Distribution of Antibiotic Resistant Bacteria that are Human Pathogens and Tritagonists in Waterways

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Abstract

Rivers draining agricultural and urban areas carry greater levels of antibiotic resistant *Escherichia coli* than rivers in unpolluted environments. The number and diversity of antibiotic resistant *E. coli* were measured in two Cantabrian rivers. Silver Stream (Māori: Whirinaki) is drains land that is used agriculture, and Avon River (Māori: Ōtākaro) draina urban environments. The rivers were sampled three times between January and November 2017. *E. coli* were enumerated on *E. coli* selective medium with or without supplementation of the antibiotics ciprofloxacin, ampicillin, rifampicin or chloramphenicol.

In summary, 60% of the *E. coli* isolates from Avon River grew on ampicillin, 1% on chloramphenicol and 1% on ciprofloxacin containing media. About 60% of the *E. coli* initially isolated on 1 µg/mL ciprofloxacin from Avon River grew on ciprofloxacin at or above 16 µg/mL, almost five times higher than the clinical breakpoint concentration.

Approximately 70% of randomly selected *E. coli* first isolated from Avon River on TBX medium with antibiotic supplementation were resistant to two or more antibiotics. Of the ciprofloxacin resistant isolates, 98% also grew on ampicillin, 33% on chloramphenicol, 88% on tetracycline, 54% on trimethoprim, 37% on gentamycin and 19% on kanamycin. In contrast, only 30% of Silver Stream isolates were resistant to at least two antibiotics. Fewer than 1% were resistant to ciprofloxacin or chloramphenicol.

The statistical analysis of this survey demonstrated that the two rivers accumulate different amounts of antibiotic resistant *E. coli* and that isolates from the two rivers are resistant to different concentrations of antibiotic.

The cause of resistance, or differential retention of resistant bacteria, is unknown. A whole genome sequencing of 50 randomly selected isolates from the two rivers was used to identify the resistance genes and alleles.

Abbreviations

µm	micrometer
µL	microliter
Amp	ampicillin
AMX-CLA	amoxicillin- clavulanic acid
ANOVA	Analysis of Variance
C	celsius
CAZ	ceftazidime
CFU	colony forming unit
Chl	chloramphenicol
Cip	ciprofloxacin
CTX	cefotaxime
ESBL	extended-spectrum beta-lactam
G	gram
Gen	gentamycin
Kan	kanamycin
Km	kilometer
Mar	multiple antibiotic resistance
MDR	multiple drug resistance
MIC	minimum inhibitory concentration
mL	milliliter
Mm	millimeter
R2A	reasoner's 2A
Rif	rifampicin

SEM	standard error of the mean
Tbx	tryptone Bile-X-glucuronide
Tet	tetracycline
Tri	trimethoprim
UK	United Kingdom
USA	United State of America
PBS	phosphate-buffered saline

Chapter 1

1.1 Introduction

Previous studies have highlighted that polluted environments habour antibiotic resistant bacteria (McArthur *et al.*, 2016; Nhung *et al.*, 2015; Rizzo *et al.*, 2013) . In this thesis, the prevalence and diversity of antibiotic resistant bacteria were measured in two Canterbury rivers that drain from agriculture land or urbanisation. Firstly, the abundance of mesophilic bacteria (bacteria that grow between 21 °C and 44 °C), *E. coli* and antibiotic resistant *E. coli* was investigated in the two rivers. Secondly, frequency of multiple-drug resistance (MDR) was determined among antibiotic resistant *E. coli* isolated from both rivers.

Antibiotics are chemicals with antimicrobial properties which are capable of inhibiting bacterial growth or causing cell death. Their discovery in medicine is one of the greatest achievements of the 20th century (Aminov, 2010; Davies & Davies, 2010; Penesyan *et al.*, 2015). Antibiotics are used globally for the prevention and treatment of both human and veterinary bacterial infectious diseases (Milic *et al.*, 2013; Nigam *et al.*, 2014). Their usage in medicine has improved life expectancy (Adedeji, 2016; Ventola, 2015). Antibiotics are important in aquaculture, agriculture and bee-keeping as growth enhancers (Gothwal & Shashidhar, 2015).

Since the discovery of antibiotics less than 100 years ago, bacterial resistance to antibiotics has emerged a big problem worldwide. In 2014, the World Health Organisation reported that the spread of antibiotic resistance in pathogenic bacteria is creating a post-antibiotic era where minor bacterial infections and diseases will easily resist treatment (World Health Organization, 2014). About two million infections related to antibiotic resistant bacteria have been reported per year. Even in a first-world country like the United States, antibiotic resistant bacteria kill approximately 23,000 people yearly at a cost of $20 billion and $35 billion additional cost for loss of productivity (Centers for Disease Control and Prevention, 2013). Likewise, in Europe, an estimated 250,000 people die with an annual treatment cost of £1.5 billion (Gelbrand *et al.*, 2015). If this situation remains unchecked, antibiotic resistance will continue to rise and most medical interventions during routine procedures, such as kidney transplants and heart surgery will have a higher probability of failing as a result of bacterial infections (The Review on Antimicrobial Resistance, 2016).

A wide range of mechanisms are responsible for the evolution of antibiotic resistant bacteria in the environment. These mechanisms are complex processes, which is part of the reason why

there is limited success in efforts to mitigate the spread of antibiotic resistance (Davies & Davies, 2010). Overall, the emergence and evolution of antibiotic resistant bacteria in the natural environment remains poorly understood. Antibiotic resistance genes that are of medical relevance have been detected in the natural environment, but it is still unknown how antibiotics used for medical treatment have influenced the environmental bacteria and how antibiotic resistant bacteria in the environment acquired resistant genes from isolates that have survived clinical treatment in patients (Gaze *et al.*, 2013).

Bacteria become resistant to antibiotics through adaptation, acquisition of antibiotic resistance genes or naturally evolve through their inherent capacity to resist antibiotic activities. Bacteria that are frequently exposed to a sub-lethal concentration of antibiotics can manifest a transient form of resistance to antibiotics (Motta *et al.*, 2015). This form of resistance is called adaptive and may be inherited epigenetically (Fernandez & Hancock, 2012). It is not a genotypical form of resistance because when the antibiotics or other triggering chemicals are removed, bacteria tend to return to an antibiotic susceptible state. Bacteria exposure to a sub-inhibitory concentration of antibiotics may also trigger adaptive responses such as an increase in efflux pumps expression or a decrease in porins (Motta *et al.*, 2015). Bacteria that intrinsically resist antimicrobial agents do so through a physiological characteristic or an inherent structure which allows for tolerance to antimicrobial agents. An example is the natural production of enzymes that deactivate drugs or the antimicrobial drug lacking affinity for the bacterial target (Giguère, 2006). Alternatively, bacteria become resistant to antibiotics by acquiring resistance genes and other resistance determinants such as transposons, integrons and plasmids from already resistant bacteria via horizontal gene transfer. This leads to a rapid increase in the dissemination of antibiotic resistance in the environment (Fernandez & Hancock, 2012). Antibiotic resistant bacteria and genes can be released from numerous of hotspots which include hospital effluent, waste treatment plants and other sites of antibiotic disposal. Marathe *et al.* (2013) showed that the discharge of wastewater containing antibiotic residues into the environment can facilitate the dissemination of antibiotic resistance among bacteria population.

Apart from antibiotics, chemicals (such as disinfectants and biocides used in hospitals, or agricultural land and in industry) may also select for antibiotic resistant bacteria and genes (Fernández *et al.*, 2011; Landers *et al.*, 2012). For instance, Kurenbach *et al.* (2015) showed that biocides used in agriculture reduce bacterial susceptibility to antibiotics. Antibiotic resistant bacteria can also be released to the environment from farms or other places where chemicals which causes antibiotic resistance can be found (Kümmerer, 2004). The emergence

of antibiotic resistant bacteria has also occurred due to the overuse of antibiotics in the clinical treatment of infections (D'Costa *et al.*, 2007; D'costa *et al.*, 2006). Despite the negative impact of antibiotic resistance, antibiotics are still globally misused in both human and veterinary medicine. Details of antibiotic usage which can ultimately result in resistance is discussed below.

1.1.1 *Use of antibiotics in animal husbandry impacts on environmental antibiotic resistance*

Globally, about 67% of antibiotics produced are used in animal husbandry (Gelbrand *et al.*, 2015). They are often added to animal feed for prophylactic, therapeutic and sub-therapeutic purposes such as fattening (Gaskins *et al.*, 2002). Antibiotic applications in animal husbandry have improved animal health, generated greater yields and improved product quality (Michael *et al.*, 2014). However, the use of antibiotics may create selective pressure on the environmental microbiome (Witte, 2000). This is evident in the prevalence of antibiotic resistance in the environment where antibiotics are used for prophylaxis and fattening (Baquero *et al.*, 2008; Zhu *et al.*, 2013). Chang *et al*. (2015) demonstrated that the use of antibiotics in animal husbandry increased the frequency of antibiotic resistant bacteria in animals such as swine and chicken. Antibiotic resistance also arises in aquaculture due to selective pressure created by the use of antibiotics for prophylaxis and treatment of bacterial infections associated with high-density production systems (Cabello *et al.*, 2013).

Apart from selective pressure created using antibiotics, antibiotic resistance genes and resistant bacteria could persist in mariculture sediment even without the use of antibiotics over a long time. For instance, trimethoprim, sulfonamide and tetracycline resistance genes have been found in the sediment of mariculture farms without input of antibiotics for several years (Han *et al.*, 2017). Aquaculture and mariculture systems have been recognised as the major sources for antibiotic resistant bacteria and transfer of resistance genes among bacteria into the environment (Watts *et al.*, 2017). This is due to the rapid spread of resistance genes among fish pathogens which increases the chance by which human pathogens acquire antibiotic resistance genes (Séveno *et al.*, 2002). The aftermath of the use of antibiotic in the aquaculture system is that even when the use of antibiotics is banned in aquaculture systems, antibiotic resistance genes persist.

1.1.2 *Use of antibiotics in crop production impacts on environmental antibiotic resistance*

In crop production, antibiotics are used to prevent bacterial infections of plants and fruit. Antibiotics are usually sprayed during crop production, as a result, antibiotic residues are often

distributed to the soil and adjacent rivers thereby selecting for antibiotic resistant bacteria strains in the environment. For instance, streptomycin was applied extensively in crop production and protection after its discovery in the 1950's. It was used to control plant pathogens such as fire blight, a bacterial disease of pomme trees. However, after 10 years of successful application of streptomycin in agriculture, streptomycin resistant bacteria evolved (Stockwell & Duffy, 2012).

1.1.3 *Use of non-antibiotic chemicals in crop production impacts on environmental antibiotic resistance*

Chemical substances such as heavy metals, biocides, disinfectants, organic and inorganic fertilisers used in agriculture for enhancing crop production can also promote the emergence of antibiotic resistant bacteria in the environment (Aktar *et al.*, 2009) . For instance, organic and inorganic fertiliser and pesticides are often used in crop production or in controlling pests and diseases have been detected in surface water (Gerber *et al.*, 2007). Most of these chemicals could also gain entrance into the ecosystem in solutions or as an emulsion and bond with water molecules or soil particles in groundwater and other environments (World Bank, 2007). Kurenbach *et al.* (2015) showed that sub-lethal concentration of herbicide in agricultural practices can induce a change in bacterial phenotype which increases the chances for spontaneous mutation and a high level of resistance in bacteria. In a similar vein, a recent study from our laboratory revealed that the active ingredients in herbicides induce a change in bacteria phenotype which can ultimately lead to resistance (Kurenbach *et al.*, 2017).

1.1.4 *Overuse of antibiotics in medicine impacts on environment antibiotic resistance*

The misuse and overuse of antibiotics in medicine is a fundamental driver of antibiotic resistance (Ventola, 2015). For instance, Bronzwaer *et al.* (2002) showed a strong relationship between the evolution of antibiotic resistant *Streptococcus pneumoniae* and the use of beta-lactam antibiotics in 11 European countries. Willemsen *et al.* (2009) also revealed that antibiotics that are frequently used among hospital patients during treatment are associated with resistant bacteria that evolve in the hospital. In their study, the use of various antibiotics, including beta-lactam antibiotics was monitored among patients. The result showed that a linear correlation between antibiotics often used among patients and antibiotic resistance that evolved. Some of the causes of antibiotic resistance in the clinical context include non-compliance of patients to complete prescribed antibiotics dosage, use of antibiotics without establishing the cause of infection to be bacteria and over-the-counter purchase without knowledge of how antibiotics work (Berglund, 2015). Insufficient antibiotic dosage may result in sub-lethal concentrations capable of inducing changes in the bacteria genetic composition via mutation or changing in gene expression.

Antibiotic resistance genes can also be transferred from one bacterium to another via horizontal gene transfer (HGT), thereby promoting the development and spread of antibiotic resistant bacteria in the environment (Viswanathan, 2014). HGT is one major mechanism that facilitates the spread of antibiotic resistance genes among bacteria of the same species and non-related bacteria. Antibiotics administered to patients in the hospital are most likely capable of killing the susceptible bacteria strains. However, when the antibiotic dosage is lower than the minimum inhibitory concentration of the bacterial strains, resistant strains may continue to reproduce and increase in population (Gullberg *et al.*, 2011; Read & Woods, 2014). Also, some antibiotics used in medical treatments are not completely metabolised in patients' body system. Un-metabolised antibiotics are excreted and pass through to the municipal wastes system where selection and emergence of antibiotic resistance can take place (Kümmerer & Henninger, 2003).

Williamson *et al.* (2014) reported a rise in the emergence of antibiotic resistant strains to common classes of antibiotics such as beta-lactams, fluoroquinolones and third generation cephalosporin which are commonly used in hospitals in New Zealand. Another study in New Zealand reported 4,000 cases of infections associated to extended spectrum beta-lactamase producing *E. coli* that are resistant to several broad-spectrum antibiotics used in hospital and environmental settings (Thomas *et al.*, 2014).

1.2 Antibiotic classification

There are different classifications of antibiotics based on their origin, spectrum, mode of action, and administration routes. Of all the various classifications of antibiotics, the mode of action is the most popular. Table 1 has an overview on the classification and function of each class of antibiotics. Antibiotics are also broadly categorised into three main classes based on their origin. The first class includes those that are naturally produced by microorganisms, for example, penicillin, tetracycline, and streptomycin. Penicillin is an antibiotic produced by the microorganism *Penicillium notatium*. It was discovered by Alexander Fleming in 1928 and introduced for therapeutic use in 1940. The second class are the synthetic antibiotics which include quinolones and sulphonamides (Martinez, 2012). The last class encompasses the semi-synthetic antibiotics, which are chemically modified from their original source. Examples include carbapenems and cephalosporin which were developed from penicillin.

Another classification of antibiotics is based on the effect they have on the bacterial cell. Some antibiotics are bactericidal, i.e., they can kill bacterial cells. Examples are aminoglycoside, vancomycin and cephalosporin. The second group is bacteriostatic, which inhibit bacterial growth and reproduction. Examples of this antibiotics groups include tetracycline, chloramphenicol and trimethoprim.

Table 1 Mode of action of antibiotics used in this study

Antibiotics	Mode of Action	Target	Species range
Fluoroquinolone (e. g ciprofloxacin)	Inhibition of nucleic acid synthesis:		
	1. Inhibition of DNA synthesis	Topoisomerase II (DNA gyrase)	Gram-negative and Gram-positive bacteria
Rifampicin	2.Inhibition of mRNA synthesis	Topoisomerase IV DNA-dependent RNA polymerase	Gram-positive and Gram-negative bacteria.
Aminoglycosides and tetracycline	Inhibition of protein synthesis:		
	1. Action on 30S ribosomal subunit	Blocking aminoacyl tRNA from binding to the ribosome acceptor site.	Gram-positive and Gram-negative bacteria
Chloramphenicol, linezolid and erythromycin	2.Action on 50S ribosomal subunit	Bind to the 50S subunit to prevent peptide bond formation Inhibit peptidyl transferase activity in ribosome	Gram-positive and Gram-negative bacteria
Ampicillin, bacitracin, Cycloserine	Inhibition of cell wall synthesis		.
	1.Inhibition of cross-linking of peptidoglycan	Penicillin-binding proteins	Anaerobic and Aerobic Gram-positive and Gram-negative bacteria
Cephalosporin and penicillin	2.Inhibition of peptidoglycan synthesis	Peptidoglycan units	Gram-positive bacteria
Trimethoprim	Inhibition of folate synthesis	Tetrahydrofolic acid synthesis inhibitors	Aerobic Gram-negative and Gram-positive bacteria

1.3 Antibiotics in the environment

Although, antibiotics are natural environmental products that predate it use by humans. However, the concentration of antibiotics are increasing in rivers, sediments, soil and wastewater since they were adopted for use by humans (Kümmerer, 2004; Zhai, 2014). Antibiotics enter the environment through various ways including discharge from production industries, and release of animal and human waste or through the disposal of unused antibiotics (Larsson, 2014a). Antibiotic discharge from industrial production of antibiotics is often in high concentrations, up to 1 mg/L, which is much higher than what can be detected in animal or human waste (Kristiansson *et al.*, 2011). Bacteria around sewage plants are often exposed to a range of antibiotics at different concentrations (Kümmerer, 2009). When slurry from sewage is used in farmland as an alternative to inorganic fertiliser, antibiotic residues are indirectly introduced to the environment (Udikovic-Kolic *et al.*, 2014). For instance, high concentrations of antibiotics have been detected on surface water adjacent to farmland as a result of runoff during irrigation or rainfall (Kümmerer, 2004).

Unused antibiotics are also often disposed into the environment in many of the developing countries. This is due to the absence of well-established take-back schemes in most of these countries (Lubick, 2010). Therefore, antibiotics mostly end up in the aquatic environment (Figure 1). The distribution of antibiotics in the environment may be affected by the property of the antibiotics itself or the physiochemical property of the receiving environment (Boxall, 2004). For instance, penicillin is easily degradable, whereas other antibiotics such as tetracycline and chloramphenicol may persist, thereby accumulating in the environment (Larsson, 2014a). Due to the hydrophilic nature of tetracycline molecules, it remains undegraded and persists in the aquatic environment (Daghrir & Drogui, 2013). Also, quinolones are not biodegradable and can remain in soil, sediment and sewage sludge for a long time, due to sorption onto particulates. Conversely, antibiotics such as virginiamycin, an antibiotic used as a growth promoter in poultry farms is biodegradable in the soil over an extended period of time (Kümmerer, 2003).

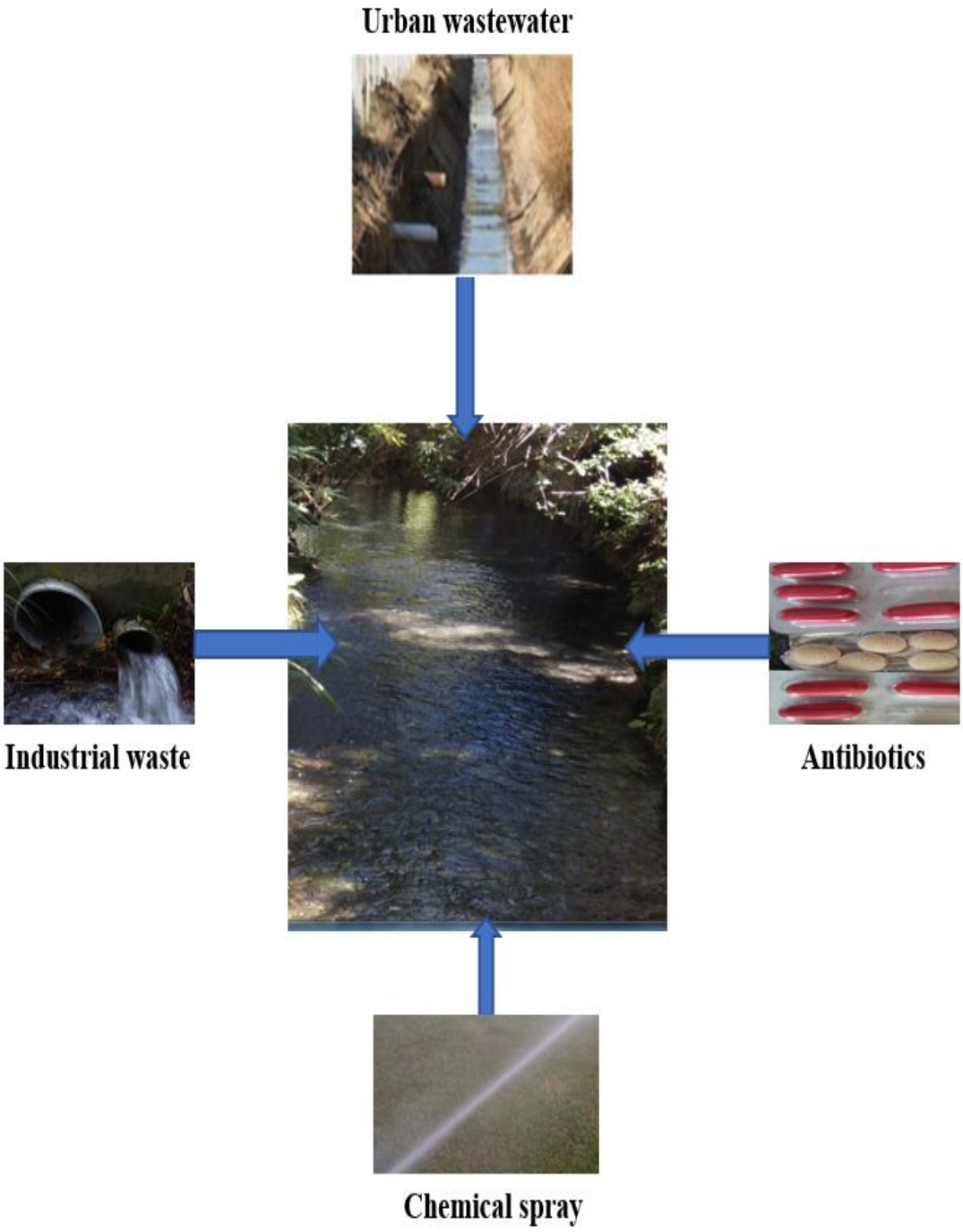


Figure 1: **Different anthropogenic practices that may be contributing to the emergence of antibiotic resistance in aquatic systems. Pictures were taken with mobile phone around the University of Canterbury, New Zealand for the purpose of illustration.**

1.4 Bacteria in the environment

Bacteria are ubiquitous and play a key role in many ecosystems such as water, soil and in other natural and artificial environments (Kümmerer, 2004). For instance, bacteria help in the nutrient cycle and in fixing of carbon dioxide in the atmosphere. They are also essential for water clarification (Kümmerer, 2004). In addition to their functions in the environment, bacteria co-exist and compete for available ecological niches in the environment. Bacteria may adopt many strategies during competition, such as production of antibiotics to compete and control the dynamics in a bacterial population (Narisawa *et al.*, 2008). Contrary, chemical substances in the environment such as synthetic and semi-synthetic antibiotics which are often used nowadays may also influence the bacterial community due to their toxicity (Ding & He, 2010).

1.5 Antibiotic resistance in the environment

Natural environments are reservoirs of antibiotic resistant bacteria and resistance genes (Martínez, 2008). Sequencing analysis of beta-lactamase, the enzyme responsible for bacterial resistance to beta-lactam antibiotics showed that beta-lactam genes exist before the use of antibiotics in both medical and veterinary medicine (Hall & Barlow, 2004). However, anthropogenic factors favour antibiotic resistance development due to the presence of antibiotics in the environment, which are exerting selective pressure on bacteria (Berendonk *et al.*, 2015). Selective pressure due to antibiotic is the major factor driving the evolution, prevalence and spread of antibiotic resistant bacteria in the environment. The diverse genetic variability of antibiotic resistance genes and bacteria habouring these genes makes the environment a reservoir for antibiotic resistance pool (Pal *et al.*, 2016). Sub-lethal concentration of antibiotics speed up the rate of selection of antibiotic resistant bacteria in the environment (Kümmerer, 2004). Moreover, HGT facilitates the prevalence of antibiotic resistance in the environment (Kaplan, 2014). Most antibiotic resistance genes are often transferred in bacterial communities in the presence of sub-lethal concentrations. Furthermore, the transfer of antibiotic resistance genes from a pathogenic bacterium allows for easy dissemination of resistant determinants to other bacteria via HGT, a process of acquisition of antibiotic resistance genes in the environment (Thomas & Nielsen, 2005). Mobile genetic elements such as integrons, transposons and plasmids harbour antibiotic resistance genes which can be transferred within bacterial communities (Davies & Davies, 2010). Generally, the emergence of antibiotic resistance in the environment is a complex process because non-

antibiotic chemical substances may be available in the natural environment that we do not have knowledge about how they cause antibiotic resistance (Kümmerer, 2004).

1.6 The role of aquatic environments in the dissemination of antibiotics and antibiotic resistance in the environment

Aquatic environment play an important role in the dissemination of antibiotic resistance (Baquero *et al.*, 2008; Berendonk *et al.*, 2015). This is due to the availability of resources such as nutrients and other growth factors that encourage the proliferation of antibiotic resistant bacteria. Aquatic environments may serve as both reservoir harbouring antibiotic resistant bacteria and a channel for the dissemination of antibiotic resistance in the environments (Suzuki *et al.*, 2017). Some of these environments include surface water, streams, rivers, wastewater treatment plants, runoff from agriculture land and other industrial wastewater effluents carrying chemical residues capable of inducing antibiotic resistance or stimulate the transfer of resistance gene from one bacteria to another. In order to understand how antibiotic resistance spreads across aquatic environments, the characterisation of antibiotic resistance profiles of different aquatic environments and the source tracking of antibiotic resistant bacteria or antibiotics is essential.

1.6.1 *Surface water*

Several concentrations of antibiotics ranging from a microgram to milligrams have been detected in surface water (Kümmerer, 2004). A recent study reported measurable concentrations of ampicillin (22.11-30.02 µg/L), cefepime (40. 84 -56.54 µg/L) and ceftazidime (4.66- 49.5 µg/L) (Soran *et al.* (2017)). Similarly, surface waters, especially those influenced by anthropogenic activities are also reservoirs for antibiotic resistant bacteria and genes (Kümmerer, 2004). There are several causes for high loads of antibiotic resistant bacteria and genes in the surface water, which may include runoff of antibiotic resistant bacteria and antibiotic residues from agriculture land. Another source may include the accidental discharge or deliberate release of antibiotics from antibiotic manufacturing environments close to the coast (Hatosy & Martiny, 2015). For instance, a study has shown the detection of tetracycline resistance genes in water run-off of a watershed of an ocean. (Barkovskii *et al.*, 2015). Also, the prevalence of *AmpC*, a gene in bacteria that induces resistance to third generation beta-lactam antibiotics have been detected on the water surface. In a related study, antibiotic resistance genes were detected in water samples. Samples were collected and investigated for the prevalence of antibiotic resistance genes using molecular approach. The results showed the

presence of *AmpC*, a gene coding for beta-lactam in the biofilm isolated from surface water (Schwartz *et al.*, 2003). Ghaderpour *et al.* (2015), in their study, also showed the abundance of multiple resistant *E. coli* present in estuaries located in a mangrove forest. Of all the *E. coli* isolates, 34% were resistant to three or more antibiotic classes, which included aminoglycosides and beta-lactams.

1.6.2 *Municipal wastewater and waste treatment plants*

Many environmental studies support the conclusion that municipal wastewater treatment plants are drivers of antibiotic resistance in the environment (Kümmerer, 2004; Williams *et al.*, 2016). For instance, Kwak *et al.* (2015) showed the prevalence of antibiotic resistant *E. coli* in wastewater. The result of their study revealed that 55% of the *E. coli* isolated from the hospital wastewater treatment plant and 34% of municipal wastewater isolates were resistant to more than three antibiotics. Similarly, the frequency of resistance among mesophilic bacteria was determined and the result showed that 64% were resistant to at least nine antibiotics. Of this, 64% are gram-negative isolates which dominate effluent of the wastewater plants (Zhang *et al.*, 2015). Odjadjare and Olaniran (2015) also showed the prevalence of antibiotic resistance bacteria in wastewater treatment plant facilities and adjacent rivers that receive impart from the waste treatment plants. At both treatment plants and receiving rivers, up to 13,803 CFU/mL of *Salmonella* isolates were detected. Of the total *Salmonella* isolates, 200 isolates were resistant to streptomycin, nalidixic acid, and sulfamethoxazole, however, all isolates were sensitive to ciprofloxacin. This suggests that wastewater treatment plants are major sources for the spread of antibiotic resistant bacteria and antibiotic resistance genes in the environment.

1.6.3 *Hospital effluent*

Hospital effluent is another route through which antibiotics are released into the environment (Laffite *et al.*, 2016; Lien *et al.*, 2016). Most hospital effluents contain a mixture of different kinds of chemical wastes and biological substances, which are highly toxic and hazardous (Tsakona *et al.*, 2007). These substances come from the medical research laboratories and diagnostic laboratories which include antibiotics, disinfectant, detergent, nutrient media and iodinated compounds (Laffite *et al.*, 2016). Other constituents of hospital effluent are human wastes (urine and feces) which contain unmetabolised antibiotics (Finley *et al.*, 2013). Ampicillin was detected in the effluent of a big hospital in Germany at a concentration ranging from 20 µg/L – 80 µg/L (Kümmerer, 2004). Also, ciprofloxacin and benzalkonium were detected in hospital wastewater at a concentration which is equivalent to the minimum inhibitory concentration (MIC) for some human pathogenic organisms (Kümmerer &

Henninger, 2003). The presence of antibiotics and waste chemicals in hospital effluent is a major facilitator for the propagation and spread of antibiotic resistant bacteria in the environment (Rowe *et al.*, 2017; Schwartz *et al.*, 2003). A recent study in Spain involving environmental surveillance for prevalence of antibiotic resistance genes in the environment has found antibiotic resistance genes and antibiotic residues in a hospital effluent as well as the downstream of receiving river (S. Rodriguez-Mozaz *et al.*, 2015). In that study, different levels of resistance were found which correlated with the concentration of antibiotics. Also, five different antibiotic resistance genes were detected from the hospital effluent, and at the upstream, a higher level of resistance genes were detected compared to downstream (S. Rodriguez-Mozaz *et al.*, 2015).

Modern hospitals have their own wastewater treatment facilities that are supposed to remove antibiotics and antibiotic resistant bacteria from the wastewater. However, most often, these systems have not been successful in effectively removing antibiotic resistant bacteria. For instance, the abundance of resistant *Pseudomonas aeruginosa* from a hospital wastewater facility using susceptibility testing and 16 rRNA gene library construct revealed that hospital wastewater treatment facilities are not always effective for the removal of antibiotic resistant bacteria (Santoro *et al.*, 2015).

1.6.4 *Industrial wastewater discharge*

Industrial discharge of chemicals is another source through which antibiotics and resistant bacteria are introduced into the environment (Wang & Yang, 2016). Waste chemical and heavy metals from industries such as pharmaceuticals, petroleum and paint have greatly contributed to the increase in antimicrobial concentrations in the environment (Figure 1). Many of these industrial wastes are not degradable and often end up in a waterbody or landfill thereby constituting widespread toxic pollution in the environment (Miao *et al.*, 2012). These pollutants may leach out from the top soil to surface and groundwater or settle on soil sediment (Gaw *et al.*, 2014).

