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Relationships Between Factors Influencing Biofilm Formation and Pathogen Retention in Complex Rhizosphere Microbial Communities

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Relationships Between Factors Influencing Biofilm Formation and Pathogen Retention in Complex Rhizosphere Microbial Communities

by

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Abstract

Riparian wetlands are unique habitats facilitating all forms of life. The riverbanks of these environments provide ideal conditions for bacteria, plants, and higher organisms. Of particular interest to this research was the variation in microbial community structure at high, intermediate and poor water quality impacted areas. Assessing the capabilities of plants to retain microbial pathogens was identified. Root systems and corresponding soil are ideal locations for bacterial deposition, resulting in attachment at these areas. Biofilm production in these regions is important for long-term establishment, leading to persistence and potential naturalization. Opportunistic pathogens originating from mammalian fecal matter are introduced into these water systems, largely due to anthropogenic impacts. Wastewater treatment facilities, agricultural operations and livestock farming all contributed to determining water quality. This research investigated the levels of *Salmonella* spp., *Enterococcus* spp. and *Escherichia coli* deposition within riparian wetlands. The objectives of this research were to 1) isolate opportunistic pathogens from the environment, 2) assess impacts of contaminant exposure on resistance profiles and how water quality may effect this, 3) compare rhizospheric, rhizoplane and waterborne isolate contaminant response behavior, 4) assess levels of adhesion mechanism and biofilm production to determine the influence of water quality and isolate source (analyte). The overarching goal of this research project was to best determine the innate capabilities of opportunistic pathogens to be retained in the rhizosphere, rhizoplane and water systems in riparian zones. Additionally, determining their abilities to generate biofilm and successfully grow at varying levels of water quality was investigated.). A range of temperatures (11^{o}C, 28^{o}C, 37^{o}C) were utilized to evaluate the ability of pathogens to synthesize adhesion mechanisms, generate biofilm and resist contaminants. At 11^{o}C, the ability to produce amyloids, biofilms and survive during antibiotic exposure was low compared to raised temperatures to higher temperatures. At 28^{o}C and 37^{o}C, a threshold was reached resulting in synthesis of curli, cellulose and extra polymeric substances as well as increased biofilm formation. Pathogens isolated from rhizospheric soil and root samples were best able to generate biofilms and adapt to contaminant stressors. Resistance profiles were more robust at high temperatures, as 80% of isolates were susceptible to most antibiotics 11^{o}C, while 70% of isolates resisted 7 or more antibiotics at 28^{o}C and 37^{o}C. Ciprofloxacin, amoxicillin and clavulanic acid, chloramphenicol and ceftazidime resulted in 70% susceptibility whereas vancomycin, tetracycline, linezolid and doxycycline were resisted by 85% of isolates. Under dynamic growth conditions, *Enterococcus faecalis* acclimated to 1mM-0.05mM of copper and chloramphenicol, however, was inhibited by 0.05-0.0125 mM of silver. Further, *E. faecalis* isolated from high water quality sites were more efficient biofilm producers at 28^{o}C under dynamic conditions. This research suggests that although water quality influences microbial behavior, temperature and varying plant communities at riparian areas may be better parameters to utilize when measuring microbial pathogen retention in the environment.

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TABLE OF CONTENTS

List of Tables:

List of Figures

LIST OF ABBREVIATONS

AM	Ampicillin	hour(s)	hr
AMC	Amoxicillin	day(s)	d
C	Chloramphenicol		
CEF	Ceftazidime		
CIP	Ciprofloxacin		
D	Doxycycline		
L	Linezolid		
SUL	Sulfasoxazole		
G	Sulfadiazine		
S	Streptomycin		
V	Vancomycin		
T	Tetracycline		
MDR	Multi Drug Resistance		
EPS	Extracellular Polymeric Substance		
ESP	Enterococcal Surface Protein		
GRWS	Grand River Water Shed		
WHO	World Health Organization		
GRCA	Grand River Conservation Authority		
ANTB	Antibiotic(s)		
mM	Millimolar		
rdar	red, dry and rough		
bdar	brown, dry and rough		
pdar	pink, dry and rough		
MOA	Ministry of Agriculture		
MOECC	Ministry of Environment and Climate Change		

1

Background & Thesis Objectives

1.1 Opportunistic Organisms

An opportunist is an organism that exploits favorable conditions. These conditions could be a change in temperature, food source, environment, and more. Opportunistic pathogens may be of fungal, protozoan, viral or bacterial origin (Katano *et al.,* 2014; Parke and Gurian-Sherman, 2001). In particular, bacterial genera such as *Pseudomonas sp*., members of *Enterobacteriaceae,* such as *Salmonella sp*., and also *Enterococcus sp*., contain pathogenic species and strains (Stover *et al.,* 2000; Lebreton *et al.,* 2012). Bacterial opportunistic organisms often are associated with a mammalian origin, with particular niche preference inside the human gastrointestinal tract (Zhang *et al.,* 2016). Initially acting as colonizers of the gut and intestinal microflora, these groupings of microorganisms possess abilities which may be detrimental to human health, with the most profound effects showing in the immunocompromised (Gordon, 2008). The ability for adaptation in unique environments and utilize these new surroundings enable these opportunists to not only survive, but become highly successful in regeneration and persistence. The environmental

persistence of opportunistic pathogens is of growing concern, requiring attention and research to best determine causes of pathogen retention.

Environmental Relevance of Bacterial Pathogens

The introduction of opportunists into the environment, especially watersheds fed by various types of surface water, results in unlimited deposition throughout the water system (Kistemann *et* al. 2002). The impacts of anthropogenic activities like agro-industrial and wastewater treatment operations generate water runoff that frequently drains into larger water systems (Hooda *et al.,* 2000). This type of effluent often contains high microbial loads, with indicator organisms, for instance coliforms, providing information regarding water contamination due to introduced fecal matter (Cook, 1979). Savichtcheva and Okabe (2006) demonstrated how mammalian feces shed into the environment through water systems is a signature reason for water pollution and pathogen introduction into the environment.

When organisms originating from mammalian GI tracts are released and introduced into various environmental settings, opportunities for persistence and naturalization are presented. Genera of bacteria that have had significant levels of environmental introduction and colonization include, but are not limited to, fecal coliforms like *Escherichia coli, Salmonella* spp*., and Enterococcus* spp. (Anderson *et al.,* 2005). In reference to microniches, mammalian digestive systems are ideal locations for bacterial colonizers, containing high amounts of nutrients and water, as well as providing conditions of consistent temperature and pH (Guarner and Malagelada, 2003).

In the environment, microorganisms that originated in a host require areas providing similar benefits [resources]. These include temperature, pH, water availability and most importantly, sources of energy (Williams *et al.,* 2005; Mawdsley *et al.,* 1995). As mentioned, digestive systems in higher organisms can provide desirable conditions, but this can also generate intense competition for resources. The competitive nature of microorganisms further advances their ability to succeed in changing surrounding environments, allowing for growth and succession of the strongest competitors (Weller, 1988). Colonizers belonging to the intestinal microflora not only require the resources mentioned previously, but also structures to which they can adhere. This is done to establish [colonize] themselves, aiding in exposure to varying biotic and abiotic factors, contributing to their efficacy for success (Dimkpa, 2009). The ability to acclimate to the new surrounding and establish themselves in a new niche as strong competitors is what may possibly lead to environmental persistence, even naturalization (Weller, 1988; DeAngelis *et al.* 2010). Abiotic factors like water, soil and plant root structure, all play key roles in facilitating environmental success and establishment of microbes. These three materials are the basis for establishing ecosystems and habitats, whether on a macro or micro scale. Types of habitats that facilitate growth and persistence of bacteria are wetlands consisting of water, soil, vegetation and aquatic plants, fundamental ecological areas (Stanely *et al*. 2003). Characteristics of freshwater wetlands, particularly at riparian zones, include types of vegetation, soil and water. Inhabitants of these ecosystems include primary producers and consumers, including microbial grazers facilitating essential cycles (nitrogen, sulphur) and fungi aiding in plant growth (Ingham *et al.* 1985).

1.2 Wetlands

Water Quality

Water quality is an important attribute measuring the degree of pollutants, namely chemical and biological, influencing biotic factors and impacting organisms using these waters.. Key biogeochemical processes occur in these environments, such as nitrogen, sulfur and phosphorous cycles which also can assist in pollutant removal from source waters (Reddy and D'Angelo, 1996). High levels of organic matter released into large water systems, for example the Grand River watershed, results in higher dissolved organic matter (DOM) and nutrient loading (Kivaisi, 2001). The Grand River Conservation Authority measures water quality based on several parameters which include dissolved oxygen, pH, conductivity and nutrient levels such as nitrogen and phosphorous (GRCA, 2016). Often, eutrophication, or excess amounts of organic nutrients, like phosphates and nitrates, may be attributed to both natural and anthropogenic sources such as organic decomposition or livestock operations (Jansen *et al.,* 1994; Johnston, 2009).

Soil Types

Soil composition is a very important characteristic of wetlands. Specifically, impacts of soil type and plant composition affect microbial community dynamiscs (Berg and Smalla, 2009). There are three common forms of soil; clay, sand and silt. Size, porosity, hydrophobicity as well as a net negative charge are important characteristics affecting soil-microbe interactions (Gannon *et al.,* 1991). Gestel and colleagues (1996) showed that loam, a mixture of all three elements, is most ideal for retaining microorganisms within the soil. Naturally, this allows bacteria to maximize

surface area and charge ratios, utilizing physiological mechanisms to form attachments within biofilm matrices to these soil particles, resulting in immobilization and subsequent environmental persistence (Stevik *et al.,* 2004). Bacteria maintain net negative charges due to outer LPS and teichoic acid membrane structures resulting from the presence of phosphate (Neuhaus and Baddiley, 2003). Gross and colleagues (2013) demonstrated the importance of surface charge in adherence, as they engineered a *Staphylococcus aureus* mutant lacking D-Aalanine synthesis in its teichoic acid synthesis, thus resulting in an increased overall negative membrane charge (Neuhaus and Baddiley, 2003). This adversely affected the organisms' ability to generate biofilms and adherence to abiotic surfaces, thus signifying the importance of electrostatic forces on attachment. Additionally, the production of extracellular polymeric substances (EPS) with net positive, or neutral (polar) charges potentially masking negatively charged surfaces, further enables bacterial adherence to negatively charged surfaces (Vu *et al.* 2009).

Rhizoplane and rhizosphere

Riparian wetlands are extremely diverse habitats, comprised of trophic levels containing numerous primary producers and consumers. Such areas are highly unique ecological environments in that they are the first interface between land and water. Particularly, they consist of aquatic (growing from riverbed and fully submerged), immersed (breaching the water surface) and terrestrial vegetation (Fritioff and Greger, 2010). Freshwater rivers carve through these ecosystems, depositing transient materials in the soil retained in the river banks. Within these river banks and in shallow waters, vegetation such as grasses, shrubs and trees provide food for consumers above and below the soil. Beneath the top soil layer, plants establish intricate root systems. The area

directly corresponding to the surface of a root is the rhizoplane, whereas the bulk soil mass surrounding the roots is known to be the rhizoplane (Figure. 1).

Various plant types, whether aquatic or terrestrial, generate oxygen and support many organisms. Further, the role of plants in ecosystems as food sources is critical. Their ability to photosynthesize and generate their own sources of food from sunlight not only enables them to grow, but to also support an extremely diverse grouping of eukaryotic and prokaryotic life. Specifically, plants generate varying types of carbohydrate compounds for their own use, as well as food sources for microbial and fungal life living in the soil horizons (A and B zones). These associations may result in symbiotic relationships with plants, working together as the microorganism offers Nitrogen in exchange for sugars (Hodge *et al.,* 2001).

The area where root surfaces directly contact soil, or the rhizoplane-rhizosphere interface, contains root exudate; organic compounds containing carbohydrates for food, in addition to amino acids, flavonoids and antimicrobial substances (Hodge *et al.* 2001). Plant type, in conjunction with water quality (ie. nutrient levels, salinity, pollution) influence the prokaryotic functional profiles found within soil (Buee *et al.,* 2009). On root surfaces (rhizoplane) and within the root-soil interface (rhizosphere), microorganisms generate adhesive substances called biofilms, utilizing specific adhesion mechanisms to persist on plant roots and the surrounding soil (Walker *et al.,* 2003). An example of this was shown by Walker and colleagues (2003) regarding the plant pathogen *Pseudomonas aeruginosa*, which not only generates biofilms, but penetrates plant roots resulting in mortality.

Furthermore, the plant root zones and surrounding soil may act as primary locations for water filtration (April and Sims, 1990; Saad *et al.* 2013). Additionally, the deposition and accumulation of environmental contaminants such as antibiotics and metals often occurs in the soil and roots in these habitats. Often in the environmental setting metal ions such as Cu^{2+}, $Fe^{2+/3+}$ and K^{2+}, are cationic (positively charged). Soil and root surfaces (exudate) are anionic (negatively charged), thus the attraction of opposing charges results in ionic binding of these molecules, resulting in adherence to root structures and soil particles, sorption and retention, notably seen with Pb, Cu and Zn (Yoon *et al.,* 2006).

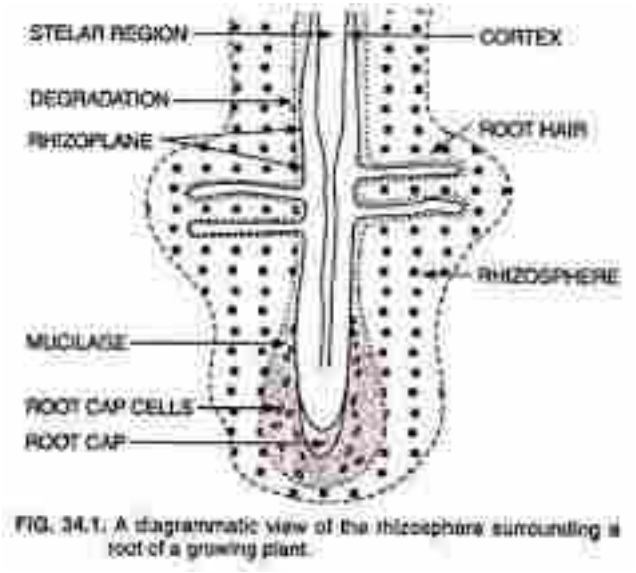


FIG. 34.1. A diagrammatic view of the rhizosphere surrounding a root of a growing plant.

