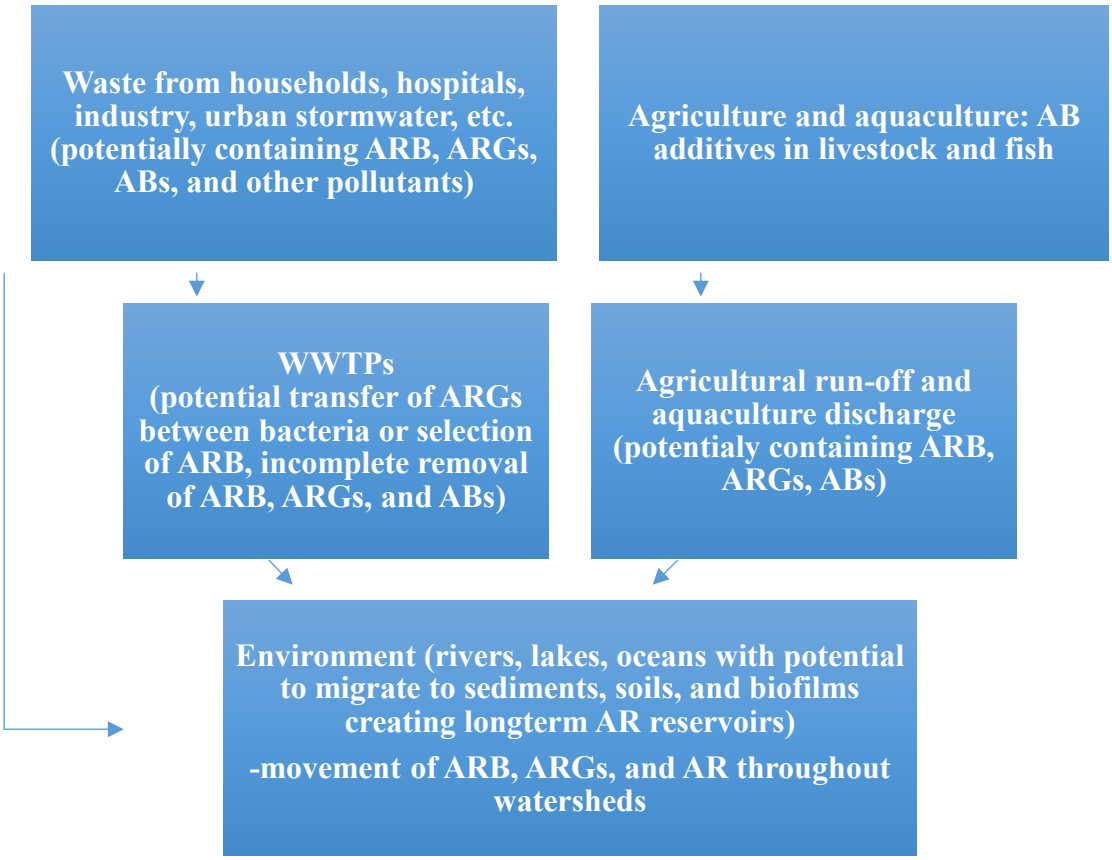


Figure 2: Summary of potential pathways for ARB, ARGs, and ABs to enter the environment through water sources. Waste from multiple sources, as well as stormwater, can enter the environment directly through runoff, septic systems, or discharge without treatment in areas that do not have municipal WWTPs. Wastewater, which enters treatment plants, is treated, however AR elements may still survive or become enriched

and be discharged into the environment. Runoff from agricultural areas as well as the use of antibiotics in aquaculture provides a vehicle for AR elements to enter water environments.

2.5 Methods and Tools to Detect and Assess Antibiotic Resistance in WWTPs and the Environment

In order to begin controlling AR, the hotspots harbouring resistant bacteria and genes must first be identified. Currently, there are two main approaches taken to determine the occurrence of ARB and ARGs in wastewater and the environment; culture-based and molecular-based methods. There are both advantages and disadvantages to each of these methods that will be discussed, however an emphasis will be given on discussing molecular methods as they were used in this thesis.

2.5.1 Cultivation-Based Methods for Determining Antibiotic Resistance in Wastewater and the Environment

Methods have been developed to adapt standardized testing of AR to waste and surface waters. These adaptions make achieving reliable and accurate results across laboratories possible (Rizzo et al., 2013). To isolate bacteria from water environments, the membrane filtration method is often used. This method can involve selective culture media which allows particular groups of bacteria to be isolated and grown, which are often coliforms and enterococci due to their correlations to water quality (APHA, 2005). Once purified, these isolates can be examined for their AR profiles. The most common

forms of this type of analysis are the dilution method and disc diffusion methods. The dilution method relies on determining the minimum concentration of AB that will inhibit a particular isolate (MIC), using either broth or agar dilutions. The disc diffusion method involves determining zones of inhibition on a plate covered with growth medium seeded with the isolate of interest. These zones determine the MIC for a particular AB (Michigan State University, 2011). Although highly standardized, both of these methods are highly time consuming and laborious.

An adaptation to the above methods was implemented to improve routine analysis. Selective culture media was supplemented with ABs in the range of their MICs, which allowed for the percentage of resistance to be calculated by comparing the ratio of bacteria growing in the presence and absence of an AB. Another advantage of this adaptation is that it enriches the ARB, which may allow for easier detection of novel or rarer genetic elements (Figueira et al., 2011; Watkinson et al., 2007). In summary, cultivation techniques allow for the enrichment of resistant indicator organisms and reveal the main sources of resistant bacteria present in wastewater. Also, it is possible to further characterize the resistance mechanisms among the isolates.

2.5.2 Molecular Biology Based Methods for Studying Abundance of ARGs in Wastewater and Environmental Samples

Not all organisms can be grown efficiently, or at all, in the laboratory, which makes it difficult to obtain a complete picture of the AR present in an environmental sample through cultivation techniques alone. Molecular biology techniques have been developed to detect microorganisms and genes that would be missed through cultivation

techniques. Molecular biology techniques rely on the assumption that resistance is caused by carrying a resistance gene and mobile elements signify mobility (Rizzo et al., 2013). Specific DNA targets are identified without the need for prior cultivation. However, it is important to remember these techniques employed alone do not differentiate between genetic targets from living or dead bacteria, or extracellular DNA (eDNA), nor do they allow for the detection of previously unknown ARGs.

Quantitative PCR (qPCR) is a widely used method that allows the detection and quantification of various resistance genes, phylogenetic groups and functional genes, and can be applied to different communities and environmental sources (Lupo et al., 2012; Volkmann et al., 2004). Quantitative PCR can be used to detect changes in abundance of resistance genes throughout a wastewater system and track its spread into the environment. This method eliminates the bias of cultivation, as DNA is extracted from a whole community and resistance elements are quantified without changing abundances of bacteria present in the initial sample (Trevors, 2011). Detecting ARGs and other genetic elements of interest involves designing suitable primers and probes, which relies on known sequences and sufficient DNA homology amongst similar organisms (Bouki et al., 2013). The ability to detect ARGs with very low copy numbers in a metagenomic sample relies on the quality of the template and the success of the nucleic acid extraction. Inhibitors such as organic salts, detergents, and humic substances are often present in wastewater, therefore an effective extraction is needed in order to reduce the impact of these contaminants (Volkmann et al., 2007). It is also important to consider varying amplification efficiencies among different DNA templates in order to avoid underestimating or overestimating the initial target amounts. Correcting for efficiencies

among environmental wastewater samples which are known to have background noise and contamination, and pure DNA standard templates, will allow for a more accurate estimation of the quantity of the template of interest (Brankatschk et al. 2012).

Quantitative PCR approaches permit both absolute and relative abundances to be quantified. Absolute quantification methods involve the use of a standard curve constructed using standards of a known concentration or copy number or using a one-point calibration calculation to quantify targets in environmental samples (Brankatschk et al., 2012). Relative abundance relies on comparing the gene copy number to a reference gene, such as the bacterial 16S rRNA gene. This measurement allows the quantification of a target gene compared to the amount of bacteria present in the sample (Brankatschk et al., 2012). These techniques are used to both quantify the amount of ARGs in wastewater and environmental samples, as well as compare the relative abundances of these genes as they progress through a WWTP system and enter the environment.

There are also newer technologies involving high-throughput (HTP) sequencing-based metagenomic analysis which overcome the problems of amplification-based techniques, such as limited primer availability, and biases in the amplification process (Rizzo et al., 2013). Metagenomics allows a study of genomic DNA from a whole microbial community. However, currently most reference bacterial genomes, which are required to perform the analysis, are limited to a few clinical isolates, which makes it difficult to compare gene sequences and gene functions of wastewater origin (Rizzo et al., 2013). As more wastewater reference genomes become available, next generation sequencing and functional genomics will allow a better understanding of vertical and HGT in wastewater environments.

In summary, molecular techniques can quantify the abundance of ARGs and other genetic elements of interest in various environmental samples. Figure 3 outlines both culture-based and molecular-based approaches for assessing AR in wastewater samples.

2.5.3 Molecular Biology Based Methods for Detecting Gene Transfer Ability Using Integrons in Wastewater and Environmental Samples

Both cultivation and molecular techniques mentioned above commonly involve looking for a known type of resistance, however these approaches may exclude unknown and unforeseen resistance events (Rizzo et al., 2013). Attention should also be paid to genetic structures that are known to host ARGs and can contribute to their mobility (Lupo et al., 2012). Integrons are defined as genetic elements that possess an *attI* site, at which gene cassettes can be integrated by site-specific recombination, and which encode the enzyme integrase, that mediates these site-specific recombination events (Bennet et al., 1999). Both chromosomal integrons (CIs) and resistance integrons (RIs) exist. Chromosomal integrons are located on chromosomes and RIs are located on MGEs which contain gene cassettes (Bennet et al., 1999). Three distinct classes of RIs have been reported; class 1, class 2 and class 3, which are divided into classes by their amino acid sequences of the integrase gene. Class 1 RIs have been the most studied of the RIs in association with AR and will be further discussed. Several studies have revealed class 1 integrons as platforms for ARG aggregation, which can lead to MDR. Class 1 integrons often co-occur with ARGs and are frequently found on MGEs such as plasmids and transposons, which favour the spread of these genes (Lupo et al., 2012). Since

integrons all share a common structure, are associated with other mobile elements and and are known to contain ARG cassettes, they are a good genetic target for assessing the potential for ARG transfer in the environment and WWTPs (Rizzo et al., 2013).

Quantitative PCR methods can be used to determine the abundances of class 1 integrons using the same methods mentioned above for ARGs. Quantifying the *int1* gene, which encodes the integrase gene in class 1 integrons, allows for an estimation of the mobility potential of a sample and gives an indication of the presence of AR, although it does not describe which exact ARGs are present (Rizzo et al., 2013). Studies have revealed that anthropological influence can enrich RIs in the environment through such factors as bacterial stress caused by the presence of ABs and heavy metals (Rizzo et al., 2013). Municipal WWTPs are known to contain factors associated with bacterial stress and are considered potential hotspots for RIs; the influent containing the highest concentrations (Ferreira Da Silva et al., 2007; Moura et al., 2007). Even though WWTPs can remove two to three log of bacteria throughout the treatment train, bacteria containing ARGs and RIs do not seem to be highly affected by the treatment process (Ferreira Da Silva et al., 2007). Therefore, class 1 integrons are a promising target in assessing the effectiveness of a WWTP's ability to remove ARGs and assess the mobility of these genes as well as track MDR in the environment.

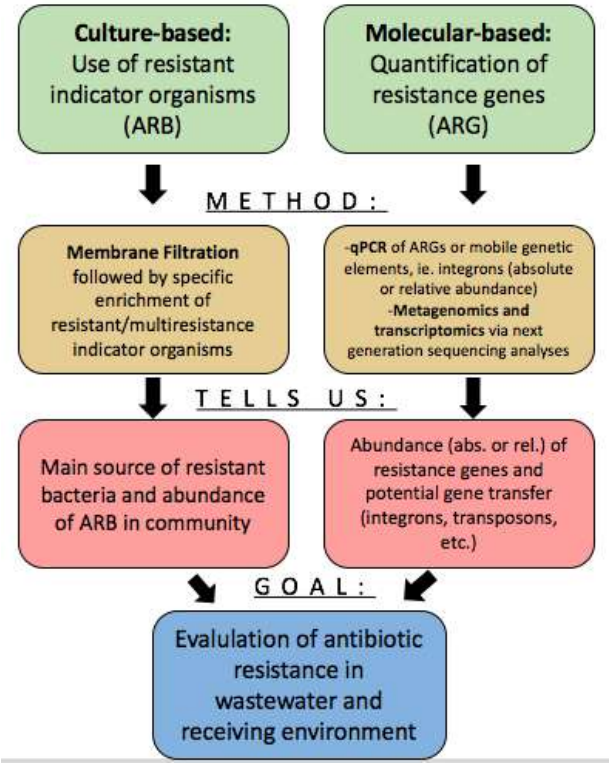


Figure 3: Methods to detect ARB and ARGs in wastewater and environmental samples. The left side details culture-based methods and outcomes, and the right side details molecular-based methods and outcomes.

2.6 Current Knowledge on ARB and ARGs in Municipal WWTPs, Receiving Water, and Surface Waters

2.6.1 Municipal WWTP Processes and the Effects on Antibiotic Resistance

2.6.1.1 Typical Municipal WWTP Design

Treatment of wastewater in municipal settings often involves three main stages of treatment: preliminary and primary, secondary, and tertiary treatment (Bouki et al., 2013). Preliminary and primary treatment consist of using physical operations to reduce the solids content in the wastewater; such as oils, sands, and settleable solids (Michael et

al., 2013). Preliminary treatment typically involves bar screens in order to remove large objects as well as grit separation that uses a vortex to separate and remove sand, gravel, and other heavy material (Mihelcic and Zimmerman, 2010). Primary treatment typically involves a settling tank that allows further separation of solids that remain after preliminary treatment. Reducing the solids load before further treatment makes secondary treatment more efficient and is an important and ubiquitous step in municipal wastewater treatment (Michael et al., 2013). It is hypothesized that primary treatment can remove 60% of total suspended solids (TSS), 30% of biological oxygen demand (BOD), and 20% of phosphorous; BOD and phosphorous mainly in the particulate phase (Mihelcic and Zimmerman, 2010).

After primary treatment a considerable amount of particulate matter has been removed, however there is still a high abundance of dissolved organic matter (Mihelcic and Zimmerman, 2010). As of 2012, Canadian WWTPs are required to achieve secondary level of treatment for their effluents (average CBOD $\leq$ 25 mg/L and average suspended solids $\leq$ 25 mg/L) (Minister of Justice, 2012), therefore there have been many upgrades to Canadian WWTPs since then. The purpose of secondary treatment is to decompose the remaining organic matter and nutrients in the wastewater. Secondary treatment is typically a biological process relying on aerobic or anaerobic microorganisms, or a combination of both (Michael et al., 2013). Aerobic treatment uses aerobes that use molecular/free oxygen to convert organic impurities and nutrients into carbon dioxide, water, and biomass. Anaerobic treatment uses anaerobes which do not require oxygen and digest organic impurities and nutrients into methane, carbon dioxide, and biomass (Mitall, 2011). Anaerobic digestion is normally followed by aerobic

digestion for the treatment of municipal wastewater. The exact form of secondary treatment can vary widely between WWTPs, however, the most common form is the conventional activated sludge method. This method uses dissolved oxygen to promote the growth of a biological floc, which is responsible for removing the majority of the organic matter and nutrients (Michael et al., 2013). In the floc, bacteria are primary responsible for assimilating the dissolved organic matter in the wastewater, while larger rotifers and protozoa consume the dispersed bacteria (i.e. predation), which otherwise would not be removed during the following sedimentation step (Mihelcic and Zimmerman, 2010). However, even when meeting standards, effluents from WWTPs using conventional biological processes often can contain an excess of nutrients such as nitrogen and phosphorous that can cause harmful eutrophication in receiving waters. Eutrophication can lead to low dissolved oxygen, death of fish, murky water, and depletion of desirable flora and fauna (Headworks, 2017). Another, more advanced, form of biological treatment is called biological nutrient removal (BNR) and is an advanced activated sludge process that incorporates the use of aerated zones, un-aerated zones and internal recycles in order to further reduce nitrogen and phosphorous from wastewater (i.e. tertiary treatment) (Hu et al., 2012). To remove an elevated amount of nutrients BNR often involves nitrification, denitrification, and phosphorous removal. In an aerobic zone, ammonia is converted to nitrate (nitrification) using *Nitrosomonas* and *Nitrosococcus* bacteria, and then is cycled back to the anoxic zone for denitrification of nitrate into nitrogen gas using *Nitrobacter* (Mihelcic and Zimmerman, 2010). Nitrification is a slow process that requires adequate oxygen and alkalinity and is sensitive to temperature, pH, and toxic chemicals, therefore an adequate solids retention

time (SRT) is required (Mihelcic and Zimmerman, 2010). Phosphorus removal (that has not already been removed in particulate form during primary treatment) occurs by using Phosphorus Accumulating Organisms (PAOs) present in anaerobic zones (Mihelcic and Zimmerman, 2010). PAOs take up organic matter using energy created from breaking phosphate bonds and subsequently releasing stored phosphate molecules. Phosphorus Accumulating Organisms and free phosphates travel through the anoxic zone to the aeration basin where stored organic matter (taken up previously in anaerobic zone) is oxidized and phosphate is taken up in increased amounts than previously stored. Phosphorus Accumulating Organisms are wasted as waste activated sludge or returned to the process as needed, therefore removed from final effluents which removes phosphorus from the effluent (Mihelcic and Zimmerman, 2010).

Aerated lagoons are often used in smaller municipalities with wastewater flows of 1 MGD or less and involve an artificial pond provided with aeration to promote the biological oxidation of wastewater (Lagoons Systems in Maine, 2008). Some other common forms of secondary treatment are membrane bioreactors, attached growth reactors, and trickling filters. The wastewater after secondary treatment flows into a settling tank or lagoon to allow settable solids (much of which is largely comprised of microorganisms) to be removed from the treated water (Mihelcic and Zimmerman, 2010). Deciding on which form of secondary treatment to use often depends on the size of the plant and the operating requirements.

Along with BNR, additional tertiary treatments can be applied in WWTPs to remove components that were not removed in previous steps. Currently, not all WWTPs systems contain tertiary treatment. Filters and disinfection can be used for further

nutrient and solids removal as well as pathogen inactivation. Common forms of disinfection in WWTPs are chlorination and ultraviolet (UV) irradiation (Michael et al., 2013). Chlorination destroys target organisms by oxidizing cellular material (USEPA, 1999). UV irradiation uses ultraviolet light to kill or inactivate microorganisms by destroying nucleic acids and disrupting DNA, which inhibits cell function (Rizzo et al., 2013). Both of these disinfection processes can result in bacteria surviving the process as well as recovery and regrowth, especially at low doses. Therefore, it is important to investigate the appropriate ranges for disinfectant dosing.

2.6.1.2 Effects of Different Design Elements and Operating Conditions

Significant changes happen to the bacterial population as it flows through the various treatment process steps. The general observation in literature is that total bacterial numbers are significantly reduced, including resistant bacteria (Guardabassi et al., 2002). However, reports have stated that both raw and treated wastewater (effluent) can contain higher proportions of various resistance populations compared to surface waters (LaPara et al., 2011; Marti et al., 2013). This may be due to favorable conditions for proliferation of ARB and HGT found in WWTPs, as well as plants' inability to totally remove resistance elements. Non-resistant bacteria may acquire resistance genes from ARB while passing through a WWTP. Therefore, although a treatment train may be effective at removing total bacteria, the proportion of remaining bacteria that have some sort of resistance may be of concern.

It is important to consider that although all WWTPs share the common goal of treating wastewater to make it cleaner and less harmful to the environment and human

health, each plant can vary in the design used and the operating conditions. It is important to examine what is coming out of WWTPs, but it is also important to consider if different designs or operating conditions can improve or add to the problem. A study by Kim et al. (2006) revealed that increases in organic loading and growth rates in an activated sludge biological treatment system resulted in an increased abundance of tetracycline resistant bacteria, demonstrating that the fate of ARB can be affected by varying operating conditions. Chen and Zhang (2013) reported ARG removal was negligible by primary clarification steps, which suggests ARGs at this stage cannot be removed by gravity (i.e. most ARGs are not contained in solids form (TSS) at this point). A study by Mezrioui and Baleux (1994) revealed that aerated lagoons removed a higher percentage of total coliforms than an activated sludge system. It was also revealed that the effluent from the aerated lagoons showed higher AR levels than the effluent from the raw sewage, suggesting that aerated lagoons may select for AR strains. However, reductions of ARGs after biological treatment using BNR systems have been observed in other studies, suggesting that biological processes targeting nutrient removal may be capable of reducing the relative abundance of ARGs (Börjesson et al., 2010). Munir et al. (2011) investigated which form of secondary treatment resulted in the lowest abundance of ARB and ARGs and found that membrane bioreactors had consistently higher removal rates than more conventional activated sludge systems. This increased reduction may have been caused by the filtration step of membrane bioreactors. These results suggest that the type of biological treatment used may impact the fate of resistant bacteria and genes, and warrants further investigation. Chen and Zhang (2013) found significant reductions of ARGs after secondary clarification that was likely related to

sludge sedimentation, implying indirectly that biosolids contain a large amount of ARGs.

Other studies have looked into different methods of disinfection and found that neither UV nor chlorination were effective at removing ARGs (Al-Jassim et al., 2015; Auerbach et al., 2007). Some studies have actually found increased ARG relative abundance after ozonation, and low doses of both UV and chlorine disinfection (Zhuang et al., 2014). These results suggest that depending on the location and the required size of the plant, WWTPs may involve varying operating conditions (i.e., seasonal changes, varying contaminants in influent, loading rate fluctuations) as well as varying designs (i.e., type of biological treatment, number and type of advanced treatment steps), that can impact the abundance of ARB and ARGs present in wastewater and treated effluents. Therefore, it is important to consider these parameters when analyzing WWTP effects on AR. Many previous studies have primarily focused on influent and effluent samples, and have not investigated the impacts of individual steps during the treatment processes (Lachmayr et al., 2009; Laht et al., 2014; Narciso-da-rocha et al., 2014; Rodriguez-Mozaz et al., 2015). Therefore a gap in knowledge exists for determining how each individual treatment step at different plants can impact AR and its determinants, suggesting the need for further investigation.

2.6.1 Culture-based Results Assessing ARB in WWTPs and Receiving Environments

It is difficult to compare the results from different studies due to an absence of standardized methods for environmental samples. However, as previously mentioned, some studies have agreed that the biological process found in WWTPs may select for

ARB strains and ARG transfer (Al-Jassim, et al., 2015; Da Silva et al., 2006; Ferreira Da Silva et al., 2007; Łuczkiewicz et al., 2009). Table 2 below compiles results from recent studies including studies listed in the review by Rizzo et al. (2013) on the ARB prevalence in WWTPs. To begin recognizing trends in resistance, it is necessary to look at different groups of bacteria commonly found in wastewater, such as enterococci, *Escherichia coli,* and *Acinetobacter*.

Enterococci are one of the most investigated bacterial groups in WWTP studies and have been long recognized as important human pathogens as well as indicator bacteria for fecal contamination (Fraser et al., 2015). In terms of the role as an indicator bacterium, *Enterococcus* sp. replaced in 2004 fecal coliforms as the new USA federal standard for water quality at public saltwater beaches and also replaced *E. coli* at freshwater beaches. This is because *Enterococcus* sp. are believed to have a higher correlation to human pathogens often found in city sewage than fecal coliforms (Jin et al., 2004). A study by Ferreira da Silva et al. (2006) assessed AR enterococci in a WWTP in Portugal that included preliminary, primary, and secondary (activated sludge) treatment followed by discharge of the effluents into a natural stream. By investigating effluents from both primary and secondary clarifiers they noted that although no resistance to vancomycin was observed, resistance to antibiotics tetracycline, gentamycin, erythromycin, and ciprofloxacin was detected in the final effluents in the ranges of 25-50% (see Table 2 for details). It is interesting to note that along with high numbers of ARB making it into the effluent, ciprofloxacin resistance was positively selected for with relative abundances increasing by 16% in the final effluents. Although this WWTP could reduce bacterial counts by 1.0-1.5 log units, it was not able to eliminate sources of AR,

and even caused some cases of positive selection of ARB. A study by Luczkiewicz et al. (2010) found similar results when examining a WWTP in Poland that operated under similar processes. Nitrofuration, erythromycin, ciprofloxacin and tetracycline resistance ranging from 20-53% was found in enterococci isolates, with cases of positive selection observed throughout the treatment process. Al-Jassim et al. (2015) observed an increase in MDR (>6 types) *Enterococcus* from the influent (3.8%) to chlorinated effluents (6.9%) in a WWTP in Saudi Arabia. This plant used preliminary, primary, secondary (activated sludge) and tertiary treatments (chlorine disinfection). They also observed positive selection for meropenem resistance (17.2% increase from the influent to the effluent).

E. coli are another group of enteric bacteria common to wastewater and are monitored closely as an indicator of the disinfection processes. Although abundances of resistance profiles may differ, *E. coli* studies have shown similar results as enterococci based studies with regards to high levels of ARB in WWTP's effluents and cases of positive selection of ARB throughout the treatment process. A study by Ferreira da Silva et al. (2007) compared final effluents to primary effluents of a WWTP and found positive selection occurring for tetracycline, amoxicillin, cephalothin and ciprofloxacin resistant *E. coli*. High levels of resistance in the final effluents ranging from 9.7-36.8% were detected as well. Cases of positive selection were also found for resistant *E. coli* by Luczkiewicz et al. (2010), in which ranges from 10-34% of bacteria were resistant to antibiotics like ampicillin, piperacillin, tetracycline, levofloxacin and ciprofloxacin in the final effluents. Galvin et al. (2010) examined a WWTP in Ireland containing a hospital influence which uses preliminary, primary and secondary (activated sludge) treatment processes. Significant positive selection of tetracycline resistant *E. coli* was observed

(26.6% increase) in final effluents, as well as high levels of ampicillin and sulfamethoxazole resistance in the final effluents (12.5-39%). Hospital effluents also contained a higher resistance rate than upstream samples, suggesting hospitals as a possible control point for reducing resistance dissemination.

Acinetobacter are widely spread in the environment and commonly occur in soil, however *Acinetobacter baumannii* is commonly isolated from the hospital environment and hospitalized patients, making this a species of interest when studying AR in WWTPs (Cunha et al., 2015). *Acinetobacter* resistant to ABs have been found to survive the treatment process and in some cases be positively selected for, following similar trends to both enterococci and *E. coli* (Zhang et al., 2009; Guardabassi et al, 2002; Al-Jassim et al., 2015). Zhang et al. (2009), examined a WWTP in the USA that included preliminary, primary, secondary (activated sludge), and tertiary (rapid sand filters and UV disinfection) treatment, before the final effluent is discharged into a river. Even with this extensive treatment train, both high numbers (11.3-100%) of *Acinetobacter* resistant to antibiotics such as trimethoprim, rifampin, chloramphenicol, and ciprofloxacin, and positive selection for some ARB was observed (see Table 2 for details). There was also increased resistance in river samples downstream from the WWTP compared to upstream samples, demonstrating how WWTPs can contribute to the dissemination of resistant bacteria into the environment.

The studies mentioned above, along with others, have shown that ARB can be present in the final effluents of WWTPs in high numbers, even with standard and advanced treatment processes, and in some cases can be positively selecting for ARB. Therefore, WWTPs appear to be a source for ARB dissemination into the environment.

Although the exact mechanisms are currently under investigation, the biological process and advanced treatments may be contributing to positive selection of some ARB.

Table 2: ARB detected in WWTPs/receiving environments from culture-dependent surveys.

