SURVEY OF ANTIBIOTIC RESISTOME IN A MIXED-USE WATERSHED IN WESTERN NEWFOUNDLAND, CANADA.

By

Emana Ifeoma Edet

A Thesis submitted to the School of Graduate Studies

in partial fulfillment of the requirements for the degree of

Master of Science in Boreal Ecosystems and Agricultural Sciences

School of Science and the Environment

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St. John’s Newfoundland and Labrador

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Approved:

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Dean of the Graduate School

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ABSTRACT

Antibiotic production is a natural phenomenon employed by microorganisms to control their environment in stress situations. Thus, antibiotic resistance has evolved in parallel to counteract naturally produced antibiotics. On the other hand, excessive use of antibiotics by humans, either for protecting human or animal health, may induce unnaturally high antibiotic stresses leading to enhanced antibiotic resistance, the so-called acquired resistance.

This study is the first to report an initial exploration into the distribution and quantification of antibiotic resistance genes, i.e. resistome, in a mixed-use watershed in Western Newfoundland, along the Humber river. The river was sampled along a gradient of increased human impact, mainly associated with the discharge of municipal wastewaters. The goals of my thesis were to (1) understand the relationship between human impact in the Humber river and the occurrence of antibiotic resistance genes in the environment, and (2) understand the role of the river in mitigating the abundance of these resistance genes, if any role is present.

Total DNA was extracted from the river water samples, sequenced by shotgun sequencing on an Illumina Hiseq platform with sequencing data quality controlled and cleaned at the sequencing facility via a QIIME pipeline, followed by identification and quantification of antibiotic resistance markers through a computational pipeline carried out in ShortBRED against a curated Antibiotic Resistance Genes Database (ARDB). About 400 antibiotic resistance genes, of variable abundance, were identified, distributed across

the tested systems, an indication of the wide distribution of antibiotic resistance in the environment. This analysis revealed the distribution and abundance of Antibiotic Resistance Genes (ARGs) within the microbial population in the Humber River.

Quantitative real-time PCR (qRT-PCR) was used to quantify absolute (copies L^{-1}) and relative abundances (copies /16S rRNA) for *TetO*, *TetM* and *AdeC* genes. Relative abundance of ARGs (copies / 16S rRNA) was higher in the upstream locations versus downstream locations.

The results showed evidence that anthropogenic impacts, especially associated with the use of antibiotics, led to an increase in the diversity and total abundance of antibiotic resistance markers in the Humber River.

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Last but not the least, thank you to my precious daughter Alero Victoria Emiko who walked this journey with me 2 years ago when she could barely stand firm on her feet. 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.

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AB	Antibiotics
ANOVA	Analysis of variance
AR	Antibiotic resistance
AMR	Antimicrobial resistance
ARG	Antibioitc resistance genes

ARB	Antibiotic resistance bacteria
CI	Chromosomal integrons
CT	Cycle threshold
DI	Deionized
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
GC	Gene copy
gDNA	Genomic DNA
HGT	Horizontal gene transfer
LB	Luria Bertani
LOQ	Limit of quantification
MGE	Mobile genetic elements
MST	Microbial source tracking
NTC	No template control
PCA	Principal component analysis
QMRA	Quantitative microbial risk assessment
qRT-PCR	Quantitative real time polymerase chain reaction
SOC	Super optimal broth
ShortBRED	Short better representative extract dataset
WQO	Water quality objectives
WWTPs	Wastewater treatment plants
Rrna	Ribosomal nucleic acid
QIIME	Quantitative Insight Into Microbial Ecology

CHAPTER 1

1 Antibiotic Resistance Genes in the Environment. An Introduction.

The persistence and spread of antibiotic resistance (AR) in the environment i.e. aquatic & terrestrial is a growing public health concern around the world (Colomer-Lluch et al., 2014; Kümmerer, 2004; Pruden & Storteboom, 2012; Pruden et al., 2013; Rodriguez-Mozaz et al., 2015). AR has been described as the temporary or permanent capability of a microorganism to remain insusceptible to the effect of treatments under conditions that would destroy or inhibit other microorganisms of the same strain (Beceiro et al., 2012; Cloete, 2003; Kümmerer, 2004, 2009).

AR is a natural phenomenon that microorganisms use to protect themselves against competitors and predators. However, the use of antibiotics in modern clinical settings, veterinary medicine and agriculture led to the emergence, selection and dissemination of AR bacteria, and thus the genes encoding resistance in many environments (Ashbolt et al., 2013; Baquero et al., 2008; Kümmerer, 2009; Sapkota et al., 2008). AR at abundances above what would be expected to be natural background is thus described as "acquired resistance". Resistance of bacteria to current antibiotics may seriously impair the prevention and treatment of an ever-increasing range of infections, thus decreasing the efficacy of conventional antibiotic therapy (Kümmerer, 2009; Levy, 2002; Marti et al., 2014).

Antimicrobial resistance (AMR) surveillance is primarily focused on the healthcare associated infections, with relatively limited, but growing, information available for its environmental dissemination (Marti et al., 2014; Moore et al., 2014). Antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have been studied and reported to be present in sewage, treated drinking water, river water, and soil (Jiang et al., 2013; Li et al., 2015; Su et al., 2014; Xu et al., 2015). The three ARGs of interest in this study; Tetracycline resistance gene *TetO* and *TetM* of the ribosome protein protection type and *AdeC* are reported to be abundant in bacteria associated with human faecal matter (Patterson et al., 2007). The gram-positive bacteria, anaerobes and nonenteric gram-negative bacteria such as *N. gonorrrhoeae* and *Haemophilus ducreyi* are known to harbour the ribosomal protection proteins (Eliopoulos et al., 2003). The *AdeC* gene has been reported in the *Acinetobacter baumannii* a pathogen known as nosocomial bacteria specific to humans (Vahdani et al., 2011). Humans and other animals are the main sources of antibiotic-resistant organisms released in to the water environments (Baquero et al., 2008). Their persistence in surface water has been reported by many authors (Garner et al., 2017; Kemper, 2008; Stange et al., 2016; Stoll et al., 2012; Zhang et al., 2016).

These bacteria can exchange their genes with water-indigenous microbes which may already contain resistance genes (Aminov & Mackie, 2007; Baquero et al., 2008). There are several mechanisms known to contribute to the AR occurrence and spread in the environment: horizontal gene transfers of ARGs among bacteria (Andersson & Hughes, 2010; Zhang, & Fang, 2009), genetic mutation, and selective pressure of antimicrobial

substances and heavy metals (Allen et al., 2010). The persistent selective pressure from antibiotic residues at sub-inhibitory concentrations in wastewater, as well as the density and diversity of microorganisms in activated sludge might favor the horizontal gene transfer of ARGs among different microbial organism. Horizontal gene transfer may be mediated by mobile genetic elements MGEs, including plasmids, transposons, bacteriophages, integrons, and a combination of MGEs (Philippe & Douady, 2003; Rizzo et al., 2013).

The extensive use of antibiotics and heavy metals in food supplements, for disease control and to facilitate growth in the livestock industry (Alexander et al., 2015), leading to long-term exposure of intestinal microbiota to antibiotics, results in natural selection of AR organisms which, when shed in feces, will contaminate the environment. On the other hand, many antibiotics are known to have relatively short half-lives (Chen et al., 2015). They may not occur in the environment regularly, but are present due to their continuous introduction into the environment. Therefore, antibiotics in the environment are regarded as pseudo-persistent (Chen et al., 2015).

The environmental contamination with antibiotics has been confirmed (Chee-Sanford et al., 2001) to contribute to the emergence of bacterial strains that are resistant to high doses of these drugs interfering with human disease treatment (Chee-Sanford et al., 2001). ARB have been found in wastewater and aquatic habitats following the introduction of antibiotics from agricultural runoff or wastewater treatment plants (Alexander et al., 2015). Notwithstanding the spread of ARGs, there has been less desired focus on the role

of environmental factors in the spread of resistance. To date, barely any comprehensive environmental data have been made available to support the development of microbiological risk assessments based on the occurrence of ARG found in the environment. (Alexander et al., 2015). Furthermore, there is a need to clearly understand the presence of antibiotics in the environment and their ultimate influence on microbes developing AMR. The evidence of seasonal patterns in the abundance of ARGs have been reported (Caucci et al., 2016). The abundance and levels of ARGs are attributed to seasons (Caucci et al., 2016), variable river flow and thus dilution rates. Also, variable concentrations of antibiotic resistances in human waste are known to occur due to variability in seasonal consumption of antibiotics (Caucci et al., 2016). The purpose of this study is to determine the relationship between human impact and the occurrence of ARGs in the Humber River, which is located in an area of relatively high human density when compared across the province of Newfoundland, but relatively low human density when compared at a global scale, and which river is perceived as pristine. Another goal of the study is to also determine if there is evidence that the river plays a role in mitigating the ARG concentrations.

1.1 Hypothesis

The central hypothesis of this research is that land-use and wastewater discharge into the Humber river watershed affects the ARG diversity and abundance in the Humber River. Thus, the underlying premise is that human activities potentiate the entry of ARGs

in the Humber River. The cumulative impact would lead to a downstream increase in ARGs absolute and relative abundance within the microbial population.

1.2 Research Objectives

1.2.1 Objectives

- To evaluate the sum of ARGs, or the resistome, along the length of the Humber river, as it encounters variable degrees of contaminant sources and pressures
- To quantify selected genes; *TetO*, *TetM* and *AdeC* by qRT-PCR assay. These genes code for antibiotic resistances known to be associated with human activities and have been extensively studied, and thus verified testing protocols are available.

Both research activities are a first survey of the diversity and concentrations of ARGs as they vary along the length of the stream as a function of the density of different potential contamination sources and types and also as a function of the seasons.

1.2.2 Approach

The research presented in this thesis is an initial exploration into the distribution of ARGs, i.e. resistome, in a mixed-use watershed in Western Newfoundland, along the Humber River. Molecular based methods, including qRT-PCR and shotgun Metagenomics sequencing were employed in this study. The activities of this study were divided into two:

- The first activity aimed at using shotgun metagenomics sequencing to examine the microbial community of the Humber River and the ARGs by

analyzing the DNA extracted from water samples collected from the Humber River.

- The second activity aimed at using qRT-PCR to assess both absolute (copies L^{-1}) and relative abundance (copies/16S rRNA copies) of three different ARGs along an intensity gradient of human activities in the Humber River and therefore to determine the impact of river use intensity on ARG levels in the river.

This provides a baseline information or reference point on the microbial community of the Humber River, revealing the putative microbial risk to human health and environment. This research is contributing to the existing body of knowledge on microbial source tracking (MST) in Newfoundland and thus helps to develop monitoring and management tools to reduce or curb fecal pollution of human origin.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Origin and environmental flows of bacterial resistance and virulence

Antibiotic-producing microbes are considered the main drivers for the maintenance of resistance genes in nature (Singer et al., 2016). Historically, there has been a continuous

battle between the development and clinical use of antibiotics and the multitude of microorganisms that cause infection and disease (Tenover, 2006). Penicillin, the first antibiotic, has been in general use since the early 1940s. However almost as soon as antibacterial drugs were deployed, bacteria responded by manifesting various mechanisms of resistance (Tenover, 2006).

The purpose of antimicrobial therapy is to facilitate the eradication of infectious microorganism from patients in a timely manner while reducing the emergence and spread of resistance. However, acquired AR often leads to treatment failure against human and animal pathogens (Freire-Moran et al., 2011; Kemper, 2008).

ARGs are emerging environmental contaminants. Bacteria can exhibit natural resistance to antibiotics acquired through mutation of normal genes and natural selection against susceptible bacteria strains, or by acquisition of genetic information that encodes for resistance (Frost et al., 2005). The location of ARGs on mobile or mobilizable genetic elements such as integrons, transposons and plasmids, facilitate the transfer of resistance to other individuals of the same or different species. The transfer of ARGs amongst bacteria through mobile genetic elements (MGEs) has been widely studied (Dobrindt et al., 2004) and demonstrated in clinically relevant isolates. The role of bacteriophages as a reservoir of ARGs has been extensively explored (Dobrindt et al., 2004). Bacteriophages are viruses that infect bacteria, that can carry genes that encode new functions or modify existing ones and thus can act as a vehicle for horizontal exchange of genetic information, which can

modify their host's genome by the insertion of their DNA into the bacterial genome (Dobrindt et al., 2004; Levy, 2002).

2.1.1 **Summary of potential pathways and cycling of AB and ARGs in the environment.**

The presence of antibiotics (AB) and antibiotic resistance genes (ARGs) in the environment has aroused an increased concern worldwide. ABs and ARGs take several routes of entry into the environment due to AB use in human and veterinary medicine. The main anticipated exposure pathways of AB and ARGs in the environment are displayed in Figures 2.1 and 2.2.

Generally, a proportion of the AB used are released into the natural ecosystem through excretion in feces and urine (household and hospital), or directly through drug disposal. These drugs may reach aquatic environments by the release of effluents from wastewater treatment plants (WWTPs), or industrial treatment plants into surface water. Sludge from WWTPs and industrial waste disposed at landfills may leach into groundwater, runoff from agricultural areas, and leaching from farmlands fertilized with manure may reach surface water. Alternative pathways into the aquatic environment derive from the use of antibiotic in aquaculture, incidental spills or discharges from industrial waste, drug production, and cemeteries.

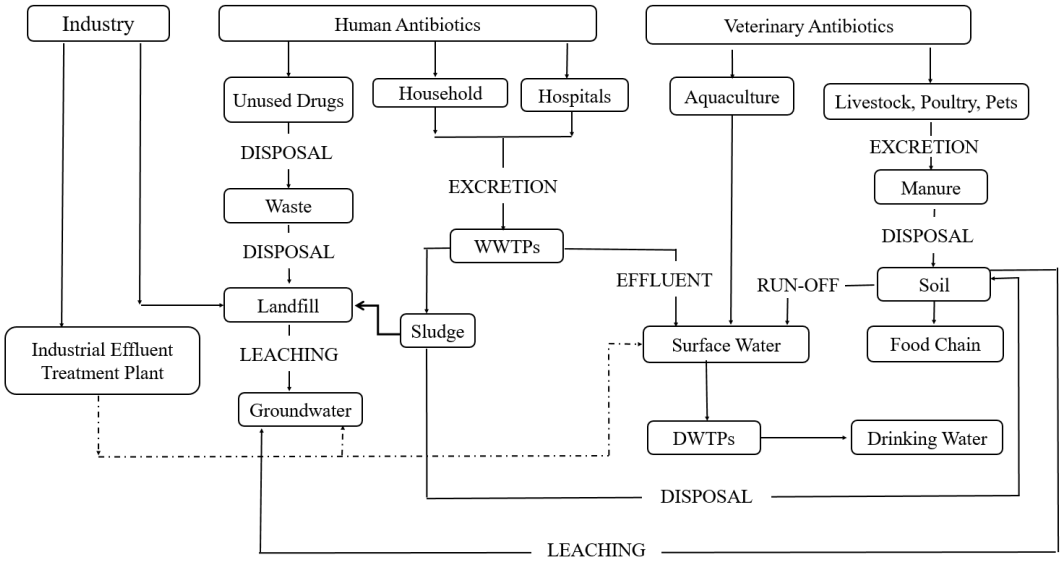

Figure 2.1 Pathways for antibiotics entering the environment (Frade et al., 2014).

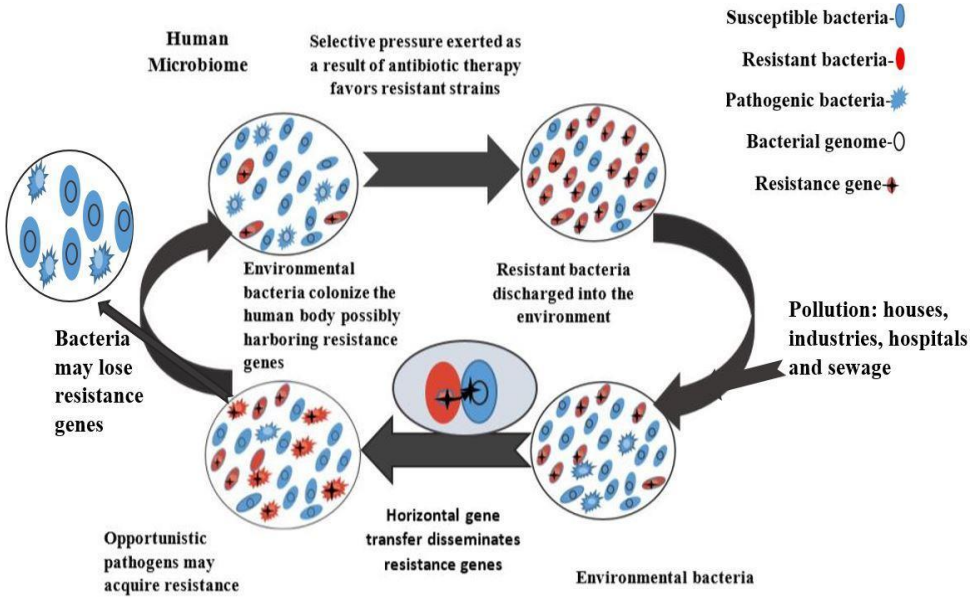

Figure 2.2 Cycling of ARGs between human and environmental microbiomes (Coutinho et al., 2013)

2.2 Virulence and Pathogenicity

The word “virulence” is derived from a Latin word “Virulentus” meaning full of poison. The quantitative measure of the degree of pathogenicity of an organism is defined as virulence (Beceiro et al., 2012). Microorganisms classified as pathogens have the ability to invade and damage the host, thereby causing illness; these pathogenic bacteria can alter their virulence to conform to the defense system of the host (Beceiro et al., 2012).

Bacteria adapt virulence mechanisms for the colonization of a new niche; virulence may occur by the acquisition of pathogenicity determinants by horizontal gene transfer (De la Cruz & Davies, 2000). As such, pathogenicity depicts another bacterial lifestyle, with the host serving merely as an additional ecological niche (Gal-Mor & Finlay, 2006). The degree of virulence is pertinent to the ability of microbes to cause disease, regardless of the host resistance mechanisms (Gal-Mor & Finlay, 2006). It is difficult to conclude that virulence is an intrinsic property of microorganisms that differentiates pathogenic from non-pathogenic microbes; there is increasing evidence that the host factors are major determinants of the outcome of host-microbe interactions. For example, virulent microbes may not be able to cause disease in a host with specific immunity hence they become non-virulent, while avirulent microbes may be able to cause disease in an immunocompromised host (Casadevall & Pirofski, 2001). Thus, virulence is an elaborate, dynamic and changeable situation involving both host and microbial factors (Casadevall & Pirofski, 2001).

Virulence factors have countless functional roles, such as the capacity to induce microbial attachment, invasion, or both, as well as the promotion of the growth of a microbe in a host through avoidance of host detection, inhibition of phagocytosis, and regulation of the capacity for intra-cellular survival. Virulence factors may or may not intensify microbial growth directly in a host. For example, the virulence of some microbes with polysaccharide capsules is interconnected to their capacity to evade host defense mechanisms and to replicate in tissues, which in turn induces damage and causes disease, largely as a by-product of the host inflammatory response to microbial growth. Conversely, for preformed toxin secreting microbes, virulence may not be associated with influencing growth or replication but, instead, with the capacity for invasion or interference with host defense because the secretion action of toxins does not require microbial growth (Casadevall & Pirofski, 2001).

2.3 Antibiotic Resistance

The undesired effects of microbial growth have long been controlled through the use of antimicrobials such as antibiotics. As a result of the principles of evolution and biology, the rise and occurrence of AR are unavoidable (Sharma et al., 2016). AR increases the morbidity caused by the bacterial infections as well as the cost of treating infectious diseases (Colomer-Lluch et al., 2011). AR as a phenomenon in itself is not surprising, nor is it new, it is a conserved natural process (Beceiro et al., 2012; Carvalho & Santos, 2016). By definition, wherever there is a change in susceptibility that renders an agent ineffective against a certain organism, this organism is referred to as resistant (Kümmerer, 2009).

Resistance to antibiotics is classified in two categories; intrinsic resistance and acquired resistance (Blair et al., 2014; Martínez & Baquero, 2002). Bacteria can be intrinsically resistant to certain antibiotics, and also these bacteria can acquire resistance to antibiotics through mutation in chromosomal genes and by horizontal gene transfer (Blair et al., 2014).

The intrinsic resistance of bacterial species to an antibiotic is the ability to resist the mechanism of action of the antibiotic due to its fundamental structural and or functional characteristics. Conventional examples of intrinsic resistance are the multi-drug resistance (MDR) phenotype by Gram-negative species, and β-lactam resistance in *Mycoplasma* spp. due to the lack of a cell wall in species of this genus or Vancomycin resistance in *Enterobacteriaceae* due to the presence of an outer membrane of these Gram-negative species (Alekshun & Levy, 2007; Blair et al., 2014; Cox & Wright, 2013). Additionally, bacteria can develop intrinsic resistance to antibiotics by three mechanisms such as, 1) through reduction of the intracellular concentrations of antibiotics due to poor penetration into the bacterium or antibiotic efflux, 2) by modification of the target of antibiotics through genetic mutation of the target, and 3) by inactivation of antibiotics through hydrolysis or structural modification (Blair et al., 2014).

Acquired resistance refers to the situation where a bacterium that used to be susceptible to an antibiotic at a given concentration is no longer susceptible at that concentration. For all practical purposes, arguably, only the relative form of AR, i.e. acquired resistance, has important clinical implications. This is because the emergence of resistance renders previously effective treatments useless, resulting in increased morbidity

and mortality, especially in the transition phase where resistance is too low to motivate a change in the empirical treatment. Acquired resistance involves changes in genes targeted by the antibiotic and the transfer of resistance determinants borne on plasmids, bacteriophage transposons and other mobile genetic elements (Alekshun & Levy, 2007).

Table 2.1 Mechanisms of action of antimicrobial agents

Mechanism of action	Antimicrobial agents	Comments
Cell wall synthesis	β-lactams- cephalosporins, penicillins, monobactams Glycopeptides: vancomycin, teicoplanin	β-lactamases, alteration of penicillin-binding proteins, permeability barrier, active efflux
Protein synthesis	Erythromycin, macrolides, chloramphenicol, clindamycin, linezolid, aminoglycoside, tetracycline, streptomycin,	Binds to 50S ribosomal subunit: erythromycin, chloramphenicol, clindamycin, linezolid Bind to 30S ribosomal subunit: aminoglycosides, tetracyclines, streptomycin
Nucleic acid synthesis	Fluoroquinolones, Rifamycin	Inhibition DNA synthesis - fluoroquinolones Inhibition RNA synthesis – rifampin
Metabolic-pathway/Intermediary metabolism	Sulfonamides, trimethoprim	
Multidrug Efflux pump		Efflux protein pump antibiotic out of the cell to confer resistance. Five (5) pump families. Located in the chromosome of both gram-positive and gram-negative bacteria

There are five major resistance mechanisms: (1) inhibition of protein synthesis, (2) interference with cell wall synthesis, (3) interference with nucleic acid synthesis, (4) inhibition of a metabolic pathway and (5) multidrug efflux pump (Tenover, 2006) (Table 2.1).

2.4 Infection

The threat from resistance to antibiotics, especially multiple resistance in bacterial strains that are widely disseminated, is serious (Colomer-Lluch et al., 2011). Bacteria are present both inside and on the surface of the human body, particularly on the skin and the mucous membranes (Beceiro et al., 2012). However, some bacteria that are categorized as pathogens are able to colonize, invade, and damage the host and thus cause illness. Pathogenic bacteria possess several factors that enable them to enhance their virulence. Most pathogens make use of a combination of two properties to cause disease: (i) toxicity and (ii) invasiveness.

The final balance of an infectious disease process will depend on the virulence or pathogenicity of the microbe as well as the host status in relation to risk. Some organisms have always been resistant to a particular agent by nature of their physiology or biochemistry (inherent or intrinsic resistance), while others acquire resistance as a result of the selective pressure/effects due to the application of antibiotics by humans (acquired resistance) (Kümmerer, 2009). Primary resistance is naturally occurring in microorganisms such as the resistance of *Pseudomonas aeruginosa* against penicillin G. The resistance is inherited by individuals of the same species through cell division (vertical resistance

transfer). In contrast, secondary resistance develops during therapy/contact of microorganism with an antibiotic. Plasmid mediated resistance is transferable between microorganisms.

2.5 Heavy metals and antibiotic resistance

Heavy metals are known to be naturally persistent in nature. The widespread distribution and recalcitrance of metal contaminants in an ecosystem may lead to a co-selection process for AR in some species, especially where resistances are co-located on the same mobile genetic elements; but the current understanding of this complex interrelationship is not extensive (Chen et al., 2015). Heavy metal contamination in the environment and resistance to antibiotics often bear some similarities, this may be because of their link to human and animal sources. (Alam & Imran, 2014; Martinez, 2009; Seiler & Berendonk, 2012). The utilization of heavy metals by humans has increased their bioavailability, leading to perceptible changes in polluted ecosystems (Seiler & Berendonk, 2012). Knowledge and awareness of heavy metal resistance in natural ecosystem therefore may help lead to a better understanding of AR in the environment.

2.6 Dissemination of ARGs in the environment

Bacteria acquire resistance genes through horizontal transfer via mobile elements such as plasmids, transposons and integrons. The mobile genetic elements (MGEs) contribute to the antibiotic resistance pattern in the aquatic habitat (Alexander et al., 2015). Plasmid genes often also code for enzymes that destroy antibiotics, or modify them in such

a way as to inactivate them. Plasmids are extrachromosomal segments of DNA that replicate independently (Cabezón et al., 2015) of the chromosome and can be exchanged among various bacteria via proteinaceous hair-like transfer appendages called pilus (Thanassi et al., 2012).

Plasmids are not essential for survival, but typically confer genes that affect some selective advantage to the host bacterium, such as virulence determinants, adhesion and AR mobility and have been found to allow the fast development of multiple AR (Brown-Jaque et al., 2015). Plasmids that carry resistance genes are called R plasmids or R factors. Transposons also known as transposable elements (TEs) on the other hand are ubiquitous, discrete segments of DNA distinguished by whether they are "autonomous" or "non-autonomous" i.e. their ability to move from one location on the chromosome to another or from the chromosome to transmissible plasmid (Brown-Jaque et al., 2015; Levy, 2002).

TEs are known to transposition for the movement along the genome, this type of recombination entails the migration of DNA sequence to a non-homologous locus. TEs are of many types, they could be simple or complex, the most important of them being the transposons and insertions (IS) (Brown-Jaque et al., 2015). Transposons and insertion elements are present in the chromosome and plasmids; they constantly reorganize the bacterial genomes. Transposase enzymes encoded by the IS elements function as catalysts for the exchange between transposon ends and genomic target sites. Transposons often contain genes, including genes for drug resistance (Brown-Jaque et al., 2015). Integrons are genetic elements that contain site-specific recombination system capable of gene

acquisition and subsequent bacterial genome rearrangement (Cambray et al., 2010). Mutation and selection coupled with the mechanism of gene exchange helps many bacterial species adapt fast to the antibacterial agents when introduced into their environment (Tenover, 2006).

Horizontal gene transfer (HGT) of AR in aquatic environments

Horizontal gene transfer or lateral gene transfer is an evolutionary process by which organisms acquire new traits that maybe useful under challenging conditions (Ashbolt et al., 2013; Martinez, 2009; Subirats et al., 2016). HGT may occur between strains of the same species or between different bacterial species and even genera (Ashbolt et al., 2013; Martinez, 2009). Horizontal mobilization expands the gene storehouse available to organisms, improving their chances to evolve. Bacterial genes may be randomly recruited from the original host into the recipient cell, but they are selectively kept only if they confer advantage on the new host strain through a marked impact on its fitness (Brown-Jaque et al., 2015).

HGT mechanism plays a role in genomic rearrangement and acquisition of ARGs by bacteria (Davies & Davies, 2010; Read & Woods, 2014; Ventola, 2015). Plasmids, integrons, and transposons are genetic mobile elements that carry antibiotic resistance genes providing additional mechanisms for gene transfer to other members of the same bacterial species. Mechanisms of HGT may be responsible for antibiotic resistance, virulence and phenotypic variability in metabolism (Brown-Jaque et al., 2015). There are three possible mechanisms of HGT; natural transformation, conjugation and transduction

(Bikard et al., 2012; Dorman, 2014; Håvarstein, 2010). For each of the processes, the incorporation of acquired resistance genes into the genome of the host or plasmid may be achieved via transposon (Tenover, 2006).

In transformation, or the uptake of naked DNA, short fragments of naked DNA from one bacterium are taken up by another. Transformation occurs naturally in the environment following death or cell lysis of a bacterium (Gal-Mor & Finlay, 2006; Tenover, 2006). Transformation is known to be the only prokaryotic HGT mechanism that fails to depend on the genetic mobile elements because the necessary genes reside in the chromosome (Sørensen et al., 2005). Conjugation (direct contact transfer of mobile plasmids) is a mechanism of gene transfer (Brown-Jaque et al., 2015) and it involves the transfer of genetic material via a proteinaceous sex pilus (pilus- represent a subset of the Type IV) secretion system between two cells (Brown-Jaque et al., 2014). Conjugation is known to be the major facilitator of ARG transfer between bacteria (Berglund et al., 2015). It is mediated by a type of plasmid, a circular piece of extrachromosomal DNA that independently replicates from the cell chromosomes.

During conjugation, the plasmid responsible for this mechanism is transmissible between cell surfaces (Grohmann et al., 2003). In gram-positive bacteria conjugation also involves the production of sex pheromones (chemical signals) by the mating pair which helps in the clumping of donor and recipient organisms enabling the exchange of DNA (Tenover, 2006). Conjugation differs from transformation in some ways; first, it involves

cell to cell contact, secondly conjugation cells must be of opposite mating types, with the donor cells usually carrying the plasmid and the recipient cells do not have a plasmid

Transduction (uptake of DNA) is the third mechanism of the horizontal gene transfer. During this process, a bacteria-specific virus (bacteriophage) transfers the bacterial DNA from a donor cell to recipient cell (Tenover, 2006). It occurs in a wide variety of bacteria and it is a common mechanism of gene transfer. HGT as a mechanism of antibiotic resistance has been validated (Davies & Davies, 2010), Especially, resistance of enteric bacteria to β-lactams drugs has been attributed to the HGT. However, some thick mycolic acid cell walls and high GC (guanine-cytosine)- containing pathogens like *Mycobacterium tuberculosis* seem to be less influenced by HGT, but developed antibiotic resistance by mutation and subsequent alteration of gene expression (Davies & Davies, 2010).

2.7 Persistence of antibiotics in water environment

Antibiotics have been used in human therapy, and agriculture as well as veterinary medicine in large quantities; and until recently, the existence of antibiotic substances in the environment has received no attention (Kummerer, 2008). Their occurrence in the environment, has resulted in the emergence and spread of antibiotic-resistance bacteria (Ashbolt et al., 2013; Kümmerer, 2009; Lekunberri et al., 2017; Lupo et al., 2012). After administration of antibiotics, large amounts of antibiotics or their metabolites are released unchanged into the environment via treated and untreated sewage, hospital waste, aquaculture discharges and agricultural run-off (Baquero et al., 2008). However, the

incomplete treatment of recalcitrant bacteria, especially pathogens and bacteria carrying some antibiotic resistance genes (ARGs) may put the receiving surface water at risk (Marti et al., 2013).

WWTPs represent a pertinent reservoir of resistance, because of the continuous influx of ARGs and antibiotic, suspended solids and dissolved organic matter which provides favorable environments for microbial growth (Rizzo et al., 2013). The coexistence of bacteria and antibiotics in wastewater treatment selects for resistance genes that spread through the microbiota, and as a result, antibiotic resistance bacteria disseminate their genes encoding resistance into water-indigenous microbes (Andersson & Hughes, 2010; Baquero et al., 2008). These antimicrobials occurring in wastewater are discharged into aquatic habitats, causing ARGs as newly emerging pollutants (Alexander et al., 2015; Bouki et al., 2013; Lapara et al., 2011; Munir et al., 2011).

The sludge from wastewater treatment plants are increasingly used as fertilizers and thus dispersed into agricultural land (Berglund et al., 2015; Su et al., 2014), thereby spreading unknown amounts of resistance genes and pharmaceuticals that are able to withstand standard wastewater treatment (Schmieder & Edwards, 2012). When manure produced in agriculture is applied to land, pollutants such as antimicrobial compounds, resistant bacteria or resistance genes concentrate and mobilize in soil (Chee-Sanford et al., 2001; Chee-Sanford et al., 2009; McKinney et al., 2010; Smith et al., 2004) and often end up in ground or surface water through runoff (Chee-Sanford et al., 2009; Tasho & Cho,

2016). Thus, it is not unexpected that many studies have found antibiotic resistant bacteria in water environments (Ribeiro et al., 2012; Schwartz et al., 2003; Watkinson et al., 2007).

The distribution of antibiotic resistant bacteria in aquatic sources in an agricultural watershed is unavoidable since water is the preponderant connection between the four major ecosystems – human, animal, soil, and aquatic – that circulate antibiotic resistance (Baquero et al., 2008; Ribeiro et al., 2012). Aquatic systems represent an environment for the release, mixing, persistence and spread of AMR bacteria and resistance genes associated with horizontally transferable genetic elements (Baquero et al., 2008; J. A. Perry & Wright, 2013; Taylor et al., 2011). It is frequently impacted by anthropogenic activities, and hence it constitutes a way of disseminating/circulating antibiotics. The presence of antibiotics in water environments is a concern because antibiotic contaminants can alter the microbial ecology, create proliferation of resistant bacteria, and thus pose a threat to human health (Martinez, 2009). Antibiotic resistance bacteria and genes might be transported into the human environment again via the use of river or pond water for domestic purposes (drinking or cooking) (Kümmerer, 2009).

Hence, the monitoring and identification of critical points in aquatic systems, which enhance the transfer and dissemination of antibiotic resistance genes, are of great importance (Marti et al., 2014).

2.8 Agricultural contribution to antimicrobial resistance in water

Agricultural and related anthropogenic activities (such as application to arable land of animal manure and sewage sludge) may act as sources of ARGs persistence and

dissemination in water (Ji et al., 2012; Threedeach et al., 2012). Antibiotics such as some beta-lactams, streptomycin, aminoglycosides and others are produced by soil bacteria (Kümmerer, 2009). The Actinomycetales phylum includes many soil bacteria such as *Streptomyces* spp. known to produce antibiotics.

There is a growing body of evidence supporting the occurrence of numerous antibiotic resistant genes in animal waste (Chee-Sanford et al., 2001; Heuer et al., 2011), in soils receiving animal manure or sewage sludge, and in aquatic environments receiving runoff from fields fertilized with manure (Wu et al., 2010).

Among many environmental compartments, agricultural soil is considered to be one of the primary receivers of released antibiotics (Kemper, 2008). When livestock manure and other animal waste spill into surface and underground water (Baquero et al., 2008), it may create a major pathway of ARGs spread, and an immediate threat to public health and water resources (Chang et al., 2015; Threedeach et al., 2012). In the livestock farming, about 70% of commercial livestock are administered antimicrobials for therapeutic, prophylactic and growth-promoting uses (Cheng et al., 2013; Xiong et al., 2015).

Manure has become a potential reservoir of resistant bacteria and antibiotic compounds, and its application in soil is assumed to significantly increase the ARGs and resistant bacterial population in soil (Heuer et al., 2011; Kümmerer, 2004). Few studies have investigated the impact of manure application on antibiotic resistance in soil, showing the effect of agricultural usage of manure containing antibiotic on the spread of antibiotic

resistance in the bacterial communities of soil ecosystem (Jechalke et al., 2013; Kyselková et al., 2015).

Nevertheless, the fate of ARGs in arable soils due to the continuous application of sewage sludge or manure remains unclear (Burch et al., 2014). The effect of antibiotics used for farming in human health has mainly focused on foodborne pathogens. These bacteria are present in the animals and can infect humans. Examples of foodborne pathogens are *Campylobacter jejuni*, *Escherichia coli*, *Salmonella* sp. or *Enterococcus faecium* among others. For these pathogens, both mutation-driven antibiotic resistance and the acquisition of ARGs are important concerns for human health, because the same strain can colonize both animals and humans, and antibiotic resistance genes can easily disseminate among bacterial species (or clones) that are phylogenetically closely related (Sundsfjord et al., 2001).

2.9 Microbial source tracking and Quantitative microbial risk assessment (QMRA)

Microbial source tracking (MST) is the application of quantitative sets of techniques aimed at identifying the sources of fecal indicator bacteria (FIB) in an environmental sample (Harwood & Stoeckel, 2011), which will in turn allow for polluted water bodies to be remediated (Mattioli et al., 2017). This source tracking method is based on molecular and biochemical methods (Meays et al., 2004; Scott et al., 2002). MST can also be called bacterial source tracking or fecal source tracking. This approach identifies

the sources of fecal bacteria introduced into the water system by human, wildlife or livestock sources (Meays et al., 2004).

Despite the need for guidelines and regulations regarding bathing and shell fishing water quality, most countries lack specific regulations. The US has a total maximum daily loads (TMDL) programs that encourage the MST studies (Santo Domingo et al., 2007). A TMDL establishes exactly the maximum amount of pollutant that a water body is able to receive and still meet the applicable water quality objectives (WQOs), and also allocates the waste loads to point source and nonpoint source. The availability of modern molecular techniques for the identification and characterization of bacteria may increase the possibilities for the source tracking of antibiotic resistance bacteria. However, the tracking of other genetic mobile elements involved with antibiotic resistance may also give a clear image of the complexity of antibiotic resistance in waterborne bacteria (Baquero et al., 2008).