Figure 1. Rhizoplane and rhizospheric zones.

Ecophysiology

Functional redundancy is a phenomenon found in some environmental ecosystems. Regarding microbial ecology, different species can assist in similar processes, such that one or more phyla may be comparable (Wohl *et al.,* 2004). In other words, although populations may differ within certain types of wetland ecosystems, similar processes will still occur. Further, microbial feedback loops exist within these environmental settings allowing for communication throughout the population, as seen during quorum sensing during biofilm formation in the rhizoplane-rhizosphere interface (Miller and Bassler, 2001).

High soil nutrient levels due to decomposition of organic matter, in conjunction with soil dynamics, play predominate roles in microbial community assemblages (ie. diazotroph congregants) (Picerno and Lovell, 2000). Key species of microorganisms are found in these communities to help generate essential compounds, such as *Nitrobacter* and *Rhizobium*, that aid in nitrogen conversion and fixation. However, wetland ecosystems not only harbor microbes native to soil and biogeochemical processes, but also serve as a potential sink for deposited opportunistic pathogens. Of interest are microorganisms released into the watershed with the ability to adversely impact human health, such as *Salmonella, Enterococcus, Escherichia coli* and *Pseudomonas* spp.

An important indicator of water quality is the measurement of fecal abundance, evaluating total coliform levels, enabling microbial source tracking (Cohen and Shuval, 1972; McQuaig *et al.,* 2012). At poor water quality wetlands for example, coliforms would be expected in higher loads than at high qulity areas. As enteric pathogens are associated with fecal-polluted water, they travel in the water system following stream flow dynamics. An example of this can be seen within the

Grand River watershed, with high quality reference sites located at the headwaters with decreasing quality as the water flows southward (downstream) in this alluvial system (Jamieson *et al.,* 2004), which results in increased pathogen carriage and deposition as the water flows downstream.

1.3 Biofilms

Biofilms are complex structures facilitating environmental persistence as well as acting as critical structures in disease utilized by infectious microorganisms (Hall-Stoodley *et al.,* 2004). Conditions such as oxygen and nutrient levels are key factors influencing microbial species present in environmental biofilm structures, contributing to complexity and size (Hall-Stoodley *et al.,* 2004). As environmental settings and conditions widely vary, the structure of microbial biofilms are reflective of their surroundings, providing an intricate, networked system facilitating communication, antibiotic resistance, but most of all, survival and persistence (Hall-Stoodley *et al.,* 2004).

Functions

As stated, biofilms assist in persistence due to adhesion to various objects, biotic and abiotic. For successful growth and maturation of the biofilm, chemical signals are required to facilitate communication. For example, acyl homoserine lactone synthase (AHL) is a necessary signaling molecule in pathogenic bacteria such as *Vibrio* sp., *Salmonella* sp. and *Enterococcus* facilitating determination of population densities (Schaefer *et al.,* 1996). Within the environment, further research has shown that some plants have the ability to exude substances mimicking AHL, resulting in adherence and population growth on plant roots (Teplitski *et al.,* 2000).

Structure

Although biofilm function remains constant, providing channels for chemical signal and nutrient delivery, the physical structure is shaped by abiotic factors such as water flow and wind (Klausen *et al.,* 2003; Stoodley, 1999). Further, nutrient levels and environmental responses influence the structure and integrity of the biofilm, promoting conditions which only prokaryotic planktonic organisms may adapt to (Sauer *et al.,* 2002; Klausen *et al.,* 2003). Identification and study of biofilms shows how intimate form and function truly are, exposing diverse delivery channels within, allowing for nutrient and waste exchange (deBeer *et al.,* 1994). deBeer and associates (1994) showed that biofilms consisted of tunnel-like structures that facilitated nutrient flow, while also identifying microbial biofilm motility capabilities.

Growth, adhesion and dispersal

Biofilm generation occurs over multiple stages, progressively becoming larger (surface area), more dense and specialized (O'Toole *et al.,* 2000). Specific physiological mechanisms aid in attachment, primarily consisting of curli fibers which aggregate together and form amyloid structures (Zhou *et al.,* 2013). In addition, cellulose and fimbriae are alternate adhesion mechanisms facilitating biofilm initiation. A study by Saldaña and colleagues (2009) demonstrated that fimbriae used to adhere to surfaces in enterohemorrhagic *E. coli* required expression of *csgA* (curli) and *bcsA* (cellulose), co-activated by the *csgD* domain. Further, it was shown that the c-di-GMP complex within some microorganisms such as *Gluconobacter* and *Salmonella* is regulated by the GGDEF and EAL domains, requiring activity for biofilm formation, which may serve as a key target site for therapeutic interventions of associated diseases (Römling *et al.,* 2005). Further, microbial

biofilms have the ability to detach from surfaces moving to more ideal, opportune environments (Figure. 2). Biofilms have been shown to detach and move in the water, slowly moving across surfaces, or utilize twitching motility to move and re-colonize (Hall-Stoodley and Stoodley, 2005).

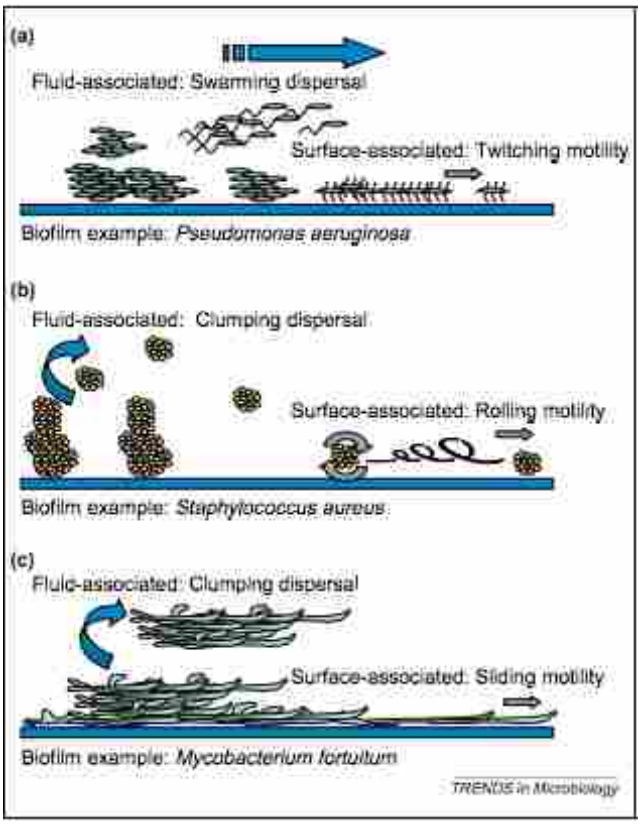


Figure 2. Biofilm dispersion methods exhibited by various pathogenic microorganisms, as described by Hall-Stoodley and Stoodley (2005).

1.4 Human pathogens and environmental naturalization

Water sources can become contaminated with infectious microorganisms. Notable enteric bacteria originating from mammalian hosts are *Salmonella, Enterococcus,* and *E. coli.* When feces are released or manure is washed into surface waters, they are transported and deposited in various

locations in the water system (Jamieson *et al.,* 2002). *Salmonella* is the causative agent of samonellosis and typhoid fever, with numerous serotypes collectively affecting millions annually (Wang *et al.,* 2013). *Enterococci* are notable microorganisms that are a major contributor to urinary tract infections, endocarditis, and sepsis. Currently, vancomycin-resistant *Enterococci* (VRE), are becoming more common due to lack of susceptibility to vancomycin, making these infections, which can be waterborne, very difficult to eradicate (Roberts, 2016).

E. coli is a common opportunistic pathogen which can cause many adverse effects. Originating from the mammalian digestive tract, it has the potential when taken in from food and water to cause conditions that range from UTI's to hemorrhagic gastroenteritis (Griffin and Tauxe, 1991). *E. coli* is associated with water contamination and fecal coliforms due to agricultural runoff, and has increasingly become persistent, almost naturalized, in the environment following retention (Ksoll *et al.,* 2007). Lastly, *Pseudomonas* is an opportunistic pathogen that may cause nosocomial infections in hospital patients, specifically burn victims or those with open wounds (Schaeburg *et al.* 1991; Hota *et al.,* 2009). Pseudomonads are indigenous to the environment and notable biofilm formers, allowing them to more readily persist or acclimate to new environments (Tymensen *et al.,* 2015). Further, infections in burn victims or patients with Cystic Fibrosis are in part due to environmental contamination of hospitals and surgical tools (Lyczak *et al.,* 2000; Oliver *et al.,* 2000).

1.5 Antibiotics

Antibiotics are chemically synthesized compounds used for therapeutic intervention. Frequently known as antibacterial or antimicrobials, they are used to reduce, prevent or eradicate diseases

attributed to microorganisms (Cars *et al.,*2001). There are two groupings of antibiotics depending on the desired outcome; bacteriostatic or bactericidal. Bacteriostatic antibiotics are used to reduce replication of organisms causing infection, however, the desired outcomes are reversible when the drugs are absent (Cioffi *et al.,* 2005). Conversely, bactericidal drugs were designed with the purpose to kill all bacterial cells- a non-reversible effect (Morones *et al.*, 2005).

First discovered by Alexander Fleming in 1928, Pencillin was the product of molds growing and secreting compounds observed to have inhibitory effects on nearby microorganisms. Not only was this revolutionary for the pharmaceutical industry for novel findings and future production, this shed light on a key event; prokaryotes generate and secrete antibiotics as survival adaptations in the environment. The family of Actinomycetes, particularly the genus *Streptomyces sp.,* has been found to synthesize over half of the antibiotic compounds utilized by the pharmaceutical industry (Walsh, 2003). Using these as a basis for further development, various classes of drugs have been designed to invoke specific responses and effects on microbes. Table 1 describes various drug types as well as their bacteriostatic or bactericidal effects, in addition to the specific action they have on bacterial cells.

It was identified that at specific life stages (stationary phase), antibiotic-producing microorganisms were releasing higher levels of antimicrobial compounds (Gramajo *et al.,* 1993). This represents a survival adaptation allowing for environmental success, enabling these organisms to outcompete other microbes in the same communities (Burgess *et al.,* 1999). On the other hand, this adaptation has potentially facilitated increasing environmental resistance to drugs, as selective adaptations

and chromosomal mutations are often going to occur in response to stress (Baker-Austin *et al.,* 2006).

Classes and modes of action

There are many types of antibiotic drug classes, however, my research focuses on a representatives of frequently prescribed drug classes (Table 2.5). With such a diverse family of pharmaceuticals, bacterial populations are managed by either direct killing, or limiting replication. The best known are antibiotics derived from B-lactams, such as Penicillin. This drug class contains beta lactam rings and act to result in a bactericidal effect on microbes in inhibition of peptidoglycan synthesis necessary for cell wall generation.

Other bactericidal drug classes commonly prescribed are Aminoglycosides and Quinolones. The former consists of specific amino sugars that facilitate protein synthesis inhibition in Gram-negative microorganisms via irreversible binding of ribosomal subunits (Hoffman *et al*., 2005). Notable prescribed aminoglycosides are Streptomycin and kanamycin. Fused aromatic rings that have attached carboxylic acid functional groups signify Quinolones. By inhibiting the function of topoisomerase and DNA gyrase, this drug class amplifies DNA supercoiling, resulting in DNA fragmentation (Ding *et al.* 2016)..

Common bacteriostatic intervention includes Tetracyclines, Amphenicols and Macrolides. The first of these three contain a core hydrocarbon ringed structure (multiple benzene rings), targeting protein synthesis (Joseph *et al.*, 2016).. Macrolides and Amphenicols also inhibit ribosomal

function, affecting protein synthesis, transpeptidation and incomplete peptide synthesis (Mingoia *et al.*, 2015).

Over recent decades, an antibiotic resistance crisis has occurred. Antimicrobial drugs which once were highly effective against all bacterial classes are now becoming ineffective. Bacterial resistance is largely attributed to three specific types of resistance mechanisms. Firstly, efflux pumps are utilized by pathogenic microorganisms to actively prevent therapeutic drugs [antibiotics] from accumulating within the cytoplasm. Often these are H+ or ATPase driven mechanisms, with domains embedded in the inner membrane that initially bind based on charged attractions, and pump outwards through the outer membrane (Levy, 2002). Secondly, replacement or modification of a target active site within the microbe may occur. This can occur either by a gene mutation or an imported gene, resulting in replacement enzymes (Leclercq and Courvalin, 1991). An example of this is observed in *Stapylococcus aureus*, which produces mecRI fragments to cleave and repress methicillin. Lastly, similar to target modification, opportunists have the ability to produce degrading enzymes specific to antibiotics (Wright, 2005).

Table 1. Antibiotic classes, modes of action, effects and their general uses.

Antibiotic Class	**Mode of Action**	**Effect**	**Examples**	**Uses**
Beta-Lactams	Inhibit cell wall synthesis	Bactericidal	Amoxicillin and Clavulanic Acid, Ampicillin	Gram +

Tetracyclines	Blocks protein synthesis	Bacteriostatic	Tetracycline	Cholera, Yersinia
Quinolones and Fluoroquinolones	Inhibits cell wall synthesis	Bactericidal Bacteriostatic	Ciprofloxacin	Clostridium, Streptococcus
Aminoglycosides	Inhibit protein synthesis	Bactericidal	Gentamycin	Gram -
Macrolides	Inhibits protein synthesis	Bactericidal Bacteriostatic	Erythromycin	Gram +, -
Cephalosporins	Disrupt peptidoglycan formation	Bactericidal	Ceftazidime, Cefazolin	Gram +, -
Sulfonamides	Inhibit folate synthesis	Bacteriostatic	Sulfadiazine, Bactrim	Gram +, -

1.6 Antibiotic resistance

Resistance to antibiotics has been trending upwards in the past few decades. Due to the consistent use of antibiotic pharmaceuticals, as well as the lack of designed and synthesized alternatives, there is decreasing susceptibility to many opportunistic bacterial pathogens (Packey and Sartor, 2010). Specifically, microorganisms causing nosocomial infections in hospitals are showing higher resistance profiles recently (Hollenbeck and Rice, 2012). This emerging phenomenon is due to acquired and naturally occurring resistance mechanisms present in various bacterial species. Resistance genes are responsible for these unique mechanisms, of which the ability to horizontally

pass this genetic information amongst other bacteria, such as within an established biofilm, further influences widespread resistance (Allen *et al.,* 2010).