High concentrations of antibiotic molecules are detected in industrialised areas in Asia. For instance, in India, ciprofloxacin was detected in a stream at concentrations above therapeutic concentrations (Gothwal & Shashidhar, 2015). Industrial ciprofloxacin released into the environment per day can be as high as 44 kg, which is sufficient enough to treat bacterial infections for five years in a country like Sweden (Larsson, 2014b). Also, several mg/mL of oxytetracycline have been found in effluent and the receiving river of a Chinese factory located

in India, which is high enough to affect the proper functionality of aquatic micro-organisms. However, comprehensive data regarding industrial effluents are limited because poor reports of companies do not get published. Also, antibiotics found in the waste effluent or receiving rivers must correspond to the one discharged during production processes. The detection of which requires analytical techniques (Larsson, 2014b).

Moreover, the discharge of antibiotics and other chemicals from industrial areas, especially pharmaceutical factories, could change bacterial phenotypes, leading to antibiotic resistance in the environment (Tahrani *et al.*, 2015). A recent example is a study on antibiotic resistant bacteria in Tunisia, where water samples from a waste treatment plant contained antibiotic resistant bacteria which include species of *Pseudomonas, Morganella and Acinetobacter*. All isolates were resistant to both amoxicillin and colistin. (Tahrani *et al.*, 2015).

1.6.5 *Agriculture waste discharge*

Waste discharge from livestock farms is also a hotspot for the spread of antibiotic resistant bacteria and antibiotics to the environment. Evidence of this is from the detection of multiple-resistant bacteria in agricultural land where a slurry of animal wastes is used for fertilisation of crops (McCarthy *et al.*, 2013). Sub-therapeutic concentrations of antibiotics present in the livestock management waste system facility could speed up the rate at which bacterial strains selected for antibiotic resistance (Kümmerer, 2004). In a study by Hsu *et al.* (2014), water samples were collected from Puzik River and wastewater from a farm in Taiwan. Seven antibiotic resistance genes that code for antibiotic resistance were detected. These included *blaTEM* gene, which is responsible for beta-lactams resistance, *tet(B)* for tetracycline, *str(A)* for streptomycin resistance, *cmlA* for chloramphenicol resistance, *sul1* gene for sulfonamide resistance and *mecA* gene which is responsible for methicillin resistance. Among the seven genes is *sul1*, which was detected in 96% of sample. The next most prominent gene was *tet(A)* which was detected in 92% of the samples (Hsu *et al.*, 2014).

1.7 Transmission of antibiotic resistance in the environment

Non-clinical environments have been identified as a key factor influencing the dissemination of antibiotic resistance (Berglund, 2015). Bacteria acquire antibiotic resistance genes from environmental pools mostly through a HGT. HGT is a common event in an aquatic ecosystem which promotes the spread of antibiotic resistance genes from bacteria harbouring such gene to non-pathogenic and pathogenic bacteria, even to clinical relevant bacterial strains (Berglund, 2015). There are three mechanisms by which HGT occurs, which include conjugation, transduction and transformation. Of these three mechanisms, conjugation is traditionally thought to be the major mechanism that influence and facilitate the transfer of antibiotic resistance genes within a bacterial population. Conjugation involves the direct transfer of genetic material (DNA) among a broad range of bacteria species. Plasmid and transposon are the most important conjugative elements conferring antibiotic resistance, which are transferred via conjugation among different bacteria in soil, sediment, wastewater plants and rivers. Once a bacterium incorporate resistance genes in its plasmid, resistance genes can then be disseminated among other bacterial population of the same species or different species, even to bacteria of different genera in the environment. For instance, the bla $_{\text{CTX-M}}$ ESBL gene has been disseminated by different host range plasmids within the enterococci family, as well as other pathogenic bacteria (Canton *et al.*, 2012).

Recently, both transformation and transduction have also been identified as important factors mediating the spread of antibiotic resistance genes among bacteria in the environment. Transformation becomes important especially in water sediment and soil where bacterial DNA can be released to the extracellular environment after cell lysis and take up by neighbouring bacteria. Also, transduction may be important in the transfer of antibiotic resistance genes between bacteria via bacteriophages in the environment. Some phages have been identified to be a broad-range host, which are capable of infesting different bacterial species, from different environments e. g., from environmental bacteria to the human microbiome (Van Hoek *et al.*, 2011). A recent study using viral metagenomes analysis has detected beta-lactamase genes in urban sewage and activated sludge (Rolain *et al.*, 2012). Moreover, *mecA*, a gene that is responsible for methicillin-resistant *Staphylococcus aureus* has been detected in bacteriophages present in wastewater treatment plant and adjacent river that receives input from the treatment plant (Colomer-Lluch *et al.*, 2011). Overall, it has become very clear that environments, especially aquatic environments are facilitators for the propagation and dissemination of antibiotic resistance genes. Measuring the number and diversity of antibiotic

resistant *E. coli* in rivers become very important to mitigate the spread and evolution of antibiotic resistance in the environment.

1.8 Objective of this study.

To fully understand the emergence, prevalence, and distribution of antibiotic resistance in *E. coli* in the aquatic environment more studies are required. The results of these studies are crucial for source-tracking antibiotic resistant *E. coli* in rivers and the public health risk it may present. *E. coli* is a gram-negative bacterium and a normal floral of the gut of all warm-blooded animals and human beings. This species is often used in environmental studies because it is easy to grow and its presence in the environment is an indication of the presence of other pathogenic bacteria. Also, the prevalence of antibiotic resistant *E. coli* in any environment is a major public health concern because *E. coli* is capable of harbouring and transferring antibiotic resistance genes among other bacteria. Before the rapid emergence of antibiotic resistance can be mitigated, it will be necessary to understand the profile and prevalence of antibiotic resistance in the environment. The study here presented will contributes to the understanding of how urbanisation and agriculture influence antibiotic resistant bacteria in rivers, specifically *E. coli* which is often used as an indicator of fecal contamination in the environment.

Prevalence and distribution of antibiotic resistant *E. coli* was investigated within three different seasons (autumn, winter and spring) in two rivers impacted by urbanisation or agricultural practice. The relative proportion of *E. coli* among mesophiles and the contribution of antibiotic resistant *E. coli* to the total *E. coli* population in the two rivers was determined. The difference in antibiotic resistance patterns between agricultural and urban rivers was compared to determine the influence land use has on the prevalence of antibiotic resistance. This work is patterned on a recent report by McArthur et al. (2015).

Hypotheses:

1. Waterways are reservoirs for antibiotic resistant *E. coli* that differ in population size and composition.
2. Antibiotic resistance patterns of *E. coli* change depending on the type of land management along the waterway.

My overall objective in this study was achieved through the following supporting objectives:

1. Isolation of *E. coli* and antibiotic resistant *E. coli* and determination of the prevalence in the selected waterways using phenotypic culture-based screening method.

2. Determination of the frequency of cross resistance to eight antibiotics using *E. coli* isolates.
3. Determination of the minimum inhibitory concentration of ciprofloxacin for *E. coli* isolates.
4. Quantification and comparison of the antibiotic resistance patterns between the two rivers.

1.9 Thesis Organisation

Chapter 2 reports the abundance of mesophiles, *E. coli* and antibiotic resistant *E. coli* isolated from Avon River and Silver Stream. The objective of this chapter is to determine the proportion of *E. coli* in the mesophilic bacterial populations in the two rivers and to evaluate the different proportion of antibiotic resistant *E. coli* in the total *E. coli* population in the two rivers in a season dependent manner. In chapter 3 multi-resistance profile of *E. coli* from both rivers was investigated by subjecting *E. coli* isolates to eight antibiotics. Based on the high frequency of resistance observed in ciprofloxacin isolates, subsets of the ciprofloxacin isolates were tested against increasing concentration of ciprofloxacin to determine the minimum inhibitory concentration.

Chapter 2

Title of the chapter "Prevalence of antibiotic resistant E. coli in two rivers in Canterbury, New Zealand."

2.1 Introduction

The rapid rise of antibiotic resistant bacteria in aquatic environments is a threat to public health (Levy, 2002). The understanding of the origin and source antibiotic resistant bacteria in the aquatic environment will provide a vital information for managing the potential danger this may present. Globally, large efforts were made to mitigate the continuous dissemination of antibiotic resistance in the environment. The World Health Organisation (WHO) and the New Zealand Ministry of Health recently introduced action plans including optimizing the use of antimicrobial drugs in human and animal health, creating awareness on prevalence of antimicrobial resistance, and creating sustainable investment for tackling antimicrobial resistance globally (Ministry of Health and Ministry for Primary Industries, 2017).

While efforts have been made towards combating antibiotic resistance prevalence and management of the use of antibiotics, there is a need for a proper understanding of the environmental origin of antibiotic resistance in order to develop management practices to mitigate the spread of antibiotic resistances in the environment (McArthur *et al.*, 2016). Freshwater environments are reservior for different kinds of pollutants from industries, agriculture and domestic activity. Water helps in the proliferation of bacterial growth and ultimately spreading antibiotic resistance throughout aquatic environments (Chen *et al.*, 2017).

Pollution of freshwater habitat is a global concern due to widespread of pollutant, their persistence, bioaccumulation and impacts on human health and microorganisms (Xu *et al.*, 2013; Zinicovscaia, 2016). Recently, pollution of freshwater has been recognized as the major problem affecting aquatic microorganisms in New Zealand (Ministry for the Environment, 2017). The increasing industrialisation and intensification in agricultural practices, especially dairy farming in New Zealand, is a major factor contributing to the pollution of fresh water due to spillage of chemical and animal waste (Schousboe *et al.*, 2015). In New Zealand, pastoral farming is currently occupying roughly 40% of the total land area and undoubtedly mobilizes most of the main pollutants into river-bodies, including nutrients, sediment, and microbes (e.g., *Escherichia coli*) (Baskaran *et al.*, 2009). Nutrient availability in water results into eutrophication and promotes microbial growth which degrades water quality and its recreational value (Ballantine & Davies-Colley, 2013).

Many regulations on freshwater quality standards are based on the total abundance of *E. coli* present in a sample (National Research Council, 2004). Depending of the country, 100 to 500 *E. coli* CFU per 100 mL may be considered as acceptable concentration for freshwater quality. Beyond certain level, water quality of different countries is considered poor. According to New Zealand Ministry of Health, the acceptable *E. coli* level should be less than 1 colony forming unit (CFU) in 100 mL of drinking water and not more than 540 CFU per 100 mL for freshwater (Ministry of Health, 2017). This standard varies from different countries. For instance, in country like Canada, *E. coli* count above 200 CFU in 100 mL of recreation water is considered unsuitable for swimming by recreational water quality standard (Lyautey *et al.*, 2010). Despite all the regulations, water quality remained unsuitable for recreational purposes, because of the increasing pollution and rise in *E. coli* concentration in freshwater. A recent report on the freshwater quality in New Zealand stated that freshwater bodies are becoming more polluted with an increasing *E. coli* concentration. *E. coli* concentrations have risen 22x and are 9.5x higher in waterways that drain from pastorial farm, compared to the *E. coli* concentration in 2013 (Ministry for the Environment, 2017). A study on freshwater quality in New Zealand revealed that the highest concentrations of *E. coli* were found in rivers that drain anthropogenic sources. *E. coli* concentrations were highest in rivers that drain through urban land (440 *E. coli*/ 100 mL), followed by rivers that drain pastoral or agriculture land (190 *E. coli*/ 100 mL) and the lowest were detected in native areas (20 *E. coli*/ 100 mL) (Larned *et al.*, 2016). Seasonal variation and environmental conditions may impact on the abundance of the microbial population in the freshwater environment (Bucci *et al.*, 2014). For instance, heavy rainfall can cause a waste treatment facility to overflow, thereby introducing fecal coliform into adjacent rivers (Shehane *et al.*, 2005). Urban septic tanks can also overflow or drain into surrounding rivers (Kümmerer, 2004). During the rainy season, animals in pastoral farming are left to graze and even drink water from the adjacent river. This allows animal waste to spread over land and wash into lakes, streams or rivers (Ballantine & Davies-Colley, 2013). However, quantitative measurement of *E. coli* concentration in rivers may be difficult to obtain because seasonal variability in environmental factors affect the information from routine sampling analysis. Therefore, such data may be insufficient to give a comprehensive evaluation of water quality. For instance, following a storm event, *E. coli* concentration in a river may be high downstream due to transport of fecal materials compared to when there is no storm event in the river (McKergow & Davies- Colley, 2010). To obtain a comprehensive assessment of water quality and to determine *E. coli* concentration in rivers, many environmental parameters such as

temperature, relative humidity, water pH, rainfall and storm events and their downstream impact need to be considered (Gotkowska-Plachta *et al.*, 2016).

2.1.1 *Measuring resistance in the environment*

Antibiotic resistance is now studied not only in the clinic but also in an environmental context. Antibiotic resistance is ancient and expected phenomenon in the environment due to the various interaction of organisms in the environment. Most antibiotic molecules are produced naturally by microorganisms in the environment, as a result, bacteria also evolved different mechanisms of resistance to maintain, proliferate and multiple. However, the quantification of resistance within bacterial populations is complex in the environment in both laboratory methodology and sampling procedure compared to the clinical settings (Davison *et al.*, 2000). In the clinical setting, antibiotic resistance is often defined as the outcome of a susceptible bacteria after exposure to antibiotic, evolve in a mechanism to survive and reproduce in spite the presence of antibiotics. Clinical breakpoint is used in medical field to define bacteria as susceptible, intermediate or resistant to antibiotic (Walsh F., 2013) . Different parameters are used in calculating resistance in clinical field, some of which include comparison with the minimum inhibitory concentration (MIC) of wild type strain, result from other clinical trials, pharmacodynamics and pharmacokinetics determination. In contrast, there is no such argument in the environmental context (Clinical and Laboratory Standard Institute, 2015; Cuenca-Estrella *et al.*, 2011). Environment harbours diverse resistant determinants such as resistance genes and mobile elements like plasmids and transposon. In most environmental studies, resistance must be quantitatively and qualitatively measured with respect to a reference bacterial population. Also, the detection method must be specific, sensitive, and reproducible. Finally, the sampling sites and method must be specific (i.e., detail how samples were collected, the sampling time, and season). The common method for measuring antibiotic susceptibility of bacteria to antibiotics concentrations could either be performed in agar culture, broth, or antibiotic disc. The least antibiotic concentration that inhibits the growth of bacteria is termed the minimum inhibitory concentration (MIC). Measuring MIC is crucial to determining bacteria resistance to a specific antibiotic.

In the study presented here, the prevalence of *E. coli* and antibiotic resistant *E. coli* at three sampling seasons in urban and rural rivers was determined. Research on the seasonal prevalence of antibiotic resistant *E. coli* in freshwater environment will be important in determining the overall antibiotic resistance genes and antibiotic resistant pathogenic and non-pathogenic bacteria. *E. coli* is an indicator organism for the presence of pathogenic bacteria. It

is a rod-like, gram-negative member of the family *Enterobacteriaceae*, and a more specific indicator of fecal pollution of the environment than other member of this family. *E. coli* is more preferably used not only in assessing drinking water quality but also in measuring water quality standard of freshwater (Ministry for the Environment, 2017), because it is universally present in human and animal feaces, easy to grow under laboratory condition and inexpensive to test (Havelaar *et al.*, 2001) Moreover, study have shown that some fecal coliform in the environment may have no fecal origin, this make *E. coli* an organism of choice in determining fecal contamination of freshwater (Odonkor & Ampofo, 2013).

2.1.2 *Research objective*

My overall objective in this study was to examine the prevalence of antibiotic resistant *E. coli* in two rivers influenced by agriculture land practices and urbanization. This is the first study to provide information on the seasonal fluctuation and characterization of antibiotic resistant *E. coli* in Avon River (Otokāro) and Silver Stream (Whirinaki) on the South Island of New Zealand in Canterbury. Some studies have shown that the *E. coli* concentration is high in the Avon River (Moriarty *et al.*, 2013; Schousboe, 2015). However, to the best of my knowledge, this is the first study in New Zealand to compare antibiotic resistant *E. coli* and total mesophilic bacteria population in urban and rural rivers during three seasons out of a year. The information from this study will contribute to our knowledge of how land use influences the prevalence of antibiotic resistant bacteria in the freshwater environment.

My hypothesis is that waterways associated with rural and urbanization are potential reservoirs for antibiotic resistant bacteria. To test this hypothesis, the following experiments were conducted:

1. The viable mesophilic and *E. coli* counts were assessed during three different seasons by conventional cultivation method.
2. Specific agar media containing antibiotics (ampicillin, rifampicin, chloramphenicol or ciprofloxacin) were used to select and enumerate resistant *E. coli*.
3. The total number of bacteria, *E. coli*, number of resistances of every isolate were then compared between the two rivers.

2.2 Materials and Methods

2.2.1 *Study sites*

Two streams of different input categories (urban or agricultural) were sampled: Avon river and Silver Stream, which are 20 km apart (Figure 2.1). The Avon River and its local tributaries flow through land characterized as residential, hospital, and various industries. The major pollutants in Avon River are domestic wastewater from Riccarton drain and industrial chemical waste from Addington drain, located along the river flow. Silver Stream drains agricultural land and is influenced by runoff from farming activities.

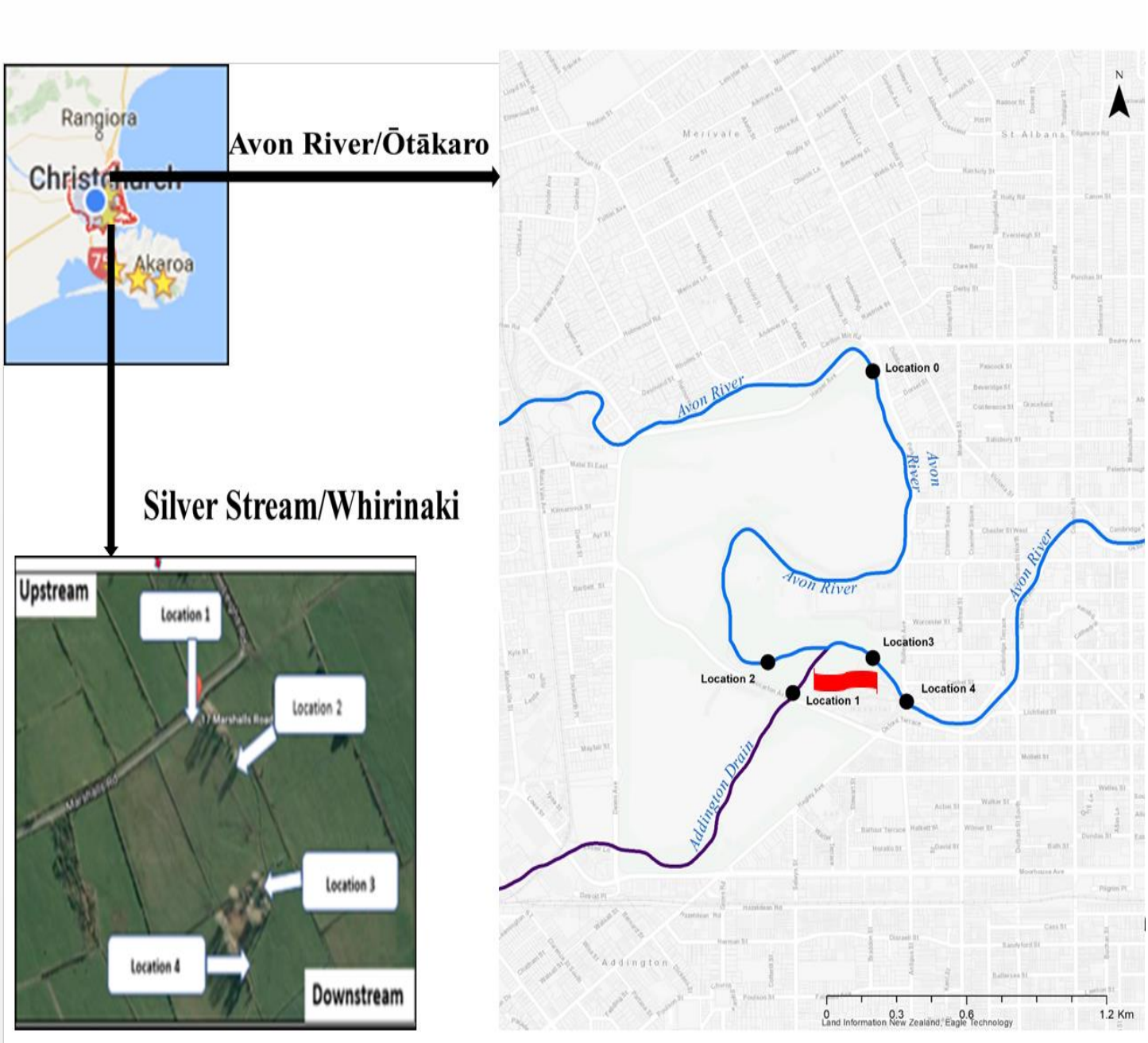


Figure 2.1: Geographical location of different sample sites (adapted from Google Maps).

2.2.2 *Sample collection*

Samples of Avon River and Silver Stream were collected on three times, between January 2017 and November 2017. For each river, water samples were collected from four locations, which are at least 100 meters apart. At each sample location, four replicate samples were collected from the water layer and the sediment. After the two sampling seasons, another location (Location 0) was sampled on Avon River together with the other four locations during spring (Figure 2.1). Sampling was conducted according to McArthur *et al.* (2015). Samples were kept cool and brought to the School of Biological Sciences at the University of Canterbury for analysis. Samples were processed within 9 hours of collection.

2.2.3 *Media, antibiotics and reagents preparation*

R2A was purchased from DIFCO (USA). Bacteriological agar and nutrient broth, a dehydrated culture medium, was purchased from Oxoid, UK. Tryptone Bile-X-glucuronide (TBX), a selective agar which contains chromogenic agent-X-glucuronide, was purchased from Himedia (India). X-glucuronide is a chemical substance that detects the activity of glucuronidase indicative of *E. coli*. The appearance of a distinct blue coloured colonies on TBX indicate *E. coli* growth. Nutrient agar was purchased from Oxoid, UK. All media were prepared according to the manufactural instructions.

The antibiotics used in this study include rifampicin (8 µg/mL), ampicillin (10 µg/mL), chloramphenicol (6 µg/mL) and ciprofloxacin (1 µg/mL). TBX agar plates were supplemented with the appropriate antibiotics. All antibiotics stock solutions were stored at -20 ºC. Ciprofloxacin was purchased from Pentax (USA), Ampicillin sodium salt from Applichem (Germany), chloramphenicol from Sigma-Aldrich (USA) and rifampicin from Life Technologies (USA). Ampicillin and ciprofloxacin were dissolved in distilled water and filtered through 0.2-micron filter. Rifampicin was dissolved methanol. Chloramphenicol was dissolved in 100% ethanol. Phosphate buffered saline was purchased from Amplichem (Germany), prepared in 10 x concentration with the pH adjusted to 7.4 using sodium hydroxide and stored at room temperature.

2.3 Isolation of bacteria from surface water sample

2.3.1 *Membrane filter procedure for E. coli enumeration*

Five 100 mL aliquots of each of the water sample replicates were filtered through 0.45 µm pore size of 47 mm cellulose nitrate filters, which were aseptically placed on the surface of TBX or TBX containing one of four different antibiotics (ciprofloxacin, chloramphenicol, rifampicin

or ampicillin) (Figure 2.2). Plates were incubated at 44 ºC for 18-24 hours. Antibiotic concentrations that were used for the first screening were in accordance with the previous study of environmental bacteria and *E. coli* isolated from streams (McArthur *et al.*, 2016). Prior to this experiment, *E. coli* BW25113 and ATCC8739 strains were tested against each of the antibiotic concentrations to ensure that the concentration used were enough to inhibit susceptible lab strains.

2.3.2 *Spot plating method for E. coli enumeration*

Water samples were serially diluted 10-fold in 96 wells plate to a final dilution of 10^{-7} (Figure 2.2). 4 µL of the diluents were used to inoculate TBX agar. After the droplets dried on the surface of TBX agars plates, plates were incubated at 44 °C for a period of 24 hours. Then, colonies were counted at the dilution where countable colonies were observed to determine the colony forming units per mL (CFU/mL).

2.3.3 *Total mesophilic population plate count*

To enumerate the total mesophilic population water samples were diluted as explained in 2.3.2 and then spotted onto R2A. Plates incubated at 30 ºC for 48 hours after which colonies were counted. Sterile water was used as a control in this experiment to confirm no growth.

2.3.4 *Spread plate method for mesophilic bacteria count*

100 µL aliquots of undiluted samples were spread on R2A using a sterile glass spreader. Plates were incubated at 30 ºC for 48 hours. Sterile water was used as a control in this experiment to check for no growth.

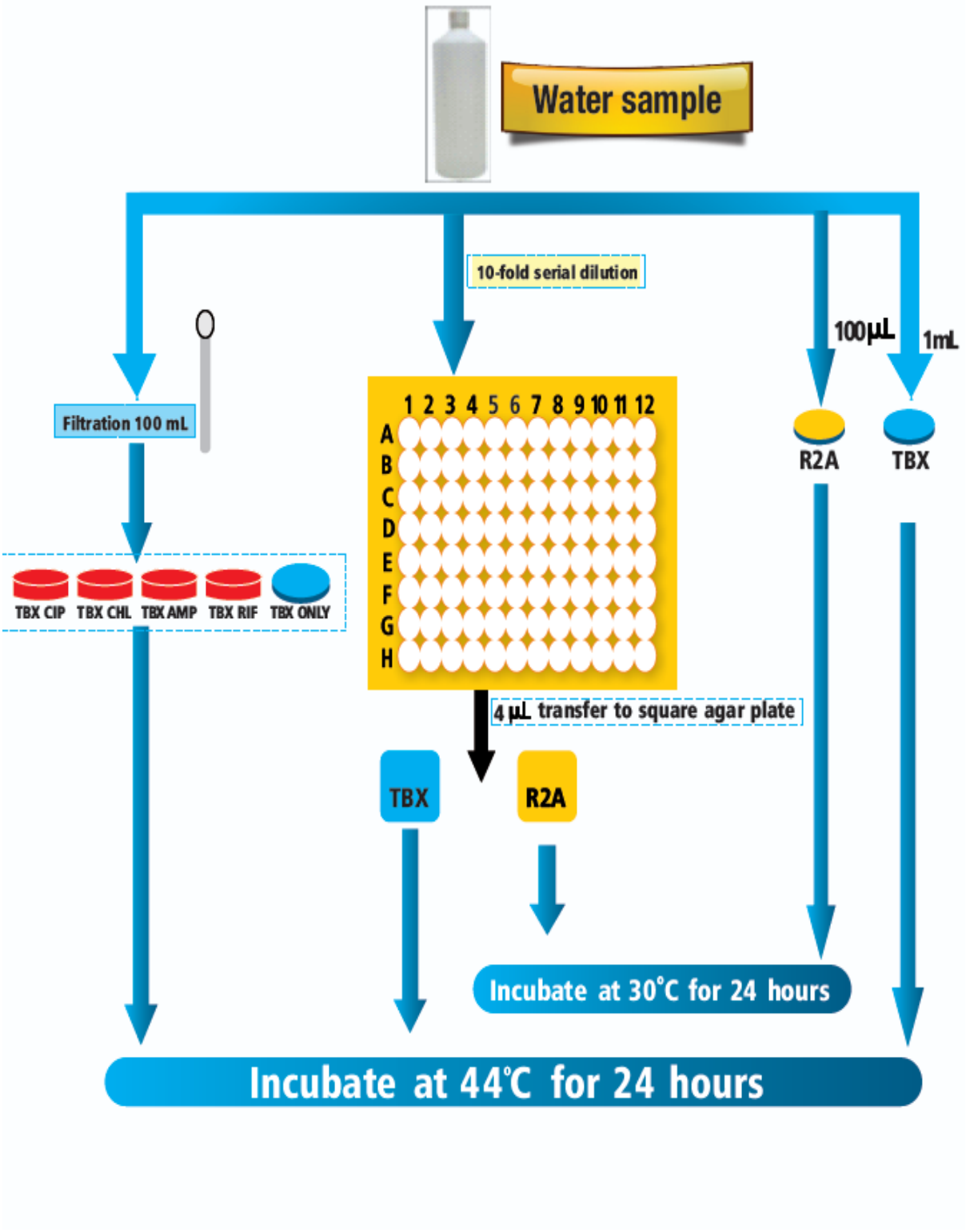


Figure 2.2: Schematic diagram showing the procedures for the isolation of mesophiles (yellow plate), *E. coli* (blue plate), and antibiotic resistant *E. coli* (red plate) from the water sample.

2.3.5 *Sediment sample processing*

100 g of sediment were weighed using a balance and 100 mL 1 x PBS were added. The mixture was vortexed vigorously to produce a slurry. 40 mL of the slurry were transferred into a sterile 50 mL centrifuge tube and then centrifuged at 120 rcf for one minute.

2.3.6 *Spread plate method for E. coli enumeration*

1 mL and 100 µL of the slurry supernatant (see 2.3.5) were plated on TBX only and TBX containing antibiotics (ampicillin, rifampicin, chloramphenicol and ciprofloxacin). The inoculum was spread around the plate using sterile glass spreader. Plates were incubated at 44 ºC for 18-24 hours to detect antibiotic resistant *E. coli* from the samples. **Error! Reference source not found.**

2.3.7 *Spot plating method for mesophilic and E. coli count*

Slurry was processed as explained in 2.3.2 and 2.3.3, respectively. For and overview of the sampling process see Figure 2.3.

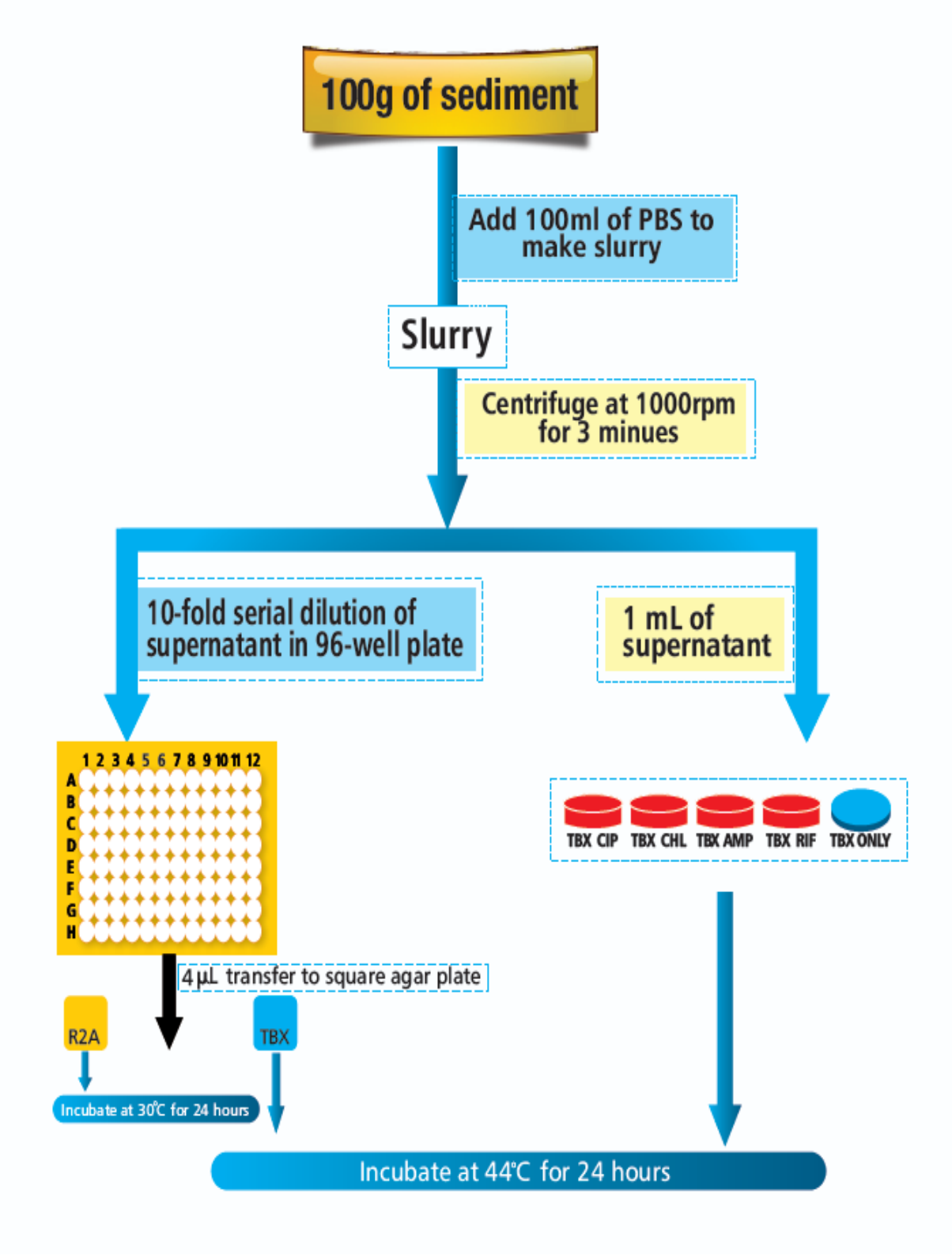


Figure 2.3: Diagram showing the procedures for the isolation of mesophile (yellow plate), *E. coli* (blue plate), and antibiotic resistant *E. coli* (red plate) from the sediment sample.