Quantitative microbial risk assessment is a method used to evaluate the relative contribution of fecal indicators and pathogens when a mixture of human sources impacts a recreational waterbody. QMRA has been developed for calculating the burden of disease from a particular pathogen (Harwood et al., 2014). The major task of QMRA includes; exposure assessment, dose-response analysis and risk characterization (Kundu et al., 2013). Assessing risk from water supplies is important when trying to make judgements regarding the level of safety required in the light of alternative and multiple routes of exposure.

Completing a QMRA for every pathogen that may be transmitted by water would be time-consuming and the necessary information is currently not available for many pathogens. To overcome this difficulty, World Health Organisation, 2003 recommended using a suite of reference pathogens in the field of QMRA to represent the possible environmental fate and transport of members of each microbial group as well the infectivity of known members of each group. A reference pathogen is an organism whose severity of impact and persistence in water is such that its control would provide confidence that health risk from pathogens of a similar nature have also been controlled (World Health Organisation, 2003). The distribution and concentration of pathogens in water are highly variable, and the use of reference pathogens introduces another layer of uncertainty into the analysis (Ramirez-Castillo et al., 2015). Hence, QMRA modelling often includes Monte Carlo simulation techniques to help capture the uncertainty and variability within frequency distributions. However, the complexity of these techniques and need for proprietary and costly software, largely limits their use to the developed world. To expand the scope of QMRA, a simplified approach has been developed using point estimates (World Health Organisation, 2003).

2.10 The Resistome

The resistance to antibiotics is not restricted to pathogenic bacteria alone (Coutinho et al., 2013). The sets of elements such as genes, environmental microbes, soil dwelling Actinobacteria that comprise a resistome are all involved in direct or indirect antibiotic

resistance (Perry & Wright, 2013, 2014; Perry et al., 2016). Thus, a resistome is the totality of AR in a system.

The resistome consists of not only genes that confer real resistance determinants, but also precursor genes that can evolve into such traits by acquired resistance. Soil and water bacteria often present resistance determinants, even in un-impacted sites. Our own concept of antibiotic resistome in the Humber river, i.e. water sample locations for examining the distribution of antibiotic resistance determinants includes the obscure and obvious i.e. genes that lie hidden and silent in the environment, having the potential to cause medical treatment failure.

CHAPTER 3

3 Methodology

3.1 Sampling locations

The Humber River in Western Newfoundland is the island's second largest watershed. It flows through the Long-Range Mountains to Deer Lake and into the Bay of Islands at Corner Brook. It is approximately 121 km long (https://www.britannica.com/place/Humber-River). It is in a humid continental climate with temperatures ranging from -25 °C to 20 °C. Snowmelt strongly influences the seasonal streamflow of the water source; and snow covers the upstream area from October to April. The river has many unique attributes, which includes (1) a significant salmon habitat and (2) a location for multiple land-use types such as forestry, agriculture, and hunting.

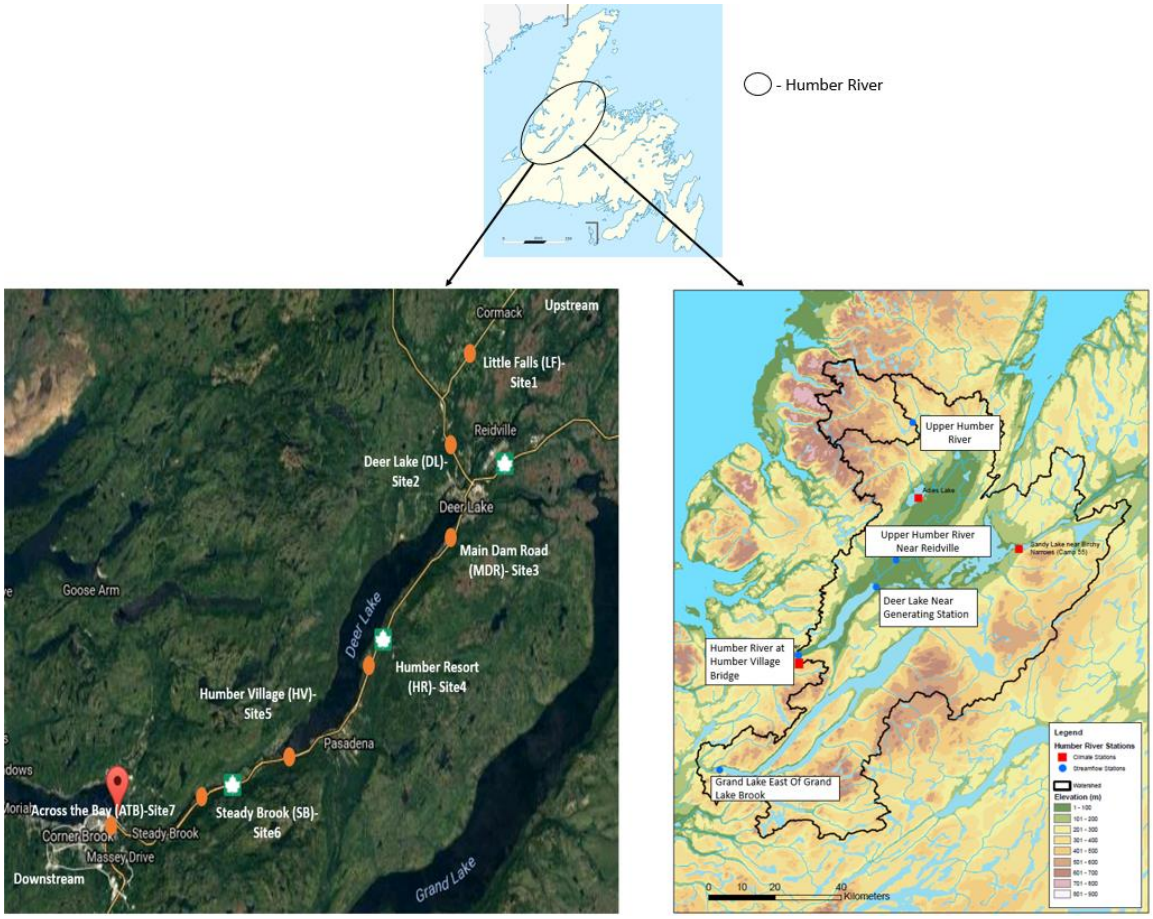

Figure 3.1 Sampling locations along the Humber River.

Table 3.1 Details of sampling locations.

Site #	Actual Locations	Choice of Sites	Description	Coordinates
Site1	Little Falls-Cormack	Minimal anthropogenic influence	Un-impacted	49°36’07.65’’ N 57°25’47.03’’ W
Site2	Deer Lake City	Minimal anthropogenic influence	Un-impacted	49°19’38.40” N 57°43’30.69” W
Site3	Main Dam Road	Tributary to Humber River/Deer Lake (discharge from the Grand Lake); drinking water source, minimal anthropogenic influence	Tributary 1	49°16’66.94’’ N 57°36’56.69’’ W
Site4	Humber Resort	Area influenced by anthropogenic activities	Impacted	49°01’20.35’’ N 57°68’90.79’’ W
Site5	Humber Village	Area influenced by human activities	Impacted	48°98’62.01’’ N 57°75’74.63’’ W
Site6	Steady Brook	Tributary to Humber River, minimal anthropogenic influence	Tributary 2	48°95’09.94’’ N 57°84’13.78’’ W
Site7	Across the Bay-Corner Brook	Anthropogenic influence	Impacted	49°14’37.02’’ N 58°13’93.82’’ W

3.2 Sampling Schedule

Over a period of over one year, seven sampling sites at selected locations along the Humber River (Figure 3.1. Table 3.1) were investigated to determine the abundance of ARGs. Samples were collected between July-December 2015 and May-September 2016 (Table 3.2; spring, summer, fall and early winter). Samples were collected in 1 L autoclaved high density polyethylene narrow mouth plastic bottles (VWR Inc.). Five litres of sample were collected from each site and sample event. Sampling was carried out early in the week to ensure samples can be handled immediately at the laboratory. Water temperature at sampling was measured in the field at the time of collection. Water pH was also measured in the field using an Oakton pH 2700 instrument. Sample was collected under the water-surface at a depth of about 30 cm by lowering the sample bottle attached to a balanced counterweight. Thus, a total number of seven (7) samples were collected on each sampling day in multiple bottles, labeled and transported to the laboratory on ice packs.

3.3 Sample Filtration

From each sample, a 250 mL aliquot, to be used for chemical analysis, was stored at -20 °C. Up to 5 L of the water samples were filtered through a cellulose-nitrate-acetate 0.45 µm membrane filter (Whatman GE Healthcare Life Science) within 24 h of collection using disposable Thermo Scientific Nalgene analytical filter funnels; membrane filters were removed with sterile forceps and stored in sterile 50 mL centrifuge tubes at -20 °C.

Table 3.2 Sampling dates

Sample date	Year	Season
July 20th	2015	Summer
July 28th	2015	Summer
August 18th	2015	Summer
September 21st	2015	Fall
October 26th	2015	Fall
November 9th	2015	Winter
November 23rd	2015	Winter
December 7th	2015	Winter
May 9th	2016	Spring
May 24th	2016	Spring
June 13th	2016	Spring
June 20th	2016	Spring
July 4th	2016	Summer
August 8th	2016	Summer
August 22nd	2016	Summer
September 19th	2016	Fall

3.4 DNA Extraction and Purification

Genomic DNA was extracted from the filtered samples using the PowerWater™ DNA isolation kit (Mo Bio Lab Inc.) according to the manufacturer's' instruction. For this, the stored filter membrane was transferred to a 5 mL bead tube. DNA was quantified and its quality verified, for each sample, by spectrophotometry in a 1.5 µL aliquot (Nanodrop ND-2000, Thermo, USA; 260/280 nm). The rest of the extract was stored at -20 °C until analysed.

3.5 Illumina Sequencing and Bioinformatics

Total genomic DNA extracts, from water samples, were sent to an external laboratory (McGill University; Genome Quebec, Montreal Quebec, Canada) for whole genome shotgun sequencing. High-throughput sequencing was conducted on an Illumina Hiseq 2500 platform. Approximately 60 µL of pooled DNA samples were used for library construction. The sequencing strategy was index PE 125 +8+258 cycles (i.e. Paired-End sequencing 258 bp reads and 8 bp index sequence). The generated metagenomics data were first filtered to remove reads containing three or more ambiguous nucleotides, or with quality score below 31 and a read length of <100 bp. Genome annotation was done with the ShortBRED pipeline (Kaminski et al., 2015); it was used to remove artificial replicates generated by the platforms of high-throughput sequencing, which may lead to incorrect conclusions in the subsequent data analysis. The remaining clean reads were used for further analysis (Appendix-A-Table 1).

3.6 Identification of ARGs via ShortBRED pipeline

The challenge in microbial community analysis is the quantification of protein families of interest from metagenomics sequencing data in a fast and accurate manner (Kaminski et al., 2015). Short Better Representative Extract Dataset (ShortBRED) is a computational system used for profiling the abundance of protein families of interest at very high specificity in shotgun metagenomics sequencing data.

This is achieved by first recognizing the short peptide markers that are stored within the protein families, and then categorize families. We applied ShortBRED pipeline to profile antibiotic resistance protein families in the antibiotic resistome of the Humber River, by employing an antibiotic resistance curated database, ARDB (Gibson et al, 2014; Kaminski et al., 2015). This method allowed for estimation of the prevalence and abundance of the DNA sequences coding for protein families of interest.

3.7 Quantification of selected ARGs

Quantitative, Real-Time PCR (qRT-PCR) was used to quantify target genes encoding tetracycline, aminoglycoside and Chloramphenicol resistance (Zhang & Fang, 2006). Three ARGs were selected for analysis, according to the result of antibiotic detection in the samples and their occurrence in the surveyed resistome. They include; *TetO*, *TetM*, and the *AdeC* gene, the latter a component of the resistance cassette *AdeC-AdeK-oprM*. An endogenous control, 16S rRNA gene, was used for normalization of the expression levels of the target genes. The primers used are listed in Table 3.3.

Table 3.3 Primers for targeting selected genes

Target	Oligonucleotide primers		Length (nt)	PCR annealing temp.	Amplicon size (bp)	References
	Primer ID	Sequence				
Tet-M	*Tet*M-FW	ACAGAAAGCTTATTATATAAC	21	55 °C	171	(Aminov, 2001)
	*Tet*M-RV	TGGCGTGTCTATGATGTTCAC	21			
Tet-O	*Tet*O-FW	ACGGARAGTTTATTGTATACC	21	60 °C	171	(Aminov, 2001)
	*Tet*O-RV	TGGCGTATCTATAATGTTGAC	21			
AdeC	*Ade*C-FW	TACACATGCGCATATTGGTG	20	52 °C	117	(Coyne et al.,2010; Modarresi et al., 2015)
	*Ade*C-RV	CGTAAAATAAACTATCCACTCC	21			

The oligonucleotides were used as previously described (Table 3.3). To verify the specificity of the primer sets the PCR products for the target genes were electrophoresed in a 1.5 % agarose gel in 1x Tris base, acetic acid and EDTA, TAE buffer, (Tris-40 mM, acetic acid-20 mM, EDTA-1 mM) with marker (100bp DNA ladder, Takara Bio, Japan). Gels were visualised with a gel imaging system (Bio-Rad Molecular imager® Gel Doc™).

The PCR reaction was performed in a total volume of 50 µL; each reaction included 1 µL of DNA template (100 ng), 5 µL of 10X PCR buffer, 0.5 µL of 50 mM-dNTP mix, 1 µL of each primer (0.1µg/ µL each), 41.5 µL of RNase/DNase free water and 1 µL of 1 U / µL Taq polymerase. In parallel, a 50 µL total volume PCR reaction was set for the same target genes using the QX200™ EvaGreen ddPCR™ Supermix. The reaction conditions for amplification of DNA were an initial 94 °C for 2 min, then 24x cycles of 94 °C for 1 min, annealing at different temperatures (*TetO*- 66 °C, *TetM*- 55 °C, *AdeC*- 52 °C)

for 1 min, 72 °C for 1 min and a final extension of 72 °C for 7 min. Upon completion, 5 µL from each reaction was used for quality analysis by agarose gel electrophoresis; and a discrete band was visible according to the amplicon size in base pairs for each gene of interest (Appendix A- Figure 1).

3.8 Amplicon cloning

PCR products of the four selected genes were cloned using a TOPO® TA cloning kit for sequencing (Invitrogen by Life Technologies) (Schmieder & Edwards, 2012), to facilitate Sanger sequencing and confirmation of gene identity. The cloning reaction was set up using the fresh PCR products from the PCR analysis on the target genes described above. A 6 µL volume of cloning reaction contained 3 µL of the PCR product, 1 µL of RNase/DNase free water, 1 µL of salt solution (200 mM NaCl; 10Mm $MgCl_2$) and 1 µL of the TOPO vector. The samples were mixed gently and incubated at room temperature for 5 min and placed on ice; 2 µL of each reaction was added into separate vials of one shot (25 µL) chemically competent *E. coli* cells, and mixed gently.

The vials were incubated on ice for 5-30 min, cells were heat shocked for 30 s at 42 °C and the tubes were immediately placed on ice. 250 µL of room temperature super optimal broth (S.O.C medium) was added to the vials, which were capped tightly and placed horizontally in an incubator shaker at 37 °C and at 200 rpm (revolutions per minute) for 1 h. Culturing was performed on one ampicillin concentration 100 µg/mL (Lee et al, 2006). 10-50 µL of the transformants were spread on a prewarmed selective substrate plate (Luria-Bertani (LB) media) and incubated overnight for about 16-18 h at 37 °C. To ensure

even spreading of small volumes, 20 µL of S.O.C medium was added. Two different volumes of the transformants were plated to ensure that at least one plate will have well-spaced colonies.

White colonies of ampicillin-resistant transformants were inoculated in LB broth containing 50 µg/ mL ampicillin and incubated at 37 °C overnight. Plasmid extraction for construction of *TetO*, *TetM*, *AdeC* and 16S rRNA libraries was done using the Qiagen mini prep kit. The presence of *Tet*, *AdeC*, and 16S rRNA fragments were checked by PCR using the same primer sets (Table 3.3).

3.9 Principles of qRT-PCR

qRT-PCR is an enzyme-driven process for amplifying short regions of DNA in vitro. This method detects and measures products generated during each cycle of the qRT -PCR process which are directly proportionate to the amount of the template prior to the start of the PCR process (Ginzinger, 2002). To accomplish this, it is necessary to have a method detecting the accumulation of PCR product and a thermocycler that is adapted to record the results after each PCR cycle in real time. Early attempts to perform quantitative PCR (qRT-PCR), prior to real-time instrument, relied on visualization of PCR products using intercalation of ethidium bromide (or other intercalating dyes) at an empirically determined PCR cycle number. qPCR is the most rapidly growing technique for use in the aquatic environment for both microbial source tracking and rapid pathogen specific quantification (Aw & Rose, 2012).

In PCR, a target DNA sequence is amplified over a number of denaturation-annealing-extension cycles. In a conventional PCR, only the final concentration of the amplicon may be monitored using a DNA-binding fluorescent dye. However, in qRT-PCR, the concentration of the amplicon is monitored throughout the amplification cycle using a group of new fluorescent reagents. These reagents bind with the amplicon without causing damage at the end of each cycle so that amplification may continue to proceed. The fluorescence intensity emitted during this process reflects the amplicon concentration in real-time (Zhang & Fang, 2006).

3.10 Standard curve design and qRT-PCR experimental design

The plasmid extracts were quantified by spectrophotometric analysis (Nanodrop 2000 Thermo Fisher scientific, USA). The efficiency of a reaction is best assessed through the design of a standard curve. In this study, the standard curves were generated by creating a dilution series from the plasmid extracts for each target gene. Seven-point calibration curves for qPCR were produced in triplicate for each assay. Target plasmid extracts were diluted from 1×10^1 to 1×10^7 target copy per reaction, while sterile RNase/DNase free water (Life technologies) was used as a negative control to design a standard curve for qPCR assays (Lee et al., 2006).

The PCR solution was prepared with 2 µL of the DNA templates and a PCR mixture to a total volume of 20 µL. The PCR mixture contained 6 µl of RNase/DNase free water (Life technologies), 10 µL of 1x SsoFastTM EvaGreen® supermix, 1µL forward and reverse primers (500 nM of each type). RNase/DNase free water (Life technologies) only

runs were employed as non-template control (NTC). All assays were carried out in triplicate (Cui et al., 2016).

All experimental procedures were done on ice. The qRT-PCR cycling condition was started with denaturation at 95 °C for 3 minutes, 40 cycles at 95 °C for 10 s and a different annealing temperature 50 °C - 60 °C for 30 s with plate read, and 5 s hold from 65 °C to 90 °C for melt-curve analysis. The setting of the annealing temperatures was designed according to qRT-PCR instrument and primer requirements. The number of gene copies was calculated by threshold cycle (CT) value and standard curve. Efficiencies ranged from 96% to 110%, with regression coefficients (R^2) of >0.998 for all calibration curves.

3.11 Real time quantitative PCR assay

Real time quantitative PCR (qRT-PCR) was used to quantify three target genes, two well-known tetracycline resistance genes *TetO, TetM,* and a gene known to participate in the multidrug resistance efflux pump mechanism (*Ade*C). The 16S rRNA gene was also quantified to allow for the calculation of the relative abundance of ARGs within the total bacterial population. The qRT-PCR assays were performed on a C1000 Touch™ Thermal cycler CFX 96™ real-time PCR system (Bio-Rad, CA) using SsoFast™ EvaGreen® supermix (Bio-Rad, CA) and specific primers. The primer sequence, annealing temperature and expected amplicon sizes of each target gene are listed in Table 3.3. All qRT-PCR reaction mixes were placed in 96-well semi skirted low profile plates. A total of 20 µL reaction volume in each well included 6 µL of RNase/DNase free water, 10 µL of 1x

SsoFastTM EvaGreen® supermix, 1µL forward and reverse primers (500 nM of each type) and 2 µL template DNA. In each qRT-PCR run, template DNA was replaced with RNase/DNase free water in other to develop a non-template control run (NTC) which was assayed in triplicate (Cui et al., 2016). Randomly selected DNA samples were assayed in triplicate to estimate method variability. All loading of reaction mixes was carried out on ice. The qRT-PCR reaction started with an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 10 s denaturation at 95 °C, annealing at different temperatures (*TetO*- 60 °C, *TetM*- 55 °C, *AdeC*- 52 °C) for 30 s with plate read. Eventually a 5 s ramp-up from 65 °C to 90 °C was monitored for melt-curve analyses. Annealing temperatures were designed according to the qPCR instrument and primer requirements. The number of gene copies was calculated from the threshold cycle (CT) value and standard curves. Apparent detection limits for the qualitative PCR assays ranged from ~10 to 1,000,000 gene copies per reaction as assessed by serial dilution until no product could be visualized on the gel.

Data obtained from the qRT-PCR assay were analyzed/presented at three different levels. To determine the relative ARG abundance (gene copies / 16S rRNA), absolute counts were normalized to 16S rRNA gene. To examine the absolute gene concentration (copies L^{-1}), gene copies were normalized to the water volume used for gDNA extractions.

To calculate the total flow rate of ARGs through a certain location of the river (copies s^{-1}), the copies per L (concentrations, copies L^{-1}) were multiplied by the flow rate data. The flow rates of only two sampling locations (Little Falls and Humber Village) were available from the web source of the Municipal Affairs and Environment, (Water

Resources Management Government of Newfoundland. Resources Management) Government of Newfoundland. (https://wateroffice.ec.gc.ca/search/historical_e.html) These were used to calculate gene flow rates.

3.12 Statistical Analysis

General statistics (Descriptive statistics, ANOVA, regression analysis) was done in Minitab 17 statistical software (Minitab, 2017) and Past3 (Hammer et al., 2001).

Generalized linear models (GLM) were used to examine significant factors across sampling locations. One-way analysis of variance (ANOVA, GLM), paired Student's t-test, Tukey HSD test, were used to assess the homogeneity of variance and statistical significance level of 5% ($p < 0.05$). Pearson correlation was used to assess significance ($p<0.05$) between ARGs and covariants (pH and temperature).

Principal component analysis (PCA) method was done for ARGs relative abundance distribution in water sample. The PCA was performed by calculating the average absolute abundance for each individual ARG and dividing that value by the sum of average absolute abundances for all ARG to obtain a ratio for each ARG target for a particular treatment (location). These proportions of each ARG for a particular location were used for PCA analysis. PCA is helpful to prioritize large data sets, thereby reducing the dimension of a data set (i.e. response variables) to a smaller number of uncorrelated variables (components or eigenvectors). A biplot is a visualization of PCA components and

describes the variability accounted for by the PCA components. Eigenvectors describe the load of each variable as eigenvalues.

CHAPTER 4

4 RESULTS AND DISCUSSION

4.1 Survey of the resistome in the Humber River watershed

Seven sites along the Humber River were sampled as previously described in Chapter 3 (Table 3.1, Figure 3.1). DNA extracts from similar sampling locations were selected based on their temperature and composited by equimolar pooling; this was done in other to achieve a higher DNA yield. Sterile water was added in other to make up the composite volume of 40-75 µL. The total amount of each composite sample sent for library construction are shown in Table 4.1.

Table 4.1 Description of composite samples for Shotgun sequence on Illumina Hi-Seq-Platform

Serial #	Sample ID*	Composite DNA ng/µL	Volume µL	Concentration (ng/µL)	Temp °C	pH
1	ATB-summer#	12.4	57	708.6	14.5-16	6.7-5.9
2	ATB-winter	8.8	57	500	7-7	6.1
3	DL-summer	11.2	57	641.2	16 – 17.5	7.9-6.7
4	HR-summer	10	57	572.3	9 – 15	6.4 -5.8
5	HV-summer	10.4	57	590.1	14- 15	6.1- 5.9
6	HV-winter	7.9	61	477.8	7-6	6.4
7	LF-summer	12.3	57	700.9	15-16.5	6.4-6.8
8	MDR-summer	9.6	57	549	10.5 -11.5	6.5-6.1
9	SB-winter	1.1	57	60.5	7 - 5	6.1

* ATB, Across the Bay; DL, Deer Lake; HR, Humber Resort; HV, Humber Village, LF, Little Falls; MDR, Main Dam Road; SB, Steady Brook

summer combines the months of July & August; winter combines the months of November & December.

4.1.1 **Physico-chemical parameters**

Water temperature at sampling varied between seasons and locations (Appendix A-Tables 2 & 3) from 4 °C to 16 °C in spring; May & June, 5 °C to 22.5 °C in summer; July & August, 5.5 °C to 17.5 °C in fall; September & October and 0 °C to 9 °C in winter (November & December). In the collection system, the pH values ranged from 4.69 to 7.89 in spring see Figure 4.2, 5.90 to 6.78 in summer, 6.17 to 6.68 in fall, and 6.05 to 8.36 in

winter. Water found in nature will generally have a pH between 6.5 and 8.5 depending on the geological and atmospheric conditions. High pH value was detected at the Deer Lake city location in the winter of 2015 sampling year (Figure 4.1), possibly because of the wastewater discharge. Wastewater that contains detergents and soap-based products may increase the pH of water source to become more basic (Addy et al., 2004). Anthropogenic activities may cause pH fluctuations usually related to pollution. The lowest pH was measured at the Humber resort location in the summer of 2016 sampling year (Figure 4.2).

Generally, several variables such as bedrock minerals (Varanka et al., 2015), acid rain, water use, wastewater discharge (Harnisz, 2013) and carbon dioxide (CO_2) may influence pH (Addy et al., 2004; Feely et al., 2003). In boreal ecosystems, the water is naturally acidic due to the accumulation and massive influx of organic acids leached from the forest floor (Lidman et al, 2016). Increases in pH may be associated with high photosynthetic conditions common in eutrophic waters (Chapman & Kimstach, 2002), or can be induced by the pollutants; however, the river water has a strong buffer capacity especially rivers rich in humic and fulvic acids and thus one would need to add massive amount of pollutant to observe any difference (Chapman & Kimstach, 2002).

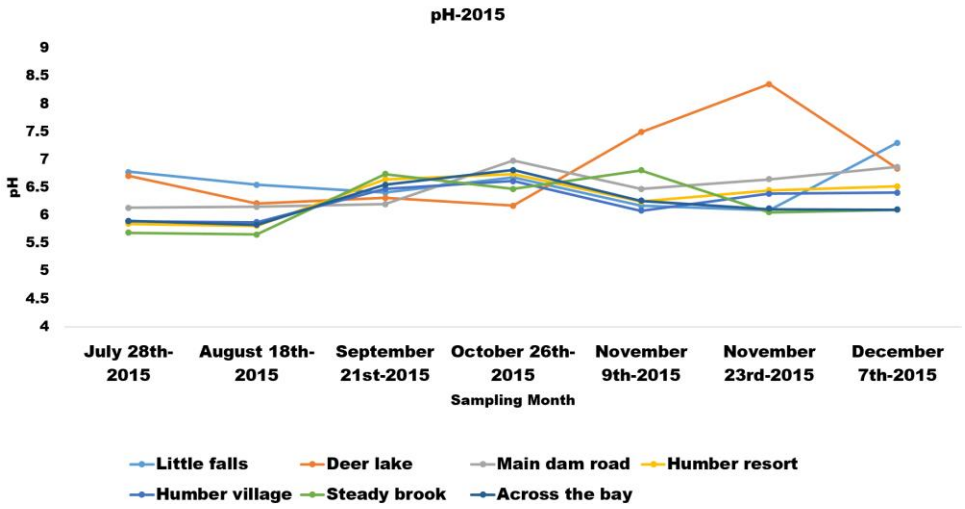

Figure 4.1 Variability in pH across sampling locations and events in 2015.

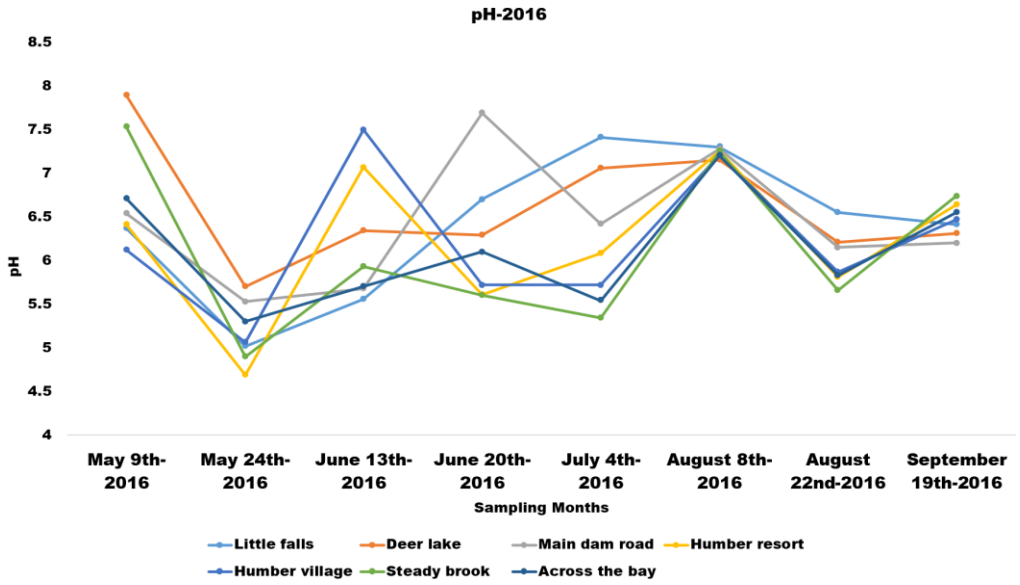

Figure 4.2 Variability in pH across sampling locations and events in 2016.

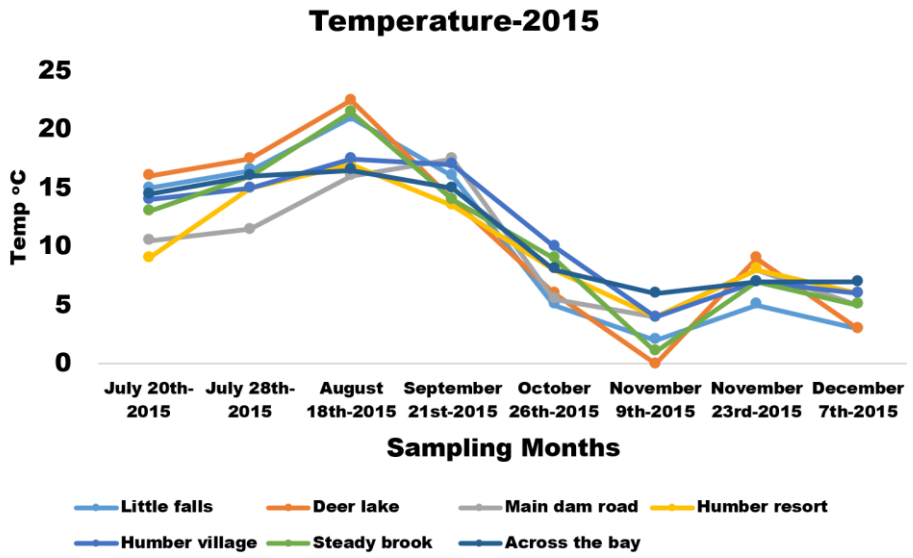

Figure 4.3 Variability in water temperature across sampling locations and events in 2015.

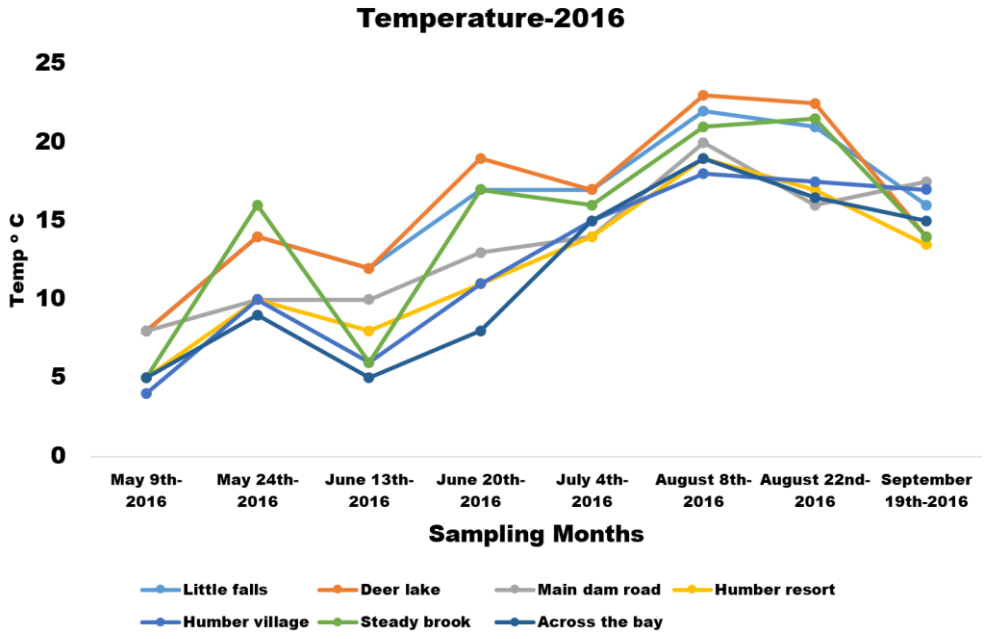

Figure 4.4 Variability in water temperature across sampling locations and events in 2016.

4.1.2 **Distribution of antibiotic resistance genes in the Humber River system.**

4.1.2.1 ARGs

ARGs are the genes in a microorganism which confer resistance to antibiotics. For example, by coding for enzymes that destroy the drug, every resistance gene is associated with a resistance profile i.e. group of antibiotics or class of antibiotics targeted by the gene. Their detection in environmental metagenomes may reveal the presence of organisms that may express antibiotic resistance traits.

Cfr-gene, *bac*A, *tsnr*, and *blaR*1 resistance genes were the most abundant and somewhat consistent across all sample locations and seasons (Figure 4.5). *Cfr*-gene had the largest proportional abundance 19% in the Little Falls location in the summer, Deer Lake location in the summer, Humber Resort location in the summer, Humber Village location in the winter, Steady Brook tributary location in the winter, Across the Bay location in the summer and Across the Bay location in the winter. *BacA* gene had the largest proportional abundance in the Little Falls location in the summer 30%, Deer Lake location in the summer, Main Dam Road tributary location in the summer and Steady Brook tributary location in the winter (Appendix A-Table 4).

*BlaR*1 gene had the largest proportional abundance in the Main Dam Road tributary in the summer 14%, Humber Village location in the summer and winter seasons 13% & 9%, and in the Across the Bay location in the summer 10%. *Cara* and *lmra* resistance genes were the most abundant in the Humber Resort location in the summer 4% & 6%, Humber Village location in the summer 7% & 6%, and Across the Bay location in the summer 5%

& 8%. *Tsnr* gene has the largest proportional abundance in the Humber village location in the summer and winter seasons 5% & 9% and Across the Bay location in the summer and winter seasons 6% & 9%. *Srmb* resistance gene was the most abundant in the Across the Bay location in the winter 1% and Humber Resort location in the summer 1%. *Bl2be_per* and *bl3_vim* genes were the most abundant in the Deer Lake location in the summer 8% & 8%. *Bl2d_oxa*1 gene was the most abundant in the Across the Bay location in the winter 1%. *Bl2a_pc* gene was the most abundant in the Humber Village location in the winter 10%.

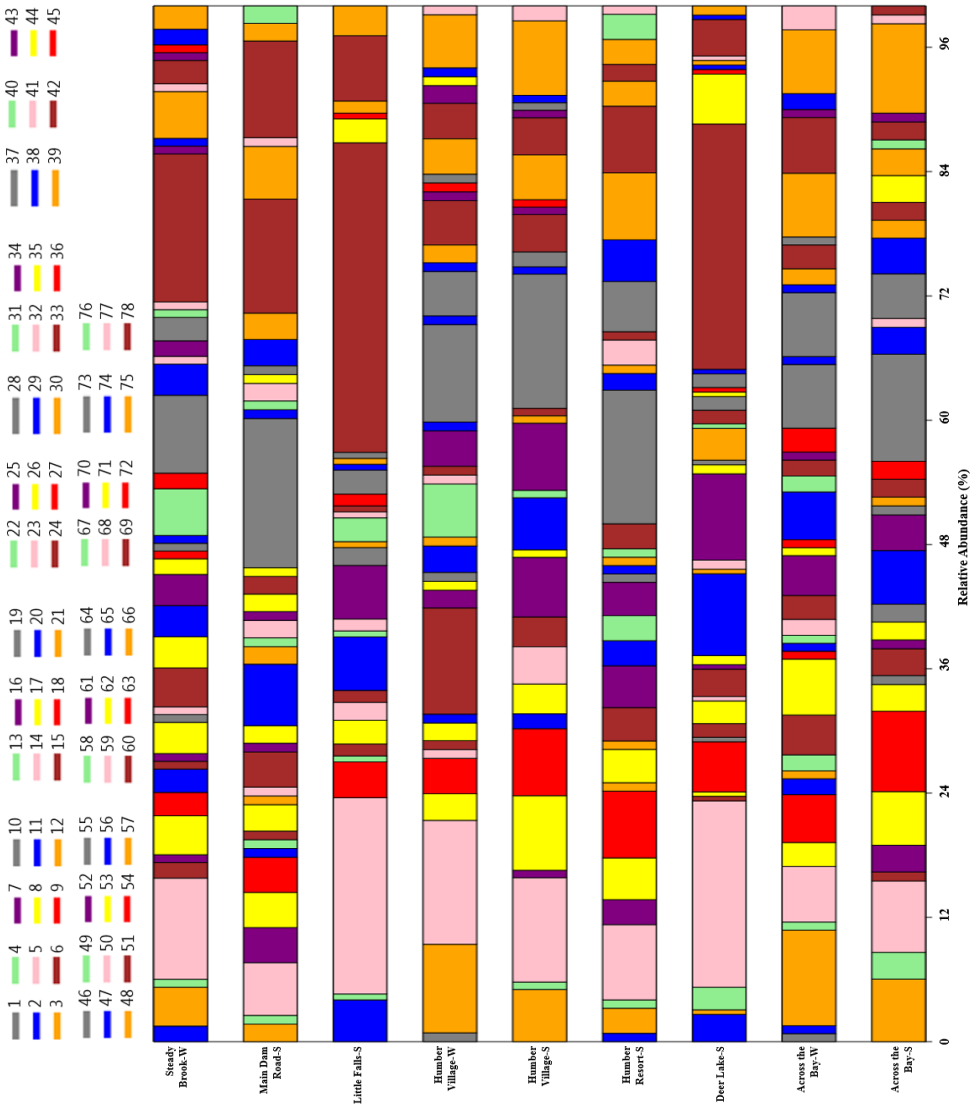

Figure 4.5 Abundance of resistance genes at the sampling locations. Gene ID's can be found in Appendix-B (Table 1)

The relative abundance of resistance genes accumulated across all locations is described in a box plot (Figure 4.6). Outliers may have occurred because of variability in measurement or heavy tailed end distribution. *BacA*, *blaR*1, *Cfr*-gene and *tsnr* genes had the largest proportional abundance among all genes.