Natural and acquired resistance

As mentioned, antibiotic resistance has been a multi-decade problem continually on the rise. Naturally acquired resistance includes genetic traits which innately are located on bacterial plasmids, conferring resistance as replication occurs. Acquired resistance is the event by which a microorganism has inherited a resistance gene from a source alternative to their plasmid, possibly through environmental DNA, or horizontal gene transfer (HGT) using pilus-mediated exchange with other bacteria. Multiple modes of exposure can result in this trend of resistance, whether as a naturally innate process due to chromosomal mutations or frequent exposure to high concentrations of antibiotics (Martinez, 2012). Additionally, previously discussed mechanisms, for instance, HGT, and early exposure in a bacterial lifecycle to organisms naturally synthesizing antimicrobials, specifically *Streptomyces,* may have environmental implications on acquired resistance profiles (Sizova *et al.*, 2001).

Antibiotic producing bacteria are equipped with the machinery necessary to both synthesize and degrade antibiotics (Canton, 2009). Further, adapted and specialized intrinsic systems to combat antibiotics (target site modification, enzyme degradation) have influenced resistance. Microbial communication is vital for exchange of genetic information and chemical cues signaling stress. Complex microbial communities form biofilms made up of varying genera of bacteria. Each may possess a gene conferring resistance to a particular antibiotic (or antibiotic producer), and can exchange this information within the biofilm (Allen *et al.,* 2010). Quorum sensing and multi-

component regulatory pathways, for instance biofilm production initiation in *Pseudomonas fluorescens* (ClpP protein synthesis pathway) have helped microbial species engineer self-protective mechanisms, likely due to increasing selective pressures (O'Toole and Colter, 1998).

At the biofilm-external environment interface, eDNA has been shown to deposit and play a very key role as a biofilm component. The significance of eDNA, or extracellular DNA, deposition is that this may also provide further opportunities for genetic exchange and increased resistance as found by Riesenfeld and colleagues (2010).

1.7 Fundamental elements for microbial growth

There are approximately 100 bioessential elements in the periodic table, containing both macro and micro nutrients when discussing bacteria. The elements C, N, S, O in particular are crucial for the development of nucleotides, amino acids and protein synthesis, as well as facilitating the structural integrity of the cell wall, particularly teichoic acid and peptidoglycan synthesis (Atilano *et al.,* 2010). Macronutrients are elements which are required in very large quantities for general growth, replication and duties various species of bacteria carry out (Berg, 2010). Micronutrients involve elements which are needed in very minimal amounts, as excess would be detrimental.

In particular, earth metals and rare earth elements are groupings of naturally occurring substances which strongly impact bacterial growth (Brantley *et al.,* 2001). Frequently known as trace metals, and can be toxic not only to bacteria, but also higher organisms that may see increased levels. Na, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and $Fe^{2+/3+}$ are examples of soft earth metals which can be utilized by bacteria for structural support, redox catalysts and Lewis Acids, as described by Brantley,

Liermann and Bau (2001). The high affinity for metal binding, particularly $Fe^{2+/3+}$, $Cu^{2+/3+}$, and Zn^{2+}, results in increased catalytic activity at various binding sites and translocation complexes (Hughes and Poole, 1989; Atilano *et al.,* 2010).

Ca^{2+} plays a strong role in Gram-negative bacterial cell wall synthesis by selectively binding and forming cross-linked membranes (Hughes and Poole, 1989). The geometry and charge of a metal is critical for binding or entering the bacterial cell, with elements such as Zn^{2+} that are flexible, more readily utilized (McHale and McHale, 1994; Bachi, 2014). Alkali and alkaline earth metals serve many important purposes and can be thought as macronutrients. Also, electron availability for metals found in the first two periods are *s* orbital elements, thus weaker binding affinity and electronegativity when compared to transition metals.

Transition metals and rare earth elements have much stronger binding affinities due to their increased electronegativity, ionization energy and smaller atomic radii. These attributes make *d* orbital elements much stronger binders to organic molecules and bacterial cells [Ligand Field Theory] (Gerloch *et al.,* 1981; Brantley *et al.,* 2001), possibly outcompeting binding of alkali and alkaline earth metals.

Additionally, transition metals in the *3d* block are thought to be the most important, however, those in the 4^{th} and 5^{th} rows of the periodic table are most toxic (Wackett *et al.,* 2004). Mercury, Tin and even Aluminum (2p) have all been proven toxic to microorganisms in low concentrations, likely do to their soft, malleable properties, allowing them to be good organic binders (DeSilve *et al.,* 2002).

1.8 Ligand Binding

Binding at essential sites, such as protein generation or nucleic acid conformation sites, can result in unwanted oxidative phosphorylation, membrane permeability and other mutagenic effects (Hughes and Poole, 1989). Metals play very crucial roles in the life cycle of microbes. Ca and Na are important activators of calmodulin, enabling flagellar activity in response to photo or chemotaxis (Vyas *et al.,* 1989). Zn and Cu have been shown to provide structural roles, such as generating heat-stable bridges and promote conformational changes of enzyme and active site complexes (Forest *et al.,* 2000). Zn^{2+}, Mg^{2+}, $Ni^{2+/3+}$ and $Fe^{2+/3+}$ aid in generating Lewis acids, enhancing the ability of acid phosphatases and push forward hydrolysis reactions (Corma and Garcia, 2002).

Lastly, Fe and Cu aid in redox catalyst activity, particularly involving enzymes needed for electron transport in cytochromes, Fe-S protein function and Cu electron transfer proteins (Wasser *et al.,* 2002). Wasser and associates also showed that Fe and Cu are critical for the function of various enzyme classes, most notably oxygenases, oxidases and hydrogenases. These metals have been shown to decrease inner and outer sphere structural reorganization during oxidation-reduction reactions, and also (re)organize electron carriers (Sigfridsson *et al.,* 2001).

Geometry, electron binding capability and pH are three of the most important attributes for metal transport and binding. Further, the presence of various essential elements N and S, favoring binding to transition metals (Hughes and Poole, 1989), with molecule size (atomic radius) affecting electron donation. With regard to transition metals, the strength of the Lewis acid will dictate binding selectivity of the bacteria (Corma and Garcia, 2002). For example, if Cu is present and in

excess, frequent ligand binding would almost always be expected, due to analytical masking [precipitation or complexation with proteins] (Hughes and Poole, 1991).

1.9 Metal uptake, transport and removal mechanisms

Bacterial cells are remarkable in their abilities to acquire and transport necessary molecules to sustain growth and function. Gram-negative bacteria have an outer membrane with porins, non-hydrophilic pores which are specially designed to allow metal ions to enter and pass through (Thurman *et al.,* 2009). Not only do these channels facilitate metal take up, but capsular and slime layer formation are also crucial for metallic binding (Chen *et al.,* 1995). This is likely due to the net negative charge exposed on the surfaces of both Gram classes of microorganisms, attracting the positively charged cations, resulting in alternative metal binding (Beveridge and Fyfe, 1985).

Primary translocation of metals can be achieved using aqueous channels or the lipid phase of bacterial membranes, dependent on the proton motive force function (Nies and Silver, 1995). Secondary transport of metals includes types of electron transport chains and terminal electron acceptors in aerobic and anaerobic microorganisms, ATP hydrolysis and also photosynthetic electron transport (Hughes and Poole, 1989). Ionophores, ion-selective channels embedded in the bacterial membrane, further obliges metal transport. One of the best studied metal acquisition and transport systems are those of siderophores, chelating Fe ions with high affinity. These siderophores are specialized ligands in microorganisms that facilitate iron acquisition and utilization, however, are absent in strict anaerobes, *Lactobacilli* and *Legionella sp.* (Pandey *et al.,* 1994; Hughes and Poole, 1989). Conversely, other genera of microbes possess varying siderophore structures, and can be seen in *Mycobacterium*, utilizing mycobactin [exochelin], and similarly,

enterobactin [enterochelin] found in the family of *Enterobacteriaceae.* Studies have also shown that along with Fe^{2+}, $Cu^{2+/3+}$ may be taken up by these secreted siderophores, as found by Koh and Henderson (2015), when showing that uropathogenic *E. coli* produced yersiniabactin capable of binding exogenous ferric and cupric ions. Additionally, other strains of *E. coli* possess citrate hydroxamate, whereas, other enteric microbes have cyclic tricatecholates facilitating ion take up (Powell *et al.,* 1983; Hughes and Poole, 1989).

2.0 Contaminant Effects on Biofilms Antibiotics

Biofilm structures are critical for microbial establishment and success. Acting as reservoirs for microbial life, facilitating communication and function between varying species is key for colonizing vertebrate and plant hosts. Antibiotic production from plants via root exudate, as well as naturally produced antimicrobial compounds (*Streptomycetes spp.)* contribute to reduced, disrupted or lack of biofilm growth (Hajipour *et al.,* 2012). Certain classes of antibiotics, namely *B*-Lactams and Quinolones, affect the ability for cell wall synthesis. Additionally, macrolides and sulfonamides reduce the ability to generate proteins necessary for biofilm formation and protein synthesis.

Mah and O'Toole (2001) characterized the differences in genetic expression between planktonic and biofilm-bound cells. They showed that microorganisms contained within the biofilm had higher tolerance to antibiotics, likely associated with the rpoS-mediated stress response regulation system, invoking biocide resistance. Biofilms act as not only a virulence factor, but also a protective layer or shield against antimicrobials. Porous channels and molecule delivery systems within the biofilm allow for incorporation of antibiotics and transport to more sensitive areas (ie.

inner portions) of the biofilm, causing more direct attacks and reduction in biofilm formation (Schafer *et al.,* 1996)

Metals

Although metals are essential to microbial life, whether required in high amounts such as $Fe^{2+/3+}$ or in trace levels as with Cu, they can be detrimental to microbial function. Rare earth elements such as Ag^{2+} and Au^{3+} have proven to be detrimental to microbial biofilm growth. Specifically, nanoparticles have been utilized as inorganic disinfectants targeted to disrupt biofilm formation and invoke toxic effects on organisms such as humans (Hajipour *et al.,* 2012). Hajipour (2012) and colleagues demonstrated that metal oxide nanoparticle toxicity inhibits varying Gram glasses. Gram-negative microbes like *E. coli* are much more susceptible to copper oxides than Gram-positive bacteria like *Staphylococcus aureus* or *Bacillus subtilis*, by protein function (Yazdankhah *et al.,* 2014).

Further, it has been shown that in freshwater microbial communities, varying copper concentrations (0, 1, 3, 10 μm) induced changes in heterotrophic metabolism and microbial physiology using Biolog™ plates (Massieux *et al.,* 2004). Additionally, as heavy metals and toxic levels of trace elements negatively impacts assembled biofilm structures, excess Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Al^{2+}, and Cr^{2+} increased microbial EPS production as well as increased corrosion rates of metals (Fang *et al.*, 2002).

1.9 Antibiotic and Metal Combinations

Na^+ and K^+ have been shown to selectively bind valinomycin, an environmentally produced antibiotic by *Streptomyces sp.*, allowing for selective transport and uptake across bacterial cell membranes (Rose and Henkens, 1974). Selective channels like ionophores within the bacterial membrane can act as antibiotic delivery systems within the environment (Sun *et al.,* 2016). Similarly, siderophore complexes generated by both Gram classes of bacteria facilitate not only iron acquisition, but also copper and other metals (Balasubramanian *et al.,* 2011).

Thus, competition for iron and other essential trace elements requires mechanisms more efficient than transferrin (vertebrates) to obtain Iron (Skaar, 2010). Although nutrients are limited, microbial pathogens are specialized and highly efficient scavengers. This is notable during host infection, increasing local body temperature and siderophore activity, improving iron uptake. In the [ecological] environmental setting, iron availability is a critical factor in ecosystem composition and stability (Tagliavini and Rombola, 2001). At low levels of iron, microorganisms have shown to generate antibiotic-like compounds that are structurally similar to siderophores, antagonizing competitors and giving them a mechanistic advantage for acquiring iron (Penyalver *et al.,* 2001). Correspondingly, iron antibiotics have been synthesized due to the efficient uptake systems made by bacteria, as albomycin and ferrimycin are transported through Fe channels (Braun and Braun, 2002).

The importance of having an understanding of charged ions is critical to assessing synergism between metals and antibiotic metabolites in the environment. Further research is required to better elucidate how metal uptake may correlate with antibiotic efficacy. Knowing siderophore and other ion transport mechanisms, in addition to charged attractions of molecules regarding biofilms,

antibiotics and metals, novel findings will ensue. Environmentally, the deposition of contaminants in wetlands within the rhizosphere not only affect microbial communities and retained opportunistic pathogens, but may change their functional profiles. Specifically, the effects of constant contaminant exposure on persisting pathogens likely facilitates changes in metal and antibiotic resistance profiles.

Research Need

Much remains to be learned regarding pathogen fate in an environmental setting following deposition. The ability and extent of harmful microorganisms to be retained in areas such as watershed wetlands is still largely unknown. Specifically, pathogen-rhizosphere interactions facilitating environmental persistence requires more focus. For example, biofilm formation in these organisms when exposed to environmental conditions is less well understood. In particular, the biofilm-forming capability of pathogenic microorganisms, which may facilitate environmental retention, requires further investigation. In addition, the enhanced synergistic effect contaminants occurring in watersheds may have on these pathogens and their ability to be retained through biofilm formation requires better understanding. Elucidating biofilm-rhizosphere interactions along with pathogens exposed to water contaminants, such as antibiotics, and metals will provide invaluable information regarding the ability of microbes with pathogenic potential to persist within conditions presented in environmental retention areas.