2.4 Isolation of *E. coli* strains

Distinct colonies of presumptive *E. coli* were picked from TBX agar or TBX containing antibiotics and sub-cultured onto plates containing nutrient agar. The transferred colony was diluted with a flame sterilized loop to distribute the colony evenly on the agar surface in a laminar flow cabinet and incubated at 37 °C for 18-24 hours. A distinct *E. coli* colony from the nutrient agar plate was transferred into 5-mL nutrient broth medium in a McCartney bottle using a sterile toothpick then incubated with aeration at 37 ºC until saturation. 700 µL of the saturated culture was then transferred into an Eppendorf tube with the addition of 300 µL of 60% sterile glycerol. Isolates were stored in a -80 °C freezer for further analysis.

2.5 Statistical analysis

R was used for statistical analysis of this data (R Core Team, 2016). A multifactor analysis of variance (ANOVA) was performed on the $\log_{10}$-transformed CFU count of data obtained to compare the statistically significant differences in mesophiles populations obtained from the two rivers. The statistical difference in mesophiles population was tested between the two rivers for the null hypothesis. For this analysis, a plot of residual for mesophiles was used for the normality and equality of variance. A similar statistical analysis was performed to compare the relationship between *E. coli* concentrations from both rivers

Antibiotic treatments were compared between two rivers samples and seasons. A linear model was generated and the log (CFU count + 0.001) for each replicate of antibiotic treatment was compared between the two rivers. A chi-squared test was performed on antibiotic resistance data obtained from the two waterways to compare antibiotic resistance levels of rivers and to see if the resistance levels were significantly different. A low p-value means that there was a statistical difference between antibiotics treatment between the two rivers. P-value level was determined at 0.01, this means that there was a low probability (less than 1%) of the observed result in this analysis occurring by chance. (See supplementary material for the analysis).

2.6 Results

Temperature and pH of the river water samples were taken at all locations (Table 2). The pH ranged from 5.2 to 8.8 in Avon River, with the lowest pH value observed at location 1 during spring and the highest value of 8.8 at location 2 during winter. In Silver stream, the highest pH value of 7.9 was recorded at location 1, while we recorded the lowest pH value during spring at location 4. The highest temperature was observed at both waterways during the autumn sampling.

Table 2: Measurement of environmental parameters at different locations in Avon River and Silver Stream.

Season	Parameter	Avon River				Silver Stream			
		L1	L2	L3	L4	L1	L2	L3	L4
Autumn	pH	7.40	6.90	7.50	7.69	7.20	7.70	7.30	7.40
	Temperature (°C)	14.5	12.4	16.4	14.0	14.8	15.8	16.8	16.0
Winter	pH	8.20	8.80	8.50	7.85	6.38	6.80	7.10	7.20
	Temperature (°C)	5.0	5.2	6.5	5.5	12.7	10.2	11.2	10.6
Spring	pH	5.20	5.90	6.50	6.80	7.90	7.00	6.60	6.12
	Temperature (°C)	9.5	9.2	8.4	8.5	6.7	8.0	9.4	6.7

2.6.1 *Enumeration of bacteria in surface water sample and sediment of Avon River and Silver Stream*

Populations of mesophilic bacteria varied considerably within seasons and between two rivers (Figure 2.4). The highest mesophilic population was recorded in autumn at Avon River (~10^7 CFU/mL, Figure 2.4 A). However, there was a seasonal variation in mesophilic population. There was ~10^4-fold increase in mesophilic population sizes in autumn compared to both winter and spring (Figure 2.4. Compare panel A with C and E). The mesophilic populations in Silver Stream were not statistically different between sampling locations at the three sampling times (Figure 2.4 B, D and F). The largest mesophilic sediment populations density was recorded in autumn sampling time at Avon River (~10^8 CFU/mL, Figure 2.5 A). The viable mesophilic populations in Avon River sediment did not differ significantly between locations in winter and spring sampling times (Figure 2.5 C and E), but did differ in autumn (Figure 2.5 A). In general, the mesophilic population was lower at all sampling times in Silver Stream compared to the Avon River.

2.6.2 *E. coli in Avon River and Silver Stream surface water and sediment*

The total *E. coli* count throughout the sampling times ranged from 10^0 to 10^2 CFU/mL (Figure 2.4). In Avon River, the highest *E. coli* count was observed at location 1 throughout the sampling times (Figure 2.4 A, C and E). The highest *E. coli* count in Silver Stream was detected in autumn (Figure 2.4 B). In sediment, *E. coli* count slightly varied at all locations and sampling times in both rivers (Figure 2.5). The lowest *E. coli* count in Avon River was detected at location 1 throughout the sampling times. *E. coli* populations in Avon River were marginaly significantly greater than Silver Stream (p-value < 0.05) (refer to supplementary material for statistics)

2.7 Distribution of antibiotic resistant *E. coli* in Avon River and Silver Stream

2.7.1 *Ampicillin resistant E. coli in Avon River and Silver Stream surface water and sediment*

Resistance to ampicillin was widespread in the two rivers. In Avon River, ampicillin resistant *E. coli* count varied across locations and seasons (Figure 2.4 A). In Silver Stream, the ampicillin resistant *E. coli* population did not vary by location in winter (Figure 2.4 D). However, there were no ampicillin resistant *E. coli* detected at some sampling locations in autumn and spring (Figure 2.4 B and F). *E. coli* resistance to ampicillin was also prevalent in water sediment at

both rivers at all the sampling times in 2017 (Figure 2.5). However, the ampicillin resistant *E. coli* populations were lower in the sediment as compared to the surface water of both sites. In general, ampicillin resistant *E. coli* populations were higher in Avon River than Silver Stream (p-value < 0.01) (refer to supplementary material for statistics).

2.7.2 *Rifampicin resistant E. coli in Avon River and Silver Stream surface water and sediment*

Recorded rifampicin resistant *E. coli* count varied considerably within seasons and between rivers (Figure 2.4). The highest rifampicin resistant *E. coli* populations in both rivers were recorded in autumn. Rifampicin resistant *E. coli* was less prevalent in the sediment at all season and rivers. No rifampicin resistant *E. coli* was detected at spring in both rivers sediments (Figure 2.5 E and F). The rifampicin resistant *E. coli* count recovered from both rivers was not statistically different (statistics shown in supplementary material).

2.7.3 *Chloramphenicol resistant E. coli in Avon River and Silver Stream surface water and sediment*

E. coli resistance to chloramphenicol was relatively low with an average count of 10^0 CFU/ 100 mL of sampled water. In Avon River, chloramphenicol resistant *E. coli* was detected at every location during the spring sampling time. Chloramphenicol resistant *E. coli* was not detected in Silver Stream during winter and spring sampling times (Figure 2.4 D and F). Overall chloramphenicol resistant *E. coli* detected in Avon River was higher compared to Silver Stream (p-value < 0.01). *E. coli* resistance to chloramphenicol was only detected once in Avon River sediment during the winter sampling time. In contrast, no chloramphenicol resistant *E. coli* was detected at any of the sampling times in Silver Stream.

2.7.4 *Ciprofloxacin resistant E. coli in Avon River and Silver Stream surface water and sediment*

In Avon River, ciprofloxacin resistant *E. coli* were more prevalent in all sampling times (Figure. 2.4). However, in Silver Stream ciprofloxacin resistant *E. coli* was only detected during winter and spring. The recovered ciprofloxacin resistant *E. coli* was significantly higher in Avon River than Silver Stream (p-value < 0.01, Figure. 2.4) (refer to supplementary material for statistics). No ciprofloxacin resistant *E. coli* was detected in Silver Stream sediment throughout the sampling times in 2017. However, resistance to ciprofloxacin was found in Avon River sediment (Figure 2.5).

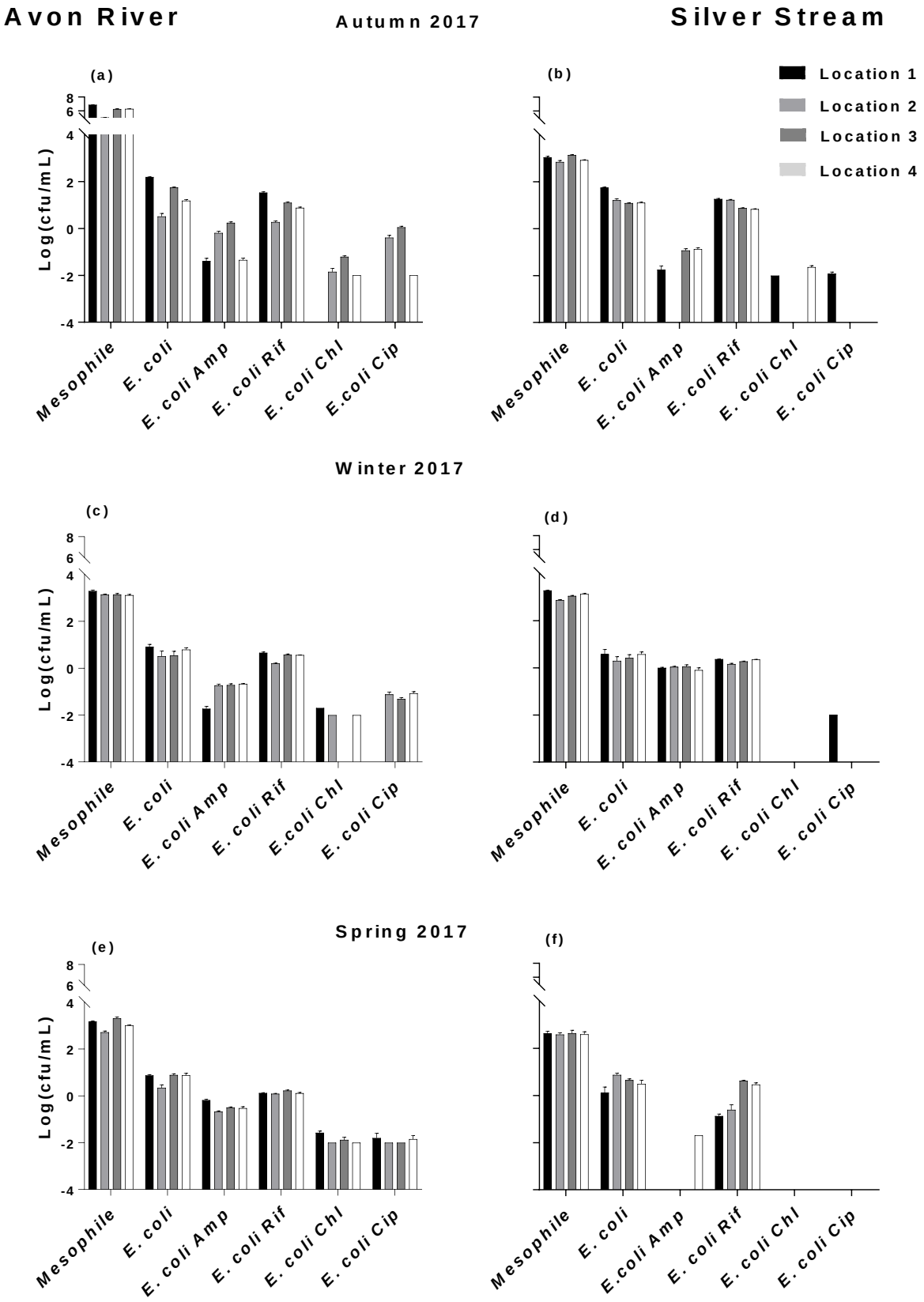


Figure 2.4: Mesophiles, *E.coli* and antibiotic resistant *E. coli* populations in surface water of Avon River (A, C, E) and Silver Stream (B, D, F). Error bar represent standard error of mean ± SEM for n = 4.

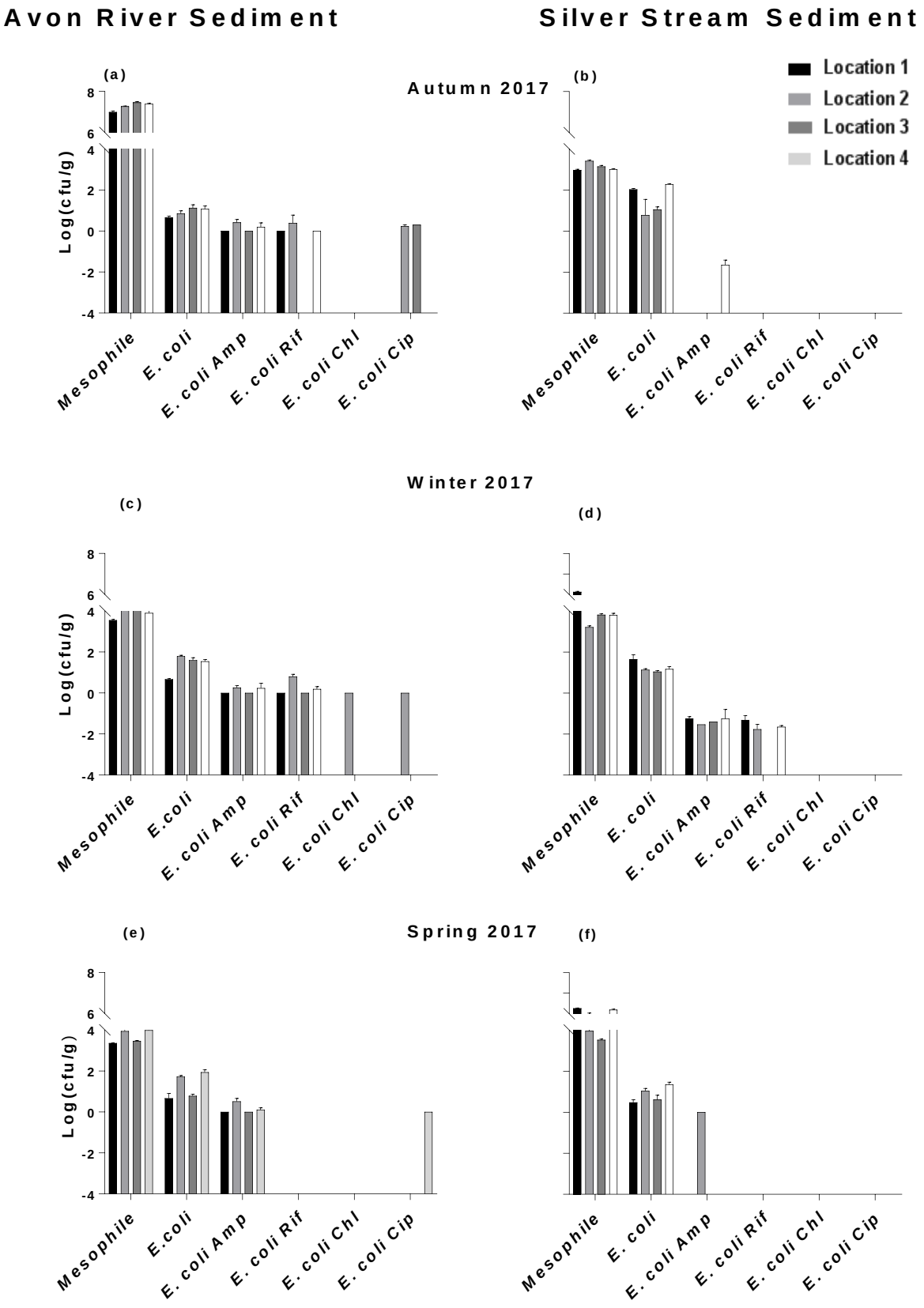


Figure 2.5: Mesophiles, *E.coli* and antibiotic resistant *E. coli* populations found in sediment of Avon River (A, C, E) and Silver Stream (B, D, F) at different sampling times. Error bar represent ± SEM for n = 4.

From the above, ciprofloxacin resistant *E. coli* were detected in Avon River at all locations within Hagley park during the three sampling seasons, but not at location 1 (Figure 2.4 A and C), the Addington drain, which flows from industrial areas and enter Avon River near Christchurch Hospital. Due to this observation, I therefore sought to determine whether the source of ciprofloxacin resistant *E. coli* in Avon River was upstream of location 0 or due to factors impacting on Avon River within Hagley Park. This hypothesis was prompted when our method could not detect ciprofloxacin resistance at the location 1 after the second sampling time. To answer this question, another location upstream location 2 at Harper Avenue (i.e., location 0) was sampled three times during spring and late spring together with the other locations within Hagley Park. The result revealed that the prevalence of ciprofloxacin resistant *E. coli* at every location within Hagley Park but not at location 0, the Harper Avenue.

2.8 Comparison of mesophiles, *E. coli* and antibiotic resistant *E. coli* population of the four locations of Avon River

2.8.1 *Comparison of viable mesophiles and E. coli populations at the four locations of Avon River*

Mesophile count range from 10^2 to 10^3 CFU/mL within locations in Avon River and the population density was not significantly different all through the sampling location at each season (Figure. 2.6). *E. coli* was detected at all the four locations in Avon River. The viable *E. coli* populations recovered from the surface water range from 10^0 to 10^1 CFU/mL at all locations throughout the sampling times (Figure 2.6). *E. coli* concentration did not differ significantly throughout sampled locations.

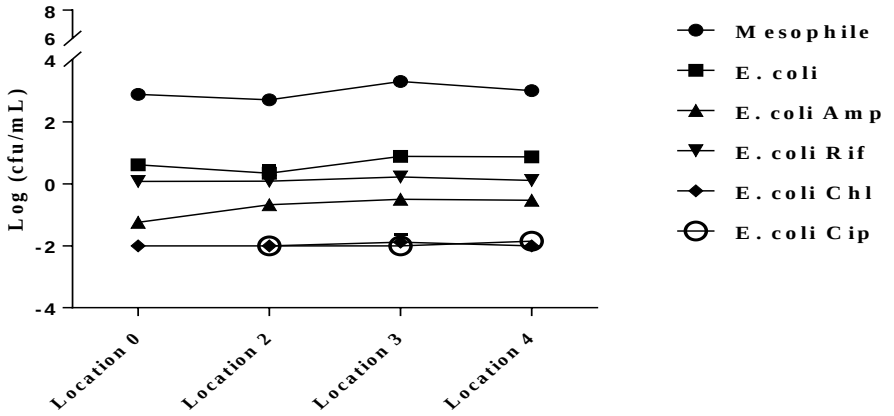


Figure 2.6 Mesophiles, *E. coli* and antibiotic resistant *E. coli* populations found in surface water at the four locations of Avon River . Error bar represent ± SEM for n = 4

Overall, the viable antibiotic resistant *E. coli* count was detected at every sampling time. Ampicillin resistant *E. coli* detected at the four sampled locations over three sampling times was on average of 10^0 CFU/100 mL (data not shown). Rifampicin resistant *E. coli* populations ranged from 10^1 to 10^2 CFU/100 mL throughout the sampling seasons at every location. The distribution of chloramphenicol resistant *E. coli* also varied across location. Chloramphenicol resistant *E. coli* was detected at every location during first and second sampling times (Figure 2.6) However, no chloramphenicol resistant *E. coli* was detected during summer sampling. Ciprofloxacin resistant *E. coli* was detected within Hagley Park but not at Harper Avenue, location 0 (Figure 2.7). Detected ciprofloxacin resistant *E. coli* concentrations within Hagley Park ranged from 2 to 3 CFU/100mL of water sample.

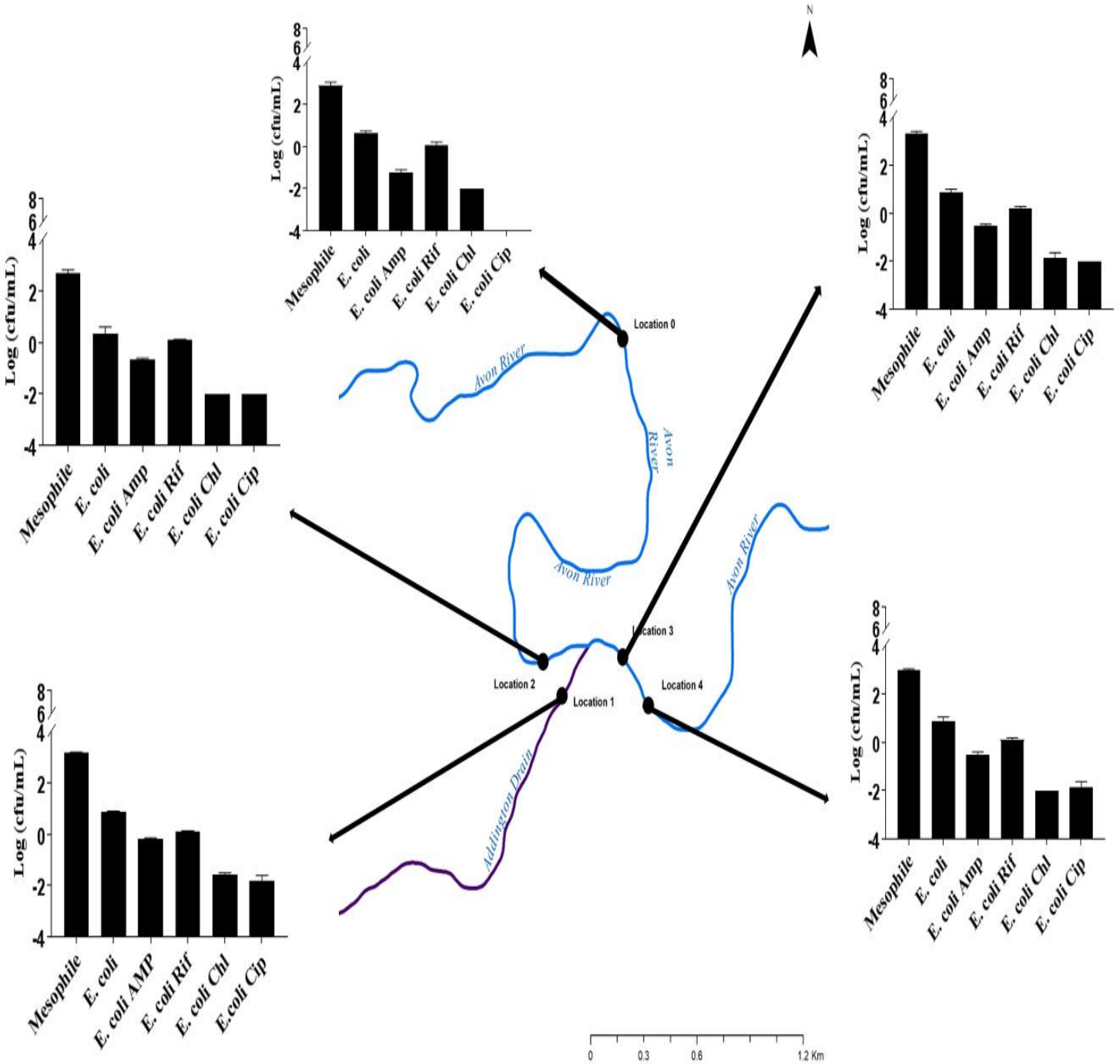


Figure 2.7 Comparison of location 0, Harper Avenue with other locations on Avon River

2.8.2 *Comparison of mesophiles, E. coli and antibiotic resistant E. coli population of the four locations of Avon River sediment*

Cultural mesophilic counts vary from one location to another at each of the sampling time. We recorded highest mesophile count at location 0 during the first and last sampling times with CFU counts of ~10^4 CFU/mL during both sampling times (data shown not shown). *E. coli* was detected at all locations and seasons. *E. coli* abundance recovered at every location was about the same at late spring sampling time. However, there was variation in culturable *E. coli* count during the spring sampling time (Figure 2.8). Antibiotic resistant *E. coli* was not prevalent in the water sediment at all locations in spring (Figure 2.8) and summer sampling seasons, except during late spring where ampicillin resistant *E. coli* was detected at location 1 and ciprofloxacin resistant *E. coli* at location 4 (data not shown)

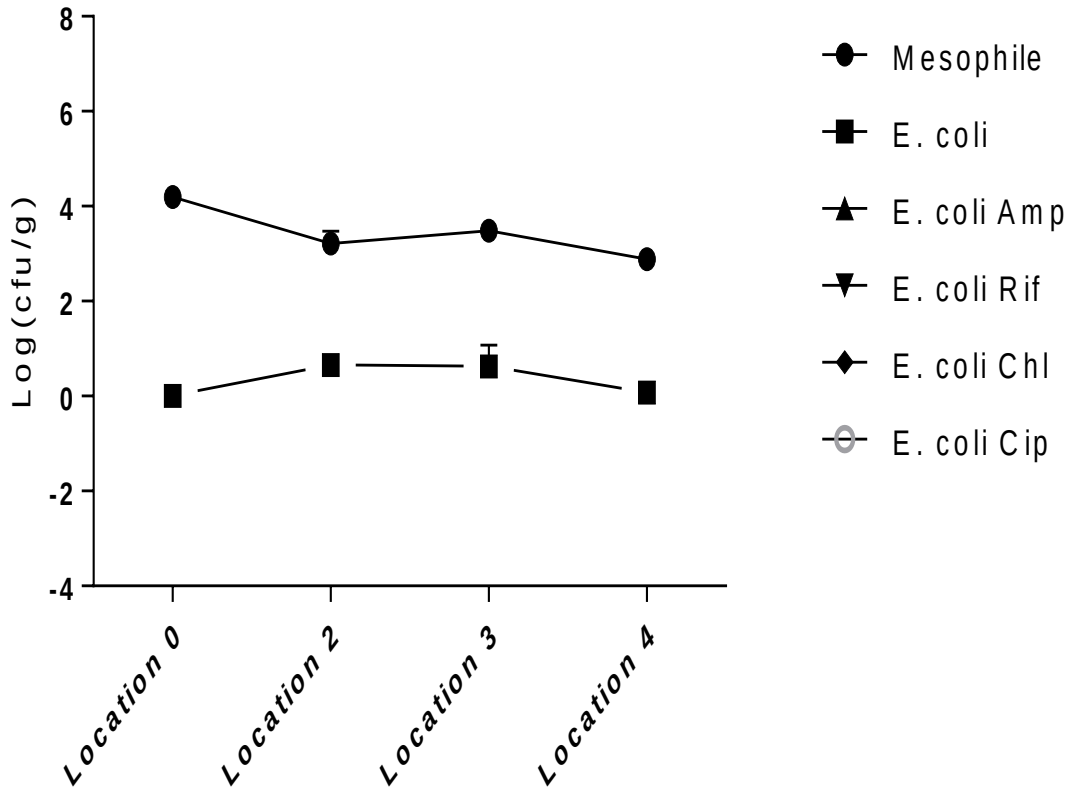


Figure 2.8: Mesophile, *E. coli* and antibiotic resistant *E. coli* populations found in sediment of the four locations of Avon River. Error bar represent ± SEM for n= 4

2.9 Discussion

The water quality assessed by pH and temperature was within the standards for measuring good water quality (World Health Organization, 2011). No other measures for water quality were assessed during this study, however we consider the overall impact of urban versus agricultural usage as different treatment. In autumn, there was a slight rise in temperature (Table 2) and this was associated with a higher culturable mesophilic bacteria population in Avon River (Figure 2.4 A). However, the variability in temperature during sampling times could not be confirmed as an important factor influencing the mesophilic populations observed in rivers during this study. Because temperature had no significant impact on mesophilic population during winter and spring sampling seasons (Figure 2.4)

It has been established that polluted aquatic environments harbor antibiotic resistant bacteria than unpolluted environment (McArthur *et al.*, 2016). However, the prevalence and pattern of antibiotic resistance across different rivers that are influenced by anthropogenic activities have not been fully explored. The findings from this study confirm that rivers associated with agriculture and urbanization are habitats for antibiotic resistant *E. coli.* This result is comparable with previous studies on the prevalence of antibiotic resistant bacteria in polluted environments (Santoro *et al.*, 2015; Schwartz *et al.*, 2003; Young *et al.*, 2013).

Notably, the *E. coli* concentrations in both rivers were above the safe levels for recreational use. *E. coli* concentrations in both rivers was comparable to those in previous study on Avon River (Environment Canterbury, 2017) (Figure 2.4). Studies have indicated that anthropogenic influence is the major factor contributing to the abundance of *E. coli* in rivers that flow through urban environment (Glinska-Lewczuk *et al.*, 2016; Lenart-Boron *et al.*, 2017). Water is only considered safe for recreational purpose when *E. coli* level is below 500 CFU/ 100 mL, according to the New Zealand ministry of environment. There are many factors that account for the increasing *E. coli* concentrations in rivers, including influx of fecal materials from contaminated soil close to the water bodies that are being washed into the river during rainfall (Chen *et al.*, 2017).

The maximum *E. coli* level was detected at location 1, the Addington main drain, which flows into Avon River from industrial and highly residential area. A previous study has shown that Addington drain is one of the most human-impacted river, full of *E. coli* and metal pollution in Canterbury region (Margetts & Marshall, 2015). The number of *E. coli* isolates recovered from

Avon River in this study was about the same as those of previous findings that showed that wildfowls and human pollution with fecal materials are the two major factors contributing to the elevated level of *E. coli* in Avon River (Moriarty *et al.*, 2013).

Additionally, the contamination of Avon River with fecal material has also been attributed to the Riccarton drain which flows through a highly populated area and enters Avon River near Hagley Park (Gasim *et al.*, 2002). My findings in Avon River are consistent with the Christchurch City council findings in 2015 on the abundance of *E. coli* on Addington Main Drain compared to other location in Avon River (Margetts & Marshall, 2015) (Figure 2.4 A, C and E). Silver Stream and Avon River sampled locations are situated close to agricultural activities and human influence, respectively. The rise in *E. coli* level seen in this study can be compared to study previously performed by the New Zealand Regional Council on freshwater quality (Ministry for the Environment, 2017). The presence of *E. coli* in freshwater is an indication of the presence of human and animal pathogenic bacteria (Harwood *et al.*, 2000).