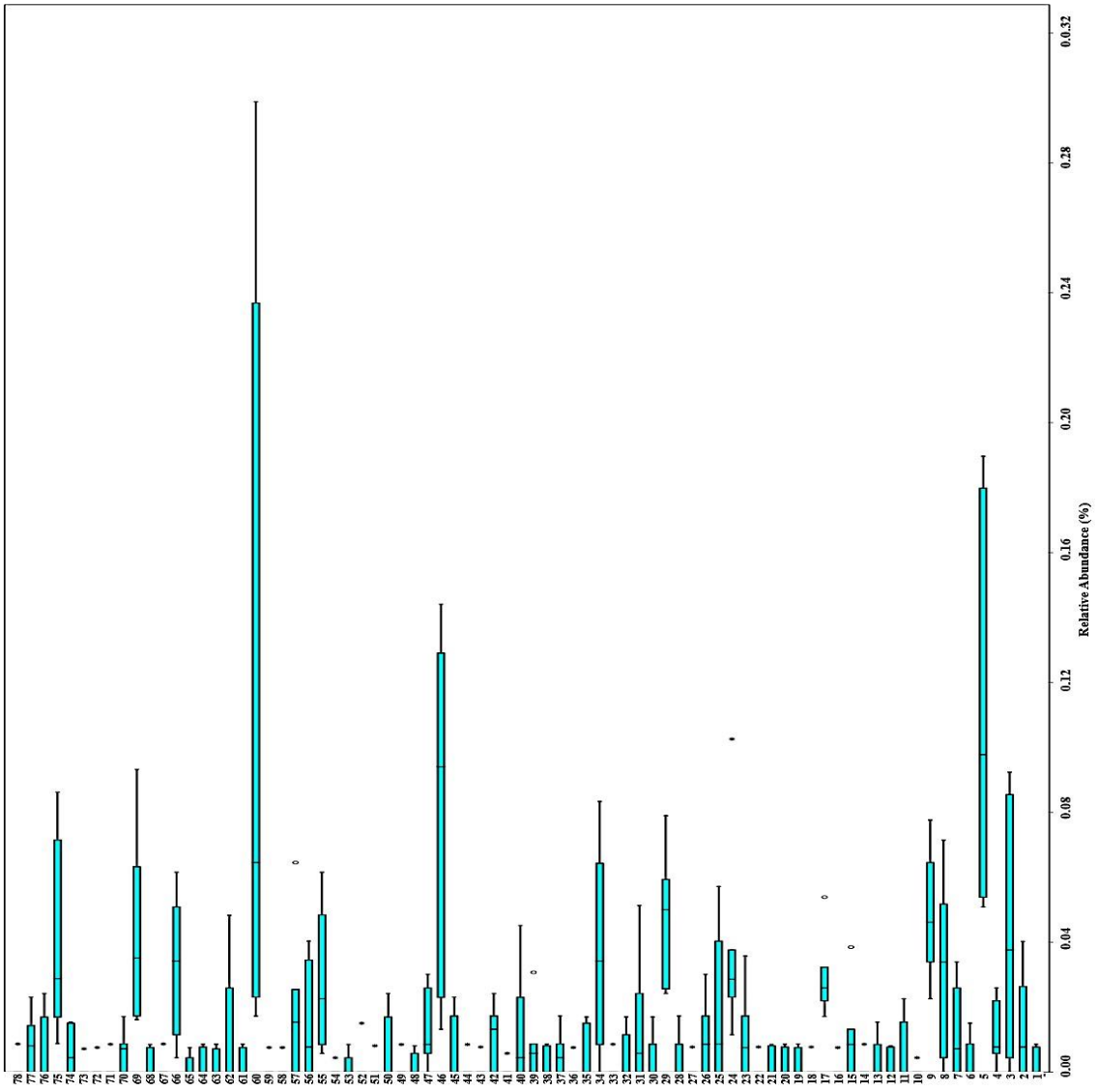

Figure 4.6 Abundance of resistance genes accumulated across all locations. Gene ID's can be found in Appendix-B (Table 1); * indicates outliers.

The proportion of resistance genes abundance across all source types (impacted, un-impacted, tributary 1 and tributary 2) is described in Figure 4.7. The proportion of *bacA* resistance gene abundance across all source types was in the range of 4-27%; impacted locations had the least proportional abundance of 4%, and un-impacted site had the largest proportional abundance of 27%. *Cfr*-gene proportion of abundance was in the range of 5-18%; tributary 1 source type had the least proportional abundance of 5%, and un-impacted site had the largest proportional abundance of 18%. *BlaR*1 proportional abundance was in the range of 2-14% across all source types; tributary 1 source type had the largest proportional abundance of 14%, and un-impacted locations had the least proportional abundance of 2%. *Tsnr* proportion of abundance across all source types was in the range of 0 - 6%; un-impacted locations had the least proportional abundance of 0%, and impacted locations had the largest proportional abundance of 6%.

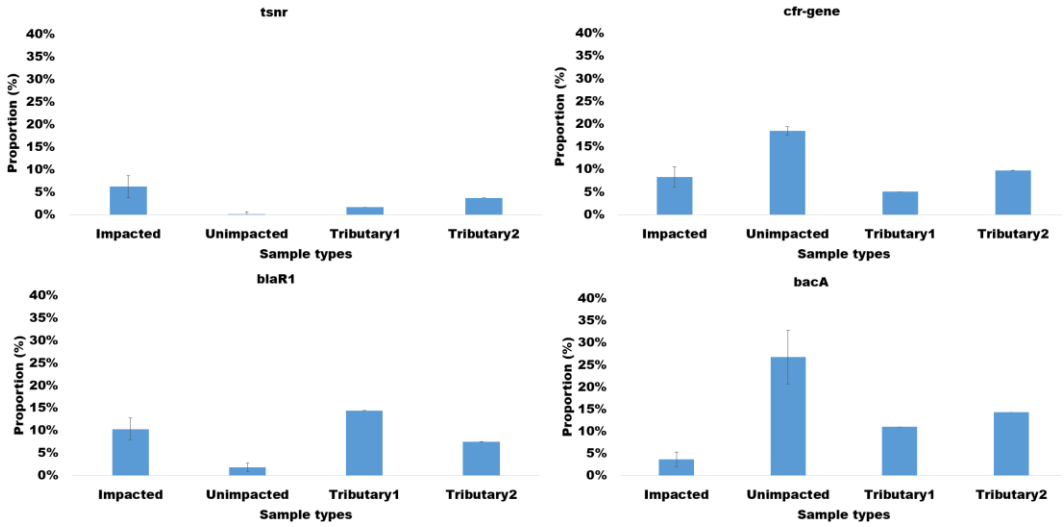

Figure 4.7 Mean abundance of dominant resistance genes; error bars describe the 95% confidence interval.

A three-pattern cluster of resistance genes across the sampling locations is demonstrated by a heat map analysis (Figure 4.8). The first cluster comprises 30 genes, *bacA*, *bl2a_iii*, *blaR1*, class D, *fosb*, *oprn*, *smea*, *dfra12*, *tnr*, *dfra13*, *tet*w, *cml_e7*, *erm38*, *tetpb*, *smec*, *oprj*, *smed*, *cml_e6*, *mex*c, *vana*, *Tetracycline_Resistance_MFS_Efflux_pump*, *FluoroquinoloneResistantDNATopoisomerase*, *srmb and aac6i* (Figure 4.8). This clustered samples from the Little Falls-summer, Deer Lake-summer, Main Dam Road tributary-summer, Humber Resort-summer, Humber Village-summer, Steady Brook tributary-winter, and Across the Bay-winter.

The second cluster included 22 resistance genes, *mexh*, *mdtk*, *cml_e5*, *amra*, *vanx*, *tcr*3, *bl2be_per*, *bacA*, *class C-AmpC*, *tsnr*, *tetj*, *pbp2b*, *and tcma*. This clustered samples from the Little Falls-summer, Deer Lake-summer, Main Dam Road tributary-summer,

Humber Resort location-summer, Humber Village-summer and winter, Steady Brook tributary- winter, and Across the Bay-summer and winter sampling seasons.

The third cluster comprised of 18 resistance genes, *otrb, aac3viii, lmra, bl2a_pc, van*a, *bacA, cara, adeC-adeK-oprM, bl1_ampc, tet*3, *vansc, and mdfra resistance genes* (Figure 4.8). This clustered samples from the Main Dam Road tributary-summer, Humber Resort-summer, Humber Village location-summer and winter, and Across the Bay location-summer and winter sampling seasons.

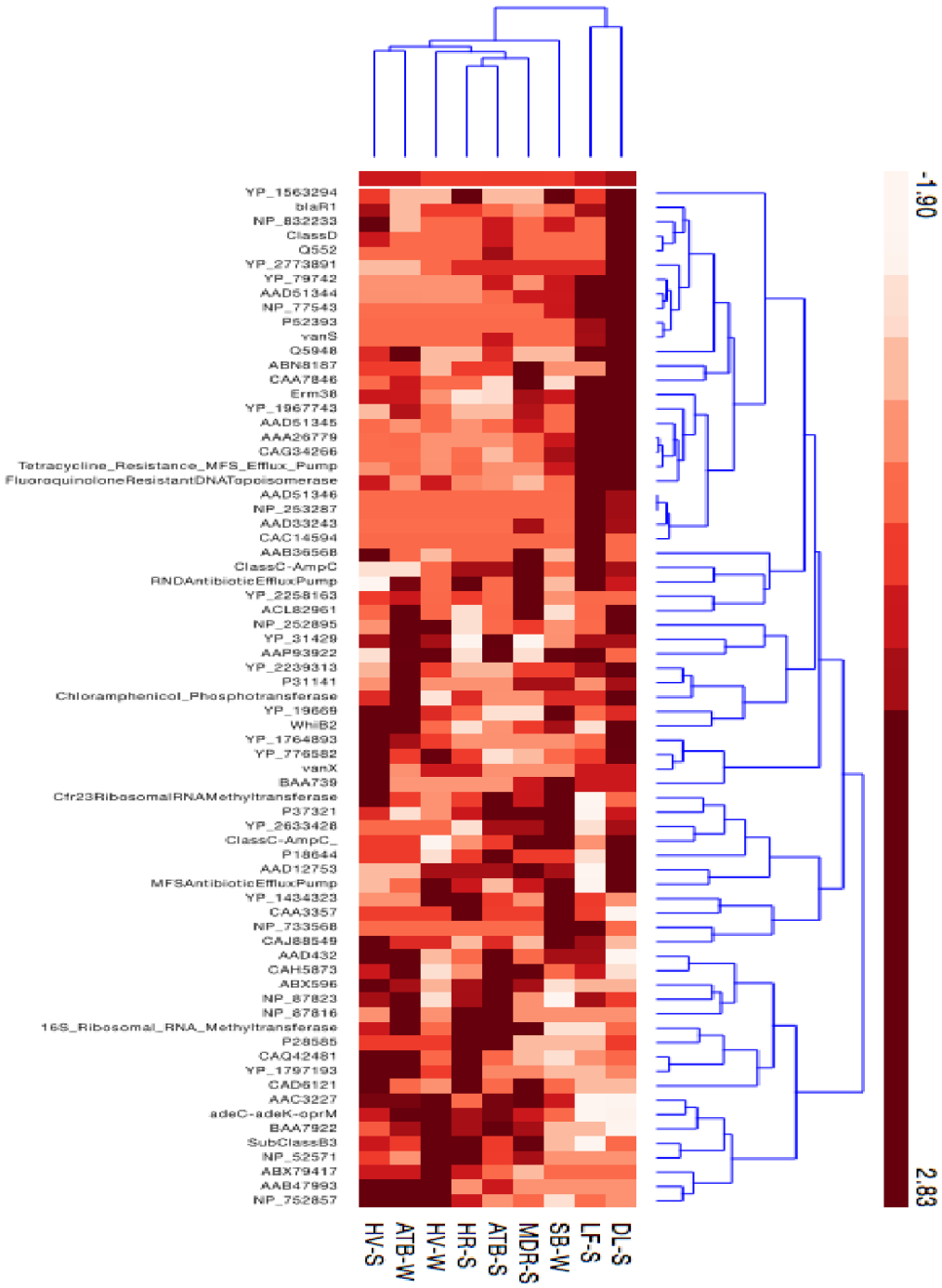

Figure 4.8 Relative abundance of antibiotic resistance genes at each sampling location. Data was normalized around the mean; the scale bar describes variation in units of standard deviation.

Principal component analysis PCA revealed that antibiotic resistance gene populations were relatively low in abundance and diversity at the impacted sampling locations; locations that clustered in the same direction are presumed to have the same resistance gene profile (Figure 4.9). *BacA* gene associated mostly with the un-impacted locations, suggesting its abundance here. The *blaR*1, *fluoroquinolone resistance DNA topoisomerase*, *bcrc*, *bacA* and *dfrb*12 were most dominant in the tributary flowing from the Grand Lake into the Deer Lake, (Main Dam Road sample location, Tributary 1). *Cfr*-gene, and *bl3_vim* resistance genes were the most dominant in the Tributary 2 location (Steady Brook).

ARGs profiles varied across source types. It appears that Tributary 1 location had the most abundance of 5 resistance genes; *Fluoroquinolone resistance DNA topoisomerase*, *blaR*1, *bcrc*, *dfrb*12 and *bacA* and Tributary 2 location had the 2 resistance genes (*Bl3_vim* and *cfr*-gene). Un-impacted locations had the most abundance of 1 resistance gene *BacA* and impacted locations had no resistance genes dominating the locations. More detailed eigenvalue can be found Appendix-B (Table 2).

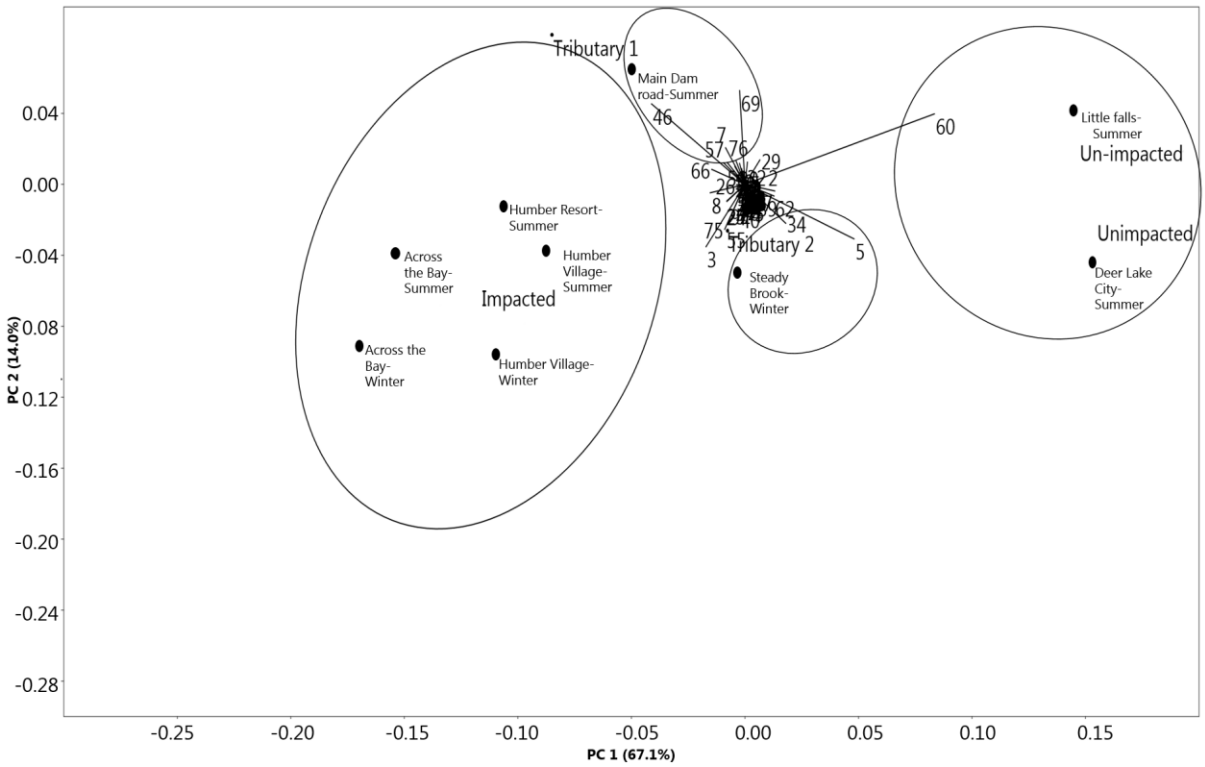

Figure 4.9 PCA biplot to assess the similarities in the profiles of antibiotic resistance genes between sample source types. Gene ID's can be found in Appendix-B (Table 1).

4.1.2.2 Relationship between abiotic parameters to resistance genes

Table 4.2 Explanatory value of factors (seasons, sample types) and covariates (pH and temperature - °C) for the abundance of *bl2d_oxa*2 resistance gene (Relative abundance-%) in the Humber River watershed.

Factors and covariates	DF	F-value	P-value
Seasons	1	14.48	0.032
Sample type	2	5.35	0.102
Temp(Season)	2	7.04	0.074
Error	3		
Total	8		
S= 0.0016	R^2=88.0%	R^2adj=68.0%	

A SIMPER analysis (similarity percentage) allowed elucidation of the contribution of the different resistance genes to the dissimilarity across sampling locations and location types. *BacA*, *cfr*-gene, *bl3_vim* resistance genes induced the most dissimilarities in the Un-impacted locations. *BlaR*1 resistance gene and *fluoroquinolone resistance DNA topoisomerase* induced the most dissimilarities in the Tributary 1 location (Appendix B-Table 3).

An analysis of variance / general linear model (ANOVA/GLM) was performed for the gene that contributed 95% of the total dissimilarity (Appendix B-Table 3). GLM analysis showed that the combination of factors (seasons, sample types and covariates (temperature and pH) and their interactions described well the variability of the dataset. Sampling seasons, sample types and temperature when nested within seasons (i.e. in a

temperature bin of summer: 9-22.5 °C and winter: 0-9 °C) were found not to relate statistically significantly to the abundance of all genes. Sampling seasons were found to relate statistically significantly to the abundance of *bl2d_oxa*2 ($\alpha < 0.05$, $r^2 = 88.0\%$) (Table 4.2). See detailed results in the table in (Appendix B- Table 4).

Table 4.3 Correlation between presence of antibiotic resistance genes (Relative abundance-%) and measured abiotic factors (pH and temperature).

Pearson Correlation	pH	Temperature	tnr	aac6i	srmb	bl2d_oxa2	aac3iia	lmrb	BacA	bl2_kpc	cata1	BacA	bl2d_oxa1	dfra12
pH	1													
Temperature	0.37	1												
tnr	-0.19	-0.62	1											
aac6i	-0.19	-0.22	-0.28	1										
srmb	-0.19	-0.20	0.31	-0.28	1									
bl2d_oxa2	-0.13	0.03	-0.19	-0.19	-0.19	1								
aac3iia	-0.13	-0.58	-0.19	0.60	-0.19	-0.13	1							
lmrb	-0.13	0.44	-0.19	-0.19	-0.19	-0.13	-0.13	1						
BacA	-0.13	0.23	-0.19	0.72*	-0.19	-0.13	-0.13	-0.13	1					
bl2_kpc	1.00*	0.37	-0.19	-0.19	-0.19	-0.13	-0.13	-0.13	-0.13	1				
cata1	-0.13	-0.44	0.70*	-0.19	-0.19	-0.13	-0.13	-0.13	-0.13	-0.13	1			
BacA	-0.13	0.10	-0.19	-0.19	0.68*	-0.13	-0.13	-0.13	-0.13	-0.13	-0.13	1		
bl2d_oxa1	-0.13	-0.38	0.62	-0.19	0.64	-0.13	-0.13	-0.13	-0.13	-0.13	-0.13	-0.13	1	
dfra12	-0.13	0.23	-0.19	-0.19	-0.19	-0.13	-0.13	-0.13	-0.13	-0.13	-0.13	-0.13	-0.13	1

* Correlation is significant at the 0.05 level.

4.1.2.3 Correlation among antibiotic resistance genes.

Correlation was found between *bacA, srmb* and *aac6i,* it appears they clustered in the same group in the heat map analysis (Figure 4.3). *Cata*1 resistance gene is significantly correlated with *tsnr* gene ($r^2 = 0.70$, $p < 0.05$). It was observed that temperature was significantly correlated with the abundance of *bl2_kpc* resistance gene ($r^2 = 1.00$, $p < 0.05$) (Table 4.3).

4.1.3 **Distribution of AR classes in the Humber River system**

4.1.3.1 AR classes

AB are categorized into classes according to the cellular function they affect. These cellular processes include cell wall synthesis (β-lactams, cephalosporin, carbapenems, glycopeptides and lipopeptides), DNA synthesis (fluoroquinolones and quinolones), RNA synthesis (rifamycin), protein synthesis (aminoglycoside, chloramphenicol, tetracycline, macrolides), and tetrahydrofolate synthesis (sulfonamides and trimethoprim). Resistance to these antibiotics can be associated to such antibiotic classes.

Bacitracin, tetracycline, multidrug (b), and transcriptional mechanism (whiB2) resistance classes were the most abundant and somewhat consistent across all locations and seasons (Figure 4.10). Genes associated with resistance for bacitracin and multidrug (b) were the most abundant in Little Falls location in the summer, Deer Lake location in the summer, in the tributary flowing from the Grand Lake, at the Main Dam Road summer location, Humber Resort location in the summer, Humber Village location in the summer and winter seasons, Steady Brook tributary in the winter, Across the Bay location in the summer and winter sampling locations.

Genes for tetracycline were the most abundant in the Little Falls location in the summer, Main Dam Road tributary location, Humber Resort location in the summer, Humber Village location in the summer and winter, and Across the Bay locations in the summer and winter. Genes for resistance to whiB2 were the most dominant in the Deer Lake location in the summer and Main Dam Road tributary in the summer. Genes

conferring resistance to penicillin were the most dominant in the Humber Resort location in the summer and Across the Bay location in the summer. Genes for resistance to carbapenems, penicillin, cephalosporin were most abundant in the Main Dam Road tributary location in the summer and the Across the Bay location in the summer. Genes for resistance to acriflavine, puromycin, t_chloride resistance classes dominate the Little Falls location in the summer.

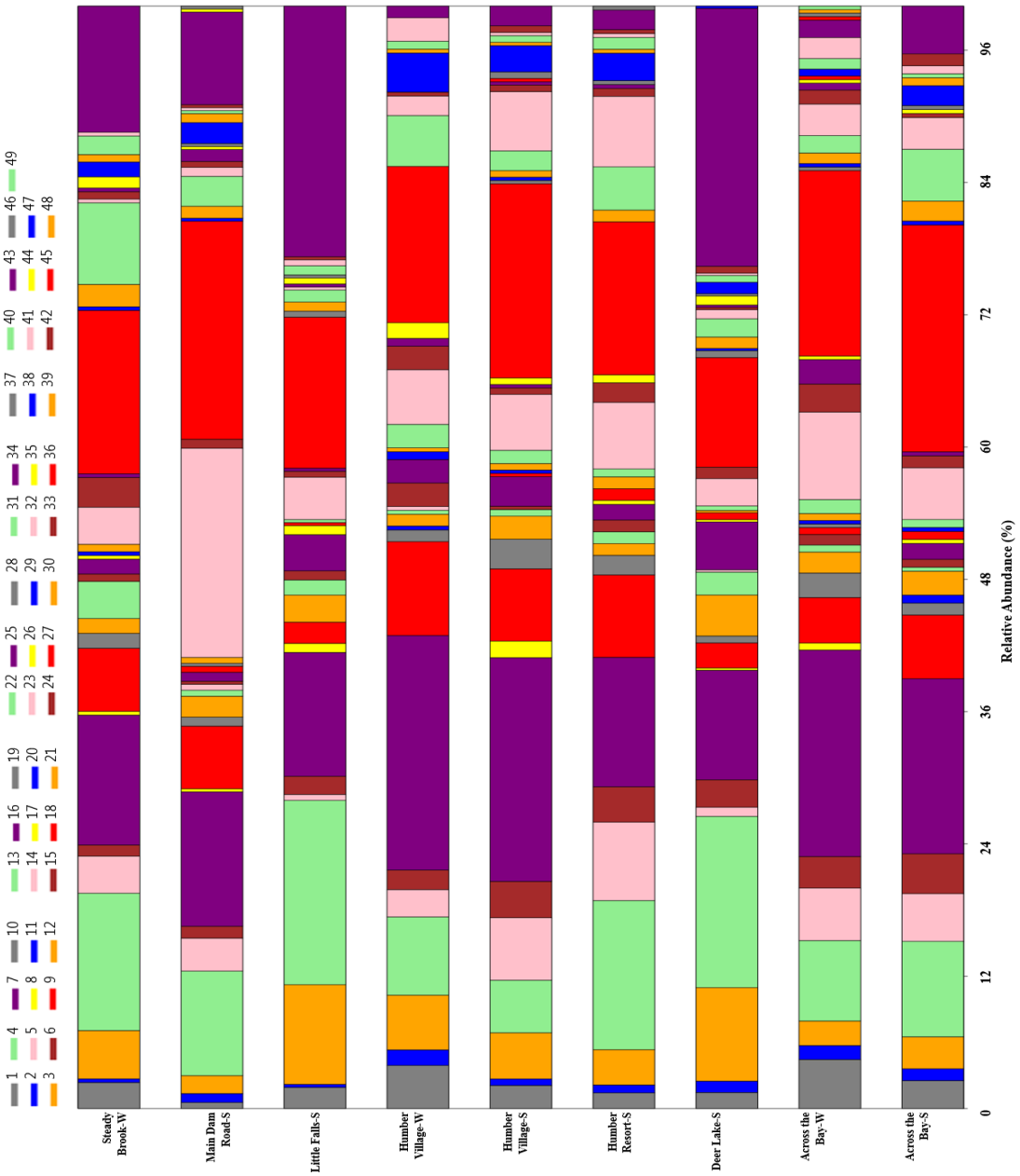

Figure 4.10 Abundance of antibiotic resistance classes at the sampling locations. Resistance classes ID's can be found in Appendix-C (Table 1).

The relative abundance rate of resistance classes is described in a box plot (Figure 4.11); outliers may have occurred because of variability in measurement or heavy tailed end distribution. Resistance classes associated with whib2, multidrug, bacitracin and tetracycline resistance classes had the largest proportional abundance among all classes.

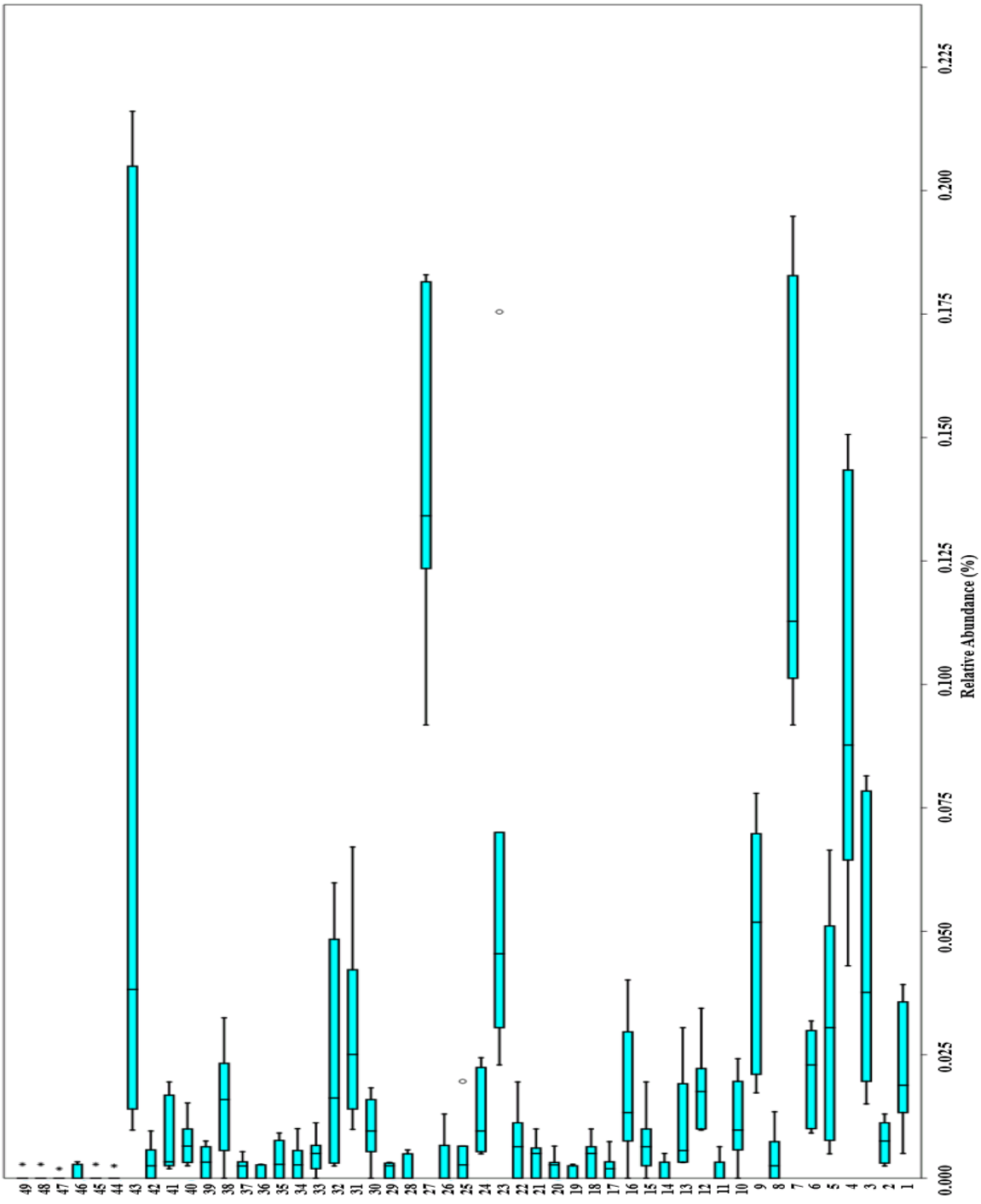

Figure 4.11 Abundance of antibiotic resistance classes accumulated across all locations. Resistance classes ID's can be found in Appendix-C (Table 1). * indicates outliers.

The proportion of abundance across all source types is described in Figure 4.12; abundance of tetracycline resistance class across the source types was in the range of 11-18%; un-impacted site had the least proportional abundance of 11%, and tributary 1 location had the largest proportional abundance. Abundance of genes associated with resistance to bacitracin class was in the range of 8-15%; impacted source type had the least proportional abundance of 8%, and un-impacted site had the largest proportional abundance of 15%.

Genes associated with resistance to multidrug (a) class was in the range of 2-8%. Tributary 1 location had the least proportional abundance and un-impacted site had the largest proportional abundance. Genes associated with resistance to multidrug (b) class was in the range of 10-16%. Un-impacted site had the least proportional abundance of 10% and impacted site had the largest proportional abundance of 16%. Genes conferring resistance to whiB2 was in the range of 2-21%; impacted source type had the least proportional abundance, and un-impacted site had the largest proportional abundance of 21%.

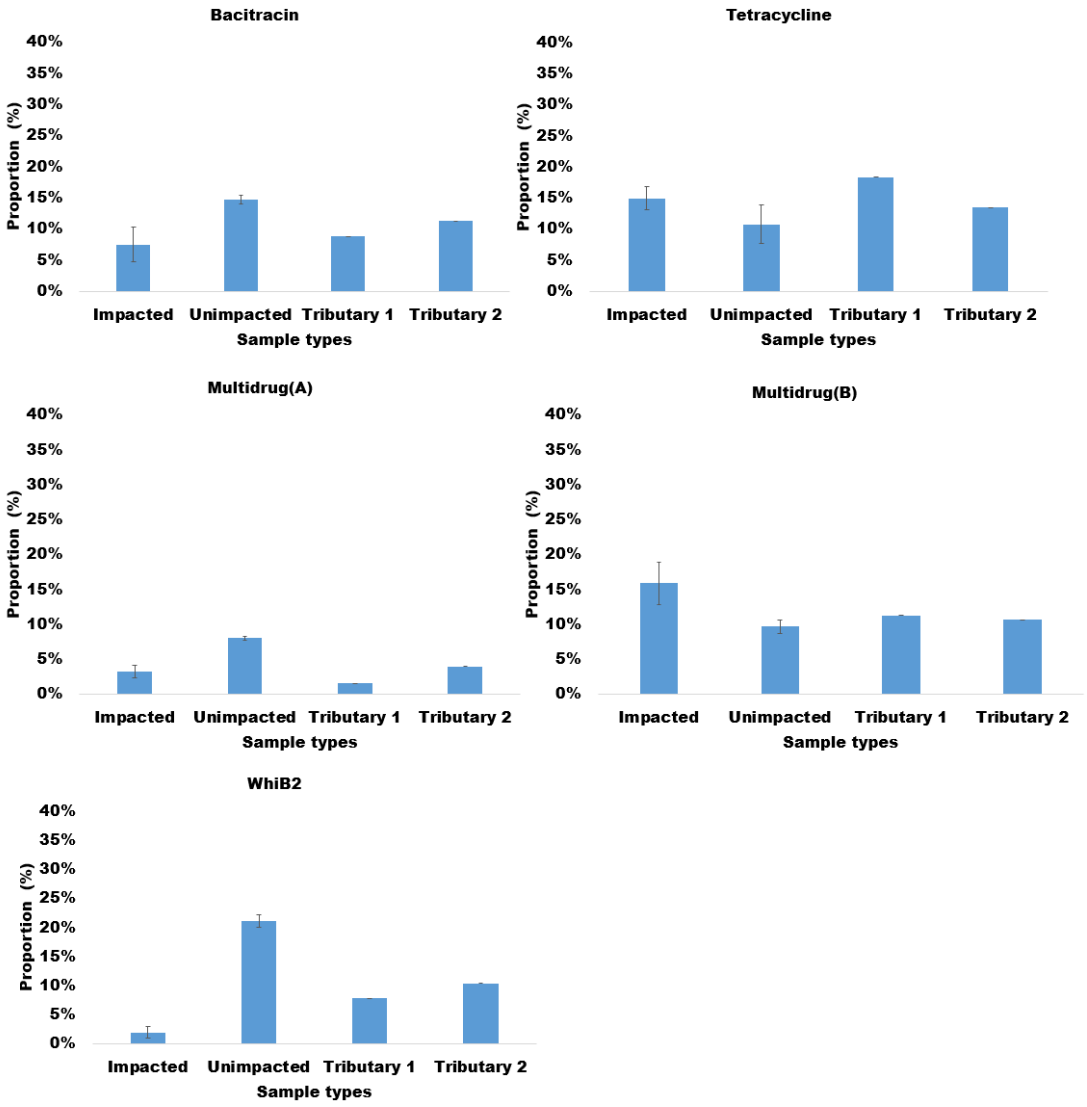

Figure 4.12 Mean abundance of dominant resistance classes, error bars describe the 95% confidence interval.

A heatmap analysis demonstrates clear patterns of clustering of resistance classes across locations. The first cluster shows Little Falls location in the summer, Deer Lake location in the summer and Main Dam Road tributary in the summer to be proportionally

dominated by genes associated with resistance to fluoroquinolone_2, aminoglycoside, glycylcycline, novobiocin, cephalosporin_1, multidrug (d), multidrug (a), and bacitracin, ampicillin, lincomycin and vancomycin classes (Figure 4.13). Humber Village location in the summer and Across the Bay location in the summer were proportionally dominated by genes conferring resistance to lincomycin, vancomycin and fluoroquinolone_1 classes. Genes conferring resistance to ampicillin were most dominant in the Humber Resort location in the summer.

The second cluster shows Main Dam Road location in the summer, Humber Resort location in the summer, Humber Village location in the summer, Steady Brook tributary location in the winter and Across the Bay location in the winter were proportionally dominated by genes associated with resistance to multidrug (g), lincosamide, macrolide, streptogramin_b, multidrug (b), trimethoprim, penicillin, chloramphenicol, fluoroquinolone, tetracycline and cloxacillin, penicillin classes.

The third cluster shows Deer Lake location in the summer, Humber Village location in the summer and winter seasons and Across the Bay location in the winter were proportionally dominated by gene conferring resistance to streptomycin, multidrug (h), neomycin, ribostamycin, fluoroquinolone, aminoglycoside, multidrug (i), macrolide, beta_lactams, multidrug (j), multidrug (k), and chloramphenicol classes. The fourth cluster shows Deer Lake location in the summer, Main Dam Road in the summer, Humber Village location in the summer, Steady Brook location in the winter and Across the Bay location in the summer to be proportionally dominated by teicoplanin, vancomycin,

cephalosporin_3, cephalosporin_2, beta_lactams, multidrug (f), and qa_compound resistance classes.