WWTP	System	Samples examined[a]	ARB detected	Resistance profile[b] and outcome*	Reference
Saudi Arabia (19700 m^3/d)	1º treatment, conventional activated sludge, and chlorine disinfection	RWW (133 isolates), after activated sludge (85 isolates), TWW (with chlorination -72 isolates)	*Acinetobacter, Aeromonas, Arcobacter, Legionella, Mycobacterium, Neisseria, Pseudomonas and Streptococcus, Enterococcus*and Enterobacter* spp* -most detected in multidrug resistance TWW isolates	Total isolates: Ampicillin (72%), erythromycin (51%), chloramphenicol (46%), kanamycin (41%), ceftazidime (32%), tetracycline (21%), meropenem (12%), ciprofloxacin (6.6%) *multidrug resistance (>6 types) percentages increased in chlorinated effluent	Al-Jassim et al. (2015)
Ripoll (45000 Inhabitants); 8000 m^3/d	Primary and secondary treatment (conventional activated sludge)	TWW, biofilm (UR, DR)	*Aeromonas, Escherichia, Shigella, Acinetobacter* and *Arthrobacter*	Ciprofloxacin (252 isolates) -17 *qnr*-positive isolates (15 *qnrS*, 2 *qnrB*) *these bacteria could be an important reservoir of ARGs in the environment as they were found downstream	Marti et al., (2014)
Poland (175000 inhabitants), 2.64x10^4 m^3/d, HE from 3 hospitals (=~2% of WWTP influent)	Preliminary treatment, primary treatment, activated sludge with secondary sedimentation. TWW discharged into Łyna River.	HE from 3 hospitals, RWW, from bioreactor, TWW (effluent), DR	*Escherichia coli*	395 strains: cefotaxime (95.7%), other cephalosporins (21.1% to 94.7%), gentamicin (61%), amikacin (48%), chloramphenicol and to trimethoprime/sulfamethoxazol (below 20%), piperacillin/ tazobactam(10.4%), imipenem(3.3%) *preliminary disinfection of hospital sewage before its inflow into the sewage system might minimize the spreading of antibiotic resistant bacteria to the environment.	Korzeniewska, et al., (2013)

WWTP	System	Samples examined[a]	ARB detected	Resistance profile[b] and outcome*	Reference
China (N.A)	N.A	2 UR, 4 DR	*fecal coliforms*	Tetracycline (average resistance from isolates: 44.7%) (pcr detected genes- *tetA, tetB, tetC, tetG, tetK, tetM, tetO, tetQ, tetX*) *increased number of tetracycline-resistant fecal coliforms related to municipal wastewater treatment plant discharge.	Zhang et al., (2015)
Portugal (100000 inhabitants)	Preliminary, primary treatment, activated sludge, discharged to natural water stream	effluents from the primary and secondary clarifiers	*Enterococcus hirae, Enterococcus faecium, E. faecalis*	Tetracycline (31/33%), gentamicin (50/50%), erythromycin (33/23%), ciprofloxacin (9/25%), amoxicillin (0/3%), sulfamethoxazole/trimethoprim (0/1%) and no resistance to vancomycin * Antibiotic-resistant strains of enterococci were not eliminated by wastewater treatment. A positive selection of ciprofloxacin-resistant *enterococci* occurred	Ferreira da Silva et al., (2006)
Poland (700000 inhabitants), 96,000 m^3/d	Preliminary, primary treatment, activated sludge	RWW, aerobic chamber, RAS, TWW	*Escherichia coli* Enterococci	153 isolates: ampicillin (34%), piperacillin (24%), tetracycline (23%), levofloxacin (10/15%), Trimethoprim/Sulphamethoxazole (11%), ciprofloxacin (10%) 199 isolates: nitrofuration (53%), erythromycin (44%), ciprofloxacin (29%), tetracycline (20%) * positive selection of isolates with antimicrobial resistance patterns was observed during the treatment processes.	Luczkiewicz et al. (2010)
Portugal (14 UWTPs)	N.A	RWW, TWW	Enterococci	983 isolates: multidrug resistance (49.4%), rifampicin (51.5%), tetracycline (34.6%), erythromycin (24.8%), nitrofurantoin (22.5%), ciprofloxacin (14%), ampicillin (3.3), vancomycin (0.6%) * WWTP processes are unable to avoid the dissemination of resistant *enterococci* into the environment.	Martins da Costa et al., (2006)

WWTP	System	Samples examined[a]	ARB detected	Resistance profile[b] and outcome*	Reference
Portugal (100000 inhabitants)	Preliminary, primary treatment, activated sludge, discharged to natural water stream	effluents from the primary and secondary clarifiers	*Escherichia*	Tetracycline (32.1/36.8%), amoxicillin (28/34.8%), sulfamethoxazole/trimethoprim (22.2/22.5%), cephalothin (10.5/20.5%), ciprofloxacin (2.5/9.7%)	Ferreira da Silva et al. (2007)
			Shigella	Tetracycline (0/25%), amoxicillin (20/12.5%), sulfamethoxazole/trimethoprim (0/12.5%), cephalothin (12/0%), ciprofloxacin (0/6.3%)	
			Klebsiella	Tetracycline (5/13%), amoxicillin (94.7/95.7%), sulfamethoxazole/trimethoprim (0/8.7%), cephalothin (0/5.9%), ciprofloxacin (0/4.4%)	
				* Positive selection occurred for many ARB	
Ireland (72000 inhabitants); hospital influence	Primary treatment, activated sludge	HE, upstream and downstream municipal effluent, secondary-treated effluent and effluent from throughout the treatment process	*Escherichia coli*	Ampicillin (24.5/12.5%), streptomycin (16.5/0%), sulfamethoxazole (11.1/12.5%), tetracycline (12.4/39%), ciprofloxacin (7.15/0%), cefoxitin (0.11/2.6%)	Galvin et al., (2010)
				* HE contained a higher proportion of antimicrobial-resistant *E. coli* than that upstream from the hospital	
				* *E. coli* resistant to cefotaxime, ciprofloxacin, and cefoxitin was present in treated effluent samples. Positive selection observed as well	
Denmark (240000 inhabitants) **Denmark (500000 inhabitants)**	Primary treatment, Activated sludge for N and P removal, tertiary treatment	RWW, TWW	*Acinetobacter*	442 isolates: ampicillin (27/22.3%), gentamicin (7.2/5.8%), tetracycline (11.5/12.9%), multidrug resistance (n.d./0.3%)	Guardabassi et al. (2002)
				* tertiary wastewater treatment did not result in a selection of ARB	
USA (210700 inhabitants), 29.5 MGD.	preliminary treatment, primary treatment secondary treatment (activated sludge process utilizing	RWW, secondary effluent and TWW, UR and DR	*Acinetobacter*	366 isolates: trimethoprim (92.2/100%), rifampin (63.1/77.5%), chloramphenicol (25.2/35%), amoxicillin/clavulanic (8.7/20%), sulfisoxazole (8.7/22.5%), ciprofloxacin (4.9/11.3%)	Zhang et al. (2009)
				*increases in resistance due to	

WWTP	System	Samples examined[a]	ARB detected	Resistance profile[b] and outcome*	Reference
	anoxic/oxic biological nutrient removal in aeration basins, tertiary treatment (rapid sand and UV), effluent into river			WWTP. Increased resistance in downstream river samples. *WWTP process contributes to the selective increase of ARB and the occurrence of multi-drug resistant bacteria in aquatic environments.	

[a] HE: hospital effluent
RWW: raw wastewater
TWW: treated wastewater
UR: upstream river
DR: downstream river
N.A.: information not available
[b] Between brackets prevalence of antibiotic resistance (%): data before and after (/) biological process where available
* main outcomes from study

2.6.2 Molecular-based Results Assessing ARGs in WWTPs and Receiving Environments

Culture-based methods give an indication of what types of ARB are present in samples, however, they do not specify which ARGs or what other genetic elements of interest (i.e. *int1*) are present. Molecular-based approaches, which were detailed above, can be used to assess which genes are present throughout a WWTP and the environment. In a review by Rizzo et al. (2013), some examples found in literature of the types of ARGs that have been detected in wastewater were listed. The findings from the compiled studies were that every known mechanism of antibiotic resistance was detected and can survive the wastewater treatment processes. Table 3 detailed below, presents some results of studies that used molecular-based methods to detect ARGs and other genetic elements in WWTPs. A study by Narciso-da-Rocha et al. (2014) examined a WWTP in Portugal consisting of preliminary, primary and biological treatment (including nitrogen and phosphorus removal) and also receives hospital effluent (HE), for *int1*, *marA, bla*$_{TEM}$

and *vanA* genes. By sampling the HE, influent, and treated effluent, it was observed that all genes were present in all samples. There was limited removal of these genes in the effluent which gets discharged into an esturay, enabling dissemination of these genes into the environment. It was observed that the *int1* gene was the most abundant in all samples, highlighting the potential for gene acquisition and transfer in the wastewater. Some other interesting results were that HE samples contained the greatest prevalence of ARGs, highlighting the important role hospitals play in the spread of ARGs into the environment. It was also observed that the relative abundance of *marA* increased in the treated effluent, indicating possible enrichment of genes throughout the treatment process. Another study by Rodriguez-Mozaz et al. (2015) yielded similar findings. By looking at the ARGs bla_{TEM}, *qnrS*, *ermB*, *sul1*, and *tetW* throughout a WWTP in Girona, which also receives hospital effluents, it was observed that although all genes were detected in all samples, HE samples contained the highest concentrations of ARGs. These findings agree to the importance of hospitals roles in the spread of resistance. Although significant reductions in copy numbers of ARGs occurred throughout the treatment process, ARGs varied in relative abundance in the effluent. Relative abundances of *ermB* and *tetW* decreased, while bla_{TEM} and *qnrS* increased, indicating possible enrichment for some ARGs. There were also higher concentrations of ARGs found in river samples downstream from the WWTP than upstream samples, which suggests a widespread dissemination of ARGs into the environment, even after treatment. A study by Lachmayr et al. (2009) looking at bla_{TEM} genes from a WWTP in Massachusetts revealed similar findings to the above studies. They found that although ARG concentrations were being decreased by WWTPs, the relative abundances were

increased. This suggests the increased survival rates of bacteria harboring ARGs through WWTPs. A study by Laht et al. (2014) which examined three WWTPs using activated sludge processes (1 in Finland, 2 in Estonia) suggested that although ARGs (*sul1, sul2, tetM, tetC*, $bla_{\text{shv-34}}$, $bla_{\text{ctx-m-32}}$, and $bla_{\text{oxa-58}}$) were being detected in treated effluents, they were being neither enriched nor reduced in relative abundance in the conventional WWTPs examined. Another study found a reduction in terms of relative abundance of *tetA* and *tetB* genes following biological treatment that focused on nutrient removal (Börjesson et al., 2010), therefore it is possible that enrichment only takes place under distinct circumstances and conditions involving particular bacteria and genes. Although the mechanisms of prevalence and enrichment of ARGs in wastewater treatment are still being studied, all these studies, among others, agree that ARGs are present in WWTP effluents (Gao et al., 2015; Al-Jassim et al., 2015; Jing Du et al., 2014).

Studies focusing on disinfection treatments found that chlorination and UV disinfection were often not helpful in significantly reducing ARGs (Al-Jassim et al., 2015; Chen and Zhang, 2013; Engemann et al., 2006; Gao et al., 2012; McKinney and Pruden, 2012; Munir et al., 2011). Some studies have suggested that chlorination, although able to reduce ARB, is not effective at removing ARGs, indicating the survival of ARGs in treated wastewater, even when ARB are inactivated (Yuan et al., 2015; Zhuang et al., 2014; Al-Jassim et al., 2015). Huang et al. (2011) even demonstrated that chlorination can contribute to positive selection of ARB, as well as demonstrating the dependence of the effects of chlorination on chlorine dosages used. One theory to explain the above results was given by Armstrong et al. (1982), suggesting that chlorination selects for stress tolerant bacteria, and genetic elements that encode stress

tolerance may be co-located with ARGs, which may contribute to the apparent positive selection of ARB during disinfection. However, the exact mechanisms of chlorine-induced antibiotic resistance selection remain unknown. UV irradiation is becoming more commonly used by wastewater facilities, for reasons such as reducing amounts of chemicals in the water and inactivation of chlorine resistant pathogens such as *Cryptosporidium* (USEPA, 1999). Munir et al. (2011) and McKinney et al. (2009) both observed slight, but not significant decreases in ARG numbers after UV treatment. Chen and Zhang (2013) also did not find significant decreases of ARGs after UV treatment. A study by Engemann et al. (2006) suggests that UV light exposure should be increased in order to maximize the gene loss rates in WWTP effluents. A bench-scale treatment study by Zhuang et al. (2014) found that relative abundances of ARGs increased after treatment by ozonation, and low doses of both UV and chlorine disinfection, suggesting disinfection may be able to select for AR. Therefore, it is important to investigate the appropriate ranges for disinfectant dosing as well as investigate disinfection's effects on resistant bacteria as it may be able to reduce total bacteria, but may be selecting for AR. WWTPs are currently not able to eliminate antibiotic resistance elements of concern, such as ARGs and mobile elements with their current processes, therefore the effluent being discharged into the environment is a contamination source of concern. It is also interesting to note that many of these studies were detecting *int1* along with ARGs, indicating that these WWTPs and receiving environments may contain HGT capacity (Chen and Zhang, 2013; Du et al., 2014; Narciso-da-Rocha et al., 2014).

There have also been studies comparing treated wastewater samples to lakes and surface water. Auerbach et al. (2007) found that wastewater contained more different

types of *tet* genes than nearby lake water that was tested and UV disinfection did not reduce the number of detectable genes. LaPara et al. (2011) found that tertiary treated wastewater contained 20-fold higher prevalence of ARGs (*tetA, tetX, tetW* and *int1*) compared to nearby surface water samples. These findings indicate that even advanced treatment cannot lower ARG levels to near background levels. Marti et al. (2013) found a significant increasing difference between the relative abundance of ARGs in biofilm samples upstream and downstream of a WWTP, highlighting the large impact of WWTPs effluent on receiving waters. Although WWTPs can reduce total amounts of bacteria, and even significantly reduce concentrations of ARGs in the effluents, a significant amount of ARGs are still being found in treated effluents along with mobility indicator genes (*int1*), and in some cases enrichment of these genes seems to be taking place.

Although ARGs are detected after advanced treatments, current knowledge about whether these genes reside in live or dead cells is lacking, therefore the fate of these ARGs is often unknown. ARGs can exist as intracellular DNA (iDNA) (i.e. DNA from within structurally intact cells), which can be spread through conjugation or transduction to other bacteria, or exist as extracellular DNA (eDNA) (i.e. DNA from outside the cell such as plasmids, DNA expelled from dead cells, and DNA released from live cells), which can be acquired by naturally competent bacteria through transformation (Mao et al., 2014; Torti et al., 2015). Although Mao et al. (2014) suggested that the majority of ARGs reside in the iDNA form in the water column (e.g. rivers), it is arguable that many ARGs leaving WWTPs may be in eDNA form as bacteria are damaged and ruptured throughout wastewater treatment. It has been shown by numerous studies that natural transformation can occur in environmental media and thus may contribute to HGT in

some species, including human pathogens (Baur et al., 1996; Hendrickx et al., 2003; Nielsen et al., 1997). This potentially makes ARGs leaving WWTPs and entering into the environment dangerous, even in if not contained in living cells. Past studies have shown that eDNA is relatively stable in soil and sediment and can persist for months, most likely due to the absorption to clay minerals, sand, and humic substances which protects the eDNA from inactivation and separation, while being able to persist only a few weeks in the water column (Mao et al., 2014). However, due to the ability of DNA to be transferred from the water column to sediment and biofilms (Marti et al., 2013), and the fact some bacteria are capable of natural transformation (Torti et al., 2015), ARGs present in the eDNA form in water can still be considered a dangerous environmental reservoir of ARGs. Therefore, it is important to consider ARGs both contained inside and outside of bacterial hosts as potential public heath threats.

Table 3: ARGs detected in WWTPs/receiving environments from molecular-based surveys

WWTP	System	Samples examined [a]	ARGs	Results and Outcome*	Reference
Portugal; 200,000 inhabitants; average monthly flow of $1.1x10^6$ m^3/day; receives HE (1000 m^3/day)	Preliminary, primary and biological treatment, including nitrogen and phosphorus removal; treated effluent is discharged to a river mouth.	HE, RWW, TWW	*Int1, marA,* bla_{TEM}*, vanA*	-HE is an important source of *vanA* and bla_{TEM}; limited removal in WWTP. -*marA* abundance increased in TWW. -*Int1* most abundant in all samples. *wastewater bacterial populations hold a high potential for gene acquisition and hospitals are contributing ARGs into environment	Narciso-da-rocha et al., (2014)

WWTP	System	Samples examined [a]	ARGs	Results and Outcome*	Reference
Wisconsin, USA; **Plant A: 250,000 inhabitants; 42 mgd** **Plant B: 62000 inhabitants; 120 mgd**	Plant A: activated sludge, biological phosphorus removal and nitrification, UV disinfection Plant B: activated sludge, chlorine disinfection	RWW, TWW, activated sludge, treated biosolids, 2 lakes	*tetA, tetB, tetC, tetD, tetE, tetG, tetM, tetO, tetS, tetQ*	-All WWTP samples contained more different types of *tet* genes than lake water. -RWW and TWW comparable numbers of *tet* genes -UV did not reduce *Tet* genes -conc. of *tetQ* highest in influent, *tetG* highest in activated sludge *WWTPs are sources of a diverse range of *tet* genes in environment.	Auerbach et al. (2007)
Girona; 800 000 inhabitant (45,000-55,000 m^3/day); receives HE (1000-1500 m^3/day)	N.A.	HE, RWW, TWW, UR, DR	*bla_{TEM}, qnrS, ermB, sul1, tetW*	-ARGs detected at highest concentrations in HE and RWW - Relative concentration of *ermB* and *tetW* genes decreased in TWW, whereas increased in the case of *bla_{TEM}*, *sul1* and qnrS genes. *treated effluents contribute to the spread ARGs in the aquatic environment; positive selection of some genes	Rodriguez-Mozaz et al. 2015)
Ripoll (45000 Inhabitants); 8000 m^3/d	primary and secondary treatment operating with conventional activated sludge	Biofilm, sediment, UR, DR	*qnrS, bla_{TEM}, bla_{CTX-M}, bla_{SHV}, ermB, sul1, sul2, tetO* and *tetW*	- relative abundances of the *qnrB, qnrS, bla_{TEM}, bla_{SHV}, ermB, sul1, sul2, tetO* and *tetW* genes were significantly higher downstream biofilm samples than upstream * WWTP may contribute to the spread of ARGs into the environment and may impact the bacterial communities of the receiving river.	Marti et al., (2013)

WWTP	System	Samples examined [a]	ARGs	Results and Outcome*	Reference
China (30000 m^3/day)	Pre- and primary treatment, A^2/O-MBR	influent (after primary settling tank) and MBR effluent	*tetG, tetW, tetX, sul1*, and *intI1*	*quantities of ARGs decreased over the treatment process, although high concentrations were discharged into the effluent.	Du et al. (2014)
Saudi Arabia (19,700 m^3/d)	1º clarifier, conventional activated sludge, and chlorine disinfection	RWW, after activated sludge, TWW (with chlorination)	*tetO, tetQ, tetW, tetH, tetZ*	All genes present in chlorinated effluent * chlorination not effective at removing ARGs * risks associated with the reuse of treated wastewater arise not from antibiotic-resistant bacteria and genes.	Al-Jassim et al. (2015)
China (320 000 inhabitants); 75,000 m^3/d	aerated grit chamber, two-stage anoxic/ aerobic activated sludge biological processes, and a secondary settling tank. The final treated effluent is discharged into a natural river.	RWW, after aerated grit chamber, after first-stage anoxic/aerobic system, after middle settling tank, after second-stage anoxic/aerobic system, after secondary settling tank (TWW)	*ereA, ereB, ermA, ermB, ermC; mefA/mefE, msrA/msrB*	All genes were detected in all wastewater samples. -The *ereA* genes were the most prevalent. -*msrA/msrB* genes in raw influent and *ermC* genes in final effluent were the least prevalent. *ARG still remained in effluent	Gao et al., (2015)
Finland (0.8 million inhabitants), **Estonia (350000 inhabitants)** **Estonia (100000 inhabitants)**	primary treatment, secondary treatment - biological treatment (activated sludge); tertiary treatment - final deep purification using a combination of methods (chemical, mechanical and biological as in secondary sedimentation, bio-filters etc.).	RWW, TWW	*sul1, sul2, tetM, tetC,* $bla_{shv\text{-}34}$, $bla_{ctx\text{-}m\text{-}32}$, and $bla_{oxa\text{-}58}$.	ARGs were detected in most samples; *sul1, sul2*, and *tetM* were detected in all samples. * neither considerable enrichment nor purification of ARGs in conventional WWTPs.	Laht et al., (2014)
USA (Massachusetts Bay)	N.A	RWW, TWW, nearfield and farfield ocean water	bla_{TEM}	*WWTPs reduce the concentrations of ARGs, however the relative abundance of bla_{TEM} genes increases with treatment,	Lachmayr et al. (2009)

WWTP	System	Samples examined [a]	ARGs	Results and Outcome*	Reference
				suggesting that bacteria harboring bla_{TEM} are positively selected	
USA (St. Louis River, Duluth-Superior Harbor, the outfall from the Western Lake Superior Sanitary District, and Lake Superior)	N.A.	TWW, Surface water and sediment samples	*tetA, tetX, tetW* and *int1*	All genes 20-fold higher in tertiary-treated wastewater than in nearby surface water samples	LaPara et al. (2011)
USA (Michigan) 5 WWTPs	Primary treatment, activated sludge/oxidation ditch/rotating biological contractors/ or membrane biological reactor (MBR), Chorine or UV disinfection	RWW, pre-disinfection, TWW	*tetW, tetO, sul1*	*MBR decreased *tetO* and *tetW* 1-3 log more than other treatments * chlorination and UV disinfection did not contribute in significant reduction of ARGs	Munir et al., (2011)
China (3 WWTPs)	Primary treatment, activated sludge, Advanced treatments: Plant A- constructed wetlands Plant B- biological aerated filter Plant C- UV disinfection	RWW, pre-advanced treatment, TWW, activated sludge, excess sludge	*tetM, tetO, tetQ, tetW, sul1, sul2, int1*	ARGs removed 1-3 orders of magnitude in all plants before advanced treatments For advanced treatments: Wetlands>biological aerated filter>UV for ARG removal	Chen and Zhang (2013)
Sweden (830 000 person equivalents)	Primary treatment, biological treatment with nutrient removal (activated sludge, trickling filters and the anaerobic digesters)	RWW, after primary clarifier, after secondary clarifier, before trickling filter, after trickling filter, reject water from centrifuges	*tetA, tetB*	ARGs detected in all samples however abundances reduced throughout treatment *Biological process reduced relative abundance of ARGs	Borjesson et al. (2010)

[a] HE: hospital effluent
RWW: raw wastewater
TWW: treated wastewater
UR: upstream river
DR: downstream river
N.A.: information not available

* main outcomes from study

2.6.2 Results of Studies Examining Anthropogenic Impacted and Pristine Environments

As previously mentioned, WWTPs have been shown to increase AR in downstream environments. However, it is interesting to note that some studies have also reported detecting AR in upstream samples, and some even in more remote or "pristine" environmental samples (Knapp et al., 2012; Ouyang et al., 2015; Proia et al., 2016; Pruden et al., 2006). Due to reasons such as spread from more anthropogenically impacted areas, ARGs have been able to disseminate and accumulate in most areas of the world, therefore it is unlikely that an area can truly be considered "pristine" anymore (Allen et al., 2010). Results from past studies finding ARGs in upstream and more pristine samples supports that statement.

Although detecting an increased abundance of ARGs in biofilm samples downstream of a WWTP, Proia et al. (2016) also detected ARGs in upstream biofilm samples, suggesting these genes' ubiquitous presence in rivers. Berlung et al. (2015) also detected ARGs in upstream sediment samples in which the upstream river is only lightly affected by humans, albeit in lower abundances than downstream samples. Other studies have detected an increased abundance of ARGs as samples were taken closer to more urbanized areas versus samples taken in more pristine environments, suggesting a link between anthropogenic activities and ARGs (Knapp et al., 2012; Ouyang et al., 2015; Storteboom et al., 2010). Sources such as farming, agricultural/urban run-off, and transport mechanisms such as soil leaching have been suggested as some contributing

factors to detecting ARG in samples not under direct influence of wastewater effluents (Proia et al., 2016). However, ARGs have still been detected in samples that are supposedly not impacted by these sources (Ouyang et al., 2015). Previously mentioned explanations such as natural AR, spread by biological and physical forces, as well as alternative cellular functions of ARGs have been suggested to explain these background levels in more pristine samples.

Determining levels of AR in the environment is important in order to assess just how ubiquitous these genes are, as well as a useful comparison to AR levels found in wastewater effluents, agriculture, etc. In summary, ARG levels seem to be increased by anthropogenic activities, however are still detected in more pristine areas suggesting widespread dissemination and presence of these genes.

2.7 Prevention and Management of Environmental Antibiotic Resistance

The evolution of bacterial AR and its emergence and spread represent one of the most threatening health care problems worldwide (Hawkey, 2008). The studies discussed above reveal that ARB and ARGs, as well as mobile elements such as resistance plasmids, are surviving wastewater treatment to one degree or another, and are contaminating receiving environments. Investigating the interaction of bacteria from human-associated microbiota with environmental microorganisms in sewage treatment systems or in natural ecosystems is an important feature to understand the emergence of novel mechanisms of resistance in human pathogens (Baquero et al., 2008). In contrast to the clinical environment, there is little data available on the epidemiology of AR in the

environment (Levy and Marshall, 2004). This makes it extremely difficult to make any predictions on the risk of spread and emergence of new ARs (Lupo et al., 2012). Therefore, enhanced knowledge and surveillance systems on the environmental reservoir of AR have become important to help predict the emergence of new resistances of clinical concern. Considering ARGs as principal contaminants of interest instead of their bacterial hosts may prove to be a promising approach towards tracking and understanding the dissemination of AR (McKinney et al., 2010).

To prevent and manage AR recommendations by Levy and Marshall (2004) have been to isolate hospitalized individuals with dangerous resistant bacteria such as MRSA, in order to limit their spread, however human waste containing ARB from these patients may ultimately end up in WWTPs. They also recommend limiting the use of ABs in both humans and animals and introducing new therapeutic approaches, such as new ABs and preventative vaccines. However, based on past studies it seems limiting the output of ARB and ARGs from WWTPs can also aid in management and prevention of the spread of resistance. Further studies to investigate the exact mechanisms and changes in ARB and ARGs throughout wastewater treatment systems can help find ways to reduce the prevalence of resistant elements leaving WWTPs. Assessing levels of AR in many different environments may also aid in developing guidelines for what should be considered dangerous levels.

2.8 Conclusions and Recommendations

Antibiotic resistance is becoming an increasing problem worldwide and detecting hotspots for its development should be of upmost importance. Based on the information

provided above, it is evident that WWTPs are harbouring ARB and ARGs. All known types of AR mechanisms have been detected in WWTPs, suggesting the relevance of WWTPs as reservoirs and suppliers of resistant genes. In some studies, it was shown that WWTPs can positively select for ARB and ARGs and promote their transfer and spread. Although the exact mechanisms are still unknown, it has been suggested that the biological process of treatment as well as advanced treatments and disinfection may be contributing to this occurrence. Secondary treatment may provide optimal conditions for HGT as well as selective pressures for resistant bacteria. Advanced treatments may be selecting for stress tolerant bacteria, in which stress tolerant genes may be co-located with ARGs, promoting their selection. It is important to consider that in cases where the relative abundances seem to be increasing, the absolute concentrations of these resistance elements are usually being reduced by several log-units throughout the treatment system. Therefore, questioning whether these lowered concentrations actually have a large impact on the environment should be considered.

It is important to improve detection techniques for ARB and ARGs and to standardize them globally in order to more accurately survey the occurrence and spread of resistance into the environment from WWTPs from around the world. Advancements in next generation sequencing may prove to be a valuable tool for this. It is also important to investigate if certain treatment steps and types are helping control or adding to the problem as there seems to be a gap in knowledge in this area currently. There are many different types of secondary treatment options available and many options for advanced treatment steps and disinfection types. It is important to investigate if adding more advanced treatments steps or switching current processes could help reduce the

spread of AR into the environment. Further studies on the forces selecting for AR in WWTPs are recommended, as well as establishing links between such selective factors as ABs or heavy metals to resistance development. Investigating and managing the potential for the reactivation of ARB and survival of ARGs after treatment may prove helpful in reducing the impact of wastewater effluents on the environment. Overall, further investigations on the presence and prevalence of ARB and ARGs in WWTPs, as well as levels that exist in the environment, is necessary in order to find ways to reduce AR's rising impact on human health.

Chapter 3: Presence and Abundance of Antibiotic Resistance Genes Throughout Treatment Stages of Two Conventional Municipal Wastewater Treatment Plants in Atlantic Canada

Abstract

Wastewater treatment plants (WWTPs) constitute a reservoir for high levels of antibiotic resistance genes (ARGs), creating public health concerns due to their presence and possible transfer between bacteria. Therefore, WWTPs may represent a critical control point in reducing the release of antibiotic resistance into the environment. The objective of this study was to investigate how the abundance of ARGs is affected by individual treatment steps in two different municipal WWTPs, located in Atlantic Canada, which use two different biological treatment technologies namely aerated lagoons (ALs) versus biological nutrient removal (BNR) reactors. Quantitative real-time PCR (qPCR) was used to quantify the absolute (copies/mL) and relative abundances (copies/copies 16S rRNA) for 9 ARGs as well as one indicator integron gene (*int1*) to assess ARG cassette presence and ARG mobility potential. Water quality assessment revealed both plants were meeting provincial effluent regulations, however eight out of ten genes (*int1*, *sul1, sul2, tetO, ermB,* $bla_{CTX\text{-}M}$, bla_{TEM}, *qnrS)* were detected in all samples at both plants *(vanA* was not detected in any samples, and *mecA* was only detected above the limit of detection in winter samples at AL plant). Although absolute ARG abundances were significantly ($p<0.05$) decreased from influent to effluent water samples at both plants, the relative abundance remained unchanged at the AL plant and decreased slightly at the BNR plant, suggesting that the BNR system may be better equipped to decrease relative ARG

abundance. Water quality variables $CBOD_5$ and nitrate showed significant ($p<0.05$) positive and negative correlations respectively with ARG concentrations at AL plant, and a genetic human fecal marker *(HF183*) was significantly ($p<0.05$) positively correlated with ARG concentrations at BNR plant, suggesting these variables may be useful in predicting ARG removal. In conclusion, although ARGs were not enriched throughout treatment, they were still found in high abundances in the effluent of the studied plants, indicating the release of these ARGs in the environment and a possible concern to public health.