This study shows that the two rivers under study here that are receiving different anthropogenic inputs may also carry different *E. coli* populations. Antibiotic resistant *E. coli* are constantly disseminated through water runoff into natural environments containing faeces of livestock that received antibiotics (Singer *et al.*, 2016). It has been established that many terrestrial habitats such as meadows, harbor antibiotic resistance which is a result of land application of manure containing resistant bacteria (Al-Bahry *et al.*, 2015). Interestingly, there is growing evidence that aquatic environments are also a reservoir of antibiotic resistant bacteria. Water is an important habitat for the proliferation and dissemination of bacteria between different environment such as surface water, wastewater, ground water and ocean. Therefore, bacteria can be mobilised from unclean environment (wastewater) to clean environment (pristine). These processes may also involve the spread of antibiotic resistant bacteria and transfer of resistance genes among bacteria in different environment (Vaz-Moreira *et al.*, 2014). Antibiotics used in this study include those commonly applied in human and veterinary medicine. Some have low application in human treatment (such as rifampicin) but used in environmental studies of antibiotic resistant *E. coli* (Berendonk *et al.*, 2015; Ghaderpour *et al.*, 2015; McArthur *et al.*, 2016).

The prevalence of antibiotic resistant *E. coli* (Figure 2.4 and 2.5) may be the effect of pollution of freshwater from agricultural runoff or discharge from hospital and waste treatment plants in urban systems (Pereira *et al.*, 2013; Pruden *et al.*, 2006). However, land use pattern may have different effect on the evolution and prevalence of antibiotic resistant *E. coli*. Also, antibiotic

molecules could create selective pressure on naturally occurring bacteria present in urban waterways thereby increasing the resistant bacterial populations (Davies *et al.*, 2006). In this study, Avon River water samples carried higher resistant *E. coli* populations to all tested antibiotics compared to Silver Stream counterpart.

Ampicillin resistant *E. coli* recovered from the two rivers in this study was higher than those of ciprofloxacin and chloramphenicol. The prevalence of ampicillin resistant *E. coli* in the study was not surprising because beta-lactam antibiotic resistance have been detected in similar aquatic environments (Blair *et al.*, 2013). One of the mechanisms used by *E. coli* is the production of beta-lactamase, an enzyme that deactivates ampicillin molecules through enzymatic breakdown of the beta-lactam ring (Zeng & Lin, 2013). Environmental microorganisms are the source of beta-lactam class of antibiotics to which ampicillin belong (Thakuria & Lahon, 2013). Also, ampicillin is widely used in veterinary practice and animal husbandry and this can potentially create a selective pressure in bacterial community thereby leading to increase in antibiotic resistance population (Economou & Gousia, 2015). Ampicillin-resistant *E. coli* levels were significantly higher in Avon River than Silver Stream at all sampling times (P-value < 0.001). While there are number of reasons for the higher level of ampicillin resistance in Avon River, the major factor might be because Avon River is more disturbed with anthropogenic activities compared to Silver Stream. Avon River flows through highly residential area and it is possible that domestic wastes including sewage materials containing ampicillin resistant *E. coli* are constantly introduced to the river. Previous studies on source tracking have reported that sewage contamination river may cause of high levels of ampicillin resistant *E. coli* in the environment (McLellan *et al.*, 2007; Parveen *et al.*, 1997).

High rifampicin resistant *E. coli* populations were observed throughout this study (Figure 2.4). This agrees with the previous study on *E. coli* isolated from surface water and sediment of Santa Ana River in California, USA (Ibekwe *et al.*, 2011). The observation from this study was also comparable with previous study which assess microbial quality and antibiotic resistance indicator bacteria Portuguse river (Bessa *et al.*, 2014). The prevalence of rifampicin resistance within the *E. coli* population was surprising. High *E. coli* populations were recovered from rifampicin supplemented plates which was almost equivalent to the number of *E. coli* isolated from plate without antibiotic. Due to this observation, it became necessary to determine and investigate the reason for high populations of rifampicin resistant *E. coli* isolates detected in this study. To answer this, selected *E. coli* isolates recovered from rifampicin containing agar

plates were grown in nutrient broth until saturation. After, 4 µL of saturated culture was transferred onto nutrient agar plate containing the same concentration of rifampicin from which isolates were initially recovered. The result showed that more than 70% of these isolates were susceptible (data not shown). One reason for this phenomenon may be that *E. coli* biomass formation on the membrane filter on rifampicin plate detoxified this antibiotic, thereby allowed susceptible population to grow in the presence of rifampicin.

Chloramphenicol is a broad-spectrum antibiotic commonly used in veterinary and human medicine as a therapeutic agent (Sørensen *et al.*, 2003). It is widely used in developing countries to treat infection of most gram-negative bacteria such as *Vibrio, Salmonella,* and *Rickettsia* (Ng, 2013). However, its application in developed countries is reducing. In this study, chloramphenicol resistance amongst *E. coli* was less common in both rivers. This is consistent to previous studies on isolation of resistant *E. coli* in waterways (Kappell *et al.*, 2015). Even though the use of chloramphenicol has significantly reduced, resistance to chloramphenicol is still being detected in aquatic environments. The reason for this may be that genes responsible for chloramphenicol resistance are distributed or transferred among the bacterial community in the absence of selective pressure (Yoo *et al.*, 2003). Additionally, chloramphenicol resistance may occur in the environment as a result of co-selection or cross-resistance of a particular resistant gene in *E. coli* (Nhung *et al.*, 2015). Poole (2005) also showed that chloramphenicol resistance could be a result of multiple efflux pump expression in bacteria.

Notably, there was significantly fewer ciprofloxacin resistant *E. coli* isolates in both rivers compared to *E. coli* population recovered from plates supplemented with other antibiotics in this study. Many factors may explain the less common ciprofloxacin resistance observed. Firstly, ciprofloxacin is a quinolone class of antibiotics and does not have any natural source. They are the first synthetic antibiotics that were introduced in the early 1970s for therapeutic use. Therefore, resistance to ciprofloxacin is generally restricted to mutation which alters target protein or activation of multiple efflux pumps. This makes the dissemination of ciprofloxacin resistance confined within a bacterial population (Hooper, 2002). However, recently, the existence of plasmid-mediated resistance have been identified (Robicsek *et al.*, 2006; Rodríguez-Martínez *et al.*, 2016). This resistance determinant includes *qnr,* a gene that encode protein responsible for protecting type IV topoisomerase and DNA gyrase from ciprofloxacin inhibition. Other plasmid-mediated resistance genes are *cr,* which is responsible for low-level

of ciprofloxacin resistance and *qepA*, responsible for extruding ciprofloxacin out of *E. coli* cell (Van Hoek *et al.*, 2011). Unlike ciprofloxacin, beta-lactam class of antibiotics are produced by microorganisms. Therefore, resistance to beta-lactam (e. g., ampicillin) has been in the environment before the use of antibiotic in the medical treatment of bacterial infections (Munita & Arias, 2016).

Secondly, ciprofloxacin is not often used in agriculture for prophylaxis, treatment or growth enhancers, and therefore the possibility of resistance development and spread among bacteria is lower compared to other antibiotics that are routinely used in agricultural practices. For instance, the emergence of vancomycin resistance was due to overuse of avoparcin, an analogue of vancomycin in chicken farming as a growth promoter (Cormican *et al.*, 1997). Another possibility for the low incidence of ciprofloxacin resistance in the environment might be due to the fact that they are expensive synthetic drugs which are largely reserved as a last resort for treatment of *E. coli* infections (Alam & Bastakoti, 2015). All these could contribute low incidence of ciprofloxacin resistance even in clinical practices and the environment at large.

Most of the ciprofloxacin resistant *E. coli* detected in Avon River were behind Christchurch Hospital, which is close to where most activities take place, including walking of domestic animals and punting (Figure 2.4 A, C and E). Avon River is susceptible to constant impact from both industrial and human pollutions which may be source of chemical selecting for ciprofloxacin resistant *E. coli*. In addition, domestic animal suffering from urinary tract infections may be another source for ciprofloxacin resistant *E. coli* in Avon River through fecal pollution. Previous study showed that dog and wildfowl are the two major sources of pollution which deteriorate the quality of Avon River through washing down of fecal materials into the waterbodies. (Moriarty *et al.*, 2013). Similarly, *E. coli* isolates may also habour resistance mechanisms such as mutation and efflux pumps systems that conferred resistance to ciprofloxacin in this study. In most countries, like Australia, ciprofloxacin is only used in medical treatment of human and to some extent in treating pets but not in livestock management (Unicomb *et al.*, 2006). This could explain the reason why there was almost no ciprofloxacin resistance in Silver Stream and significantly more ciprofloxacin resistant *E. coli* in Avon River (p-value<0.001) (Figure 2.4 and 2.5).

The observation from location 0 (e.g., Harper Avenue) (Figure 2.7) showed that antibiotic resistance *E. coli* was less common compared to other locations within Hagley Park. During the sampling times, no ciprofloxacin resistant *E. coli* was detected at location 0 (Figure 2.7). The detection of antibiotic resistance especially ciprofloxacin resistance within Hagley Park showed that some factors are responsible for this observation. One possibility may be that other tributaries such as Riccarton drain (not sampled) and Addington drain (location 1) which join Avon River before and after location 2 are carrier of ciprofloxacin resistant *E. coli* or chemicals that can select for such resistance. Another possibility may be because Avon River is more influenced by human activity and domestic animals within Hagley Park compared to other sampled location upstream Hagley Park. However, this result was not substantial enough to show whether there was a significant difference between the antibiotic resistance populations at different locations in Avon River. Because antibiotic resistant bacteria detected during spring sampling time generally lower at all locations compared to other sampling seasons. Based on the current observation of antibiotic resistance patterns at different locations in Avon River, future work will be required to investigate more on the sources of the of antibiotic resistance especially ciprofloxacin resistant *E. coli* detected in Avon River within the Hagley Park locations.

2.10 Conclusions

The result of this study showed that rivers that are influenced by different anthropogenic source reservoirs for antibiotic resistant *E. coli.* The number of rivers tested in an urban and rural area was only 1 each in this study, so it is not possible to generalise that different land uses were the explanation for the differences between the rivers. However, this study provides a baseline measurement of resistance in these rivers which were different. Moreover, these results are consistent with other studies (Allen *et al.*, 2011; McArthur *et al.*, 2016) that suggest that anthropogenic activities and environmental pollution can promote the evolution and dissemination of antibiotic resistance. The data obtained in this study suggest that urban and agriculture waterways are also habitats for antibiotic resistant *E. coli* which are differ in population density and structure. Future studies should look at the seasonal prevalence of resistance across forest streams, urban streams and agriculture streams to assess the diversity of antibiotic resistant bacteria, different resistance genes in various environment and how human activities influence waterways.

Chapter 3

Study on the diversity and prevalence of multiple antibiotic resistant E. coli in two Canterbury Rivers, New Zealand

3.1 Introduction

Multiple drug-resistant (MDR) strains of *E. coli* in the environment, especially freshwater, is a threat to public health (Amaya *et al.*, 2012; Centers for Disease Control and Prevention, 2013), as there are limited or no new antibiotics available with novel mechanisms of action against such strains (Chang *et al.*, 2015). As the prevalence MDR bacteria continues to increase, posing threat even beyond medical environments, there is a need for an ecological understanding on the evolution and distribution of resistance in order predict and counteract the effect of environmental MDR (Allen *et al.*, 2010; Kümmerer, 2004; McArthur *et al.*, 2016). Resistance to a single antibiotic may be considered a manageable problem where several broad-spectrum antibiotics are available as a substitute. However, the overuse of antibiotics has resulted in the emergence of bacterial populations that are resistant to more than one antibiotic (Al-Bahry *et al.*, 2015). The implication of MDR is, therefore, not limited to human and veterinary medicine, as antibiotic resistance genes from both origins can be mobilised into bacteria in the environment, especially freshwater environment (Chee-Sanford *et al.*, 2001).

There is no standard definition for MDR. For the purpose of this study, MDR is defined as the ability of a bacterial species (e.g., *E. coli*) to tolerate lethal doses of more than one class of antibiotics capable of killing susceptible bacterial strains (Abia *et al.*, 2015). *E. coli* that are constantly exposed to antibiotic residues in the environment may develop series of defence strategies to survive and replicate in the presence of antibiotics (Alves *et al.*, 2017). In principle, there are two mechanisms by which MDR occur in *E. coli*. One of such mechanism involves the accumulation of multiple genes in which each of the gene is responsible for resistance to a particular class of antibiotics. These resistance genes are usually transferred from one *E. coli* strain to another via plasmid or transposon (Nikaido, 2009). For instance, conjugative transposon (TN1545) confers resistance to kanamycin and tetracycline. Also, TN21, is a transposon that confers resistance to aminoglycosides and sulfanamides (Nikaido, 2009). The second mechanism of MDR involves over expression of genes that code for multiple efflux pumps, a transport protein used in expelling wide range of antibiotics out of the cell. Example includes ArcAB-TolC of *E. coli*, a housekeeping efflux pump, which is responsible for

extrusion of antibiotics such as quinolones, chloramphenicol, beta-lactams, fusidic acid and tetracycline (Sun *et al.*, 2014).

As a result of different resistance mechanisms, some bacterial strains are now resistant to almost all the major classes of antibiotics and chemotherapeutic agents (Fair & Tor, 2014). Generally, *E. coli* isolates have high resistance to older generation antibiotics such as ampicillin, tetracycline and streptomycin (Fair & Tor, 2014; Tadesse *et al.*, 2012). Some strains are also resistant to newer generation antibiotics, such as cephalosporins and fluoroquinolones (Collignon, 2009). Furthermore, MDR *E. coli* are now firmly established in environments (Amaya *et al.*, 2012; Ghaderpour *et al.*, 2015). For instance, *E. coli* strains that are resistant to fluoroquinolone, amoxicillin, gentamycin and trimethoprim have been identified in hospital environment (Rzewuska *et al.*, 2015).

Freshwater environments are key facilitator for the evolution and dissemination of MDR bacteria and resistance genes (Kappell *et al.*, 2015). This may be due to the presence of antibiotic residues or chemical molecules that can exert a selective pressure on bacteria, thereby contributing to the emergence of MDR (Li *et al.*, 2010). Freshwater environments have also become reservoirs for resistance genes and potential source where non-pathogenic and pathogenic bacteria acquire resistance genes (Dantas *et al.*, 2008). Animal waste used as landfill in agricultural land often contains antibiotic resistant *E. coli*. These bacterial strains may reach rivers via runoff or leaching of soil, thereby increasing the prevalence of antibiotic resistance in rivers (Larsson, 2014a). Wastewater treatment plant system-borne MDR *E. coli* may also overflow during rainfall and washed down to the adjacent rivers, thereby increasing bacterial populations of MDR *E. coli* freshwater environment (Marti *et al.*, 2013).

In this Chapter, the frequency of MDR *E. coli* from Avon River (Māori: Ōtākaro) and Silver Stream (Māori: Whirinaki) in the Canterbury region of New Zealand was estimated. In Chapter 2, I isolated *E. coli* strains from these rivers. In this Chapter, I tested *E. coli* isolates against an additional eight antibiotics

E. coli resistance to ciprofloxacin were routinely found in water samples (Chapter 2). Ciprofloxacin is an effective antibiotic against *E. coli*. This antibiotic has no natural source, and it is not produce by any know microorganism. Thus, it was surprising to find that Avon River and Silver Stream were reservoirs of this kind of resistance, reaching levels up to 8 times higher than the clinical breakpoint. If this kind of resistance is prevalent, I hypothesised that *E. coli* in both rivers might also have MDR. In addition, despite the sampling locations being close

on Avon River, there appeared to be a difference between the frequencies of resistant *E. coli* at the confluence of the Addington Brooke, location 1 compared sample locations 2 - 4. I hypothesised that the difference might be due to the influence of human activities in Hagley Park.

3.1.1 Research objectives

The objective of this chapter is to test whether antibiotic resistant *E. coli* strains from both rivers are also be resistant to several other classes of antibiotics. Furthermore, based on the detection of ciprofloxacin resistant *E. coli* isolates from both rivers in Chapter 2, I also sought to determine the minimum inhibitory concentration of ciprofloxacin that will stop the growth of *E. coli* isolates from both rivers. Finally, I determined whether *E. coli* isolates from both rivers are extended-spectrum beta-lactam (ESBL) resistant

Specific objectives were to

1. Test whether *E. coli* isolated directly from Avon River and Silver Stream exhibit MDR phenotypes.
2. Compare resistance patterns between *E. coli* isolates from the two rivers.
3. Determine the minimum inhibitory concentration of ciprofloxacin resistant *E. coli* from both rivers.
4. Investigate whether ampicillin resistant *E. coli* from Avon River and Silver Stream are extended-spectrum beta-lactam resistant isolates.

3.2 Materials and Methods

E. coli isolates from rivers (supplementary material) and lab strains (Table 3.1) were used. *E. coli* strains were revived from -80 ºC storage and routinely grown on nutrient agar plates. Bacteria were inoculated in a laminar flow cabinet and incubated at 37 ºC for 18-24 hours. To test antibiotic resistance phenotypes, cells were grown on nutrient agar supplemented with one or a combination of the following antibiotics: 10 µg/mL ampicillin, 8 µg/mL rifampicin, 6 µg/mL chloramphenicol, 1 µg/mL ciprofloxacin, 5 µg/mL tetracycline, 10 µg/mL trimethoprim, 5 µg/mL gentamicin and 5 µg/mL kanamycin.

Table 3.1: Bacterial strains used in this study

Bacteria	**Reference**
ATCC8739	(Baba *et al.*, 2006)
BW25113	(Baba *et al.*, 2006)
S17-1	(Simon *et al.*, 1983)
SM10	(Simon *et al.*, 1983)
JB570	Laboratory strain
JB644	Laboratory strain
DE1491	Laboratory strain
CSH121	Laboratory strain

3.2.1 *Determining Multiple Drug-Resistant in Escherichia coli isolates from Avon River and Silver Stream*

301 river-borne strains of *E. coli* previously isolated on either antibiotic-supplemented Tryptone Bile X-glucuronide agar (TBX) plates or plates without antibiotic were grown on nutrient broth in a 96 well plate at 37 °C until saturation. Then, 4 µL of the cultures were transferred aseptically onto nutrient agar plates supplemented with and without antibiotics. Plates were dried in a laminar flow cabinet for one hour and then incubated for 18-24 hours at 37 °C. Antibiotic resistant *E. coli* was determined macroscopically by comparing the control plate with antibiotic-supplemented plates. Prior to this experiment, a preliminary test was conducted to determine the minimum inhibitory concentrations of all antibiotics (ciprofloxacin, chloramphenicol, rifampicin, ampicillin, kanamycin, gentamycin, trimethoprim and tetracycline) that inhibit the growth of the control strains *E. coli* BW25113 and *E. coli* ATCC8739. Antibiotic resistant laboratory strains were used as positive control as shown in (**Table** 3.1).

3.2.2 *Determination of the minimal inhibitory concentration of laboratory E. coli strains and ciprofloxacin resistant E. coli isolates from Avon and Silver Stream*

A dose-response assay was performed to determine the minimal inhibitory concentration of ciprofloxacin. Different dilutions of saturated culture of ciprofloxacin resistant *E. coli* isolates recovered from both rivers was plated on nutrient agar plates supplemented with increasing concentrations of ciprofloxacin. *E. coli* BW25113 and ATCC8739 were used as negative controls while ciprofloxacin resistant *E. coli*, JB644 was used as positive control (Figure 3.1). Nutrient broth in a 96 well plate was inoculated with purified *E. coli* colony and incubated at 37º C with agitation on a rotary table to provide aeration until saturation. Nutrient agar plates supplemented with a range of ciprofloxacin concentrations was poured and dried for at least one hour in a laminar flow cabinet. Serial dilutions from 10^{-1} to 10^{-7} of the saturated culture were made and 4 µL droplet of the diluents were transferred onto a plate. The concentrations used in this assay were 2 µg/mL, 4 µg/mL, 8 µg/mL, and 16 µg/mL ciprofloxacin. Plates were dried for at least one hour and incubated at 37 ºC for 24 hours. Colony counts on all agar plates supplemented with antibiotics were compared to plates without antibiotics.

3.2.3 *Determination of extended beta lactam resistance among ampicillin resistant E. coli isolates from Avon River and Silver Stream*

Fifty randomly-selected *E. coli* isolates from the rivers grown on ampicillin were revived from -80 ºC storage onto solid nutrient agar plates in a laminar flow cabinet and incubated at 37 ºC until visible colonies were seen. Afterwards, single *E. coli* colonies were picked and used to inoculate nutrient broth in a 96 well plate. The antibiotic susceptibility test was performed using the method explained in 3.2.2 and spotted on nutrient agar plates containing rising concentrations of cefotaxime or no antibiotics. The plates were incubated at 37 ºC for 18-24 hours before growth on antibiotic supplemented plate was compared to growth on plates without antibiotic. Prior to this experiment, MIC of laboratory *E. coli* strains (BW25113 and ATCC8739) and a cefotaxime resistant *E. coli* was determined by plating out 10 µL saturated culture of the three *E. coli* control strains on varying concentrations of cefotaxime.

All ampicillin resistant *E. coli* isolates that grew at 8 µg/mL of cefotaxime were further screened for ESBL phenotype confirmation using double-disc synergy method in accordance to CLSI guidelines (Clinical and Laboratory Standard Institute, 2010). Pure colony of ampicillin resistant *E. coli* isolate was inoculated in a freshly prepared nutrient broth as explained in 3.2.2. 1 mL of the saturated culture was poured onto the surface of solidified nutrient agar. Plates were dried in a laminar flow cabinet for 1 hour and antibiotic discs (cefotaxime and ceftazidime (30 µg), and amoxicillin/clavulanic acid 20 + 10 µg) were placed aseptically on the surface of the agar containing culture with amoxicillin-clavulanic acid disc placed at the middle. Plates were incubated aerobically at 37 ºC for 18-24 hours, and the observation on plates were recorded the next day. A zone of inhibition of amoxicillin-clavulanic acid greater than 5 mm was compared with both cefotaxime and ceftazidime. The control used in this experiment are laboratory *E. coli* BW25113 and ATCC8739.

3.3 Results

3.3.1 *Antibiotic resistance profiles of E. coli isolated from Avon River and Silver Stream*

301 *E. coli* isolates from Avon River and Silver Stream were tested against a panel of antibiotics (ampicillin, rifampicin, chloramphenicol, ciprofloxacin, tetracycline, trimethoprim, gentamycin, and kanamycin, data shown in supplementary material and Figure 3.1). Among the tested isolates, 18% were directly isolated on TBX medium with no antibiotic. The others were isolated on one of four different antibiotics (ciprofloxacin, chloramphenicol, ampicillin and rifampicin) as shown in (Table 3.2). *E. coli* strains BW25113, ATCC8739, JB644, JB570, S17-1 and SM10 were used as controls.

Among the 48-ciprofloxacin resistant *E. coli* isolates recovered from Avon River, varying percentages: 97.92% to 18.75% grew on all different antibiotics used in this experiment as shown in (Table 3.2). In contrast, ciprofloxacin resistant *E. coli* isolates from Silver Stream did not grow on some antibiotics. Similarly, among the chloramphenicol resistant *E. coli* from Avon River, a high percentage of isolates grew on all the antibiotics tested, whereas all chloramphenicol resistant *E. coli* recovered from Silver Stream were susceptible to ciprofloxacin, trimethoprim, kanamycin and gentamycin (Table 3.2). Ampicillin resistant *E. coli* isolates recovered from both rivers were resistant to other antibiotics at varied levels as shown in (Table 3.2). Higher percentage of ampicillin resistant *E. coli* isolates from Avon River were also resistant to all other antibiotics tested compared to those from Silver Stream.

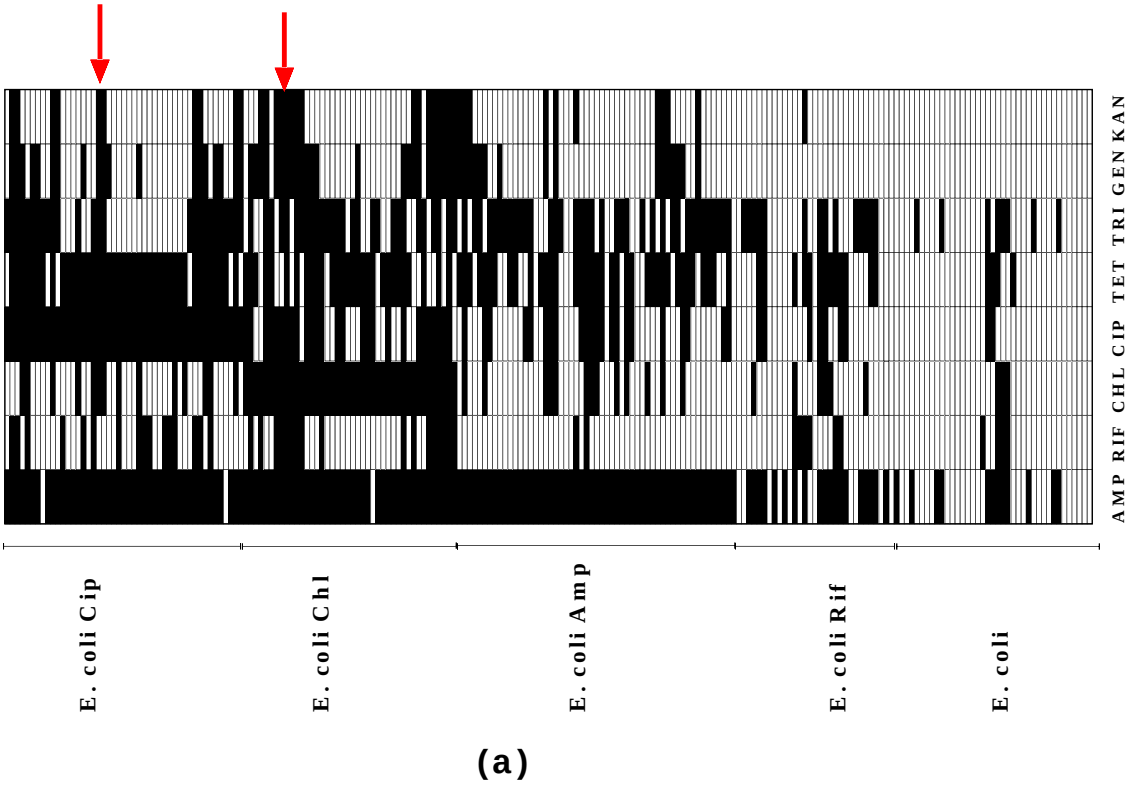
Table3.2: Profile of multi-drug resistant *E. coli* isolates from Avon River and Silver Stream.

			Resistance* %							
isolates	**total**	**Source**	**AMP**	**RIF**	**CHL**	**CIP**	**TET**	**TRI**	**GEN**	**KAN**
***E. coli* CIP**	**52**	**Avon 48**	**97.92**	**33.33**	**31.25**	**100**	**87.50**	**54.17**	**37.5**	**18.75**
		Silver 4	**25**	**0**	**0**	**100**	**100**	**50**	**0**	**0**
***E. coli* CHL**	**47**	**Avon 41**	**97.56**	**41.46**	**100**	**58.54**	**68.29**	**68.29**	**53.66**	**36.59**
		Silver 6	**83.33**	**33.33**	**100**	**0**	**100**	**0**	**0.00**	**0**
***E. coli* AMP**	**90**	**Avon 55**	**100**	**3.64**	**25.45**	**40**	**63.64**	**65.45**	**29.09**	**16.36**
		Silver 35	**62.86**	**20**	**8.57**	**2.86**	**28.57**	**14.29**	**0**	**0**
***E. coli* RIF**	**58**	**Avon 32**	**62.50**	**18.75**	**18.75**	**21.88**	**40.63**	**43.75**	**0**	**3.13**
		Silver 26	**26.92**	**7.69**	**0**	**7.69**	**7.69**	**3.85**	**0**	**0**
E. coli	**54**	**Avon 38**	**21.05**	**10.53**	**7.89**	**5.26**	**10.53**	**21.05**	**0**	**0**
		Silver 16	**25**	**12.50**	**12.50**	**0**	**12.50**	**0**	**0**	**0**

*** Resistance is defined as the detection of visible bacterial growth on antibiotics supplemented plates which is equivalent to the plate without antibiotic. AMP, ampicillin (10 µg/mL); RIF, rifampicin (8 µg/mL); CHL, chloramphenicol (6 µg/mL); CIP, ciprofloxacin (1 µg/mL); TRI, trimethoprim (10 µg/mL); GEN, gentamycin (5 µg/mL) and KAN, kanamycin (5 µg/mL).**

A high percentage of *E. coli* isolates were recovered from $TBX_{rifampicin}$ (data shown in chapter 2). However, upon susceptibility testing, 81.25% from Avon River and 92.3% from Silver Stream were found to be susceptible to rifampicin (Table 3.2). All rifampicin resistant *E. coli* isolates from Silver Stream were also susceptible to chloramphenicol, gentamycin and kanamycin. However, 19% of the Avon River rifampicin resistant *E. coli* were resistant to chloramphenicol (Table 3.2). The reason for the difference in the result of rifampicin resistant *E. coli* isolates recovered from chapter 2 and the observation in this chapter may be that *E. coli* isolates from rifampicin supplemented plate were not actually resistant to rifampicin, because when individual isolate was tested against the same concentration of rifampicin higher percentage were susceptible. This result indicated that high biofilm formation, a first line of defense in bacteria allowed the susceptible population to grow in the presence of rifampicin (Zhou *et al.*, 2015). *E. coli* isolated directly from plates without antibiotic were also tested for susceptibility against the antibiotics panel. All *E. coli* isolates from both rivers showed higher susceptibility to all antibiotics tested compared to *E. coli* recovered from antibiotic supplemented plates (Table 3.2 and Figure 3.1).

MDR profile of *E. coli* isolates from Avon River and Silver Stream



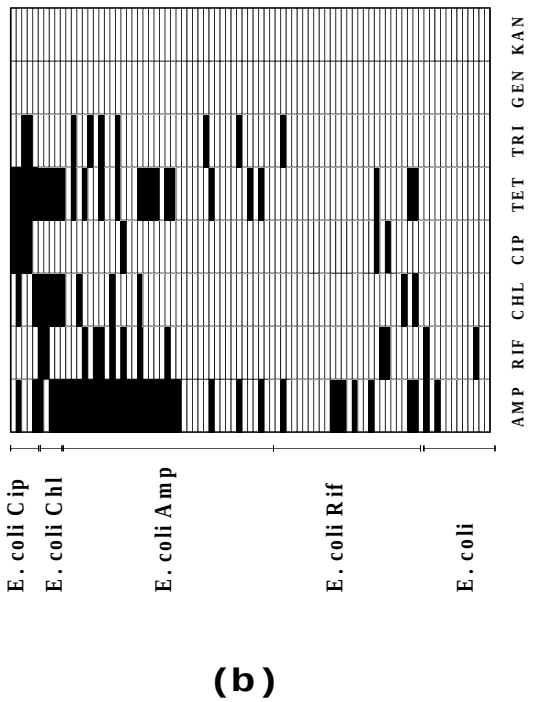


Figure 3.1. MDR profile of *E. coli* from (a) Avon Ricer and (b) Silver Stream. Black box indicates resistant *E. coli* phenotype and white indicates susceptible *E. coli* phenotype. The two red arrows indicate isolates that are resistant to all tested antibiotics.