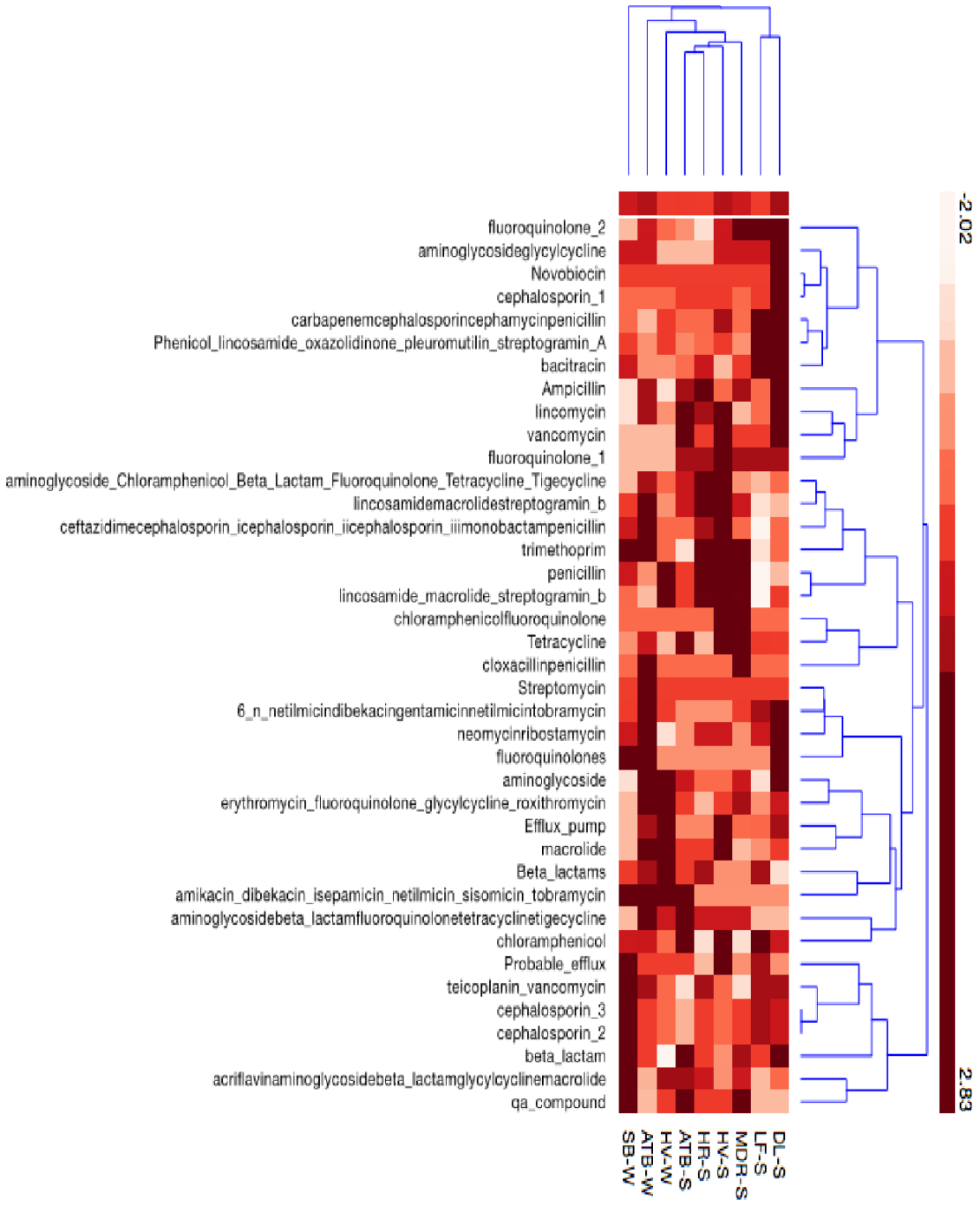

Figure 4.13 Relative abundance of antibiotic resistance classes at each sampling location. Data were normalized around the mean; the scale bar describes variation in units of standard deviation.

PCA revealed differences in the abundance of resistance classes profile across all source types. Unimpacted locations were dominated by genes associated with resistance to bacitracin, lincosamide, macrolide, streptogramin_b, and ampicillin classes. Fewer resistance classes were presented at the impacted locations with genes conferring resistance to multidrug (b) dominating (Figure 4.14). Genes for resistance to tetracycline and whiB2 were the most dominant in the Tributary1 location. Genes conferring resistance to penicillin and multidrug (g) were the most dominant classes in the Tributary 2 location. More detailed eigenvalues can be found in Appendix-C (Table 2).

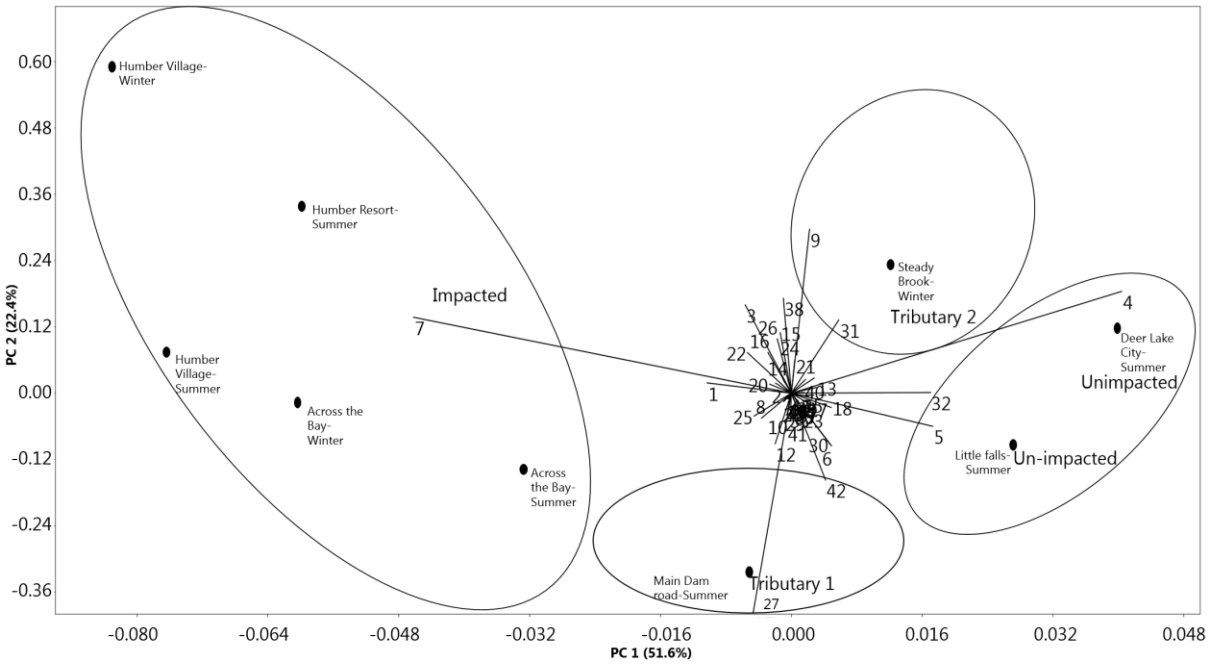

Figure 4.14 PCA biplot to assess the similarities in the profiles of antibiotic resistance classes between sample source types. Resistance classes ID's can be found in Appendix-C (Table 1).

4.1.3.2 Relationship between abiotic parameters to resistance class associated gene

Table 4.4 Explanatory value of factors (seasons, sample type) and covariates (pH and temperature (°C) for the abundance of genes for selected antibiotic resistance classes (Relative abundance %) in the Humber River watershed"

Factors and covariates	DF	Trimethoprim		Macrolide specific		Cloxacillin, penicillin		Quinolone		WhiB2	
		F-value	p-value	F value	p-value	F value	p-value	F value	p-value	F value	p-value
Season	1	17.39	0.025	13.32	0.03	11.58	0.042	14.48	0.032	7.17	0.075
Sample type	2	21.59	0.017	5.18	0.106	2.95	0.196	5.35	0.102	23.70	0.015
Temp (Season)	2	24.63	0.014	6.57	0.080	6.96	0.075	7.04	0.074	9.89	0.048
Error (S)	3	0.0017		0.0013		0.0007		0.0047		0.0195	
Total	8	r^2- 99.0%	r^2(adj)- 97.4%	r^2- 85.9%	r^2(adj)- 62.7%	r^2- 83.8%	r^2(adj)- 56.7%	r^2- 88.0%	r^2(adj)- 68.0%	r^2- 97.9%	r^2(adj)- 94.3%

The SIMPER analysis (similarity percentage) allowed elucidation of the contribution of the different antibiotic resistance class genes to the dissimilarity across sampling locations and sample types (impacted, un-impacted, tributary 1 and tributary 2). Genes conferring resistance to vancomycin, bacitracin, multidrug (a) induced the most dissimilarities in the un-impacted locations. Genes associated with tetracycline and carbapenems, penicillin, cephalosporin resistance induced the most dissimilarities in the tributary 1 location. multidrug (b) resistance induced the most dissimilarities in the tributary 1 and tributary 2 locations.

An analysis of variance / general liner model (ANOVA / GLM) was performed for the classes that contributed to 95% of the total dissimilarity see (Appendix C- Table 3). The GLM analysis showed that the combination of factors (seasons, sample types) and covariates (temperature and pH) and their interaction described well the variability in the dataset. Sampling season, sample types and temperature, when nested within seasons (i.e. in a temperature bin of summer: 9-22.5 °C and winter: 0-9 °C), were found to relate statistically significantly to abundance of the genes conferring resistance to the trimethoprim class, (α <0.05, r^2 = 99.0%) see Table 4.4. The sampling of seasons was also found to be statistically significant for quinolones (α <0.05, r^2 = 88.0%), cloxacillin, penicillin (α <0.05, r^2 = 83.7%) and macrolide specific resistance classes (α <0.05, r^2 = 85.8%). The temperature nested with season and the sample type was found to be statistically significant for transcriptional regulator resistance (α <0.05, r^2 = 97. 8%). The full table of results can be seen in Appendix-C (Table 4).

4.1.3.3 Correlation among antibiotic resistance classes.

Correlation was also found between thiostrepton, fosfomycin and multidrug (c). Macrolide specific resistance is significantly correlated with teicoplanin, cloxacillin, penicillin and quinolones.

Tigecycline is significantly correlated with multidrug (a), transcriptional mechanism and multidrug (b).

The Transcriptional mechanism is correlated with multidrug (a) and multidrug (c). Fosfomycin and multidrug (c) are significantly correlated. Ampicillin, penicillin and quinolones are significantly correlated. Multidrug (b) is significantly correlated with novobiocin and transcriptional mechanism. Teicoplanin and cloxacillin, penicillin is significantly correlated. Cloxacillin, penicillin and puromycin are significantly correlated. Multidrug (d) and transcriptional mechanisms are significantly correlated.

It was observed that temperature was significantly correlated with abundance of genes associated with fluoroquinolone specific ($r^2 = 0.75$, $p < 0.05$) and multidrug (b) ($r^2 = 0.70$, $p < 0.05$) resistance classes (Table 4.5).

Table 4.5 Correlation between the presence of genes conferring resistance to antibiotics (Relative abundance-%) and measured abiotic factors (pH and temperature).

Pearson Correlation	pH	Temperature	Ampicillin	Thiostrepton	Macrolide specific	Tigecycline	Multidrug (a)	Fosfomycin	Ampicillin, penicillin	Fluoroquinolone specific	Monobactams, penicillin	Multidrug (b)	Polymyxin	Teicoplanin	Multidrug (c)	Cloxacillin, penicillin	Multidrug (d)	Novobiocin	Quinolone	Transcriptional mechanism	Puromycin
pH	1																				
Temperature	0.37	1																			
Ampicillin	-0.20	0.47	1																		
Thiostrepton	-0.12	-0.61	-0.34	1																	
Macrolide specific	-0.09	-0.10	0.21	-0.39	1																
Tigecycline	0.51	0.56	0.00	-0.06	-0.11	1															
Multidrug(a)	0.63	0.45	-0.27	-0.13	-0.46	0.76*	1														
fosfomycin	-0.34	-0.65	-0.58	0.78*	-0.49	-0.42	-0.24	1													
Ampicillin, penicillin	-0.23	-0.08	-0.14	-0.27	0.46	-0.21	-0.14	0.01	1												
Fluoroquinolone specific	0.08	0.75*	0.39	-0.57	-0.01	0.11	-0.02	-0.41	-0.18	1											
Monobactam, penicillin	-0.18	0.01	0.00	0.30	-0.56	-0.41	-0.20	0.54	-0.01	0.01	1										
Multidrug(b)	0.22	0.70*	0.15	-0.33	-0.04	0.82*	0.60	-0.49	0.03	0.22	-0.14	1									
Polymyxin	-0.48	-0.13	-0.03	0.00	0.26	-0.11	-0.46	0.07	-0.10	0.04	0.12	0.20	1								
Teicoplanin	-0.25	-0.15	0.67*	-0.10	0.67*	-0.28	-0.60	-0.37	0.13	-0.03	-0.35	-0.34	-0.08	1							
Multidrug(c)	-0.32	-0.38	-0.33	0.70*	-0.24	-0.26	-0.37	0.81*	0.02	-0.02	0.34	-0.41	0.05	-0.10	1						
Cloxacillin penicillin	-0.19	-0.28	0.20	0.12	0.82*	-0.08	-0.57	-0.14	0.38	-0.28	-0.27	-0.05	0.35	0.66*	0.10	1					
Multidrug(d)	-0.59	-0.29	0.15	0.32	0.08	-0.26	-0.58	0.29	-0.35	0.24	-0.15	-0.39	0.35	0.33	0.58	0.17	1				
Novobiocin	-0.13	0.44	0.18	-0.25	-0.16	0.63	0.58	-0.34	0.16	-0.04	-0.18	0.82*	0.03	-0.25	-0.41	-0.19	-0.30	1			
Quinolone	-0.13	0.03	0.09	-0.54	0.83*	-0.29	-0.41	-0.34	0.78*	0.09	-0.18	-0.02	0.19	0.40	-0.21	0.61	-0.21	-0.13	1		
Transcriptional mechanism	0.58	0.48	-0.13	-0.44	-0.03	0.71*	0.82*	-0.57	0.04	-0.06	-0.32	0.74*	-0.13	-0.40	-0.74*	-0.23	-0.75*	0.63	0.00	1	
Puromycin	-0.13	-0.38	0.16	0.64	0.29	0.16	-0.34	0.13	-0.23	-0.43	-0.18	-0.05	0.27	0.48	0.31	0.71*	0.40	-0.13	-0.13	-0.29	1

*Correlation is significant at the 0.05 level.

4.1.4 **Distribution of AR mechanisms in the Humber River system**

4.1.4.1 AR mechanism

Bacteria can acquire resistance to antibiotics either through genetic mutation or by acquiring antimicrobial genes from other bacteria (Blair et al., 2014). These resistance mechanisms, multidrug efflux pump, inhibition of protein synthesis, interference with cell wall synthesis, interference with nucleic acid synthesis and inhibition of a metabolic pathway are the adopted by bacteria to fight antibiotics (Tenover, 2006)

RNA Methylation, efflux pump, drug enzymatic destruction resistance mechanisms were the most abundant and somewhat consistent across all locations and seasons (Figure 4.15). Resistance to beta-lactams, efflux pump and drug enzymatic destruction resistance mechanisms were the most dominant in the Little Falls location in the summer, Deer Lake location in the summer, Main Dam Road tributary in the summer, Humber Resort location in the summer, Humber Village location in the summer, Humber Village in the winter, Steady Brook tributary in the winter and Across the Bay locations in the summer and winter sampling seasons.

Resistance to beta_lactams was the most abundant in the Little Falls location in the summer, Deer Lake location in the summer, Main Dam Road tributary in the summer, Humber Resort location in the summer, Humber Village location in the summer, Humber Village in the winter and Across the Bay locations in the summer and winter sampling seasons. A distinct difference in the abundance of resistance mechanisms was seen for the Steady Brook tributary location in the winter. Transcriptional mechanisms and RNA methylation were the most abundant.

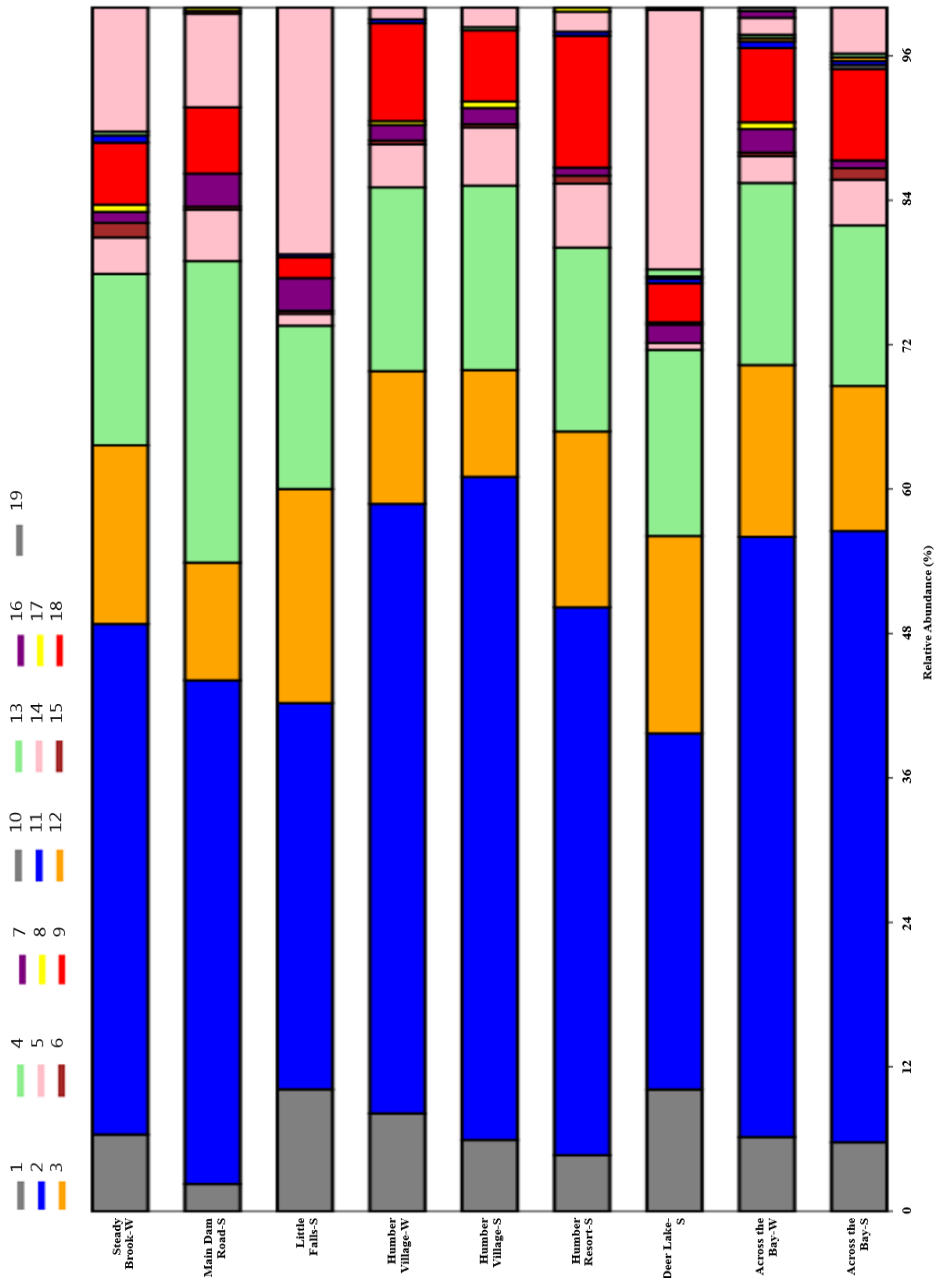

Figure 4.15 Abundance of genes associated with resistance mechanism at the sampling locations. Resistance mechanism ID's can be found in Appendix-D (Table 1).

The relative abundance rate of resistance mechanisms is illustrated in a box plot. (Figure 4.16). (*) indicates outliers which may have occurred because of variability in

measurement or heavy tailed end distribution. Efflux pump, transcriptional mechanism, RNA methylation, drug enzymatic destruction resistance mechanisms had the largest proportional abundance among all resistances mechanisms.

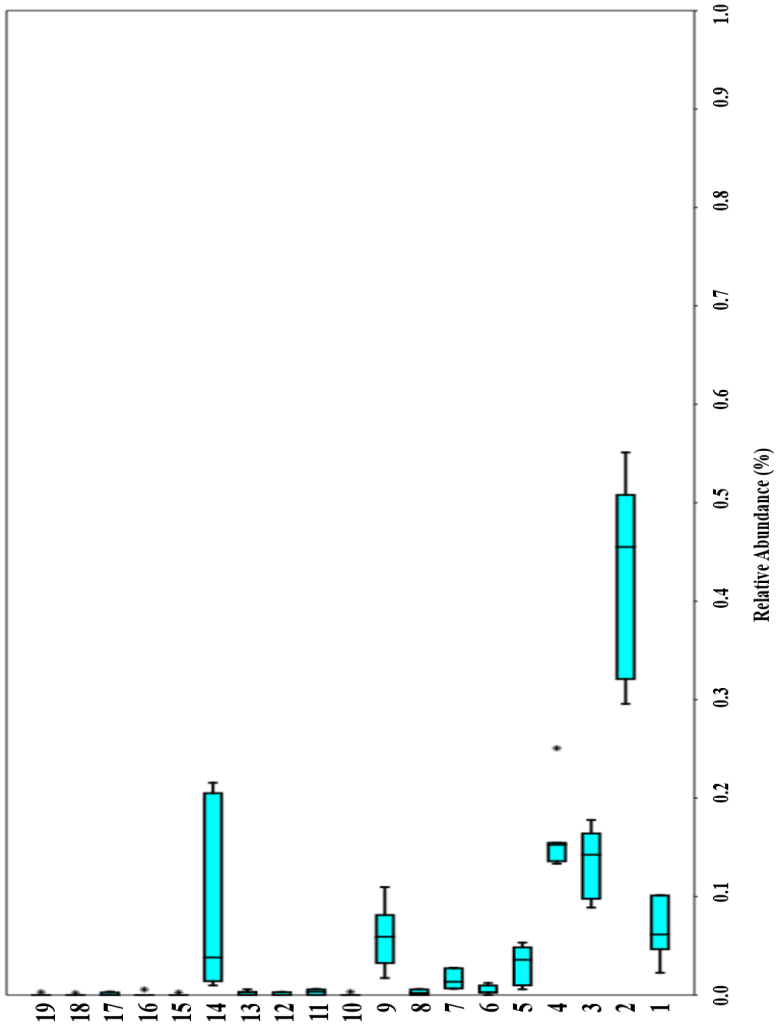

Figure 4.16 Abundance of genes associated with resistance mechanism accumulated across all locations. Resistance mechanism ID's can be found in Appendix-D (Table 1); (*) indicates outliers.

Figure 4.17 shows the proportion of the resistance mechanisms across the source types. Transcriptional mechanism proportion of abundance across all source types was in the range of 2-21%. Impacted locations had least proportional abundance of 2%, and un-impacted locations source types had the largest proportional of abundance of 21%.

Efflux pump resistance mechanism proportional abundance across all source types was in the range 31-50%; the un-impacted locations had the least proportional abundance of 31%, and the impacted locations had the largest proportional abundance of 50%. Drug enzymatic destruction resistance mechanism proportion of abundance was in the range of 10-17%. Tributary 1 source type had the least proportional abundance of 10%. Un-impacted locations had the largest proportional abundance of 17%. RNA_methylation proportional abundance was in the range of 2-10%; Tributary 1 had the least proportional abundance of 2%, and un-impacted locations had the largest proportional abundance of 10%.

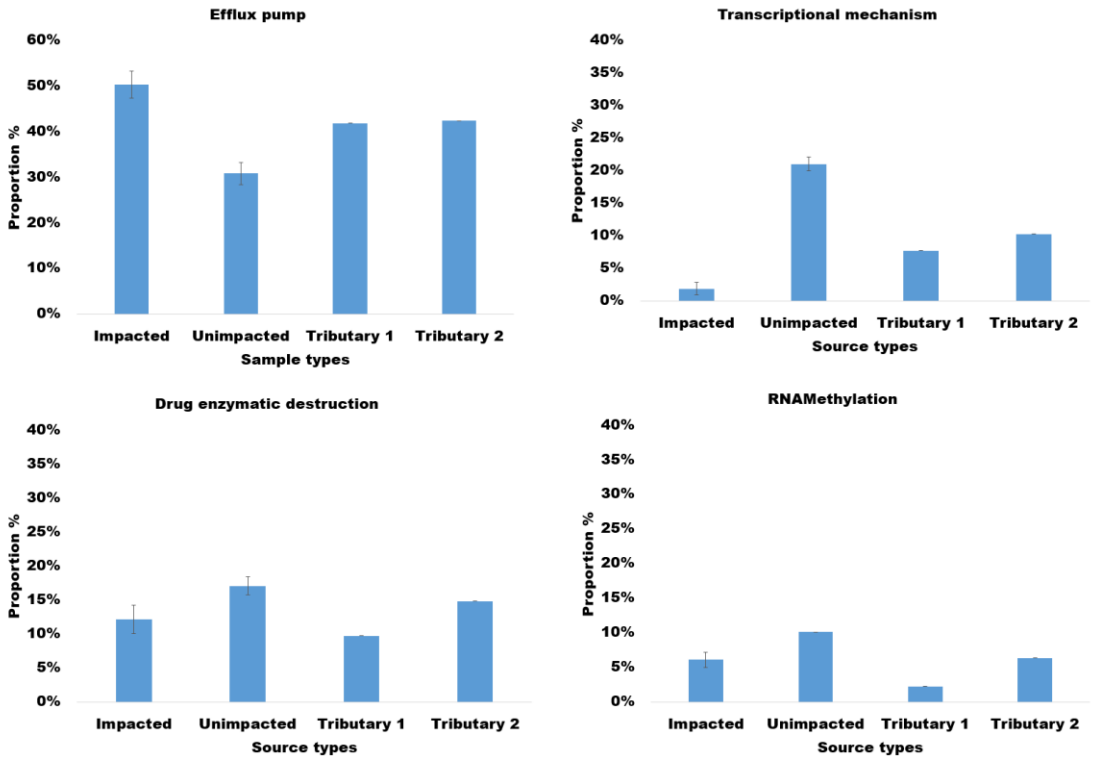

Figure 4.17 Mean abundance of dominant resistance mechanism, error bars describe the 95% confidence interval.

Three clear patterns of clustering of antibiotic resistance mechanism across locations are demonstrated in a heatmap analysis. The first cluster comprised of 7 antibiotic resistance mechanisms; Humber Village location in the summer, Steady Brook tributary location in the winter, and Across the Bay in the winter to be proportionally dominated by efflux pump, trimethoprim _resistance, and cell wall/membrane/envelope biogenesis resistance mechanisms (Figure 4.18).

The second cluster comprised 8 antibiotic resistance mechanisms which showed that Little Falls location in the summer, Deer Lake location in the summer, Main Dam

Road tributary in the summer were proportionally dominated by fluoroquinolone, novobiocin, RNA_methylation, drug_enzymatic_destruction, transcriptional mechanism, drug_target_protection/modification_(mutation) and resistance to beta lactams.

The third cluster comprised of 5 antibiotic resistance mechanisms which showed Main Dam Road tributary in the summer, Humber Resort location in the summer, Humber Village location in the summer and winter seasons, Steady Brook location in the winter, and Across the Bay in the summer were proportionally dominated by teicoplanin, drug_target_protection/modification, penicillin, inhibition metabolites and macrolide phosphotransferase resistance mechanisms.

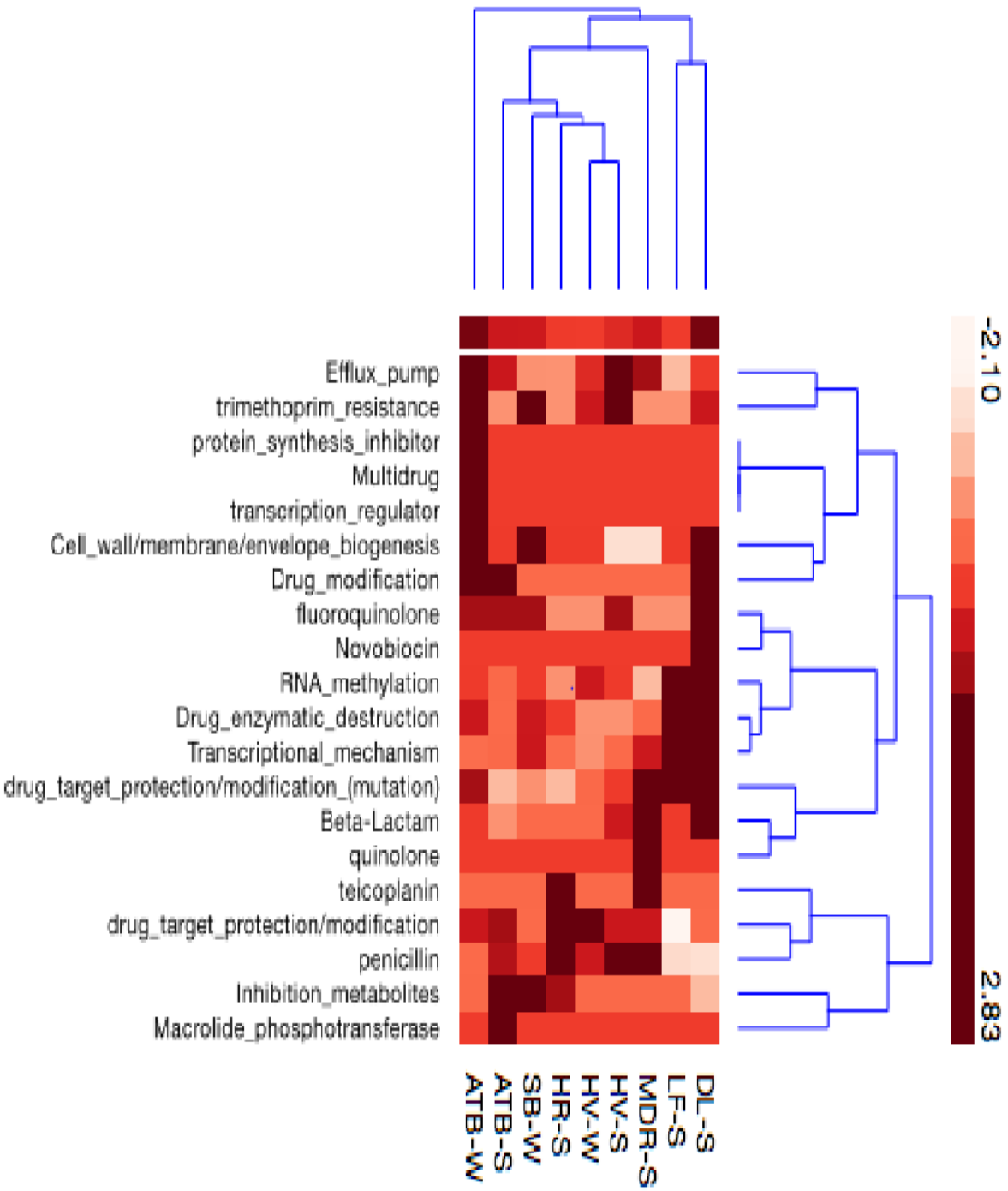

Figure 4.18 Relative abundance of genes associated with resistance mechanism at each sampling location. Data were normalized around the mean; the scale bar describes variation in units of standard deviation.

The PCA revealed differences in the abundance of resistance mechanism profiles across all source types (Figure 4.19). The un-impacted site was dominated by transcriptional mechanism, while the impacted sites was dominated by the efflux pump. Tributary 1 was dominated by resistance to beta lactams. Tributary 2 were dominated by RNA_methylation and drug enzymatic destruction resistance mechanisms.

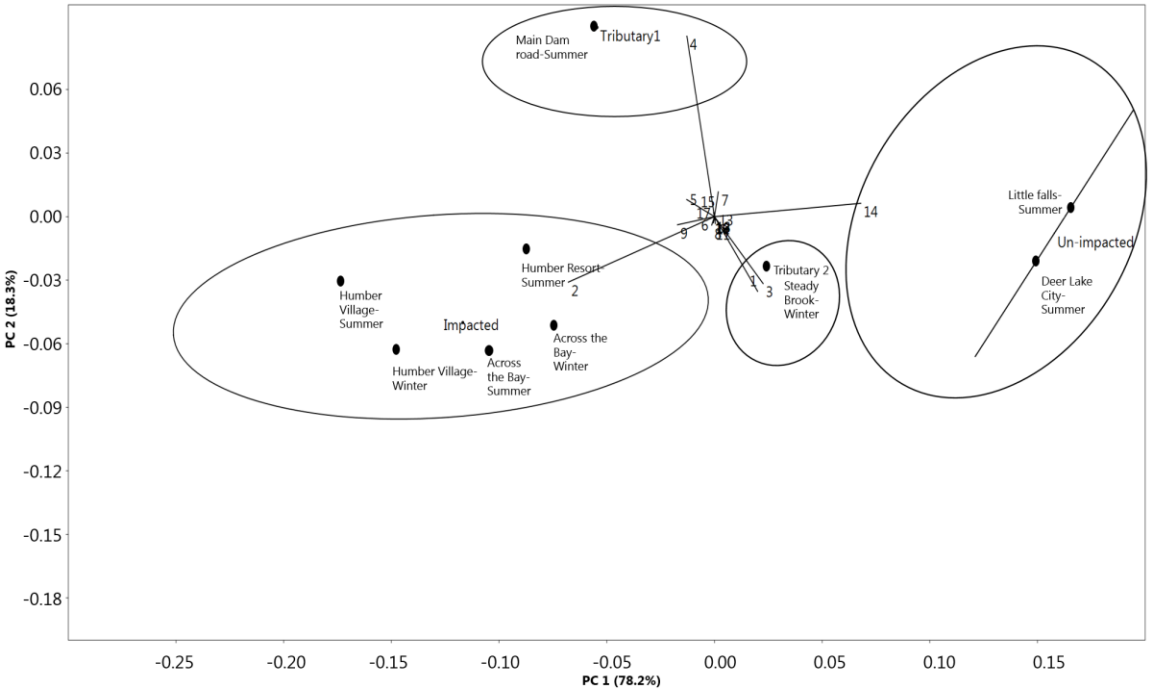

Figure 4.19 PCA biplot to assess the similarities in the profiles of gene associated with biological processes between sample source types. Biological processes ID's can be found in Appendix-D (Table 1).

Table 4.6 Explanatory value of factors (Seasons, sample type) and covariates (pH and temperature (°C) on the abundance of different biological processes (Relative abundance %) in the Humber River watershed.

Factors and covariates	DF	Drug target protection/modification		Quinolones	
		F-values	P-values	F-values	P-values
Seasons	1	8.33	0.063	14.48	0.032
Sample type	2	16.53	0.024	5.35	0.102
Temp(Season)	2	12.0	0.037	7.04	0.074
Error (S)	3				
Total	8	S-0.004 r^2=93% r^2adj=80.5%		S-0.0005 r^2-88.0% r^2adj=68.0%	

The SIMPER analysis (Similarity percentage) allowed elucidation of the contribution of the different resistance mechanisms to the dissimilarity across sampling locations and source types (impacted, un-impacted, tributary 1 and tributary 2). Resistance to beta_lactams and efflux pump resistance mechanisms induced the most dissimilarities across the impacted, un-impacted, tributary 1 and tributary 2. Genes associated with the transcriptional mechanism and drug enzymatic destruction mechanisms induced the most dissimilarities in the un-impacted locations.

An analysis of variance / general liner model (ANOVA / GLM) was performed for the resistance mechanisms that contributed to 95% of the total dissimilarity (Appendix D-Table 3.). The GLM analysis showed that the combination of factors (seasons, sample types) and covariates (Temperature and pH) and their interaction described well the variability in the dataset (Table 4.6). Sampling seasons were found to relate statistically

significantly to the abundance of quinolones resistance mechanism ($\alpha < 0.05$, $r^2 = 88.0\%$). Sample types were found to relate statistically significantly to the abundance of drug_target protection/modification resistance mechanism ($\alpha < 0.05$, $r^2 = 92.7\%$). Temperature, when nested within seasons (i.e. in a temperature bin of summer: 9-22.5 °C and winter: 0-9 °C), was found not statistically significant to the abundance of the resistance mechanisms. Full table of result can be seen in Appendix- D (Table 4).

4.1.4.2 Correlation among antibiotic resistance mechanisms.

A strong correlation was found between protein synthesis inhibitors and transcriptional regulators and the multidrug efflux pump resistance mechanism ($r^2 = 1$, $p < 0.05$). (Table 4.7).

Table 4.7 Correlation between the presence of genes associated with biological processes (Relative abundance-%) and measured abiotic factors (pH and temperature).