Hypothesis

Based on current research, pathogens expressing genes responsible for enhanced biofilm production, which may lead to rhizosphere persistence, are expected. Increased biofilm-forming potential should be expressed by these pathogens, which will influence contaminant resistance profiles. Specifically, in the presence of waterborne contaminants such as antibiotics, enhanced biofilm production is also expected. A synergistic response between antibiotics and other contaminants is possible, influencing biofilm formation. Changes in pathogen behavior will occur as adaptation to new roles in these environmental microbial communities is expected. This will lead to enhanced functions, such as increased biofilm formation and contaminant resistance. Rich amounts of nutrients released from the plant roots may enhance conditions within the rhizosphere that will contribute to environmental persistence and enhanced resistance profiles. The overarching hypothesis for this research paper is that enhanced biofilm production resulting from environmental pressures will enable opportunistic pathogens, such as *Salmonella* spp., *Enterococcus* spp., and *E. coli* to be retained in the rhizosphere of watershed wetlands.

Objectives

1) Isolate opportunistic pathogens from rhizoplane, rhizosphere and water samples of riparian wetlands.
2) Evaluate the ability of these isolated microorganisms to respond to adverse conditions caused by contaminant stress, including antibiotics and metals.
3) Quantify the amount of contaminant-resisting opportunistic pathogens from each sampling area and determine how water quality may influence resistance profiles.

4) Determine the ability of isolated opportunistic pathogens to produce adhesion mechanisms and biofilm to assess impacts of water quality on these capabilities

2

Methods

Experimental Approach

The integrative nature of this project can be seen to some extent in the experimental approach (Figure 2.1.). A series of culture-based and molecular-based methods were used to assess antibiotic effects and biofilm formation in changing environments (ie. temperatures) as well as both static and dynamic conditions. Biofilm formation was studied on an organismal level, and considered at a cellular level.

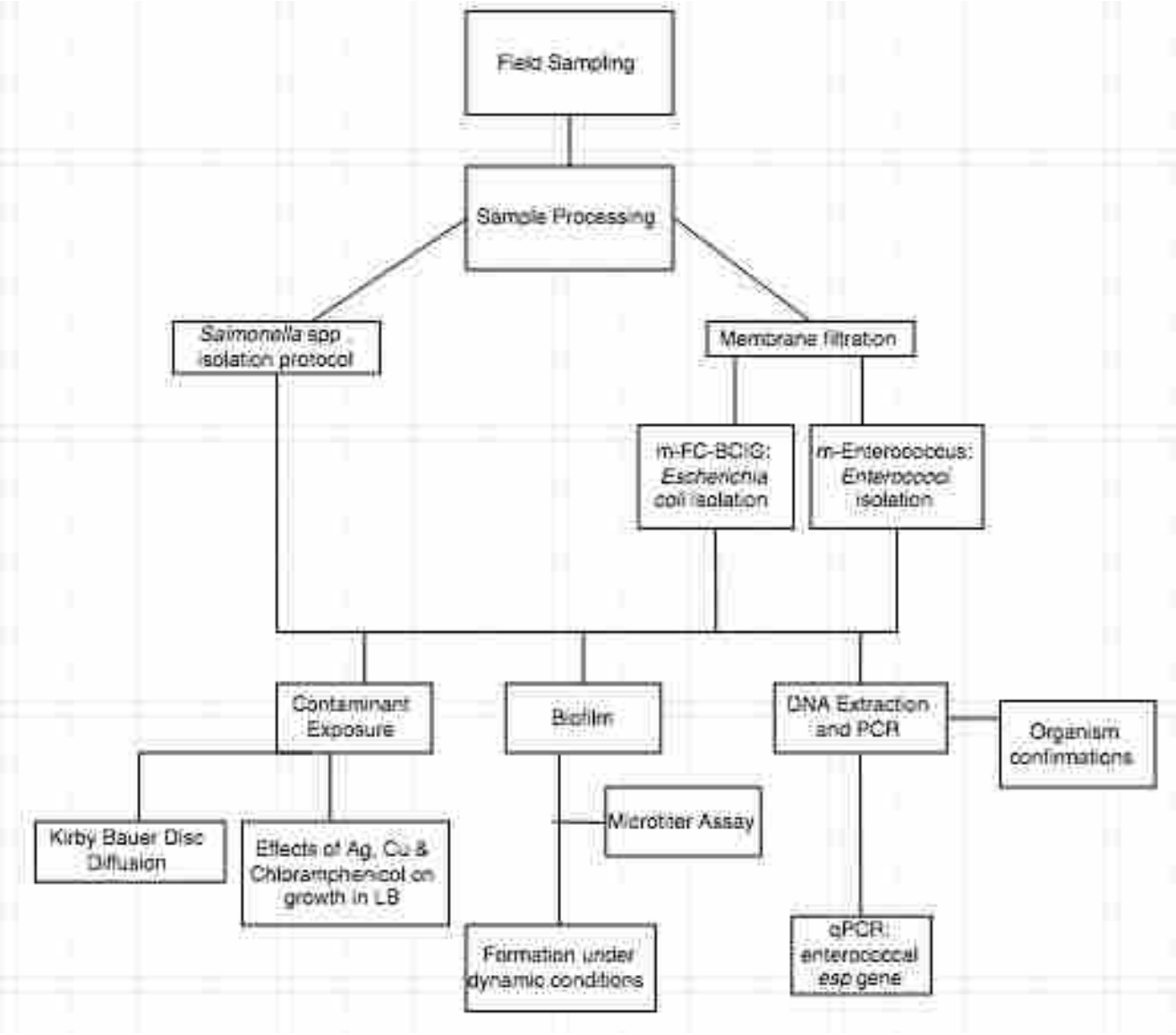


Figure 2.1. Schematic representation of experimental approach. This diagram details the specific processes used to test a linkage between biofilm formation and the stressors that mimic host and/or non-host environments.

Field Sites and Isolation Parameters

In order to better understand patterns of biofilm formation and bacterial expression of biofilm components, it is important to have samples from a variety of different locations. *Salmonella* spp., *E. coli,* and *Enterococcus* spp. samples were each isolated from locations of high, medium and low water quality at freshwater locations (Table 2.1). The isolates from low quality sites were expected to have recently been in a host environment and more tolerant to contaminant-induced stress (Mbuthia *et al.,* 2013; Wang *et al.*, 2014). The sampling locations were subsequently divided into rhizosphere, rhizoplane and water locations (Table 2.1). The different sites were chosen in order to compare the biofilm-forming capabilities and resistance profiles of bacteria isolated from different zones and environmental conditions. Locations of isolation are described below, and specific methods of *Escherichia coli, Enterococcus* spp. and *Salmonella* spp. isolation are outlined in Sections 2.3 and 2.4.

All riparian wetland samples were obtained from sites located in the Grand River watershed. The river, with head waters beginning in Dufferin, ON and draining into Lake Erie at Port Maitland, is an expansive system extending over 300 km. Throughout the watershed, much effluent is brought into the river via surface runoff from agriculture (livestock), as well as hospital waste, and municipal waste water discharge. All isolates of *E. coli, Salmonella* spp., and *Enterococcus* spp. were obtained at areas along the river. High water quality sites such as Shand Dam and West Montrose were determined to be minimally impacted by anthropogenic influence, and determined to be the most pristine as dictated by lowest amounts of introduced discharge. Canagagigue, Conestogo and Bridge and Lancaster sites were intermediate (medium) water quality, having

effluent from hospital and treated municipal waste water discharge, as well as surface agricultural runoff, influencing water conditions. Lastly, Rare and Doon sites were considered the poorest (low) water quality areas, being the most heavily impacted by anthropogenic-source effluent discharge into Grand River surface waters. These sampling locations were determined based on nutrient levels as measured by the Grand River Conservation Authority. Areas exceeding designated criteria values for N and P were considered low water quality, and conversely low values of N and P considered high water quality.

Table 2.1. Locations of *E. coli, Enterococcus* spp. and *Salmonella* spp. isolation, water quality and human impacts.

Site Name	**Water Quality**	**Isolates**			**Anthropogenic Impact**
		Enterococcus spp.	*Salmonella* spp.	*E. coli*	
Shand Dam	High	9	1	1	Wastewater treatment facilities
West Montrose	High	6	5	1	Wastewater treatment facilities
Canagagigue Creek	Intermediate	0	3	0	Agriculture and livestock operations
Conestogo	Intermediate	0	3	0	Agriculture and livestock operations
Bridge and Lancaster	Intermediate	13	2	1	Wastewater treatment facilities
Doon	Poor	12	3	1	Wastewater treatment, agriculture, livestock
Rare	Poor	0	0	1	Wastewater treatment, agriculture, livestock

Table 2.2. List of organisms isolated from rhizoplane, rhizosphere and water analytes.

Sampling Zone	**Microorganism**		
	Enterococcus spp.	*Salmonella* spp.	*E. coli*
Rhizospheric soil	23	5	1
Rhizoplane	13	3	3
Water	6	7	2
Total	**42**	**15**	**6**

Control Organisms:

In addition to the environmental isolates, *Salmonella enterica, E.coli, and Enterococcus faecalis* lab strains (Cassandra Helt, Janice Thomas) were used as biological controls for assessing the presence of biofilm components. As well, each organism's DNA was used for positive controls when carrying out PCR on environmental isolates. These controls were obtained from the Wilfrid Laurier Teaching Lab (Emily McConnel) and also the American Type Culture Collection (Virginia, USA).

As described by Tutulan (Unpublished, 2015) and Römling (2003), engineered strains of *Salmonella enterica* were used for adhesion mechanism analysis. A wild-type strain, UMR1, expressed both curli fimbriae and cellulose. The three remaining controls utilized were MAE14, MAE299, and MAE755. These engineered strains were all mutants, expressing only curli fimbriae (MAE299), only cellulose (MAE14) or neither (MAE755).

General Growth Conditions

Note: All media recipes are included in Appendix A. Media was prepared as described on manufacturer's bottle.

E. coli, Enterococcus spp., and *Salmonella* spp. strains were routinely maintained in Luria-Bertani broth (Fisher Scientific, Markham, ON) and LB agar at 35±2°C. The isolates were grown at temperatures of 11°C, 28°C and 37°C when testing for biofilm formation and expression of biofilm components. These temperatures were chosen to represent both environmental and host environments and to assess optimal expression of biofilm components. A temperature of 11°C is often encountered in the environment during cooler seasons, 37°C is a mammalian host temperature and 28°C is the optimal temperature for expression of curli fimbriae and cellulose for the *S. enterica* controls and may be at the higher end of environmental temperatures (Römling *et al.*, 2003). Antibiotic exposure assays utilized the same temperatures. The duration at each condition varied, as incubation at 11^{o}C ranged from 72-96 h, 28-36 h at 28^{o}C and 18-24 h at 37^{o}C.

Salmonella spp. Isolation

Salmonella spp. were collected from the environment using a swab collection technique as specified by Standard Methods (APHA, 2005). A swab was constructed using sterilized cheesecloth, and placed under water using a pig-tailed spike. After 3 d, the swab was collected along with approximately 100 mL of water. Processing of the samples began the same day of collection. The *Salmonella* spp. isolation protocol used is similar to the procedure described by McEgan and colleagues (2014), as well as Cassandra Helt (Wilfrid Laurier, 2014). All media used for the isolation of *Salmonella* spp. was purchased from BD Difco^{TM} Missisauga, ON. Upon arrival to the lab, 90 mL of the samples were inoculated in 10 mL of 10x buffered peptone water (BPW),

which allows for non-selective recovery of *Salmonella* spp. by acting as a buffer and providing a nutrient rich environment (BD™, 2015). The flasks were then placed incubator-shaker (Fisher Scientific, Whitby, Canada) at 37°C for 24 h at 140 rpm (Helt, 2014). Root and soil samples were taken from each site using a Hori all-in-one knife and placed in a sterile Whirlpak™ bags. Samples were weighed into 5g samples and mixed with 100 mL of BPW, sonicated for 60 s and then filtered through 0.45 µm, 47 mm mixed cellulose filters (Difco, Fisher Scientific; Ottawa, Canada).

Following the recovery period, samples underwent selective enrichment using Tetrathionate Broth (TB) with 2% (v/v) iodine solution. The media promotes *Salmonella* spp. growth because of their ability to reduce tetrathionate, while the iodine and oxgall in the medium prevents the growth of coliforms (BD™b, 2015). From the BPW sample solution, 1 mL was inoculated into 9 mL of the TB with 2% (v/v) iodine. As a negative control, 1mL of *E. coli* BL21 grown in LB broth was inoculated into the TB media, and *S. enteritidis* grown in LB was inoculated as a positive control. These controls were carried out throughout the rest of the isolation process. The isolation step was performed in duplicate, and samples were incubated in a New Brunswick incubator for 24 h at 37°C.

The next selective enrichment medium was Modified Semisolid Rappaport-Vassiliadis (MSRV). This media is for the detection of motile *Salmonella* spp., to distinguish this organism from the closely related, non-motile *Shigella* (BD™, 2015). From the TB- Sample mix, 100 µL was inoculated into the center of the MSRV plate, in triplicate, and incubated with the agar-side facing up at 44 ± 2°C for 24 h. If samples were motile, they would leave a halo of growth around the point of inoculation.

The third selective enrichment medium used was MacConkey (MAC) agar. This medium allows for the differentiation between lactose fermenters and non-lactose fermenting organisms, such as *Salmonella* spp. (BD™, 2015). On this agar, *Salmonella* spp. is expected to appear as colorless colonies, while lactose fermenters, such as *E. coli,* would appear pink. Using a small micropipette tip, about 2 μL of culture were picked up from the most outer edge of the halo on MSRV. The culture was then transferred to MAC, in duplicate, and quadrant streaked using a flame-sterilized loop, and incubated at 37°C for 24 h.