3.1 Introduction

The overuse and misuse of antibiotics (ABs) in medical and agricultural settings have resulted in their continuous release into the environment, which has led to the development of antibiotic resistant bacteria (ARB) due to the selective pressure of the AB residues (Davies and Davies, 2010; Rizzo et al., 2013). The efficacy of ABs may be reduced if antibiotic resistant genes (ARGs) are picked up by dangerous pathogens (Allen et al., 2010; Sanderson et al., 2016). Even in the absence of selective pressures, ARGs can persist in the environment (Martinez, 2009; Tamminen et al., 2011), and have been recognized as emerging environmental pollutants (Pruden et al., 2006; Sanderson et al., 2016). Interestingly, while antibiotic resistance (AR) can be found in naturally occurring bacteria (Storteboom et al., 2010), environmental areas that are more exposed to human related activities have shown higher concentrations of ARB and ARGs (Allen et al., 2010; Galán et al., 2013; Pruden et al., 2006).

The effluent from wastewater treatment plants (WWTPs) is thought to be a main anthropogenic source for the release of AB residues, ARB, and ARGs into the environment (Auerbach et al., 2007; Ferreira Da Silva et al., 2007; Gao et al., 2015; Lupo et al., 2012). Although WWTPs are designed to remove nutrients and pathogenic bacteria, they are not designed to remove AB residues and resistance elements (Narciso-da-rocha et al., 2014). Many ARGs have been located on integrons, transposons, or plasmids which allow mobilization and transfer to other bacteria (Allen et al., 2010). Biological wastewater treatment may create an ideal environment for selection or horizontal gene transfer (HGT) of ARGs. Contributing factors include the close proximity of bacteria originating from multiple sources, as well as a selective, nutrient-rich environment containing both ABs and heavy metals (Auerbach et al., 2007; Baquero et al., 2008; Davies and Davies, 2010; Ferreira Da Silva et al., 2007; Levy and Marshall, 2004; Rizzo et al., 2013). Therefore, due to possible ARG enrichment during the treatment process (Łuczkiewicz et al., 2010; Narciso-da-rocha et al., 2014; Zhang et al., 2009), and their subsequent discharge into the environment, understanding these reservoirs are crucial to assessing the risk of transfer between human dominated and natural environments (Laht et al., 2014).

Antibiotics, ARBs and ARGs can be affected differently within WWTPs due to variations in the mechanical, biological and physical properties of the plant (Batt et al., 2007; Rizzo et al., 2013). Previous studies have primarily focused on influent and effluent samples, without analyzing the impact of individual steps in treatment processes (Lachmayr et al., 2009; Laht et al., 2014; Narciso-da-rocha et al., 2014; Rodriguez-Mozaz et al., 2015). In order to better design plants to increase removal of resistance

determinants and decrease their subsequent release into the environment, it is important to assess the effect of each step in the treatment process. In Canada, WWTP effluent quality is monitored through measurement of parameters such as total suspended solids (TSS), carbonaceous biological oxygen demand ($CBOD_5$), or nutrient levels. Currently, neither antibiotics nor resistance elements are monitored. Past studies have found significant correlations between water quality factors, such as the removal of nutrients and organic matter throughout treatment, and ARG concentrations (Nõlvak et al., 2013; Novo and Manaia, 2010). Therefore, examining these correlations may aid in finding ways to assess potential proxies for monitoring ARG levels in treated wastewater.

The objective of this study was to examine how the individual treatment steps in two WWTPs using different biological treatment trains affected the abundance of nine ARGs and one genetic indicator of ARG cassettes and mobility potential in the wastewater as it underwent treatment (Norman et al., 2009). Water quality (*E. coli*, ammonia, phosphorous, $CBOD_5$, *HF183* human *Bacteroides* marker, etc.) and presence of selective an AR factor (antibiotics) were also assessed in order to examine their potential relationships to ARG abundances.

3.2 Materials and Methods

3.2.1 Treatment plants and sample collection sites

Samples were collected from two different types of biological treatment plants in Atlantic Canada; an aerated lagoon (AL) system in Greenwood, NS, and a biological nutrient removal (BNR) system (Plant B). The WWTPs will be referred to as Greenwood and Plant B for the remainder of this chapter. Greenwood was sampled on July 7, 2015,

July 28, 2015, August 18, 2015, and February 23, 2016 in order to conduct a comparison over time and seasons to assess the water quality, ARG presence, abundance, and consistency of treatment. Plant B was sampled on July 12, 2016 to compare general similarities and differences between treatment plants using different biological treatment systems and designs.

3.2.1.1. Greenwood

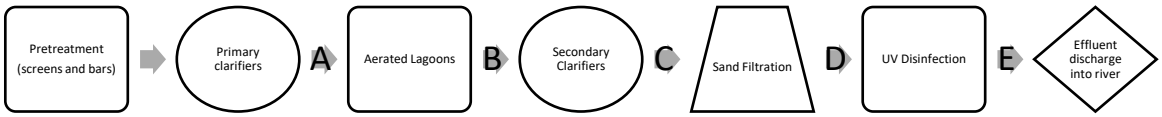
Greenwood is a medium sized plant that discharges approximately 1 MGD (3.8×10^6 L/day) into Fales River, a tributary to the Annapolis River, which is used for recreational activities in the community. There are around 600-800 customer connections, including Greenwood's airbase which contains a small hospital unit. Treatment steps include primary clarifiers, two aerated lagoons, two secondary clarifiers, sand filtration, and UV disinfection. The hydraulic retention time (HRT) in the lagoons ranges between 18 and 24 hours (personal communications Bill MacLellan at King's County, Berwick, NS, Canada). The plant must adhere to a regulatory effluent discharge requirement of a maximum of 5 mg/L for $CBOD_5$ and 5 mg/L for TSS. Water samples were collected in pre-sterilized 1 L Nalgene collection bottles (Thermo Fisher Scientific, Waltham, MA, United States) after the primary clarifiers (A) [referred to as the influent as it was the first accessible sampling site], after the aerated lagoons where water from the two lagoons was mixed (B), after the secondary clarifiers where the effluent from two clarifiers was mixed (C), after sand filtration (D) and after UV disinfection (E) as shown in Figure 4A. Water samples were kept on ice while being transported back to the

laboratory at Dalhousie University in Halifax, NS (a distance of approximately 150 km) and stored at 4°C overnight and processed the following morning.

3.2.1.2 Plant B

Plant B's treatment plant services approximately 13,000 people in an urban area, including a local hospital. The effluent discharge of approximately 3 MGD (11.4×10^6 L/day) is released into a harbour, which is also used for shellfish harvesting. The plant is a tertiary BNR system that is designed to remove ammonia, phosphorous, and nitrogen. Treatment steps include primary clarifiers, BNR process reactors, two secondary clarifiers, and UV disinfection. The HRT in the BNR process reactors is approximately 21 hours. The regulatory discharge requirements are that the effluent should contain a maximum of 25 mg/L for $CBOD_5$, 25 mg/L for TSS, and 200 MPN/100 mL of faecal coliforms (Plant B's Wastewater treatment plant report, 2011; personal communications with plant operators). Samples were collected in pre-sterilized 1 L Nalgene collection bottles (Thermo Fisher Scientific) and collected from the raw influent (A), after the primary clarifiers (B), after BNR treatment (C), after the secondary clarifiers where out-going water from the two clarifiers was mixed (D), and after UV disinfection (E) as shown in Figure 4B. Samples were kept on ice and transported back to the laboratory at Dalhousie University in Halifax, NS (a distance of approximately 300 km) for immediate processing.

A) Greenwood WWTP



B) Plant B

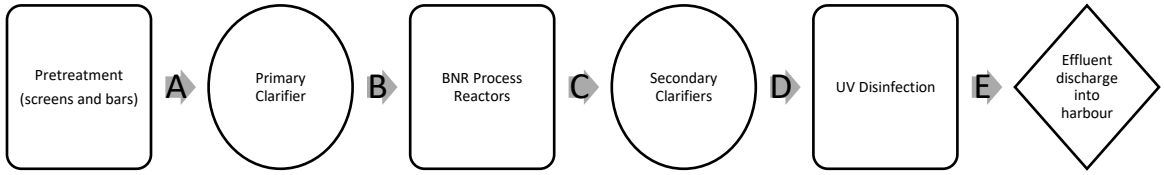


Figure 4: A) Greenwood's WWTP treatment train B) Plant B's WWTP treatment train with sampling sites indicated by letters (A-E).

3.2.2 Analysis of the Abundance of Antibiotic Resistance Gene, Human Fecal Markers and 16S rRNA Gene Copies

3.2.2.1 Genomic extraction

Wastewater samples in quantities ranging from 50 to 500 mL were either filtered through 0.45 µM membranes and a vacuum manifold (Millipore, Inc., Bedford, MA) or centrifuged at 3200 x g for 10 minutes depending on the sampling date (Greenwood samples from July 7, 2015 and July 28, 2015 were subjected to centrifugation; Greenwood samples from August 18, 2015 and February 28, 2016, and Plant B samples were filtered). Filters or pellets were stored at -20°C until DNA extraction. Genomic DNA was extracted from the entire filter or cell pellet using the MoBio Powersoil DNA extraction kit (VWR International, Ville Mont-Royal, QC, Canada) according to manufacturer's specifications. The concentration and purity of the DNA were evaluated by ultraviolet absorbance spectrophotometry at 260/280 nm and 260/230 nm (Biotek EL

808 Absorbance Microplate reader, Fisher Scientific).

3.2.2.2. Quantitative real-time PCR

Assessment of gene targets was performed using quantitative real-time PCR (qPCR; Bio-rad, Hercules, CA, United States). Primer and probe sequences and cycling conditions for nine ARGs and one integrase gene used as a proxy to assess ARG cassettes and mobility potential (Toleman et al., 2006), were obtained from literature and are listed in Table 5. The genetic targets assessed in this study include class 1 integrase (*int1*), class A β-lactamase ($bla_{CTX\text{-}M}$ and bla_{TEM}), erythromycin resistant gene (*ermB*), fluoroquinolone resistant gene (*qnrS*), sulphonamide resistant genes (*sul1* and *sul2*), tetracycline resistance gene (*tetO*), methicillin resistant gene (*mecA*), and vancomycin resistant gene (*vanA*). These ARGs were chosen to represent a variety of different antibiotic classes and resistance mechanisms as well as clinically relevant genes (Szczepanowski et al., 2009; Volkmann et al., 2004). Human-specific *HF183* *Bacteroides* 16S rRNA genetic marker was also quantified to examine a potential correlation between human faecal pollution and ARG abundance (Sauer et al., 2011). All targets were quantified in all water samples, except for one sampling run (Greenwood, July 28/2015) where *tetO*, *mecA*, *vanA* and *HF183* were not tested due to limitations in sample DNA quantities.

Control plasmids for *int1*, bla_{TEM}, *sul1*, and *sul2* were obtained from Dr. E. Topp (University of Western Ontario, London, ON, Canada) and described in Rahube et al. (2014). Control plasmids for $bla_{CTX\text{-}M}$, *ermB*, *qnrS*, *tetO*, *mecA*, and *vanA* were obtained from Dr. K. Neudorf and are described in Neudorf et al. (2017). Concentrations of

plasmid DNA were quantified using Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific). Standard curves were constructed for each assay using tenfold serial dilutions of positive controls in triplicate. Quality assurance for standard curves were performed using recommendations from Biorad Real-Time PCR Applications Guide (BioRad, 2013). Efficiencies ranged from 87.3% to 114.5% and R^2 values were >0.99 for all calibration curves. Limit of quantification (LOQ) (copies/reaction) were as follows: *int1*=14.4, *sul1*=11.7, *sul2*=9.6, *tetO*=69.0, *ermB*=13.8, $bla_{CTX\text{-}M}$=6.2, bla_{TEM}=243.0, *qnrS*=112.0, *mecA*=69.0, *vanA*=138.0, 16S rRNA=67000, *HF183*=3630. Limit of detection (LOD) was 5 copies/reaction.

TaqMan qPCR on a Bio-Rad CFX96 Touch system (Bio-Rad, Hercules, CA, USA) was used in quantification of *int1* and ARGs. The following reaction mixture was used: 1 x SsoAdvanced™ Universal Probes Supermix (Bio-Rad), 0.9 µM of each primer, 0.25 µM TaqMan probe, 2 µl template DNA, and 2 µl of sterile nuclease-free water (Thermo Fisher) to a final volume of 20 µl. Samples and negative controls without template DNA reactions were run in duplicate, while standards (control plasmid) reactions were run in triplicate. Taqman probes were also used in quantification of the human faecal *HF183* marker. The following reaction mixture was used: 1 x SsoAdvanced™ Universal Probes Supermix (Bio-Rad), 0.6 µM of each primer, 0.25 µM TaqMan probe, 2 µl template DNA, and 6.5 µl of sterile nuclease-free water (Thermo Fisher Scientific) to a final volume of 25 µl. SYBR Green qPCR was used to quantify universal 16S rRNA gene fragments. The following reaction mixture was used: 1x Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, United States, or Bio-rad), 0.4 µM primers, and 1 µl of template DNA, and 7.4 µl of sterile

nuclease-free water (Thermo Fisher Scientific) to a final volume of 20 µl. All reactions were run in triplicate including negative controls without template DNA.

Raw fluorescence data were exported from the Bio-Rad qPCR system and imported into the LinRegPCR program (v 11.4) (Ruijter et al., 2009). All samples within each qPCR run, which were analyzed with a specific primer set (e.g. *int1*), were treated as one amplicon group in order to set a common window of linearity. For each run, the fluorescence threshold was set in reference to obtaining a cycle threshold (CT) value for primer specific internal positive control that matched the concentration specific CT value from the standard curve, which has been previously established. Following that the LinRegPCR program automatically calculated the individual sample's CT and E_{fi} (efficiency estimated from fluorescence increase) values. The mean E_{fi} of each amplicon group was calculated as the arithmetic mean of all sample replicates, excluding any replicates that were 5% above or below the median efficiency of all replicates (such deviating replicates were rerun). The same process was performed for determining E_{fi} values for the triplicate standards run on the same plate (sample efficiency range 63.7% to 112.3%; standard efficiency range 71.2% to 123.1%). In order to account for differences in efficiencies between samples and standards, a one-point calibration (OPC) method for absolute quantification was used (Brankatschk et al., 2012). Any values that fell between the LOD and LOQ were set to ½ LOQ value. Any values that fell below the LOD were set to ½ LOD. For calculation of the relative abundance of genes, the gene copy (GC) number of each gene was normalized to the GC number of the 16S rRNA gene in each sample and log transformed (log(GC/16S rRNA GC)). For calculation of the absolute abundance of genes, gene copies were normalized to the water volume used

for gDNA extractions to generate GC per mL of water. Only sampling events from August and February were used in the calculation of absolute abundance.

3.2.3 Assessment of Water Quality

Water samples were held at 4°C and processed for further analysis within the recommended holding times for each parameter (24-48 hours). Each sample was tested for the following water quality parameters: $CBOD_5$, TSS, volatile suspended solids (VSS), total nitrogen (TN), ammonia, nitrate, total phosphorous (TP), chemical oxygen demand (COD), total coliforms, and *Escherichia coli*.

Analysis of $CBOD_5$ was performed in duplicate according to the APHA standard method 5210B (American Public Health Association, 1999). TSS and VSS were performed using Whatman™ 934-AH 47 mm glass fiber filters (Thermo Fisher Scientific) according to APHA standard methods 2540 D (American Public Health Association, 1999). TN was analyzed using Hach® TN Test 'N Tubes™ (0.5 to 25.0 mg/L N, Hach Company, Loveland, CO, United States), according to the manufacturer's procedure. Ammonia and nitrate were measured using high performance ammonia and nitrate electrodes, respectively, as directed by the manufacturer (Thermo Fisher Scientific). The electrodes were attached to an Orion Star™ series meter (Thermo Fisher Scientific). TP was analyzed using Hach® TP Test 'N Tube™ ranging from 1.0 to 100.0 mg/L $PO4^{3-}$ (Hach Company), following the manufacturer's procedure. COD was analyzed using Hach® COD TNT plus Vial™ Test ranging from 20 - 1500 mg/L COD (Hach Company), following the manufacturer's procedure. Total coliforms and *E. coli* were measured using IDEXX Colilert®-18 and Quanti-Trays® (IDEXX Laboratories,

Inc., Westbrook, ME, United States) according to the manufacturer's instructions (IDEXX Laboratories, Inc., 2012).

3.2.4 Antibiotics

The samples were also tested for a range of 10 antibiotics consisting of amoxicillin, cefaclor, cefprozil, cefdinir, levofloxacin, ciprofloxacin, azithromycin, clindamycin, clarithomycin, and triclocarbon by Dr. Anthony Tong at Acadia University.

3.2.4.1 Sample Preparation

A vacuum filter apparatus equipped with 3 mL Chromabond® HR-X (200 mg sorbent) solid phase extraction (SPE) columns. These SPE columns were conditioned with 6 mL methanol and 6 ml of deionized (DI) water. The pH of the samples (100 mL) were adjusted to pH 2.5±0.5 with 1 M HCl. The sample were then pumped through the column at 5 mL/min under vacuum. Cartridges were washed with 3 mL of 10% methanol in DI and dried for 5 min under vacuum. Analytes were eluted using 3 mL of methanol two times (a total of 6 mL). Eluent was collected and reduced to 1 mL with gentle nitrogen blowdown at approximately 37 °C.

3.2.4.2 Analysis of Antibiotics

Antibiotic analysis was performed using an Agilent 1200 HPLC coupled with an Agilent 6410 triple quadrupole mass spectrometer. Chromatographic separation was performed using a 25-cm Agilent Poroshell Eclipse C18 column with a 4.6-mm internal diameter and 2.7-µm particles. The mobile phase consisted of 60% acetonitrile and 40% water. The flow rate of mobile phase was 0.5 mL/min. A solvent gradient was programed to start at 20 % methanol for 30 seconds, which increased to 100 % by 20 minutes with a

maximum flow gradient of 100 mL/min^2. The column was held at a constant temperature of 40 °C. Following separation, ionization was conducted with an electrospray ionization (ESI) source under 35-psi nebulizer pressure. Drying gas temperature was set to 350 °C with a flow rate of 12 L/min. The MS was operated in the positive mode and the capillary voltage was held at 4000 V. Nebulizing gas and collision gas were 98 % nitrogen and ultra-high purity (UHP, 99.999 %) nitrogen. Precursor-to-product ion transitions were established for all target antibiotics. To boost the sensitivity of analysis, only one transition was monitored for each compound. Fragmentor voltage and collision energy were carefully optimized to achieve maximum response for each transition. The MS parameters and transitions are shown in Table 4.

Table 4 Target antibiotics

Name	Acronym	Transition Precursor Ion (m/z) to Product Ion (Da)
Amoxicillin	AMC	398→349
Azithromycin	AZM	750→591
Cefaclor	CFL	368→106
Cefdinir	CFD	396→126
Cefprozil	CFP	390→114
Clarithromycin	CTM	749→158
Clindamycin	CDM	425→126
Ciprofloxacin	CPX	332→314
Levofloxacin	LFX	362→318
Triclocarban	TCC	315→128

3.2.4.3 Quality assurance and quality control

To assist with quality assurance and quality control, solvent blank samples were included. Sampling bottles were pre-cleaned by manufacturers. These bottles and other glassware were thoroughly cleaned using DI water and methanol. Salinization of glassware was not performed because spiked samples did not show loss of target compounds.

Laboratory control samples were prepared by spiking a 100 mL DI sample containing 5 g of sodium chloride with an antibiotic mixture and subjecting the sample to the same analytical method. The recoveries of the target antibiotics were analyzed. These recoveries in control samples were reported. An internal standard was used to ensure the quality of instrumental analysis and sample preparation. Duplicates were included to verify the reproducibility of the analytical method.

3.2.5 Data Analysis

One-way analysis of variance (ANOVA), Tukey HSD test, and t-tests (Interactive Statistical Pages, 2016; GraphPad Software, Inc, 2016) were used to assess statistically significant differences ($p<0.05$) among samples. Correlation coefficients were used to assess significant ($p<0.05$) correlations between ARGs and water quality parameters (Excel, 2016). When performing analyses based on the average of all tested ARGs, abundances for each individual ARG were pooled and then averaged at each WWTP for each treatment step. Principal Component Analyses (PCA) were performed by calculating the average ARG absolute abundance for each individual ARG and dividing that value by the sum of average absolute abundances for all ARGs to obtain a ratio for

each ARG target for a particular treatment step (i.e. for the influent). These ratio values for each ARG for a particular WWTP step were the fingerprints used for PCA analysis.

Table 5 Real-time PCR primer sequences and reaction conditions.

Target gene	Primer	Sequence (5'-3')	Conditions	Reference
16S rRNA	1369F 1492R	CGGTGAATACGTTCYCGG GGWTACCTTGTTACGACTT	95°C for 10 mins; 40 cycles of 95°C for 15s, 55°C for 30s, and 72°C for 30s	Suzuki et al., 2000
erm(B)	ermBF ermBR ermBP	GGATTCTACAAGCGTACCTTGGA GCTGGCAGCTTAAGCAATTGCT FAM-CACTAGGGTTGCTCTTGCACACTCAAGTCBHQ-1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Böckelmann et al., 2009
mecA	mecAF mecAR mecAP	CATTGATCGCAACGTTCAATTTAAT TGGTCTTTCTGCATTCCTGGA FAM-CTATGATCCCAATCTAACTTCCACATACCBHQ-1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Böckelmann et al., 2009; Francois et al., 2003
tetO	tetOF tetOR tetOP	AAGAAAACAGGAGATTCCAAAACG CGAGTCCCCAGATTGTTTTTAGC FAM-ACGTTATTTCCCGTTTATCACGGAAGCG-BHQ-1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Böckelmann et al., 2009
bla*CTX-M*	*BLACTX-M* UP *BLACTX-M* LP CTX-probe	ACCAACGATATCGCGGTGAT ACATCGCGACGGCTTTCT FAM-TCGTGCGCCGCTG-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Colomer-Lluch et al., 2011
***bla*TEM**	TEM UP TEM LP TEM Probe	CACTATTCTCAGAATGACTTGGT TGCATAATTCTCTTACTGTCATG FAM-CCAGTCACAGAAAAGCATCTTACGG-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Lachmayr et al., 2009
sul1	qSUL653f qSUL719r tpSUL1	CCGTTGGCCTTCCTGTAAAG TTGCCGATCGCGTGAAGT FAM -CAGCGAGCCTTGCGGCGG-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Czekalski et al., 2012; Heuer et al., 2008
sul2	qSUL2_595f qSUL2_654r tpSUL2_614	CGGCTGCGCTTCGATT CGCGCGCAGAAAGGATT FAM -CGGTGCTTCTGTCTGTTTCGCGC-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Czekalski et al., 2012; Heuer et al., 2008
qnrS	*qnrS* UP *qnrS* LP *qnrS* probe	CGACGTGCTAACTTGCGTGA GGCATTGTTGGAAACTTGCA FAM –AGTTCATTGAACAGGGTGA-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Colomer-Lluch et al., 2014
vanA	vanAF vanAR vanAP	CTGTGAGGTCGGTTGTGCG TTTGGTCCACCTCGCCA FAM-CAACTAACGCGGCACTGTTTCCCAAT-BHQ-1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Volkmann et al., 2004

Target gene	Primer	Sequence (5'-3')	Conditions	Reference
int1	intI1-LC1 intI1-LC5 intI1-probe	GCCTTGATGTTACCCGAGAG GATCGGTCGAATGCGTGT FAM-ATTCCTGGCCGTGGTTCTGGGTTTT-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Barraud et al., 2010
HF183	HF183-F HF183-R HF183-p	ATCATGAGTTCACATGTCCG CTTCCTCTCAGAACCCCTATCC CTAATGGAACGCATCCC	95°C for 3 min; 40 cycles of 95°C for 30s, 58°C for 30s	Haughland *et al*., 2010; Layton *et al*., 2013

3.3 Results and Discussion

3.3.1 Changes in gene abundances and *E. coli* throughout treatment

3.3.1.1 ARG presence

In Greenwood, all gene targets with the exception of *mecA* (only detected above LOD in February 2016) and *vanA* (never detected above LOD) were observed in all samples on every sampling event (Figure 5). Although other studies have identified highly clinically relevant genes (e.g. *mecA* and *vanA*) which confer resistance to ABs of last resort (e.g. methicillin and vancomycin) in WWTP samples (Narciso-da-rocha et al., 2014; Szczepanowski et al., 2009; Volkmann et al., 2004), their presence was expected to be low or undetected due to only a small clinic being present on the WWTP connection as opposed to a full-scale hospital (Narciso-da-rocha et al., 2014). Hospitals are a major contributor of highly clinically relevant genes in WWTPs (Narciso-da-rocha et al., 2014; Volkmann et al., 2004). Similar results were observed at Plant B, where the *mecA* and *vanA* markers remained below the LOD (Figure 6). However this was a onetime sampling event, therefore it is possible these genes could be present on other occasions as hospital effluents contribute to the WWTP influent volume. Since *mecA* and *vanA* genes can be present on chromosomal DNA (versus being present on broad-host-range plasmids) (Biavasco et al., 2007; Cetinkaya et al., 2000; Colomer-Lluch et al., 2011; Katayama et

al., 2000) their low presence may also be partially caused by lower presence/survival of bacteria who carry these genes in their chromosomes (Goldstein et al., 2012). ARGs belonging to families such as *tet,* and *sul*, have been commonly assessed and detected in WWTPs in the past, and represent resistance genes to some of the oldest, high-consumption ABs (Al-Jassim et al., 2015; Auerbach et al., 2007; Du et al., 2014; Laht et al., 2014; Lapara et al., 2011; Marti et al., 2013; Rodriguez-Mozaz et al., 2014). Other ARGs (*qnrS, ermB,* $bla_{CTX\text{-}M}$, bla_{TEM}) although less studied, have also been commonly detected in wastewater, and represent genes that are often plasmid-borne and confer resistance to newer ABs (Marti et al., 2013; Narciso-da-Rocha et al., 2014; Rodriguez-Mozaz et al., 2014). The detection of ARGs in wastewater is dangerous to public health as many of these genes have been found associated with plasmids, conjugative genetic elements, or class 1 integrons, which can contain other resistance genes and are able to spread between bacteria, including pathogens (Rodriguez-Mozaz et al., 2014).

3.3.1.2 Absolute abundance of ARGs

3.3.1.2.1 Greenwood

There was a significant ($p<0.05$) decrease in the average ARG absolute abundance (copies/mL) of 1.63 log units from the influent sewage to the treated effluent (Figure 5A), indicating that the treatment train decreased ARG concentrations successfully. These findings suggesting a significant decrease of ARGs throughout treatment agree with past studies (Chen and Zhang, 2013; Gao et al., 2015; Laht et al., 2014; Munir et al., 2011). Throughout the treatment train, average ARG concentrations decreased by the following log units after each treatment step: 0.18 (lagoon), 1.44

(clarifier), 0.18 (sand filters), -0.18 (UV). This indicates that the clarifiers are responsible for causing the largest incremental removal of ARGs (significant decrease from previous step ($p<0.05$)) from the wastewater and this removal most likely occurs through the sedimentation process, indirectly suggesting biosolids contain a large amount of the ARGs (Börjesson et al., 2010; Chen and Zhang, 2013). The AL and sand filters treatment steps contributed little to the removal of ARGs. There is also an increase (although not significant ($p>0.05$)) of ARGs after UV treatment, which indicates that UV disinfection does not remove ARGs from the wastewater.