Biological process	pH	Temperature	drug target protection/ modification (mutation)	Inhibition, metabolites	Trimethoprim resistance	fluoroquinolone	Cell wall/membrane/ envelope biogenesis	Drug modification	teicoplanin	protein synthesis inhibitor	Novobiocin	quinolone	Macrolide phosphor-transferase	Transcription regulator	Multidrug
pH	1														
Temperature	0.37	1													
drug target protection/modification (mutation)	0.55	0.18	1												
Inhibition, metabolites	-0.21	-0.43	-0.64	1											
trimethoprim resistance	-0.35	-0.63	-0.19	0.13	1										
fluoroquinolone	-0.36	0.17	-0.32	0.01	0.38	1									
Cell wall/membrane/envelope biogenesis	-0.11	-0.54	-0.38	0.44	0.39	0.36	1								
Drug modification	-0.24	0.13	-0.20	0.03	-0.01	0.61	0.39	1							
teicoplanin	-0.19	0.10	0.02	0.03	-0.52	-0.53	-0.32	-0.36	1						
protein synthesis inhibitor	-0.13	-0.38	0.20	-0.18	0.44	0.16	0.45	0.53	-0.19	1					
Novobiocin	-0.13	0.44	-0.00	-0.44	-0.08	0.70	0.13	0.29	-0.19	-0.13	1				
quinolone	-0.13	0.03	0.57	-0.21	-0.35	-0.36	-0.55	-0.24	0.54	-0.13	-0.13	1			
Macrolide phosphotransferase	-0.13	0.23	-0.42	0.46	-0.35	0.23	0.02	0.64	-0.19	-0.13	-0.13	-0.13	1		
transcription regulator	-0.13	-0.38	0.20	-0.18	0.44	0.16	0.45	0.53	-0.19	1*	-0.13	-0.13	-0.13	1	
Multidrug	-0.13	-0.38	0.20	-0.18	0.44	0.16	0.45	0.53	-0.19	1*	-0.13	-0.13	-0.13	1*	1

* Correlation is significant at the 0.05 level.

4.1.5 **Discussion**

An improved understanding of abundance and distribution of the ARGs in the aquatic environment is essential to manage the spread of ARB and protect human health. Metagenomics shotgun sequencing of ARGs was performed on nine composite water samples collected monthly from seven locations along the Humber river under varying source types e.g. un-impacted, impacted, tributary1, and tributary2 derived from an intensity gradient of human activities.

Cfr-gene, *Bac*A, *tsnr* and *blaR1* genes were the most abundant resistance genes in the river water samples collected. *Bac*A gene is known to confer resistance to bacitracin, this gene has been reported to be dominant in drinking water and river water (Jia et al., 2015; Li et al., 2015). A previous survey has reported that about 52% of swine operations use bacitracin (Sarmah et al, 2006). Bacitracin resistance genes were also found to be much higher in human faeces and commonly distributed in waters samples (Li et al., 2015) and therefore, as expected, the abundance of bacitracin resistance genes are usually associated with the antibiotics used extensively as human medicine or veterinary medicine for growth and prophylaxis (Li et al., 2015), so it is not surprising that the resistance gene is present in drinking water, river water, influent and effluents in high abundances (Li et al., 2015).

Chlorination has been found to be a contributing factor to the increase of total ARGs abundance (Shi et al., 2013). Chlorination may eliminate bacteria by destroying cell wall, but the *bac*A gene product is essential for the biosynthesis of peptidoglycan and other cell wall components, which results in the survival of the bacteria harboring *bac*A gene

under chlorine stress (Jia et al., 2015). *Tsnr* resistance gene proportion of abundance was low across source types; un-impacted locations had the least proportional abundance of 0%, and impacted locations had the largest proportional abundance of 6%.

BlaR1 resistance gene proportion of abundance was in the range of 2-14% across all source types; tributary 1 location had the largest proportional abundance of 14%, and un-impacted locations had the least proportional abundance of 2%. *BlaR*1 codes for a β-lactam-recognizing protein. Its transmembrane structure exposes its penicillin-sensitive domain to the external medium, and allows the protein to detect the presence of extracellular antibiotics. *Cfr*-gene proportion of abundance was in the range of 5-18%; Tributary1 location (Main Dam Road) had the least proportional abundance of 5%, and un-impacted locations had the highest proportional abundance of 18%.

The resistance classes across all source types, tetracycline, bacitracin, multidrug and whiB2 can be seen to vary in proportional abundances between source types. In a study by Stoll et al. (2012), tetracycline found in river water samples from Germany and Australia at a range of 55 and 45% respectively, suggests a wide occurrence of tetracycline genes in aquatic ecosystems. In this study, lower levels of proportional abundance genes conferring resistance to tetracycline were found across all source types with a range of 9-15%; the tetracycline class of antibiotic is one of the most commonly used therapeutics in human and veterinary medicine, its wide spread in these source types may be due to its importance in human and veterinary medicine and animal husbandry (Chopra & Roberts, 2001; Mazaheri Nezhad Fard et al., 2011).

Genes conferring resistance to tetracycline are often carried on conjugative plasmids or transposons which allow for mobilization via horizontal gene transfer (Auerbach et al., 2007). Genes associated with resistance to bacitracin had a proportional abundance range of 8-14% across source types. Genes for resistance to multidrug (a) had a low proportional of abundance across all source types; tributary 1 location had the least proportional abundance and un-impacted locations had the largest proportional abundance.

Genes conferring resistance to multidrug (b) class was in the range of 10-16%; un-impacted locations had the least proportional abundance and impacted locations had the largest proportional abundance. Genes conferring resistance to whiB2 was in the range of 2-21%. Un-impacted locations had the least proportional abundance, and impacted locations had the largest proportional abundance. WhiB2 in *M. tuberculosis* (also called whmD) which by amino acid similarity is most similar to *M. smegmatis and* found everywhere, strongly suggesting its foundations for common core phenotype that are retained throughout a bacterial taxon (Goldsworthy et al., 2011; Raghunand & Bishai et al., 2006).

Understanding the mechanisms by which bacteria successfully defend themselves against antibiotic assault is essential. Efflux pumps, transcriptional mechanisms, RNA_methylation, and drug enzymatic destruction resistance mechanism had the largest proportional abundance across source types and sampling locations. Proportional abundance of genes coding for an efflux pump was found to be less abundant in the un-impacted locations versus the impacted sites. Efflux pump genes are found in almost all

bacterial species contributing significantly to the increased resistance to multiple antibiotics.

Efflux pumps are in the membrane and actively export antibiotics out of the bacterial cell. It is a mechanism of resistance to tetracycline. This mechanism of resistance plays a major role in the intrinsic resistance of gram-negative bacteria to many drugs that may be used for the treatment of bacterial infections caused by gram-positive bacteria. Genes coding for resistance against antibiotics that affect transcriptional mechanisms had a proportional abundance of 2-21% across source types. The drug enzymatic destruction resistance mechanism was found to have lower proportional abundance in the tributary1 site and a higher proportional abundance in the un-impacted site. Genes for the resistance through RNA methylation had the least proportional abundance in tributary 1 and had the largest in the un-impacted location.

The PCA revealed the relationship between ARGs and locations. Firstly, one may conclude that *bacA, tsnr, blaR*1 and *cfr*-genes dominated across all source types, it is fair to assume that the presence of these genes at source types are linked to human and animal sources, because these genes are used in human and veterinary medicine. *BlaR*1, *tsnr* and *bac*A genes confer resistance to penicillin, thiostrepton and bacitracin respectively. Genes associated with whiB2, bacitracin, tetracycline, multidrug (a) and multidrug (b) dominated the source types among other resistance classes. The distribution of resistance mechanisms across locations showed that the efflux pump, RNA methylation, drug enzymatic

destruction, and transcriptional mechanisms were the most dominating mechanisms across all source types.

The ANOVA analysis showed that season, sample types, temperature and pH had no effect on the resistance genes. Sampling seasons were found to relate statistically significantly to the abundance of *bl2d_oxa*2 resistance gene. Sampling seasons were found to relate statistically significantly to the abundance of genes associated with resistance to trimethoprim, macrolide specific, quinolone, cloxacillin and penicillin classes. Sample type and temperature(seasons) were found to relate statistically significantly to the abundance of trimethoprim and whiB2. The effect of factors and covariates on the abundance of biological processes revealed sampling seasons were found to relate statistically significantly to the abundance of quinolones resistance mechanisms. Sampling type and temperature (seasons) were found to relate statistically significantly to the abundance of drug target protection modification.

4.1.6 **Conclusion**

Antibiotic resistance distribution and abundance were analyzed, sampling locations were grouped into contamination intensity and, although the ARGs were found in all source types, antibiotic resistance gene contamination was more abundant in the un-impacted and tributary 1 location and varied over seasons.

Tributary 2 and the impacted locations had lower abundance and diversity of antibiotic resistance genes and classes. The un-impacted locations had the largest

proportional abundance of bacitracin and *bac*A resistance gene. Tributary 1 location had the largest abundance of and *blaR*1 gene, and tetracycline.

A generally consistent relation was observed for the proportional abundance of *bac*A and bacitracin genes, *blaR*1 genes with penicillin abundance, which may suggest that the fate and transport of antibiotic resistance genes maybe correlated with certain degrees of antibiotic residues in the environment or more likely the wastewater source. All types of antibiotic resistance mechanisms were detected in all source types. The above results draw attention to a need to understand the pathways and mechanism of ARGs released into the aquatic environment in the study area, and seek effective ways to reduce their spread for the concern of public health.

4.2 Detection and quantification of selected antibiotic resistance genes (ARGs) by real-time qRT-PCR

The three selected ARGs of interest in this study are; two tetracycline resistance genes which encode ribosome protection protein *TetO*, *TetM* and *AdeC*, a gene known to participate in the multidrug efflux pump mechanism. These genes were chosen due to their prevalence (Munir et al., 2011; Wu et al., 2010) and high detection frequency in the study site.

4.2.1 Amplification specificity (confirmation of primer sets).

Target genes and the specificity of primers were initially verified by PCR and an electrophoresis analysis of the amplicon (Figure 4.20). Strong, single electrophoresis bands were obtained for the target genes, which confirmed the sizes and putative identity of the amplicons. Amplification specificity was also checked by the melting curve analysis, carried out at the end of the qRT-PCR analyses, followed by secondary gel electrophoresis analysis; the latter employed the DNA amplified during qRT-PCR (Figures 4.21-4.24).

In order to determine the percentage of target genes per 16S rRNA gene, the absolute values from the qRT-PCR runs were normalized to 16S rRNA genes, which further provided a means to assess the level of resistance proportional to the size of the overall population (Pei et al., 2006).

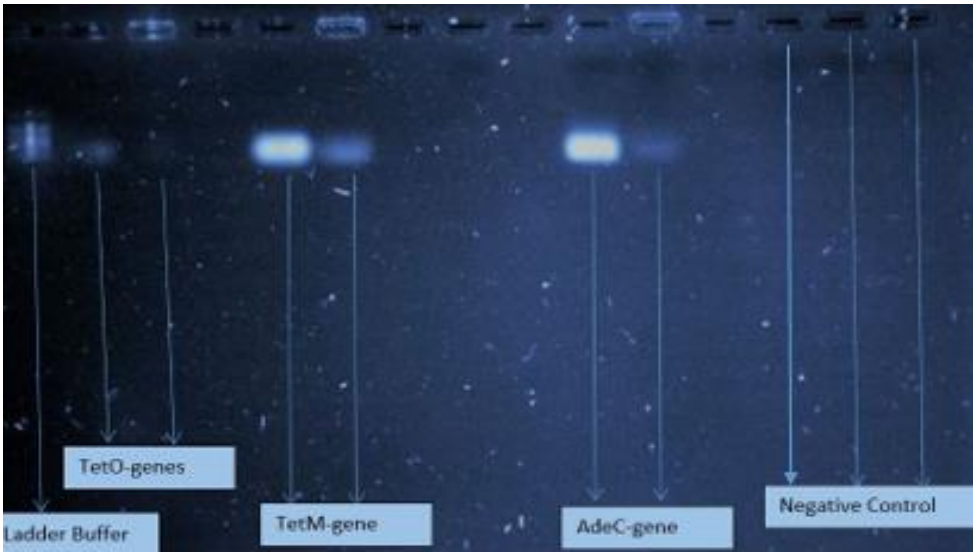

Figure 4.20 Gel electrophoresis bands for three target genes. See Appendix-A Figure 1 for labeled fragment lengths of target genes. Two lanes for each gene was designed for validation and confirmation.

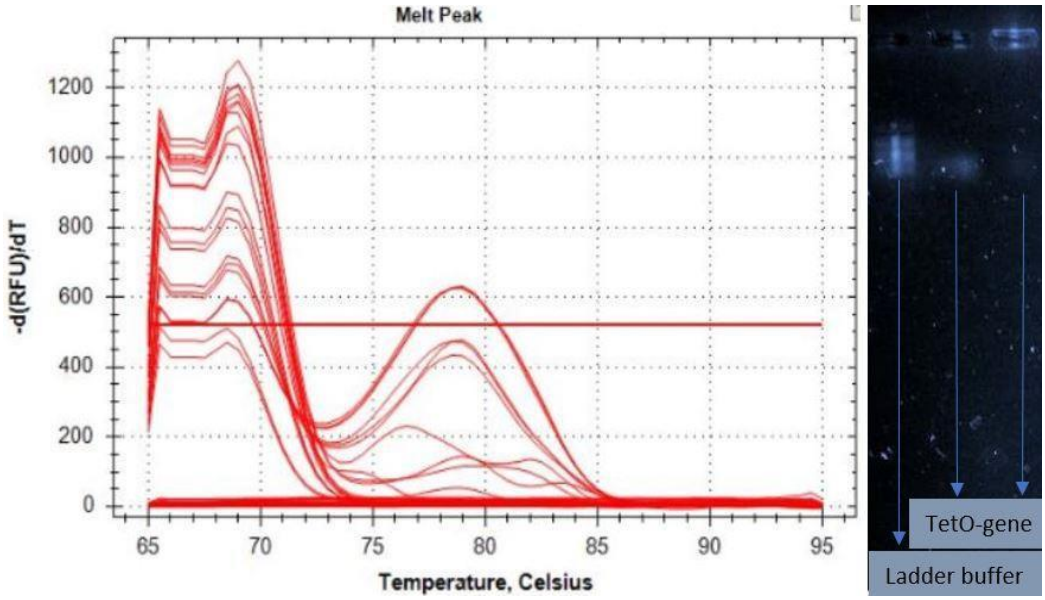

Figure 4.21 qRT-PCR melting peaks and electrophoresis gel band for *Tet*(M) gene.

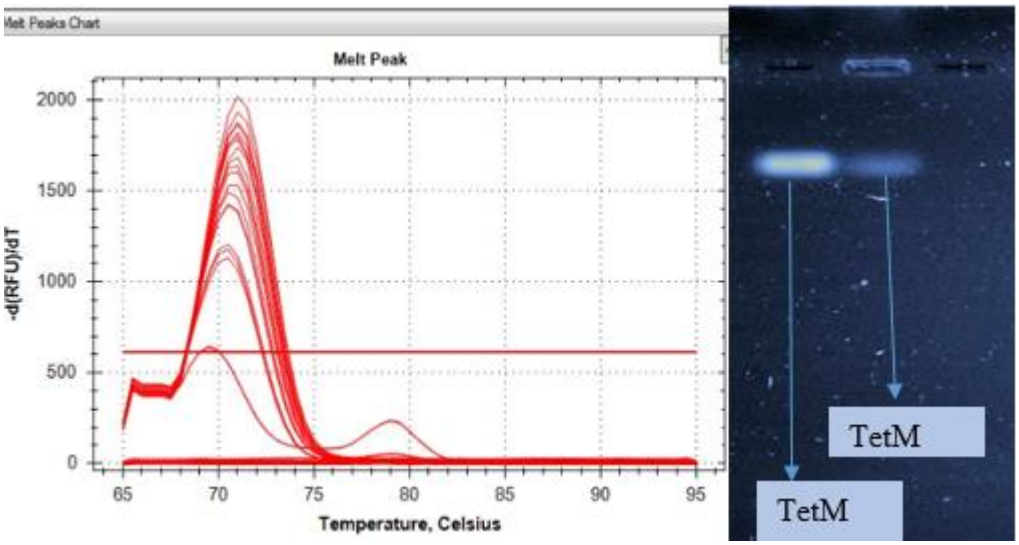

Figure 4.22 qRT-PCR melting peaks and electrophoresis gel band for *Tet*(M) gene.

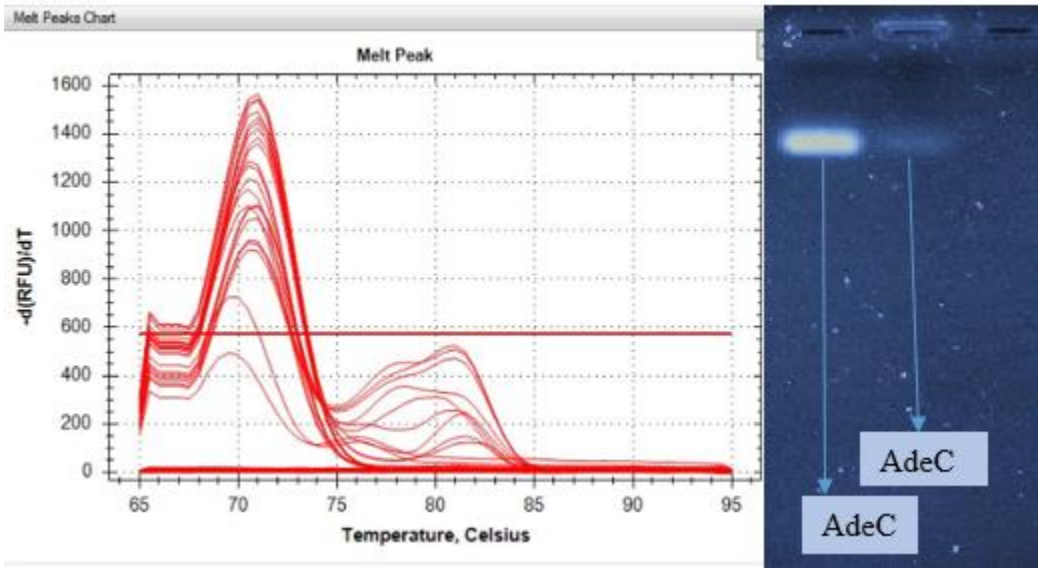

Figure 4.23 qRT-PCR melting peaks and electrophoresis gel band for *Ade*C gene.

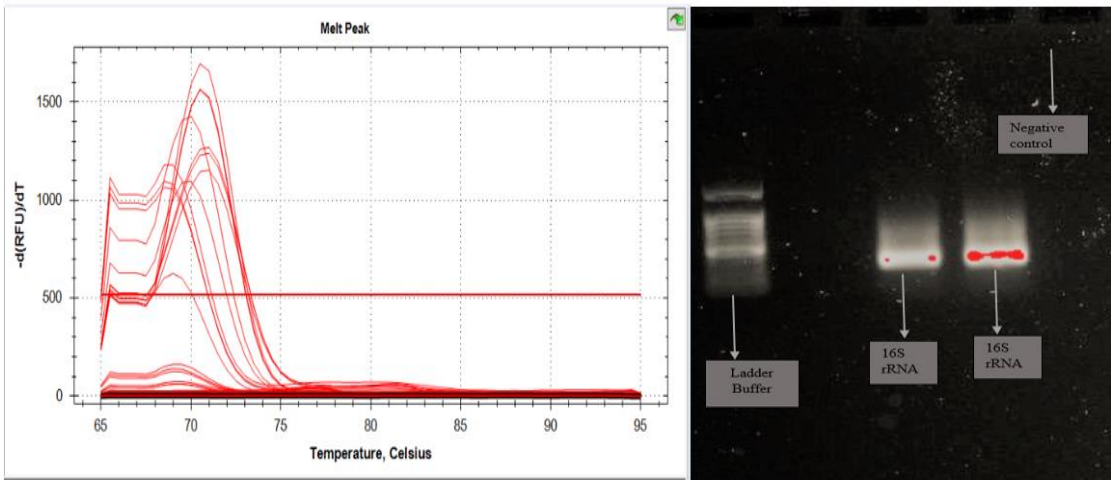

Figure 4.24 qRT-PCR melting peaks and electrophoresis gel band for 16S rRNA gene.

4.2.2 **Absolute gene concentration (copies L^{-1}) analysis.**

qRT-PCR was used to determine the concentrations of tetracycline *TetO* and *TetM* and *AdeC* genes present in each sample. The amount of template was held constant at 2 µL in each qRT-PCR reaction to ensure comparable amplification efficiency. For the calculation of absolute concentration of the genes, gene copies were normalized to water volume used for gDNA extraction to generate the value of GC per L of water. All sampling events from July-2015 and September-2016 were used in the calculation of absolute gene concentration.

4.2.2.1 Presence and concentration of *TetO TetM* and *AdeC* resistance genes in the Humber river watershed

Concentration of the three selected *TetO*, *TetM* and *AdeC* genes was evaluated in the Humber river (Chapter 3, figure 3.1) for samples collected in July-December 2015 and

May-September 2016. The concentrations of the ARGs copies L^{-1} in the river samples displayed no seasonal differences (weather event effects) for Tetracycline genes, but showed seasonal differences for the *AdeC* gene.

4.2.2.1.1 TetO

TetO gene was detected at all locations and all seasons, at concentrations ranging from ND to 3.68 x 10^{0} copies L^{-1} (Figure 4.25). Humber Resort location had the highest concentration of *TetO* which was not detected at the Little Falls location. Concentrations were lowest in the summer. Steady Brook tributary had the highest concentration of *TetO* 6.58 x 10^{-1} copies L^{-1} and Humber Village location had the lowest concentration 7.1 x 10^{-2} in the summer (Figure 4.25).

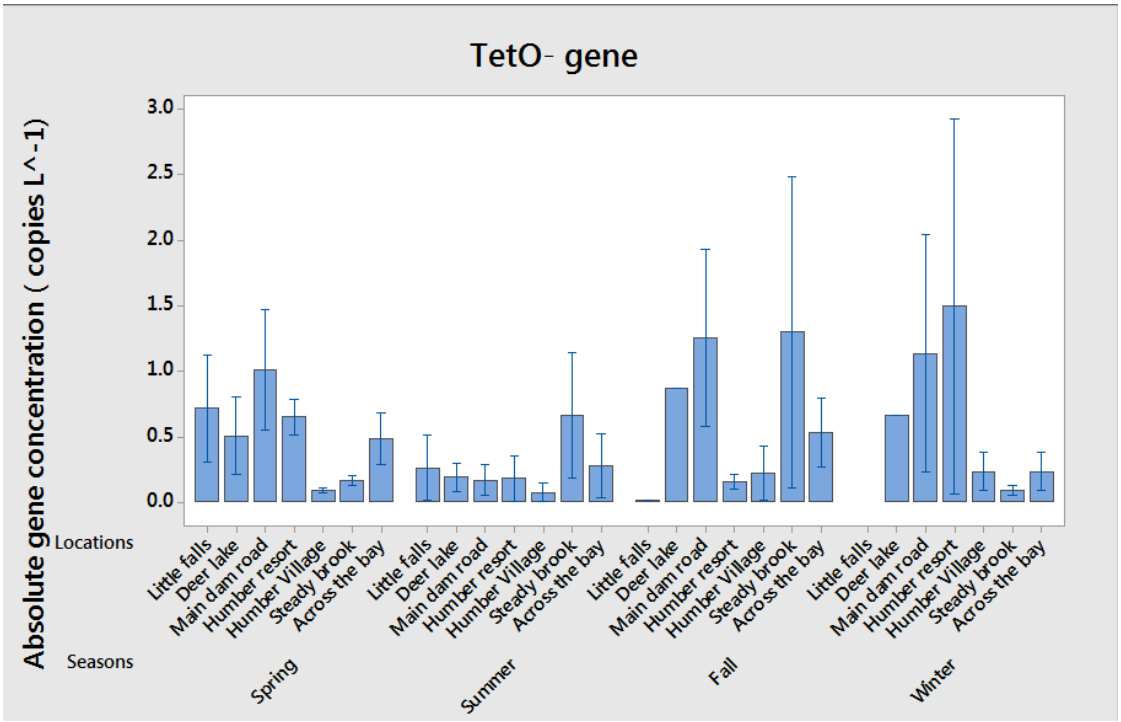

Figure 4.25 Abundance of *TetO* -gene copies (copies L^{-1}) at different locations and seasons. Error bars represent the standard error of the mean.

For the spring season, the concentration ranged from 8.67×10^{-2} to 1.01×10^{0} copies L^{-1}; Main Dam Road tributary location had the highest concentration, of 1.01×10^{0} copies L^{-1} and Humber Village location had the lowest concentration 8.67×10^{-2} copies L^{-1}.

In the fall sampling season, *TetO* was found to be higher at Deer lake, 8.72×10^{-1} copies L^{-1}, Main Dam Road tributary location, 1.25×10^{0} copies L^{-1}, Steady Brook tributary, 1.30×10^{0} copies L^{-1} and Across the Bay, 5.28×10^{-1} copies L^{-1}. Little Falls location, Humber Resort location, Humber Village location were found to have very low concentrations of the *TetO* gene, ranging from 1.56×10^{-1}, 2.19×10^{-1} copies L^{-1} and 1.51×10^{-2}.

Concentration of *TetO* in the winter season ranged from ND to 1.5×10^{0} copies L^{-1}. *Tet*(O) was not detected at Little Falls location, and the Humber Resort location had the highest concentration of 1.5×10^{0} copies L^{-1}.

4.2.2.1.2 *TetM*

The concentration of *TetM* gene across locations and seasons ranged from ND to 7.9×10^{0} copies L^{-1}. Humber Resort location had the highest concentration of 7.9×10^{0} copies L^{-1} while it was ND at the Little Falls location (Figure 4.26).

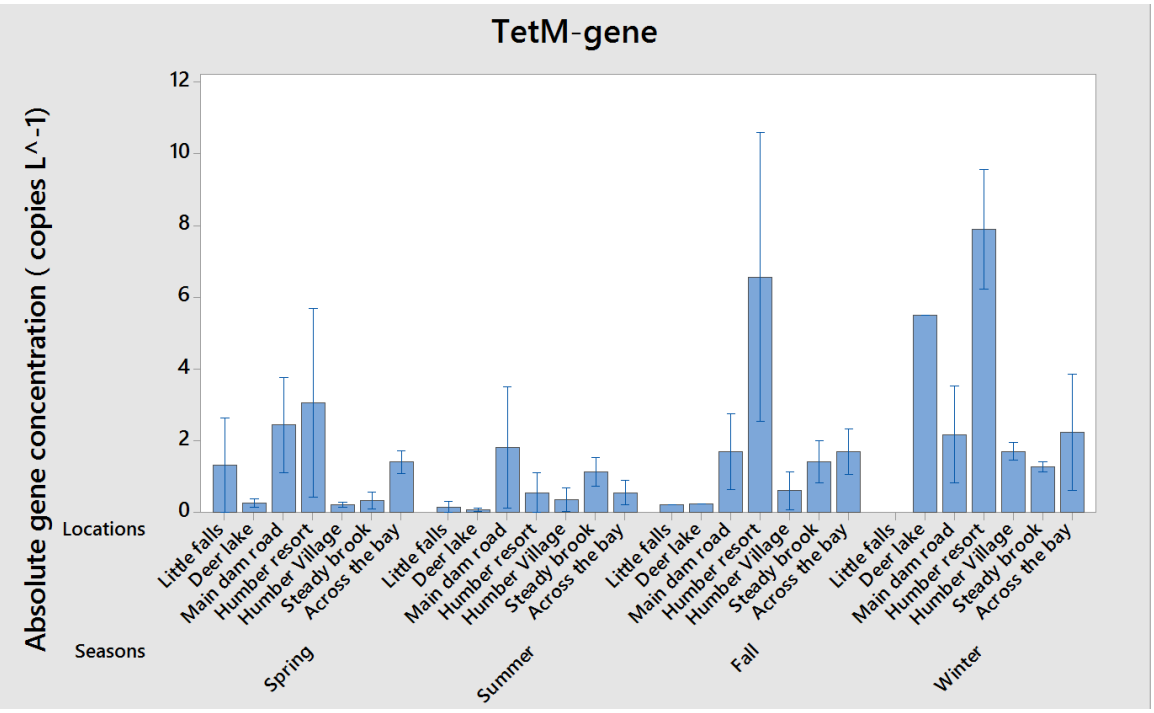

Figure 4.26 Abundance of *TetM* -gene copies (copies L $^{-1}$) at different locations and seasons. Error bars represent the standard error of the mean.

Concentrations were lowest during the summer sampling season across all locations among other sampling seasons 6.5 x 10^{-2} to 1.8 x 10^{0} copies L^{-1}; Main Dam Road tributary location had the highest concentration of 1.8 x 10^{0} copies L^{-1}, and the Deer Lake location had the lowest concentration of 6.5 x 10^{-2} copies L^{-1} of *TetM* gene. In the spring season, the concentration of *TetM* across locations ranged from 2.61 x 10^{-1} to 3.05 x 10^{0} copies L^{-1}; Humber Resort location had the highest concentration and Humber Village location had the lowest concentration of *TetM* gene.

Fall season concentrations ranged from 2.21 x 10^{-1} to 6.55 x 10^{0} copies L^{-1}; Humber Resort location had the highest concentration and Little Falls location had the lowest concentration. The concentration of *TetM* across locations in the winter season ranged from ND to 7.89 x 10^{0} copies L^{-1}; Humber Resort location had the highest concentration of 7.89 x 10^{0} copies L^{-1}, while it was not detected at Little Falls (Figure 4.26).

4.2.2.1.3 *Ade*C

The total abundance concentration of *Ade*C gene across locations and sampling seasons was found to be somewhat stable when compared to the tetracycline genes. Figure 4.27. The concentration was in the range of ND to 1.28 x 10^{1} copies L^{-1}; Deer Lake location had the highest concentration and *Ade*C gene was not detected at Little Falls.

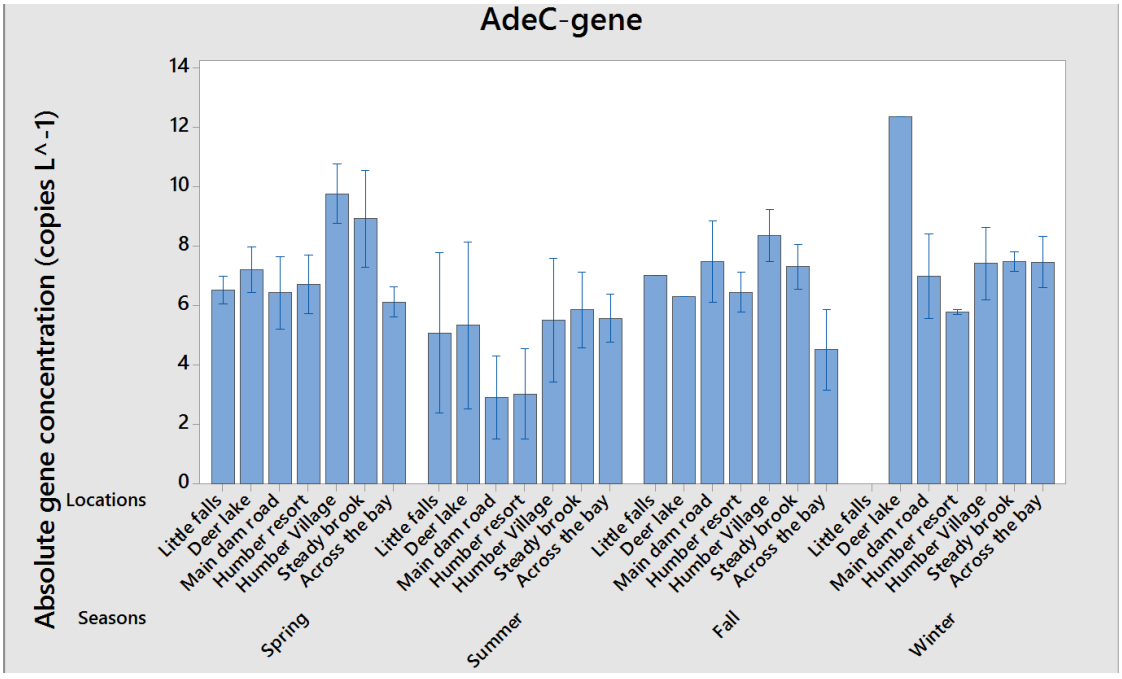

Figure 4.27 Abundance of *Ade*C -gene copies (absolute gene concentration, copies L^{-1}) at different locations and seasons. Error bars represent the standard error of the mean.

In the summer, the concentration of *AdeC* gene ranged from 2.9 $x10^0$ to 5.9 x 10^0; Steady Brook tributary location had the highest concentration of 5.9 x 10^0 copies L^{-1}, Main Dam Road tributary location had the lowest concentrations of 2.9 $x10^0$ copies L^{-1} (Figure 4.27).

In the spring sampling season, the concentration range was 6.1 x 10^0 to 9.76 $x10^0$ copies L^{-1}; Humber Village location had the highest concentration of 9.76 $x10^0$ copies L^{-1}, Across the Bay location had the lowest concentration of 6.1 x 10^0 copies L^{-1}. The concentration of *AdeC* gene in the fall sampling season ranged from 4.5 x 10^0 to 8.3 x 10^0 copies L^{-1}; Humber Village location had the highest concentration of 8.3 x 10^0 copies L^{-1} and Across the Bay had the lowest concentration of 4.5 x 10^0 copies L^{-1}. In the winter season, the concentration of *AdeC* ranged from ND to 1.24 x 10^1 copies L^{-1}; Deer Lake location had the highest concentration of 1.24 x 10^1 copies L^{-1}, while it was not detected at Little Falls.

4.2.2.1.4 Variation in *TetO*, *TetM* and *AdeC* gene concentrations (copies L^{-1}) along the Humber River

Figure 4.28 shows the concentrations (copies L^{-1}) of *TetO*, *TetM* and *AdeC* across sample sites. To compare the population means of target genes along the river, the paired t-test was performed. The result showed that the 95% CI mean difference between *TetO* and *TetM* genes was -1.579, -0.546, and the t-test of mean difference =0. The t-value= -

4.09 and the p-value = < 0.05 which suggest there is a significant difference between the means of *TetO* and *TetM* genes. The 95% CI for mean difference between *TetO* and *AdeC* genes was -6.619, -5.377, and the t-test of mean difference = 0. The t-value = -19.21 and the p-value = < 0.05, the means of *TetO* and *AdeC* genes are significantly different. The 95% CI for mean difference between *TetM* and *AdeC* genes was -5.681, -4.190, and the t-test of mean difference = 0. The t-value = -13.17 and the p-value = < 0.05 there is no significant difference between the means of *TetM* and *Ade*C genes.

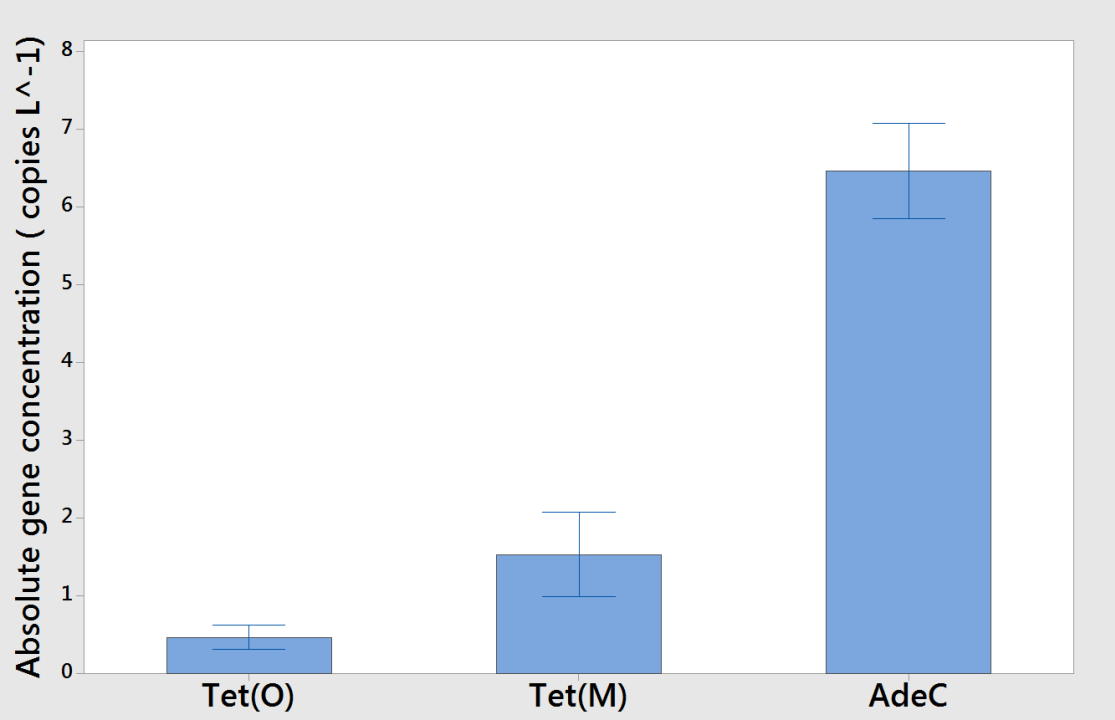

Figure 4.28 Absolute concentration of target genes across sample sites. Error bars represent the standard error of the mean.

4.2.2.1.5 Impact of location and abiotic factors on *TetO*, *TetM* and *AdeC* genes concentration (copies L^{-1}).

ANOVA was carried out employing a GLM to assess the influence of factors (year, seasons and sample locations) and covariates (Temperature and pH) on the *TetO*, *TetM* and *AdeC* gene concentrations (copies L^{-1});

4.2.2.1.5.1 Tetracycline genes

Sampling years, seasons and locations were found not to be significantly correlated to the tetracycline genes concentrations, pH and temperature ranges were binned across seasons, spring: 4-9 °C, summer: 9-23 °C, fall: 5-17.5 °C, winter: 0-9 °C and were found not to be significantly correlated with *TetO* and *TetM* genes (Table 4.8).