Biochemical Testing

Following the selective enrichment procedures, three biochemical tests were performed in parallel in order to determine which environmental isolates were presumptive *Salmonella* spp. isolates (isolates yielding characteristics of *Salmonella* spp.). The three biochemical tests were carried out using Triple Sugar Iron (TSI), Lysine Iron Agar (LIA) and Urea broth. All three were inoculated using a single colony picked from MAC. The TSI agar was inoculated using a stab and streak method, and a positive *Salmonella* spp. result was determined by the formation of blackening of media (H_2S precipitation), gas production and dextrose fermentation (discoloration of media). LIA was inoculated using the double stab and streak method. *Salmonella* spp. also produces H_2S in this medium due to reducing ferrous sulfate (blackening of media). Finally, isolates were inoculated into Urea broth. *Salmonella* spp. does not contain the urease enzyme, therefore, a negative Urea broth test result is considered positive for *Salmonella* spp.. A positive *Salmonella* spp. control was used for each test, and a negative control made using *Klebsiella pneumoniae*. All inoculated tests

were incubated for 48 h at 37°C. A positive result for *Salmonella* spp. in urea broth is a no-change reaction, which is where the broth does not undergo color change, as the urease enzyme is not present in *Salmonella* species.

PCR Colony Confirmation

The presumptive *Salmonella* spp. isolates were confirmed using colony PCR. The primers used for *Salmonella* spp. confirmation are genus specific primers of the *invA* invasion protein Sal-F 5′CGTTTCCTGCGGTACTGTTAATT 3′ and Sal-R 5′AGACGGCTGGTACTGATCGATAA 3′ (Shannon *et al.,* 2007). DNA was isolated using Qiagen ™ Soil DNA isolation kits, using provided Qiagen ™ Soil DNA Isolation protocols. The PCR master mix was prepared for the 25 µL reactions using reaction solutions from Sigma-Aldrich (Oakville, ON, CAN). Each reaction contained 1x Go-Taq™ Flexi Green PCR Buffer, 1.5 µM $MgCl_2$, 0.5 µM of Sal-F and Sal-R, 200µM dNTP, Milli-Q water (enough to complete a 25 µL reaction) and 0.2 µL Go-Taq™ Flexi. The PCR Reaction was carried out using the iCycler Thermal Cycler System (Bio-Rad, CA, United States). The PCR conditions began with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 20 sec at 94°C and 1 min at 65°C and a final extension step of 7 minutes at 72°C. Once the reaction ended, the PCR products were held at 4°C until they were stored at -20°C. *S. enteritidis* and *E. coli* were used as the positive and negative controls, respectively. To observe the PCR products, 7 µl were then loaded onto a 2% (w/v) agarose gel in 1X tris-acetate-EDTA (TAE) buffer and run for 60 min at 100V. The gels were then stained in ethidium bromide (0.5 µg/Ml EtBr) solution for 20 min, and decolorized in water for 5 min. The gels were imaged using a

BioRad™ GelDoc™ XR (CA, United States). *Salmonella* spp. isolates were confirmed by the presence of an 82 base pair band.

E. coli Isolation

E. coli was isolated using the membrane filtration technique as described by Food and Drug Administration (FDA, 2002). Water samples were diluted to 10^{-1} and 10^{-2} and 10mL were filtered through 0.45 µm, 47 mm mixed cellulose filters (Difco, Fisher Scientific; Ottawa, Canada). Additionally, 1 mL and 10 mL of the undiluted samples were also filtered. As a positive control, 100 µL of 18 hr *E. coli* culture was filtered. Root and soil samples were weighed into 5g samples and mixed with 100 mL of buffer, sonicated for 60 seconds and then filtered through 0.45 µm, 47 mm mixed cellulose filters (Difco, Fisher Scientific; Ottawa, Canada). The filters were then placed onto mFC media (Difco, Fischer Scientific) supplemented with 100 µg/L BCIG (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide) as per Lyautey and colleagues (2010) (Sigma-Aldrich, Oakville, ON, Canada). The plates were then incubated at 44 ± 0.5°C for 24 h in a New Brunswick™ Scientific Classic Series C76 Waterback Shaker (Edison, New Jersey, USA), full beaker of water. After incubation, colonies were checked for color, with positive *E. col* growth confirmed if blue. The selected isolates were streaked for purity onto LB agar, DNA extracted using Qiagen™ DNA extraction kits, and molecular confirmation using Eco-F and Eco-R primers were used (Table 2.3).

Enterococcus spp. Isolation

Enterococcus spp. isolates were obtained using similar sampling and analytical processing techniques (membrane filtration) as described American Public Health Association (APHA, 1998) similar to *E. coli.* Root and soil samples were weighed into 5g samples and mixed with 100 mL of buffer, sonicated for 60 s and then filtered through 0.45 µm, 47 mm mixed cellulose filters (Difco, Fisher Scientific; Ottawa, Canada). Water samples were diluted to 10^{-1} and 10^{-2} and 10 mL were filtered through 0.45 µm, 47 mm mixed cellulose filters (Difco, Fisher Scientific; Ottawa, Canada). The filters were then placed onto m-Enterococcus (Difco, Fischer Scientific) media and incubated at 37^{o}C for 48 h in a New Brunswick™ incubator with a large, full beaker of water. Positive isolates appeared maroon as maroon colonies, which were then streaked for purification on Bile Esculsin Agar (Difco, Fischer Scientific) and incubated for 24 h at 37^{o}C. Black colonies surrounded by darkened media were considered positive for group D streptococci. The selected isolates were streaked for purity onto LB agar, grown in LB for 18 h at 35±2^{o}C and DNA extracted using Qiagen^{TM} DNA extraction kits. Molecular confirmation using genus and species specific primers for *Enterococcus* spp. (Table 2.3).

Molecular confirmation of *Enterococcus spp.* and *E. coli (*Methods adapted from Helt, 2012)

Enterococcus spp. isolates were identified using genus- and species-specific multiplex PCR in order to distinguish three common species of *Enterococcus,* including; *E. faecalis, E. faecium,* and *E. durans*. Genus-specific PCR primers to 16S rRNA genes were designed previously and used in each reaction to confirm the genus Enterococci (Deasy *et al*., 2000). For species-specific identification, the enterococcal superoxide dismutase (sodA) gene sequences were used. A previous report identified the manganese-dependent superoxide dismutase gene sodA as an ideal

gene for species identification of enterococci (Poyart *et al*., 2000). Four sets of PCR primers (Table 2.2) were used as previously published (Deasy *et al*., 2000; Jackson et al., 2004) and synthesized by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). The PCR master mix consisted of 1.25 µL of genus and species-specific primers (16 µM) for *E. durans,* and *E. faecium*, with the exception of *E. faecalis* (FL1, FL2 primers), in which 2.5 µL was added to the base mix, consisting of 3 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mix, 5 X GoTaq Flexi Buffer (4.5 µL), and 2.5 U of GoTaq Flexi DNA Polymerase (Promega, Fisher Science). PCR was performed using a BioRad™ Icycler iQ PCR machine. The PCR mixtures were performed in a final volume of 22.5 µL consisting of 20 µL of master mix and 2.5 µL of template (or a single isolated colony). Following an initial denaturation at 95°C for 4 min, products were amplified by 30 cycles of denaturation at 95°C for 30 s, annealing at 55° for 1 min, and elongation at 72°C for 1 min. Amplification was followed by a final extension at 72°C for 7 min. Ten microliters of PCR product was electrophoresed on a 1.6% (w/v) sodium borate agarose gel and confirmed under UV light after ethidium bromide (EB) staining. Similarly, *E. coli* isolates were confirmed using species-specific PCR primers as previously developed by Lee *et al*., (2006) (Table 2.3). PCR mixture (20 µL) contained 5 X GoTaq Flexi Buffer (4.0 µL), 2.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphate mix, 1.0 U of GoTaq Flexi DNA Polymerase (Promega), and 2.0 µL of each primer (10 µM). The PCR conditions were 95°C for 5 min for pre-denaturing, followed by 35 cycles at 95°C for 20 s, 60°C for 1 min, and a final extension at 72°C for 10 min. PCR products were examined on 1.6% (w/v) sodium borate agarose gels and confirmed under UV light after Ethidium Bromide (EB) staining.

Table 2.3. Genus and species specific primers for multiplex PCR molecular confirmation of *Enterococcus* spp. and *E. coli.*

Strain	Primer	Sequence (5'-3')	Product Size (bp)	Reference
Enterococcus spp.	E1 E2	TCAACCGGGGAGGGT ATTACTAGCGATTCCGG	733	Deasy *et al.* (2000)
E. faecalis	FL1 FL2	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	360	Jackson *et al.* (2004)
E. faecium	FM1 FM2	GAAAAAACAATAGAAGAATTAT TGTTTTTTGAATTCTTCTTTA	215	Jackson *et al.* (2004)
E. durans	DU1 DU2	CCTACTGATATTAAGACAGCG TAATCCTAAGATAGGTGTTTG	295	Jackson *et al.* (2004)
Escherichia coli	Eco-F Eco-R	GTCCAAAGCGGCGATTTG CAGGCCAGAAGTTCTTTTTCCA	<100	Lee *et al.* (2006)

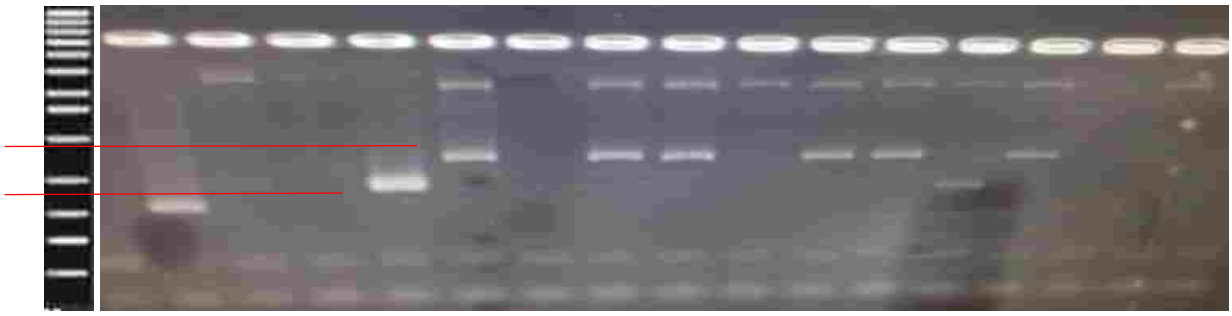


Figure 2.2. Confirmation bands of *Enterococcus faecalis* (top line; 360 base pairs) and *Enterococcus faecium* (bottom line;215 base pairs).

2. Biofilm

Adhesion Mechanisms

Calcofluor-infused agar plates were used for the initial detection of cellulose, or similar exopolysaccharides. Cultures were inoculated on LB plates containing 0.025% (w/v) Calcofluor white (Fluorescent Brightener 28, Sigma-Aldrich, MO, USA). If an exopolysaccharide, such as cellulose, is present it binds to the Calcofluor dye and fluoresces under UV light (Römling *et al.*, 2003). The isolates were streaked onto the agar and incubated at temperatures of 10°C, 21°C, 28°C and 37°C. After incubation, the colonies were observed for fluorescence under 300 nm UV light using a Hoefer Scientific Mighty Bright UV light box. For positive and negative controls, UMR1, MAE14, MAE299 and MAE775 were plated and incubated at 28°C. Each isolate was tested in triplicate.

Congo red (Fluka Analytical, Sigma Aldrich, MO, USA and Fisher Scientific, Ottawa, Canada) and Coomassie Brilliant Blue (Fisher Scientific) infused LB agar (final concentrations of 40 μg ml^{-1}, 15μg ml^{-1}, respectively) were used to detect the presence of curli fimbriae and cellulose produced by bacterial colonies (Römling *et al.*, 2003). After incubation, the colonies exhibit different morphotypes based on the presence of curli fimbriae and/or cellulose. The *rdar* (red, dry and rough) morphotype is expressed by organisms that produce both curli fimbriae and cellulose as part of their extracellular matrix; the *pdar* (pink, dry and rough) morphotype expresses only cellulose, *bdar* (brown, dry and rough) only curli. A *saw* (smooth and white) morphotype expresses

neither component (Römling *et al.*, 2003). Similar to the Calcofluor plates, isolates were incubated on Congo Red plates at different temperatures. The previously mentioned controls, UMR1, MAE14, MAE299 and MAE775 were plated and incubated at 28°C and used as a guide while comparing morphotypes of environmental isolates. This assay was completed in triplicate.

Static Biofilm Generation

This assay was modelled after Merritt and researchers (2011) with some modifications. This assay allowed for qualitative and quantitative observation of the amount of biofilm formed. Bacterial isolates were grown in LB broth overnight. The samples were then diluted 1:100 in fresh medium and 100 µL of each sample were transferred in quadruplicate to a 96-well microtiter plate (Non-Tissue Culture Treated, Flat Bottom with Low Evaporation Lid, Corning Inc., NY, USA). Each of the isolates were inoculated in four different plates for incubation at various temperatures. The plates were then incubated; at 37°C for 18-24 h, 28°C for 36-48 h, and 11°C for 96 h. After incubation, the microtiter plates were washed twice with distilled water and left to air-dry. The plates were then stained with 125 µL of 0.1% (w/v) crystal violet solution. The crystal violet stains the bacterial cells that are adhered to the sides of the wells; these cells are those that typically produce extracellular substances (*i.e.,* proteins and exopolysaccharides) which facilitate attachment to the surface and can lead to biofilm formation. The crystal violet in the wells was then solubilized using 200 µL 30% (v/v) acetic acid and measured for absorbance at 600nm in a xMark ^{TM} Bio Rad Microplate Absorbance Spectrophotometer. A high absorbance reading corresponded with heavy biofilm growth whilst minimal absorbance indicated scarce biofilm

formation (Merrit *et al.,* 2011). This experiment was repeated 3 times to ensure results were consistent between trials.