3.3.2 *Ciprofloxacin MIC determination among resistant E. coli isolates from Avon River and Silver Stream*

Minimum inhibitory concentration was determined for a subset of *E. coli* isolates that were initially recovered on solid nutrient agar supplemented with 1 µg/mL ciprofloxacin in chapter two. The concentration of ciprofloxacin used in this assay ranged from 2 to 16 µg/mL as shown in (Table 3.3). Ciprofloxacin resistant *E. coli* isolates from Avon River tolerate higher concentration of ciprofloxacin compared to those from ciprofloxacin resistant *E. coli* isolated from Silver Stream. Most of the tested ciprofloxacin resistant *E. coli* isolates from Avon River were growing at ciprofloxacin concentrations of 16 µg/mL (Figure 3.2). The growth pattern of ciprofloxacin resistant isolates from both rivers was different even in the absence of antibiotics (Figure 3.2). Avon River ciprofloxacin resistant *E. coli* grew to saturation in the absence of antibiotics whereas ciprofloxacin resistant isolates from Silver Stream were only grew up to an approximate of 10^6 CFU/mL.

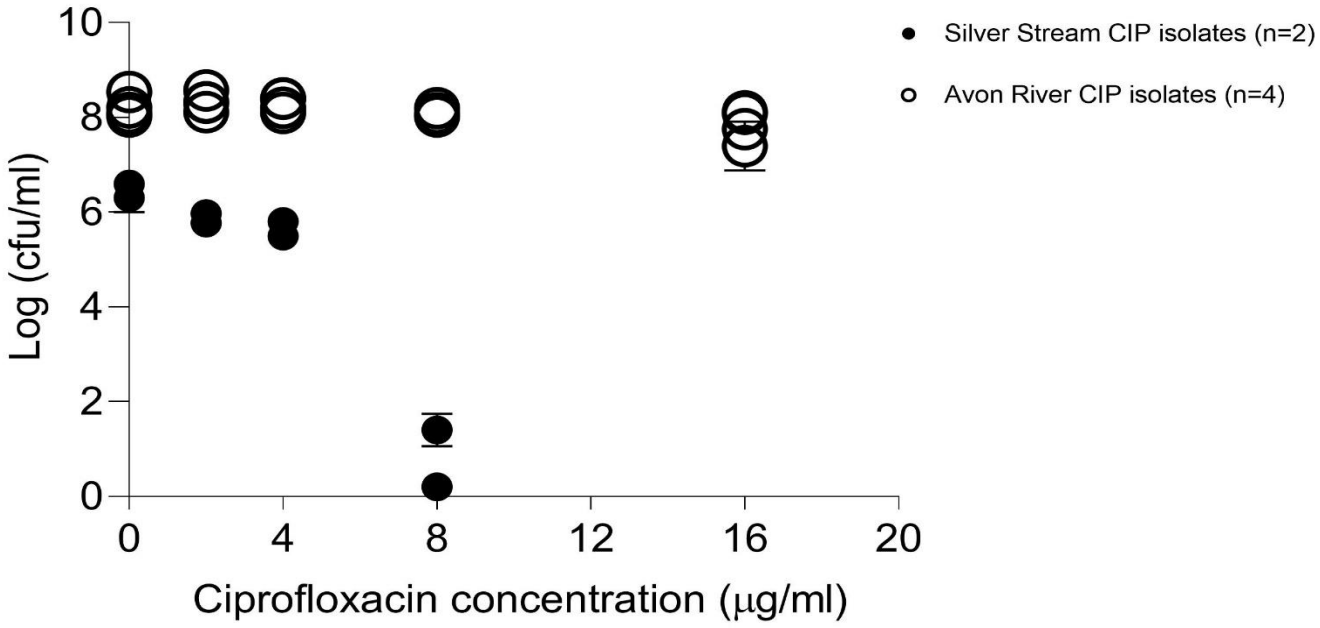


Figure 3.2. Dose response curve of ciprofloxacin resistant *E. coli* from Avon River (opened dot) and Silver Stream (closed dot). Dose curve is reported as the log-transformed CFU/mL count ± SEM (n = 4). This curve was used to determine the difference in the ciprofloxacin resistant *E. coli* from the two rivers.

Table 3.3: Ciprofloxacin minimum inhibitory concentration (MIC) for *E. coli* isolates from Avon River and Silver Stream.

Ciprofloxacin concentration (µg/mL)					
***E. coli* Isolate**	**Site**	**2**	**4**	**8**	**16**
B1CIPL2:3	**Avon**	**R**	**R**	**R**	**R**
A3CIPL2:1	**Avon**	**R**	**R**	**R**	**R**
E4CIPL3:3	**Avon**	**R**	**R**	**R**	**R**
G6CIPL3:3	**Avon**	**R**	**R**	**R**	**R**
F6CIPL3:2	**Avon**	**R**	**R**	**R**	**R**
E6CIPL3:4	**Avon**	**R**	**R**	**R**	**R**
D6CIPL4:1	**Avon**	**R**	**R**	**R**	**R**
H8CIPL3:4	**Avon**	**R**	**R**	**R**	**R**
G8CIPL2:4	**Avon**	**R**	**R**	**R**	**R**
C8CIPL2:4	**Avon**	**R**	**R**	**R**	**R**
L14CIP	**Silver**	**R**	**S**	**S**	**S**
L11CIP	**Silver**	**R**	**I**	**S**	**S**
BW25113	**Lab**	**S**	**S**	**S**	**S**
ATCC	**Lab**	**S**	**S**	**S**	**S**
CIP resistant JB644	**Lab**	**R**	**R**	**R**	**R**

S = No CFU count at lowest dilution as compared to plate without treatment; R = *E. coli* CFU count at the same dilution with the plate without antibiotic and I = intermediate *E. coli* lesser CFU count as compared with plate without antibiotics

3.3.3. *Antibiotic susceptibility pattern of Avon River and Silver Stream ampicillin resistant E. coli on cefotaxime*

To further characterise antibiotic resistant *E. coli* isolates from both Rivers, ampicillin resistant *E. coli* isolates from Avon River and Silver Stream were tested for extended-spectrum beta-lactamase (ESBL) production. I also sought to further support my hypothesis that antibiotic resistance pattern of *E. coli* changes depending on the type of land management along the five locations in Avon River. To test this hypothesis, a total of 50 ampicillin resistant *E. coli* isolates that where initially isolated from $TBX_{\text{-ampicillin}}$ plates were randomly selected. Of the 50 ampicillin resistant *E. coli*, seven isolates were from location 5 and 28 from the other locations within Hagley Park in Avon River. The remaining 15 ampicillin resistant *E. coli* isolates were from Silver Stream. The prevalence of cefotaxime resistant *E. coli* was widespread in Avon River. Twelve out of thirty-five ampicillin resistant *E. coli* from Avon River grew on 8 µg/mL of cefotaxime. However, none of the *E. coli* isolates that grew at this concentration were from location 5 of Avon River as shown in (Table 3.4). Instead, fourteen out of fifteen ampicillin resistant *E. coli* from Silver Stream were susceptible at 2 µg/mL of cefotaxime.

Table 3.4:Cross-resistance testing of ampicillin resistant *E. coli* to varying concentrations of cefotaxime.

Isolate	Location	Cefotaxime concentration			
		2 µg/mL	4 µg/ml	6 µg/mL	8 µg/mL
AMPL31	Avon	R	R	R	I
AMPL23	Avon	R	R	R	R
AMPL34	Avon	I	I	I	I
AMPL43	Avon	S	S	S	S
AMPL23	Avon	S	S	S	S
AMPL11	Avon	I	I	I	I
AMPL22	Avon	S	S	S	S
AMPL34	Avon	S	S	S	S
AMPL14	Avon	S	S	S	S
AMPL31	Avon	S	S	S	S
L13AMP	Silver	S	S	S	S
L32AMP	Silver	S	S	S	S
L32AMP	Silver	S	S	S	S
L32AMP	Silver	S	S	S	S
L14AMP	Silver	S	S	S	S
L33AMP	Silver	S	S	S	S
L41AMP	Silver	R	S	S	S
L12AMP	Silver	S	S	S	S
L43AMP	Silver	S	S	S	S
L34AMP	Silver	S	S	S	S
AMPL22	Avon	R	S	S	S
AMPL31	Avon	R	R	R	R
AMPL14	Avon	R	R	R	R
AMPL21	Avon	S	S	S	S
AMPL23	Avon	S	S	S	S
AMPL41	Avon	R	R	R	R
AMPL43	Avon	R	R	R	R
AMPL33	Avon	I	I	I	S
AMPL12	Avon	S	S	S	S
SAMPL24	Avon	S	S	S	S
AMPL13	Avon	S	S	S	S
AMPL12	Avon	R	R	R	I
AMPL43	Avon	R	R	R	R
SAMPL22	Avon	S	S	S	S
AMPL42	Avon	R	R	R	I
AMPL53	Avon	S	S	S	S
AMPL42	Avon	I	I	I	I
AMPL32	Avon	S	S	S	S
AMPL23	Avon	S	S	S	S
AMPL51	Avon	S	S	S	S
AMPL51	Avon	S	S	S	S
AMPL52	Avon	S	S	S	S
AMPL51	Avon	S	S	S	S
AMPL53	Avon	S	S	S	S
AMPL52	Avon	S	S	S	S
L31AMP	Silver	S	S	S	S
L33AMP	Silver	S	S	S	S
L13AMP	Silver	S	S	S	S
L14AMP	Silver	S	S	S	S
SL14AMP	Silver	S	S	S	S
	Total Avon =35 Silver =15	% R = 30	% R = 26	% R = 26	% R = 24

R, resistant, I, intermediate resistant and S, susceptible

3.3.4. *Confirmation test for ESBL producing E. coli*

A total of 16 ampicillin resistant *E. coli* isolates were screened against cefotaxime, ceftazidime and amoxicillin-clavulanic acid. Twelve (75%) of these isolates were resistant to cefotaxime at 8 µg/mL, as shown in (Table 3.4). Of the 75% cefotaxime resistant *E. coli* isolates, 5 (41.67%) were found to have zone of inhibition greater than 5 mm around amoxicillin-clavulanic acid antibiotic disc, which confirmed the phenotypically ESBL producing *E. coli* (Table 3.5 and Figure 3.3).

Table 3.5: Confirmatory test result for ESBL phenotype using double disc method.

***E. coli* Strain**	**CTX zone diameter (mm)**	**CAZ zone diameter (mm)**	**AMX/CLA zone diameter (mm)**	**Location**
AMPL33	0	15	15	Avon
AMPL34	16	22	15	Avon
AMPL31	0	6	10	Avon
AMPL43	15	15	10	Avon
AMPL23	0	15	15	Avon
AMPL33	20	20	20	Avon
AMPL12	0	0	15	Avon
AMPL14	12	15	8	Avon
AMPL22	12	15	10	Avon
AMPL32	12	25	15	Avon
AMPL41	0	8	8	Avon
33AMP	18	20	10	Silver
32AMP	22	22	18	Silver
33AMP	22	22	16	Silver
13AMP	15	22	17	Silver
BW25113	22	16	15	Lab

ESBL production phenotypic confirmation test using combined disk method. Zone of inhibition diameters were measured in mm for different *E. coli* isolates. Note: Cefotaxime (CTX), Ceftazidime (CAZ), and Amoxicillin-clavulanic acid (AMX/CLA).

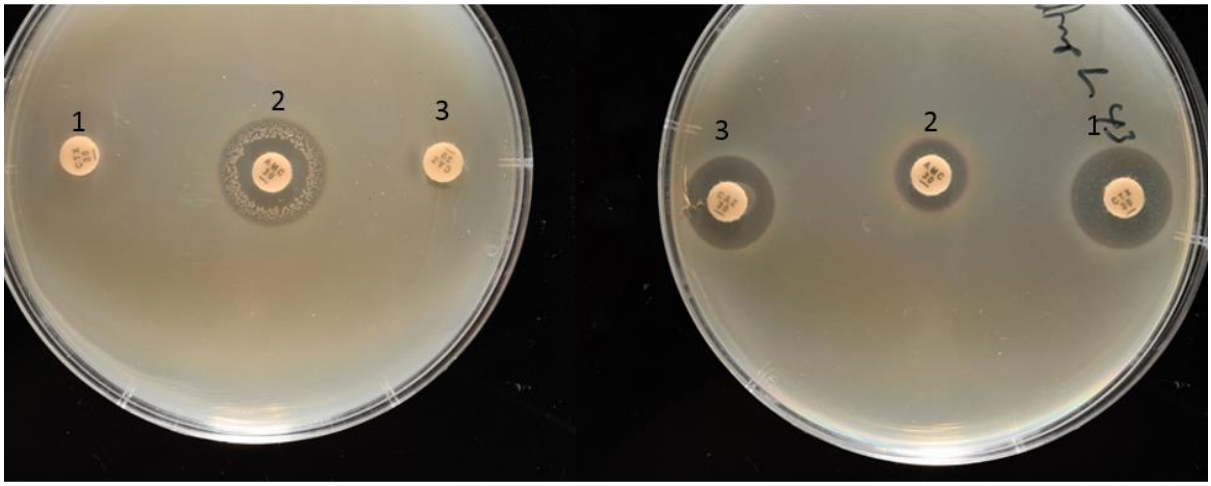


Figure 3.3. Confirmation of extended-spectrum beta-lactamase producing *E. coli* isolate using amoxicillin-clavulanic acid, cefotaxime and ceftazidime. Note: 1-cefotaxime, 2-amoxicillin-clavulanic acid, 3-ceftazidime.

3.4 Discussion

3.4.1 *Multiple antibiotic resistant E. coli in Canterbury Rivers*

The rapid emergence of MDR bacteria has reduced the efficacy of antibiotics in human and veterinary medicine (Kappell *et al.*, 2015). As a consequence of rampant use of antibiotics, increasing number of antibiotic resistant bacteria have been reported from environmental, clinical and veterinary sources, making antibiotic resistance a global threat to human health (Von Baum & Marre, 2005). Previous studies have shown the prevalence of bacteria harbouring multiple antibiotic resistance genes in the aquatic environment (Baquero *et al.*, 2008; Martinez, 2009). In this study, the MDR *E. coli* was found in almost all antibiotic resistant *E. coli* isolates from two rivers in Canterbury, New Zealand. Overall, high population (over 80%) of MDR were detected among *E. coli* isolates that were already resistant to one class of antibiotics (Table 3.2 and Figure 3.1). This result is consistent with previous studies in India and Mexico (Chandran *et al.*, 2008; Ramirez Castillo *et al.*, 2013; Varghese & Roymon, 2013). Many natural environments harbour antibiotic resistance genes and resistance determinants such as plasmid, transposons, and integrons, which may be conferring different kind of resistance to antibiotics. The prevalence of MDR as presented in this study may also be due to activation of multiple efflux pumps responsible for transporting different antibiotic molecules (Li & Nikaido, 2009; Nikaido, 2009). Another possibility might be the influence of agriculture and urbanisation practices, which involve discharge of antibiotic residues and biocides that can co-select for resistance in a situation where the resistance determinant is present on the same genetic element (Berkner *et al.*, 2014). Antibiotics contamination of rivers may create a selective pressure within bacterial population, leading rise in MDR strains (Kümmerer & Henninger, 2003; Lien *et al.*, 2016). This result is also consistent with studies on the frequencies of antibiotic resistant bacteria in the environment (D'costa *et al.*, 2006; Schmitt *et al.*, 2006). Notably, the prevalence of MDR *E. coli* was higher in Avon River than Silver Stream (Figure 3.1). This also follow from the result of chapter 2 where ciprofloxacin and chloramphenicol resistant *E. coli* isolates were more frequently isolated from Avon River than Silver Stream. This may be the consequence human activities, impact from domestic and industrial waste which are directly or indirectly selecting for higher prevalence of MDR *E. coli* in Avon River. This finding is consistent with previous study in a Matang mangrove estuaries where anthropogenic source is the major factor contributing to the abundance of MDR *E. coli* (Ghaderpour *et al.*, 2015).

Among the ciprofloxacin resistant *E. coli* isolates from rivers, higher percentage those from Avon River were also resistant to ampicillin, tetracycline, gentamycin and trimethoprim (Table 3.2). The cross resistance of ciprofloxacin resistant *E. coli* isolated from Avon River to other antibiotics tested in this study is consistent with clinical observations of multiple resistance in ciprofloxacin resistant *E. coli* (Pépin *et al.*, 2009). Notably, almost all ciprofloxacin resistant *E. coli* isolates had cross-resistance to ampicillin. This observation in this study agrees with the findings of Amaya *et al.* (2012), where 100% of ciprofloxacin resistant *E. coli* isolates grew on ampicillin. The cross resistance of ciprofloxacin to other unrelated classes of antibiotics may be due to the multiple antibiotic resistance (mar) locus present in most Gram-negative bacteria like *E. coli*. Mutation in the mar locus of *E. coli* may be responsible for the association for high frequency of resistance among ciprofloxacin resistant *E. coli* to other antibiotics used in this study (Tolun *et al.*, 2004). The association of ciprofloxacin resistance with ampicillin in this study may be due to the presence of beta-lactam resistance genes and plasmid-mediated quinolone resistance genes (*qnr*) in most of the *E. coli* isolates as reported in previous studies (Ramirez Castillo *et al.*, 2013; Wang *et al.*, 2001). *E. coli* isolates resistant to ciprofloxacin recovered from Silver Stream were uniformly susceptible to kanamycin, gentamycin, chloramphenicol and rifampicin (Figure 3.1b). The reason for these observations may be that ciprofloxacin resistant *E. coli* from Avon River have different resistance mechanisms to other antibiotic such as plasmid linked resistance that is not present in Silver Stream counterpart. Alternatively, ciprofloxacin resistant *E. coli* from Avon River may be cross selecting for resistance to kanamycin and gentamycin, which is not the case of ciprofloxacin resistant *E. coli* from Silver Stream.

There are many reasons for the different resistance pattern among ciprofloxacin resistant *E. coli* isolate from the two study sites. Firstly, the number of ciprofloxacin resistant *E. coli* isolates detected in Silver Stream was lower (less than 5 isolates throughout the sampling time) compared to those detected in Avon River. This makes the number ciprofloxacin resistant *E. coli* isolates tested in this assay not equal which suggests that ciprofloxacin resistance may not be common in Silver Stream as compared with Avon River. Secondly, the result obtained here may be due to more anthropogenic selective pressure in Avon River, thereby contributing to the prevalence and persistence ciprofloxacin resistant *E. coli* (Tacao *et al.*, 2012). This also suggests that the impact of wastewater discharge from both domestic and industrial sources along the flow of Avon River may directly or indirectly favour or co-select ciprofloxacin resistant *E. coli* isolates (Amabile-Cuevas *et al.*, 2010).

Chloramphenicol resistant *E. coli* isolates were also resistant to other antibiotics tested. Chloramphenicol is a broad-spectrum antibiotic with high inhibitory property against bacteria. It is used in both medical and agriculture fields as a therapeutic agent in controlling bacterial diseases or to prevent infections (Lu *et al.*, 2009). Chloramphenicol residues enter aquatic environment via aquaculture wastewater, creating a selective pressure within bacterial community which in turn favours the emergence of antibiotic resistance (Lu *et al.*, 2009). Also, most of the genes conferring resistance to chloramphenicol are usually found in mobile genetic determinant, such as plasmid, integron, and transposon. These elements, often carry additional antibiotic genes which may be responsible for cross-resistance to other class of antibiotics (Schwarz *et al.*, 2004). In this study, chloramphenicol resistant *E. coli* isolates had high level of resistance to ampicillin (98% and 83%) and tetracycline (68% and 100%) for Avon River and Silver Stream respectively as shown in (Table 3.2). Chloramphenicol resistant *E. coli* isolates from Avon River also showed different levels of resistance to trimethoprim, ciprofloxacin, gentamycin and kanamycin. However, these resistances were not found in Silver Stream chloramphenicol resistant *E. coli* isolates. This finding suggests that rivers that are more influenced by industrial and human activities harbour high level of antibiotic resistant bacteria (Chandran *et al.*, 2008).

MDR was also detected in some of ampicillin resistant *E. coli* in both rivers (Figure 3. 1). Higher levels of resistance were detected among ampicillin resistant *E. coli* isolates from Avon River compared to Silver Stream (Table 3.2). Out of the ampicillin resistant *E. coli* from both rivers, some were also resistant to other antibiotics (Figure 3.1). One of the antibiotics used in this study was tetracycline, commonly used in treatment of bacterial infections in both clinical and veterinary settings. The cross-resistance of ampicillin resistant *E. coli* to tetracycline is not surprising because of its widespread use as a first-line drug in treating animal and human bacterial infections, thereby contributing to high prevalence of resistance in the environment (Roberts, 1996). Also, tetracycline resistance is one of the most naturally occurring antibiotic resistance in the environments, there is however possibility that *E. coli* isolates in this study are inherently resistant to tetracycline (Munita & Arias, 2016; Speer *et al.*, 1992).

The observation among rifampicin resistant *E. coli* in this study was quite surprising. Over 70% of *E. coli* isolates recovered from rifampicin supplemented agar plates from both rivers water was found to be susceptible to the same rifampicin concentration when re-tested for MDR. One possible explanation for this phenomenon may be that these isolates were not originally

antibiotic resistant but the method employed in this study allowed biofilm formation which makes large population of susceptible *E. coli* to grow on rifampicin (data shown in chapter 2).

3.4.2 *Minimum inhibitory concentration of ciprofloxacin resistant E. coli isolated from Avon River and Silver Stream*

Some of the tested antibiotics are clinically more important than others. For example, rifampicin is not used for *E. coli* infections while ciprofloxacin is a core drug (Hickerson & Carson, 2006). The level of antibiotic dosing in a patient follows pharmacokinetic models based partially on minimum inhibitory concentration (MIC) estimates for susceptible strains of the intended target organism (Defife *et al.*, 2009). Usually, these estimates are provided by the Ministry of Health or similar agencies. Patients infected with *E. coli* with higher tolerances than the MIC are at risk of developing severe pathologies/symptoms. Bacteria with an MIC above a certain level are considered too high to treat at any achievable antibiotic dosing. I defined MIC as the lowest concentration of ciprofloxacin that inhibited the formation of visible growth after incubation period. The revised ciprofloxacin MIC concentration for clinical breakpoint resistance in all enterobacteria is ≥ 2 µg/ml (Humphries *et al.*, 2012). The MIC of ciprofloxacin among a subset of *E. coli* isolates that were initially selected on 1 µg/mL was determined. *E. coli* isolates were tested on concentrations of ciprofloxacin ranging from 2 µg/mL to 16 µg/mL.

Ciprofloxacin resistant *E. coli* were detected in both rivers. However, the population sizes and the extremes in MIC were different. Due to the difference in population of ciprofloxacin-resistant *E. coli* recovered from both rivers, the minimum concentration of ciprofloxacin that will stop the growth *E. coli* isolates recovered from agar plate supplemented with ciprofloxacin was determined. This hypothesis was tested using a subset of ciprofloxacin resistant *E. coli* isolates from Avon River and Silver Stream. Out of the ciprofloxacin resistant *E. coli* isolates tested from the Avon River, isolates were resistant to up to eight times the clinical breakpoint concentration, while Silver Stream counterpart were all susceptible at 4 µg/mL. (Figure 3.2 and Table 3.3). The reason for resistance to higher concentration of ciprofloxacin among Avon River *E. coli* isolates than Silver Stream counterpart is unknown. One possible explanation may be that ciprofloxacin is rarely used around Silver Stream being an agricultural region, but has an extensive application in the treatment bacterial infections associated human and domestic animals that often found within Avon River. Previous study has shown that the major sources of *E. coli* in Avon River to human and domestic animals faecal contamination (Moriarty *et al.*, 2015), which may also be the source of ciprofloxacin resistant *E. coli* as seen in the Avon River

in this study. Moreover, it has been established that environments that receive high input of antibiotic residue or other chemical substances that can exert selective pressure may also harbour high population of antibiotic resistant bacteria (Li *et al.*, 2010). Hospital, municipal, and industrial waste have been identified as the major sources of antibiotics and chemicals that can select for antibiotic resistance (Segura *et al.*, 2009). Interestingly, location where high level of ciprofloxacin resistance was detected in this study are influenced by some of the source listed above, if not all.

The growth pattern of ciprofloxacin resistant *E. coli* isolates from both rivers also differ even in the absence of ciprofloxacin. Ciprofloxacin resistant *E. coli* from Avon River grew to saturation while those from Silver Stream did not. This was observed when different dilutions of *E. coli* culture from both rivers were plated out on nutrient agar (Figure 3.2). One possibility for this observation may be because of limited knowledge on the physiology and nutrient requirement of environmental isolates. Some bacteria could grow rapidly under normal environmental condition while others may grow slowly due to poor nutrient and heterogeneity in bacterial population. When such slow growth bacteria are cultured under laboratory condition, they may require more time to adjust to the growth condition.

3.4.3 *Cross-resistance to cefotaxime among of ampicillin resistant E. coli isolate from Avon River and Silver Stream*

Following the abundance of ampicillin resistant *E. coli* recovered from Avon River and Silver Stream, I assessed the prevalence of ESBL producing *E. coli* among *E. coli* isolates to further characterise antibiotic resistance in the two rivers. This was done preliminarily by screening selected ampicillin resistant *E. coli* isolates on a third-generation cephalosporin antibiotic (cefotaxime). Cefotaxime is a third-generation beta-lactam antibiotic with high inherent activities against *E. coli*. Resistance to any antibiotic of cephalosporin class is an indication of extended-spectrum beta-lactamases (ESBLs) producing *E. coli* (Jonathan, 2005). ESBLs are enzymes produced by *E. coli*, which hydrolyse the amide bond on the antibiotics (e.g., cefotaxime, ceftazidime and ceftriaxone) beta-lactam ring (Rawat & Nair, 2010).

Prevalence of cefotaxime resistance was detected among ampicillin resistant *E. coli* from Avon River in this study. The result revealed that twelve out of thirty-five tested of ampicillin resistant *E. coli* isolates from Avon River were also resistant to 8 µg/mL of cefotaxime, whereas over 90 % (14 out of 15) of the Silver Stream ampicillin resistant *E. coli* isolates were susceptible at 2 µg/mL of cefotaxime (Table 3.4). The result presented here on cross resistance

of ampicillin resistant *E. coli* to other beta-lactam, cefotaxime is consistent to the findings of Ash *et al.* (2002). The occurrence of beta-lactam associated resistance in the urban rivers is not surprising (Hu *et al.*, 2008; Sara Rodriguez-Mozaz *et al.*, 2015), since beta-lactams are mostly used antibiotics in clinical setting and animal husbandry (Li *et al.*, 2007). Similarly, antibiotic resistance genes encoding resistance to beta-lactam antibiotics may be inherently present in *E. coli* genetic make-up or acquired via horizontal gene transfer thereby contributing to the prevalence of beta-lactam resistance (Gang & Jie, 2016). These findings are also comparable with previous studies that have detected beta-lactam resistance, especially the penicillin and cephalosporin group in aquatic environment (Alouache *et al.*, 2012; Ash *et al.*, 2002). The detection of cefotaxime resistance among ampicillin resistant *E. coli* may be an indication that *E. coli* isolates are producing ESBL. To confirm the presence of ESBL-producing *E. coli* among ampicillin-resistant *E. coli*, a phenotypic confirmatory test was performed using cefotaxime, amoxicillin/clavulanic acid and ceftazidime disc. Out of 12 cefotaxime resistant *E. coli* from Avon River five (41.67%) was found to be ESBL producer among the isolates from Avon River. The zone of inhibition around amoxicillin-clavulanic acid was larger than 5 mm for *E. coli* isolates producing ESBL (Figure 3.3). The detection of ESBL-producing *E. coli* may reflect human and animal input along the flow of Avon River where resistance to cefotaxime was detected. Another possibility may be due to contamination of antibiotics residues or chemical agents selecting for cefotaxime resistance in *E. coli*. Screening of *E. coli* isolates for ESBL production become necessary in aquatic environment that constantly receives input from anthropogenic source because water serves as important reservoir of resistance genes where bacteria of different origin can acquire resistance. However, it is worthwhile to consider that the disc method may not be sufficient to confirm the presence of ESBL producing *E. coli* due to the co-existence of ESBL with other beta-lactamases such as AmpC beta-lactamase enzymes present in most *E. coli* chromosome (Jacoby, 2009), which also hydrolyse beta lactams, thereby interfering with the interpretation of ESBL detection in *E. coli* (Poulou *et al.*, 2014). Due to the lack of sensitivity of the different phenotypic methods, a molecular approach using specific polymerase chain reaction (PCR) amplification of beta-lactamase genes will more accurate in identifying ESBL producing *E. coli* (Krishnamurthy *et al.*, 2013). The information about different range of resistance to antibiotics in environmental *E. coli* isolates will be useful in understanding the threshold and dissemination of antibiotic resistance in the environment. Moreover, understanding the pattern of resistance in bacteria from different polluted aquatic sources will be required to mitigate the spread of resistance to bacterial pathogens that are a public health concern

3.5 Conclusions.

The result obtained from the frequency of MDR determination among *E. coli* isolates from Avon River and Silver Stream indicate that anthropogenic influence on rivers may lead to the prevalence of antibiotic resistant *E. coli* that may not be resistant to only one antibiotic but also to other class antibiotics. The findings of ciprofloxacin resistant *E. coli* isolates showing high prevalence of MDR support previous study which suggest that ciprofloxacin resistance promote resistance to other antibiotic with different mechanism of action (Fung-Tomc *et al.*, 1993). More studies will be needed to investigate associated resistance to all classes of antibiotic in any environmental antibiotic resistant *E. coli* isolates in rivers within Canterbury region. (Kappell *et al.*, 2015).

Chapter 4

4.1 General discussion and future direction

Antibiotic resistance has been in the environment even before the discovery of antibiotic for therapeutic use several decades ago (D'Costa *et al.*, 2011; Davies & Davies, 2010). However, the threat of antibiotic resistance was only recognised within human and veterinary medicine(Cantas *et al.*, 2013). Several studies have attributed the crises of antibiotic resistance to be overuse and misuse of antibiotic in medical treatment of bacterial infections (Chung *et al.*, 2007; Lewy, 2013), with little or no attention to the environment (Gaze & Depledge, 2017). Recently, there is a growing evidence that the environment, especially aquatic environments, may act as reservoirs for antibiotic resistant bacteria (Kappell *et al.*, 2015; McArthur *et al.*, 2016; Santoro *et al.*, 2015; Schwartz *et al.*, 2003), as discussed in the introductory session, chapter 1 of this thesis. Understanding the factors that contribute to the emergence of antibiotic resistance in various environmental hotspot will be important information for public health.

The studies described in this thesis were inspired by a published study on the prevalence and pattern of multi-antibiotic resistant bacteria in aquatic environments in Unites States. The monitored rivers had no known input of antibiotics (McArthur *et al.*, 2016). My thesis focused on monitoring and measuring the distribution and prevalence of antibiotic resistant *E. coli* in rivers that are influenced by different human activities such as urbanization and agricultural practises. In chapter 2 of this thesis, I measured the abundance and pattern of antibiotic resistant *E. coli* in Avon River and Silver Stream that receive different anthropogenic input. The Avon River is an urban river which flows through the city of Christchurch, New Zealand. The river also drains two major tributaries namely Addington Main Drain and Riccarton Drain. Addington Drain flows from an industrial area to join Avon River at Hagley Park just upstream of the Christchurch Hospital. Similarly, Riccarton Drain flows from a highly residential area and joins the Avon River just before the Hospital. In contrast, Silver Stream drains mainly agriculture land. No study in Canterbury region of New Zealand has investigated and measured the prevalence of antibiotic resistant *E. coli* in two rivers simultaneously. I investigated and compared these rivers within three seasons in 2017 in order understand whether different anthropogenic activities during sampling seasons affected the prevalence and pattern of antibiotic resistant *E. coli*. Water and sediment samples were collected to quantify and characterised *E. coli* and antibiotic resistant *E. coli* population. My overall objective was to monitored and measured *E. coli* populations in Avon River and Silver Stream between January and November 2017. This main objective was achieved by the following supportive objectives:

1. Isolation of antibiotic resistant *E. coli* and determination of the prevalence in the selected rivers using phenotypic culture-based screening method.
2. Determination of the frequency of multiple resistance to eight antibiotics using *E. coli* isolates.
3. Determination of the minimum inhibitory concentration of ciprofloxacin for *E. coli* isolates.
4. Quantification and comparison of the antibiotic resistance patterns between the two rivers.