4.2.2.1.5.2 AdeC

Sampling years, seasons and temperature ranges binned within seasons, spring: 4-9 °C, summer: 9-23 °C, fall: 5-17.5 °C, winter: 0-9 °C were found to relate statistically significantly to the concentrations of the *AdeC* gene. Sampling location and pH were found not to be significant to the concentrations of *AdeC* gene. (Table 4.8).

Table 4.8 Explanatory value of factors (Year, seasons, sample location) and covariates (pH and Temperature (°C) on the concentration of *TetO*, *TetM* and *AdeC* genes (copies L^{-1}) in the Humber river watershed.

Factors and Covariates	Source	DF	TetO		TetM		AdeC	
			F-value	p-value	F-value	p-value	F-value	p-value
Factor	Year	1	0.01	0.935	0.51	0.478	8.77	0.005
	Seasons	3	0.13	0.940	0.77	0.518	4.31	0.008
	Locations	6	0.44	0.851	0.14	0.991	0.80	0.576
Covariates	Temp(Season)	4	0.97	0.431	0.32	0.861	2.78	0.035
	pH (location)	7	0.94	0.485	0.33	0.936	1.21	0.311
	Error	55						
	Total	76						
	S		0.736109		2.41505		2.13518	
	r^2.		25.30%		36.41%		51.64%	
	r^2 (adj)		0.00%		12.12%		33.18%	

4.2.2.1.6 Mean comparison test for *TetO*, *TetM*, and *AdeC* genes (copies L^{-1}).

To determine the significant differences between the means of the target genes and factors (sampling seasons, locations, and year), Post Hoc (Tukey comparison test) analysis was carried out. Results showed a significant difference in the means of *TetM* gene concentrations between sampling locations; Humber Resort location is significantly different from Little Falls, Deer Lake, Humber Village, Steady Brook and Across the Bay sampling locations. The means for sampling seasons (Figure 4.29) and sampling years were

found to be significantly different for the *AdeC* gene; summer sampling season was significantly different from the spring, fall and winter seasons (Figure 4.29).

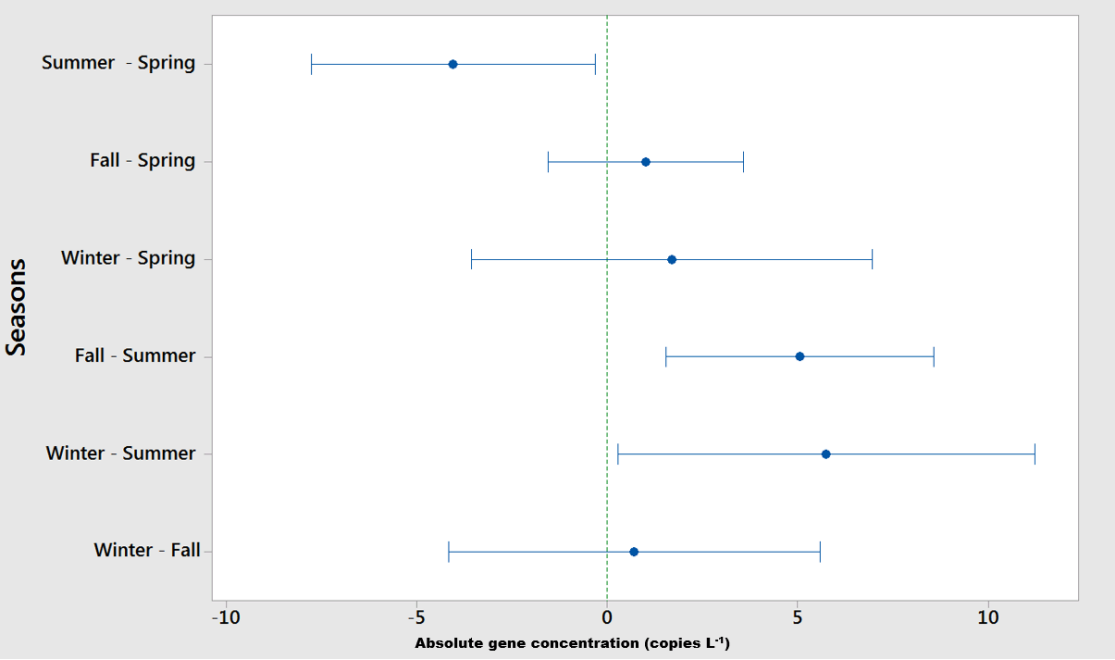

Figure 4.29 Post-Hoc analysis shows significant difference between the means of sampling seasons for *Ade*C gene.

4.2.3 **Correlation of target genes (copies L^{-1}).**

Table 4.9 Pearson correlation of target genes (Absolute gene concentration (copies L^{-1}).

	Temperature	pH	TetO	TetM	AdeC
Temperature	1				
pH	0.27662*	1			
Tet(O)	0.002738	0.25422*	1		
Tet(M)	0.2424*	0.16888	0.30839*	1	
AdeC	0.24267*	0.27477*	0.074324	0.17209	1

* Correlation is significant at 0.05 level.

The extensive usage of antibiotics is a primary reason for the increase of bacterial resistance and the corresponding concentrations of resistance genes in the environment (Xu et al., 2015). To study the correlations between resistance genes is essential in other to assess their individual effects. The Pearson correlation analysis of target genes performed

showed the correlation results for ARGs and corresponding covariant (Temperature and pH).

A correlation was found between *TetO* and *TetM*, which is similar to the study by (Harnisz et al, 2015). It was observed that temperature and pH are correlated. Temperature is correlated with *Tet*M and *Ade*C genes. pH is correlated with *TetO* and *AdeC* genes (Table 4.9).

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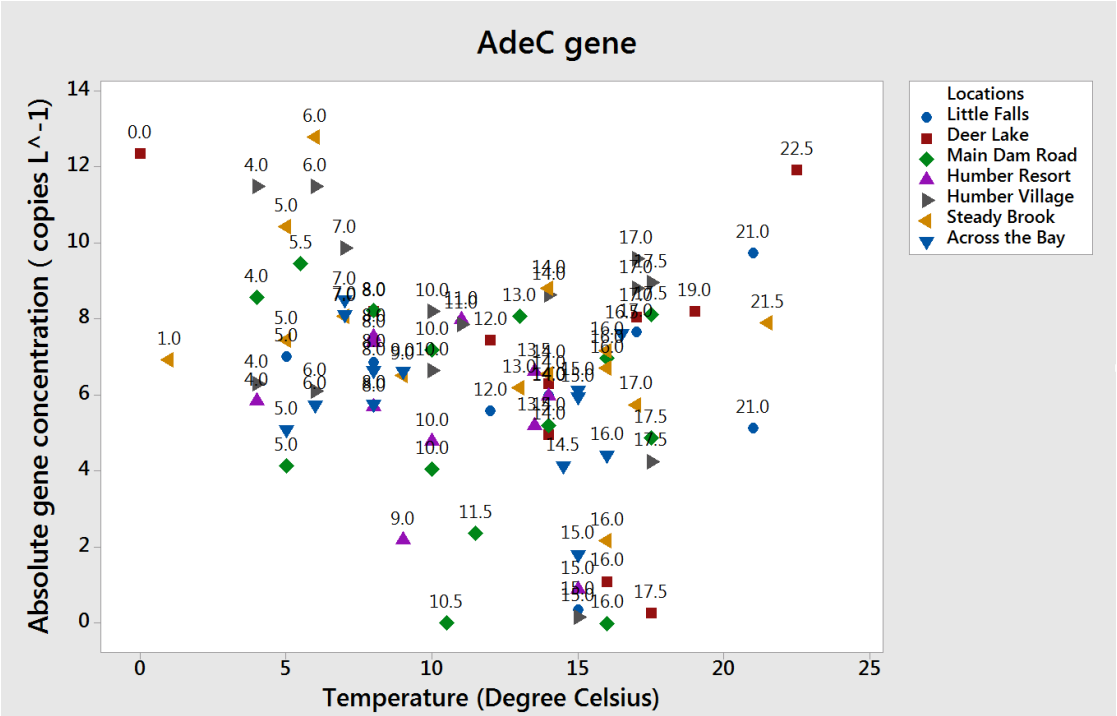

Figure 4.30 Relationship between temperature and the concentration of *AdeC* gene.

4.2.4 **Abundance of *TetO, TetM* and *AdeC* within the bacterial population sampled along the Humber river watershed (ARG copies / 16S rRNA copies).**

To examine the relative abundances of all three genes; *TetO, TetM* and *AdeC* for each sample location, the absolute counts obtained from qRT-PCR analysis were normalized to 16S rRNA gene (Ji et al., 2012). The total abundance of all three genes are illustrated in (Figures 4.31 to 4.33). 16S rRNA gene represents a fundamental method of study of bacterial evolution and ecology in environmental samples, because of its abundant distribution in all cells (Kembel et al., 2012). However, the number of copies of 16S rRNA gene within an individual bacterium varies (Kembel et al., 2012; Tao et al., 2014) among species and as such the relationship between ARGs and 16S rRNA gene copies is not truly a description of ARGs per bacterial cells.

Calculation of the relative abundance of ARG's versus the total 16S rRNA counts allows for an estimate of the intensity of the respective resistance among the entire bacterial population (Kembel et al., 2012).

4.2.4.1 Presence and relative concentration of *TetO*, *TetM* & *AdeC* genes in the bacterial population sampled in the Humber River watershed.

4.2.4.1.1 TetO

The abundance of *TetO* gene along the locations showed a low detection in few locations and sampling seasons, the abundance range was from ND to 1.96 x 10^{-4} copies / 16S rRNA. Humber Resort location, Humber Village location and Across the Bay locations had the lowest abundance of the *TetO*, and Little Falls location had the highest abundance (Figure 4.31).

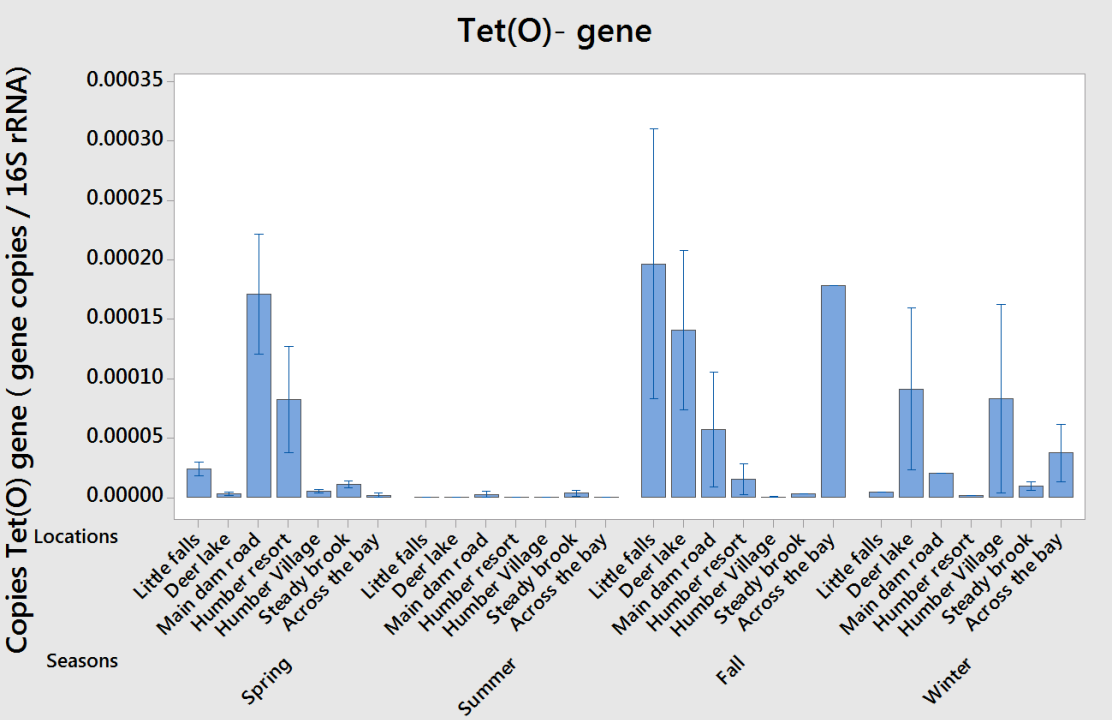

Figure 4.31 Abundance of *TetO* gene copies (copies / 16S rRNA) at different sample locations and seasons. Error bars represent the standard error of the mean.

Summer sampling season had the lowest abundance among all seasons, and the Fall season had the highest abundance. In the summer sampling season, Humber Resort location, Humber Village location and Across the Bay location had the lowest abundance of the *TetO* gene and Steady Brook tributary had the highest abundance. In the spring the abundance of *TetO* gene was in the range of 1.8 $x10^{-6}$ to 1.7 x 10^{-4} copies / 16S rRNA. Across the Bay location had the lowest abundance and Main Dam Road tributary location had the highest abundance.

The abundance of the *TetO* gene in the fall season showed the highest abundance across locations. Humber Village location had the lowest abundance of 2.6 x 10^{-7}copies / 16S rRNA and Little falls location had the highest abundance of 1.9 x 10^{-4} copies / 16S rRNA. In the winter sampling season, the abundance range of *TetO* across locations was 1.94 x 10^{-6} to 9.12 x 10^{-5} copies / 16S rRNA.

4.2.4.1.2 TetM

The abundance of *TetM* gene along the locations and seasons was in the range of ND to 1.28 x 10^{-3} copies / 16S rRNA (Figure 4.34). Little Falls location, Deer Lake location, Humber Resort location and Humber Village location had the lowest abundance of *TetM* gene, and Across the Bay location had the highest abundance of the *TetM* gene 9.94 x 10^{-4} copies / 16S rRNA (Figure 4.32).

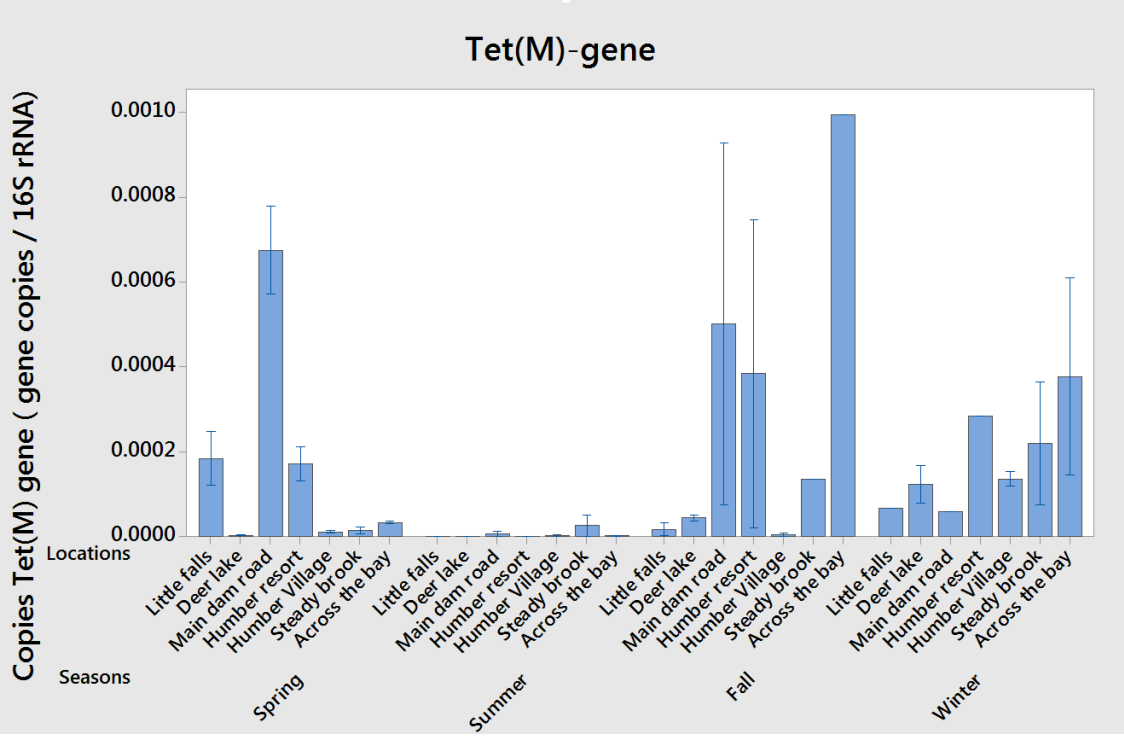

Figure 4.32 Abundance of *TetM* gene copies (copies / 16S rRNA) at different sample locations and seasons. Error bars represent the standard error of the mean.

The summer sampling season had the lowest abundance of the *TetM* gene across locations, the fall season had the highest abundance of the gene. In the summer sampling season, *TetM* gene was not detected in the Little Falls location, Deer Lake location, Humber Resort location; the Steady Brook tributary had the highest abundance. In the spring season, the abundance of *TetM* across locations was in the range of 2.2 x 10^{-6} to 6.74 x10^{-4} copies / 16S rRNA; The Deer Lake location had the lowest abundance and the Main Dam Road tributary had the highest abundance.

In the fall sampling season, the concentration distribution of *TetM* across location was 4.0 x 10^{-6} to 9.94 x 10^{-4} copies / 16S rRNA. Humber Village location had the lowest abundance, and Across the Bay location had the highest abundance of the *TetM* gene. The winter sampling season showed an abundance range of 6.61 x 10^{-5} to 3.77 x 10^{-4} copies / 16S rRNA across locations for the *TetM* gene; the Little Falls location had the lowest abundance and Across the Bay location had the highest abundance (Figure 4.32).

4.2.4.1.3 AdeC

Among the three ARGs; *TetO, TetM, AdeC* the relative abundance of the *AdeC* gene in all Sample locations were quite distinct and relatively stable. *AdeC* gene had the highest abundance in all locations and seasons ranging from 1.84 x 10^{-5} to 2.89 x 10^{-3} copies / 16S rRNA. Deer Lake location in the summer had the lowest abundance and Deer Lake location in the fall had the highest abundance of *AdeC* gene (Figure 4.33).

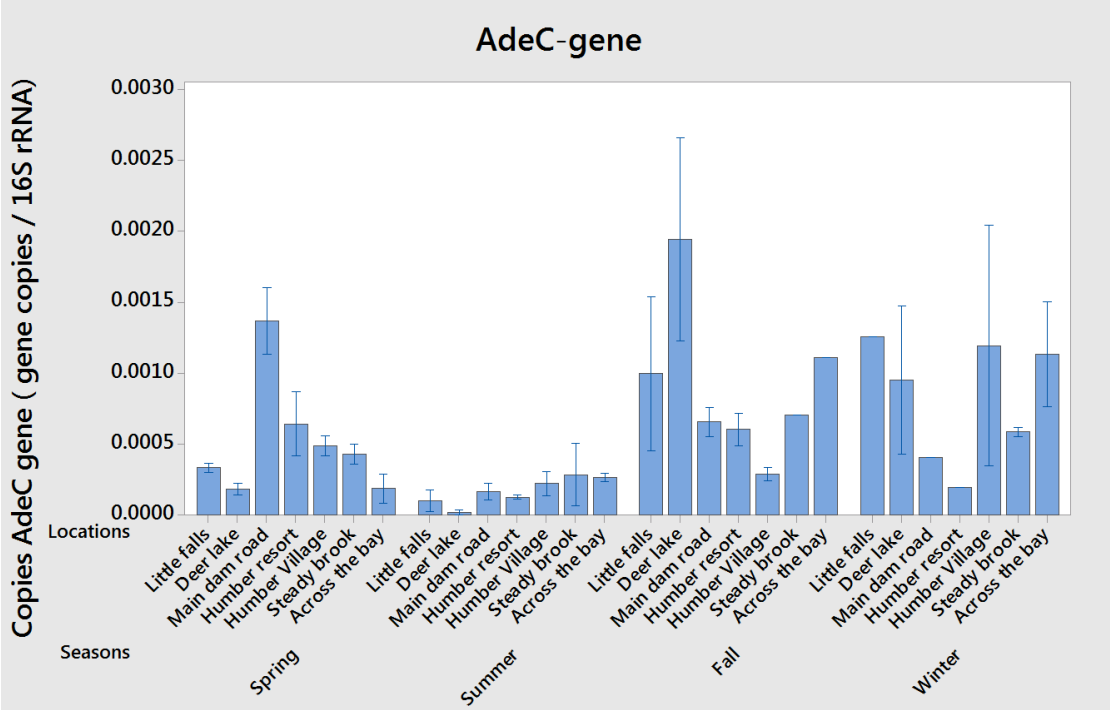

Figure 4.33 Abundance of *AdeC*-gene copies (copies / 16S rRNA) at different sample locations and seasons. Error bars represent the standard error of the mean.

The summer sampling season had the lowest abundance of *AdeC* gene across locations and the fall season had the highest abundance. In the summer, Deer Lake had the lowest abundance, and Steady Brook tributary had the highest abundance. In the fall season, the range of *AdeC* gene was 2.88×10^{-4} to 1.94×10^{-3} copies / 16S rRNA. Humber village location had the lowest abundance, and Deer Lake location had the highest abundance. In the winter, *AdeC* gene was in the range of 1.96×10^{-6} to 1.25×10^{-3} copies / 16S rRNA. Humber Resort location had the lowest abundance, and Little Falls location had the highest abundance of the *AdeC* gene (Figure 4.33).

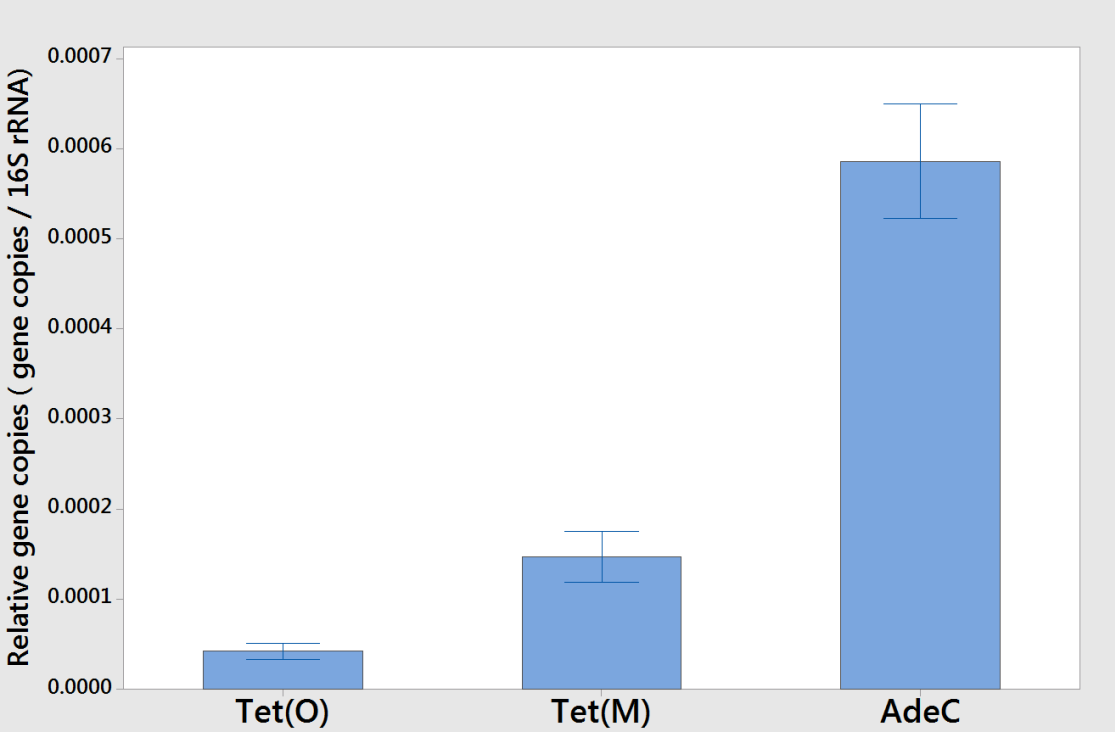

Figure 4.34 Abundance of target genes. Error bars represent the standard error of the mean.

4.2.4.2 Difference in *TetO*, *TetM* and *AdeC* gene concentrations (copies / 16S rRNA)

A paired t-test was performed to determine if the relative abundance concentrations along the river watershed are significant and consistent. The result showed that the 95% CI mean difference between *TetO* and *TetM* genes was -0.0001581, -0.000051, and the t-test of mean difference = 0. The t-value = -3.91 and the p-value = < 0.05 which suggest there is a significant difference between the means of *TetO* and *TetM* genes. The 95% CI for mean difference between *TetO* and *AdeC* genes was -0.000658, -0.000430, and the t-test of mean difference = 0. The t-value = -9.50 and the p-value = < 0.05, the means of *TetO* and *AdeC* genes are significantly different. The 95% CI for mean difference between *TetM*

and *AdeC* genes was -0.000559, -0.000319, and the t-test of mean difference = 0. The t-value = -7.26 and the p-value = < 0.05 there is a significant difference between the means of *TetM* and *AdeC* genes.

4.2.4.3 Impact of location and abiotic factors on *TetO, TetM* & *AdeC* gene concentration (Copies / 16S rRNA).

An analysis of variance was carried out employing a GLM to assess the influence of factors (year, seasons and sample locations) and covariates (Temperature and pH) on the ARGs concentration (copies/ 16S rRNA).

4.2.4.3.1 Tetracycline genes

Sampling years, seasons were found to be significantly correlated with the *TetO* gene concentration. pH and temperature ranges binned across seasons, spring: 4-9 °C, summer: 9-23 °C, fall: 5-17.5 °C, winter: 0-9 °C were found not to be significantly correlated with *TetO* and *TetM* gene concentrations (Table 4.10).

4.2.4.3.2 *Ade*C

Sampling years, seasons and temperature ranges binned within seasons, spring: 4-9 °C, summer- 9-23 °C, fall: 5-17.5 °C, winter: 0-9 °C were found to relate statistically significantly to the abundance of *AdeC* gene. Sampling location and pH were found not to be significant to the abundance of *AdeC* gene (Figure 4.10).

Table 4.10 Explanatory value of factors (Year, seasons, sample location) and covariates (pH and Temperature (°C) on the concentration of *TetO*, *TetM* & *AdeC* genes (copies /16S rRNA) in the Humber river watershed

Factors and Covariates	Source	DF	TetO F-value	p-value	TetM F-value	p-value	AdeC F-value	p-value
Factors	Year	1	5.56	0.022	0.14	0.710	5.88	0.018
	Seasons	3	3.05	0.035	0.98	0.410	3.40	0.023
	Locations	6	0.69	0.662	2.21	0.053	0.81	0.568
Covariates	Temp(Season)	4	2.51	0.050	0.66	0.619	3.07	0.022
	pH (location)	7	0.58	0.768	1.66	0.134	0.62	0.737
	Error(S)	63						
	Total	84						
	S		0.00008		0.0002		0.00054	
	r^2		36.22%		43.45%		41.39%	
	r^2 (adj)		14.96%		24.60%		21.85%	

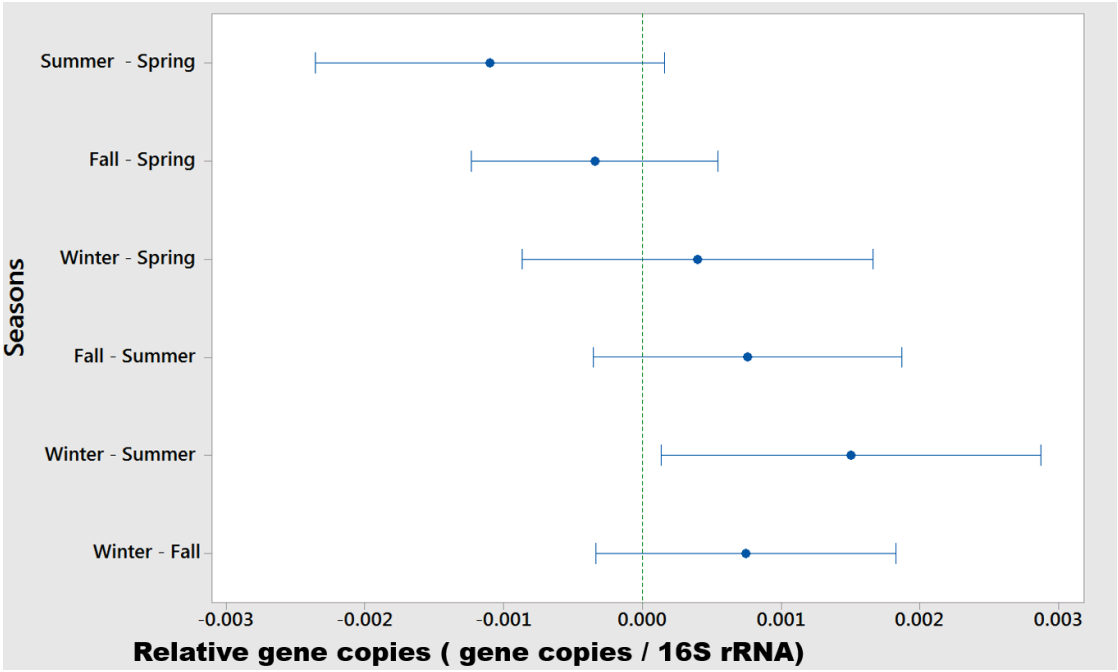

Figure 4.35 Post Hoc analysis show significant difference between the means of sampling seasons for *AdeC* gene.

4.2.4.4 Mean Comparison of target genes (copies / 16S rRNA).

4.2.4.4.1 Tetracycline genes

Tukey comparison test showed that sampling year was significantly different for the *TetO* gene concentration. The means of sampling locations had a significant difference for *TetO* and *TetM* genes.

4.2.4.4.2 *Ade*C

The means of sampling years were significantly different for the *AdeC* gene. Sampling seasons were significantly different; summer was significantly different from winter season (Figure 4.35).

4.2.5 **Total mass of *Tet*(O), *Tet*(M) & *Ade*C in the Humber river (copies s^{-1})**

The available flow rates m^3 s^{-1} data of the Humber river, on the dates of sampling events from two sampling locations. Little Falls and Humber Village) which represents the upstream and downstream, were used to examine the gene copies s^{-1}) of all three target genes (*TetO*, *TetM* and *AdeC*) see Appendix D-Table 5. The copies per L (concentrations, copies L^{-1}) were multiplied by the flow rate data. The total flow rate of all three genes are shown in Figures 4.38 to 4.39.

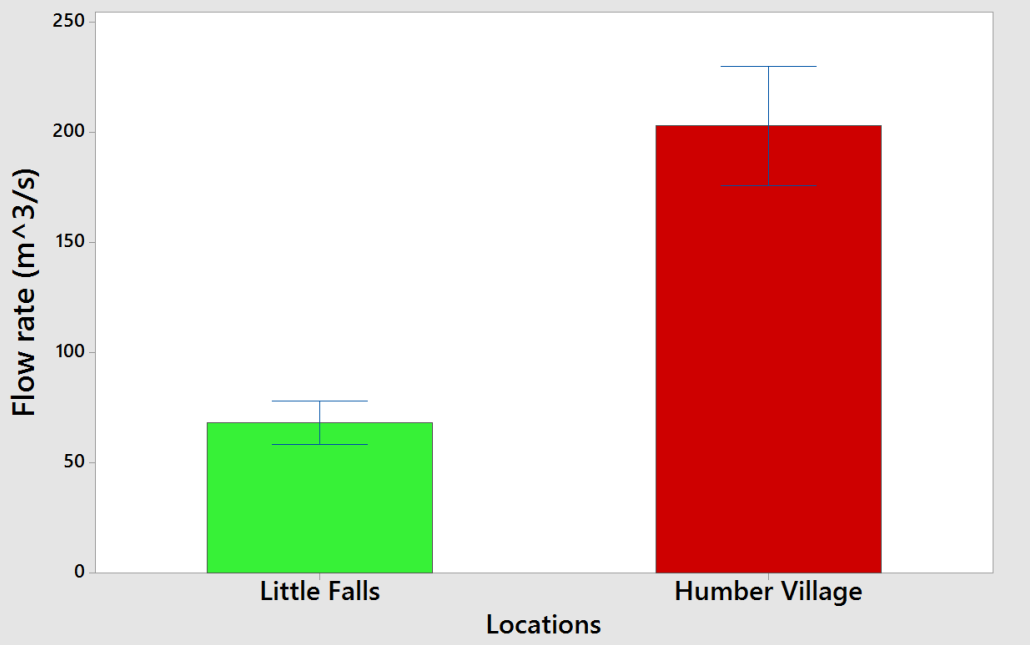

Figure 4.36 Water flow rate m^3 s^{-1} of the Humber river at an upstream and a downstream location. Error bars represent the standard error of the mean

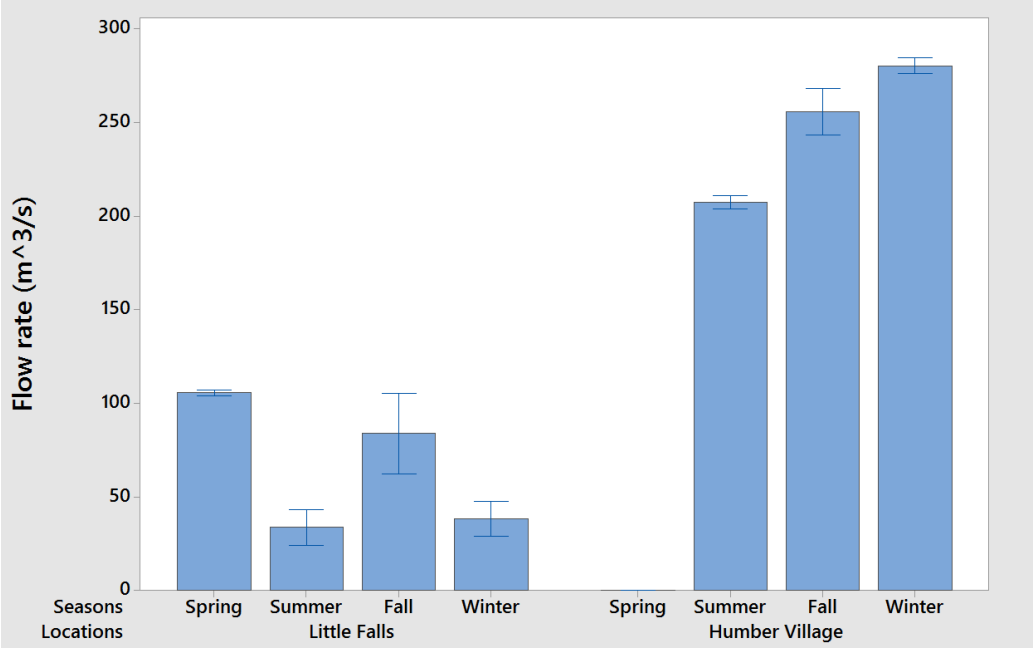

Figure 4.37 Water flow rate m^3 s^{-1} of the Humber river across sampling seasons and locations. Error bars represent the standard error of the mean

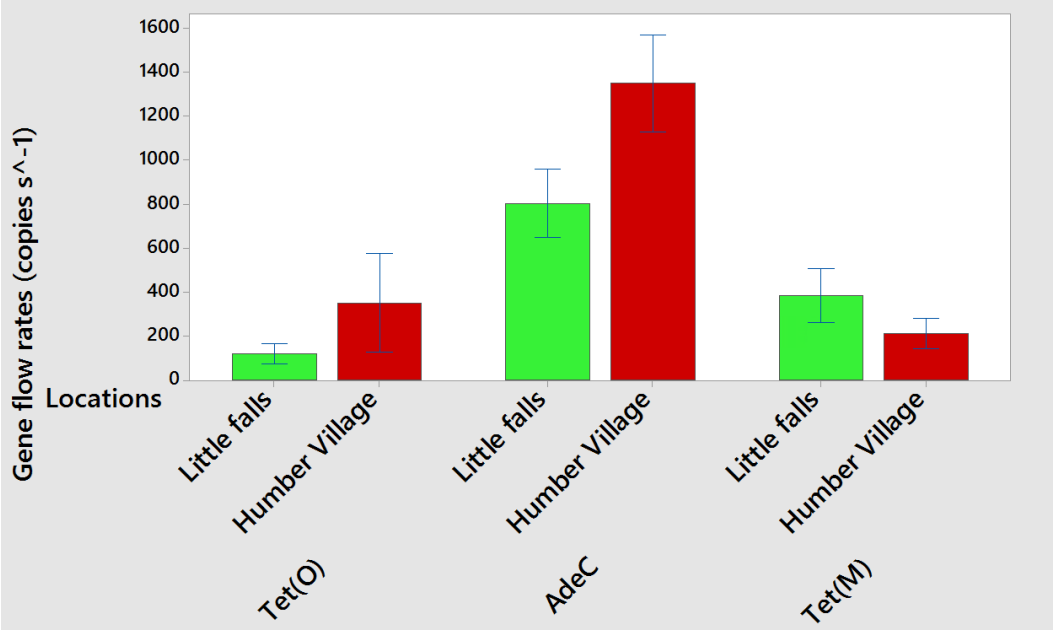

Figure 4.38 Abundance of target genes copies s^{-1} at two sampling locations. Error bars represent the standard error of the mean.