All individually assessed genes, with the exception of *sul1* and $bla_{CTX\text{-}M}$, exhibited significant ($p<0.05$) decreases from influent to effluent water samples. Previous research suggests that sulfamethoxazole-resistant bacteria are difficult to remove, which may explain the non-significant reduction of *sul1* (Gao et al., 2012). Decreases from influent to effluent ranged from 0.94 log (*mecA*) to 2.23 log (*qnrS*). Börjesson et al. (2010) observed similar log decreases of 0.5-2.5 log when looking at *tet* genes in an activated sludge system designed for biological nutrient removal, while Munir et al. (2011) observed higher log decreases of 2.37-4.56 looking at *tet* and *sul1* genes in conventional activated sludge systems. 16S rRNA gene absolute abundance was significantly decreased ($p<0.05$) by 1.73 log units, demonstrating the WWTP's ability to reduce total bacterial levels similarly to ARGs. ARG concentration log(copies/mL) levels in the influent ranged from 3.27 log (*mecA*) to 5.85 log (*qnrS*), while effluent ranges were 2.32 log (*mecA*) to 4.15 log (*int1*). Du et al. (2014), observed a range of 4.61-7.32 log copies/mL in the influent and 2.71-4.97 log copies/mL in the effluent of a membrane bio-reactor (MBR) system (looking at *sul1, intI1, tetX, tetG, tetW* genes), therefore having

similar effluent values but higher influent values, while other studies observed higher influent and effluent values (Auerbach et al., 2007; Munir et al., 2011). These results indicate that Greenwood is capable of significantly reducing ARG concentrations for most genes and that Greenwood may receive and discharge less ARG concentrated wastewater compared to other plants. This may be partially due to Greenwood being a smaller plant receiving wastewater from a more rural area that does not include a full-scale hospital.

3.3.1.2.2 Plant B

There was a significant ($p<0.05$) decrease in absolute abundance of 2.22 log units from influent to effluent, indicating the plant reduced ARG concentrations successfully (Figure 6A). Throughout the treatment train average ARGs decreased by the following log (copies/mL) units after treatment steps: -0.05 (primary clarifier), 1.57 (BNR), 0.28 (secondary clarifiers), 0.41 (UV). This indicates that the BNR secondary treatment is responsible for causing the largest incremental removal of ARGs from the wastewater (significant decrease from previous step; $p<0.05$). Reductions of ARGs after biological treatment have been observed in other studies (Börjesson et al., 2010). The effect of primary clarification on removal of ARGs was found to be negligible, supporting findings from Chen and Zhang (2013). Secondary clarification contributed little to the removal of ARGs, while UV treatment reduced levels slightly compared to the previous treatment step, however not significantly ($p>0.05$).

All individual genes decreased from influent to effluent with ranges from 1.56 log (*sul2*) to 3.16 log ($bla_{CTX\text{-}M}$). Helt (2012) observed slightly lower log removals of

between 1.26-1.62 when looking at a pilot-scale BNR system as did Börjesson et al. (2010) investigating a full-scale BNR system. 16S rRNA gene absolute abundance was significantly decreased ($p<0.05$) by 0.99 log units, indicating the WWTP's ability to reduce total bacteria, albeit this system removed 1 order of magnitude fewer bacteria than Greenwood, and fewer bacterial genes than ARGs. Gene concentration levels (copies/mL) ranged from 3.50 log (*bla*$_{TEM}$) to 6.42 log (*int1*) in the influent and 1.10 log (*bla*$_{CTX-M}$) to 4.06 log (*int1*) in the effluent.

3.3.1.3 Relative abundance of ARGs at Greenwood's and Plant B's WWTPs

At Greenwood's WWTP, the ARG relative abundances at each tested treatment step did not vary significantly ($p>0.05$) between summer and winter sampling trips, indicating no seasonal difference in gene relative abundances within the plant, except for the seasonal presence of *mecA* which was observed. Therefore, data from all four sampling trips were averaged for analysis. No significant differences ($p>0.05$) were observed in the relative abundance of any of the individual ARG markers between wastewater treatment steps, except for *sul2*, which significantly increased ($p<0.05$) its relative abundance from influent to AL samples (Figure 5B). Throughout the treatment train the average ARG relative abundance changed by the following log units after treatments: 0.13 (lagoon), -0.12 (clarifier), -0.02 (sand filters), 0.00 (UV); none of which were significant changes ($p>0.05$). This indicates that on average there was neither a relative enrichment nor reduction in gene abundances throughout the treatment train. This result likely means total bacteria and ARB are being reduced at comparable rates and the plant neither selects for or against bacteria harbouring ARGs. Other studies support these

findings for the majority of ARGs throughout different wastewater treatment plants using activated sludge treatment (Gao et al., 2012 [for *sul1* averaged from mulitple plants]; Laht et al., 2014 [multiple ARGs; activated sludge]).

There was also no significant difference ($p>0.05$) in average relative ARG abundance between steps at Plant B (Figure 6B) (however large standard deviations caused by large differences in individual ARG abundances may have led to this result). Throughout the treatment train average ARG relative abundance decreased by the following log units after treatments: -0.02 (primary clarifier), 0.79 (BNR), 0.01 (secondary clarifiers), -0.11 (UV). Although not statistically significant when all ARGs were averaged, when looking at each gene individually it appeared the relative abundance of the majority of genes decreased after the BNR step, with the exception of *sul2,* which increased slightly (also observed at Greenwood). These results support the reduction trends of ARG abundance throughout WWTPs observed in other studies (Börjesson et al., 2010; Gao et al., 2012; Munir et al., 2011). BNR biological treatment may more efficiently decrease bacteria carrying ARGs than AL treatment based on these results. However, since this was a onetime sampling event, it is not known if these changes after BNR were statistically significant or if they would be found consistently. Börjesson et al. (2010) also observed reductions in ARG abundances following activated sludge treatment designed for nutrient removal, suggesting processes designed to remove nutrients may target ARGs more effectively. Börjesson et al. (2010) suggested that bacteria harbouring ARGs may not be favoured in nutrient removal targeted biological processes, explaining the lower abundances of some ARGs after treatment.

However, other studies have found some varying results. Czekalski et al. (2012)

found an increase in relative abundance for *sul1*, but a slight decrease for *sul2*, which demonstrates the variability of ARGs' relative abundances throughout different plants. Rodriguez-Mozaz et al. (2015) found both an increase (bla_{TEM}, *sul1*, and *qnrS*) and decrease (*ermB* and tetW) in relative abundance of genes throughout wastewater treatment, while other studies found only reductions (usually *tet* family) or no changes (normally *sul* family) (Börjesson et al., 2010; Gao et al., 2012; Munir et al., 2011). A possible explanation for apparent increases in relative abundances after treatment in some cases could be HGT of genes amongst bacterial cells, or selection of AR resistant bacteria during biological treatment (Rodriguez-Mozaz et al., 2015; Szczepanowski et al., 2009). Relative abundance of ARGs seems to vary from plant to plant and between different ARGs, however Greenwood's plant consistently shows no significant changes in relative abundances for majority of genes tested. Different selective pressures throughout treatment at WWTPs may contribute to some of these differences found between studies (i.e. varying presence and retention time of certain antibiotics) as suggested in other studies (Laht et al., 2014; Rodriguez-Mozaz et al., 2015).

Ranges of levels of relative abundance (log(ARG GC/16S rRNA GC)) of individual ARGs in the influent were -4.81 log (*mecA*) to -2.51 log (*qnrS*), while ranges in the effluent were -4.09 log (*mecA*) to -2.36 log (*int1*) at Greenwood's WWTP. Similar to slightly higher levels were found in other studies (Laht et al., 2014; Mao et al., 2015; Narciso-da-rocha et al., 2014), which shows ARGs are present in similar relative abundances in multiple WWTPs. At Plant B the ranges of relative abundance levels in the influent were -5.82 log (bla_{TEM}) to -2.90 log (*int1*), while ranges in the effluent were -6.75 log ($bla_{CTX\text{-}M}$) to -3.79 log (*int1*). These values were lower than observed in both

Greenwood and other similar studies (Laht et al., 2014; Mao et al., 2015; Narciso-da-rocha et al., 2014), indicating that Plant B may receive and discharge lower abundances of ARB compared to previously studied plants. At Greenwood WWTP, effluent values were slightly, but not significantly ($p>0.05$) higher than influent values. However, at Plant B the effluent values were lower than influent, indicating this type of WWTP may be able to decrease the relative abundance of individual ARGs, although whether or not these decreases are consistent or statistically significant is unknown due to limitations in the number of sampling events. Future sampling of this WWTP is recommended to examine the consistency of these trends.

At both plants, *int1* became the most detected gene out of the tested genes in the effluent, which was also observed by Narciso-da-Rocha et al. (2014), who suggested that *int1* may be more stable in wastewater. However, *int1* can be detected from empty integrons as well as full integrons, which may explain their consistent presence in wastewater (Narciso-da-rocha et al., 2014). The presence of *int1*, which has been found to significantly drive evolution and proliferation of multiple ARB, has been reported in both influent and effluent samples, as well as in association with other ARGs, of multiple WWTPs (Chen et al., 2015; Lapara et al., 2011; Moura et al., 2007; Zhang et al., 2009). Since integrons have been associated with MDR bacteria, their detection in wastewater may provide a global perspective on AR and ARGs as integrons have the ability to carry and allow the transfer of multiple ARGs (Rizzo et al., 2013).

3.3.1.4 *E. coli* and *HF183* marker

At both plants *E. coli* was significantly ($p<0.05$) decreased from influent to effluent, indicating successful disinfection (6.74 log unit reduction Greenwood; 6.70 log unit reduction Plant B). Effluent values for *E. coli* were 0.53 +/- 0.17 log units (MPN/100mL) at Greenwood and <1 MPN/100mL at Plant B. *HF183* maker was also decreased significantly ($p<0.05$) at both plants, by 2.20 log units from influent to effluent at Greenwood and 1.49 log units at Plant B, indicating both WWTPs' capacity to the reduce the number of human anaerobic fecally derived bacteria. At Greenwood, the Pearson correlation coefficients for *E. coli* and *HF183* marker in relation to ARG and *int1* gene concentrations averaged from August and February were $r=0.79$ ($p>0.05$) and $r=0.81$ ($p>0.05$) respectively, both of which were not statistically significant. This indicates moderate but not statistically significant correlation between the removal of ARG concentrations and *E. coli* or the *HF183* marker. At Plant B, Pearson correlation coefficients for *E. coli* and *HF183* marker in relation to ARG and *int1* gene concentrations were $r=0.90$ ($p<0.05$) and $r=0.95$ ($p<0.05$), respectively, indicating significant correlations between the removal of gene concentrations and *E. coli*, and significant correlations between the removal of gene concentrations and *HF183* marker. Further research investigating the correlations between ARGs and and these factors in other WWTPs is recommended to assess the strength of these trends.

Both plants exhibit a decrease of over 6 log units for *E. coli*, but only around 2 log unit decreases for ARGs and *HF183;* ARGs and *HF183* being removed at similar rates. Performing metagenomic sequencing to determine what types of bacteria are in the effluent is recommended to determine potential ARG carriers at these plants. Chern et al.

(2015) reported that using qPCR for detection of genetic elements, such as *HF183*, to monitor inactivation of fecal indicator bacteria in wastewater is not suitable as bacteria levels were decreased significantly while genetic markers were not. Genetic detection from dead cells and exogenous DNA is possible therefore accurate live counts from qPCR are not feasible using this method. This phenomenon is observed in Figures 5C and 6C, where after UV disinfection *E. coli* is significantly reduced, however gene abundances remain unchanged, which is likely the case for bacteria harbouring ARGs (i.e. qPCR may be detecting ARG copies from dead bacteria). However, exogenous DNA can still be transferred to other bacteria through transformation (Frost et al., 2005), especially while in presence of selective pressures (e.g. AB and metals), therefore ARGs entering the environment through effluent discharge can still be a threat to public health. In summary, these results suggest that using methods to assess ARG abundances that rely on live bacteria may not be an accurate representation of the total presence of these genes as the log reductions were quite different. The *HF183* marker is more highly correlated to ARGs levels as a similar log removals were found for both, and may suggest this marker is removed from wastewater in similar mechanisms as ARGs.

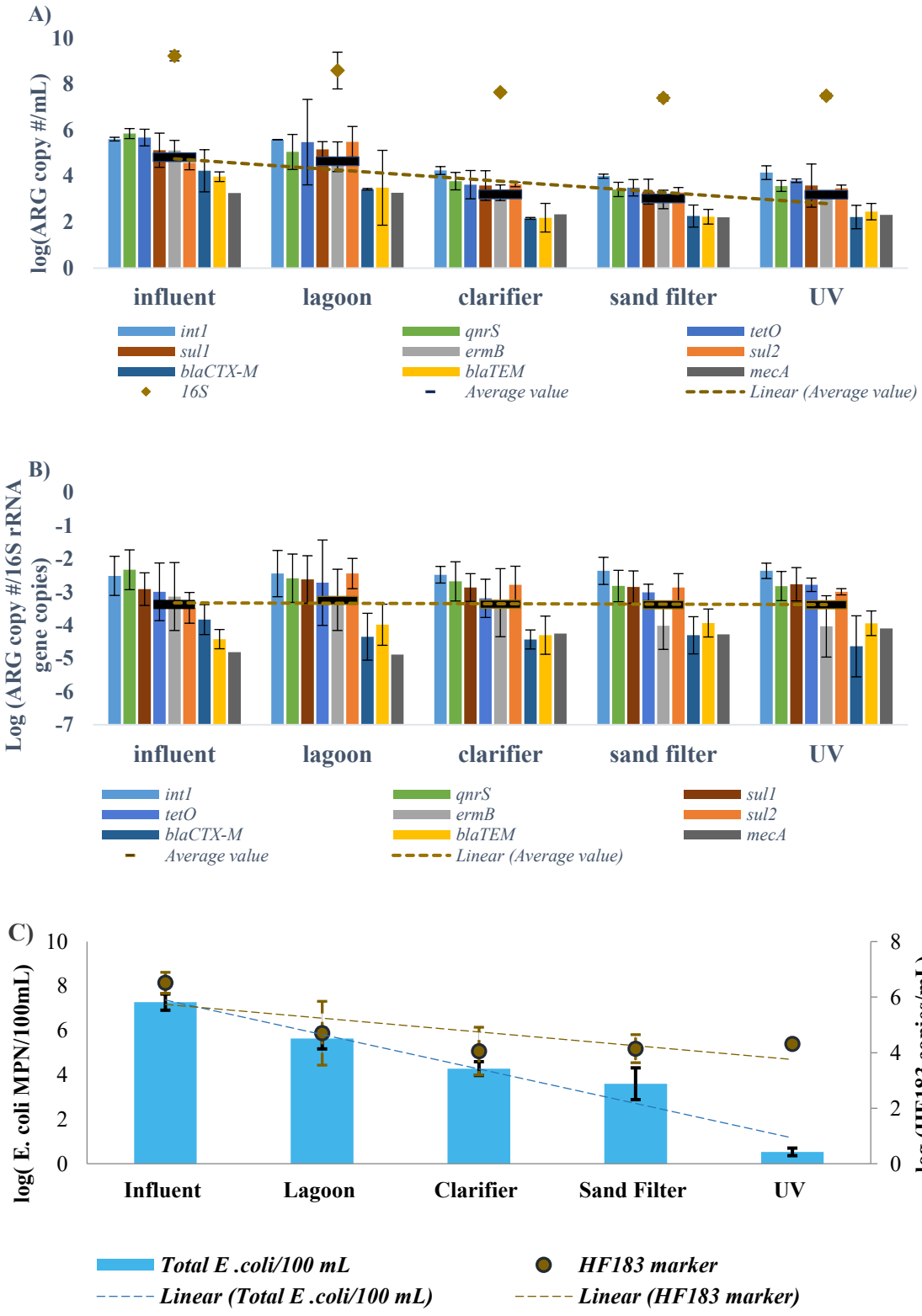
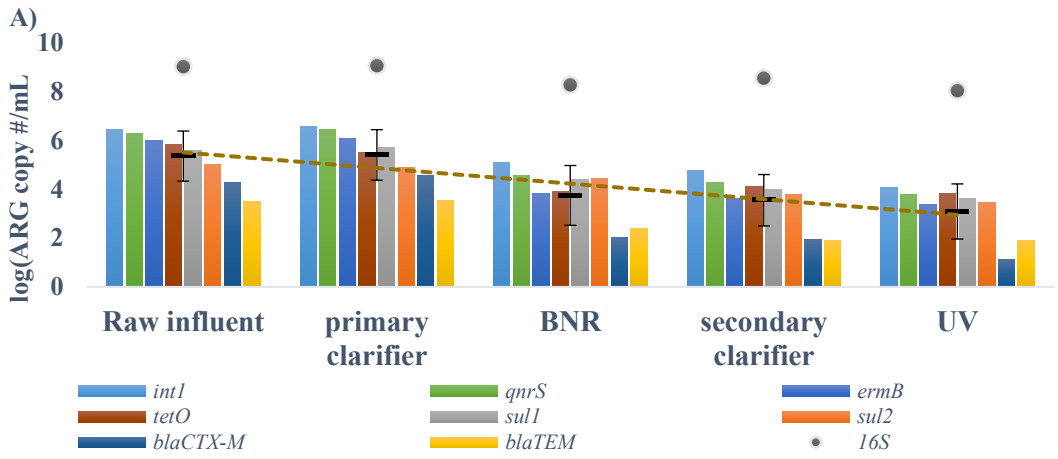

A)
log(ARG copy #/mL)
10
8
6
4
2
0
influent
lagoon
clarifier
sand filter
UV
int1
sul1
blaCTX-M
16S
qnrS
ermB
blaTEM
Average value
tetO
sul2
mecA
Linear (Average value)
B)
Log (ARG copy #/16S rRNA gene copies)
0
-1
-2
-3
-4
-5
-6
-7
influent
lagoon
clarifier
sand filter
UV
int1
tetO
blaCTX-M
Average value
qnrS
ermB
blaTEM
Linear (Average value)
sul1
sul2
mecA
C)
log(E. coli MPN/100mL)
10
8
6
4
2
0
log (HF183 copies/mL)
8
6
4
2
0
Influent
Lagoon
Clarifier
Sand Filter
UV
Total E .coli/100 mL
Linear (Total E .coli/100 mL)
HF183 marker
Linear (HF183 marker)

Figure 5: Abundances of ARGs and fecal indicators at Greenwood's WWTP A) Absolute abundances of each ARG and *int1* and each WWTP step tested averaged from August 2015, and February 2016 sampling dates. Error bars indicated standard deviation between the sampling trips. Black lines indicate the average. 16s rRNA values for each step are also presented in dot form. B) Relative abundances of each ARG and int1 at each WWTP step tested averaged from July 7, July 28, August 2015, and February 2016. Error bars indicated standard deviation between the four sampling trips. C) *E. coli* MPN/100mL levels from all four sampling trips, and HF183 marker concentrations averaged from August and February, are presented. Error bars indicate standard deviations between sampling trips. Dashed lines indicate the linear trend lines.



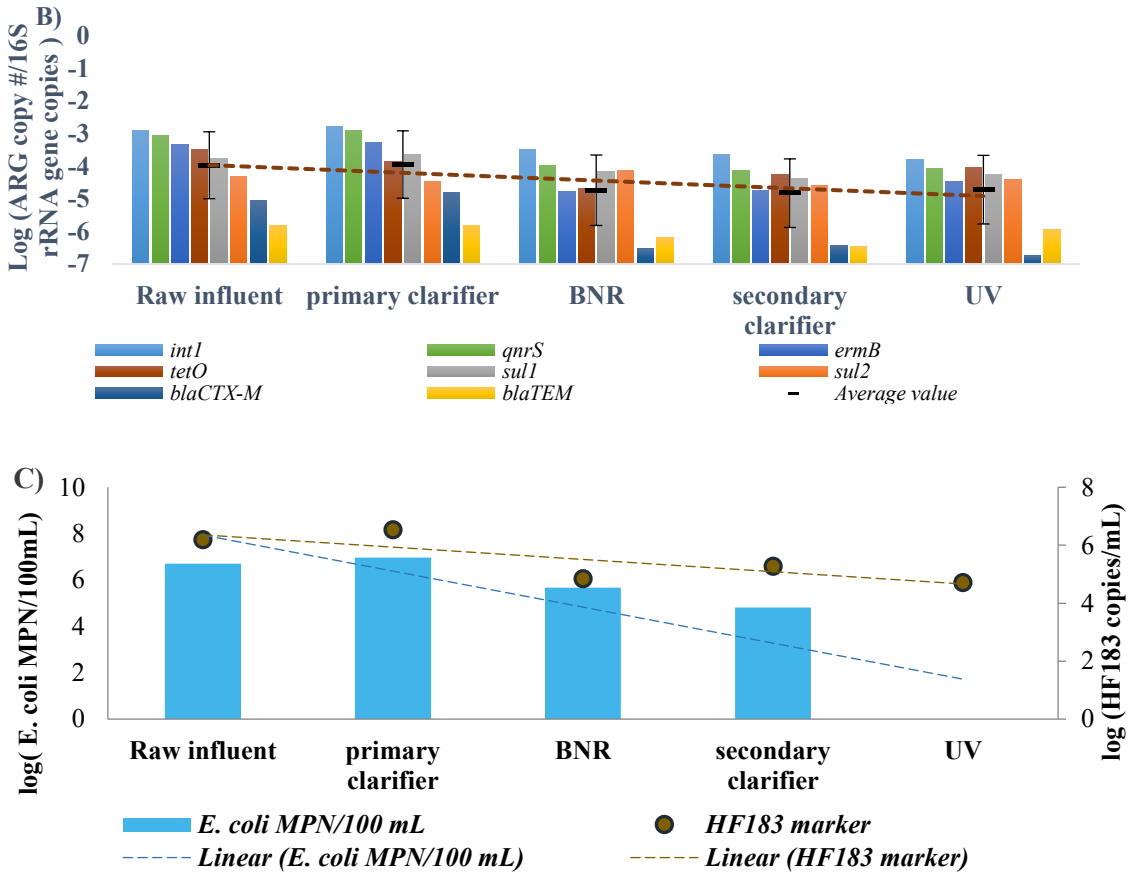


Figure 6: Abundances of ARGs and fecal indicators at Plant B. A) Absolute abundances of each ARG and *int1* and each WWTP step tested in July 2016. Black lines indicate the average and error bars represent the standard deviation from averaging the eight genes presented. 16s rRNA values for each step are also presented in dot form. B) Relative abundances of each ARG and *int1* at each WWTP step tested in July 2016. Black lines indicate the gene average relative abundance for each treatment step and error bars represent the standard deviation from averaging the eight genes presented. C) *E. coli* MPN/100mL levels and HF183 marker concentrations from July 2016 are presented. Dashed lines indicate linear trends for each factor.

3.3.2 Water quality assessment and correlation to gene abundance

Greenwood's plant is efficient at nutrient and solids removal as all measured parameters were decreased between 0.5 and 2 orders of magnitude as seen in Table 6 (except nitrate which was increased as expected (due to conversion of ammonia into nitrate (nitrification)). The plant met the regulatory target for both TSS and $CBOD_5$ (both regulated at 5 mg/L) in the effluent. In regards to correlations between ARG concentrations and water quality at the tested sites, nitrate ($r=-0.92$, $p<0.05$) and $CBOD_5$ ($r=0.95$, $p<0.05$) showed statistically significant correlations (Table 6). Decreasing $CBOD_5$ levels while increasing the conversion of ammonia to nitrate may help predict the concentration of ARGs in wastewater effluent in this type of plant design. Positive relationships between removal of nutrients and ARG abundance have been suggested in other studies (Börjesson et al., 2010; Nõlvak et al., 2013; Novo and Manaia, 2010). Nolvak et al. (2013) found significant negative correlations between nitrate and positive correlations between organic matter and ARG concentrations, indicating that treatment efficiency plays an important role in determining the amount of ARGs entering the environment via the effluent from wastewater treatment facilities.

Plant B was efficient at nutrient and solids removal as all measured parameters decreased between 1 and 2 orders of magnitude as seen in Table 7. Tested parameters were all decreased from influent to effluent demonstrating the plant's ability to reduce these nutrients and solids before being discharged into the harbour. The plant fulfilled the targeted regulatory removal for both TSS and $CBOD_5$ (both regulated at 25 mg/L). There were no statistically significant correlations ($p>0.05$) between ARG concentrations and water quality at the tested sites, however higher correlation coefficients were observed

for ammonia (r=0.86) and $CBOD_5$ (r=0.64), which suggests there may be some weaker relationships between these factors at this plant, which may become more apparent with repeated sampling.

Table 6: Average water quality data and Pearson correlation coefficients from Greenwood. Standard deviations represent variation between four sampling trips (July 7, July 28, August 2015, and February 2016). Bolded values indicate water quality parameters under provincial regulation. The starred correlation coefficients are statistically significant at $p<0.05$.

	Average Nitrate (mg/L)	Average Ammonia (mg/L)	Average TN (mg/L)	Average TSS (mg/L)	Average VSS (mg/L)	Average COD (mg/L)	Average TP (mg/L)	$CBOD_5$ (mg/L)
Influent	1.5 ±1.7	18.8 ± 5.8	31.3 ± 6.76	160.9 ± 65.3	151.2 ± 59.6	406.1 ± 70.3	9.4 ± 2.1	169.3 ± 42.2
Lagoon	5.5 ± 2.7	1.0 ± 0.55	201.1 ± 41.83	2861.3 ± 1006.0	2332.0 ± 812.5	4272.3 ± 1478.0	255.6 ± 69.0	242.3 ± 92.3
Clarifier	8.1 ± 3.7	0.7 ± 0.61	10.5 ± 7.57	5.9 ± 1.9	4.8 ± 1.2	21.9 ± 5.4	3.3 ± 3.7	3.6 ± 3.3
Sand Filter	8.6 ± 3.6	0.3 ± 0.40	7.5 ± 5.00	1.6 ± 1.2	1.4 ± 0.66	21.3 ± 0.50	3.1 ± 3.7	2.9 ± 1.3
UV	8.3 ± 3.4	0.3 ± 0.39	6.7 ± 4.00	**1.6 ± 0.66**	1.7 ± 0.52	18.9 ± 2.0	3.2 ± 3.8	**1.3 ± 1.3**
Pearson correlation coefficient (p-value) with ARG gene concen-trations	-0.92 * ($p<0.05$)	0.69 ($p>0.05$)	0.65 ($p>0.05$)	0.60 ($p>0.05$)	0.60 ($p>0.05$)	0.62 ($p>0.05$)	0.57 ($p>0.05$)	0.95 * ($p<0.05$)

Table 7: Water quality data and Pearson correlation coefficients from Plant B July 2016 sampling event. Bolded values indicate water quality parameters under provincial regulation.

	Average Ammonia (mg/L)	Average TN (mg/L)	Average TSS (mg/L)	Average VSS (mg/L)	Average COD (mg/L)	Average TP (mg/L)	$CBOD_5$ (mg/L)
Raw influent	30.6	48.8	321.0	219.0	964.0	49.7	454.3

	Average Ammonia (mg/L)	Average TN (mg/L)	Average TSS (mg/L)	Average VSS (mg/L)	Average COD (mg/L)	Average TP (mg/L)	$CBOD_5$ (mg/L)
Primary Clarifier	13.0	22.7	45.7	35.0	488.7	13.9	224.1
BNR	0.94	161.0	1901.0	1445.5	2060.0	184.0	404.8
Secondary Clarifier	0.19	4.0	4.2	2.2	48.0	0.10	9.5
UV	0.25	4.4	**4.6**	1.0	16.0	0.00	**8.1**
Pearson correlation coefficient (p-value) with gene concentrations	0.86 (p>0.05)	0.01 (p>0.05)	-0.12 (p>0.05)	-0.13 (p>0.05)	0.19 (p>0.05)	-0.02 (p>0.05)	0.64 (p>0.05)

3.3.4 Summary and comparisons between WWTPs

Nearly all tested ARGs were observed in both WWTPs, demonstrating their widespread prevalence in Atlantic Canadian treatment systems. Both plants statistically significantly reduced absolute abundance of ARGs throughout treatment. When comparing the plants at equivalent treatment steps, there were no significant differences in absolute abundances ($p>0.05$). This indicates that the plants contain similar concentrations of genes and these concentrations decrease following similar patterns. However, Greenwood's plant seems to afford the largest decrease in the concentration of ARGs after the secondary clarifiers, indicating that the majority of ARGs are being removed during the sedimentation process, while Plant B's largest decreasing step is after the BNR secondary treatment, suggesting removal during biological treatment. Little ARG removal was observed after the AL treatment in Greenwood and Plant B's secondary clarifiers also removed minimal ARGs; although this could be due to most ARGs being removed in the previous BNR step. This demonstrates the differences between plants in terms of ARG reduction processes. However, in both plants ARGs are still present in the effluent and are entering water sources. In Greenwood the effluent

discharges into a small river that is used for recreational activities downstream. Plant B's effluent discharges into a harbour that is used for shell fish harvesting. The presence of ARGs in both of these water sources could be of public health concern and therefore warrants further investigations. Furthermore, UV disinfection, which was used at both plants, did not significantly remove ARGs when compared to previous treatment steps, however a larger reduction in ARG numbers was observed after Plant B's UV treatment. These results demonstrate how although UV disinfection reduces live *E. coli* and other live bacteria, ARGs are still able to survive the treatment, as supported by previous studies looking at ARG markers (Chen and Zhang, 2013; McKinney and Pruden, 2012; Munir et al., 2011). Additional tertiary treatment steps may be required if attempting to target ARG removal.