4.1.1 Antibiotic resistance profile of *E. coli* in Avon River and Silver Stream surface water and sediment.

One of the main objective of this thesis was to show that polluted rivers are reservoirs for antibiotic resistant *E. coli* that are different in population size and composition. No information was available on the prevalence of antibiotic resistant *E. coli* in both rivers as at the start of this research. Therefore, the information gathered from this study will be useful when comparing the spatial distribution of antibiotic resistant *E. coli* in rivers.

The results obtained on the distribution and prevalence of antibiotic resistant *E. coli* in chapter 2 support the idea that rivers that are anthropogenically influenced are habitats for different levels of antibiotic resistance. Antibiotic resistant *E. coli* were detected in both rivers during the different sampling seasons. However, the population size and composition of antibiotic resistant *E. coli* detected in the two rivers varied. A significant higher level of antibiotic resistant *E. coli* was detected in Avon River at all sampling seasons compared to Silver Stream, which is an agriculture river. The increased antibiotic resistant *E. coli* populations in Avon River may be attributed to the fact that this river receive different anthropogenic impacts compared Silver Stream which drains agriculture land. For instance, Avon River drains different polluted tributaries, one of which include Addington Drain which flows from highly industrial environment. Previous studies have highlighted that Addington drain is one of the most polluted rivers in New Zealand (Charters, 2016). Chemical pollutants in Addington drain may be selecting for antibiotic resistant *E. coli* or antibiotic resistance genes which could be transfer from one *E. coli* to another (Seiler & Berendonk, 2012), thereby leading to higher prevalence of antibiotic resistant *E. coli*.

4.1.2 Diversity and prevalence of multiple antibiotic resistant *E. coli* in Avon River and Silver Stream

From chapter 2, where I recovered varied antibiotic resistant *E. coli* populations from both rivers on antibiotic supplemented plates, I sought to better understand the antibiotic resistance profile of *E. coli* in both rivers, and to elaborate on the characterisation of antibiotic resistant *E. coli* in these two rivers. An additional study was conducted in chapter 3 to determine whether *E. coli* isolates that are resistant to one class of antibiotics in chapter 2 of this study are also resistant to other classes of antibiotics. This idea was motivated as a result of high frequencies of antibiotic resistant *E. coli* recovered from both rivers at all sampling times in chapter 2. Antibiotic resistant *E. coli* isolates from both rivers were tested on eight additional antibiotics include ciprofloxacin, a frontline antibiotic in the treating *E. coli* infections. Higher proportion of *E. coli* isolates that were already resistant to one antibiotic showed different levels of resistance to other antibiotics tested in this assay in chapter 3. Generally, there was higher prevalence of MDR *E. coli* detected in Avon River compared to Silver Stream counterpart. Specifically, approximately 98% of ciprofloxacin resistant *E. coli* were also resistant to ampicillin, 88% to tetracycline, and 54% to trimethoprim. Also, certain percentage of ciprofloxacin resistant *E. coli* were resistant to kanamycin and gentamycin. In contrast, Silver Stream ciprofloxacin resistant *E. coli* were uniformly susceptible to kanamycin and gentamycin. The widespread of MDR among bacterial species is now considered a global concern (Van Duin & Paterson, 2016), some bacteria strains are now resistant to almost all antibiotics (Al-Bahry *et al.*, 2015). The result obtained from the multiple resistant testing suggested that polluted rivers as discussed in chapter 1 of this thesis may harbour *E. coli* that are not only resistant to one class of antibiotic but other unrelated classes of antibiotics.

4.1.3 Determining the minimum ciprofloxacin inhibitory concentration that will stop the growth of ciprofloxacin resistant *E. coli* isolates from Avon River and Silver Stream

From the observations of chapter 2 and MDR testing in chapter 3, I noticed that there was significantly higher ciprofloxacin resistant *E. coli* recovered from Avon River compared to Silver Stream (Chapter 2). Similarly, the result of MDR tested clearly revealed that ciprofloxacin resistant *E. coli* from Avon exhibit higher cross-resistance to other antibiotics tested compared to Silver Stream counterpart. As a result of the difference in MDR, I therefore hypothesised that ciprofloxacin resistant *E. coli* from both rivers may tolerate different concentrations of ciprofloxacin. Information about levels of ciprofloxacin resistance among isolates will aid to better understand the antibiotic resistance pattern of *E. coli* isolates from

both rivers, I determined the minimum ciprofloxacin concentration that will inhibit the growth of ciprofloxacin resistant *E. coli* isolates from Avon River and Silver Stream. Ciprofloxacin resistant *E. coli* isolates from both rivers showed different levels of resistance to increasing concentration of ciprofloxacin. Isolates from Silver Stream grew at 2 µg/mL, but were all susceptible at 4 µg/mL. However, ciprofloxacin resistant isolates from Avon River grew at 16 µg/mL, which is 8-times the clinical breakpoint resistance (Humphries *et al.*, 2012). At clinical breakpoint, bacterial infection is considered untreatable.

4.1.4 Determining the prevalence of extended spectrum beta-lactams resistant *E. coli* among ampicillin resistant *E. coli*

Owing to the high prevalence of ampicillin resistant *E. coli* recovered from both rivers in chapter 2, I hypothesised that *E. coli* isolates from Avon River and Silver Stream may also be resistant to other beta-lactams. I investigated this by randomly selecting ampicillin resistant *E. coli* recovered from both rivers and tested against cephalosporin (cefotaxime), a beta-lactam class of antibiotics. Resistance to cefotaxime is an indication to extended spectrum beta-lactam ESBL-producing *E. coli*. One mechanism *E. coli* used to become resistant to this class of antibiotic is through production of beta-lactamase, an enzyme which hydrolyse beta-lactam ring of the antibiotics (Shaikh *et al.*, 2015). *E. coli* may acquire antibiotic resistance genes that confer resistance to beta-lactam antibiotics (Shaikh *et al.*, 2015; Vaidya, 2011), thereby contributing to the prevalence of beta-lactam resistance as seen in this study. It was observed that higher proportion of *E. coli* isolates from Avon River survived higher concentration of cefotaxime while Silver Stream counterparts were uniformly susceptible at concentration < 2 µg/mL. I further performed a phenotypic confirmation test on all *E. coli* isolates that grew at 8 µg/mL of cefotaxime using the double disk method in accordance to the CLSI guidelines. Zones of inhibition were measured, and (5 out of 12) isolates from Avon River had an inhibition zone greater than 5 mm around amoxicillin-clavulanic acid which confirmed the presence of ESBL-producing *E. coli*. The spread of ESBL producing *E. coli* in the environment, especially aquatic environment has tremendously increased as a result of anthropogenic motivated activities (Tacao *et al.*, 2012). Although, the emergence of ESBL producing *E. coli* environment remains unclear, but study has revealed that these ESBL resistant *E. coli* was first detected in the environment two decades after its outbreak in clinical settings (Kitzis *et al.*, 1988). This suggests that environmental ESBL producing *E. coli* may have been resulted from fecal pollution in the environments that are highly influenced by human activities (Guenther *et al.*, 2011).

4.1.5 Sequencing of phenotypically antibiotic resistant *E. coli*

The work presented in this study has clearly supported the notion that environment that receives anthropogenic impacts may inhabit diverse *E. coli* populations that are multi-drug resistant compared to a less impacted environment. The isolated multiple resistant *E. coli* can form the foundation of future studies. This study in this thesis showed that phenotypic traits displayed by *E. coli* (resistance to different antibiotics), is likely an indication of genotypic traits which are often acquired via horizontal gene transfer. The study here presented could be followed up and expanded upon in many ways. One way future work can expand on this research would be to analyse the genomic sequence of the *E. coli* isolated in this study to investigate the genetic basis for antibiotic resistance and the genetic variability of *E. coli* isolates from both rivers. I created a foundation for future research by collaborating with the Institute of Environmental Science and Research (ESR). 50 *E. coli* isolates were sent to ESR for whole genome sequencing. The sequences strains were compared with the international database (Forde *et al.*, 2014). All isolates have closer identity to *E. coli* than other Enterobacteriaceae. Sequencing of *E. coli* isolates is one of the methods that could enable me to identify different resistance genes that are present in each of the isolates. However, the sequencing result came back late and cannot meet up with the deadline of this thesis.

Another way future research can expand on this study is to use the information available from the whole genome sequencing of these isolates to investigate the diversity, types of resistance and mechanisms of resistance used by *E. coli* to survive in the presence of different classes of antibiotics. Also, sequencing information could shed light on the different origins and sources of the *E. coli* isolates. This can be done by comparing each of the *E. coli* genome sequence with a reference genome of *E. coli* from different sources, which could be achieved by producing phylogenetic and proteomics tree to identify the important genome of the sequence. Through the use hierarchical clustering, variable genes could be classified in to cluster of know origin or other classification that might be of interest (Lukjancenko *et al.*, 2010). For instance, there are species that originated from human (Law, 2000), some other from animals such as cattle, sheep or goat (Callaway *et al.*, 2009), while some could originate from birds (Hubálek, 1994). With the availability of whole genome sequence, future research in this field can trace the source and origin of *E. coli* in rivers.

Finally, future study can expand on this research by characterising isolated *E. coli* based on both phenotypic and genotypic antibiotic resistance. One way this can be done is to investigate whether if any of the gene encoding resistance is knocked out, *E. coli* isolate will remain

resistant to the same number and concentrations antibiotics initially resistant to during MDR testing. This kind of investigation will be useful in the understanding of the discrepancies associated with phenotypic-genotypic antibiotic resistance. Furthermore, this will shed light on other mechanisms that may be responsible for the prevalence of antibiotic resistance other than the resistance genes present in *E. coli* isolates. There are several mechanisms used by *E. coli* to protect itself from antibiotic activity. Some of such mechanisms include modification of antibiotic. This involves prevention of antibiotic molecules from entering the target site for binding to *E. coli* cell. For example, the beta-lactamase produced by *E. coli* binds to the beta-lactam rings of beta-lactam antibiotics and render them inactive (Rawat & Nair, 2010). Another mechanism *E. coli* use in resisting antibiotics is through prevention of antibiotics from entering the cell. *E. coli* uses a water filled membrane protein called porin in expelling antibiotics from it cell (Delcour, 2009). Finally, is through production of an alternative target site for antibiotic binding. The alternative targets are usually enzymes, which are produced to bypass the toxicity of antibiotics. A good example of this mechanism is the production of alternative penicillin binding protein produced together with the normal penicillin binding protein in bacteria (Hawkey, 1998)

4.1.6 Limitations and future perspectives

One major limitation of this study is that samples were collected once during each of the sampling season, as this is what was achievable within the scope of this thesis. Therefore, my result could not tell whether there is a significant difference within season. Future study should consider multiple samplings within different seasons in order to see whether there will be a significant different in antibiotic resistance profile of *E. coli* within each season. However, what was achievable within the time frame of this thesis was replicates samples collected from four different locations during each sampling season from both rivers as explained in the material and methods section. Another limitation of this study is that there was no additional river that was less anthropogenically impacted. Avon River and Silver Stream were the only two rivers accessible within Canterbury region of New Zealand for this study. Therefore, I was unable to compare my result with no other river. However, the results presented in this study clearly showed that the rivers studied here host different populations of antibiotic resistant *E. coli*. Future study in this area should consider sampling the headwaters of Avon River and additional locations before and after Hagley Park. This will help to further elucidate why antibiotic resistant *E. coli* were more prevalent within the Park compared to other location. I attempted to investigate the source of antibiotic resistance observed within Hagley Park by

sampling an additional location upstream of location 2. But during the sampling times, *E. coli* population detected in Avon River was generally low. Furthermore, there was no statistical difference between antibiotic resistance populations detected at the Harper Avenue, upstream location 2 and locations within Hagley Park.

Conclusions

Polluted rivers have been reported to be habitat for multiple resistant bacteria. This study has showed the prevalence of MDR *E. coli* in two Canterbury rivers, namely Avon River and Silver Stream. In particular, higher frequency of resistance was recorded in Avon River, which could be attributed different anthropogenic impact. The prevalence of antibiotic resistance *E. coli* in Avon River also suggests the presence of other pathogenic bacteria. It will be commendable if the Ministry of Environment New Zealand and the Ministry of Health could incorporate antibiotic resistance assessment and *E. coli* count in assessing the environmental health risk of rivers in New Zealand.

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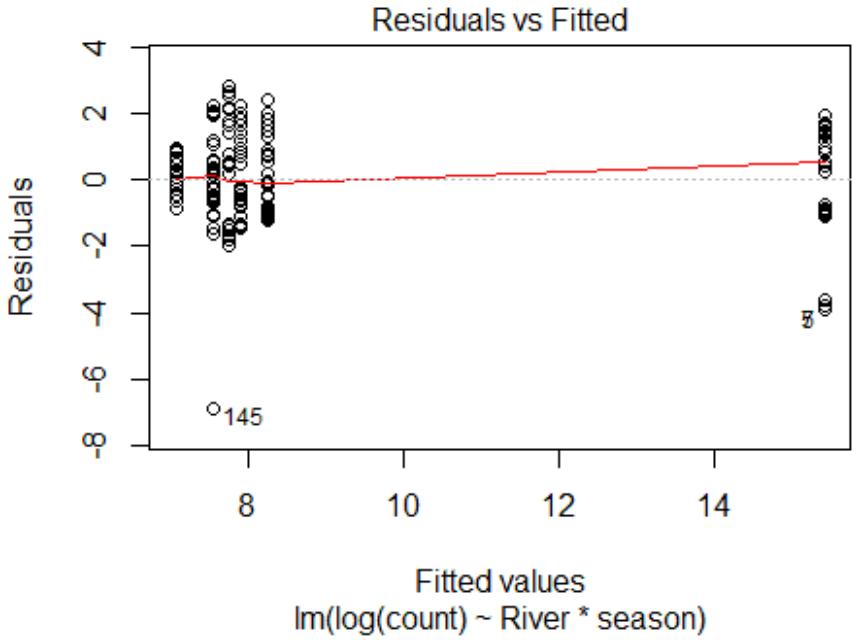
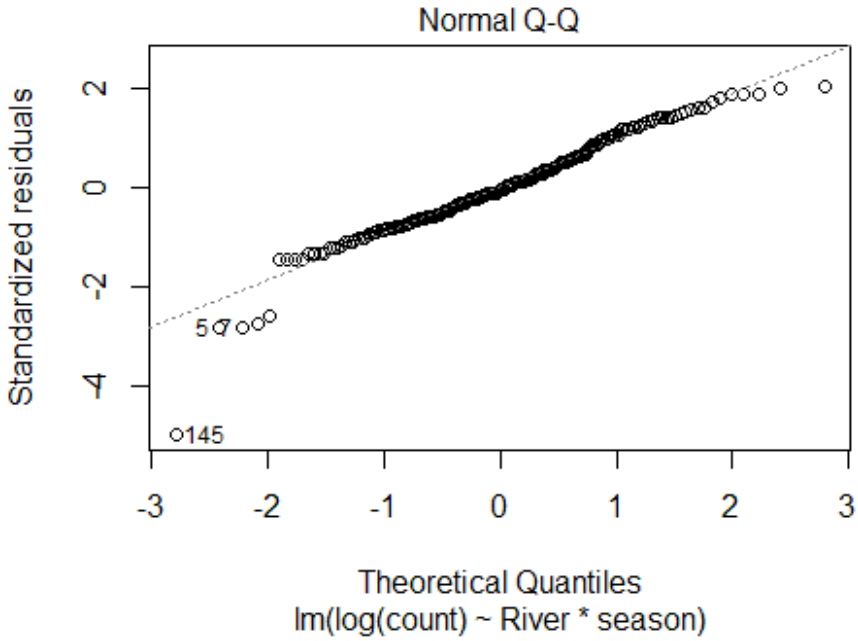
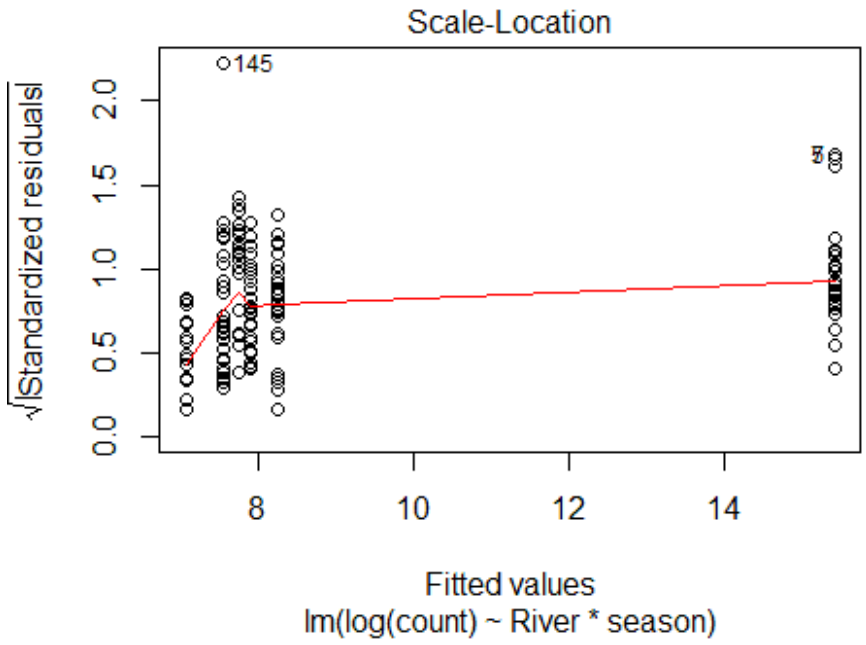
Supplementary material

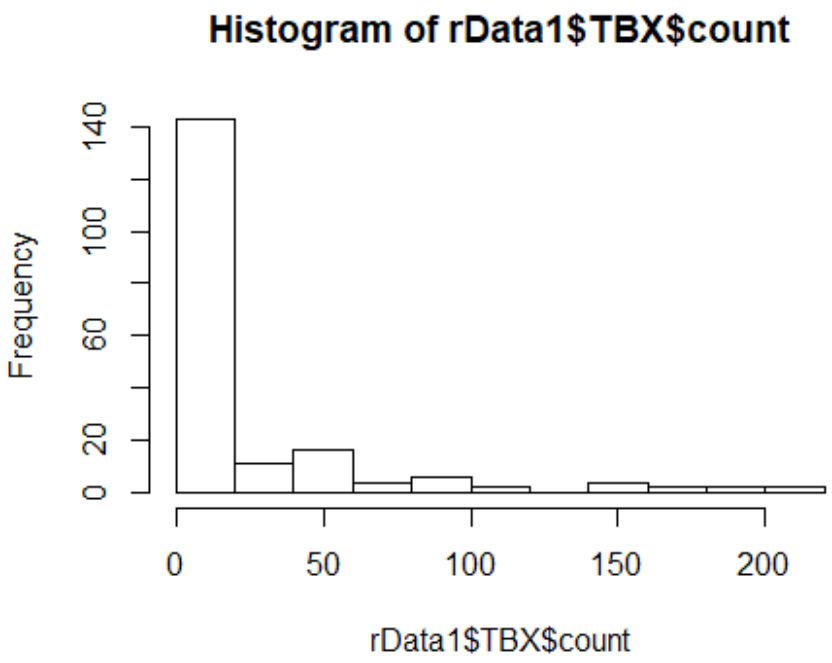
River analysis

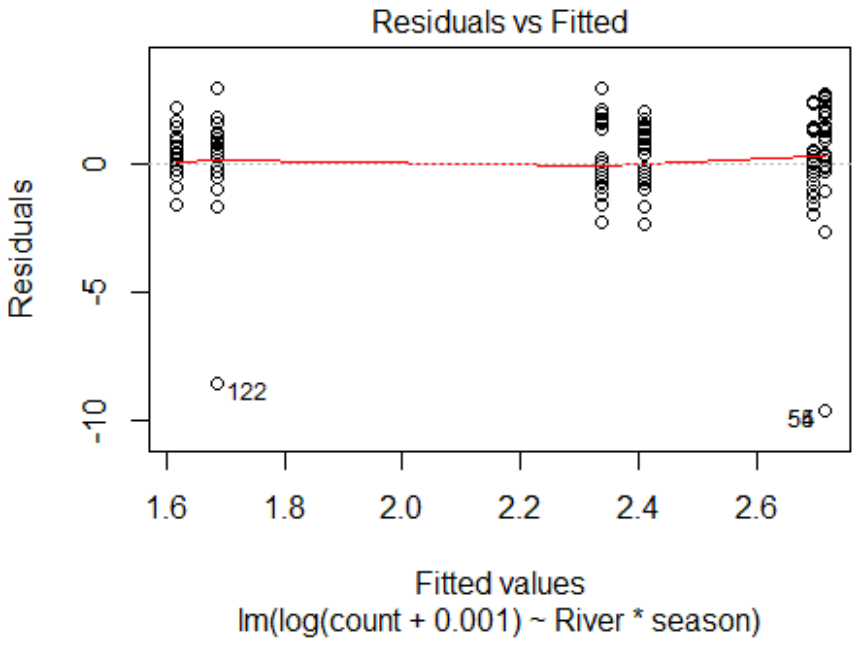


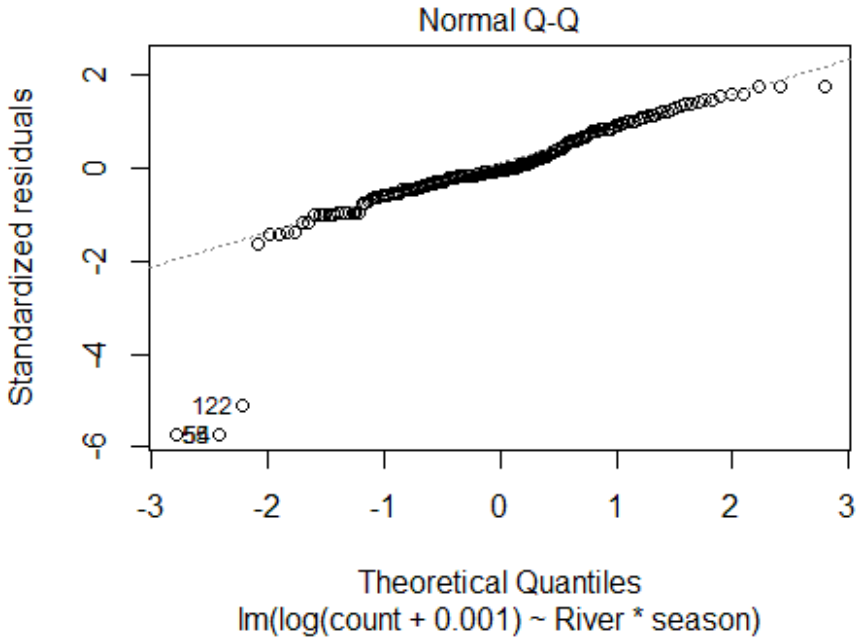


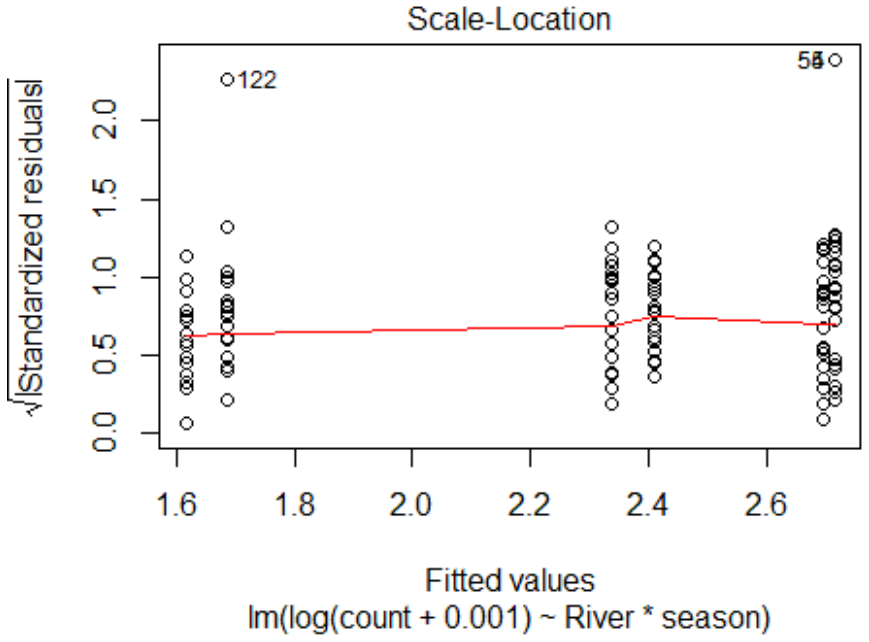

```
## hat values (leverages) are all = 0.03125
##  and there are no factor predictors; no plot no. 5
```

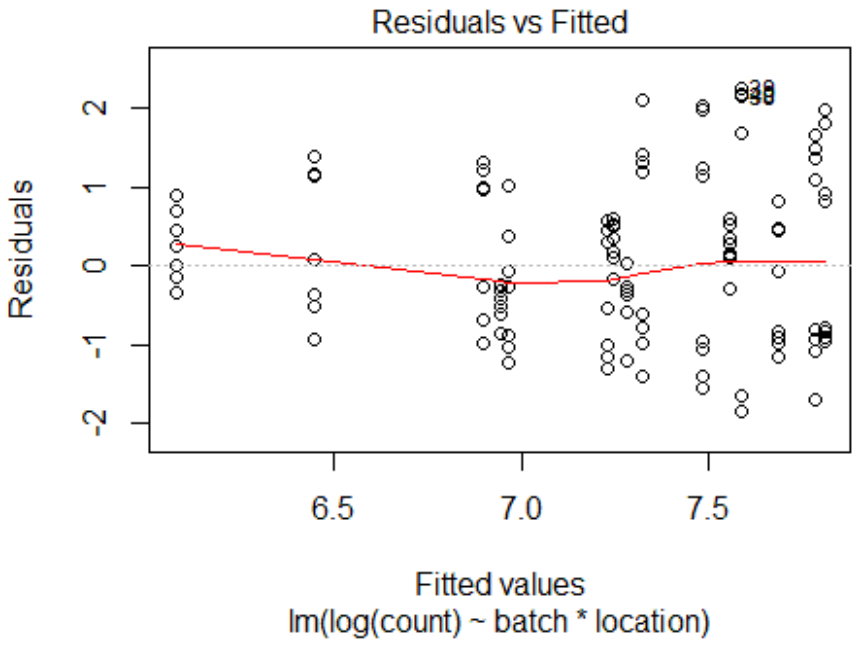

Scale-Location
145
√|Standardized residuals|
0.0
0.5
1.0
1.5
2.0
8
10
12
14
Fitted values
lm(log(count) ~ River * season)

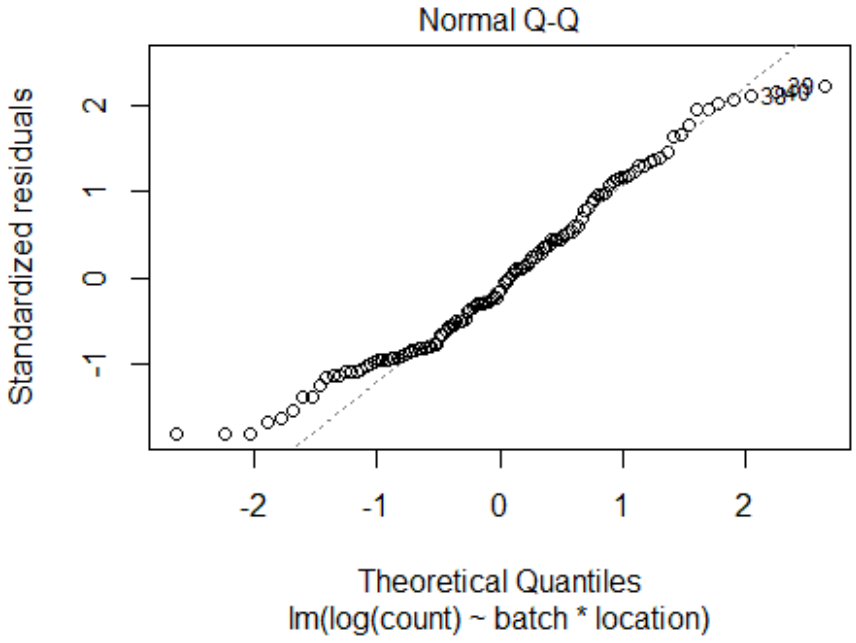

Histogram of rData1TBXcount
Frequency
0
20
60
100
140
0
50
100
150
200
rData1TBXcount

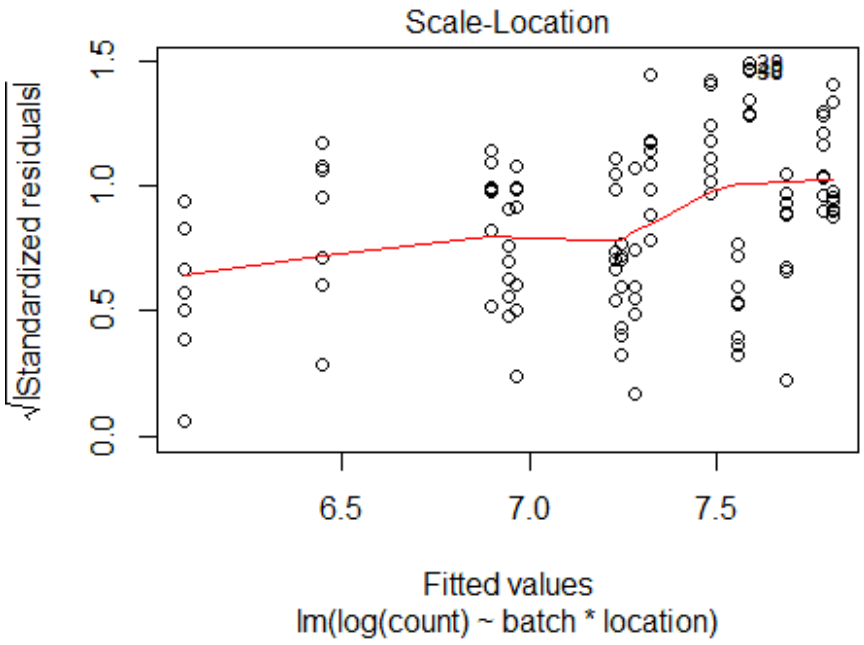

Residuals vs Fitted
Residuals
0
-5
-10
122
1.6
1.8
2.0
2.2
2.4
2.6
Fitted values
lm(log(count + 0.001) ~ River * season)