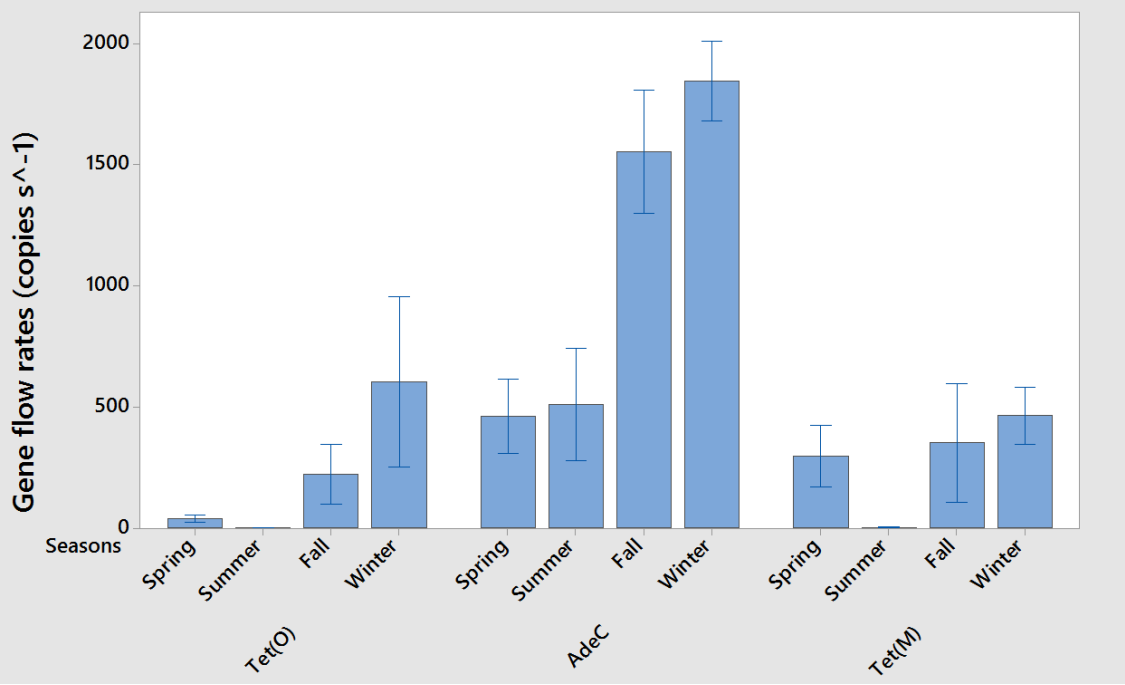

Figure 4.39 Genes flow rates copies s^{-1} across sampling seasons. Error bars represent the standard error of the mean.

4.2.5.1 Presence and concentration of ARGs in the Humber River watershed copies s^{-1}.

4.2.5.1.1 *TetO*

TetO gene was detected at the two locations and seasons at different rates. Humber Village location had the highest rate of *TetO* and Little Falls location had the lowest rate (Figure 4.38). The summer sampling season had the lowest rate and the winter sampling season had the highest rate (Figure 4.39).

4.2.5.1.2 *TetM*

The rate of *TetM* gene was higher in the Little Falls location and Humber Village location had the lowest rate (Figure 4.38). Summer sampling season had the lowest rate and the winter season had the highest rate (Figure 4.49).

4.2.5.1.3 *AdeC*

The *AdeC* gene among target genes had the highest abundance rate at the locations. The Humber Village location had the highest rate, and the Little Falls had the lowest rate (Figure 4.38). The spring sampling season had the lowest rate and the winter sampling season had the highest rate of *AdeC* gene (Figure 4.39).

4.2.5.2 Difference in ARGs flow rates (copies s^{-1}).

A paired t-test was performed to compare the means of target genes copies s^{-1} along the river watershed. The result showed that the 95% CI mean difference between *TetO* and *Tet*M genes was -330, 243, and the t-test of mean difference = 0. The t-value = -0.31 and the p-value = < 0.05 which suggests there is no significant difference between the means of *TetO* and *TetM* genes.

The 95% CI for mean difference between *TetO* and *AdeC* genes was -1196, -513 copies s^{-1}, and the t-test of mean difference = 0. The t-value = -5.09 and the p-value = < 0.05, the means of *TetO* and *AdeC* genes are significantly different. The 95% CI for mean difference between *TetM* and *AdeC* genes was 553, 1068, and the t-test of mean difference = 0. The t-value = 6.42 and the p-value = < 0.05 there is a significant difference between the means of *TetM* and *AdeC* genes.

4.2.5.3 Impact of abiotic factors and target genes (copies s^{-1}).

An analysis of variance was carried out employing a GLM to assess the influence of factors (year, seasons and sample locations) and covariates (Temperature and pH) on the ARGs concentration (copies s^{-1}).

4.2.5.3.1 Tetracycline genes

Sampling years, seasons were found to be significantly correlated to the *TetO* gene concentration. pH and temperature ranges binned across seasons spring: 4-9 °C, summer: 9-23 °C, fall: 5-17.5 °C, winter: 0-9 °C were found not to be significantly correlated with *TetO* and *TetM* gene concentrations (Table 4.11).

4.2.5.3.2 *AdeC*

Sampling years, seasons and temperature ranges binned within seasons, spring: 4 - 9 °C, summer: 9-23 °C, fall: 5-17.5 °C, winter: 0-9 °C were found to relate statistically significantly to the abundance of *AdeC* gene. There was no significant correlation between sampling location and *AdeC* gene. pH was found to be significant to the abundance of *AdeC* gene (Figure 4.11).

Table 4.11 Explanatory value of factors (Year, seasons, sample location) and covariates (pH and Temperature (°C) on the concentration of *TetO*, *Tet*(*M* and *AdeC*- genes (copies s^{-1}) in Humber river.

			Tet-O		Tet-M		AdeC	
Factors and covariates	Sources	DF	F-value	p-value	F-value	p-value	F-value	p-value
Factors	Year	1	0.11	0.745	2.00	0.174	9.29	0.007
	Season	3	0.30	0.825	1.97	0.152	1.08	0.380
	Location	1	0.39	0.541	1.70	0.208	2.08	0.165
Covariates	Temp (Season)	4	0.73	0.580	1.94	0.145	4.83	0.007
	pH (Location)	2	0.21	0.814	1.61	0.226	5.65	0.012
	Error	19						
	Total	30						
	S		785		308		409	
	r-sq.		27.31%		62.76%		84.79%	
	r^2 (adj)		0.00%		41.21%		75.98%	

4.2.6 **Discussion**

4.2.6.1 Presence and concentrations of ARGs

There is a strong evidence that ARGs accumulate in the environment due to anthropogenic activity (Pei et al., 2006; Storteboom et al., 2007). Nevertheless, little quantitative data can be found on the attenuation and fate of such genes in aquatic systems (Pruden et al., 2006). Studies have shown that ARGs can be increased in sediments in areas of intensive agricultural and urban activity (Pei et al., 2006), although identity of their bacterial hosts and the rates and mechanism (s) by which ARGs enter and attenuate in such settings have not been defined (Engemann et al., 2008). As such, knowing the proportion of ARGs that enter a receiving water and degrade within the water column versus migrate to peripheral compartments of the watershed is important because it influences modeling of the fate of ARGs in aquatic systems (Pei et al., 2006).

Total community DNA was successfully extracted from each sample. As expected, samples collected from different sample locations yielded different quantities of DNA per volume of original sample. To account for this, standard amount of DNA was used as template in each qRT-PCR reaction to assess the number of different types of ARGs, i.e. *TetO, TetM* and *AdeC* per sample.

The qRT-PCR methods used in this study provided a useful means to quantify ARGs in the water samples and thus bioassay and monitor the relative impact of antibiotic use. qRT-PCR also allowed for direct quantification of "pollutants" in and of themselves, while all three levels of data gathered in this study (Absolute gene concentrations, relative gene copies and total mass of genes) revealed similar overall trends, but with some differences observed. For example, the absolute gene concentration (copies L^{-1}) varied over a higher abundance concentration between locations and seasons, while the quantities per 16S rRNA genes were relatively lower and consistent between locations and seasons. The latter suggests that the contamination is relatively constant and of uniform quality and that changes in concentration might be driven by variability in the river flow rates and therefore dilution rates. Thus, expectedly the gene flow rates data showed an overall trend similar to the relative gene abundances.

The target ARGs were all detected in every location at different concentrations, the *AdeC* gene was found with the highest concentration in almost every sample location and season in all levels of data, this gene was followed by *TetM* and *TetO*. Overall, the

concentrations of *AdeC* gene were significantly higher than tetracycline genes; differences in usage and chemical properties might be a major reason for this observation.

4.2.6.1.1 Tetracycline resistance genes

The total mass of *TetO* gene flow rate from the Little Falls location (upstream) to the Humber Village location (downstream) in the Humber river was in the range of 119 to 349 copies s^{-1}. *TetM* flows from an upstream rate of 383 to 210 copies s^{-1}.

TetM was detected in higher concentrations in sample locations compared to *TetO*. *TetM* was detected at seven sampling sites at a concentration of about 7.00 x 10^{-1} to 4.20 x 10^{0} copies L^{-1} and *TetO* was detected in all seven sampling locations at a concentration range of 1.42 x 10^{-1} to 7.88 x 10^{-1} copies L^{-1}.

TetM has been studied and shown to be dominant in the upstream of other rivers, e.g. Cache la Poudre (Poudre river), northern Colorado (Storteboom et al, 2010), the upstream of the Poudre river has a pristine source arising from snowmelt in the Rocky Mountains with few tributaries or anthropogenic influences. Dominant Tet gene in this study differs from those observed in other regions, for example, *TetA*, *TetC*, *TetG*, *TetW* and *TetX* in the Huangpu river and drinking water sources in Shanghai, China (Jiang, et al., 2011), *TetC*, *TetH*, *TetO*, and *TetW* in the Poudre river, US (Pruden et al., 2006; Storteboom et al., 2010), and *TetA*, *TetB* in the Wenyu river, China (Hu et al., 2008). The difference in *Tet* gene types may be caused by the differential use patterns and host bacteria species in these regions (Jiang et al., 2011). This study obtained the primary *Tet* genes in the surface

water of the Humber River (western Newfoundland area), which to our knowledge, is the first report of the detection of *TetM* and *TetO* in natural water samples.

Concentrations (copies L^{-1}) of tetracycline genes in this study showed a higher average concentration of *TetM* compared to *TetO* across sampling locations. Our concentrations are lower than the concentrations detected in other rivers; e.g.; *TetO* and *TetM* have been detected at higher levels of 10^4 copies L^{-1} in the Huangpu river Shanghai China (Jiang et al., 2013). The concentrations (copies L^{-1}) in the Huangpu river Shanghai were found to be five orders of magnitude higher than the concentrations (copies L^{-1}) in the Humber river.

In a study by Harnisz et al., 2015, *TetM* in the upstream Lyna river in Olsztyn (Poland) was detected at higher levels of 10^8 copies L^{-1} and the downstream was detected in higher levels of 10^9 copies L^{-1}. The upstream concentration of *TetO* was detected at higher levels of 10^9 copies L^{-1}, and the downstream concentration of *TetO* was in the range 10^9 copies L^{-1}. These concentrations (copies L^{-1}) were higher than the concentrations (copies L^{-1}) in the Humber river.

In addition, *TetM* was not detected in surface water samples collected from Brisbane river in Australia and Rhine, Danube river in Germany (Stoll et al., 2012). It was also observed that *Tet* genes with both high detection frequencies and concentrations e.g. *TetA*, *TetC*, *TetX* and *TetG* are those coding for efflux protein, whereas, *Tet* genes with low detection frequencies and concentrations like those in this study *TetM*, *TetO*, and *TetW*, *Tet Q* used in the studies of (Jiang et al., 2013) and (Jia et al., 2015) belong to ribosomal

protection protein group. This phenomenon may be understood on the basis of the mobility of Tet gene and their host bacteria species. Most efflux protein *Tet* genes such as *TetA*, *TetB*, *TetC* and *TetG* are carried by mainly mobilizable vectors such as self-transmissible transposons and smaller plasmids, and thus readily disseminate among bacteria species. In contrast, ribosomal protection *Tet* genes such as *TetO*, *TetM*, *TetQ* and *TetS* are commonly found largely in conjugative plasmids or chromosomes where genes are not self-mobile (Chopra & Roberts, 2001), so that they seldom transfer and spread.

In addition, gram-negative species are dominant bacteria populations prevalent in aquatic environments; however, most ribosomal protection *Tet* genes are only stable and transferable in gram-positive bacteria species. The relationship between the levels of antibiotic resistance genes and physico-chemical parameters (temperature and pH) of river water sample were analyzed by Pearson's correlation showed that tetracycline genes are correlated, pH was correlated with *TetO* abundance, while temperature was correlated with the abundance of *TetM*

4.2.6.1.2 *AdeC*

The total mass of *AdeC* gene flow rate from the Little Falls location (upstream) to the Humber Village location (downstream) in the Humber river was in the range of 801 to 1347 copies s^{-1}.

AdeC resistance gene was detected at all seven sampling locations. This gene exhibited concentrations greater than for two detected tetracycline genes, with an average concentration of 6.50×10^{0} copies L^{-1}. The high detection concentration of *AdeC* gene in

the water samples may be linked to the role of its known mechanism of resistance "Multidrug efflux pump''. Multidrug pumps have functional roles as antibiotic resistance mechanisms (Martinez et al., 2009). This gene belongs to the resistance nodulation division (RND) family, known to be present only in gram-negative bacteria and are chromosomally located (Lupo et al., 2012). Efflux pumps implicated in antibiotic resistance are increasingly being detected in association with transferable elements (Lupo et al., 2012). Chromosomally encoded efflux pump and their determinants present in mobile elements can spread and evolve rapidly as a consequence of antibiotic selective pressure (Martinez, 2009). Pearson's correlation showed temperature and pH are correlated with the abundance of *AdeC* gene.

4.2.6.2 Quantification of ARGs versus 16S rRNA

Figures 4.31, 4.32 and 4.33 present the data obtained for three target genes *TetO, M* and *AdeC*. It summarizes the relative abundance for the genes in the Humber river. Although *TetO* and *TetM* were both encoding a ribosomal protection protein, the distribution of these genes was different. In general, the levels of *AdeC* gene were higher than the *Tet* genes. with the mean values of 4.86 x 10^{-4} copies / 16S rRNA, *TetM*-1.47 x 10^{-4} copies / 16S rRNA and Tet*O*- 4.20 x 10^{-5} copies / 16S rRNA. The proportion of detected *AdeC* was in the range of 4.86 x 10 -^{4} to 7.21 x 10^{-4} copies / 16S rRNA, and its distribution along locations was relatively stable.

The relative abundance i.e. copies/ 16S rRNA of *TetO* and *TetM* genes in the Humber river, in comparison to the relative abundance of *TetO* and *TetM* genes in a

different study, Beijing river (Ling et al., 2013), showed that the values are comparable to the values in the Humber river, pointing out that the low concentrations (copies L^{-1}) in Humber river are not necessarily due to the lack of contamination but to the relatively large dilution (i.e. lower proportion of contaminant flow versus the entire water volume in the river).

4.2.7 **Conclusion**

The overall patterns of the molecular signature of ARGs watershed scales may serve to provide insight into the mechanisms of ARGs proliferation in anthropogenically impacted rivers.

Selection of ARGs due to antibiotic pollution from urban sources is commonly assumed to be one of the driving factors elevating ARG concentrations in impacted aquatic environments. If this were the case, then it would be expected that ARG distribution at impacted river sites would be similar to the pristine origin but with higher concentrations and thus higher frequency of detection. Conversely, if antibiotic resistant bacteria are selected in upstream sources and subsequently transported, then impacted river sites would be expected to exhibit distinct ARG patterns relative to the origin.

This study has investigated the occurrence of *TetO*, *TetM* and *AdeC* genes in the Humber river in western Newfoundland. All three ARGs were detected in the river at different concentrations. *AdeC* gene had higher levels than *Tet* genes. The concentrations of the *AdeC* gene were often stable in upstream and downstream samples. *TetM* was the most frequently detected tetracycline gene.

Some patterns of ARG occurrence were also noteworthy, in particular the detection of the target genes at the sample locations presumed to have less intensity of human activities were found to be somewhat higher than sample locations with more human activities. In contrast, distributions of these ARG did not vary relative to the anthropogenic impact. Tet*M* detection distribution were found to be similar in un-impacted and impacted sample locations. This is similar to results from a study by (Storteboom et al., 2010), the work demonstrated similar distribution of *TetM* in pristine and impacted river sites. Their study area, the Poudre river in northern Colorado is a watershed including pristine, urban and agricultural impacts, it has its source arising from snowmelt in the Rocky Mountains with few tributaries or anthropogenic influences. Contextualizing these results with results from more contaminated rivers, e.g. Beijing River, suggests that the Humber river dilutes the contaminant flow significantly and this ensures its relative level of pristineness nature, not necessarily the lack of contamination.

5 Chapter 5

5.1 Summary

Using culture-independent methods, the presence and relative abundance of antibiotic resistance genes were surveyed in the antibiotic resistome of a mixed-use watershed in the Humber River, Western Newfoundland, Canada, along an intensity gradient of human activities. Shotgun sequencing (whole-genome sequencing) on Illumina Hi-seq platform was performed which allowed for the survey of the bacterial diversity and resistance genes abundance in the resistome. Quantitative Real Time PCR was performed to quantify three selected antibiotic resistance genes, *TetO*, *TetM*, and *AdeC* that confer resistance to Tetracyclines antibiotic class and multidrug resistance respectively.

5.2 Conclusion

The majority of the antibiotic resistance genes examined in this study were detected repeatedly along sample locations and source types, suggesting many of these genes are commonly present in the Humber river environment. These genes were detected at the upstream and downstream sample locations; *TetO* was least detected. The distribution and abundance of resistance genes varied differently between source types (impacted, un-impacted, tributary 1 and tributary 2). Un-impacted source locations and tributary 1 location source type were dominated by resistance genes and antibiotics known to be linked with human and animal sources, these two source locations are presumed to have less

human impact, and the latter is used as a source of drinking water. Impacted source and tributary 2 source location had minimal abundance of resistance genes.

The absolute concentration i.e. copies L^{-1} of *Tet*(O), *Tet*(M) and *Ade*C genes in sampling locations were considerably higher in the upstream locations than the downstream locations. The summer sampling seasons for all three genes showed the lowest concentrations across sampling locations, this might be due to photolysis and high dilution effect caused by increased river runoff in the summer. The concentrations of the three genes in the Humber River were lower when compared to reports from literature.

Furthermore, the relative abundance i.e. copies / 16S rRNA of *TetO*, *TetM* and *AdeC* across the locations showed the proportional abundance of target genes at the upstream sampling locations were higher, but the cumulative impact lead to a downstream increase of the target genes, as the total bacterial population, as described by 16s rRNA, increased. For *TetO* and *AdeC* the total gene mass flow rates expressed as the total gene count flowing from a specific river location (counts s^{-1}) were higher at the downstream location (Humber Village) than at the upstream location (Little Falls), while for the *TetM* gene flow rates were higher at the upstream location than at the downstream location. This might be explained by the wide range of hosts for this gene in the environment. The differences between the target gene mass flow rates at the upstream and downstream may confirm contamination; nevertheless, overall the dilution factor of the Humber river is sufficiently large to lower the concentrations of contamination to very low level. Moreover, the relative abundance of target genes in this study, is comparable to the relative abundance

reported in literature for more contaminated sites, suggesting that while the absolute contamination level in Humber river is lower, the density of antibiotic resistance genes per putative bacterial cell is not very different, clearly pointing out to contamination but also to dilution in the large volume of water in the Humber river.

The relationship between the transport of antibiotics and the transport of ARGs in the environment has not been exactly defined in the literature and we did not measure the concentration of antibiotics. Another important area of future work will be to build a better understanding and the separation of human and animal sources. Source tracking methods for resistance genes need to be further developed and applied to accomplish this. Even if it becomes possible to clearly distinguish human and agricultural sources of ARGs, it will still be important to consider that even antibiotics used exclusively for agriculture have been demonstrated to co-select for microbial resistance to a variety antibiotic.

A better understanding of the sources and fate of antibiotics and ARGs will facilitate improved modeling of the ultimate impact of antibiotic use on human, animal and environmental health and may ultimately be applied in developing strategies to mitigate potentially adverse impacts.

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APPENDICES

1.0 APPENDIX-A

Table 1- List of clean reads from Shotgun Illumina Hi-Seq analysis.

Location	Ab	Season	Reads
Little falls	LF	Summer	25,848,162
Main dam road	MDR	Summer	28,179,279
Deer Lake	DL	Summer	30,789,255
Humber Summer	HR	Summer	26,548,695
Humber village	HV	Summer	32,141,974
Humber village	HV	Winter	25,921,141
Steady Brook	SB	Summer	23,754,639
Across the Bay	ATB	Summer	24,886,258
Across the Bay	ATB	Winter	28,406,276

Table 2. List of physio-chemical parameters for the 2015 sampling year.

July 20th 2015		July 28th 2015		August 18th 2015		September 21st 2015		October 26th 2015		November 9th 2015		November 23rd 2015		December 7th 2015	
Temp (°C)	pH	Temp (°C)	pH	Temp (°C)	pH	Temp (°C)	pH	Temp (°C)	pH	Temp (°C)	pH	Temp (°C)	pH	Temp (°C)	pH
15	ND	16.5	6.78	21	6.55	16	6.41	5	6.68	2	6.17	5	6.09	3	7.3
16	ND	17.5	6.71	22.5	6.21	14	6.31	6	6.17	0	7.49	9	8.36	3	6.84
10.5	ND	11.5	6.14	16	6.15	17.5	6.2	5.5	6.98	4	6.47	8	6.64	5	6.87
9	ND	15	5.84	17	5.81	13.5	6.64	8	6.74	4	6.25	8	6.45	6	6.52
14	ND	15	5.89	17.5	5.87	17	6.47	10	6.62	4	6.08	7	6.39	6	6.41
13	ND	16	5.68	21.5	5.66	14	6.74	9	6.47	1	6.8	7	6.05	5	6.1
14.5	ND	16	5.9	16.5	5.83	15	6.55	8	6.81	6	6.26	7	6.11	7	6.1

Table 3. List of physio-chemical parameters (2016) sampling year.

May 9th-2016		May 24th-2016		June 13th-2016		June 20th-2016		July 4th-2016		August 8th-2016		August 22nd-2016		September 19th-2016	
Temp. (°C)	pH	Temp	pH	Temp	pH	Temp	pH	Temp	pH	Temp	pH	Temp	pH	Temp	pH
8	6.37	14	5.02	12	5.56	17	6.7	17	7.41	22	7.3	21	6.55	16	6.41
8	7.89	14	5.7	12	6.34	19	6.29	17	7.06	23	7.15	22.5	6.21	14	6.31
8	6.54	10	5.53	10	5.68	13	7.69	14	6.42	20	7.28	16	6.15	17.5	6.2
5	6.41	10	4.69	8	7.07	11	5.61	14	6.08	19	7.25	17	5.81	13.5	6.64
4	6.12	10	5.06	6	7.5	11	5.72	15	5.72	18	7.21	17.5	5.87	17	6.47
5	7.53	16	4.9	6	5.93	17	5.6	16	5.34	21	7.25	21.5	5.66	14	6.74
5	6.71	9	5.3	5	5.7	8	6.1	15	5.54	19	7.2	16.5	5.83	15	6.55

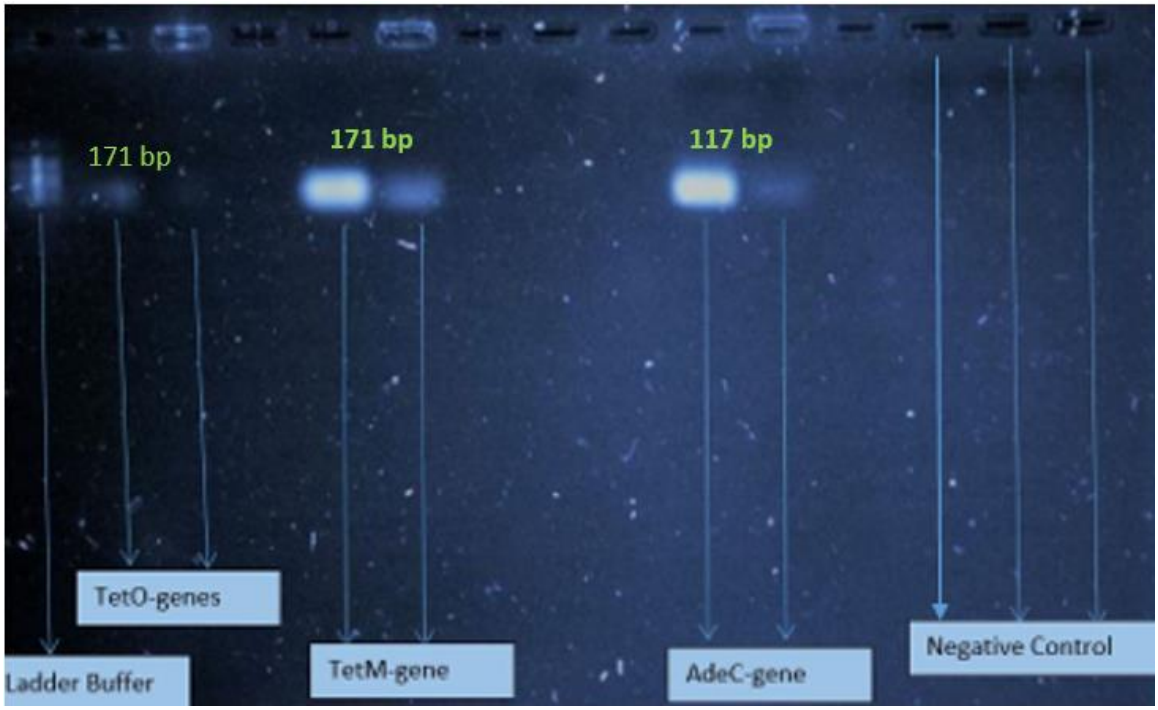

Figure 1. Gel electrophoresis bands for *TetO*, *TetM* and *AdeC* genes and their amplicon size.

Table 4. Relative abundance (%) of resistance genes.

Sample ID	*Cfr-gene*	*BacA*	*Blar1*	*Cara*	*Imra*	*Tsnr*	*Smrb*	*Bl2be_per*	*Bl3_vim*	*Bl2d_oxa1*	*Bl2a_pc*
ATB-S	7%	2%	10%	5%	8%	6%	0%	5%	3%	0%	3%
ATB-W	5%	2%	6%	2%	5%	9%	1%	5%	0%	1%	2%
DL-S	18%	24%	1%	0%	5%	0%	0%	8%	8%	0%	3%
HR-S	7%	6%	13%	4%	6%	2%	1%	2%	3%	0%	3%
HV-S	10%	4%	13%	7%	6%	5%	0%	5%	6%	0%	3%
HV-W	12%	4%	9%	3%	3%	9%	0%	3%	3%	0%	10%
LF-S	19%	30%	2%	0%	3%	0%	0%	5%	5%	0%	1%
MDR-S	5%	11%	14%	3%	3%	2%	0%	6%	1%	0%	3%
SB-W	10%	14%	8%	4%	2%	4%	0%	3%	3%	0%	4%

2.0 APPENDIX-B

Table 1. List of antibiotic resistance genes according to ShortBRED.

#	Target genes	Gene type
1	P52393	tnr
2	P18644	tnr
3	P52391	tsnr
4	16S_Ribosomal_RNA_Methyltransferase	Unknown
5	Cfr23RibosomalRNAMethyltransferase	Cfr-gene
6	YP_1177494	bcr
7	P42334	bcrc
8	AAC3227	cara
9	ABX596	lmra
10	NP_388149	lmrb
11	AAA5325	oleb
12	CAA455	srmb
13	P25256	tlrc
14	ABCAntibioticEffluxPump	unknown
15	NP_214776	aac2ic
16	P13246	aac3iia
17	CAH5873	aac3viii
18	YP_182184	aac3x
19	AAC41391	aac6i
20	AAL5121	aac6ib
21	NP_37559	aadd
22	ANT6_1	ant6ia
23	NP_832233	bl2a_iii
24	NP_87823	bl2a_pc
25	CAQ4248	bl2be_ctxm
26	CAQ42481	bl2be_ctxm
27	P28585	bl2be_ctxm
28	P22391	bl2be_oxy1
29	P37321	bl2be_per
30	TEM	bcr
31	LRA	LRA
32	CAA71441	bl3_cpha
33	CAE48334	bl3_imp
34	CAD6121	bl3_vim
35	BAA7922	bl1_ampc
36	YP_857635	bl1_ceps
37	YP_111962	bl1_cmy2

#	Target genes	Gene type
38	YP_672241	bl1_ec
39	CAC17625	bl1_och
40	NP_252799	bl1_pao
41	AAG1341	bl2_kpc
42	ClassC-AmpC	ClassC-AmpC
43	Q6778	bl2d_oxa1
44	NP_511223	bl2d_oxa1
45	ClassD	Class D
46	blaR1	penicillin
47	Chloramphenicol_Phosphotransferase	unknown
48	EEQ9652	BacA
49	NP_7456	BacA
50	NP_8859	BacA
51	YP_114148	BacA
52	YP_115544	BacA
53	YP_1187667	BacA
54	YP_1252958	BacA
55	YP_141679	BacA
56	YP_1434323	BacA
57	YP_1563294	BacA
58	YP_157958	BacA
59	YP_1696597	BacA
60	YP_1797193	BacA
61	YP_2421432	BacA
62	YP_2633428	BacA
63	YP_264987	BacA
64	YP_289644	BacA
65	YP_349555	BacA
66	YP_55152	BacA
67	YP_58946	BacA
68	YP_698526	BacA
69	Fluoroquinolone Resistant DNA Topoisomerase	Unknown
70	YP_2773891	fosb
71	NP_ 5293	BacA
72	Q2736	BacA
73	NP_77543	dfra12
74	Q5948	*dfra*13
75	NP_249397	Catb4
76	P384	dfrb2
77	L1	unknown

#	Target genes	Gene type
78	BAA3454	mphc

Table 2. PCA Eigenvalues for resistance genes.

Variables	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Tnr	-0.01	-0.02	-0.01	0.06	0.04	0.03
Tnr	0.11	-0.03	0.04	0.06	-0.04	-0.09
Tsnr	-0.15	-0.31	0.07	0.33	0.09	0.09
16S_Ribosomal_RNA_Methyltransferase	0.01	-0.02	-0.08	-0.11	-0.15	0.13
Cfr-gene	0.42	-0.27	-0.20	0.00	0.32	-0.18
Bcr	0.00	-0.05	0.12	-0.10	-0.05	0.05
Bcrc	-0.07	0.18	-0.04	-0.12	-0.06	0.01
Cara	-0.13	-0.04	0.07	-0.24	-0.07	-0.26
Lmra	-0.02	-0.09	-0.31	-0.11	-0.18	-0.14
Lmrb	0.01	0.00	-0.02	-0.02	-0.01	0.04
Oleb	-0.03	-0.02	0.19	-0.02	-0.08	-0.01
Srmb	-0.01	-0.01	-0.01	0.02	-0.02	0.00
Tlrc	-0.01	0.05	-0.01	0.13	-0.05	0.01
ABCAntibioticEffluxPump	0.00	-0.01	-0.01	0.02	0.07	0.02
aac2ic	0.01	-0.02	0.00	0.23	-0.09	0.15
aac3iia	0.00	-0.02	0.07	-0.04	-0.01	0.01
aac3viii	-0.03	-0.04	0.05	0.11	-0.17	0.00
aac3x	0.00	-0.01	0.00	0.05	-0.03	0.01
aac6i	0.00	-0.03	0.06	-0.05	-0.03	0.00
aac6ib	-0.01	-0.02	-0.01	0.06	0.04	0.03
Aadd	-0.02	0.06	-0.02	-0.02	0.01	0.01
ant6ia	0.00	-0.01	0.00	0.05	-0.03	0.01
bl2a_iii	0.01	0.00	-0.01	0.11	-0.06	-0.27
bl2a_pc	-0.07	-0.08	0.04	-0.02	0.58	0.29
bl2be_ctxm	-0.07	-0.08	-0.19	0.07	-0.03	-0.26
bl2be_ctxm	-0.03	0.00	0.20	-0.13	-0.06	0.15
bl2be_ctxm	0.00	-0.01	0.00	0.05	-0.03	0.01
bl2be_oxy1	-0.01	-0.03	-0.03	-0.01	0.01	0.01
bl2be_per	0.06	0.12	-0.27	0.01	-0.24	0.25
Bcr	-0.02	0.10	-0.04	0.02	0.06	0.11
LRA	-0.03	-0.01	-0.08	0.06	0.42	-0.01
bl3_cpha	0.01	0.11	-0.06	0.06	0.07	0.05
bl3_imp	0.00	-0.01	-0.01	0.02	0.07	0.02

Variables	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
bl3_vim	0.16	-0.19	-0.24	-0.34	0.03	-0.03
bl1_ampc	-0.01	0.08	0.10	-0.09	-0.03	0.15
bl1_ceps	0.00	-0.02	0.07	-0.04	-0.01	0.01
bl1_cmy2	0.03	-0.02	0.04	0.00	-0.01	-0.12
bl1_ec	-0.01	-0.03	0.06	-0.05	0.07	0.01
bl1_och	0.05	-0.04	-0.15	-0.13	-0.08	0.17
bl1_pao	0.04	-0.10	0.41	-0.05	-0.07	-0.10
bl2_kpc	0.01	0.01	0.00	0.03	0.01	-0.05
ClassC-AmpC	-0.02	0.06	-0.13	-0.01	-0.11	0.05
bl2d_oxa1	0.00	-0.01	0.00	0.05	-0.03	0.01
bl2d_oxa1	-0.01	0.06	-0.01	0.01	0.00	0.03
ClassD	0.00	-0.07	0.12	0.10	-0.14	-0.05
penicillin	-0.36	0.40	-0.03	-0.26	0.23	-0.34
Chloramphenicol_Phosphotransferase	-0.03	-0.06	0.21	-0.14	-0.05	-0.05
BacA	0.01	0.00	-0.01	0.01	0.01	-0.07
BacA	-0.01	0.06	-0.01	0.01	0.00	0.03
BacA	-0.04	0.09	0.02	-0.11	-0.01	0.01
BacA	0.00	0.00	-0.01	-0.02	0.01	-0.02
BacA	0.00	-0.03	0.15	-0.08	-0.01	0.02
BacA	-0.01	0.05	-0.03	-0.01	-0.01	0.07
BacA	0.01	0.00	-0.02	-0.02	-0.01	0.04
BacA	-0.07	-0.20	-0.02	0.11	-0.04	0.12
BacA	-0.07	0.11	-0.15	-0.10	-0.04	0.05
BacA	-0.08	0.11	-0.14	-0.07	0.08	0.00
BacA	0.00	-0.02	0.07	-0.04	-0.01	0.01
BacA	0.00	-0.02	0.07	-0.04	-0.01	0.01
BacA	0.73	0.35	0.20	0.02	0.02	-0.14
BacA	-0.01	-0.03	0.06	-0.03	0.06	-0.02
BacA	0.11	-0.06	-0.22	-0.10	-0.16	0.23
BacA	0.01	-0.02	-0.03	0.02	0.06	-0.04
BacA	-0.01	-0.02	-0.01	0.06	0.04	0.03
BacA	0.01	-0.02	0.06	-0.05	-0.02	0.05
BacA	-0.13	0.07	0.21	0.16	-0.05	0.01
BacA	0.00	-0.01	-0.01	-0.01	-0.03	-0.01
BacA	-0.01	0.04	0.05	-0.05	-0.01	0.08
Fluoroquinolone Resistant DNA Topoisomerase	-0.02	0.46	-0.13	0.47	0.03	0.00
Fosb	-0.02	-0.06	0.04	0.02	0.07	0.01
BacA	0.00	-0.01	-0.01	0.02	0.07	0.02

Variables	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
BacA	0.00	-0.02	0.07	-0.04	-0.01	0.01
dfra12	0.00	-0.01	-0.01	-0.01	0.00	-0.04
dfra13	-0.01	-0.07	0.11	0.01	-0.02	0.06
Catb4	-0.08	-0.22	-0.13	0.23	-0.10	-0.41
dfrb2	-0.03	0.11	-0.04	-0.06	0.03	0.01
L1	-0.03	-0.06	-0.05	0.11	-0.04	-0.05
Mphc	0.00	-0.01	-0.01	-0.01	-0.03	-0.01
Contribution to variability	67.1%	14.0%	7.6%	3.1%	2.7%	2.1%

Table 3. Contribution of resistance genes of dissimilarity among sampling sites (SIMPER-similarity analysis) (Hammer et al., 2001).