Although there was no statistical ($p>0.05$) difference between individual treatment steps in terms of average ARG relative abundance at either plant (however the range of abundances of individual ARGs was large at Plant B causing possible difficulties detecting differences between steps during statistical analysis), Plant B decreased individual gene relative abundance after secondary treatment in most cases, which was not observed in Greenwood. Comparison, using t-tests, between each plant at equivalent treatment steps revealed that there was not a significant difference ($p>0.05$) in relative abundance of genes after the primary clarifier step between plants, but there was after biological treatment (AL and BNR steps), secondary clarifiers, and UV disinfection ($p<0.05$). This indicates that both plants start with similar relative abundance of ARGs, but Plant B is able to reduce the abundance of ARGs more efficiently. Since the significant decrease starts after the BNR step, it suggests that the BNR system causes the

significant difference in abundance. However, there have not been many studies comparing BNR systems to ALs therefore it is unknown if these trends have been seen in other plants. Helt (2012) compared ARG abundances between a bench scale BNR system and constructed lagoons, and found little difference between the two systems. It's possible that the increased nutrient removal process in BNR systems aids in the reduction of ARGs (Börjesson et al., 2010; Nõlvak et al., 2013), however there were stronger correlations between nutrient reduction and ARGs at Greenwood than at Plant B. Further sampling at Plant B to determine the BNR's impact on ARGs abundances is recommended. Other studies observed membrane biological reactors (MBR) as having greater treatment efficiencies for reducing ARGs compared to other conventional treatment systems such as activated sludge, suggesting MBRs can target resistant bacteria more efficiently (Du et al., 2014; Munir et al., 2011). Therefore, comparing more types of biological treatments at WWTPs is recommended in order to determine the most appropriate choice of secondary treatment in order to target ARG removal.

In both plants *sul2* was enriched after the biological treatment step, which suggests that bacteria carrying this gene may practice horizontal gene transfer or selection more often. Chen and Zhang (2013) also reported *sul* genes as increasing in relative abundance throughout treatment. It is also important to consider that when sampling, each sampling site is independent from the previous site since the wastewater that is collected is not the same water that was sampled at the previous site, therefore each sample represents a snapshot of the characteristics that can observed after a particular treatment step (e.g. the hydraulic retention time (HRT) usually ranges between 18-24 hours). Variations in influent quality and flow, and treatment efficiencies could change

the ARG abundances observed between samples collected at the same site at different times. Therefore, *sul2* might not actually be getting enriched and may be a phenomenon of individual samples. Investigating the persistence of sulphonamide ABs as selective pressures as well as investigating the mode of transfer of these genes may help in understanding this apparent increase in abundance.

Table 8 lists the ranks of each gene found in each plant by summing the relative abundance and the absolute abundance at each step measured for each gene. Rankings were quite similar between plants; *int1*, *qnrS* and *sul1* being the most detected gene throughout treatment and bla_{TEM}, $bla_{CTX\text{-}M}$, *mecA,* and *vanA* being the least detected in relative abundance throughout treatment. These data suggest similar mechanisms involved with entering and surviving in wastewater for those genes that exhibit similar abundances. These results support findings by Laht et al. (2014), who suggest that older, more commonly used ABs are also represented by higher levels of their corresponding ARGs in the environment, stressing the need to study the selective pressure of AB presence. Nõlvak et al. (2013) also saw higher abundances of more commonly used ABs' resistant genes.

A Principal Component Analysis was performed comparing ARG fingerprints (i.e. the proportions of each gene type out of total genes) between water type (i.e. influent or effluent) and WWTP process type (i.e. AL biological treatment plant or BNR treatment plant) in order to evaluate the variance in results (Metsalu et al., 2016) (Figure 7). It was determined that 80.8% of the total variance can be explained by WWTP and 16.7% can be explained by water type. These results suggest that although the ARG fingerprint did change slightly from influent to effluent at each plant, the different

WWTPs explained more of the variance in fingerprints. This could be caused by the different WWTP designs or may also be due to local differences as the influent seems to have different ARG profiles.

Table 8: Features and ranking sum order of relative abundance and absolute abundance of genes within the WWTPs

Gene	Greenwood WWTP Rank Relative/ absolute abundance	Plant B WWTP Rank Relative/ absolute abundance
int1 (ARG cassette and mobility potential)[a]	1/1	1/1
qnrS (often plasmid borne; resistance evolves rapidly)[b]	2/3	2/2
sul1 (high consumption and long use)[c]	3/4	3/3
sul2 (high consumption and long use)[c]	4/5	6/6
tetO (high consumption and long use)[c]	5/2	4/4
ermB (macrolides second more prescribed if penicillins can't be used)[d]	6/6	5/5
bla$_{TEM}$ (high consumption, newer use)[c]	7/7	8/8
bla$_{CTX-M}$ (high consumption, newer use)[c]	8/8	7/7
mecA (clinically relevant; risk to human health)[c]	9/9	<LOD
vanA (clinically relevant; risk to human health)[c]	<LOD	<LOD

[a](Allen et al., 2010)
[b](Varela et al., 2016)
[c](Laht et al., 2014)
[d](Brunning, 2015)

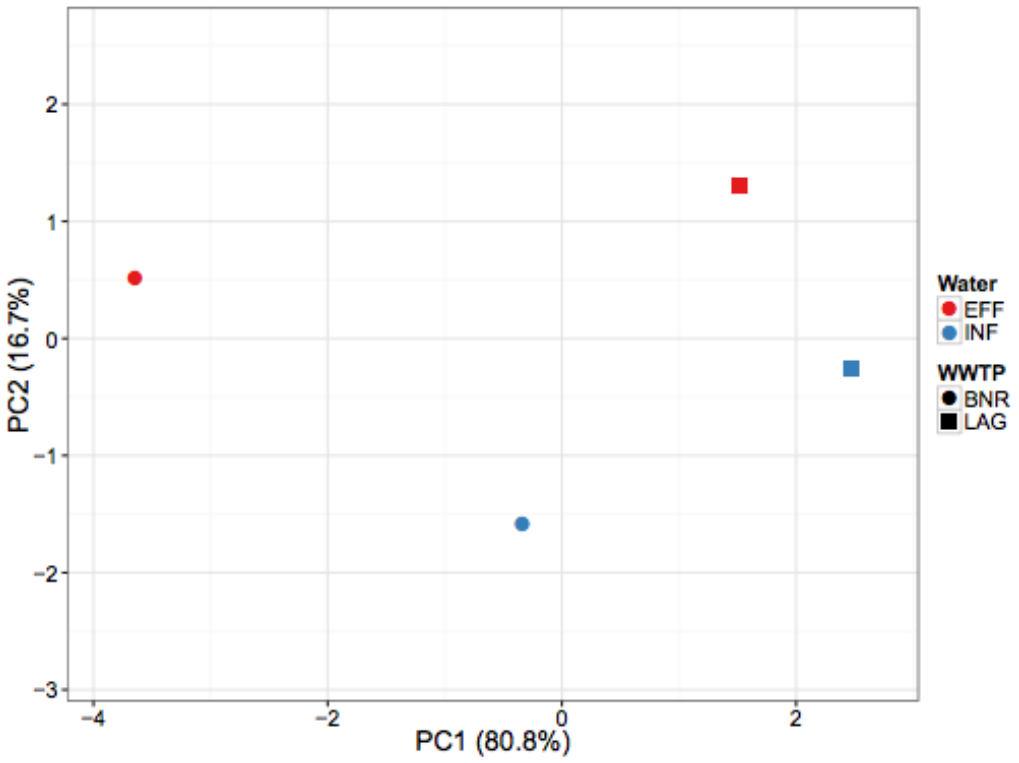


Figure 7: Principal Component Analysis (PCA) plot of the ARG content comparing water type (i.e. influent or effluent), and biological treatment systems (i.e. BNR (Plant B) or AL (Greenwood)). These results suggest that although the ARG fingerprint did change slightly from influent to effluent at each plant, the different WWTPs explained more of the variance in fingerprints.

3.3.6 Antibiotic Results and correlations

Antibiotic results from samples can be found in Appendix A. All ABs besides cefaclor, and cefprozil were detected in various samples at both plants. Most penicillin antibiotics were not detected despite their high consumption, which may be due their chemical instability (Graham et al., 2011). The most consistently present antibiotic was ciprofloxacin, which was also found by Rodriguez-Moraz et al. (2015), however in the

clarifier samples at Plant B, amoxicillin was detected in high concentrations. Correlation analysis was performed on the August and February samples and also the Plant B samples between the ABs and ARG absolute concentrations. No significant ($p<0.05$) correlations were observed between ARGs and the ABs they confer resistance to. Other studies have found correlations in the past (Gao et al., 2012; Rodriguez-Mozaz et al., 2015). It is possible that antibiotics could have degraded or further sampling may reveal antibiotics correlation to increased ARGs, however with a limited sample size no correlations were found in this study. However, these results show that most ABs tested for are present throughout wastewater treatment at both plants indicating that selective pressures are present in the wastewater.

3.4 Conclusions

With the exception of *vanA* and *mecA,* a consistent presence of all tested genes was observed in both treatment plants. Older and more commonly prescribed antibiotics were more commonly detected in both plants, showing their widespread presence in wastewater. There was a high presence of *int1* at both plants, which suggests ARG cassettes may be present in the plants and these cassettes may be located on mobile genetic elements which can promote their spread. There was a significant decrease in absolute abundance of ARGs after treatment in both plants, as well as a significant decrease in nutrients, solids and *E. coli*. However, some ARGs remained present in effluent discharge, leading to their subsequent release into the receiving environments of both plants. Since UV treatment was not able to significantly decrease ARG levels in either plant, additional advanced treatment options should be explored for ARG

reduction. Therefore, both WWTP designs could be better optimized to increase ARG removal. On average, relative abundance of total ARGs were neither highly reduced nor enriched in either plant, however Plant B's BNR system step may be able to decrease individual ARG levels more effectively than ALs. The highest correlations to ARG concentrations were found for organic matter and nutrients, as well as the *HF183* marker, demonstrating that these factors may be useful in predicting ARG levels in the future. Although no significant correlations were found between ABs and ARG concentrations, a larger sample size may show selective behaviour in these plants. In summary, further investigation on Plant B's BNR system for its role in reducing ARG abundances, and advanced treatment steps that not only kill pathogens, but eliminate ARGs, are recommended.

3.5 Acknowledgments

We would like to thank both the Municipality of Kings County and the City of Plant B. We would also like to thank our team members for their assistance in the field and laboratory (Dr. Kara Neudorf, Audrey Hiscock, Robert Johnson, Amy Jackson, and Joanna Poltarowicz). Funding for this project was provided by the Natural Science and Engineering Research Council of Canada.

Chapter 4: Impacts of Municipal Wastewater Effluent and Seasonal Variations on the Presence and Abundance of Antibiotic Resistance Genes in a Nova Scotia River.

Abstract

From a public health standpoint increasing ARGs in the environment is problematic due to the risk of HGT and development of ARBs. The objective of this study was to investigate the abundance of ARGs both upstream and downstream of a WWTP discharge site to assess if the WWTP contributes to increased ARG levels downstream. Furthermore, the upstream watershed was also investigated in order to determine background levels of ARGs in samples both closer to and further away from urbanization during different seasons and weather events. Quantitative real-time PCR (qPCR) was used to quantify the absolute (copies/mL) of nine ARGs as well as one indicator gene (*int1*) to assess presence of ARG cassettes and potential of mobility. Water quality assessments revealed the plant was meeting effluent regulations while also decreasing ARG levels around 2 log units, however there was a significant ($p<0.05$) increase in average absolute and relative abundances (*int1*, *sul1, sul2, tetO, ermB,* $bla_{CTX\text{-}M}$, bla_{TEM}, *qnrS, mecA)* downstream from the plant, indicating the plant impacts the ARG abundances in the receiving water. As a general trend, ARG concentrations were found to decrease the further the water sample was taken away from human-impacted areas, however ARGs were still detected in more "pristine" samples. Wet weather/early spring river water samples contained significantly ($p<0.05$) more ARGs than samples taken during drier weather events in late spring and summer seasons, however there seemed to be a greater impact of the WWTP effluent on downstream water samples in summer

seasons. Significant correlations were found between water quality variables such as ammonia, TSS, VSS, *HF183* human fecal marker, *E. coli*, conductivity, and some ABs, suggesting these factors may be useful in predicting ARG concentrations in water from the WWTP and receiving river. In summary, WWTP effluent increased ARG levels in this river, however ARGs were also detected in various upstream samples indicating AR's ubiquitous nature in the watershed.

4.1 Introduction

Effluents from WWTPs have been shown in previous studies to contribute to increased levels of AR in receiving environments by increasing levels of ARB, ARGs, and AR selective factors such as ABs downstream (Berglund et al., 2015; Makowska et al., 2016; Proia et al., 2016; Rodriguez-Mozaz et al., 2015). Although WWTPs have been shown to decrease levels of ARGs throughout treatment, effluents have been shown to still contain considerable levels of ARGs, which are subsequently discharged into the environment (Rodriguez-Mozaz et al., 2015). Furthermore, other anthropogenic activities, aside from municipal wastewater discharge, have also been shown to increase levels of AR and its determinants in the environment. Some of these other sources include antibiotic additives in agricultural practices (followed by the application and release of livestock wastes to soil and water environments), and additives in the fishery industry (followed by aquaculture discharge into the environment), as well as effluent from on-site wastewater treatment systems (Storteboom et al., 2010; Tamminen et al., 2011). Rapid urbanization has caused increased levels of the above sources and the waste products they create, therefore proximity to and increased levels of urbanization is

often associated with higher levels of AR in the environment (Ouyang et al., 2015). Studies have also found correlations between declining water quality (e.g. increased conductivity, and increased concentrations of ammonia, nitrate, TSS, and *E. coli*) caused by increased urbanization/agricultural activities, and increased levels of AR in the environment, suggesting anthropogenic related pollution from multiple sources contributes to AR reservoirs (Al-badaii and Shuhaimi-Othman, 2015).

Even sub-lethal concentrations of ABs can sustain or exert a selective force for AR in the environment, and once ARGs are established they can be replicated and spread among bacteria (Martinez, 2009). Along with increased selection due to medical and agricultural uses of ABs, AR also exists and is selected for naturally due to pressures on bacteria from endogenous and naturally occurring ABs, as well the use of ARGs for alternate cellular functions in nature (Allen et al., 2010). Antibiotic resistance has been shown to be able to spread throughout the environment from the above sources through forces such as urban and agricultural runoff into rivers and watersheds, wind, as well as biological forces such as animals and humans (Allen et al., 2010). Therefore, due to urban sources and a natural presence of AR in the environment, ARGs have been detected in many environmental samples, including from more pristine environments located further away from anthropogenic impacts (Knapp et al., 2012; Ouyang et al., 2015; Pruden et al., 2006; Storteboom et al., 2010). Antibiotic resistance genes may persist even after selective pressures such as ABs are removed or the host bacteria has died making them extremely difficult to get rid of (Salyers and Amábile-Cuevas, 1997). Antibiotic resistance genes are often present on mobile genetic elements such as

plasmids, transposons, and integrons, which allows for their HGT between bacteria (Davies and Davies, 2010).

Seasonal conditions can also contribute to variations in ARG abundances in the environment as shown by Knapp et al. (2012). They found that ARG concentrations were higher and more equally distributed in wet seasons versus dry seasons river water samples in Cuba, suggesting that higher flows and re-entrainment of ARG from sediments along the river increased ARG transport during the wet season. However, they also found ARG levels were more spatially variable in the dry season, finding wastewater effluents strongly influencing local downstream water column ARG levels, suggesting that drier seasons allow a better picture of WWTP impacts on receiving water to be seen. They suggested that localization of ARGs develops in dry seasons due to lower flow rates, increased local influence of outfalls, and reduced sediment transport causing the differences observed between seasons and sites. Therefore, it is useful to examine ARG abundance in the environment during different seasons and weather events in order to obtain a more complete assessment of environmental AR.

The objective of this study was to examine through qPCR if the effluent from a municipal AL WWTP affected ARG levels in a receiving river by collecting samples both upstream and downstream of the outlet site. Furthermore, the upstream watershed (both closer to urbanization, and so-called "pristine" areas further away) was examined to assess background levels of ARGs in the environment. Sampling occurred both during wet and dry events (wet event in early spring, dry events in late spring and summer) to assess seasonal variations in ARG abundances. Water quality (*E. coli*, ammonia, phosphorous, conductivity, *HF183* human *Bacteroides* marker, etc.), flow rates, and

presence of selective AR factors (antibiotics) were also assessed in order to examine their potential relationships to ARG abundances.

4.2 Materials and Methods

4.2.1 Sampling sites and sample collection

The wastewater treatment plant that was sampled is located in Greenwood, Nova Scotia and is described in chapter 3 under the methods section. Greenwood is a village located in Nova Scotia's Annapolis Valley and has a population of around 6500 people (Statistics Canada, 2011). The WWTP discharges its effluent into Fales River, which is a tributary to the larger Annapolis River that is used for recreational activities in the community during summer months. Sampling was divided into two main sections. Sampling that included influent, effluent, downstream, and upstream 1 water samples was performed on August 18/2015 (summer/dry weather event), February 23/2016 (winter/dry weather event), April 12/2016 (early spring/wet weather event), May 10/2016 (late spring/dry weather event), and August 2/2016 (summer/dry weather event). Samples were labeled as a dry weather event if there was no precipitation on the day of the sampling event, i.e. base flows, while samples were labeled as wet weather event if there was precipitation on the day of sampling event, i.e. event conditions causing peak flows. These sampling dates and collection sites were used to exam the effect of the WWTP on the ARG content and water quality in the receiving river. For the sampling dates April 12/2016, May 10/2016, and August 2/2016, an additional six upstream water sites located within the watershed of the discharge point of the WWTP were sampled (UP2 to UP7). Figure 8 details the locations of these collection sites and describes each

sampling location, which were used in the analysis of Fales River upstream watershed in order to determine background ARG levels. Upstream sample collection sites were chosen to represent points along both primary tributaries in closer proximity of (UP2, UP3, UP4, UP6) and further away (UP5, UP7) from urbanization and human-impacted areas. Accessibility was also a factor in choosing sampling locations. The watershed delineation and stream network was created by Jenny Hayward (Centre for Water Resource Studies, Dalhousie University) using ESRI ArcGIS ArcMap 10© software. Samples were collected in pre-sterilized 1 L Nalgene collection bottles (Thermo Fisher Scientific). Water samples were kept on ice while being transported back to the laboratory at Dalhousie University in Halifax, NS (a distance of approximately 150 km), stored in the fridge at 4°C overnight and processed the following morning.

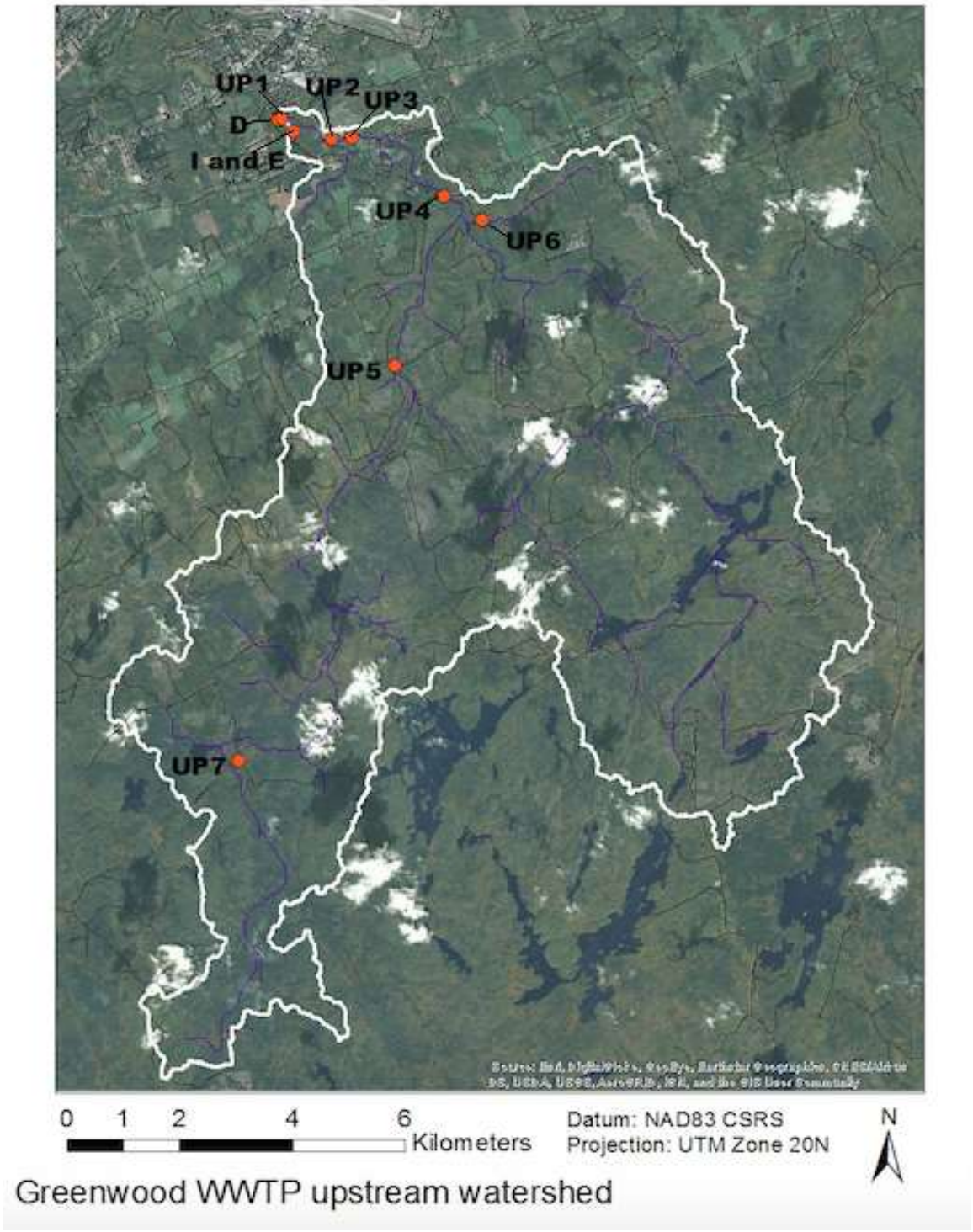


Figure 8: Greenwood WWTP and watershed sampling locations. Watershed boundaries are represented by white lines, while the stream network is represented by blue lines. Grey lines represent roads. Sampling locations are labeled as I and E (influent and

effluent), D (downstream), and UP1 to UP7 (Upstream 1 to Upstream 7). The D site was directly downstream from the effluent discharge pipe where the river water had a chance to mix with the effluent. UP1 lay directly upstream from the WWTP effluent discharge pipe. UP2 and UP3 were close to UP1 and located in more residential/urban areas (note: there is a side stream entering the river before UP2 which could account for differences between UP2 and UP3). UP4 is near a walking trail which is located on a dirt road surrounded by a few houses (UP4 also is just downstream of the confluence of the 2 primary tributaries). UP5 is on the left side of the watershed further away from urbanization. UP6 is located on a side stream from the main river that flows into UP4 and is along dirt road used as a walking/dog walking trail; there also looks to be more "urban" areas along that side stream (note: the upstream right side of the watershed had limited motor access therefore this collection site represents a proxy for the right side of watershed for the purposes of this study). UP7 is further upstream on the left side of the watershed (upstream from UP5) where there is very little urbanization and represents a sampling site deemed "pristine" for the purposes of this study. Map created by Jenny Hayward using ESRI ArcGIS ArcMap 10© software.

4.2.2 Analysis of the Abundance of Antibiotic Resistance Gene, Human Fecal Markers and 16S rRNA Gene Copies

4.2.2.1 Genomic extraction

Wastewater and river samples in quantities ranging from 50 (influent) to 500 mL (effluent and river samples) were filtered through 0.45 μM membranes and a vacuum manifold (Millipore, Inc., Bedford, MA). Filters were stored at -20°C until DNA

extraction. Genomic DNA was extracted from the entire filter using the MoBio Powersoil DNA extraction kit (VWR International, Ville Mont-Royal, QC, Canada) according to manufacturer's specifications and stored at -20°C.

4.2.2.2 Quantitative real-time PCR

Assessment of gene targets was performed using quantitative real-time PCR (qPCR; Bio-rad, Hercules, CA, United States). Primer and probe sequences and cycling conditions for nine antibiotic resistance genes (*sul1, sul2, tetO, ermB,* $bla_{CTX\text{-}M}$*,* bla_{TEM}*, qnrS, vanA, mecA)* and one integrase gene (*int1*) used as a proxy to assess ARG cassettes and mobility potential (Normal et al., 2009; Toleman et al., 2006), were obtained from literature and are listed in Table 5 in Chapter 3 of this thesis. Human-specific *HF183 Bacteroides* 16S rRNA genetic marker was also quantified to examine a potential correlation between human faecal pollution and ARG abundance (Sauer et al., 2011). All targets were quantified in all water samples and were run in duplicates for samples, and triplicates for positive control standards and no template controls (NTCs). Control plasmids, standard curve construction, LOQs and LOD, and qPCR reaction mixtures are all described in Chapter 3 and the same methods were used for analysis of samples in this chapter.

Raw fluorescence data were exported from the Bio-Rad qPCR system and imported into the LinRegPCR program (v 11.4) (Ruijter et al., 2009). The same process as detailed in Chapter 3 was used to obtain CT values and E_{fi} values. Samples efficiencies ranged from 79.5% to 123.9% and standard efficiencies ranged from 83.4% to 128.7%. The OPC method was again used for quantification of each ARG in each

sample (Brankatschk et al., 2012). For statistical calculations, any values that fell between the LOD and LOQ were set to ½ of LOQ value. Any values that fell below the LOD was set to ½ of the LOD. For calculation of the relative abundance of genes, the gene copy (GC) number of each gene was normalized to the GC number of the 16S rRNA gene in each sample and log transformed (log(GC/16S rRNA GC)). For calculation of the absolute abundance of genes, gene copies were normalized to the water volume used for gDNA extractions to generate GC per mL of water.

4.2.3 Assessment of Water Quality

Water samples were processed for further analysis within the recommended holding times for each parameter (24-48 hours). All samples were tested for the following water quality parameters: total suspended solids (TSS), volatile suspended solids (VSS), ammonia, nitrate, total coliforms, and *Escherichia coli*. Wastewater samples were also tested for $CBOD_5$, total nitrogen (TN), total phosphorous (TP), and chemical oxygen demand (COD). TSS and VSS were performed using Whatman™ 934-AH 47 mm glass fiber filters (Thermo Fisher Scientific) according to APHA standard methods 2540 D (American Public Health Association, 1999). Ammonia and nitrate were measured using high performance ammonia and nitrate electrodes, respectively, as directed by the manufacturer (Thermo Fisher Scientific). The electrodes were attached to an Orion Star™ series meter (Thermo Fisher Scientific). Total coliforms and *E. coli* were measured using IDEXX Colilert®-18 and Quanti-Trays® (IDEXX Laboratories, Inc., Westbrook, ME, United States) according to the manufacturer's instructions in IDEXX Laboratories, Inc. (2012). Analysis of $CBOD_5$ was performed in duplicate according to

the APHA standard method 5210B (American Public Health Association, 1999). TN was analyzed using Hach® TN Test 'N Tubes™ (0.5 to 25.0 mg/L N, Hach Company, Loveland, CO, United States), according to the manufacturer's procedure. TP was analyzed using Hach® TP Test 'N Tube™ ranging from 1.0 to 100.0 mg/L $PO4^{3-}$ (Hach Company), following the manufacturer's procedure. COD was analyzed using Hach® COD TNT plus Vial™ Test ranging from 20 - 1500 mg/L COD (Hach Company), following the manufacturer's procedure.

Water quality measurements for temperature (°C), conductivity (µS/cm), dissolved oxygen (DO (both % and mg/L)), pH, and oxidation reduction potential (ORP (mv)), were conducted on samples *in-situ*. The measurements were made with a handheld YSI 600R or 600QS multi-parameter water quality sonde (YSI Inc., Yellow Springs, OH United States). The sondes were calibrated prior to sampling according to the manufacturer's specifications. River flow rates were determined using the velocity – area method (Dingman, 2002) when the river was deemed safe to enter based on flow rates and water levels. Velocity and depth were measured using a Gurley Precision Instruments (Troy, NY, United States) 625DF2N digital pygmy meter.

4.2.4 Antibiotics Analysis

The samples were also tested for a range of 10 antibiotics consisting of amoxicillin, cefaclor, cefprozil, cefdinir, levofloxacin, ciprofloxacin, azithromycin, clindamycin, clarithomycin, and triclocarbon. See chapter 3 for detailed methods of analysis.

4.2.5 Data Analysis

One-way analysis of variance (ANOVA), Tukey HSD test, and t-tests were used to assess statistically significant differences ($p< 0.05$) among samples. Pearson correlation coefficients were used to assess significant ($p<0.05$) correlations between ARGs and water quality parameters. Analysis was performed using GraphPad Prism version 7.00 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com, and Mircosoft Excel, 2016. When performing analyses based on the average of all tested ARGs, abundances for each individual ARG were pooled and then averaged for each sampling site. Principal Component Analyses (PCA) were performed by calculating the average ARG absolute abundance for each individual ARG and dividing that value by the sum of average absolute abundances for all ARGs to obtain a ratio for each ARG target for a particular treatment step (i.e. for the effluent). These ratio values for each ARG for a particular WWTP step were the fingerprints used for PCA analysis.