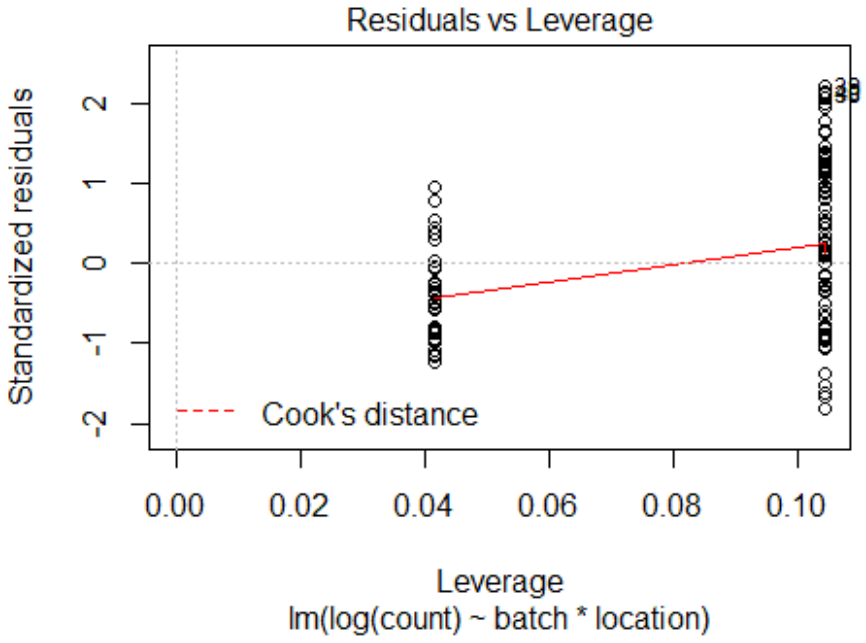

Normal Q-Q
Standardized residuals
Theoretical Quantiles
lm(log(count + 0.001) ~ River * season)

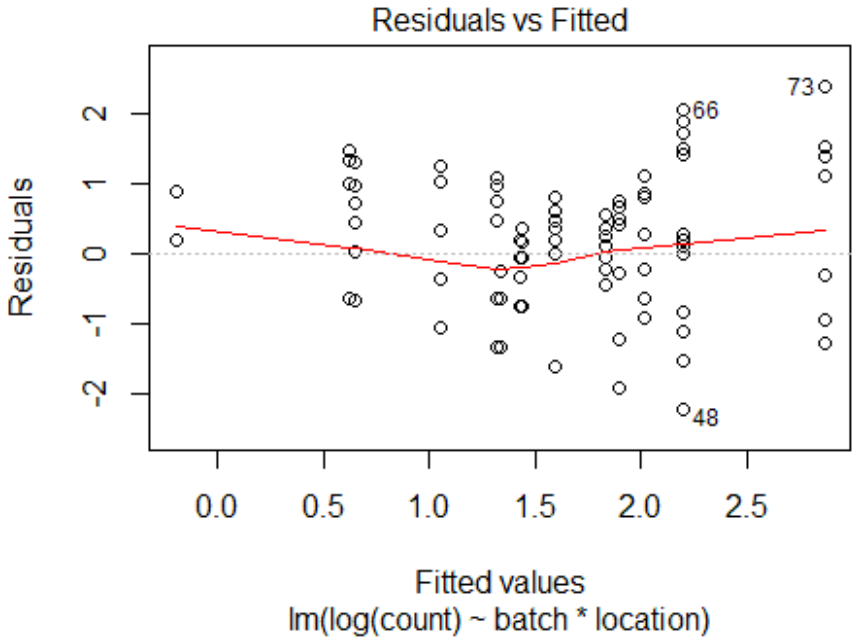

Scale-Location
√|Standardized residuals|
Fitted values
lm(log(count + 0.001) ~ River * season)

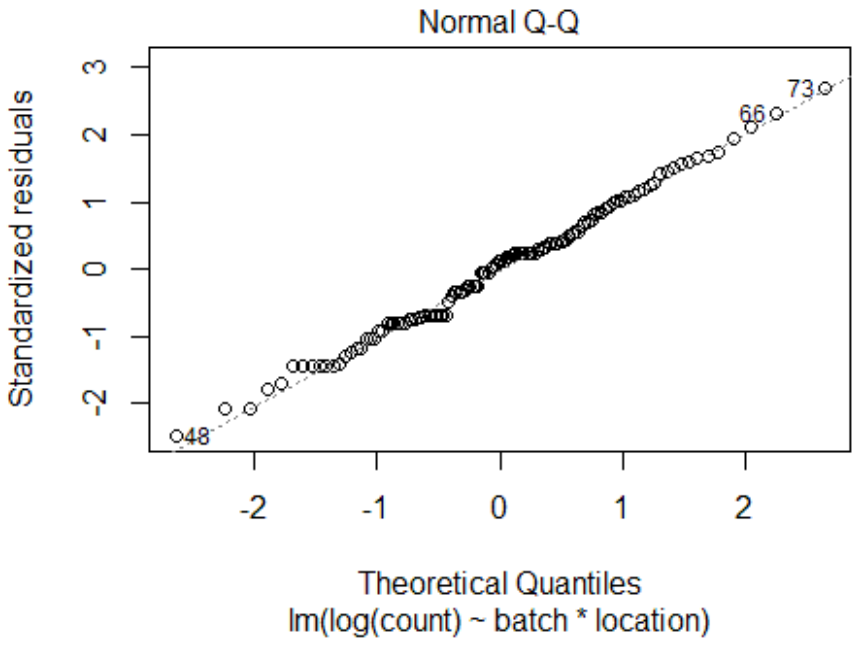

Residuals vs Fitted
Residuals
2
1
0
-1
-2
6.5
7.0
7.5
Fitted values
lm(log(count) ~ batch * location)

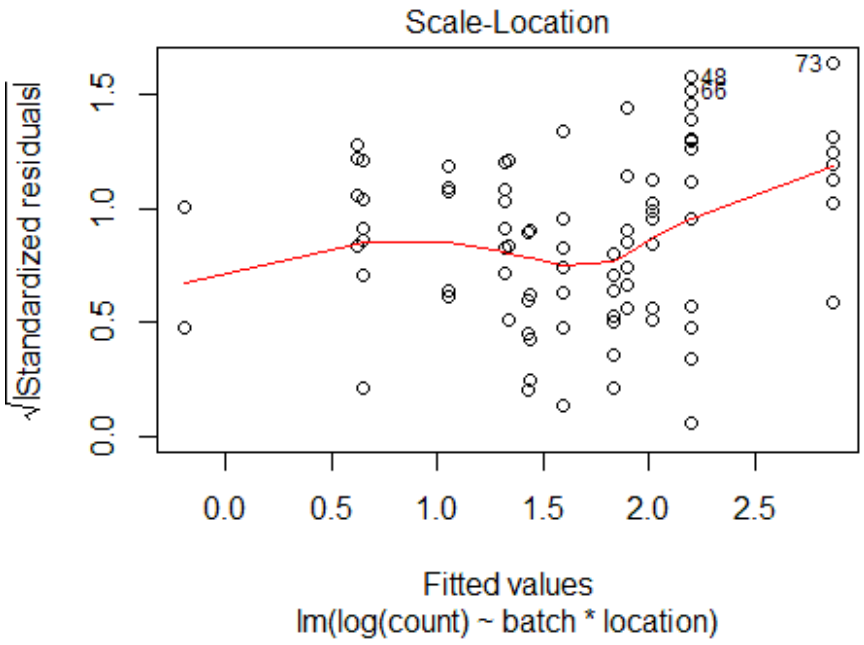

Normal Q-Q
Standardized residuals
2
1
0
-1
-2
-1
0
1
2
Theoretical Quantiles
lm(log(count) ~ batch * location)

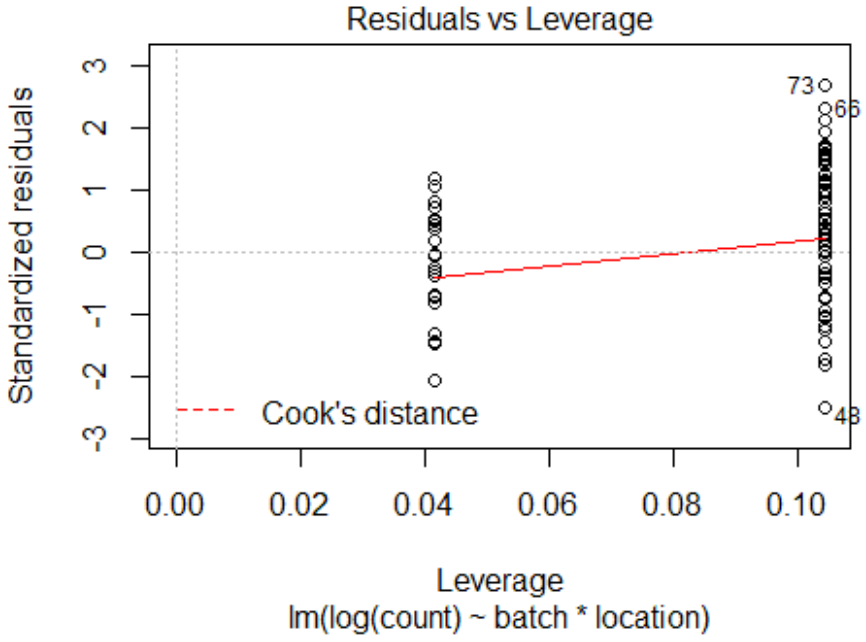

Scale-Location
√|Standardized residuals|
0.0
0.5
1.0
1.5
6.5
7.0
7.5
Fitted values
lm(log(count) ~ batch * location)


Residuals vs Leverage
Standardized residuals
-2
-1
0
1
2
Cook's distance
0.00
0.02
0.04
0.06
0.08
0.10
Leverage
lm(log(count) ~ batch * location)


Residuals vs Fitted
Residuals
Fitted values
lm(log(count) ~ batch * location)
73
66
48


Normal Q-Q
Standardized residuals
Theoretical Quantiles
lm(log(count) ~ batch * location)
73
66
48



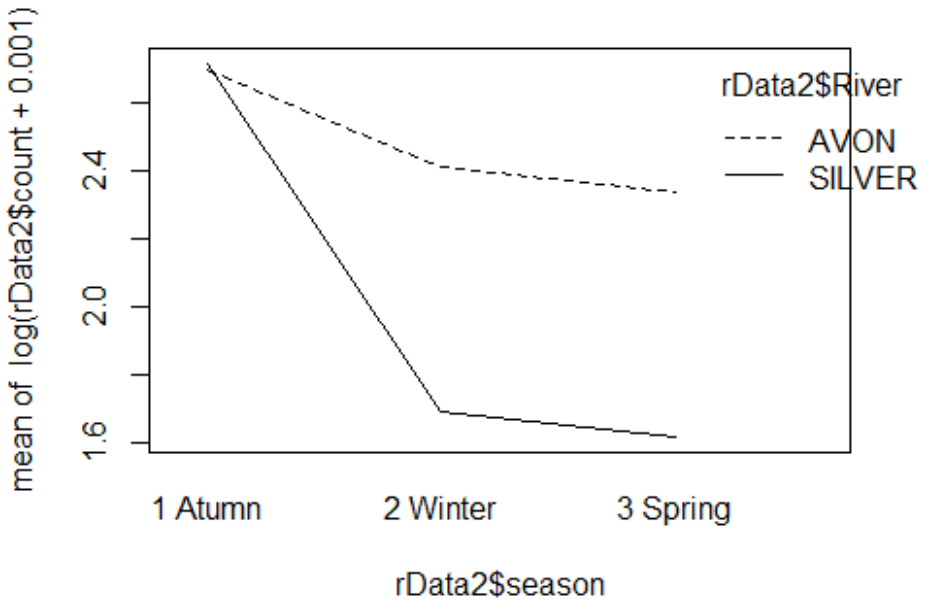


Mesophiles count Analysis

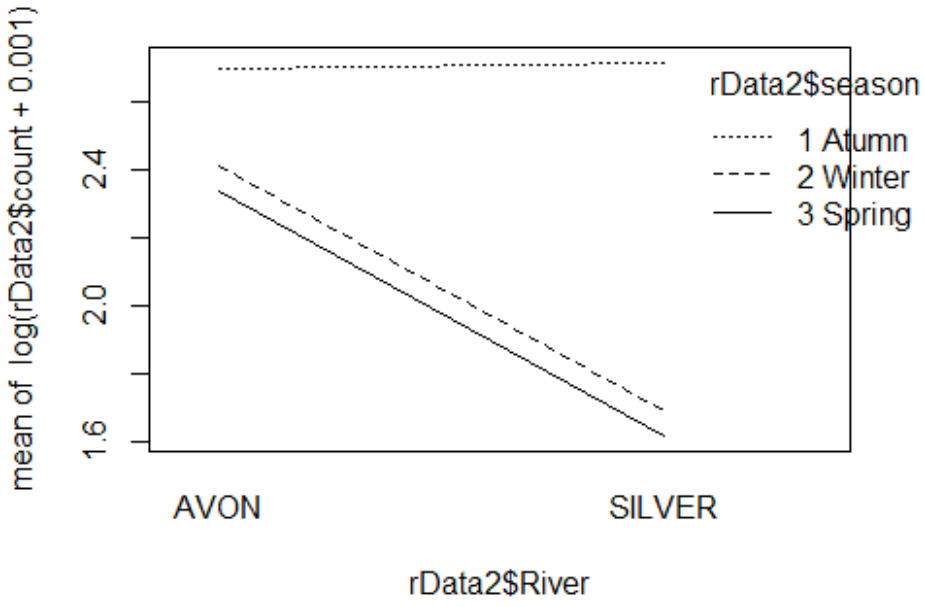
```
anova(m1)
```

```
## Analysis of Variance Table
##
## Response: log(count)
##               Df Sum Sq Mean Sq F value    Pr(>F)
## River          1 386.83  386.83  195.79 < 2.2e-16 ***
```

```
## season          2 494.54  247.27  125.15 < 2.2e-16 ***
## River:season    2 733.06  366.53  185.51 < 2.2e-16 ***
## Residuals     186 367.49    1.98
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```




```
interaction.plot(rData2$River,rData2$season,log(rData2$count+0.001))
```



E. coli count Analysis

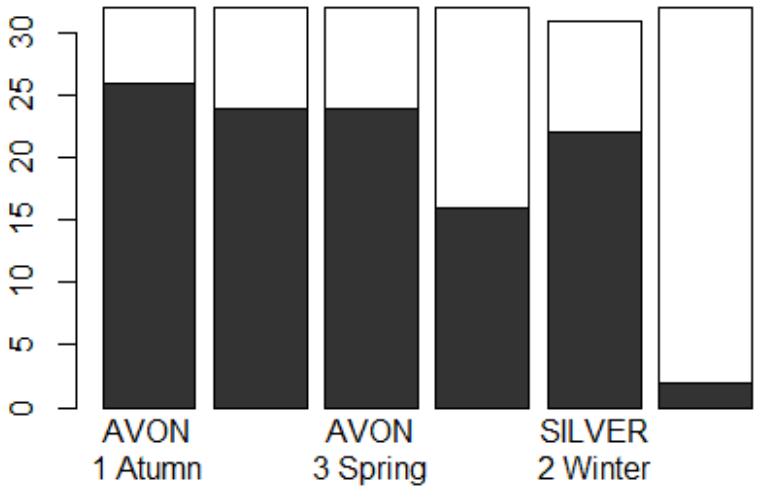
```
summary.aov(m2a)
```

```
##                Df Sum Sq Mean Sq F value Pr(>F)
## River           1   10.8  10.819   3.711 0.0556 .
## season          2   20.6  10.315   3.538 0.0310 *
## River:season    2    5.9   2.936   1.007 0.3673
## Residuals     186  542.3   2.916
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

E. coli AMP Analysis using glm

```
anova(m3, test="Chisq")
```

```
## Analysis of Deviance Table
##
## Model: binomial, link: logit
##
## Response: countMoreThan0
##
## Terms added sequentially (first to last)
##
##
##              Df Deviance Resid. Df Resid. Dev  Pr(>Chi)
## NULL                           190     257.57
## River         1   24.902       189     232.67 6.031e-07 ***
## season        2   17.508       187     215.16 0.0001579 ***
## River:season  2   15.620       185     199.54 0.0004056 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```



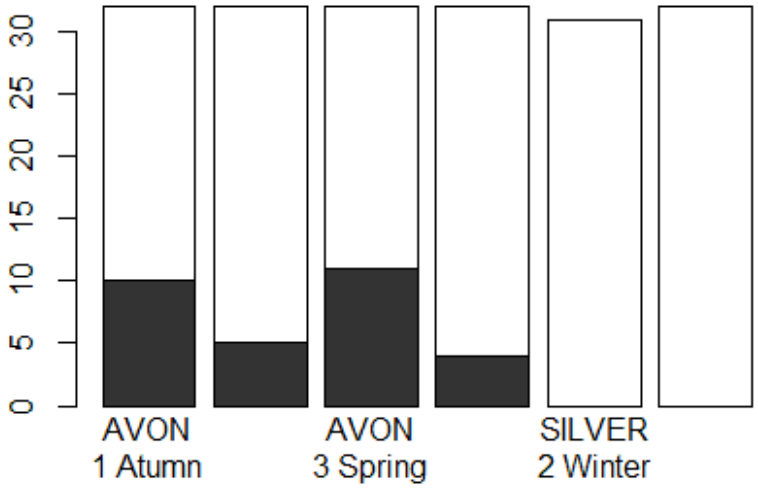

```
table((rData1$`E. coli AMP`$countMoreThan0),tc)
```

```
##         tc
##          AVON\n1 Atumn AVON\n2 Winter AVON\n3 Spring SILVER\n1 Atumn
##   FALSE              6              8              8              16
##   TRUE              26             24             24              16
##         tc
##          SILVER\n2 Winter SILVER\n3 Spring
##   FALSE                9               30
##   TRUE                22                2
```

```
                        E. coli CHL Analysis
                       anova(m4, test="Chisq")
```

```
## Analysis of Deviance Table
##
## Model: binomial, link: logit
##
## Response: countMoreThan0
##
## Terms added sequentially (first to last)
##
##
##              Df Deviance Resid. Df Resid. Dev  Pr(>Chi)
## NULL                            190     166.08
## River         1  20.7701       189     145.31 5.179e-06 ***
## season        2   5.9187       187     139.40   0.05185 .
## River:season  2   6.6118       185     132.78   0.03667 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
#tc<-paste(rData1$`E. coli CHL`$River,rData1$`E. coli CHL`$season)
barplot(table((1-rData1$`E. coli CHL`$countMoreThan0),tc))
```




```
table((rData1$`E.coli CHL`$countMoreThan0),tc)
```

```
##        tc
##         AVON\n1 Atumn AVON\n2 Winter AVON\n3 Spring SILVER\n1 Atumn
##   FALSE            22             27             21               28
##   TRUE             10              5             11                4
##        tc
##         SILVER\n2 Winter SILVER\n3 Spring
##   FALSE               31               32
##   TRUE                 0                0
```

E. coli RIF Analysis

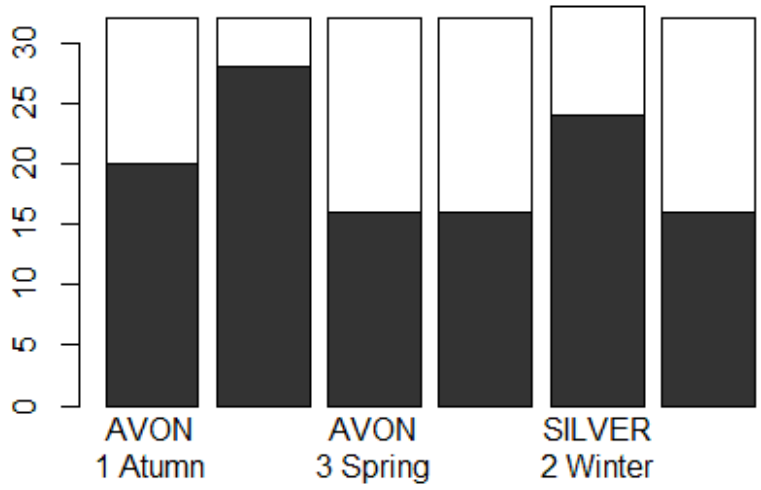
```
anova(m5, test="Chisq")
```

```
## Analysis of Deviance Table
##
## Model: binomial, link: logit
##
## Response: countMoreThan0
##
## Terms added sequentially (first to last)
##
##
##              Df Deviance Resid. Df Resid. Dev  Pr(>Chi)
## NULL                           192     255.99
## River         1   1.6410       191     254.35 0.2001931
## season        2  14.6992       189     239.65 0.0006428 ***
## River:season  2   1.4426       187     238.21 0.4861077
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
tc<-paste(rData1$`E. coli RIF`$River,rData1$`E. coli RIF`$season,sep="\n")
```

```
barplot(table((1-rData1$`E.colI RIF`$countMoreThan0),tc))
```




```
table((rData1$`E. coli RIF`$countMoreThan0),tc)
```

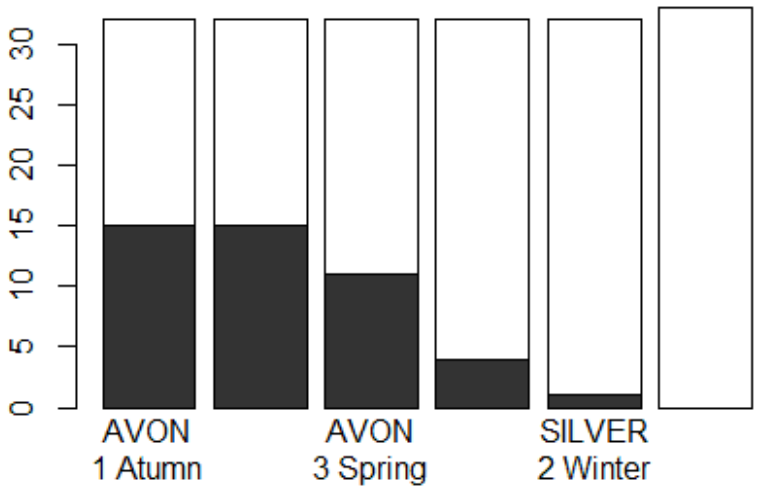
```
##         tc
##          AVON\n1 Atumn AVON\n2 Winter AVON\n3 Spring SILVER\n1 Atumn
##   FALSE             12              4             16              16
##   TRUE              20             28             16              16
##         tc
##          SILVER\n2 Winter SILVER\n3 Spring
##   FALSE                9               16
##   TRUE                24               16
```

```
                          E. coli CIP Analysis
                         anova(m6, test="Chisq")
```

```
## Analysis of Deviance Table
##
## Model: binomial, link: logit
##
## Response: countMoreThan0
##
## Terms added sequentially (first to last)
##
##
##              Df Deviance Resid. Df Resid. Dev  Pr(>Chi)
```

```
## NULL                           192     211.98
## River         1   41.550       191     170.43 1.149e-10 ***
## season        2    3.585       189     166.84    0.1666
## River:season  2    4.172       187     162.67    0.1242
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
tc<-paste(rData1$`E. coli CIP`$River,rData1$`E. coli CIP`$season,sep="\n")
barplot(table((1-rData1$`E. coli CIP`$countMoreThan0),tc))
```

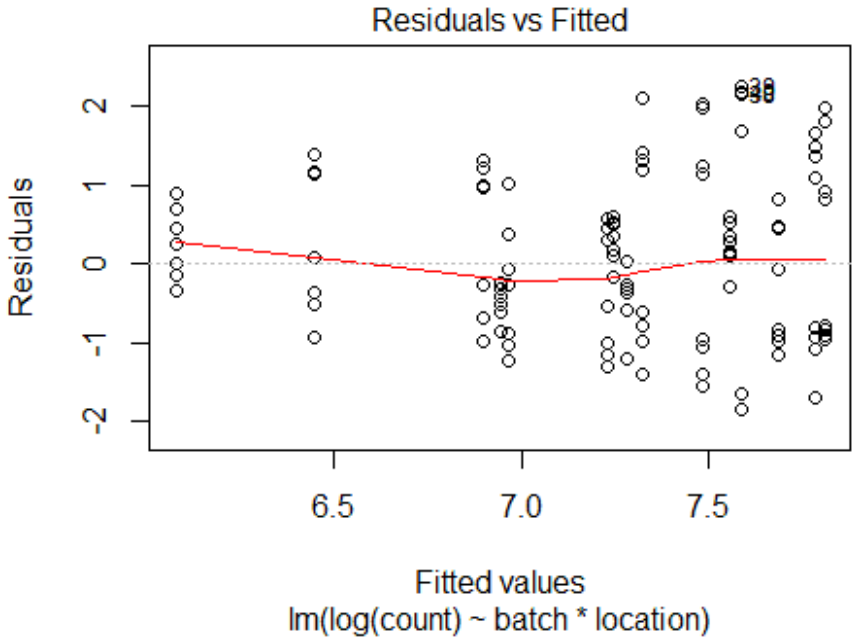


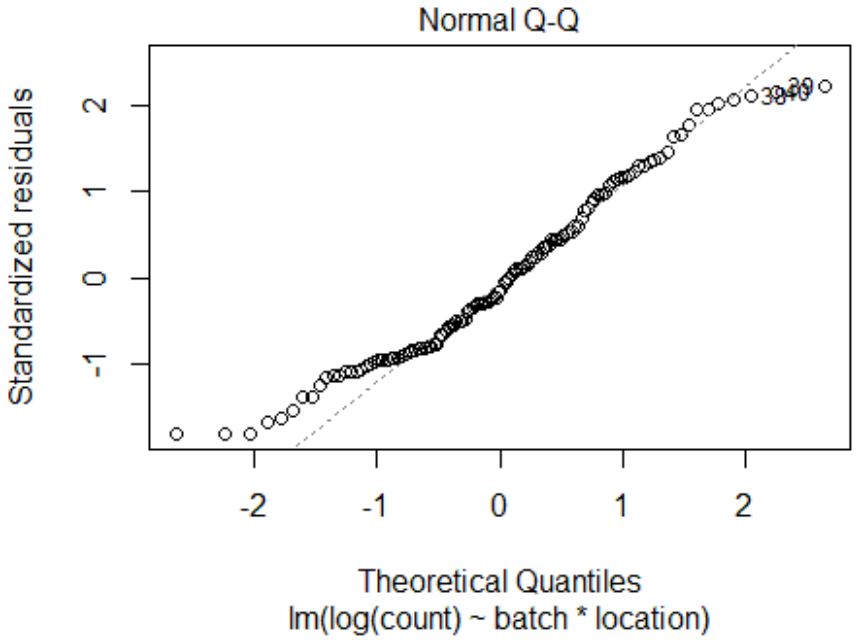

```
table((rData1$`E. coli CIP`$countMoreThan0),tc)
```

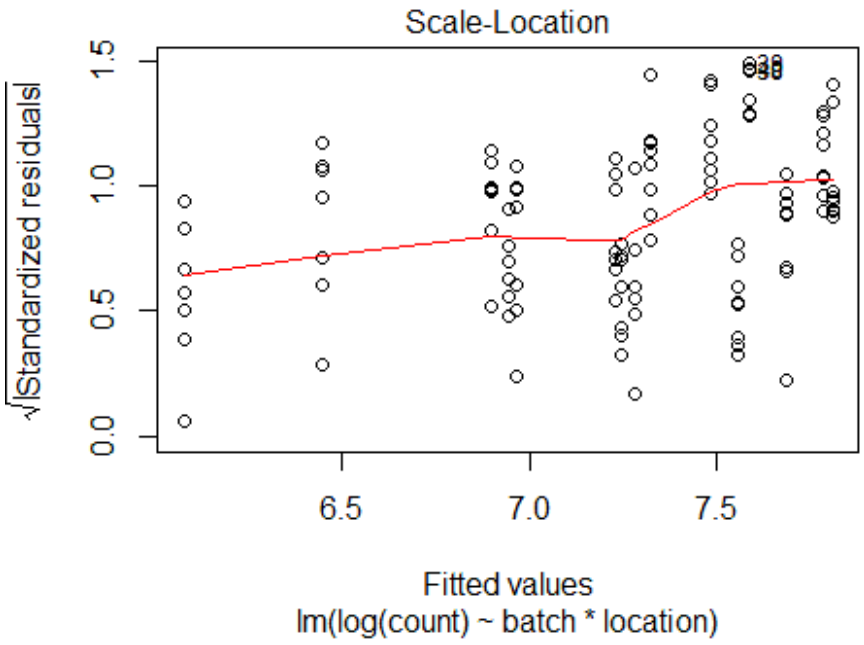
```
##         tc
##          AVON\n1 Atumn AVON\n2 Winter AVON\n3 Spring SILVER\n1 Atumn
##   FALSE             17              17              21               28
##   TRUE              15              15              11                4
##         tc
##          SILVER\n2 Winter SILVER\n3 Spring
##   FALSE                31                33
##   TRUE                  1                 0
```

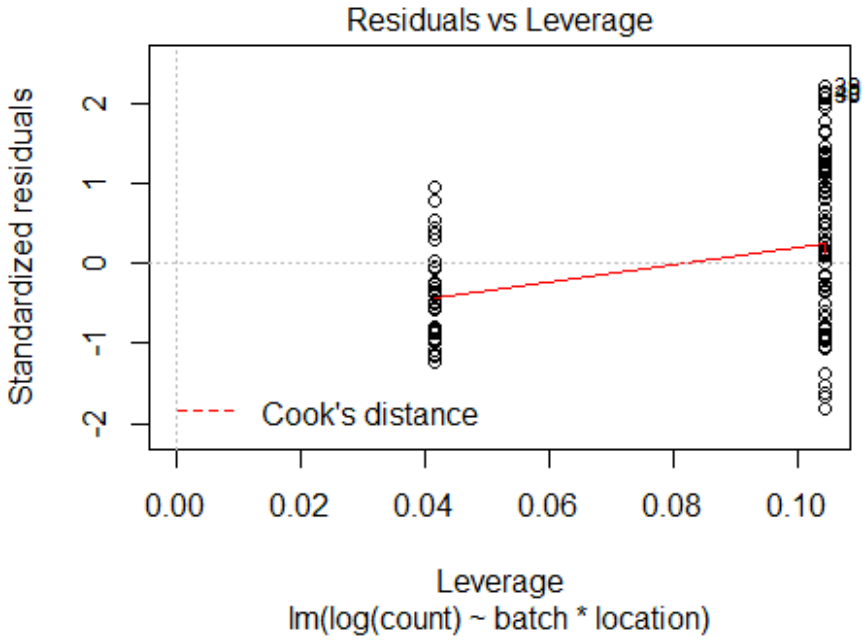
Question 3: analysis of avon longitudinal study