Resistance genes	Av. dissimilarity	Contrib. %	Cumulative %	Mean Impacted	Mean Un-impacted	Mean Tributary 1	Mean Tributary 2
BacA	6.12	13.09	13.09	0.04	0.30	0.11	0.14
Cfr-gene	3.55	7.60	20.69	0.08	0.19	0.05	0.10
blaR1	3.37	7.21	27.90	0.10	0.02	0.14	0.08
Fluoroquinolone Resistant DNA Topoisomerase	1.92	4.10	32.00	0.03	0.06	0.09	0.02
bl3_vim	1.70	3.63	35.64	0.03	0.05	0.01	0.03
Tsnr	1.66	3.54	39.18	0.06	0.00	0.02	0.04
BacA	1.23	2.64	41.82	0.04	0.01	0.05	0.05
Cara	1.19	2.55	44.37	0.04	0.00	0.03	0.04
bl1_pao	1.19	2.54	46.91	0.00	0.02	0.00	0.05
Catb4	1.18	2.53	49.44	0.06	0.03	0.02	0.02
bl2be_per	1.16	2.49	51.93	0.04	0.05	0.06	0.03
bl2be_per	1.02	2.19	54.12	0.01	0.02	0.00	0.00
Tnr	0.96	2.05	56.16	0.00	0.04	0.00	0.02
BacA	0.95	2.03	58.20	0.04	0.01	0.01	0.02
Lmra	0.93	1.99	60.18	0.06	0.03	0.03	0.02
BacA	0.89	1.91	62.10	0.02	0.00	0.03	0.00
Bcrc	0.88	1.88	63.98	0.01	0.00	0.03	0.01
bl2a_pc	0.85	1.82	65.80	0.04	0.01	0.03	0.04
BacA	0.81	1.73	67.54	0.02	0.00	0.03	0.00
bl2be_ctxm	0.81	1.73	69.27	0.03	0.00	0.01	0.00
bl2be_ctxm	0.72	1.54	70.81	0.01	0.00	0.02	0.03
Chloramphenicol_Phosphotransferase	0.70	1.50	72.31	0.01	0.01	0.01	0.03

Resistance genes	Av. dissimilarity	Contrib. %	Cumulative %	Mean Impacted	Mean Un-impacted	Mean Tributary 1	Mean Tributary 2
Oleb	0.59	1.27	73.59	0.01	0.00	0.01	0.02
bl1_och	0.57	1.23	74.82	0.00	0.01	0.00	0.00
LRA	0.52	1.10	75.92	0.02	0.01	0.01	0.00
bl1_ampc	0.50	1.08	77.00	0.00	0.00	0.02	0.02
ClassC-AmpC	0.50	1.08	78.07	0.01	0.01	0.02	0.00
BacA	0.49	1.05	79.12	0.01	0.00	0.02	0.01
ClassD	0.48	1.04	80.16	0.01	0.01	0.00	0.02
bl3_cpha	0.47	1.01	81.16	0.00	0.01	0.02	0.00
dfrb2	0.45	0.97	82.13	0.00	0.00	0.02	0.00
bl2a_iii	0.43	0.92	83.05	0.01	0.02	0.01	0.01
Bcr	0.43	0.91	83.96	0.00	0.00	0.02	0.00
dfra13	0.41	0.87	84.84	0.01	0.00	0.00	0.02
Bcr	0.38	0.82	85.66	0.00	0.00	0.00	0.02
bl1_cmy2	0.37	0.80	86.46	0.00	0.02	0.00	0.01
aac2ic	0.36	0.77	87.23	0.01	0.01	0.01	0.01
aac3viii	0.36	0.76	87.99	0.03	0.02	0.03	0.03
16S_Ribosomal_RNA_Methyltransferase	0.35	0.76	88.75	0.01	0.01	0.01	0.01
BacA	0.35	0.74	89.49	0.00	0.00	0.00	0.02
L1	0.29	0.62	90.10	0.01	0.00	0.00	0.00
Fosb	0.27	0.59	90.69	0.01	0.00	0.00	0.01
Tlrc	0.27	0.58	91.27	0.00	0.01	0.01	0.00
BacA	0.25	0.54	91.81	0.00	0.00	0.01	0.01
BacA	0.23	0.48	92.29	0.00	0.00	0.01	0.00
Aadd	0.21	0.45	92.74	0.00	0.00	0.01	0.00
bl1_ec	0.21	0.44	93.18	0.00	0.00	0.00	0.01
BacA	0.20	0.43	93.61	0.00	0.00	0.00	0.01
BacA	0.20	0.43	94.05	0.00	0.00	0.00	0.01
BacA	0.19	0.42	94.46	0.00	0.00	0.01	0.00
bl2d_oxa1	0.19	0.42	94.88	0.00	0.00	0.01	0.00
aac6i	0.19	0.41	95.29	0.00	0.00	0.00	0.01

Table 4. Explanatory value of factors (Seasons, sample type) and covariates (pH and temperature - °C) on the abundance of different antibiotic resistance genes (Relative abundance %) in the Humber River watershed

Resistance Types	Source	DF	F-value	P-value	S	r^2	r^2 (adj)
tnr	Seasons	1	1.36	0.328	0.0015374	93.11%	81.63%
	Sample type	2	0.09	0.917			
	Temp (Seasons)	2	4.55	0.123			
	Error	3					
	Total	8					
aac6i	Seasons	1	1.42	0.319	0.0042714	46.29%	0.00 %
	Sample type	2	0.31	0.756			
	Temp (Seasons)	2	1.13	0.432			
	Error	3					
	Total	8					
Srmb	Seasons	1	0.75	0.450	0.0043766	40.53%	0.00%
	Sample type	2	0.09	0.912			
	Temp (Seasons)	2	0.42	0.691			
	Error	3					
	Total	8					
bl2d_oxa2	Seasons	1	14.48	0.032	0.0015980	88.00%	68.00%
	Sample type	2	5.35	0.102			
	Temp (Seasons)	2	7.04	0.074			
	Error	3					
	Total	8					
aac3iia	Seasons	1	8.33	0.063	0.0011270	92.42%	79.78%
	Sample type	2	0.09	0.917			
	Temp (Seasons)	2	8.04	0.062			
	Error	3					
	Total	8					
bacA	Seasons	1	0.21	0.677	0.0039978	27.42%	00.00%
	Sample type	2	0.44	0.681			
	Temp (Seasons)	2	0.16	0.858			
	Error	3					

Resistance Types	Source	DF	F-value	P-value	S	r^2	r^2 (adj)
	Total	8					
bl2_kpc	Seasons	1	2.36	0.222	0.0010647	88.42%	69.11%
	Sample type	2	8.40	0.059			
	Temp (Seasons)	2	7.35	0.070			
	Error	3					
	Total	8					
cata1	Seasons	1	0.02	0.908	0.0038435	31.75%	0.00%
	Sample type	2	0.09	0.917			
	Temp (Seasons)	2	0.00	0.999			
	Error	3					
	Total	8					
bacA	Seasons	1	0.19	0.693	0.0034727	37.42%	0.00%
	Sample type	2	0.33	0.741			
	Temp (Seasons)	2	0.43	0.687			
	Error	3					
	Total	8					
bl2d_oxa1	Seasons	1	0.97	0.396	0.0023061	69.67%	19.11%
	Sample type	2	0.09	0.917			
	Temp (Seasons)	2	1.88	0.296			
	Error	3					
	Total	8					
dfra12	Seasons	1	0.21	0.677	0.0033125	27.42%	0.00%
	Sample type	2	0.44	0.681			
	Temp (Seasons)	2	0.16	0.858			
	Error	3					
	Total	8					

3.0 APPENDIX-C

Table 1. List of antibiotic resistance classes according to ShortBRED

Serial #	Resistance classes
1	Thiostrepton
2	Aminoglycoside
3	Multidrug (A)
4	Bacitracin
5	Lincosamide,macrolide,streptogramin_b
6	Lincomycin
7	Multidrug (B)
8	Penicillin
9	Penicillin
10	Multidrug (C)
11	Monobactams,penicillin
12	Multidrug (D)
13	Cephalosporin
14	Ampicillin, penicillin
15	Beta lactams
16	Multidrug(E)
17	Multidrug(F)
18	Ampicillin
19	Cloxacillin, penicillin
20	Fosfomycin
21	Trimethoprim
22	Macrolide
23	Carbapenems,penicillins,cephalosporin
24	Multidrug transporter
25	Deoxycholate fosfomycin
26	Enoxacin, norfloxacin
27	Tetracycline
28	Tigecycline
29	Polymyxin
30	Beta-Lactam
31	Multidrug(G)
32	Ampicillin
33	Acriflavine, aminoglycoside, macrolide
34	Macrolide specific
35	Doxorubicin, erythromycin
36	Acriflavine, Puromycin, t_chloride

Serial #	Resistance classes
37	Fluoroquinolone specific
38	Lincosamide, macrolide, Streptogramin_b
39	qa_compound
40	Vancomycin, Teicoplanin
41	Vancomycin
42	Transcriptional mechanism
43	Quinolone
44	Puromycin
45	Teicoplanin
46	Novobiocin
47	Transcription regulator
48	Streptomycin

Table 2. PCA Eigenvalues for resistance classes.

Variables	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Thiostrepton	-0.15	0.03	-0.18	-0.28	0.00	0.08
Aminoglycoside	-0.03	0.03	-0.01	-0.11	0.07	-0.21
Multidrug (A)	-0.08	0.23	0.15	0.15	0.05	-0.15
Bacitracin	0.58	0.27	-0.02	-0.25	-0.27	0.52
Lincosamide,macrolide,streptogramin_b	0.25	-0.09	-0.16	0.37	0.08	0.02
Lincomycin	0.07	-0.14	0.04	0.09	0.06	0.01
Multidrug (B)	-0.67	0.20	-0.06	0.13	-0.06	0.49
Penicillin	-0.05	-0.07	-0.07	0.19	-0.01	0.01
Penicillin	0.03	0.43	0.27	0.15	0.06	0.28
Multidrug (C)	-0.01	-0.06	-0.15	0.20	0.05	-0.02
Monobactams, penicillin	-0.01	-0.01	0.09	-0.06	-0.02	-0.02
Multidrug (D)	-0.03	-0.13	0.02	0.03	-0.06	0.03
Cephalosporin	0.04	0.01	-0.05	0.04	0.03	0.00
Ampicillin, penicillin	-0.01	0.03	0.01	-0.02	0.01	-0.01
Beta lactams	-0.02	0.16	0.01	-0.14	0.05	-0.04
Multidrug(E)	-0.04	0.11	0.21	0.25	-0.03	-0.04
Multidrug(F)	0.03	-0.01	0.03	-0.01	0.00	-0.01
Ampicillin	0.07	-0.04	-0.02	-0.01	0.03	0.02
Cloxacillin penicillin	0.00	-0.01	-0.04	-0.02	0.00	0.02
Fosfomycin	-0.04	0.02	0.02	-0.03	0.00	-0.02
Trimethoprim	0.04	0.04	-0.09	0.06	0.04	0.02
Macrolide	-0.08	0.11	-0.02	-0.03	0.03	-0.04
Carbapenems,penicillins,cephalosporin	0.04	-0.04	-0.43	-0.11	0.15	0.19
Multidrug transporter	0.01	0.09	-0.17	-0.19	0.07	0.00
Deoxycholate fosfomycin	-0.07	-0.06	-0.22	-0.15	0.03	0.01
Enoxacin norfloxacin	-0.02	0.14	-0.01	0.02	0.04	0.00
Tetracycline	-0.07	-0.58	0.39	-0.03	-0.38	0.31
Tigecycline	-0.01	-0.02	-0.04	0.03	0.02	0.03
Polymyxin	-0.01	-0.05	0.00	0.00	-0.01	-0.01

Variables	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Beta-Lactam	0.07	-0.13	0.06	-0.05	-0.02	-0.05
Multidrug(G)	0.08	0.19	0.38	-0.25	0.05	-0.27
Ampicillin	0.25	0.00	-0.09	0.55	0.05	0.05
Acriflavine, aminoglycoside, macrolide	0.01	-0.03	-0.12	-0.01	0.02	0.02
Macrolide specific	0.01	-0.02	-0.09	0.02	0.00	0.05
Doxorubicin, erythromycin	0.00	-0.04	0.00	-0.04	-0.01	-0.01
Acriflavine, Puromycin, t_chloride	-0.01	-0.02	-0.04	0.03	0.00	0.01
Fluoroquinolone specific	0.01	-0.02	0.04	0.08	-0.01	-0.02
Lincosamide, macrolide, streptogramin_b	-0.01	0.25	0.19	0.10	0.00	0.04
qa_compound	0.01	-0.01	0.08	-0.01	-0.02	-0.04
Vancomycin, Teicoplanin	0.03	0.04	-0.08	0.00	0.04	0.07
Vancomycin	0.02	-0.07	0.11	0.03	-0.02	0.00
Transcriptional mechanism	0.06	-0.23	0.28	-0.11	0.84	0.33
Quinolone	0.00	0.00	0.00	0.00	0.00	0.00
Puromycin	0.00	-0.01	-0.04	-0.02	0.00	0.02
Teicoplanin	0.02	0.00	-0.05	-0.01	0.01	0.02
Novobiocin	0.00	0.00	0.00	0.00	0.00	0.00
Transcription regulator	0.00	-0.01	-0.04	-0.02	0.00	0.02
Streptomycin	0.00	-0.01	-0.04	-0.02	0.00	0.02
Contribution to variability	51.6%	22.4%	13.8%	12.1%	0.00%	0.00%

Table 3. Contribution to dissimilarity of resistance classes (SIMPER-similarity analysis) (Hammer et al., 2001)

Resistance classes	Av. dissim	Contrib. %	Cumulative %	Mean Impacted	Mean Unimpacted	Mean Tributary 1	Mean Tributary 2
Vancomycin	5.48	17.16	17.16	0.02	0.22	0.08	0.10
Carbapenems,penicillins,cephalosporin	3.98	12.47	29.62	0.05	0.02	0.18	0.03
Bacitracin	2.36	7.40	37.02	0.08	0.14	0.09	0.11
Tetracycline	2.25	7.05	44.08	0.15	0.09	0.18	0.13
Multidrug (A)	1.87	5.87	49.95	0.03	0.08	0.02	0.04
Multidrug (B)	1.71	5.35	55.30	0.16	0.09	0.11	0.11
Multidrug (G)	1.55	4.86	60.16	0.03	0.02	0.03	0.07
Penicillin	1.24	3.87	64.03	0.06	0.02	0.05	0.05
Lincosamide,macrolide,streptogramin_b	1.10	3.45	67.48	0.04	0.01	0.03	0.03
Multidrug (E)	0.87	2.72	70.19	0.01	0.04	0.01	0.01
Ampicillin	0.86	2.68	72.87	0.04	0.01	0.01	0.00
Cephalosporin	0.77	2.43	75.30	0.01	0.02	0.01	0.03
Thiostrepton	0.64	2.00	77.30	0.03	0.01	0.01	0.02
Lincomycin	0.56	1.76	79.06	0.03	0.02	0.01	0.01
Cloxacillin, penicillin	0.55	1.73	80.80	0.02	0.01	0.02	0.01
Multidrug Transporter	0.55	1.73	82.52	0.01	0.01	0.01	0.02
Multidrug (D)	0.53	1.65	84.17	0.01	0.03	0.02	0.01
Multidrug (C)	0.42	1.33	85.50	0.02	0.01	0.01	0.01
Teicoplanin, vancomycin	0.35	1.09	86.59	0.01	0.01	0.00	0.02
Beta_lactams	0.34	1.06	87.65	0.01	0.01	0.01	0.02

Resistance classes	Av. dissim	Contrib. %	Cumulative %	Mean Impacted	Mean Unimpacted	Mean Tributary 1	Mean Tributary 2
Macrolide	0.31	0.97	88.62	0.01	0.00	0.00	0.00
Monobactams, penicillin	0.28	0.86	89.49	0.01	0.00	0.00	0.01
Doxorubin,erythromycin	0.25	0.78	90.26	0.00	0.01	0.00	0.01
Aminoglycoside	0.24	0.74	91.01	0.01	0.01	0.01	0.00
Macrolide specific	0.23	0.73	91.74	0.00	0.00	0.01	0.00
Qa_compound	0.23	0.72	92.46	0.00	0.00	0.01	0.01
Deoxycholate, fosfomycin	0.20	0.64	93.09	0.01	0.00	0.00	0.00
Ampicillin, penicillin	0.19	0.61	93.70	0.00	0.01	0.01	0.00
Puromycin	0.19	0.60	94.30	0.00	0.00	0.00	0.00
Acriflavine,puromycin,t_chloride	0.19	0.59	94.88	0.01	0.00	0.01	0.01
Trimethoprim	0.18	0.58	95.46	0.00	0.00	0.01	0.01

Table 4. Explanatory value of factors (Seasons, sample type) and covariates (pH and temperature (°C) on the abundance of different antibiotic resistance classes (Relative abundance %) in the Humber River watershed"

Resistance Classes	**Source**	**DF**	**F-value**	**P-value**	**S**	**r^2**	**r^2 (adj)**
Ampicillin resistance	Season	1	2.01	0.251	0.0031666	63.98%	3.94%
	Sample type	2	0.53	0.637			
	Temp (Seasons)	2	0.77	0.539			
	Error	3					
	Total	8					
Trimethroprim resistance	Season	1	17.39	0.025	0.0017411	99.03%	97.42 %
	Sample type	2	21.59	0.017			
	Temp (Seasons)	2	24.63	0.014			
	Error	3					
	Total	8					
Macrolide specific resistance	Season	1	13.32	0.035	0.0018842	85.86%	62.69%
	Sample type	2	5.18	0.106			
	Temp (Seasons)	2	6.57	0.080			
	Error	3					
	Total	8					
Tigecycline resistance	Season	1	0.79	0.439	0.0012926	88.43%	69.15%
	Sample type	2	1.13	0.431			
	Temp (Seasons)	2	5.90	0.091			
	Error	3					
	Total	8					
Multidrug resistance	Season	1	4.54	0.123	0.0160580	83.05%	54.81%
	Sample type	2	0.31	0.752			

Resistance Classes	Source	DF	F-value	P-value	S	r^2	r^2 (adj)
	Temp (Seasons)	2	3.66	0.157			
	Error	3					
	Total	8					
Fosfomycin resistance	Season	1	1.32	0.333	0.0018362	74.59%	32.23%
	Sample type	2	1.47	0.360			
	Temp (Seasons)	2	0.39	0.707			
	Error	3					
	Total	8					
Ampicillin, penicillin resistance	Season	1	2.78	0.194	0.0020451	54.91%	0.00%
	Sample type	2	1.40	0.372			
	Temp (Seasons)	2	1.51	0.353			
	Error	3					
	Total	8					
Fluoroquinolone specific resistance	Season	1	0.01	0.921	0.00101117	88.61%	69.64%
	Sample type	2	1.88	0.295			
	Temp (Seasons)	2	0.43	0.687			
	Error	3					
	Total	8					
Monobactams, penicillin resistance	Season	1	0.24	0.658	0.0031475	27.22%	0.00%
	Sample type	2	0.55	0.627			
	Temp (Seasons)	2	0.14	0.878			
	Error	3					
	Total	8					
Multidrug resistance	Season	1	0.05	0.835	0.0036148	91.43%	77.13%
	Sample type	2	3.23	0.179			

Resistance Classes	Source	DF	F-value	P-value	S	r^2	r^2 (adj)
	Temp (Seasons)	2	2.35	0.243			
	Error	3					
	Total	8					
Polymyxin resistance	Season	1	0.00	0.982	0.0021997	6.35%	0.00%
	Sample type	2	0.04	0.964			
	Temp (Seasons)	2	0.08	0.923			
	Error	3					
	Total	8					
Teicoplanin resistance	Season	1	6.50	0.084	0.0012752	71.12%	22.98%
	Sample type	2	0.40	0.701			
	Temp (Seasons)	2	3.25	0.177			
	Error	3					
	Total	8					
Acriflavine, puromycin, t_chloride resistance	Season	1	0.03	0.902	0.0340097	70.44%	21.17%
	Sample type	2	1.48	0.356			
	Temp (Seasons)	2	0.13	0.885			
	Error	3					
	Total	8					
Kanamycin, tobramycin resistance	Season	1	5.94	0.093	0.0009935	78.14%	49.69%
	Sample type	2	0.50	0.651			
	Temp (Seasons)	2	3.82	0.150			
	Error	3					
	Total	8					
Multidrug resistance	Season	1	0.03	0.884	0.0080762	55.67%	0.00%

Resistance Classes	Source	DF	F-value	P-value	S	r^2	r^2 (adj)
	Sample type	2	1.15	0.426			
	Temp (Seasons)	2	0.28	0.775			
	Error	3					
	Total	8					
Cloxacillin, penicillin resistance	Season	1	11.58	0.042	0.0007718	83.75%	56.67%
	Sample type	2	2.95	0.196			
	Temp (Seasons)	2	6.96	0.075			
	Error	3					
	Total	8					
Quinolones resistance	Season	1	14.48	0.032	0.004726	88.00%	68.00%
	Sample type	2	5.35	0.102			
	Temp (Seasons)	2	7.04	0.074			
	Error	3					
	Total	8					
Transcriptional regulator resistance	Season	1	7.17	0.075	0.0195525	97.87%	94.31%
	Sample type	2	23.70	0.015			
	Temp (Seasons)	2	9.89	0.048			
	Error	3					
	Total	8					
Puromycin resistance	Season	1	0.97	0.396	0.0008398	69.67%	19.11%
	Sample type	2	0.09	0.917			
	Temp (Seasons)	2	1.88	0.296			
	Error	3					
	Total	8					

4.0 APPENDIX-D

Table 1. List of antibiotic resistance mechanism according to ShortBRED.

1	RNA methylation
2	Efflux pump
3	Drug enzymatic destruction
4	Beta-Lactam
5	Penicillin
6	Inhibition, metabolites
7	drugtargetprotection/modification (mutation)
8	trimethoprim resistance
9	drug target protection/modification
10	Macrolide phosphotransferase
11	Cell wall/membrane/envelope biogenesis
12	Drug modification
13	Fluoroquinolone
14	Transcriptional mechanism
15	quinolone
16	protein synthesis inhibitor
17	Teicoplanin
18	Novobiocin
19	transcription regulator

Table 2. List of Eigenvalues of antibiotic resistance mechanisms

Biological processes	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**	**PC 6**
RNA methylation	0.19	-0.34	0.20	-0.74	-0.12	0.08
Efflux pump	-0.65	-0.30	0.48	0.16	0.05	-0.01
Drug enzymatic destruction	0.22	-0.30	-0.42	0.37	-0.51	0.02
Beta_lactams	-0.12	0.82	0.04	-0.13	-0.19	-0.13
Penicillin	-0.12	0.08	-0.12	0.19	0.33	0.67
Inhibition metabolites	-0.01	-0.04	-0.05	0.29	0.12	-0.13
7Drug target protection/modification(mutation)	0.02	0.11	0.14	-0.06	-0.45	0.51
Trimethoprim resistance	-0.01	-0.03	0.02	0.07	0.02	-0.28
Drug target protection/modification	-0.17	-0.04	-0.61	-0.33	0.42	0.04
Macrolide phosphotransferase	0.00	0.00	0.00	0.00	0.02	0.00
Cell wall/membrane/envelope biogenesis	0.00	-0.03	-0.04	0.06	0.00	-0.23
Drug modification	0.00	-0.01	0.00	-0.03	0.00	-0.11
Fluoroquinolone	0.01	-0.01	0.01	-0.01	0.09	-0.29
Transcriptional mechanism	0.65	0.06	0.38	0.19	0.40	0.06
Quinolone	0.00	0.02	0.00	0.01	-0.01	0.01
Protein synthesis inhibitor	0.00	0.00	0.00	-0.01	-0.10	-0.11
Teicoplanin	0.00	0.02	-0.03	0.00	0.00	0.07
Novobiocin	0.00	0.00	0.00	-0.03	0.02	-0.06
Transcriptional regulator	0.00	0.00	0.00	0.00	-0.05	-0.05
Contribution to variability	78%	18.3%	1.8%	0.76%	0.5%	0.1%

Table 3. Contribution to dissimilarity of resistance mechanisms (SIMPER-similarity analysis)

Resistance mechanisms	**Av. dissimilarity**	**Contrib. %**	**Cumulative %**	**Mean-Impacted**	**Mean-Unimpacted**	**Mean-Tributary1**	**Mean-Tributary2**
Transcriptional mechanism	5.20	24.34	24.34	0.02	0.21	0.08	0.10
Efflux pump	5.12	23.97	48.31	0.50	0.31	0.42	0.42
Beta_lactams	2.80	13.12	61.43	0.15	0.15	0.25	0.14
Drug enzymatic destruction	2.11	9.90	71.33	0.12	0.17	0.10	0.15
RNA methylation	2.10	9.82	81.15	0.06	0.10	0.02	0.06
Drug target protection/modification	1.39	6.51	87.66	0.08	0.02	0.06	0.05
Penicillin	1.07	5.01	92.67	0.04	0.01	0.04	0.03
Drug target protection/modification (mutation)	0.56	2.61	95.28	0.01	0.02	0.03	0.01

Table 4. Explanatory value of factors (Seasons, sample type) and covariates (pH and temperature (°C) on the abundance of different resistance biological processes (Relative abundance %) in the Humber River watershed"

Biological process	Source	DF	F-value	P-value	r^2	r^2 (adj)
Drug target protection/ modification	Season	1	8.33	0.063	92.70%	80.53%
	Sample type	2	16.53	0.024		
	Temp (Seasons)	2	12.06	0.037		
	Error	3				
	Total	8				
Inhibition metabolite	Season	1	2.16	0.238	73.48%	29.28%
	Sample type	2	1.00	0.466		
	Temp (Seasons)	2	2.84	0.203		
	Error	3				
	Total	8				
Trimethoprim resistance	Season	1	0.65	0.478	56.92%	0.00%
	Sample type	2	0.13	0.880		
	Temp (Seasons)	2	0.17	0.850		
	Error	3				
	Total	8				
fluoroquinolone	Season	1	0.73	0.457	62.75%	0.68%
	Sample type	2	1.11	0.435		
	Temp (Seasons)	2	0.34	0.735		
	Error	3				
	Total	8				
Cell wall/membrane/envelope biogenesis	Season	1	0.79	0.441	60.33%	0.00%
	Sample type	2	0.10	0.905		
	Temp (Seasons)	2	0.19	0.839		
	Error	3				
	Total	8				
Drug modification	Season	1	0.01	0.938	35.61%	0.00%
	Sample type	2	0.15	0.864		
	Temp (Seasons)	2	0.20	0.830		
	Error	3				
	Total	8				
Teicoplanin	Season	1	5.94	0.093	78.14%	41.69%
	Sample type	2	0.50	0.651		
	Temp (Seasons)	2	3.82	0.150		

Biological process	Source	DF	F-value	P-value	r^2	r^2 (adj)
	Error	3				
	Total	8				
Protein synthesis inhibitor	Season	1	0.97	0.396	69.67%	19.11%
	Sample type	2	0.09	0.917		
	Temp (Seasons)	2	1.88	0.296		
	Error	3				
	Total	8				
Quinolone	Season	1	14.48	0.032	88.00%	68.00%
	Sample type	2	5.35	0.102		
	Temp (Seasons)	2	7.04	0.074		
	Error	3				
	Total	8				
Macrolide phosphotransferase	Season	1	0.21	0.677	27.24%	0.00%
	Sample type	2	0.44	0.681		
	Temp (Seasons)	2	0.16	0.858		
	Error	3				
	Total	8				
Transcription regulator	Season	1	0.97	0.396	69.67%	19.11%
	Sample type	2	0.09	0.917		
	Temp (Seasons)	2	1.88	0.296		
	Error	3				
	Total	8				

Table 5. List of locations and flow rates (m^3 s^{-1})

Year	Months	Locations	Flow rate(m^3 s^{-1})
2015	July	Humber Village	202
2015	July	Humber Village	214
2015	August	Humber Village	206
2015	September	Humber Village	243
2015	October	Humber Village	268
2015	November	Humber Village	289
2015	November	Humber Village	279
2015	December	Humber Village	249
2015	November	Humber Village	289
2015	November	Humber Village	289
2015	November	Humber Village	289
2015	November	Humber Village	279
2015	November	Humber Village	279
2015	November	Humber Village	279
2015	July	Little falls	26.6
2015	July	Little falls	55.6
2015	August	Little falls	56.6
2015	September	Little falls	62
2015	October	Little falls	127
2015	November	Little falls	29
2015	December	Little falls	47.3
2015	September	Little falls	62
2016	May	Humber Village	0
2016	May	Humber Village	0
2016	May	Humber Village	0
2016	May	Humber Village	0
2016	May	Little falls	107
2016	June	Little falls	99.2
2016	July	Little falls	19.2
2016	August	Little falls	9.74
2016	May	Little falls	107
2016	May	Little falls	107
2016	May	Little falls	107

Table 6. Actual values used for analysis (Relative gene copies / 16S rRNA

Temperature	pH	Years	Sampling months	Seasons	Sampling dates	Source types	Sampling location	Tet (O)	Tet (M)	AdeC
16	6.41	2016	September	Fall	19-Sep	un-impacted	Little falls	0.0000	0.0000	0.0002
2	6.17	2015	November	Winter	9-Nov	un-impacted	Little falls	0.0000	0.0001	0.0013
5	6.68	2015	October	Fall	26-Oct	un-impacted	Little falls	0.0000	0.0000	0.0001
16	6.41	2015	September	Fall	21-Sep	un-impacted	Little falls	0.0004	0.0000	0.0014
16	6.41	2015	September	Fall	21-Sep	un-impacted	Little falls	0.0004	0.0001	0.0024
21	6.55	2015	August	Summer	18-Aug	un-impacted	Little falls	0.0000	0.0000	0.0003
12	5.56	2016	June	Spring	13-Jun	un-impacted	Little falls	0.0000	0.0000	0.0003
15		2015	July	Summer	20-Jul	un-impacted	Little falls	0.0000	0.0000	0.0000
8	6.37	2016	May	Spring	9-May	un-impacted	Little falls	0.0000	0.0002	0.0003
8	6.37	2016	May	Spring	9-May	un-impacted	Little falls	0.0000	0.0002	0.0004
8	6.37	2016	May	Spring	9-May	un-impacted	Little falls	0.0000	0.0002	0.0004
8	6.37	2016	May	Spring	9-May	un-impacted	Little falls	0.0000	0.0004	0.0004
22	7.3	2016	August	Summer	8-Aug	un-impacted	Little falls	0.0000	0.0000	0.0000
17	7.41	2016	July	Summer	4-Jul	un-impacted	Little falls	0.0000	0.0000	0.0001
14	5.02	2016	May	Spring	24-May	un-impacted	Little falls	0.0000	0.0000	0.0002
9	8.36	2015	November	Winter	23-Nov	Deer lake	Deer lake	0.0002	0.0002	0.0015
0	7.49	2015	November	Winter	9-Nov	Deer lake	Deer lake	0.0000	0.0001	0.0004
6	6.17	2015	October	Fall	26-Oct	Deer lake	Deer lake	0.0002	0.0000	0.0012
14	6.31	2015	September	Fall	21-Sep	Deer lake	Deer lake	0.0001	0.0001	0.0027
17.5	6.71	2015	July	Summer	28-Jul	Deer lake	Deer lake	0.0000	0.0000	0.0000
16		2015	July	Summer	20-Jul	Deer lake	Deer lake	0.0000	0.0000	0.0001
23	7.15	2016	August	Summer	8-Aug	Deer lake	Deer lake	0.0000	0.0000	0.0000
12	6.34	2016	June	Spring	13-Jun	Deer lake	Deer lake	0.0000	0.0000	0.0002
14	5.7	2016	May	Spring	24-May	Deer lake	Deer lake	0.0000	0.0000	0.0001
17.5	6.2	2016	September	Fall	19-Sep	un-impacted	Main dam road	0.0001	0.0009	0.0008
4	6.47	2015	November	Winter	9-Nov	un-impacted	Main dam road	0.0000	0.0001	0.0004
20	7.28	2016	August	Summer	8-Aug	un-impacted	Main dam road	0.0000	0.0000	0.0003
5.5	6.98	2015	October	Fall	26-Oct	un-impacted	Main dam road	0.0000	0.0001	0.0006
16	6.15	2015	August	Summer	18-Aug	un-impacted	Main dam road	0.0000	0.0000	0.0000
10	5.68	2016	June	Spring	13-Jun	un-impacted	Main dam road	0.0000	0.0009	0.0006

Temperature	pH	Years	Sampling months	Seasons	Sampling dates	Source types	Sampling location	Tet (O)	Tet (M)	AdeC
10	5.68	2016	June	Spring	13-Jun	un-impacted	Main dam road	0.0001	0.0004	0.0016
10	5.68	2016	June	Spring	13-Jun	un-impacted	Main dam road	0.0003	0.0007	0.0019
11.5	6.14	2015	July	Summer	28-Jul	un-impacted	Main dam road	0.0000	0.0000	0.0003
10.5		2015	July	Summer	20-Jul	un-impacted	Main dam road	0.0000	0.0000	0.0000
8	6.54	2016	May	Spring	9-May	un-impacted	Main dam road	0.0003	0.0005	0.0017
14	6.42	2016	July	Summer	4-Jul	un-impacted	Main dam road	0.0000	0.0000	0.0003
10	5.53	2016	May	Spring	24-May	un-impacted	Main dam road	0.0001	0.0008	0.0010
4	6.25	2015	November	Winter	9-Nov	impacted	Humber resort	0.0000	0.0003	0.0002
8	6.74	2015	October	Fall	26-Oct	impacted	Humber resort	0.0000	0.0007	0.0005
13.5	6.64	2015	September	Fall	21-Sep	impacted	Humber resort	0.0000	0.0000	0.0007
15	5.84	2015	July	Summer	28-Jul	impacted	Humber resort	0.0000	0.0000	0.0001
9		2015	July	Summer	20-Jul	impacted	Humber resort	0.0000	0.0000	0.0001
11	5.61	2016	June	Spring	20-Jun	impacted	Humber resort	0.0000	0.0002	0.0003
11	5.61	2016	June	Spring	20-Jun	impacted	Humber resort	0.0000	0.0001	0.0003
11	5.61	2016	June	Spring	20-Jun	impacted	Humber resort	0.0001	0.0002	0.0003
8	7.07	2016	June	Spring	13-Jun	impacted	Humber resort	0.0000	0.0001	0.0004
8	7.07	2016	June	Spring	13-Jun	impacted	Humber resort	0.0000	0.0002	0.0014
10	4.69	2016	May	Spring	24-May	impacted	Humber resort	0.0000	0.0001	0.0002
5	6.41	2016	May	Spring	9-May	impacted	Humber resort	0.0003	0.0004	0.0016
6	6.41	2015	December	Winter	7-Dec	impacted	Humber Village	0.0002	0.0001	0.0029
7	6.39	2015	November	Winter	23-Nov	impacted	Humber Village	0.0000	0.0001	0.0004
4	6.08	2015	November	Winter	9-Nov	impacted	Humber Village	0.0000	0.0002	0.0003
10	6.62	2015	October	Fall	26-Oct	impacted	Humber Village	0.0000	0.0000	0.0003
17.5	5.87	2015	August	Summer	18-Aug	impacted	Humber Village	0.0000	0.0000	0.0003
15	5.89	2015	July	Summer	28-Jul	impacted	Humber Village	0.0000	0.0000	0.0001
14		2015	July	Summer	20-Jul	impacted	Humber Village	0.0000	0.0000	0.0003
17	6.47	2016	September	Fall	19-Sep	impacted	Humber Village	0.0000	0.0000	0.0002
11	5.72	2016	June	Spring	20-Jun	impacted	Humber Village	0.0000	0.0000	0.0004
6	7.5	2016	June	Spring	13-Jun	impacted	Humber Village	0.0000	0.0000	0.0006
10	5.06	2016	May	Spring	24-May	impacted	Humber Village	0.0000	0.0000	0.0003
10	5.06	2016	May	Spring	24-May	impacted	Humber Village	0.0000	0.0000	0.0004
10	5.06	2016	May	Spring	24-May	impacted	Humber Village	0.0000	0.0000	0.0003
4	6.21	2016	May	Spring	9-May	impacted	Humber Village	0.0000	0.0000	0.0006

Temperature	pH	Years	Sampling months	Seasons	Sampling dates	Source types	Sampling location	Tet (O)	Tet (M)	AdeC
4	6.21	2016	May	Spring	9-May	impacted	Humber Village	0.0000	0.0000	0.0008
5	6.1	2015	December	Winter	7-Dec	un-impacted	Steady brook	0.0000	0.0000	0.0006
5	6.1	2015	December	Winter	7-Dec	un-impacted	Steady brook	0.0000	0.0009	0.0005
5	6.1	2015	December	Winter	7-Dec	un-impacted	Steady brook	0.0000	0.0001	0.0005
5	6.1	2015	December	Winter	7-Dec	un-impacted	Steady brook	0.0000	0.0001	0.0007
7	6.05	2015	November	Winter	23-Nov	un-impacted	Steady brook	0.0000	0.0001	0.0007
1	6.8	2015	November	Winter	9-Nov	un-impacted	Steady brook	0.0000	0.0001	0.0005
16	5.34	2016	July	Summer	4-Jul	un-impacted	Steady brook	0.0000	0.0000	0.0001
14	6.74	2015	September	Fall	21-Sep	un-impacted	Steady brook	0.0000	0.0001	0.0007
17	5.6	2016	June	Spring	20-Jun	un-impacted	Steady brook	0.0000	0.0000	0.0003
17	5.6	2016	June	Spring	20-Jun	un-impacted	Steady brook	0.0000	0.0000	0.0003
6	5.93	2016	June	Spring	13-Jun	un-impacted	Steady brook	0.0000	0.0001	0.0008
6	5.93	2016	June	Spring	13-Jun	un-impacted	Steady brook	0.0000	0.0000	0.0005
6	5.93	2016	June	Spring	13-Jun	un-impacted	Steady brook	0.0000	0.0000	0.0005
16	5.68	2015	July	Summer	28-Jul	un-impacted	Steady brook	0.0000	0.0001	0.0005
16	4.9	2016	May	Spring	24-May	un-impacted	Steady brook	0.0000	0.0000	0.0003
5	7.53	2016	May	Spring	9-May	un-impacted	Steady brook	0.0000	0.0000	0.0004
7	6.1	2015	December	Winter	7-Dec	impacted	Across the bay	0.0001	0.0001	0.0022
7	6.1	2015	December	Winter	7-Dec	impacted	Across the bay	0.0000	0.0013	0.0015
7	6.1	2015	December	Winter	7-Dec	impacted	Across the bay	0.0000	0.0002	0.0013
7	6.11	2015	November	Winter	23-Nov	impacted	Across the bay	0.0000	0.0003	0.0003
6	6.26	2015	November	Winter	9-Nov	impacted	Across the bay	0.0000	0.0000	0.0003
19	7.2	2016	August	Summer	8-Aug	impacted	Across the bay	0.0000	0.0000	0.0003
15	6.55	2015	September	Fall	21-Sep	impacted	Across the bay	0.0002	0.0010	0.0011
16	5.9	2015	July	Summer	28-Jul	impacted	Across the bay	0.0000	0.0000	0.0003
14.5		2015	July	Summer	20-Jul	impacted	Across the bay	0.0000	0.0000	0.0002
5	5.7	2016	June	Spring	13-Jun	impacted	Across the bay	0.0000	0.0000	0.0001
9	5.3	2016	May	Spring	24-May	impacted	Across the bay	0.0000	0.0000	0.0003