4.3. Results and Discussion

4.3.1 Presence and abundance of ARGs in WWTP samples

All ARGs were detected in both the influent and effluent samples from all sampling events (August 18/2015, February 23, April 12, May 10, and August 2 2016), except *mecA,* which was below the LOD in samples from August 18/2015, as well as the effluent sample from August 2/2016, and *vanA* which was below the LOD in all samples in this chapter and will not be included in further analysis (Figure 9). The potential presence of *mecA* and *vanA* genes on chromosomal DNA (versus being present on broad-

host-range plasmids) (Biavasco et al., 2007; Cetinkaya et al., 2000; Colomer-Lluch et al., 2011; Katayama et al., 2000) may explain the lower presence of these genes, as bacteria who carry these genes in their chromosomes may not survive/be present in wastewater treatment as often as other bacteria (Goldstein et al., 2012). Furthermore, these genes confer resistance to antibiotics used as a last resort in clinical practices which may also contribute to their lower occurrence. There was a decrease of 1.93 log units from the influent to the effluent based off the pooled ARG averages from the five sampling events (pooled since similar results were observed for all events; also similar results as observed in chapter 3, indicating the WWTP continues to operate consistently throughout time and additional seasons). This indicates that the WWTP can decrease overall ARG absolute abundance significantly ($p<0.05$) throughout treatment. These results indicating a decrease of ARGs throughout treatment agree with previous studies (Chen and Zhang, 2013; Gao et al., 2015; Laht et al., 2014; Munir et al., 2011). However, the effluent still contained around 3 log units of ARGs, therefore although reduced, ARGs are still being discharged into Fales River and may be impacting the levels of ARGs in the river.

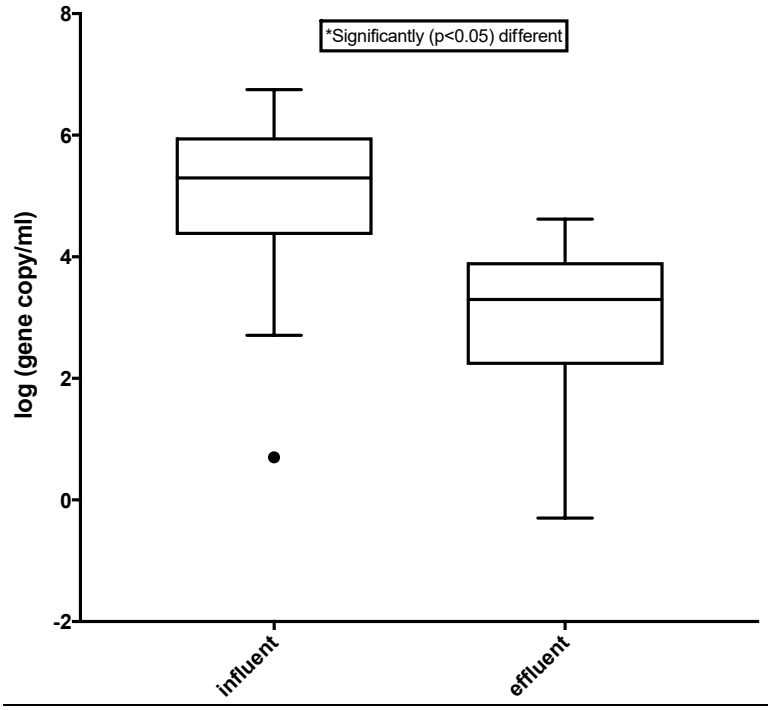


Figure 9: Box plots (box and whiskers Tukey method) of influent and effluent of the absolute abundance for all ARGs tested and int1 from August 2015 and February, April, May and August 2016. Whiskers are 75th percentile plus 1.5 times interquartile range (IQR), and 25th percentile minus 1.5 times the IQR. Median is represented by a line. Outliers are represented by points. Samples below the previously stated LOQ for each ARG were set to one half of the LOQ. Samples below LOD were set to one half of LOD (2.5 copies/rxn or 0.70 log (copies/mL) for influent samples or -0.30 log (copies/mL) for effluent samples, except *vanA* which was not included in analysis due to all samples being below detection. T-test was performed to compare influent vs. effluent and a significant ($p<0.05$) difference was found.

4.3.2 Presence and abundance of ARGs in water samples collected upstream and downstream of the WWTP effluent discharge point

Downstream (D) and upstream 1 (UP1) samples from August, 2015 and February, April, May, and August 2016 sampling events were analyzed for their abundances of ARGs. The results showed that when looking at the change in log units of gene copies/mL for each gene from UP1 to D, that there was a higher concentration of ARGs downstream from the WWTP than upstream (Figure 10A), meaning Greenwood's WWTP impacted on the levels of ARGs found in the river downstream from the plant. All ARGs were detected in both UP1 and D samples, except for bla_{TEM} (<LOD in UP1 and D samples in May), $bla_{CTX\text{-}M}$ (<LOD in UP1 and D samples in April, as well as UP1 sample in May), *sul2* (<LOD in D sample in April), *mecA* (<LOD in both UP1 and D samples in August 2015 and August 2016 UP1 sample).

When comparing each gene in UP1 and D samples with t-tests, only *ermB* was significantly ($p<0.05$) different, for both absolute and relative abundance. However, when examining abundance graphically (Figure 11), D samples look to harbour more genes than UP1 samples per mL. Furthermore, when pooling all ARGs for UP1 and D samples, there is a significant difference ($p<0.05$) in absolute abundance (Figure 12), which indicates that the WWTP does contribute to increased ARG levels in the river. This trend of increased ARG levels in samples taken downstream of WWTP outlets have been observed in other studies (Amos et al., 2014; Berglund et al., 2015; Proia et al., 2016; Zhang et al., 2015). Rodriguez-Moraz et al. (2015) found a significant increase in the absolute abundance of *ermB*, *qnrS*, and *sul1* genes when examining water samples taken during autumn/winter seasons upstream and downstream of a WWTP in Spain that

services around 800 000 inhabitants. Median ranges of around 1.8-2.1 log units upstream and 2.2-3.0 log units downstream were found, which is similar to what was found in this study even though the plant serviced many more inhabitants than Greenwood's plant, however river also size would impact these concentrations. Although increased levels were also found for bla_{TEM} and *tetW*, the differences were not significant, following similar statistical trends observed for many ARGs in this study where downstream ARG levels tended to be higher than upstream, however were not statistically significantly different. Makowska et al. (2016) also found significant increases in abundance of ARGs (*tetA* and *sul1*) downstream from a WWTP (servicing 120 000 inhabitants) outlet in a river in Poland during multiple seasons, finding similar levels to the current study, while downstream levels of *int1, tetB, tetM,* and *sul2* genes were not statistically significantly higher. The WWTP used activated sludge biological treatment with nutrient removal, however similar results were observed in the receiving river as for Greenwood's AL based plant, which suggests that WWTPs contribute to rising ARG levels in rivers regardless of the treatment system.

This study examined multiple ARGs in multiple seasons capturing both peak flow and base flow conditions in the receiving water of a smaller WWTP shown to significantly reduce ARG concentrations throughout treatment, and we have shown that a broad range of ARGs can still be detected with some increasing in abundance downstream of the effluent outlet even when meeting strict effluent discharge targets. Along with previous studies, these results highlight the need for further investigations into ways to reduce WWTPs' impacts on the receiving environment, as WWTPs located

in different locations around the world and under different waste loads and processes seem to be affecting the environment.

Looking at the relative ARG abundance (Figure 10B), here represented as the change in log units of gene copies/16s RNA gene copies for each gene from UP1 to D samples, it was apparent that the relative abundance of ARGs increased downstream from the WWTP, clearly indicating the impact of the WWTP on the proportion of bacteria carrying ARGs in water obtained downstream from the plant. Furthermore, when pooling all ARGs for UP1 and D samples, there was a significant difference ($p<0.05$) in relative abundance between the two sites. Interestingly, Marti et al. (2013) observed an increased relative abundance of ARGs in biofilm samples collected downstream of a WWTP discharge, and suggested a migration of ARGs from the water column to biofilms contributed to this, however this would be governed by the hydrodynamics of the river and the potential for deposition. They also suggested biofilms acting as long term reservoirs for ARGs. Other studies have examined WWTP effluents coming from treatment trains servicing from 1035 to 500 000 inhabitants using different types of treatment including conventional activated sludge and advanced tertiary nutrient removal systems, and also found increased relative abundance of ARGs in downstream biofilm, sediment, and water samples (Amos et al., 2014; Makowska et al., 2016; Proia et al., 2016). Taken together with the results from the present study, it appears that WWTPs regardless of the treated sewage volumes and treatment technology unavoidably increase the relative ARG abundances in downstream water samples in most receiving environments.

The impact of the WWTP on the ARG content in the river was higher during August sampling events, as there were larger differences between upstream and downstream samples for most ARGs (Figure 10A). This is likely due to lower river levels and flows (see Table 10 for flow rates (m^3/s)), which was observed during the summer season events, causing the effluent discharge to contribute more to the water levels downstream, as well due to lower levels of ARGs upstream during these sampling events. A study by Knapp et al. (2012) which examined a river in Cuba during both wet and dry seasons also found that waste outfalls more strongly influenced ARG levels in local downstream river samples in a drier season. They suggested lower seasonal water levels and increased local influence of outfalls, as well as reduced ARG transport due to lower flows in the river as potential reasons for the larger impacts observed, which corroborates what was observed in this study. The summer season (or dry-season in Cuba) is when the rivers are more likely to be used for recreational activities such as swimming and fishing, therefore high ARG concentrations are likely to have a more direct impact on public health. Colder and higher flow conditions such as what was observed in February and April (See Table 10) in Nova Scotia would dilute ARGs in the downstream water samples and have less impact on public health, also having less impact since recreational usage is limited during that time of the year in our study area. There also seemed to be higher levels of ARGs in the water in the upstream portions of the river during colder seasons and wet weather events as some negative values (i.e., no increase in the downstream sample) existed during February and April for some genes. This may be due to higher flows in the upstream watershed causing re-entrainment of ARGs from sediment, or storm run-off in the watershed. *Sul2* was much higher in the upstream

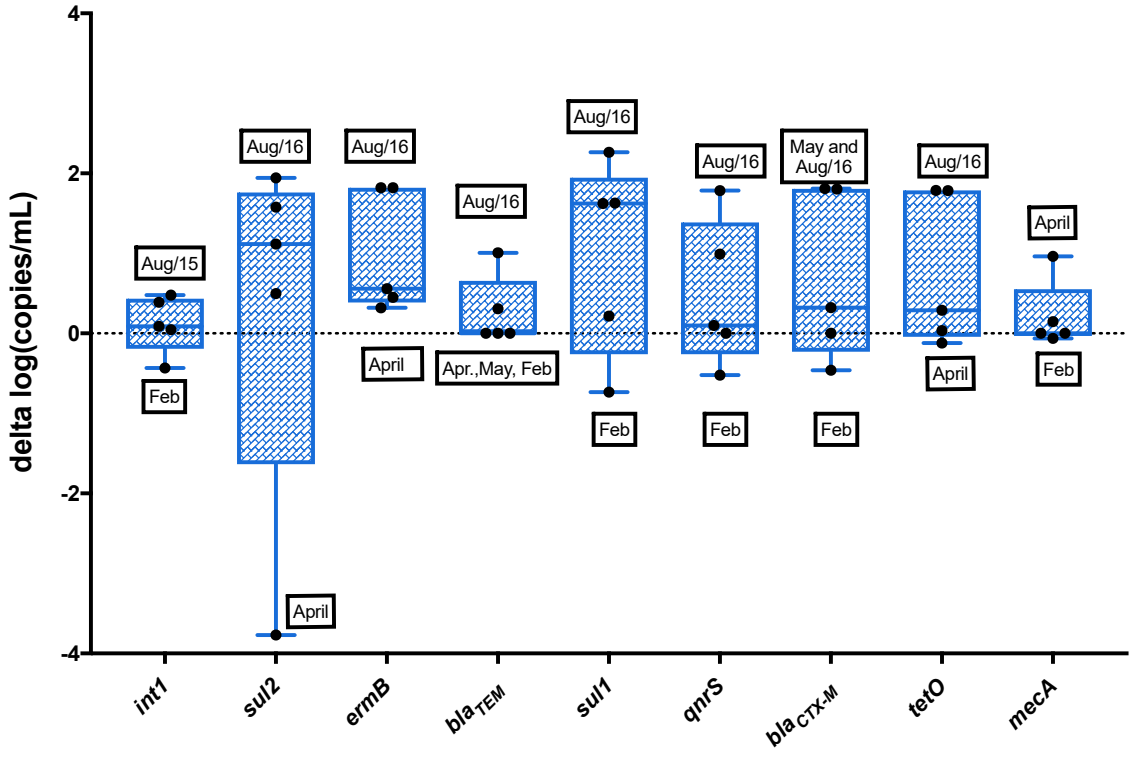
sample (3.47 log units) than downstream sample (-0.30 log units) in April, which could be related to the higher flow during the sampling period and activation of transport mechanisms in the upstream watershed. It is important to remember that when comparing samples upstream and downstream, that the grab samples represent snapshots of the river ARG levels. The above results highlight the importance of seasonal consideration when assessing ARG results in river samples, especially in areas in the world where seasons greatly impact water levels and environmental conditions. Many studies do not sample over multiple seasons nor consider flow rates or weather events when assessing ARG levels in surface waters, which have been shown in this study to significantly impact results.

It is interesting to note that UP1 samples contain similar abundances of genes (i.e. often ranging between 0-3 log units per mL) as D samples indicating an upstream influx of these genes into the river. Marti et al. (2013) detected ARGs in biofilm and sediment samples located 100 m upstream from a WWTP, suggesting an existing background level of AR naturally occurring in the environment. Likely a source of ARGs is other anthropogenic activities, such as livestock rearing, as was suggested by Marti et al. (2013). Other studies have also detected ARGs in upstream samples (Proia et al., 2016; Rodriguez-Mozaz et al., 2014; Storteboom et al., 2010), suggesting an existing background level of ARGs in most rivers; Proia et al. (2016) detecting ARGs in samples taken from a river impacted by limited anthropogenic activity 100 m upstream of a WWTP, Rodriguez-Mozaz et al. (2014) in samples taken 250 m upstream from a WWTP in a river which is an important source of drinking water for around $5.5x10^6$ inhabitants, and Stortebloom et al. (2010) in samples taken from a river in a pristine location with few

anthropogenic influences. Existing background levels of AR naturally occurring in the environment have been observed in other studies as well (Allen et al., 2010; Berglund et al., 2015; D'Costa et al., 2011). Sources such as livestock rearing, agricultural run-off, and transport mechanisms such as soil leaching have been suggested as some contributing factors (Proia et al., 2016), which may have contributed to levels upstream in Greenwood. Mobile organisms such as fish and invertebrates could also potentially contribute to some transport mechanisms of ARGs in rivers adding to upstream ARG levels. More on ARGs presence in the environment not impacted directly by wastewater will be discussed later in this chapter. Furthermore, UP1 samples varied greatly in ARG abundance as shown in the box plot, while downstream values seemed more consistent (Figure 12). This may be due to the consistent levels of ARGs reaching the downstream river from the WWTP, while the upstream river received a more variable influx of ARGs due to a variety of environmental conditions, which will be discussed in more detail later in this chapter.

In summary, the WWTP increased the abundance of ARGs in the river, however there were also high levels upstream from the plant indicating these ARGs are already present in this river. Therefore, while the WWTP did increase ARG levels in the river, it was not responsible for all of the ARGs in the river pointing to the existence of other contributing sources.

A)


delta log(copies/mL)
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2
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-2
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Aug/16
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Aug/16
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ermB
blaTEM
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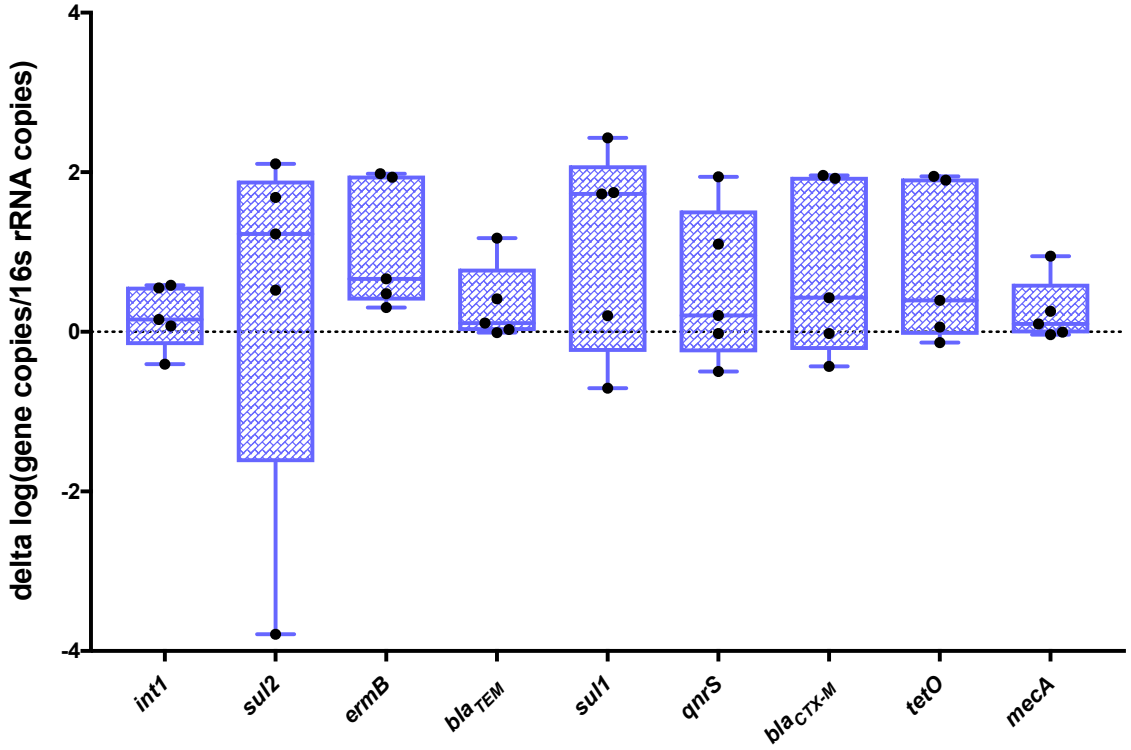
qnrS
blaCTX-M
tetO
mecA

B)


delta log(gene copies/16s rRNA copies)
4
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-2
-4
int1
sul2
ermB
blaTEM
sul1
qnrS
blaCTX-M
tetO
mecA

Figure 10: Change in ARG abundances from upstream 1 to downstream sites A) Box plots (Box and whiskers; min to max (show all points)) of the change in absolute abundance (gene copies/mL) from August 2015, February, April, May and August 2016 from UP1 to D. Positive and negative values indicate that there were more ARGs per mL downstream and upstream, respectively, from the WWTP. Blue lines represent median values. Highest and lowest points are labeled by sampling month to visualise seasonal trends. Samples below the LOQ for each ARG were set to one half of the LOQ. Samples below LOD were set to one half of LOD (2.5 copies/rxn or -0.30 log (copies/mL) for river samples). B) Box plots (Box and whiskers; min to max (show all points)) of the change in relative abundance (gene copies/16S rRNA gene copies) from August 2015, February, April, May and August 2016 from UP1 to D. Positive and negative values indicate that there were more ARGs per 16s rRNA copy downstream and upstream, respectively, from the WWTP. Purple lines represent median values. Highest and lowest points are represented by whiskers.

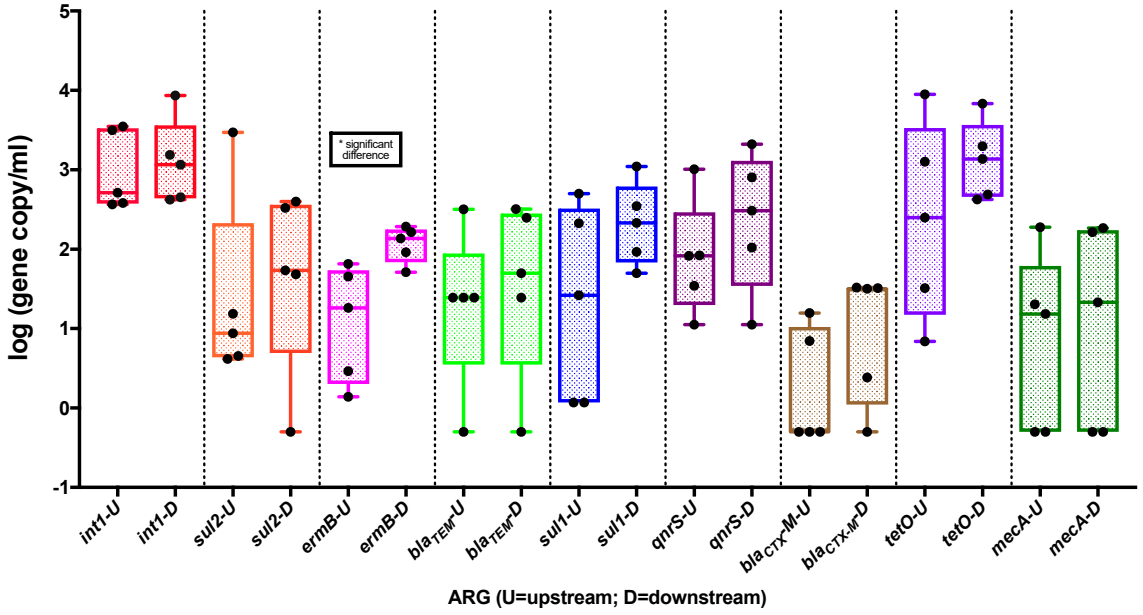


Figure 11: Absolute abundance of individual ARGs in water samples from August 2015, February, April, May and August 2016 in UP1 and D samples showing each gene. The box plots show all points from each sampling event, while the whiskers represent the minimum and maximum values for each gene. Lines indicate the median values. Samples below LOQ for each ARG were set to one half of the LOQ. Samples below below LOD and set to one half of LOD (-0.30 log (copies/mL) for river samples). T-tests showed that only *ermB* was significantly ($p<0.05$) different between up- and downstream samples.

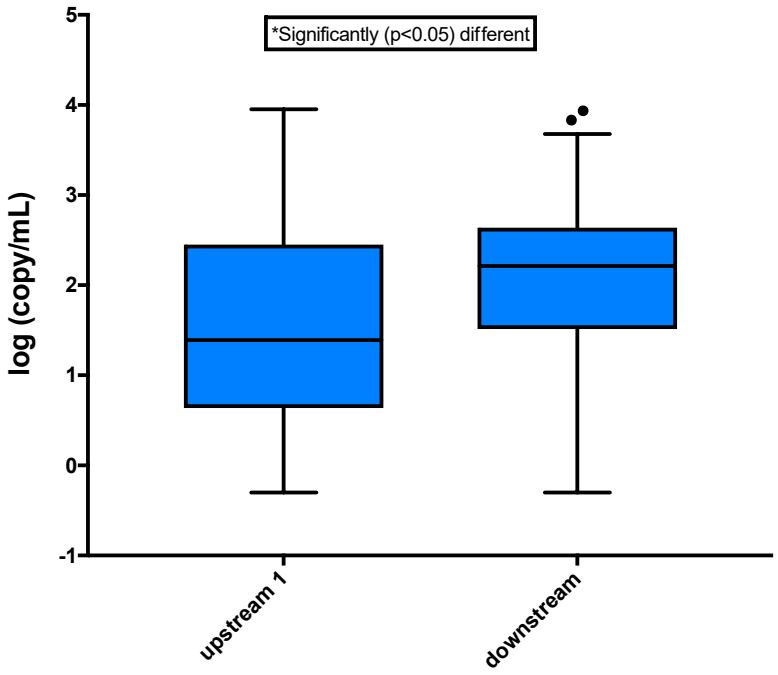


Figure 12: Box plots (Box and Whiskers Tukey Method) of the concentration of all ARGs (including *int1*) in water samples obtained in August 2015 and February, April, May and August 2016 from the upstream UP1 and D sampling sites. Outliers are represented by dots. Whiskers are 75th percentile plus 1.5 times interquartile range (IQR), and 25th percentile minus 1.5 times the IQR. Median is represented by a line.

Samples below the LOQ for each ARG were set to one half of the LOQ. ARG concentrations below the LOD were set to one half of LOD (-0.30 log (copies/mL) for river samples). T-test was performed to compare upstream vs. downstream and a significant ($p<0.05$) difference was found.

4.3.3 Presence and abundance of ARGs and 16S rRNA in Fales River upstream watershed

Sampling locations upstream in the watershed as shown in Figure 8 were also assessed for their absolute abundance of each ARG on April 12, May 10, and August 2 2016 sampling events. Initial sampling in August 2015 and February 2016 revealed the presence of ARGs in samples from the upstream site (UP1), therefore additional upstream sampling sites were added to further investigate these ARG levels. The abundance of the ARG copies/mL in the river samples displayed seasonal/weather event differences, i.e., April samples contained significantly ($p<0.05$) higher ARG copies/mL compared to May and August water samples (Figure 13). The peak in ARG abundances in April coincided with the highest stream flows (Table 10) compared to May, and then August, and also the lowest temperatures (April river average = 7.1°C) followed by May (river average = 10.9°C) and then August (river average = 19.1°C). During the April 12 sampling event, there was also snow melt likely happening (mean temperature of the day was 11.2°C, which was warmer than previous 7 days, and snow on the ground was reported within 7 previous days), and moderate rain fall occurred (17.4 mm/day, and also rained 5 out of 7 previous days) which likely caused peak flow conditions (Government

of Canada, 2016). In contrast, the mean temperatures for May 10 and August 2 were 9.9°C and 19.9°C, respectively, with 0 mm of rain on both of those days (Government of Canada, 2016). This suggests that during the April sampling, there was a flushing event (spring freshet) which increased chance of contaminants entering the river that may have contributed to the increased ARG abundances. Knapp et al. (2012) also found seasonal variations in ARG levels in a river in Cuba, finding wet seasons to have an increased abundance of ARGs compared to dry seasons. Snow melts and wet seasons appear to allow the movement of ARGs from soils or sediments into the water column to increase transport down the river, thus causing elevated ARG levels.

Figure 14 shows boxplots of ARG absolute abundance results at each sampling site separated by sampling event. As shown previously, this figure also shows that the river seems to be influenced by seasonal conditions, while the WWTP seems to remain relatively stable through seasons. Levels in the upstream river were higher at most sites in the April samples with obvious "hotspots" in April observed in UP4 and UP6 samples (on same side of watershed) but not in the UP5 sample (different side of watershed), suggesting that ARG contamination may have came from the eastern side of the watershed or the side stream where UP6 is located. UP4 and UP6 are located near more urbanized areas, therefore that may have contributed to these seasonal peaks in ARG abundance, as urbanization has been suggested to increase ARG levels (Ouyang et al., 2015).

Excluding "hotspots" in April, gene concentrations follow a general trend of decreasing the further upstream and away from urbanization the sample was taken (note that since there are two primary tributaries in the watershed UP6 does not necessarily

follow the ranking system for UP1-UP7). However, concentrations seem to remain at a consistent level even at the "pristine" site (i.e. UP7) not surrounded by urbanization. This suggests ARGs are present and can persist in the environment even without typical selective pressures, as supported by other studies. Ouyang et al. (2015) found a higher abundance (2 orders of magnitude higher) and diversity of ARGs and MGEs in urban stream samples than in more "pristine" (remote suburban mountain free of human activities) samples. However, they still detected ARGs in the "pristine" samples. Stortebloom et al. (2010) and Pruden et al. (2006) also found similar results of higher ARG detection in more urban effected areas vs. more "pristine" environments (upstream river), however still detecting low levels of ARGs in "pristine" samples as well. Taken together our findings and previous reports suggest that anthropogenic activities do increase the levels of ARGs in the environment, however, ARGs also exist in the environment with less obvious human inputs.