Assessing Biofilm Growth

After determining the absorbance values of the crystal violet bound to attached cells, the degree of biofilm formation was assessed. The biofilms were characterized as previously described by Stepanovic and colleagues (2000). The values were classified as no biofilm, weak biofilm, moderate biofilm and strong biofilm, by comparing the absorbance of the Crystal Violet solubilized in 30% acetic acid (v/v) (AB) to the negative control, or blank, which was 30% acetic acid (v/v) (AB_c). The categories are outlined in Table 3.2.

Table 2.4. Biofilm Classification System

No Biofilm	AB*$\leq AB_c$**
Weak Biofilm	$AB_c < AB \leq 2x\ AB_c$
Moderate Biofilm	$2xAB_c < AB \leq 4x\ AB_c$
Strong Biofilm	$4x\ AB_c < AB$

*AB= absorbance at 600nm reading of crystal violet solubilized in 30% acetic acid (v/v)

**AB_c= absorbance at 600nm reading of the negative control, or blank (30% acetic acid (v/v)

Dynamic Biofilm Formation

Using a BioFlux 200© (Fluxion Biosciences™), constant conditions (flow; dyne) mimicked environmental conditions that may influence microbial biofilm formation. *E. faecalis* isolates obtained from high, intermediate and poor water quality sites were grown up in LB Broth for 18 h. All isolates were extracted from rhizoplane and rhizosphere samples. Aliquots of 100 µL of fresh media were used to prime inlet wells. Lid interface was screwed on using a torque wrench, and a force of 1 dyne was used to push media through wells (inlet to outlet; approximately 5 minutes). Next, 20 µL of grown culture was placed in the outlet wells. At a force of 2 dynes for 5 seconds, cultures were pushed into viewing area. Using a Nikon™ Ti E Eclipse [bright field] microscope, biofilm production was visualized and captured (Figure 4.24). After waiting 10 minutes, 700 µL of fresh LB Broth was added to the inlet wells, and another 10 minutes was allowed for incubation. Videos and images were taken at 0, 18 and 24 h. Using Calcofluor (0.025% w/v), extra polymeric substances were stained, and a DAPI used for fluorescent imaging.

Antibiotic Response

Antibiotics for susceptibility testing were chosen based on suggested groupings by the Clinical and Laboratory Standards Institute (2015) of antibiotics, World Health Organization list of most important drugs, as well as relevance in the Grand River Watershed (generally used for *E. coli, Enterococcus* spp., and *Salmonella* spp. infections, and previous research on *Salmonella* susceptibility completed by Janis Thomas and Cassandra Helt in the Applied Microbiology Research Lab supervised by Dr. Robin Slawson (Thomas, 2011; Helt, 2012).

Table 2.5. Susceptibility Testing Antimicrobial Compounds

Class	**Antimicrobial Name**	**Drug Code**	**Disk Potency (µg)**	**Action**
Penicillin's	Ampicillin	A	10	bactericidal
	Amoxicillin/ Clavulanic acid	AmC	20/10	bactericidal
Cephalosporin's	Ceftazidime	Ctx	30	bactericidal
Aminoglycosides	Streptomycin	S	10	bactericidal
Tetracycline's	Tetracycline Doxycycline	T D	30	bacteriostatic
Fluoroquinolones	Ciprofloxacin	Cip	5	bactericidal
Glycopeptide	Vancomycin	V	30	bactericidal
Amphenicol	Chloramphenicol	C	30	bacteriostatic
Sulfonamides	Sulfasoxazole	Sul	30	bactericidal

	Sulfadiazine	G		
Oxazolidinones	Linezolid	L	30	bacteriostatic

Disc-Diffusion Assay

Environmental isolates were tested for antimicrobial susceptibility using antibiotic discs (BD) listed in Table 2.5. Antibiotic resistance was determined as described by the Clinical and Laboratory Standards Institute, using the Kirby-Bauer disk diffusion method (CLSI, 2007). Isolates were grown in LB Broth to a 0.5 McFarland standard of turbidity, after which 100 µL were transferred onto Mueller-Hinton (MH) agar. Disks of antibiotics listed in Table 2.4 (purchased from BD, MD, USA) were then placed on the inoculated plates using sterilized tweezers. Following incubation at 37°C for 18-24 h, 28^{o}C for 48 h and 11^{o}C for 96 h, the zones of inhibition were measured and used to categorize the isolates as susceptible, intermediately resistant, or resistant according to the CLSI zone diameter interpretive standards for each antibiotic (CLSI, 2007). This was done in triplicate and the average zone of inhibition was used to determine resistant or susceptible behavior.

Dynamic Contaminant Response

Environmentally isolated *Enterococcus faecalis* was grown overnight (18 h) to a McFarland standard of 0.5. Silver nitrate ($AgNO_3$), Copper Nitrate ($Cu[NO_3]_2$) and Chloramphenicol stock solutions were made at a concertation of 0.1 M then diluted to environmentally relevant values (Cooke, 2014; Pileggi *et al.*, 2016). Concentrations varied by chemical, which were diluted with

50 mL LB broth during exposure trials and can be found in Table 2.6. Using a shaking chest incubator (New Brunswick Scientific, Edison, NJ), 250 mL Erlenmeyer flasks were incubated at 120 rpm and 28^0C for 24 h. Absorbance (450 nm) was taken every 2 h, for 24 h, using a spectrophotometer (Jenway, UK). Data was plotted using Microsoft Excel (2013).

Table. 2.6. Concentrations (millimolar) of metals and antibiotics used in contaminant response exposure trials.

$AgNO_3$	**$Cu[NO_3]_2$**	**Chloramphenicol**	**$AgNO_3$ + Chloramphenicol**	**$Cu[NO_3]_2$ + Chloramphenicol**
0.5 mM	10 mM	0.5 mM	0.2 mM + 0.2mM	1 mM + 0.2 mM
0.25mM	1 mM	0.25 mM	0.1 mM + 0.2 mM	0.5 mM + 0.2 mM
0.0125 mM	0.5 mM	0.0125 mM	0.2 mM + 0.1 mM	0.2 mM + 0.2 mM
0.0625 mM	0.25 mM	0.0625 mM	-	-

3

Contaminant Response Measurements in Environmentally Isolated Opportunistic Pathogens

The ability of environmentally isolated microbial pathogens to respond to contaminant stress was assessed. Isolates from high, intermediate and poor water quality areas found at riparian zones along the Grand River Watershed were obtained from root, soil and water samples. The following sections will present findings of how enteric opportunistic pathogens such as *Salmonella* spp., *Escherichia coli,* and *Enterococcus* spp., colonize environmental settings. Exposure to a range of temperatures in the presence of stressors, particularly antibiotics and metals, will also be evaluated.

This chapter addresses the research question of how contaminant exposure, particularly antibiotic metabolites and metals, may influence microbial behavior in an environmental setting. At each water quality site, rhizosphere soil, roots (rhizoplane) and water samples were collected, processed and analyzed. The objectives evaluated in this chapter were as follows.

1) Isolate opportunistic pathogens from rhizoplane, rhizosphere and water samples of riparian wetlands.

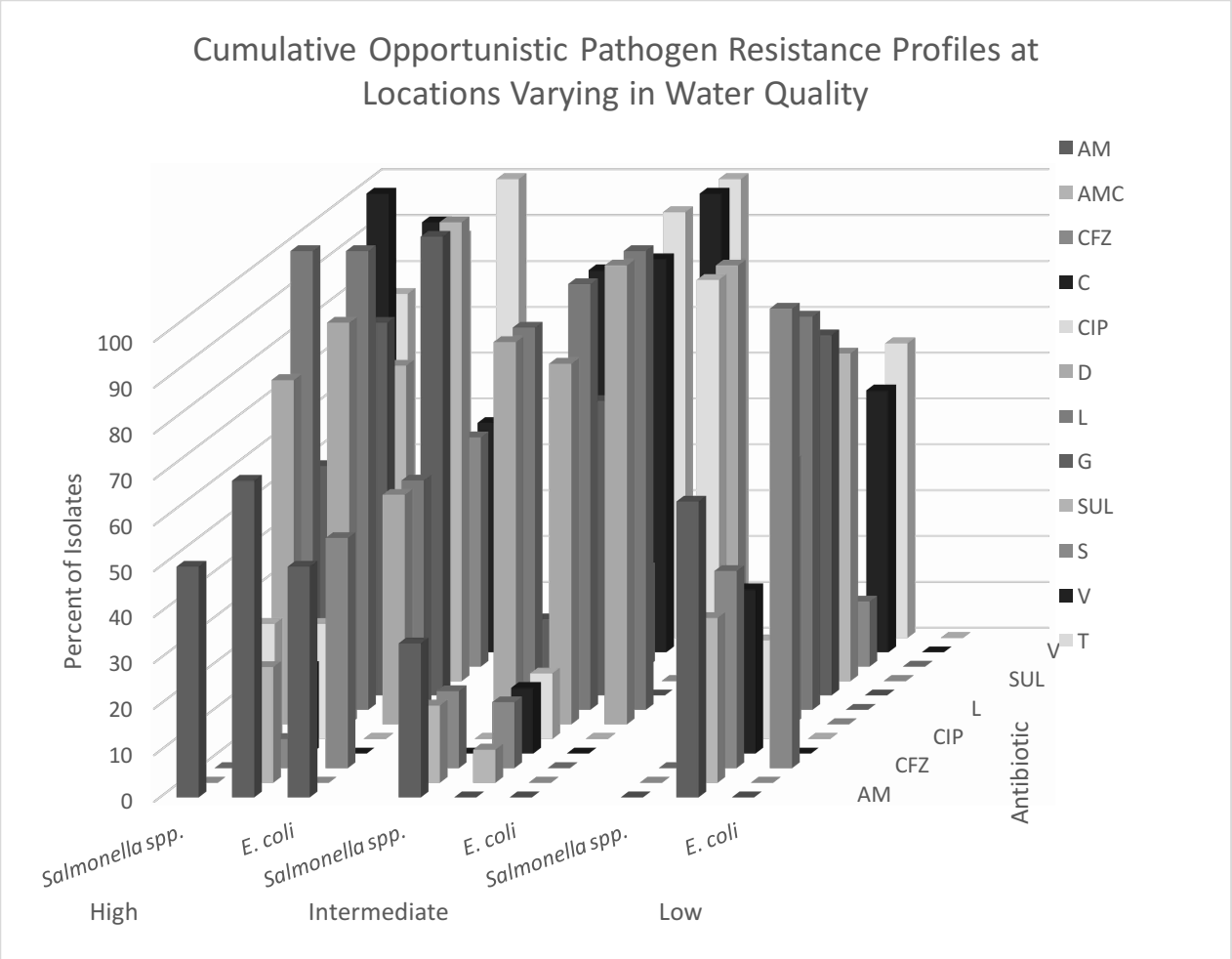
2) Evaluate the ability of these isolated microorganisms to respond to adverse conditions caused by contaminant stress, including antibiotics and metals.
3) Quantify the amount of contaminant-resisting opportunistic pathogens from each sampling area and determine how water quality may influence resistance profiles.

By considering a range of temperatures which could include optimal and stressful conditions, the ability to replicate and grow in the presence of antimicrobials was assessed. Microbial responses to individual stressful stimuli (antibiotics or metal) as well as in combination were assessed. Specifically, the effect exposure to these stressful conditions has on the growth of environmental *E. faecalis, Salmonella* spp., and *E. coli* isolates was measured. Firstly, antibiotic exposure using the disc diffusion method at 11ºC, 28ºC and 37ºC are presented. Each assay is subdivided to show comparisons of antibiotic profiles between isolated genera of microbes and also environmental zones these organisms were re-isolated from. Rhizospheric soil, rhizoplane [root] and water samples were collected and processed as discussed in Chapter 2 to obtain these organisms. Contaminant response trials were conducted using an *Enterococcus faecalis* isolate extracted from rhizospheric samples obtained from a low water quality sampling area.

3.1 Effectivity of Antibiotic Exposure at 37ºC

Antibiotic tolerance and the ability to measure microbial growth in the presence of antimicrobials was carried out using the Kirby-Bauer Disc Diffusion assay. As described in Table 2.5, 12 different antibiotics were chosen for usage. They were selected based on their environmental relevance, particularly in the GRWS, as well as their ranking on the necessary antibiotics list compiled by World Health Organization. Microorganisms were grown for 18 h in LB and then spread-plated

on Mueller-Hinton agar, incubated at 37ºC, 28ºC, and 11ºC in the presence of antibiotic-soaked discs, then analyzed after 24 h, 48 h and 96 h. Zones of inhibition were measured (mm) and compared to known values using data charts supplied in BD BBL™ Sensi-Disc™ Antimicrobial Testing Kits. Each was done in triplicate and was designated as resistant or susceptible based on known values.



<u>Figure 3.1.</u> Resistance profiles of pathogen isolates grown at 37°C. Kirby-Bauer disc diffusion results of microorganisms isolated from high quality wetlands (left), intermediate water quality impacted wetlands (center) and low water quality impacted wetlands (right). In order

from front, first, are the antibiotics chose. AM= ampicillin, AMC = amoxicillin and clavulanic acid, CFZ = ceftazidime, C = chloramphenicol, CIP = ciprofloxacin, D = doxycycline, L= linezolid, G= sulfadiazine, SUL= sulfasoxazole, S= streptomycin, V= vancomycin, and T= tetracycline.

Antibiotic profiles for all isolates obtained from impacted riparian wetlands are depicted in Figures 3.1. As seen, there is a general decrease in resistance profiles from high, to low water quality impacted wetlands. *Enterococcus* spp. was most prominent at each site (Table 2.1) and had more robust resistance profiles, whereas *E. coli* and *Salmonella* spp. were lesser (Table 2.1, 2.2). Interestingly, susceptibility increased as site quality decreased when isolates grew in the presence of antibiotics at host body temperature (37^{o}C). All isolates from high water quality impacted wetlands showed greater than 50% resistance against 75% of antibiotics.