```
#First analysis considers R2A. Counts were always above zero, as a result
we used linear regression to test for a relationship between log(EOP) and
batch, season and the interaction between the two.
plot(m7)
```


Residuals vs Fitted
Residuals
2
1
0
-1
-2
6.5
7.0
7.5
Fitted values
lm(log(count) ~ batch * location)


Normal Q-Q
Standardized residuals
2
1
0
-1
-2
-1
0
1
2
Theoretical Quantiles
lm(log(count) ~ batch * location)



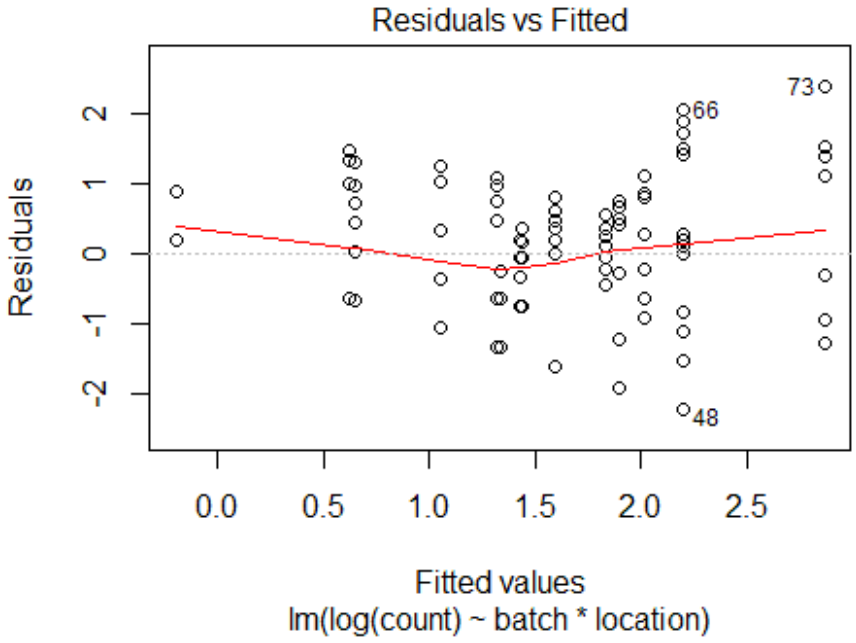


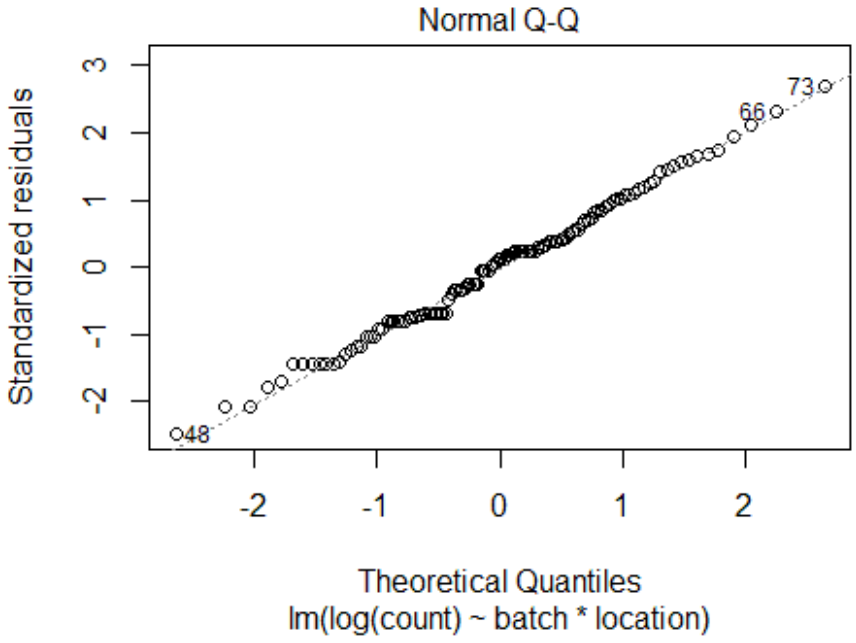

```
#residuals look ok
```

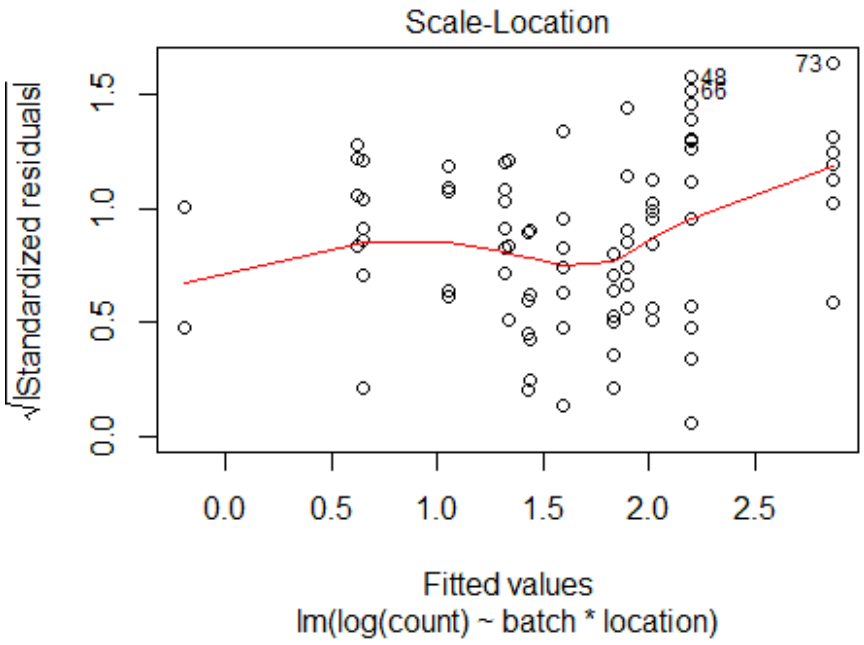
```
summary.aov(m7)
```

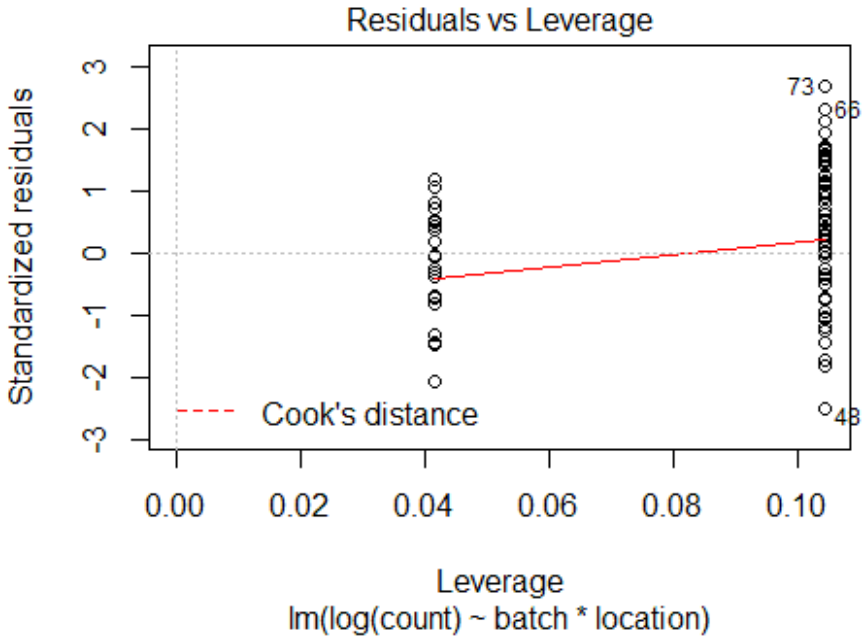
```
##                  Df Sum Sq Mean Sq F value  Pr(>F)
## batch             1  10.08  10.079   8.728 0.00383 **
## location          4   8.65   2.162   1.872 0.12041
## batch:location    4   8.14   2.036   1.763 0.14140
## Residuals       110 127.03   1.155
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

#nothing is significant in anova

R2amultcomp

##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: User-defined Contrasts
##
##
## Fit: aov(formula = log(count) ~ mergedFactors, data = rDataQ3_1$R2A)
##
## Linear Hypotheses:
##                       Estimate Std. Error t value Pr(>|t|)
## 2,3,4 versus 1 == 0  -0.2366     0.2465  -0.960   0.3392
## 2,3,4 versus 5 == 0  -0.6387     0.2465  -2.591   0.0218 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- holm method)

#in contasts 5 is significantly different from 2,3,4

#second analysis consdiers TBX, counts were always above zero, a result we
used linear regression to test for a relationship between log(EOP) and
batch, season and the interaction between the two.

plot(m8)
```


Residuals vs Fitted
Residuals
Fitted values
lm(log(count) ~ batch * location)
73
66
48


Normal Q-Q
Standardized residuals
Theoretical Quantiles
lm(log(count) ~ batch * location)
73
66
48






```
summary.aov(m8)
```

```
##                  Df Sum Sq Mean Sq F value   Pr(>F)
## batch             1  30.66  30.658  35.045 3.73e-08 ***
## location          4   5.37   1.343   1.535    0.197
## batch:location    4  28.39   7.097   8.112 9.05e-06 ***
## Residuals       110  96.23   0.875
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
TBXmultcomp
```

```
##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: User-defined Contrasts
##
##
## Fit: aov(formula = log(count) ~ mergedFactors, data = rDataQ3_1$TBX)
##
## Linear Hypotheses:
##                       Estimate Std. Error t value Pr(>|t|)
## 2,3,4 versus 1 == 0 -0.49583    0.21243  -2.334    0.043 *
## 2,3,4 versus 5 == 0  0.07843    0.21243   0.369    0.713
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- holm method)
```

```
#Third analysis consdiers TBX AMP.

table(rDataQ3_1$`TBX AMP`$countMoreThan0)
```

```
##
## FALSE  TRUE
##    55    65
```

```
#Half of the observations have counts of 0 as a result it won't be
possible to use linear regression.
#here are teh counts broken down by category

tLabel<-paste(as.factor(rDataQ3_1$`TBX
AMP`$batch),as.factor(rDataQ3_1$`TBX AMP`$season))
table(rDataQ3_1$`TBX AMP`$countMoreThan0,tLabel)
```

```
##        tLabel
##         1 spring 2 late spring 3 summer
##   FALSE       12            21       22
##   TRUE        28            19       18
```

```
#Instead
#we use logistic regression/glm to test if the number of counts>0 varies
with category.

anova(m9, test="Chisq")
```

```
## Analysis of Deviance Table
##
## Model: binomial, link: logit
##
## Response: countMoreThan0
##
## Terms added sequentially (first to last)
##
##
##                Df Deviance Resid. Df Resid. Dev Pr(>Chi)
```

```
## NULL                              119     165.52
## batch           1   5.0899        118     160.43  0.02407 *
## location        4   2.4601        114     157.97  0.65179
## batch:location  4   1.2005        110     156.77  0.87802
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
TBXAMPmultcomp
```

```
##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: User-defined Contrasts
##
##
## Fit: glm(formula = countMoreThan0 ~ mergedFactors, data =
rDataQ3_1$`TBX AMP`)
##
## Linear Hypotheses:
##                     Estimate Std. Error z value Pr(>|z|)
## 2,3,4 versus 1 == 0  0.04167    0.11925   0.349    0.727
## 2,3,4 versus 5 == 0  0.16667    0.11925   1.398    0.324
## (Adjusted p values reported -- holm method)
```

```
#in contrasts 1 is significantly different from 2,3,4

#fourth analysis considers TBX CHL

table(rDataQ3_1$`TBX CHL`$countMoreThan0)
```

```
##
## FALSE  TRUE
##   103    17
```

```
#the vast majority of the counts are equal to 0.
#here are the counts broken down by category

tLabel<-paste(as.factor(rDataQ3_1$`TBX
CHL`$batch),as.factor(rDataQ3_1$`TBX CHL`$season))
table(rDataQ3_1$`TBX CHL`$countMoreThan0,tLabel)
```

```
##        tLabel
##         1 spring 2 late spring 3 summer
##   FALSE       28            35       40
##   TRUE        12             5        0
```

```
#As a result we do a glm (see TBX Amp)

anova(m10, test="Chisq")
```

```
## Analysis of Deviance Table
##
## Model: binomial, link: logit
##
## Response: countMoreThan0
```

```
##
## Terms added sequentially (first to last)
##
##
##                Df Deviance Resid. Df Resid. Dev  Pr(>Chi)
## NULL                             119     97.915
## batch           1  16.6525       118     81.262 4.489e-05 ***
## location        4   4.3110       114     76.951    0.3655
## batch:location  4   5.4325       110     71.519    0.2457
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
#Nothing is significant
```

```
TBXCHLmultcomp
```

```
##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: User-defined Contrasts
##
##
## Fit: glm(formula = countMoreThan0 ~ mergedFactors, data =
rDataQ3_1$`TBX CHL`)
##
## Linear Hypotheses:
##                      Estimate Std. Error z value Pr(>|z|)
## 2,3,4 versus 1 == 0 -0.01389    0.07672  -0.181    0.856
## 2,3,4 versus 5 == 0  0.06944    0.07672   0.905    0.731
## (Adjusted p values reported -- holm method)
```

```
#fifth analysis considers TBX RIF
```

```
table(rDataQ3_1$`TBX RIF`$countMoreThan0)
```

```
##
## FALSE  TRUE
##    60    60
```

```
#Half of the observations have counts of 0 as a result it won't be
possible to use linear regression. Instead
#we use logistic regression/glm to test if the number of counts>0 varies
with category.
anova(m11, test="Chisq")
```

```
## Analysis of Deviance Table
##
## Model: binomial, link: logit
##
## Response: countMoreThan0
##
## Terms added sequentially (first to last)
##
##
##                Df Deviance Resid. Df Resid. Dev Pr(>Chi)
```

```
## NULL                              119     166.35
## batch           1        0        118     166.35        1
## location        4        0        114     166.35        1
## batch:location  4        0        110     166.35        1
```

```
#note that the p-values are 1, this is a little wierd, after a bit of
digging i noticed that there are exactly 20
#0s and 20 1's in each treatment combination. Is this right???
```

```
TBXRIFmultcomp
```

```
##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: User-defined Contrasts
##
##
## Fit: glm(formula = countMoreThan0 ~ mergedFactors, data =
rDataQ3_1$`TBX RIF`)
##
## Linear Hypotheses:
##                         Estimate Std. Error z value Pr(>|z|)
## 2,3,4 versus 1 == 0  3.786e-16  1.260e-01       0        1
## 2,3,4 versus 5 == 0 -8.565e-17  1.260e-01       0        1
## (Adjusted p values reported -- holm method)
```

```
tLabel<-paste(as.factor(rDataQ3_1$`TBX
RIF`$batch),as.factor(rDataQ3_1$`TBX RIF`$season))
table(rDataQ3_1$`TBX RIF`$countMoreThan0,tLabel)
```

```
##        tLabel
##         1 spring 2 late spring 3 summer
##   FALSE       20            20       20
##   TRUE        20            20       20
```

```
###next CIP
```

```
tLabel<-paste(as.factor(rDataQ3_1$`TBX
CIP`$batch),as.factor(rDataQ3_1$`TBX CIP`$season))
table(rDataQ3_1$`TBX CIP`$countMoreThan0,tLabel)
```

```
##        tLabel
##         1 spring 2 late spring 3 summer
##   FALSE       29            37       38
##   TRUE        11             3        2
```

```
anova(m12, test="Chisq")
```

```
## Analysis of Deviance Table
##
## Model: binomial, link: logit
##
## Response: countMoreThan0
##
```

```
## Terms added sequentially (first to last)
##
##
##                Df Deviance Resid. Df Resid. Dev Pr(>Chi)
## NULL                            119     94.242
## batch           1   9.3825       118     84.859 0.002191 **
## location        4   8.7936       114     76.066 0.066470 .
## batch:location  4   6.3227       110     69.743 0.176312
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
#effect of location is marginally significant
```

```
CIPmultcomp
```

```
##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: User-defined Contrasts
##
##
## Fit: glm(formula = countMoreThan0 ~ mergedFactors, data =
rDataQ3_1$`TBX CIP`)
##
## Linear Hypotheses:
##                      Estimate Std. Error z value Pr(>|z|)
## 2,3,4 versus 1 == 0 -0.05556    0.07454  -0.745   0.4561
## 2,3,4 versus 5 == 0  0.15278    0.07454   2.050   0.0808 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported
```

***E.coli* isolates used in MDR testing**

E. coli isolate	Amp	Rif	Chl	Cip	Tet	Tri	Gen	Kan
CIP 14	1	0	0	1	0	1	0	0
CIP12	1	1	0	1	1	1	1	1
CIP11	1	1	0	1	1	1	1	1
CIP23	1	0	1	1	1	1	1	0
CIP24	1	1	1	1	1	1	0	0
SCIP22	1	0	0	1	1	1	1	0
CIP 42	1	0	0	1	1	1	1	0
CIP32	0	0	0	1	1	1	0	0
CIP41	1	0	0	1	0	1	0	0
SCIP41	1	0	1	1	1	1	1	1
CIP41	1	0	0	1	0	1	1	1
CIP L2:2	1	1	0	1	1	0	0	0
CIP L2:4	1	0	0	1	1	0	0	0
CIP L2:3	1	0	0	1	1	0	0	0
CIP L3:3	1	0	1	1	1	1	0	0
CIP L3:2	1	1	0	1	1	0	1	0
CIP L3:4	1	0	0	1	1	0	0	0
CIP L4:1	1	1	1	1	1	1	0	0
SCIP3:4	1	0	1	1	1	1	1	1
SCIP 2:1	1	0	1	1	1	1	1	1
CIP L3:4	1	0	0	1	1	0	1	0
CIP L2:4	1	0	0	1	1	0	0	0
CIP L2:1	1	1	1	1	1	0	0	0
CIP L2:3	1	0	0	1	1	0	0	0
CIP L4:4	1	0	0	1	1	0	0	0
CIP L4:1	1	0	0	1	1	0	0	0
CIP L3:2	1	1	1	1	1	0	1	0
CIP L4:3	1	1	0	1	1	0	0	0
CIP L2:2	1	1	0	1	1	0	0	0
CIP L3:1	1	0	0	1	1	0	0	0
CIP L3:4	1	0	0	1	1	0	0	0
CIP L2:1	1	1	0	1	1	0	0	0
CIP L4:2	1	1	0	1	1	0	0	0
SCIP2:4	1	1	1	1	1	0	0	0
CIP L3:3	1	0	0	1	1	0	0	0
CIP L2:4	1	0	1	1	1	0	0	0
CIP 1:4	1	0	0	1	0	1	0	0
CIP 1:2	1	1	0	1	1	1	1	1
CIP 1:1	1	1	0	1	1	1	1	1
CIP 2:3	1	0	1	1	1	1	1	0
CIP2:4	1	1	1	1	1	1	0	0

SCIP 2:2	1	0	0	1	1	1	1	0
CIP 4:2	1	0	0	1	1	1	1	0
CIP 3:2	0	0	0	1	1	1	0	0
CIP 4:1	1	0	0	1	0	1	0	0
SCIP 4:1	1	0	1	1	1	1	1	1
CIP 4:1	1	0	0	1	0	1	1	1
SCIP2:3	1	0	1	1	1	0	0	0
L13CIP	0	0	0	1	1	0	0	0
L14CIP	1	0	1	1	1	0	0	0
L14CIP	0	0	0	1	1	1	0	0
L14CIP	0	0	0	1	1	1	0	0
CHL52	1	1	1	1	1	1	1	0
CHL13	1	0	1	0	1	0	1	0
CHL13	1	1	1	0	0	0	1	1
CHL12	1	0	1	1	1	1	1	1
CHL32	1	0	1	1	1	1	0	0
CHL34	1	1	1	1	0	0	1	1
CHL31	1	1	1	1	0	1	1	1
CHL22	1	1	1	1	1	1	1	1
CHL44	1	1	1	1	0	0	1	1
CHL41	1	1	1	1	1	1	1	1
CHL42	1	1	1	0	0	1	1	1
CHL L4:2	1	0	1	1	1	1	1	0
CHLL3:2	1	0	1	1	1	1	1	0
CHL L3:4	1	0	1	1	1	1	1	0
CHL Ll3:4	1	1	1	1	1	1	0	0
CHL L3:3	1	0	1	0	0	1	0	0
CHL L2:1	1	0	1	0	1	1	0	0
CHL L3:1	1	0	1	1	1	1	0	0
CHL L3:3	1	0	1	1	1	1	0	0
CHL L4:1	1	0	1	0	1	0	0	0
CHL L2:2	1	0	1	0	1	1	0	0
CHL L3:2	1	0	1	0	1	1	1	0
CHL L3:1	1	0	1	1	1	0	0	0
CHL L1:1	1	0	1	1	1	0	0	0
CHL L4:4	0	0	1	1	1	1	0	0
CHL L2:3	1	0	1	0	0	1	0	0
CHL L4:2	1	0	1	0	1	0	0	0
CHL L1:1	1	0	1	1	1	0	0	0
CHL L2:3	1	0	1	0	1	1	0	0
CHL L2:4	1	0	1	0	1	1	0	0
CHL 5:2	1	1	1	1	1	1	1	0
CHL 1:3	1	0	1	0	1	0	1	0
CHL 1:3	1	1	1	0	0	0	1	1
CHL 1:2	1	0	1	1	0	1	1	1

CHL 3:2	1	0	1	1	1	1	0	0
CHL 3:4	1	1	1	1	0	0	1	1
CHL 3:1	1	1	1	1	0	1	1	1
CHL 2:2	1	1	1	1	1	1	1	1
CHL 4:4	1	1	1	1	0	0	1	1
CHL 4:1	1	1	1	1	1	1	1	1
CHL 4:2	1	1	1	0	0	1	1	1
L41CHL	1	0	1	0	1	0	0	0
L44CHL	1	1	1	0	1	0	0	0
L12CHL	0	1	1	0	1	0	0	0
L44CHL	1	0	1	0	1	0	0	0
L11CHL	1	0	1	0	1	0	0	0
L11CHL	1	0	1	0	1	0	0	0
AMP13	1	0	0	0	1	0	1	1
AMP12	1	0	1	1	1	1	1	1
AMP14	1	0	0	0	1	0	1	1
AMP31	1	0	0	0	0	1	1	0
AMP43	1	0	0	0	1	1	1	0
AMP53	1	0	1	1	1	0	1	0
SAMP22	1	0	0	1	1	1	0	0
AMP52	1	0	0	0	1	1	0	0
AMP34	1	0	0	0	0	1	1	0
AMP21	1	0	0	0	0	1	0	0
SAMP41	1	0	0	0	1	1	0	0
AMP41	1	0	0	0	1	1	0	0
AMP42	1	0	0	0	0	1	0	0
AMP32	1	0	0	1	0	1	0	0
AMP23	1	0	0	1	1	1	0	0
AMP31	1	0	0	0	0	0	0	0
AMP L4:3	1	0	0	0	1	0	0	0
AMP L4:4	1	0	1	1	1	0	1	1
AMP S1:2	1	0	1	1	1	1	0	0
AMP L3:2	1	0	1	1	1	1	1	1
AMP L1:4	1	0	0	0	0	1	0	0
AMP L3:1	1	0	0	0	0	0	0	0
AMP L2:2	1	0	0	0	0	0	0	0
AMP L3:4	1	1	0	0	1	1	0	1
AMP L2:3	1	0	0	1	1	1	0	0
AMP L1:1	1	1	1	1	1	1	0	0
AMP L3:4	1	0	1	1	1	1	0	0
AMP L2:3	1	0	1	1	1	0	0	0
AMP L3:1	1	0	0	1	1	1	0	0
AMP L2:2	1	0	0	0	0	0	0	0
AMP L3:1	1	0	0	1	1	0	0	0
AMP L1:4	1	0	1	1	1	1	0	0

AMP L2:1	1	0	0	0	0	1	0	0
AMP L2:3	1	0	1	1	1	1	0	0
AMP L4:1	1	0	0	1	1	0	0	0
AMP L4:3	1	0	0	0	0	0	0	0
AMP L3:3	1	0	0	0	0	1	0	0
AMP L1:2	1	0	1	0	1	0	0	0
SAMP 2:4	1	0	0	0	1	1	0	0
AMP 1:3	1	0	0	0	1	0	1	1
AMP 1:2	1	0	1	1	1	1	1	1
AMP 1:4	1	0	0	0	1	0	1	1
AMP 3:1	1	0	0	0	0	1	1	0
AMP 4:3	1	0	0	0	1	1	1	0
AMP5:3	1	0	1	1	1	0	1	0
SAMP2:2	1	0	0	1	1	1	0	0
AMP 5:2	1	0	0	0	1	1	0	0
AMP 3:4	1	0	0	0	0	1	1	1
AMP 2:1	1	0	0	0	1	1	0	0
SAMP 4:1	1	0	0	0	1	1	0	0
AMP 4:1	1	0	0	0	1	1	0	0
AMP 4:2	1	0	0	0	0	1	0	0
AMP 3:2	1	0	0	1	0	1	0	0
AMP 2:3	1	0	0	1	1	1	0	0
AMP 3:1	1	0	0	0	0	0	0	0
L32AMP	1	0	0	0	0	0	0	0
L32AMP	1	0	0	0	1	1	0	0
L14AMP	1	0	1	0	0	0	0	0
L11AMP	1	1	0	0	1	0	0	0
L13AMP	1	0	0	0	0	1	0	0
L32AMP	1	1	0	0	0	0	0	0
L33AMP	1	1	0	0	1	1	0	0
L41AMP	1	0	0	0	0	0	0	0
L12AMP	1	1	1	0	0	0	0	0
L43AMP	1	0	0	0	1	1	0	0
L34AMP	1	1	0	1	0	0	0	0
L14AMP	1	0	0	0	0	0	0	0
L33AMP	1	0	0	0	0	0	0	0
L12AMP	1	1	1	0	1	0	0	0
L14AMP	1	0	0	0	1	0	0	0
L42AMP	1	0	0	0	1	0	0	0
L34AMP	1	0	0	0	1	0	0	0
L13AMP	1	0	0	0	0	0	0	0
L31AMP	1	1	0	0	1	0	0	0
L33AMP	1	0	0	0	1	0	0	0
L13AMP	1	0	0	0	0	0	0	0
L14AMP	0	0	0	0	0	0	0	0

S14AMP	0	0	0	0	0	0	0	0
L21AMP	0	0	0	0	0	0	0	0
L24AMP	0	0	0	0	0	0	0	0
S11AMP	0	0	0	0	0	1	0	0
L22AMP	1	0	0	0	1	0	0	0
L12AMP	0	0	0	0	0	0	0	0
L14AMP	0	0	0	0	0	0	0	0
L42AMP	0	0	0	0	0	0	0	0
L41AMP	0	0	0	0	0	0	0	0
S33AMP	1	0	0	0	0	1	0	0
L44AMP	0	0	0	0	0	0	0	0
S44AMP	0	0	0	0	1	0	0	0
L23AMP	0	0	0	0	0	0	0	0
RIF31	0	0	0	0	0	0	0	0
RIF41	0	0	0	0	0	1	0	0
RIF12	1	0	0	0	0	1	0	0
RIF51	1	0	1	0	0	1	0	0
RIF33	1	0	0	1	1	1	0	0
RIF24	1	0	0	1	1	1	0	0
RIF42	0	0	0	0	0	0	0	0
RIF54	1	0	0	0	0	0	0	0
RIF13	0	0	0	0	0	0	0	0
RIF21	1	0	0	0	0	0	0	0
RIF L2:2	0	0	0	0	0	0	0	0
RIF L1:4	1	1	1	0	1	0	0	0
RIF L3:2	0	1	0	0	0	0	0	0
RIF L3:2	1	1	0	0	1	1	0	1
RIF L4:3	0	1	0	1	1	0	0	0
RIF L2:2	0	0	0	0	0	0	0	0
RIF L2:4	1	0	1	1	1	1	0	0
RIF L2:1	1	0	1	1	1	1	0	0
RIF L1:2	1	0	1	0	1	0	0	0
RIF L2:2	1	1	0	0	1	1	0	0
RIF L2:4	1	1	0	1	1	0	0	0
RIF L2:1	1	0	0	1	1	0	0	0
RIF 3:1	0	0	0	0	0	0	0	0
RIF 4:1	0	0	0	0	0	1	0	0
RIF 1:2	1	0	0	0	0	1	0	0
RIF 5:1	1	0	1	0	0	1	0	0
RIF 3:3	1	0	0	0	1	1	0	0
RIF 2:4	1	0	0	0	1	1	0	0
RIF 4:2	0	0	0	0	0	0	0	0
RIF 5:4	1	0	0	0	0	0	0	0
RIF 1:3	0	0	0	0	0	0	0	0
RIF 2:1	1	0	0	0	0	0	0	0

L12RIF	1	0	0	0	1	0	0	0
L11RIF	0	0	0	0	0	0	0	0
L23 RIF	0	0	0	0	0	0	0	0
L33 RIF	0	0	0	0	0	0	0	0
L43 RIF	1	0	0	0	0	1	0	0
L41 RIF	0	0	0	0	0	0	0	0
S12RIF	0	0	0	0	0	0	0	0
L24RIF	0	0	0	0	0	0	0	0
S44RIF	0	0	0	0	0	0	0	0
L21RIF	0	0	0	0	0	0	0	0
L31RIF	0	0	0	0	0	0	0	0
L44RIF	0	0	0	0	0	0	0	0
L41RIF	0	0	0	0	0	0	0	0
L44RIF	1	0	0	0	0	0	0	0
L41RIF	1	0	0	0	0	0	0	0
L12RIF	1	0	0	0	0	0	0	0
L22RIF	0	0	0	0	0	0	0	0
L12RIF	1	0	0	0	0	0	0	0
L33RIF	0	0	0	0	0	0	0	0
L44RIF	0	0	0	0	0	0	0	0
L31RIF	1	0	0	0	0	0	0	0
L14RIF	0	0	0	1	1	0	0	0
L21RIF	0	1	0	0	0	0	0	0
L23RIF	0	1	0	1	0	0	0	0
L41RIF	0	0	0	0	0	0	0	0
L21RIF	0	0	0	0	0	0	0	0
CON12	0	0	0	0	0	0	0	0
CON11	0	0	0	0	0	0	0	0
SCON52	1	0	0	0	0	0	0	0
CON31	0	0	0	0	0	1	0	0
CON33	0	0	0	0	0	0	0	0
SCON24	0	0	0	0	0	0	0	0
CON23	0	0	0	0	0	0	0	0
CON14	1	0	0	0	0	0	0	0
SCON32	1	0	0	0	0	1	0	0
CON24	0	0	0	0	0	0	0	0
CON21	0	0	0	0	0	0	0	0
CON22	0	0	0	0	0	0	0	0
SCON14	0	0	0	0	0	0	0	0
CON44	0	0	0	0	0	0	0	0
SCON41	0	0	0	0	0	0	0	0
CON L1:2	0	0	0	0	0	0	0	0
CON L1:2	0	1	0	0	0	0	0	0
CON L2.1	1	0	0	1	1	1	0	0
CON L2:2	1	0	0	1	1	0	0	0

CONL4:2	1	1	1	0	1	1	0	0
CON L3:3	1	1	1	0	0	1	0	0
CON L3:3	1	1	1	0	0	1	0	0
CON L4:1	0	0	0	0	1	0	0	0
CON 1:2	0	0	0	0	0	0	0	0
CON 1:1	0	0	0	0	0	0	0	0
SCON 5:2	1	0	0	0	0	0	0	0
CON 3:1	0	0	0	0	0	1	0	0
CON 3:3	0	0	0	0	0	0	0	0
SCON2:4	0	0	0	0	0	0	0	0
CON 2:3	0	0	0	0	0	0	0	0
CON 1:4	1	0	0	0	0	0	0	0
SCON 3:2	1	0	0	0	0	1	0	0
CON 2:4	0	0	0	0	0	0	0	0
CON 2:1	0	0	0	0	0	0	0	0
CON 2:2	0	0	0	0	0	0	0	0
SCON 1:4	0	0	0	0	0	0	0	0
CON 4:4	0	0	0	0	0	0	0	0
SCON 4:1	0	0	0	0	0	0	0	0
L12CON	0	0	1	0	0	0	0	0
L33CON	1	0	0	0	1	0	0	0
L34CON	1	0	1	0	1	0	0	0
L44CON	0	0	0	0	0	0	0	0
L11CON	1	1	0	0	0	0	0	0
L22CON	0	0	0	0	0	0	0	0
L13CON	1	0	0	0	0	0	0	0
L33CON	0	0	0	0	0	0	0	0
L14CON	0	0	0	0	0	0	0	0
L41CON	0	0	0	0	0	0	0	0
L22CON	0	0	0	0	0	0	0	0
L13CON	0	0	0	0	0	0	0	0
S43CON	0	0	0	0	0	0	0	0
S22CON	0	1	0	0	0	0	0	0
S12CON	0	0	0	0	0	0	0	0
S23CON	0	0	0	0	0	0	0	0