It's been suggested that ABs have been produced naturally for over 500 million years (Baltz, 2008). Antibiotics and ARGs have therefore existed in the environment before human AB therapeutic uses came into effect, however human activities have most likely increased the prevalence and spread of AR (Allen et al., 2010). Studies reported in a review by Allen et al. (2010) suggest that animals living in closer proximity to humans have a higher abundance of ARGs and ARB and may represent vehicles for the spread of these AR determinants around the world and even to remote locations (i.e. migratory birds). Similar results were observed for humans, i.e. higher levels of AR found in humans living more closely to urbanized areas. However even isolated human populations still had some level of AR detected, suggesting harbourage of AR even in the

absence of obvious selective pressures (Allen et al., 2010). Also, it has been suggested that no environment can be considered truly "pristine" anymore, which may explain the finding of low ARG levels in "pristine" environments reported here and other studies. Antibiotic resistance genes in the environment may also have alternative cellular and environmental roles in bacteria (Allen et al., 2010). It is possible that genes that confer resistance to antibiotics in pathogens may have an alternative purpose in their original host explaining their presence in different compartments of the environment without typical selective pressures (Martinez, 2009). In this review, it is suggested that ARGs may not always place a metabolic burden on bacteria as once thought, and in some cases can even be beneficial for bacterial growth. Therefore resistance does not always disappear in the absence of selective pressures, and once established can spread and invade different ecosystems. Therefore, with a combination of the above factors (i.e. natural pre-antibiotic era ARG presence, spread through animals and humans from urban areas, alternative functions in bacteria, and low cost to bacteria), it is not surprising that ARGs are found in areas not under direct influence of modern selective factors and seem to increase the more closely the area is located to urbanization (Chen et al., 2015).

The variations in individual ARG levels among the sampling sites in the watershed are shown for some different ARGs in Figure 15 (for remaining ARGs, see Appendix B Figure B1). Most ARGs decreased as the samples were collected further away from urbanization (except the April "hotspots"). However, *mecA* behaved in unexpected ways having high presence in April and some May upstream samples. *MecA* confers resistance to antibiotics used as a last resort in clinical practices, therefore presence of this resistance gene was expected to be low in the environment due to lower

clinical use. *MecA* is also often present on chromosomal DNA and therefore only carried by certain bacteria which would also likely limit its environmental presence (Katayama et al., 2000). It is possible that during these higher flow conditions *mecA* entered the river through releases from melting snow, septic system discharges, storm runoff, presence of selective factors, and re-entrainment from sediment during the increased flow. *Int1*, *sul2*, *ermB*, *sul1*, $bla_{CTX\text{-}M}$ and *mecA* were the genes that seemed to be most affected by the wet weather sampling event "hotspot" phenomenon in April. *Sul2*, *ermB*, *sul1*, $bla_{CTX\text{-}M}$, and *mecA* were also not consistently present in upstream samples, indicating the genes that flushed into the river in high abundances in April seemed to be less commonly found in the river during other seasons. *Int1* and *tetO* remained present in consistently higher levels in both the river and plant compared to other ARGs. *TetO* confers resistance to tetracycline ABs, an older group of highly consumed ABs (Laht et al., 2014), which may explain its high presence. Studies have suggested that integron abundance is increased by human contaminations and may explain *int1*'s high presence in more urbanized areas (Chen et al., 2015). This suggests that more urbanized areas are more capable of HGT and ARG spread.

A principal component analysis was performed in order to determine if the variance in ARG fingerprint (i.e. the proportion of each ARG in a sample out of total ARGs) was better explained by sample type (wastewater effluent or river sample) or sampling month (April, May, or August) (Figure 16) (Metsalu et al., 2016). It was found that the water type explained 62.9% of the variance in fingerprint, while the sampling month explained 21.9% of the fingerprint variance, indicating that most of the variance in fingerprint is caused by sample type. This analysis also showed that the WWTP effluent

ARG fingerprint is less affected by seasons compared to the river samples. Furthermore, an additional principal component analysis was performed in order to determine if the variance in ARG fingerprint was better explained by river sample location (i.e. upstream or downstream of the WWTP) or sampling month (April, May, or August) (Figure 17) (Metsalu et al., 2016). It was found that the difference in ARG fingerprints is better explained by the location of the river sample (upstream or downstream of the WWTP) (42.7%) than the sampling month (35.8%), suggesting that the WWTP effluent discharge affects ARG fingerprints in the river.

ARG levels in the river ranged from below detection to 3.95 log units (copies/ml), with the highest concentration detected for *tetO* in the UP1 sample from April. Downstream samples often ranged between 2-3 log units, while upstream samples ranged around 0-3 log units. Other studies have found even larger ranges. Jiang et al. (2013) found a range of around 1 to 5 log units of ARGs (*sul1, sul2, tetA, tetB, tetC, tetG, tetM, tetO, tetW, tetX,* bla_{TEM}*;* the majority being detected in all river samples) in a river in China, interestingly finding higher ARG levels in suburban vs. urban samples. However, there were agricultural activities and animal production in the suburban area likely contributing to the increased abundance of ARGs. Similarly, Makowska et al. (2016) detecting a range of 0-3 log in upstream river samples and 1-3 log in downstream samples in Poland while Ouyang et al. (2015) found high levels of 8 log unit total abundance of ARGs copies/mL in an urban stream in China and 5.9 log in pristine samples. It seems ARG levels in rivers vary based on the country and amount of urbanization/agricultural activity nearby. Berglund et al. (2015) suggested that factors such as temperature and nutrient availability in rivers may affect resistance development

and therefore may explain why different locations around the world yield different results. Other important factors determining AR development may be population density, the presence of sewage treatment discharge, the burden of infectious disease in the area, as well as the tradition for AB usage in agriculture, aquaculture, and human medicine.

It should be noted that the genes detected could come from both extracellular DNA (eDNA) and genes from within intact live bacteria, a fact which could affect the future of these ARGs. If viable ARG harbouring bacteria were decreased at the same rate as *E. coli* by the wastewater treatment (see Figure 18B), then many of the ARGs leaving the plant may be associated with dead bacteria, or in extracellular form such as plasmids from ruptured cells. However, a study by Mao et al. (2014) showed that ARGs can persist in both forms (eDNA and iDNA) up to two weeks in the water column, potentially allowing time for HGT and transformations (i.e. risk of uptake of ARGs by competent pathogens such as Pseudomonas in the environment (Demanche et al., 2001)). Furthermore, extracellular ARGs are able to be transferred from the water column to biofilms and sediment (Chen et al., 2015; Marti et al., 2013; Torti et al., 2015), and therefore persist even longer. Transformation of eDNA (kanamycin resistance genes) to indigenous bacteria was also observed by Mao et al. (2014). Therefore the presence of ARGs in water samples, even if they are not contained in live bacteria, is still able to contribute to reservoirs of ARGs in the environment, and allow potential transformation into environmental bacteria and pathogens, and therefore should be further investigated.

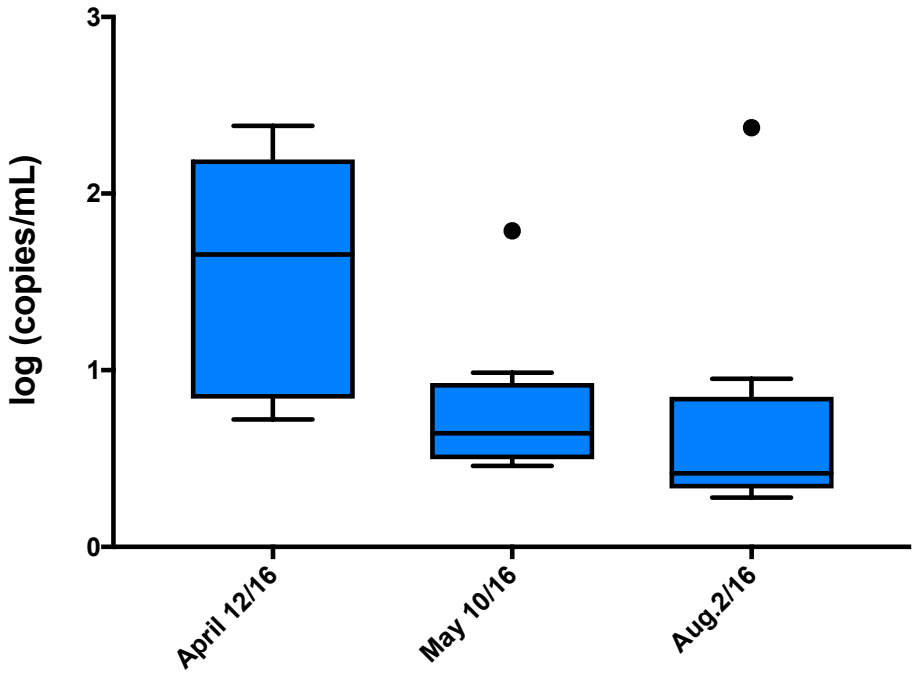


Figure 13: Boxplot (box and whiskers Tukey) of absolute abundance of all ARGs from downstream and upstream samples (UP1-UP7) separated by month, from April, May and August 2016. Outliers are shown as dots. There is a significant difference between April and May samples and between April and August samples ($p<0.05$) however not between the May and August samples.

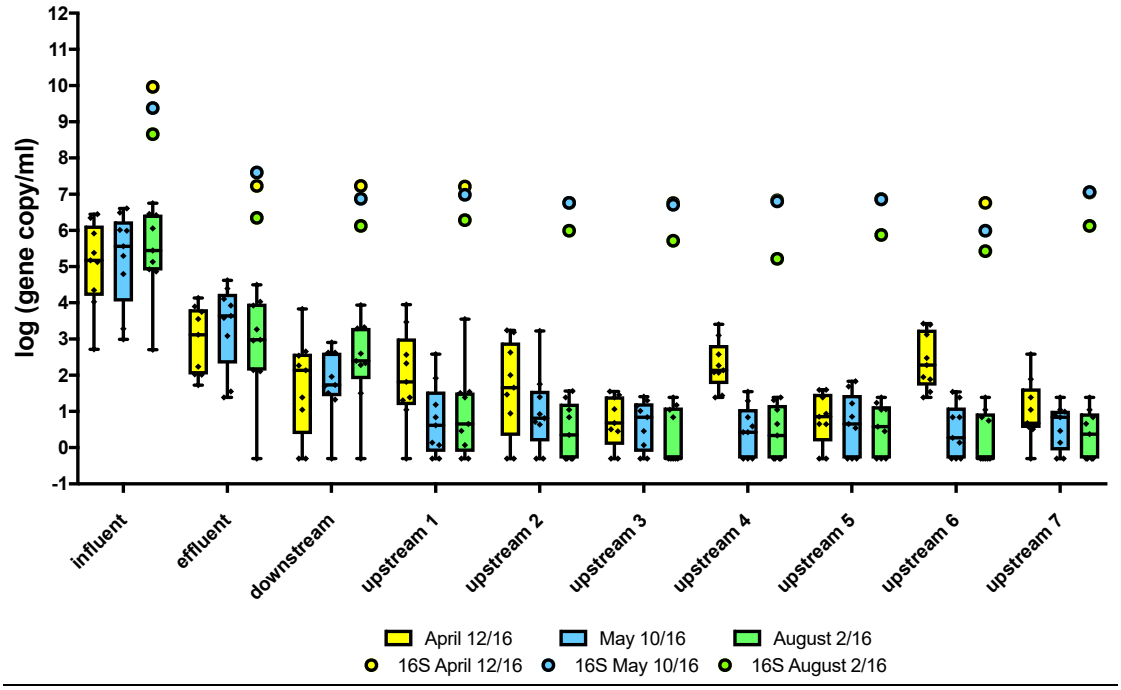


Figure 14: Box plots (min to max showing all ARGs) showing the seasonal ARG absolute abundances in the influent and effluent from the WWTP and water samples obtained downstream and upstream from the WWTP in Fales River. Dots represent each ARG marker, while lines represent the median log (gene copies/mL) of each sampling location. Samples below the LOQ for each ARG were set to one half of the LOQ. Samples below the LOD were set to one half of LOD (-0.30 log (copies/mL) for river samples). 16S rRNA log (gene copies/mL) levels are also shown.

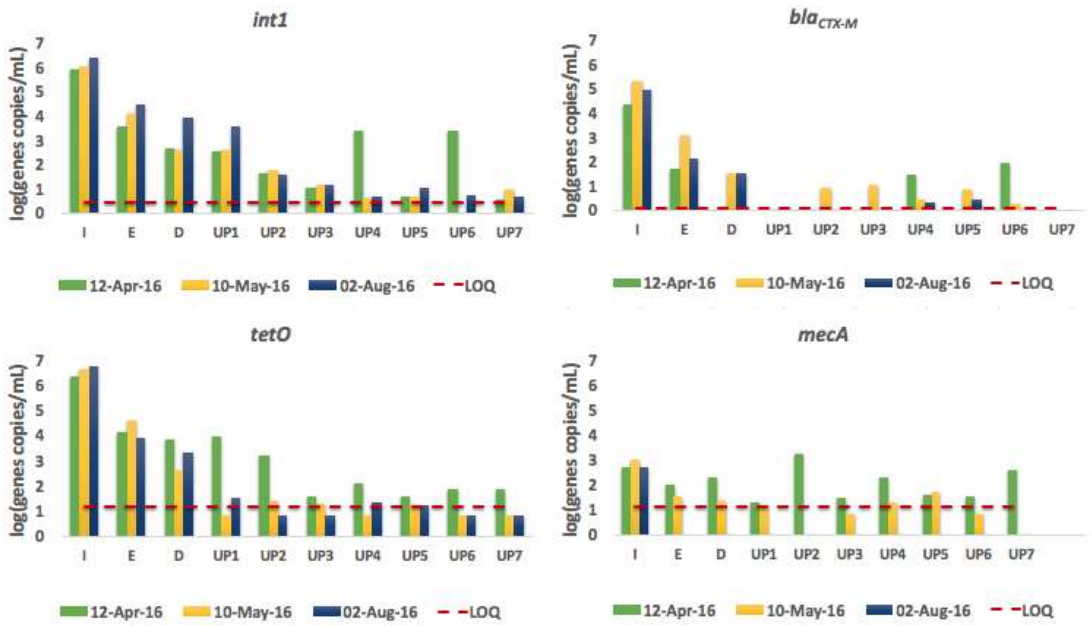


Figure 15: Seasonal ARG absolute abundances (log (copies/mL)) for selected ARGs in the influent (I) and effluent (E) from the WWTP and water samples obtained downstream (D) and upstream (UP) of the WWTP. Samples below the LOQ (shown as a red dashed line) for each ARG were set to one half of the LOQ. Samples below below LOD were set to one half of LOD (-0.30 log units) for river samples).

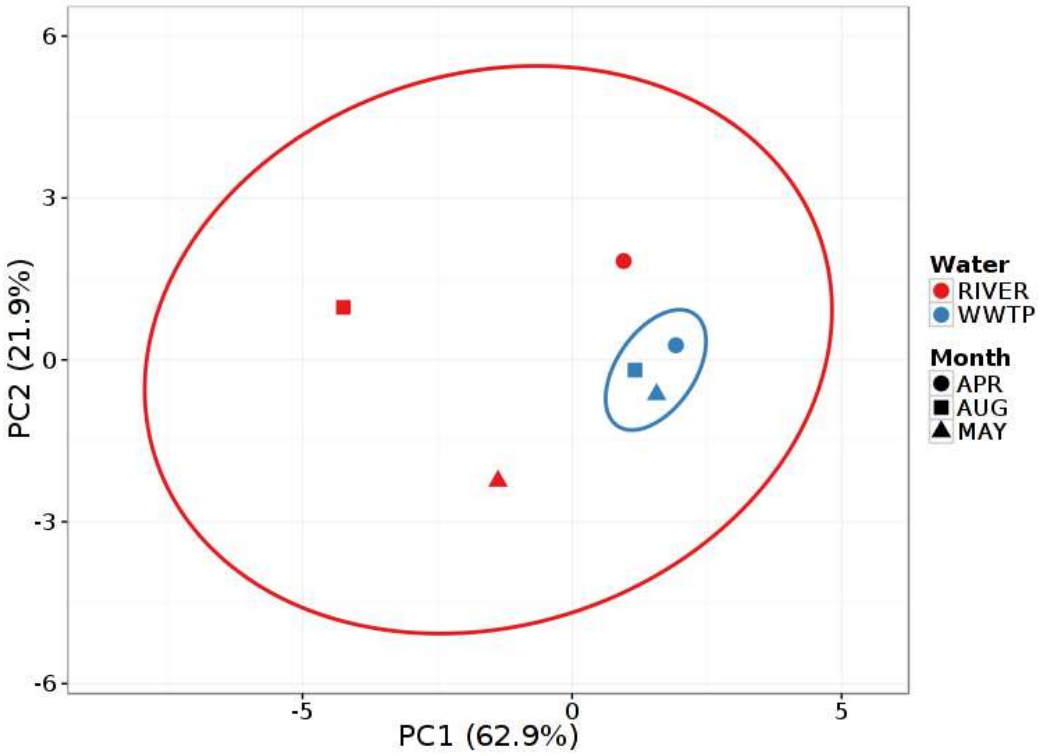


Figure 16: Principal Component Analysis (PCA) plot of the ARG profiles comparing water type i.e. WWTP effluent or river sample (D, UP1-7), and sampling month, i.e. April, May, or August.

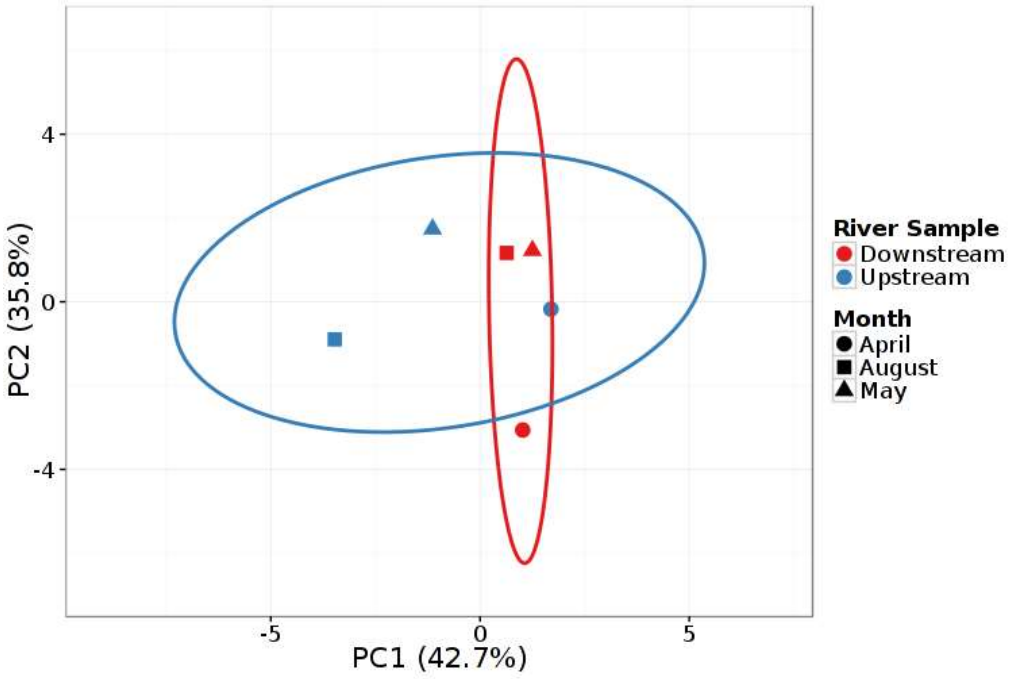


Figure 17: Principal Component Analysis (PCA) plot of the ARG profiles comparing river samples (i.e. upstream (UP1 to UP7) vs. downstream (D)), and sampling month, i.e. April, May, or August.

4.3.4. Total coliforms, *E. coli*, and *HF183* marker levels and correlation to ARG abundance

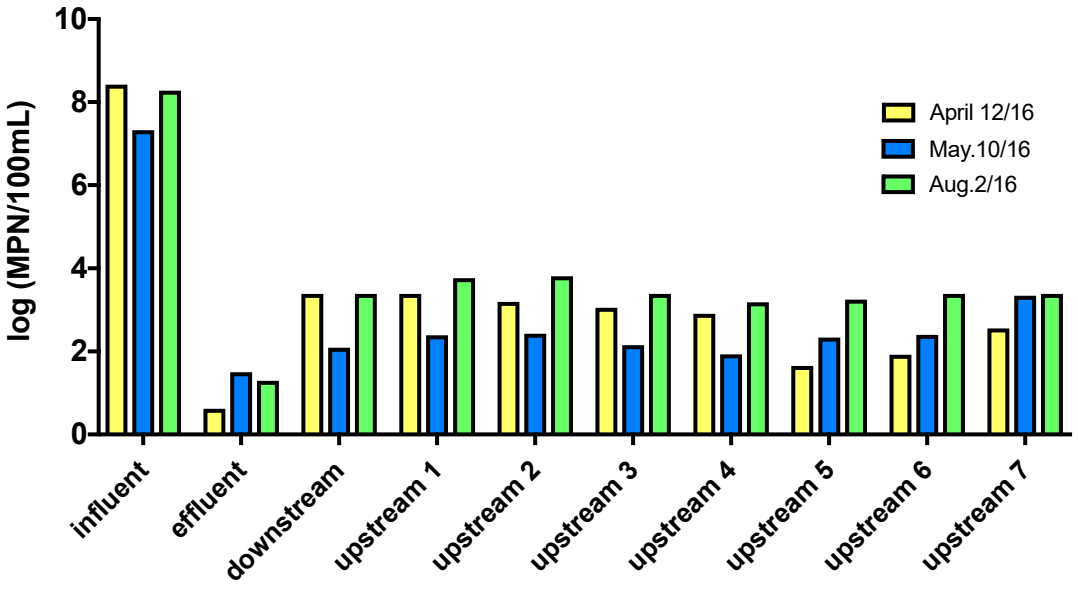
Figure 18 shows the content of fecal indicator markers, total coliforms, *E. coli*, and *HF183* human *Bacteroidales* marker in water samples obtained on the April, May and August sampling trips in 2016. Total coliforms decreased throughout the treatment of the wastewater and were found in fairly consistent levels throughout the river sites in all sampling events (Figure 18A). Significant ($p<0.05$) Pearson coefficient correlations

were obtained between average ARG levels and total coliforms for April and May (April r=0.660; May r=0.690) when examining the WWTP and river samples (n=10). Levels of *E. coli* similarly decreased throughout the WWTP treatment and remained close to the detection limit in the more remote portions of the watershed (after upstream 3) for all seasons (Figure 18B), indicating that the majority of ARGs being detected in the upstream river, and especially April's hits, are not coming from live *E. coli*. Significant ($p<0.05$) correlations were also found for ARG levels and *E. coli* for all months (April r=0.710; May r=0.820; August r=0.710). The *HF183* marker, which detects human types of *Bacteroidales* and can be used as a proxy to measure human faecal pollution (Sauer et al., 2011), decreased throughout wastewater treatment following a similar log reduction as ARGs (Figure 18C and Figure 14 for *HF183* and total ARGs, respectively), indicating the removal process may be similar between the two genetic marker types. *HF183* also seemed to decrease the further the samples were taken upstream, however increased abundances were observed around UP6 and UP7 (Figure 18C), pointing to an upstream source of human fecal contamination in the watershed. There were significant ($p<0.05$) correlations between *HF183* levels and ARG levels for each month (April r=0.879, May r=0.845, August r=0.860). This suggests the *HF183* marker could be used as an indicator of ARGs in rivers as well as wastewater. *HF183* was the only factor analyzed that seemed to "peak" in the April samples containing high ARG levels, indicating it may be useful in predicting spikes in ARG levels. A study by Sauer et al. (2011) found the *HF183* marker present in the majority of samples taken at urban stormwater run-off outfalls, suggesting that *HF183* is ubiquitous in an urban environment. Levels ranged in most samples between 1-2 log (*HF183* copies/mL) and were similar to the levels found in

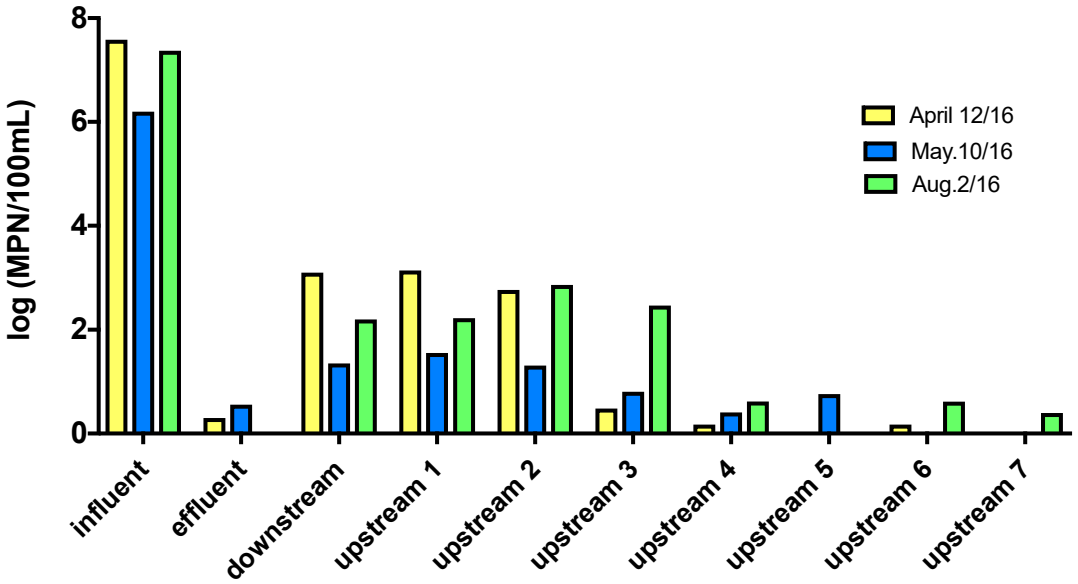
the current study for upstream samples, suggesting human sewage is widespread in rivers impacted by urbanization in this study as well. Sauer et al. (2011) also detected *E. coli* in ranges of around 3-6 log (MPN/100 mL) in majority of samples. While *E. coli* levels were on the lower end, results from the current study were similar for downstream and upstream samples closer to urbanization.

In summary, total coliforms, *E. coli*, as well as *HF183* all correlated with ARG levels to some degree, with *HF183* being the strongest predictor of ARGs in both the plant and river. *HF183* marker is a genetic marker as are ARGs and can be detected from both living and dead bacteria, while total coliforms and *E. coli* are detected from living organisms, which may contribute to *HF183* more accurately predicting ARG levels. The more "pristine" sampling sites also seem to contain both ARGs and *HF183* marker, which suggests that even areas not surrounded by high levels of urbanization are still vulnerable to contamination by these factors. It's possible that there could be septic systems from nearby houses or cabins that are contributing to human fecal contamination and ARGs in these areas.

A)


log (MPN/100mL)
10
8
6
4
2
0
April 12/16
May.10/16
Aug.2/16
influent
effluent
downstream
upstream 1
upstream 2
upstream 3
upstream 4
upstream 5
upstream 6
upstream 7

B)


log (MPN/100mL)
8
6
4
2
0
April 12/16
May.10/16
Aug.2/16
influent
effluent
downstream
upstream 1
upstream 2
upstream 3
upstream 4
upstream 5
upstream 6
upstream 7

C)

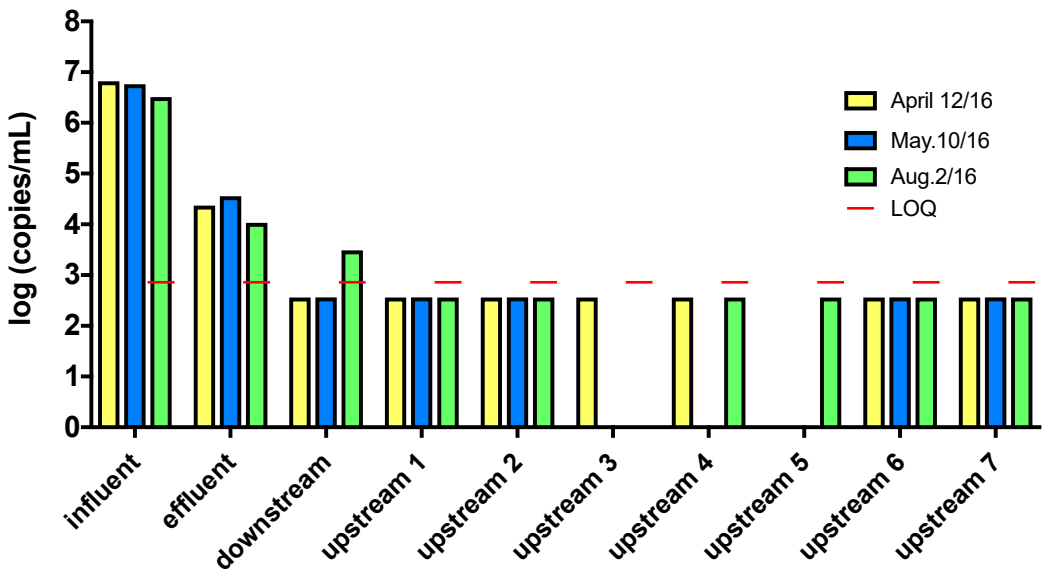


Figure 18: Content of fecal indicator markers in influent and effluent samples from the WWTP and water samples downstream and upstream from the discharge point on Fales River. A) Total coliforms log (MPN/100mL) on April, May and August 2016 (LOD 1 MPN/100 mL). B) *E. coli* values on April, May and August 2016 (LOD 1 MPN/100 mL) and C) HF183 human *Bacteroidales* marker on April, May and August 2016. *HF183* values were corrected if below the LOQ (values <LOQ set to half of the LOQ). Samples that appear not to have a bar are <LOD and therefore contained <0.5 HF183 copies/mL or less.