Amoxicillin and clavulanic acid, followed by chloramphenicol and ciprofloxacin, had the lowest percentage of resistance with values of 20-37%, with the exception of *Salmonella* isolated at poor water quality sites. At intermediate quality wetlands, there are increases in resistance to doxycycline and linezolid, however decreases were observed in the majority of other antimicrobials. Both tetracycline and vancomycin invoked high resistance in the isolated pathogens from high and intermediate water quality impacted sites, whereas at lower water quality sampling sites showed there was less observed resistance. Evaluating each site shows the affinity of *Enterococcus* spp. to successfully grow in the presence of stress-inducing chemicals like antibiotics. Interestingly, *Salmonella* spp. isolates showed higher levels of resistance to doxycycline and ciprofloxacin (100%) at poor quality sites when compared to those of better water quality.

The next series of figures depicts antibiotic resistance profiles based on environmental sample type. Figure 3.2 (left column)corresponds with isolated pathogens from high water quality areas, whereas (center column) represents those from intermediate water quality and (right column) from poor water quality. Additionally, environment samples retrieved included soil (rhizosphere soil), roots (rhizoplane) and water, represent the specific location these pathogens were isolated from.

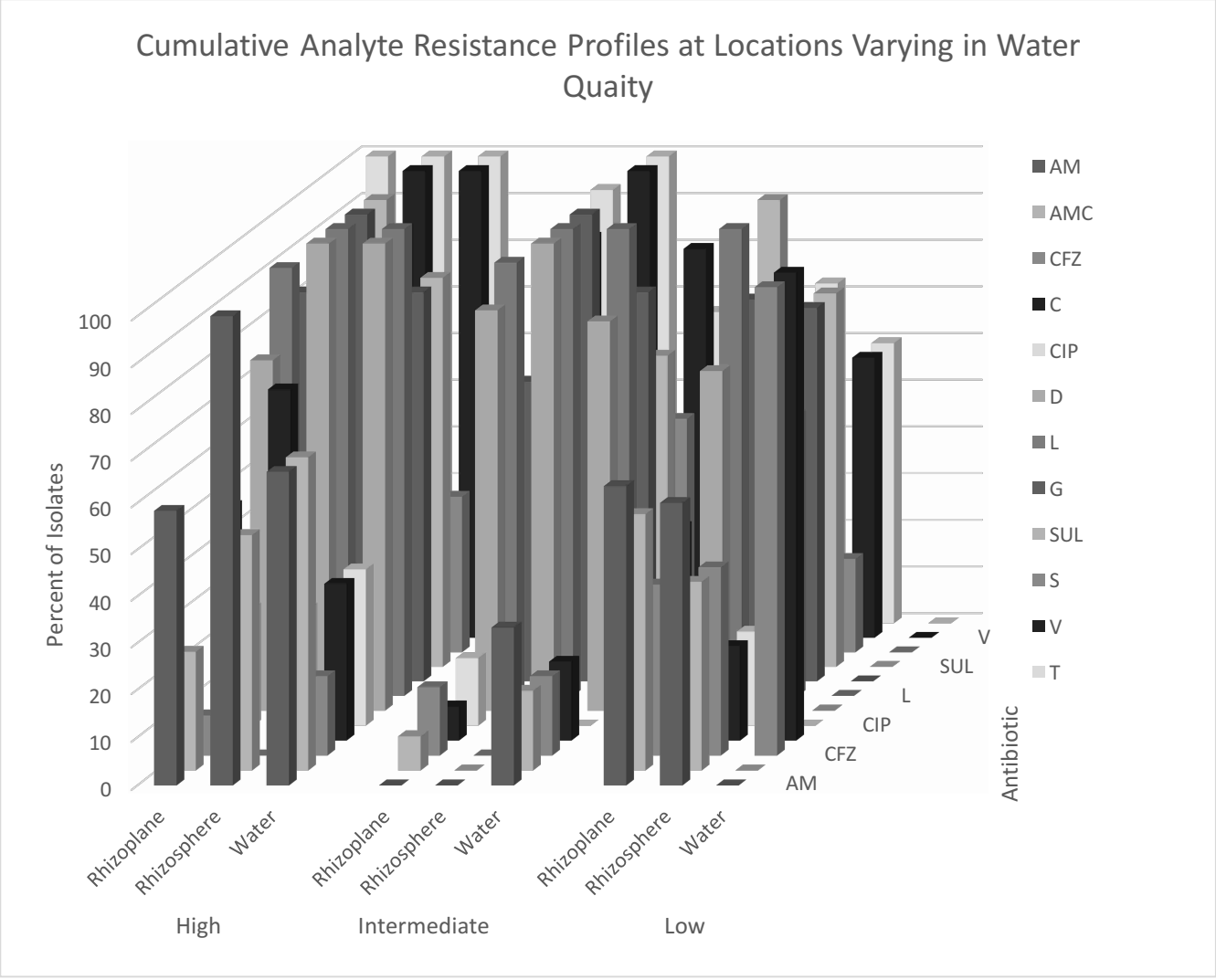
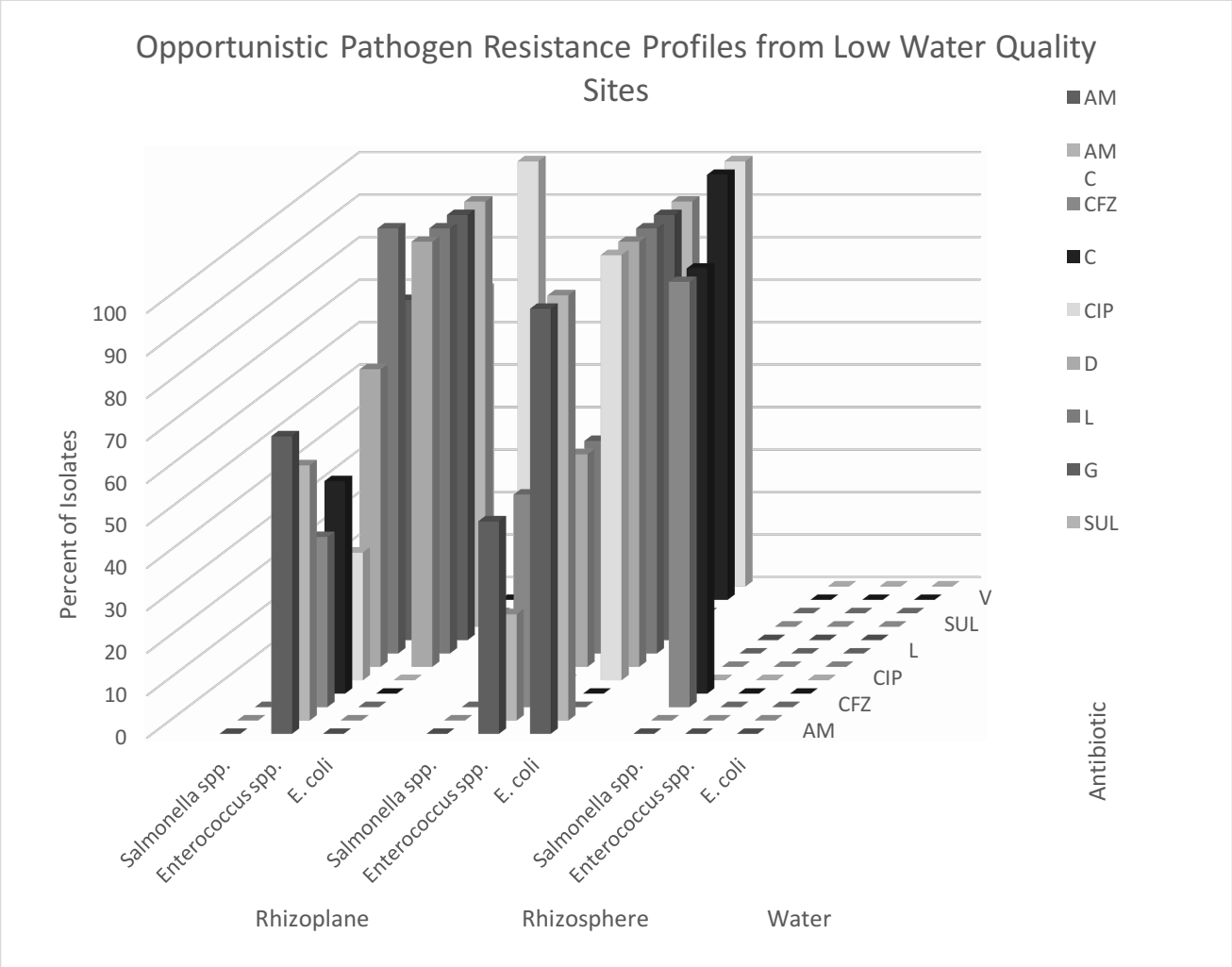


Figure 3.2. Percentage of opportunist resistance during antibiotic exposure at 37°C. Kirby-Bauer disc diffusion results of microorganisms isolated from the roots, soil and water of riparian zones sampled at high quality wetlands are shown (left), intermediate water quality impacted sites (center) and poor water quality impacted sites (right).

An initial trend that is apparent is that rhizospheric analytes yielded the highest percentages of isolates showing resistance when grown at 37°C. At high water quality impacted sites (Shand Dam and West Montrose) 100% of isolates were resistant to tetracycline, and 8 other antibiotics with resistance equal to or greater than 50 percent. Isolates obtained from water samples showed poorest resistance values, whereas rhizospheric isolates had intermediate to complete resistance profiles. In high and intermediate quality wetlands, resistance was highest for doxycycline, linezolid, tetracycline, vancomycin, sulfisoxazole and sulfadiazine. Isolates at intermediate sites were most susceptible to ciprofloxacin, chloramphenicol, ceftazidime, ampicillin and amoxicillin which shows differing trends when compared to both high and low quality sites. At low quality sites, 40-100% of isolates originating from rhizospheric samples displayed 100% resistance to ciprofloxacin and ceftazidime.

A more detailed representation of specific pathogen resistance profiles originating from the rhizosphere of high water quality impacted sites is examined in Figure 3.3. After growth at 37°C for 18 h, the rhizosphere was again the most densely populated region with opportunistic bacteria, followed by rhizoplane and water samples. *Enterococcus* spp. organisms were consistently found in each sample type in highest numbers, followed by *Salmonella* spp. and *E. coli.* All three genera were 100% resistant to tetracycline when isolated from the rhizosphere with at least 50% of isolates showing multidrug resistance to greater than 4 antimicrobials

(vancomycin, sulfisoxazole, sulfadiazine, linezolid and doxycycline). The least amount of resistance displayed by organisms isolated in the rhizosphere was to ceftazidime and ciprofloxacin, with greater than 60% susceptibility as see in Figure 3.3.



<u>Figure 3.3.</u> Percentage of resistant opportunistic pathogens isolated from high water quality areas exposed to antibiotics at 37°C. Organisms isolated from the rhizoplane (left), rhizosphere (center) and water (right).

Salmonella spp. and *Enterococcus* spp. originating from the rhizosphere showed 100% resistance to several antibiotics; tetracycline, vancomycin, sulfisoxazole, doxycycline and linezolid. At high

quality sites, *Salmonella* spp. resistance was most prevalent in the rhizoplane. *E. coli* recovery was minimal in rhizosphere and water samples, however *Enterococcus* spp. found in abundance, in all sample types. Similar resistance profiles were seen in the water, root and soil (MDR to 5 or more antibiotics), as well as increased survivability when exposed to ampicillin and amoxicillin.

Figure 3.4 shows that at intermediate water quality wetlands, *Enterococcus* spp. isolates were more prominent than other isolated pathogens and had more diverse resistance profiles. *Salmonella* spp., surprisingly, was the only organism isolated from the rhizoplane which showed resistance, whereas, all organisms except *E. coli* isolated from water displayed resistance. Similar resistance profiles are seen across the sampled genera of microbes, as resistance to tetracycline, vancomycin, doxycycline, linezolid and sulfadiazine was greater than 60%. Amoxicillin (80-100% susceptibility), ampicillin (60-100%), streptomycin (75-100%), ciprofloxacin (85-100%) and ceftazidime (80-100%) were effective inhibitors of microbial growth in isolates from all sample type