4.3.5 Water quality assessment and correlations to ARG abundance

4.3.5.1 Nutrients and solids correlations to ARG abundance

Physico-chemical characteristics of the wastewater and river water are shown in Table 9. The effluent leaving the WWTP consistently met the regulatory target values for both TSS and $CBOD_5$ (both 5 mg/L) during the sampling period, indicating that the plant operated efficiently. For all sampling months there was a significant correlation

between average ARG concentrations and ammonia, TSS and VSS (except May where there was a lack of data for TSS and VSS, and August where there was a lack of data for ammonia). Absolute abundance of ARGs correlates moderately with these water quality indicators, however there were no obvious spikes in any water quality test that correlated with April's "hotspots" abundances, indicating that these factors are not indicative of independent spikes of ARGs.

UP7 samples contained more TSS than some more downstream samples. UP7 sampling site was often lower in water levels allowing sediment to potentially enter the collection bottles, which may contribute to these higher levels of solids as well as some unexpected ARG detection (i.e. ARGs may have been contained in sediment). Downstream samples contained an elevated level of nitrate, however, this was not statistically ($p>0.05$) different from upstream 1 samples.

Table 9: Water Quality data from Greenwood including influent and effluent samples from the WWTP and down- and upstream river samples. Pearson Correlation coefficient is listed for each sampling month assessing ARG concentrations with water quality from the three sampling events. Asterisk indicates significant correlation ($p<0.05$); n=10. Bolded values represent factors that have provincial effluent regulations. COD, TN, TP, and $CBOD_5$ were only measured within the plant.

Sample (2016)	Average Nitrate (mg/L)	Average Ammonia (mg/L)	Average TSS (mg/L)	Average VSS (mg/L)	Average COD (mg/L)	Average TN (mg/L)	Average TP (mg/L)	CBO D_5 (mg/ L)
April								
I	3.02	13.37	122.30	110.70	197.67	34.90	5.27	68.75

Sample (2016)	Average Nitrate (mg/L)	Average Ammonia (mg/L)	Average TSS (mg/L)	Average VSS (mg/L)	Average COD (mg/L)	Average TN (mg/L)	Average TP (mg/L)	CBO D_5 (mg/L)
E	11.70	0.75	**1.00**	0.00	10.00	27.20	0.00	**3.64**
D	0.44	0.05	33.20	0.00				
UP1	0.25	0.02	1.60	0.00				
UP2	0.19	0.01	3.20	0.00				
UP3	0.15	0.08	1.70	0.20				
UP4	0.12	0.05	0.20	0.00				
UP5	0.12	0.04	0.00	0.00				
UP6	0.29	0.03	0.00	0.00				
UP7	0.10	0.02	11.40	0.00				
May								
I	4.67	20.00	N/A	N/A	119.00	28.00	4.13	83.50
E	16.90	0.00	N/A	N/A	64.00	20.00	0.00	**1.78**
D	0.12	0.00	N/A	N/A				
UP1	0.05	0.20	N/A	N/A				
UP2	0.03	0.00	N/A	N/A				
UP3	0.02	0.60	N/A	N/A				
UP4	0.01	0.08	N/A	N/A				
UP5	0.01	0.03	N/A	N/A				
UP6	0.00	0.30	N/A	N/A				
UP7	0.00	0.30	N/A	N/A				
August								
I	N/A	N/A	179.00	162.67	449.67	N/A	10.77	140.38
E	N/A	N/A	**1.50**	1.25	16.00	N/A	5.70	**1.52**
D	N/A	N/A	1.80	0.80				
UP1	N/A	N/A	2.20	0.80				
UP2	N/A	N/A	1.00	0.60				
UP3	N/A	N/A	1.50	5.00				
UP4	N/A	N/A	0.80	0.40				
UP5	N/A	N/A	1.20	0.40				
UP6	N/A	N/A	2.00	1.40				
UP7	N/A	N/A	30.00	15.60				

Sample (2016)	Average Nitrate (mg/L)	Average Ammonia (mg/L)	Average TSS (mg/L)	Average VSS (mg/L)	Average COD (mg/L)	Average TN (mg/L)	Average TP (mg/L)	CBO D_5 (mg/L)
April r=	0.449	0.836*	0.784*	0.819*				
May r=	0.635*	0.821*	N/A	N/A				
August r=	N/A	N/A	0.776*	0.789*				

N/A: data not available

4.3.5.2 River physical parameters from Sonde measurements and flow and correlations to ARG abundance

Table 10 shows results from *in-situ* Sonde measurements for each month. In all sampling months, conductivity was positively correlated (r-values ranging between 0.85 to 0.97) with ARG levels. This indicates that the higher conductivity of the water, the more ARGs are likely to be detected. Conductivity is often associated with levels of total dissolved solids, which can be increased by wastewater and urban runoff, therefore it is not surprising these measurements increase in sampling areas more impacted by urbanization (Behar, 1996). Negative correlations were found between DO levels and ARGs, indicating that the lower the DO levels in the water, the more ARG are likely to be found. Negative correlations were also found for ORP and ARGs. A positive ORP reading indicates the presence of oxygen, while negative values indicate the absence of oxygen (Aqua Health Products Inc., 2016). Therefore, these results suggest the more oxygen present in a sample, the lower the levels of ARGs. This may have to do with the longevity of the ARG markers in the water as one would expect faster degradation in an oxygenated environment. pH was found to be positively correlated with ARGs in May

indicating a higher pH correlated with higher levels of ARGs during that sampling event. Al-Badaii et al. (2015) also observed positive correlations between AR coliform bacteria and conductivity, and negative correlations with DO, however finding negative correlations to pH in their study.

When comparing average water quality data between downstream and upstream 1 samples there were no significant differences ($p>0.05$), however there were larger averages for conductivity, and ORP in downstream samples, which may suggest the WWTP does increase these factors downstream slightly. Using upstream 3 as an indicator of seasonal changes in flow, it can be seen that the peak flow in April (3.33 m^3/s), falls to 1.15 and 0.02 m^3/s in May and August, respectively, showing that flows can range over several orders of magnitude during the year.

4.3.5.3 Summary of water quality

Al-Badaii et al. (2015) suggested that AR may be related to inferior water quality from such sources as untreated sewage in rural areas, WWTP effluents, urban runoff, and agricultural and industrial activities, as increased levels of AR were found as water samples were taken closer to these sources. Since correlations between some water quality indicators and ARG levels were also found in this study, it supports the above findings. However, repeated sampling to determine the strength of these correlations in other seasons and and other areas in the watershed is recommended.

Table 10: Water quality data from on site sonde measurements. Significant ($p<0.05$) correlations to ARG concentrations are marked with an asterisk. N/A signifies a lack of a measurement.

Sample (2016)	Temp-erature (°C)	Conductivity (µS/cm)	DO (%)	DO (mg/L)	pH	ORP (mv)	Flow (m^3/s)
April							
I	9.76	687	32.4	3.67	7.23	107.9	N/A
E	9.48	547	68.8	7.84	6.61	278	N/A
D	6.80	37	N/A	N/A	6.82	471.4	>safe
UP1	6.70	28	N/A	N/A	7.65	452.4	>safe
UP2	7.25	24	8.0	13.00	5.67	356.1	>safe
UP3	7.12	40	105.9	12.80	6.37	375.2	3.22
UP4	7.05	22	105.9	12.84	5.64	380.3	>safe
UP5	6.97	20	104.6	12.71	5.18	400.9	>safe
UP6	7.23	27	104.9	12.66	5.19	383.3	Too shallow and rocky
UP7	8.07	21	99.6	11.78	4.04	404.9	>safe
May							
I	11.8	704	27.2	2.92	7.12	73.7	N/A
E	12.23	537	73.8	7.90	6.51	286.3	N/A
D	10.68	44	106.2	11.80	5.98	255.0	1.06
UP1	10.68	44	106.2	11.99	6.26	213.8	1.21
UP2	11.00	25	107.9	11.87	5.89	278.5	>safe
UP3	10.08	25	107.2	11.87	5.90	293.6	1.15
UP4	10.08	23	106.6	11.79	5.74	302.5	>safe
UP5	10.88	21	105.6	11.66	5.24	355.8	0.63
UP6	10.05	27	107.0	12.07	5.75	364.4	Too shallow and rocky
UP7	13.77	21	103.2	10.67	4.28	370.0	0.13
August							
I	18.93	816	3.0	0.30	7.42	86.5	N/A
E	22.29	596	110.3	9.57	7.45	160.8	N/A
D	19.26	148	159.9	14.75	7.34	113.2	0.04
UP1	18.42	58	155.1	14.55	7.50	50.0	0.06
UP2	19.47	48	135.6	12.48	5.70	6.4	>safe
UP3	18.43	41	130.2	12.23	5.72	342.8	0.02
UP4	18.71	33	153.9	14.37	7.15	158.7	Too low
UP5	19.29	28	146.8	13.54	6.94	167.7	Too low

Sample (2016)	Temp-erature (°C)	Conductivity (µS/cm)	DO (%)	DO (mg/L)	pH	ORP (mv)	Flow (m^3/s)
UP6	16.76	51	106.5	10.32	6.94	167.7	Too low
UP7	22.75	26	114.2	9.82	5.94	213.4	Too low
April r=	0.735*	0.847*	-0.493	-0.876*	0.490	-0.826*	
May r=	0.339	0.967*	-0.957*	-0.950*	0.726*	-0.784*	
August r=	0.139	0.945*	-0.749*	-0.756*	0.558	-0.499	

4.3.6 Antibiotics and correlations with ARG abundance

Table 11 shows the content of ABs analysis in water samples from April, May and August of 2016. There were significant positive correlations in April ($r=0.805$, $p<0.05$) and May ($r=0.906$, $p<0.05$) between the total ABs (sum of all antibiotics (ng/L) for each sampling site) and total ARGs (sum of log(copies/mL) of all ARGs at each sampling site), however there was not a significant positive correlation in August. This indicates that when an increased quantity of antibiotics is present in a sample, there also seems to be increased quantities of ARGs, as a general trend in these samples, which makes sense as ABs select for ARGs (Martinez, 2009).

In order to assess individual AB effects on ARG levels Pearson correlation analysis was also performed on ARGs and the ABs they confer resistance to (i.e. $bla_{CTX\text{-}M}$, $bla_{TEM,}$ and *mecA* confer resistance to amoxicillin, cefaclor, cefprozil, and cefdinir; *qnrS* confers resistance to levofloxacin, and ciprofloxacin; *ermB* confers resistance to azithromycin, clindamycin, and clarithromycin; *tetO* confers resistance to triclocarbon). In April there were significant correlations found between *qnrS* and ciprofloxacin ($r=0.726$, $p<0.05$), and *ermB* and clarithromycin ($r=0.936$, $p<0.05$) when examining all

sampling locations (n=10), linking presence of these ABs and their associated resistance genes. *Int1* also had significant correlations with ciprofloxacin ($r=0.684$, $p<0.05$) and clarithromycin ($r=0.779$, $p<0.05$). It is also interesting to note that UP2, UP6, and UP7 contained more ABs than the other upstream sites, or even the downstream site. These sites in April often had elevated levels of ARGs, therefore it is possible that increased AB pollution at these sites contributed to increased ARG abundance. Although not to be found significantly correlated, *mecA* levels were quite elevated in the upstream samples (especially UP2 and UP7), as are cefdinir levels at these sites, which *mecA* confers resistance to. This is interesting as *mecA* was not expected to be found in such high levels in upstream rivers samples, however these spikes in April coincided with high levels of cefdinir. As discussed earlier, snow melt was likely occurring during this sampling event and may have washed ABs into the river that had been preserved during times of low temperature (Dolliver and Gupta, 2008). In May there were significant ($p<0.05$) correlations between $bla_{CTX\text{-}M}$, $bla_{TEM,}$ and *int1*, and amoxicillin ($r=0.871$; $r=0.675$; $r=0831$), *qnrS* and ciprofloxacin ($r=0.963$), and *ermB*, *int1*, and azithromycin ($r=0.926$; $r=0.816$), suggesting co-occurrences of these ABs and their respective ARGs. In August no significant ($p>0.05$) correlations between ARGs and the ABs they confer resistance to were found. There also seemed to be less ABs (besides amoxicillin) detected in the further upstream samples in August compared to the other months, suggesting a seasonal trend with ABs presence or persistence due to altered environmental conditions (e.g., temperatures of 19-22°C) in the river.

Berglund et al. (2015) also found ABs present in wastewater effluents and downstream water samples, however they did not detect any ABs upstream from the

WWTP. They detected ABs in effluent and downstream samples in ranges of below detection to 47 ng/L (ciprofloxacin, clarithromycin, clindamycin, and trimethoprim), which were lower than what was found in this study. Rodriguez-Moraz et al. (2015) detected ABs in wastewater and both upstream and downstream samples when looking at 9 different ABs. More comparable results were found in terms of concentration of ABs detected in samples, however azithromycin was not found in upstream samples, but clarithromycin was, which was not found in the current study. Therefore, it seems AB presence and levels vary between sample type, location, and sampling event.

In summary, a number of different ABs were present in both wastewater and river samples, especially ABs such as amoxicillin, azithromycin, and ciprofloxacin. This indicates that even in upstream, less urbanized areas, ABs can be detected and therefore are potentially able to exert selective pressures for ARGs. A potential source of ABs upstream may be septic systems effluents. There also may be a seasonal trend with ABs as more seemed to be present in the spring versus the summer. There were some significant correlations between ARGs and the ABs they confer resistance to, suggesting selection of ARGs or co-occurrences between antibiotic usage and the presence of ARG containing organisms in both wastewater and the river.

Table 11: Antibiotics detected in samples (ng/L) in April 12, May 10, and August 2, 2016 samples

Sample	Month (2016)	Amox-icillin	Cefa-clor	Cef-dinir	Levo-floxacin	Cipro-floxacin	Azithro-mycin	Clinda-mycin	Clarithro-mycin	Triclo-carbon
April MDL (ng/L)		**16**	**542**	**50**	**28**	**63**	**39**	**1**	**1**	**28**
May/August MDL (ng/L)		**35**	**29**	**95**	**91**	**71**	**40**	**30**	**74**	**50**
Influent	April	<DL	<DL	<DL	156	2246	196	13	406	48

Sample	Month (2016)	Amox-icillin	Cefa-clor	Cef-dinir	Levo-floxacin	Cipro-floxacin	Azithro-mycin	Clinda-mycin	Clarithro-mycin	Triclo-carbon
	May	3949	<DL	<DL	<DL	383	300	33	115	158
	August	<DL	77	<DL	<DL	<DL	<DL	<DL	<DL	75
Effluent	April	<DL	<DL	<DL	78	747	54	36	181	<DL
	May	4600	<DL	<DL	<DL	201	342	37	192	65
	August	<DL	<DL	<DL	99	195	<DL	<DL	<DL	68
Down-stream	April	<DL	<DL	<DL	<DL	593	<DL	5	6	<DL
	May	741	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
	August	739	<DL	<DL	<DL	<DL	82	<DL	<DL	<DL
Upstream 1	April	<DL	<DL	<DL	29	581	<DL	3	1	<DL
	May	674	<DL	<DL	<DL	91	84	<DL	<DL	<DL
	August	634	<DL	<DL	<DL	259	42	<DL	<DL	56
Upstream 2	April	<DL	<DL	535	173	969	<DL	4	7	<DL
	May	743	<DL	<DL	<DL	<DL	113	<DL	<DL	<DL
	August	648	<DL	<DL	<DL	72	40	<DL	<DL	<DL
Upstream 3	April	<DL	<DL	<DL	<DL	576	<DL	3	2	<DL
	May	903	<DL	<DL	<DL	<DL	105	<DL	<DL	<DL
	August	597	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Upstream 4	April	<DL	<DL	<DL	<DL	600	<DL	4	1	<DL
	May	798	<DL	<DL	<DL	<DL	118	<DL	<DL	<DL
	August	622	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Upstream 5	April	<DL	<DL	<DL	<DL	560	<DL	4	1	<DL
	May	772	<DL	<DL	<DL	<DL	97	<DL	<DL	<DL
	August	1032	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Upstream 6	April	<DL	<DL	844	33	606	<DL	5	1	<DL
	May	410	<DL	<DL	<DL	120	120	<DL	<DL	55
	August	639	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Upstream 7	April	<DL	<DL	211	67	734	<DL	4	1	<DL
	May	696	<DL	<DL	<DL	<DL	90	<DL	<DL	58
	August	766	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL

*Cefprozil consistently not detected aboce the LOD

4.4 Conclusions

Greenwood's WWTP has very strict effluent discharge requirements compared to most plants, however ARGs are still present in the effluent and the plant subsequently impacts the downstream river ARG levels. Correlations with water quality parameters and the presence of AB residues (selective factors), including an increased presence in more urbanized areas, suggests that ARG levels are impacted by anthropogenic activities and ways to potentially reduce these reservoirs should be assessed. Although levels of ARGs that represent a threat to human and ecosystem health are not currently established (Proia et al., 2016), their consistent presence in the environment and even in areas not highly impacted by urbanization warrants further investigation. These background ARG levels suggest that even areas thought to be "pristine" are likely more affected by urbanization than originally thought, or that there are larger natural reservoirs of these materials or other contaminating factors exist. Even though a portion of these ARGs may be being detected from eDNA or dead cells, genetic material can persist for weeks and even longer if attachment to sediment or biofilm occurs, and are therefore at risk of being transformed by competent bacteria, which includes pathogens. Therefore, ARGs can be a threat to public health in both living and from dead bacteria, which makes WWTP effluent an important source of these ARGs in receiving waters.

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Chapter 5: Conclusions

5.1 Project Summary

Using qPCR, the presence and abundance of nine ARGs and an integron gene (*int1*), used as a proxy to evaluate ARG cassette presence, were assessed throughout multiple treatment steps at two Atlantic Canada WWTPs that use different biological treatments (ALs vs. BNR reactors). The majority of ARGs were detected repeatedly along the treatment trains during sampling events of both municipal WWTPs, which suggests these genes are common in Atlantic Canadian wastewater and reduced but not removed by current treatment practices. Antibiotic resistance genes (e.g. *tetO* and *sul1*) that confer resistance to older and more highly prescribed ABs (e.g. tetracyclines and sulfonamides) were more commonly detected at both plants, while ARGs (e.g. *vanA* and *mecA*) conferring resistance to ABs of last resort (e.g. vancomycin and methicillin) were the least detected. *MecA* and *vanA* are not always present on broad-host-range plasmids and may be associated with bacteria not common to WWTPs which may also explain their lower presence. There was also a high presence of *int1* in water samples from both plants, suggesting the existence of ARG gene cassettes and potential HGT capacity in the systems.

Although both plants were able to reduce the absolute abundance (copies/mL) of ARGs throughout treatment, there were still several log units of ARGs remaining in the effluent which is discharged into the environment. At the Greenwood WWTP, the secondary clarification step caused the largest incremental removal of ARGs, while the BNR step was responsible for this at Plant B, demonstrating how varying treatment

designs can affect ARG removal. Furthermore, the unchanged relative abundance of ARGs (copies/16S rRNA gene copies) in Greenwood samples suggested the WWTP reduces resistant bacteria and total bacteria at a similar rate. Conversely, there were slight reductions in relative abundance of many ARGs after the BNR step at Plant B, and the BNR treatment systems designed for nutrient removal may therefore be better equipped to remove ARBs. Further investigations into BNR treatments effects on ARG abundances are recommended. UV disinfection at both plants was not able to reduce ARG levels, suggesting additional tertiary treatment steps may be required to target ARG removal. Correlations existed between nutrient, organic matter, and human fecal marker (*HF183)* removal and ARG concentrations, which suggests that WWTP effectiveness in terms of removal of typically monitored parameters and anaerobic fecal indicator bacteria may be linked to ARG removal.

Greenwood's WWTP discharges its effluent into a small river. Although the WWTP is under very strict effluent discharge requirements, a considerable amount of ARGs were still detected in the treated effluents. Water samples both upstream and downstream of the WWTP discharge point were assessed for ARGs and significant increases were observed downstream for both absolute and relative abundances. This indicates that the WWTP is increasing ARG levels in the river, which can consequently spread throughout the environment. However, a considerable amount of ARGs were also detected in upstream samples which suggested other sources contributed to the presence of these genes in this river. By sampling further upstream in the watershed, it was found that ARG levels increased at sample sites located closer to anthropogenic impacted areas. However, even low ARG levels were consistently detected in samples taken in more

"pristine" areas, which highlighted the ubiquitous nature of these genes in the environment.

Higher levels of ARGs were often detected in months with higher flow rates and colder temperatures, suggesting a seasonal relationship as well as a potential weather event relationship. Contaminations from snow melt, runoffs and re-entrainment from sediments and biofilms may contribute to these increased levels. Conversely, the downstream samples were more greatly impacted by the WWTP effluents in warmer months, where the effluents contributed a higher proportion of the water volume as well as ARGs to the river in lower flow conditions. Correlations to common water quality indicators, as well as AR selective factors (e.g. antibiotics) suggest that ARG levels are higher in areas with decreased water quality, which may be caused by wastewater effluents and anthropogenic pollution.

In summary, these WWTPs are not able to totally reduce ARGs in the effluents, which are ultimately discharged into the environment. Further investigations into the effects of different designs are recommended in order to limit their impacts on the receiving environment. These ARGs are also present in the watershed either naturally and/or from other sources likely related to anthropogenic activities. Rather present in live bacteria, or in eDNA form, ARGs have the potential to partake in HGT and spread throughout the environment. The potential uptake and transfer of these genes by dangerous pathogens threatens public health if humans are to come in contact with these pathogens. Therefore, finding ways to manage and decrease environmental ARGs reservoirs, especially from WWTP effluents, is of importance.

5.2 Future Directions

1. Further investigation of effects of biological treatment systems on ARG abundance: Additional sampling at Plant B is recommended in order to enhance our understanding of the BNR treatment's effect on AR. Sampling other plants that use different forms of biological treatment or have different treatment trains may also increase our understanding of how treatment impacts removal and/or development of resistance elements.
2. Investigating AR selective factors: Testing for more antibiotics/biocides that relate to the ARGs investigated, and monitor heavy metals levels throughout wastewater treatment and in the environment may increase of understanding of how these elements drive bacteria to obtain and maintain resistance genes.
3. Metagenomic sequencing: Sequencing of wastewater and river samples will allow for the profile of bacterial groups present in the samples to be obtained, as well as allow for an assessment of which bacterial groups are carrying ARGs. Investigating how these proportions change throughout treatment trains as well as upstream and downstream may increase our understanding of how the WWTP process affects resistant strains of bacteria. Sequencing may also increase our knowledge on which ARGs are co-located within integrons.
4. Distinguishing between DNA from viable and non-viable cells: Using techniques (e.g. sample pre-treatment with propidium monoazide) to determine the proportion of ARGs detected from living cells versus dead cells may increase our understanding of the fate these genes.
5. Investigation of long term aquatic ARG reservoirs: Sampling sediments and

biofilms along with the water column may increase our understanding of the persistence of ARGs downstream of WWTPs and in the environment.

6. Evaluating on-site septic systems effect on ARG abundance: In more rural areas, not all houses are connected to a municipal WWTP, therefore waste is treated by on-site systems. Evaluating these systems' effect on ARGs may increase our understanding of their impacts on the environment and if they should also be considered hotspots for AR.
7. Investigating the presence of additional ARGs and genetic markers: Including additional ARG markers may give a more complete picture of what resistance genes are present in wastewater and the environment. Adding additional genetic markers such as ruminant or bird fecal markers and assessing their correlations to ARG levels may increase our understanding of what types of pollution may be contributing to environmental ARG reservoirs.

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Appendix A: Antibiotic Concentrations within WWTPs

Table A1: Antibiotic concentrations from Greenwood WWTP

Sample	Amox-icillin	Cef-prozil	Cef-dinir	Levo-floxacin	Cipro-floxacin	Azithro-mycin	Clinda-mycin	Clari-thromycin	Tricl o-carbo n
MDL ng/L	**16**	**142**	**50**	**28**	**63**	**39**	**1**	**1**	**28**
Influent	<DL to 28	<DL	<DL to 2082	105 to 491	367 to 2139	<DL to 189	14 to 30	60 to 827	34 to 65
Lagoon	<DL	<DL	<DL to 1717	67 to 218	302 to 1214	<DL to 88	20 to 144	13 to 143	<DL
Clarifier	<DL	<DL	<DL	36 to 88	144 to 787	<DL to 43	13 to 53	53 to 151	<DL
Sand Filter	<DL to 17	<DL	<DL to 2343	36 to 265	163 to 1665	<DL to 56	15 to 45	57 to 188	<DL to 45
UV	<DL	<DL	<DL to 1821	55 to 1298	194 to 2528	46 to 136	15 to 49	64 to 355	<DL to 36

*Cefaclor not detected

Table A2: Plant B Antibiotic Concentrations

Sample	Amox-icillin	Cef-aclor	Cef-prozil	Cef-dinir	Levo-floxacin	Cipro-floxacin	Azithro-mycin	Clinda-mycin	Clari thro-mycin
MDL (ng/L)	**35**	**29**	**36**	**95**	**91**	**71**	**40**	**30**	**74**
Raw	<DL	65	<DL	<DL	<DL	130	<DL	<DL	<DL
Primary Clarifier	5249	<DL	<DL	<DL	174	544	304	40	127

Sample	Amox-icillin	Cef-aclor	Cef-prozil	Cef-dinir	Levo-floxacin	Cipro-floxacin	Azithro-mycin	Clinda-mycin	Clari thro-myci n
BNR	<DL	<DL	<DL	<DL	<DL	277	<DL	<DL	<DL
Secondary Clarifier	4600	<DL	<DL	<DL	<DL	201	342	37	192
UV	<DL	<DL	<DL	<DL	<DL	<DL	66	<DL	<DL

Appendix B: Greenwood ARG individual plots for influent to Upstream 7 sites in April 12, May 10 and August 2, 2016

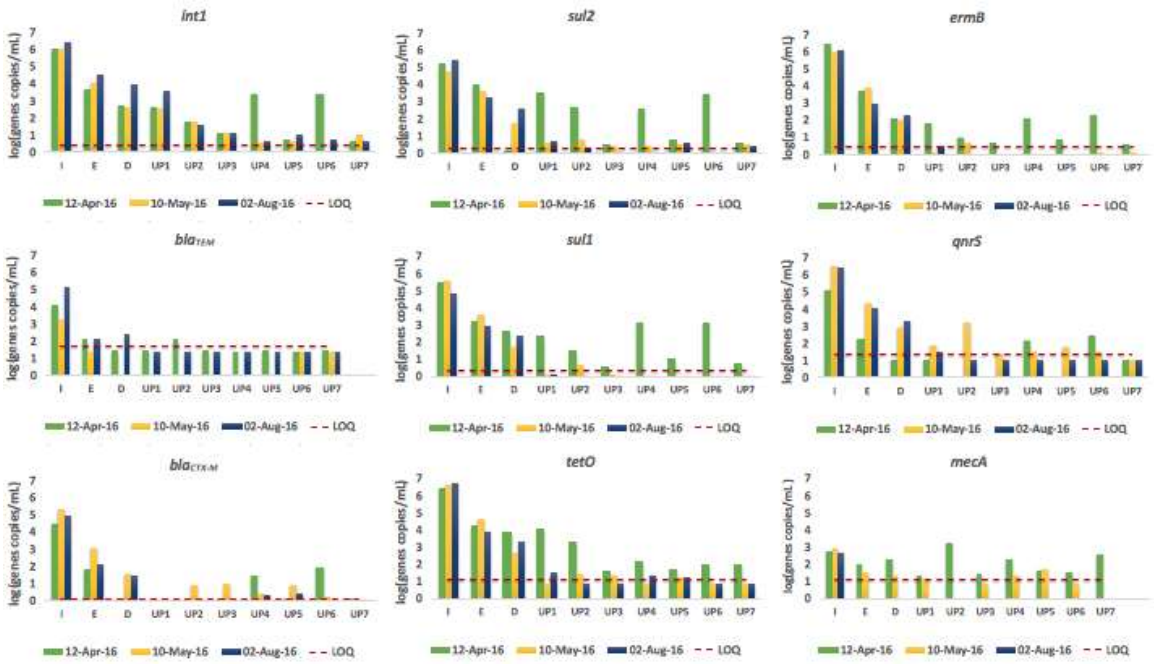

Figure B1: ARG absolute abundances (log (copies/mL)) for each gene at each site for each sampling event. Samples below the LOQ (shown in log(copies/mL) form as red dashed line) for each ARG were set to one half of the LOQ and log transformed to copies/mL. Samples below the LOD were set to one half of LOD (-0.30 log copies/mL for river samples), therefore samples that appear to not to have a bar are actually below -0.30 log copies/mL. *VanA* marker was not detected in any samples above the LOD and therefore not shown.