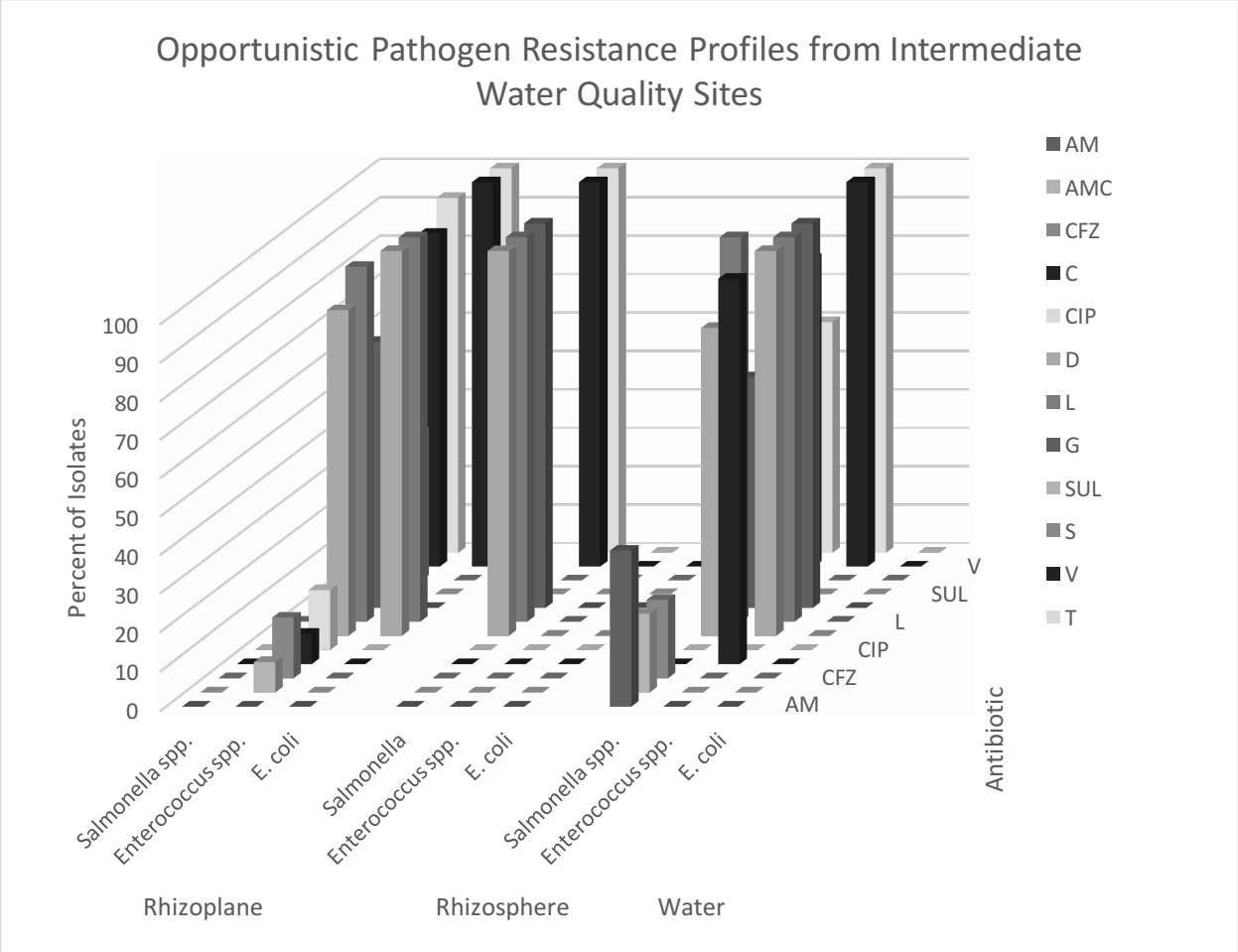
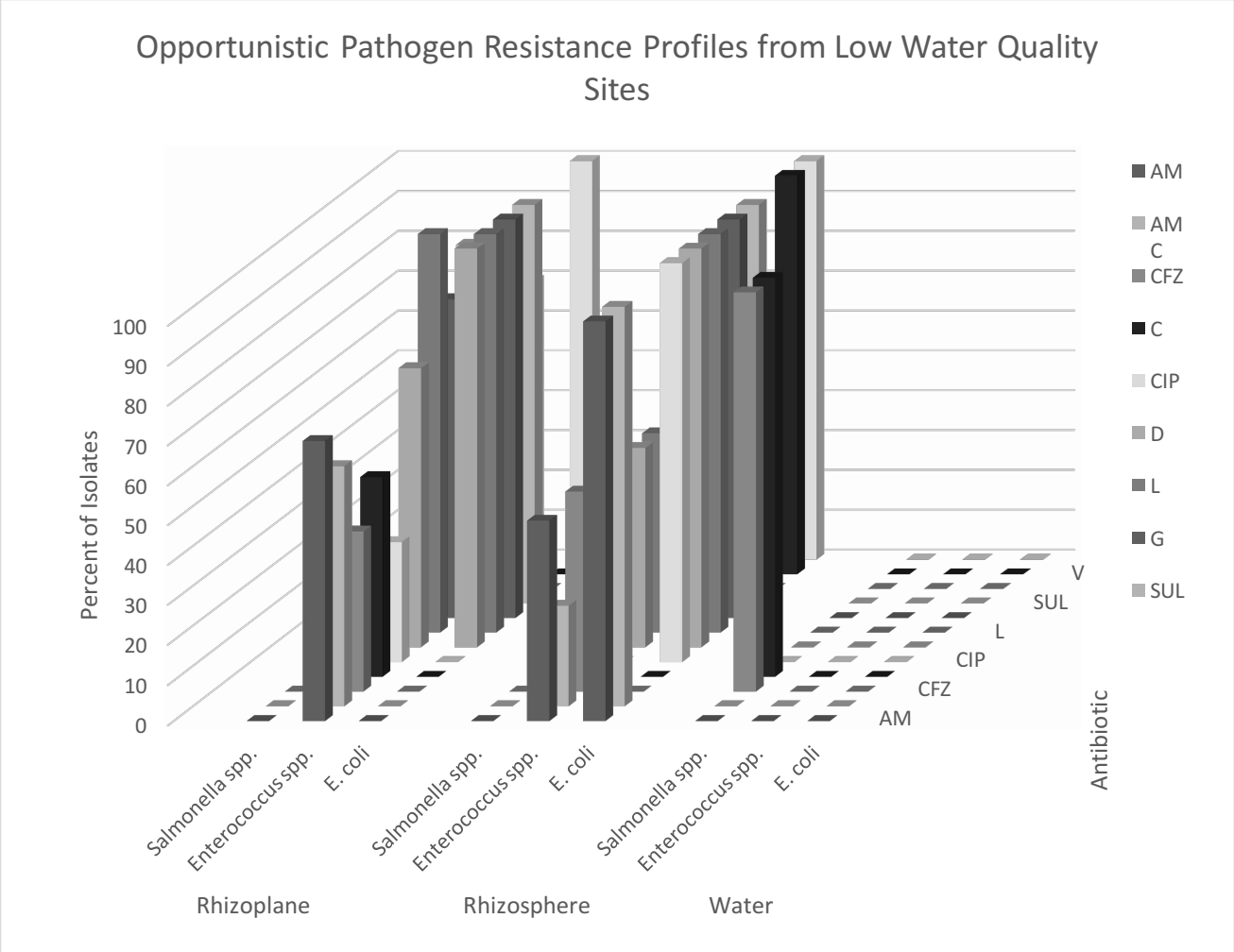


Figure 3.4. Percentage of resistant opportunistic pathogens isolated from intermediate water quality areas exposed to antibiotics at 37°C. Organisms isolated from the rhizoplane (left), rhizosphere (center) and water (right).

Chloramphenicol was an effective inhibitor of growth when comparing rhizosphere and rhizoplane isolates, however evoked less of a response against enterocccal isolates from water (100% resistance). Ciprofloxacin elicited the strongest degree of inhibitory effects (85-100% susceptibility), as isolates regardless of source struggled to grow in the presence of this antibiotic. *Enterococcus* spp. isolated from the rhizosphere were the only group of microorganisms displaying

phenotypic resistance as heavy growth on media, whereas most other isolates that were susceptible to ciprofloxacin resulted as clear and no growth.

In Figure 3.5, our analysis of results indicates that at poor water quality sites, *Salmonella* spp. isolates were less resistant than *E. coli* and *Enterococcus* spp. in the water, but in no other zones. Rhizosphere samples yielded *E. coli* isolates 100% resistant to tetracycline, sulfasoxazole, sulfadiazine, linezolid and doxycycline. Enterococcal isolates showed a broad range of resistance, with 75% of the antibiotics used showing ineffective responses. Ciprofloxacin was less likely to inhibit growth, with only 30% effectivity in *Enterococcus* spp. isolates. *E. coli* was 100% susceptible to vancomycin, streptomycin, ciprofloxacin, chloramphenicol, ceftazidime, ampicillin and amoxicillin whilst, again, 50% of enterococcal isolates showed resistance to each drug the organisms were measured against.



<u>Figure 3.5</u> Percentage of resistant opportunistic pathogens isolated from low water quality areas exposed to antibiotics at 37°C. Organisms isolated from the rhizoplane (left), rhizosphere (center) and water (right).

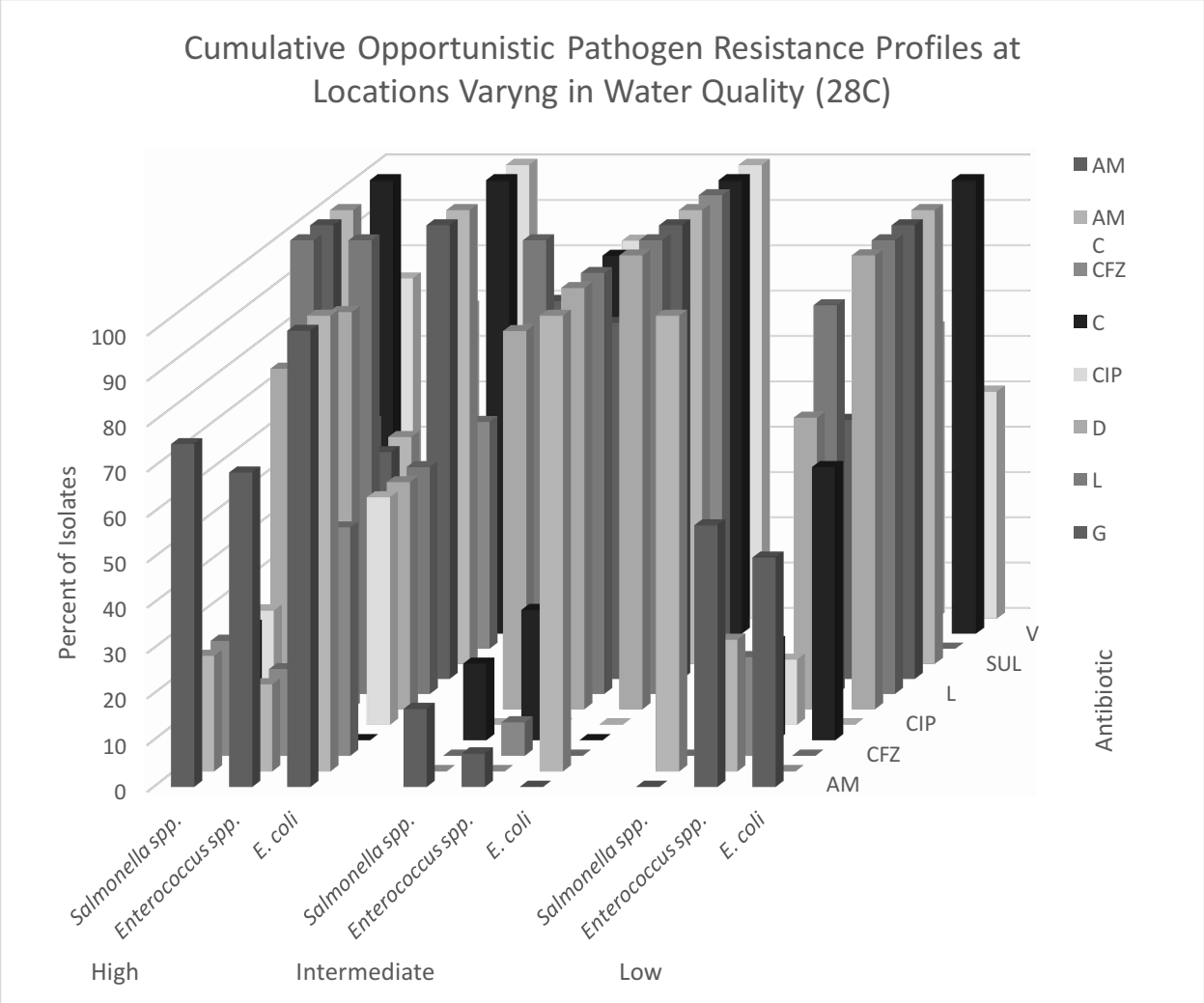
E. coli isolates from the rhizoplane were 100% resistant to several drugs; tetracycline, vancomycin, sulfasoxazole, sulfadiazine, linezolid, doxycycline, ciprofloxacin, ampicillin and amoxicillin. Unlike at higher water quality areas, *E. coli* isolates from low water quality impacted areas resulted in increased MDR profiles. Enterococci showed a diverse resistance profile, but again was least affected by sulfadiazine and sulfasoxazole, whereas other antibiotics resulted in resistance to 50% or less of all enterococcal isolates (Table 2.2, 2.3 show locations and water quality).

Lastly, *Salmonella* were the only organisms isolated from water samples at low quality sites to show any resistance. Surprisingly, these isolates were susceptible to all drugs used except for chloramphenicol and ceftazidime, which had proved effective at sampling locations of higher water quality.

In this next series of exposure trials, the same 12 antibiotics were used, however the temperature growth conditions were changed to 28^{o}C. Incubation occurred over 48 h, with analysis again examining for zones of resistance or susceptibility. This temperature was chosen as it is reflective of both environmental and adhesion mechanism plus exopolysaccharide promoting conditions (Romling, 2003; Barnhart and Chapman, 2006).

3.2 Effectivity of Antibiotic Exposure at 28^{o}C

The following figures correspond to the total pathogen resistance of all opportunistic pathogen isolates from high, intermediate and low water quality impacted sampling locations. Again, the three genera are measured against each other to gain an understanding how both wetland condition and water quality impacts the degree of resistance observed. All 3 genera of organisms were resistant to tetracycline (65+%), vancomycin (80+%), sulfasoxazole (at least 50% of isolates) and sulfadiazine (58+%), showing at an environmentally relevant temperature of 28^{o}C resulted in similar outcomes as at a host body temperature, 37^{o}C.



<u>Figure 3.6.</u> Resistance profiles of pathogen isolates grown at 28°C. Kirby-Bauer disc diffusion results of microorganisms isolated from high quality wetlands (left), intermediate water quality impacted wetlands (center) and low water quality impacted wetlands (right). In order from front, first, are the antibiotics chose. AM= ampicillin, AMC = amoxicillin and clavulanic acid, CFZ = ceftazidime, C = chloramphenicol, CIP = ciprofloxacin, D = doxycycline, L= linezolid, G= sulfadiazine, SUL= sulfasoxazole, S= streptomycin, V= vancomycin, and T= tetracycline.

Unlike exposure at 37°C (Figure 3.1), *Salmonella* spp. showed the strongest potential for success when exposed to antibiotics, followed by *Enterococcus* spp. and *E. coli.* At high water quality sites, all *E. coli* and *Salmonella* spp. isolates showed 80% or greater resistance to tetracycline,

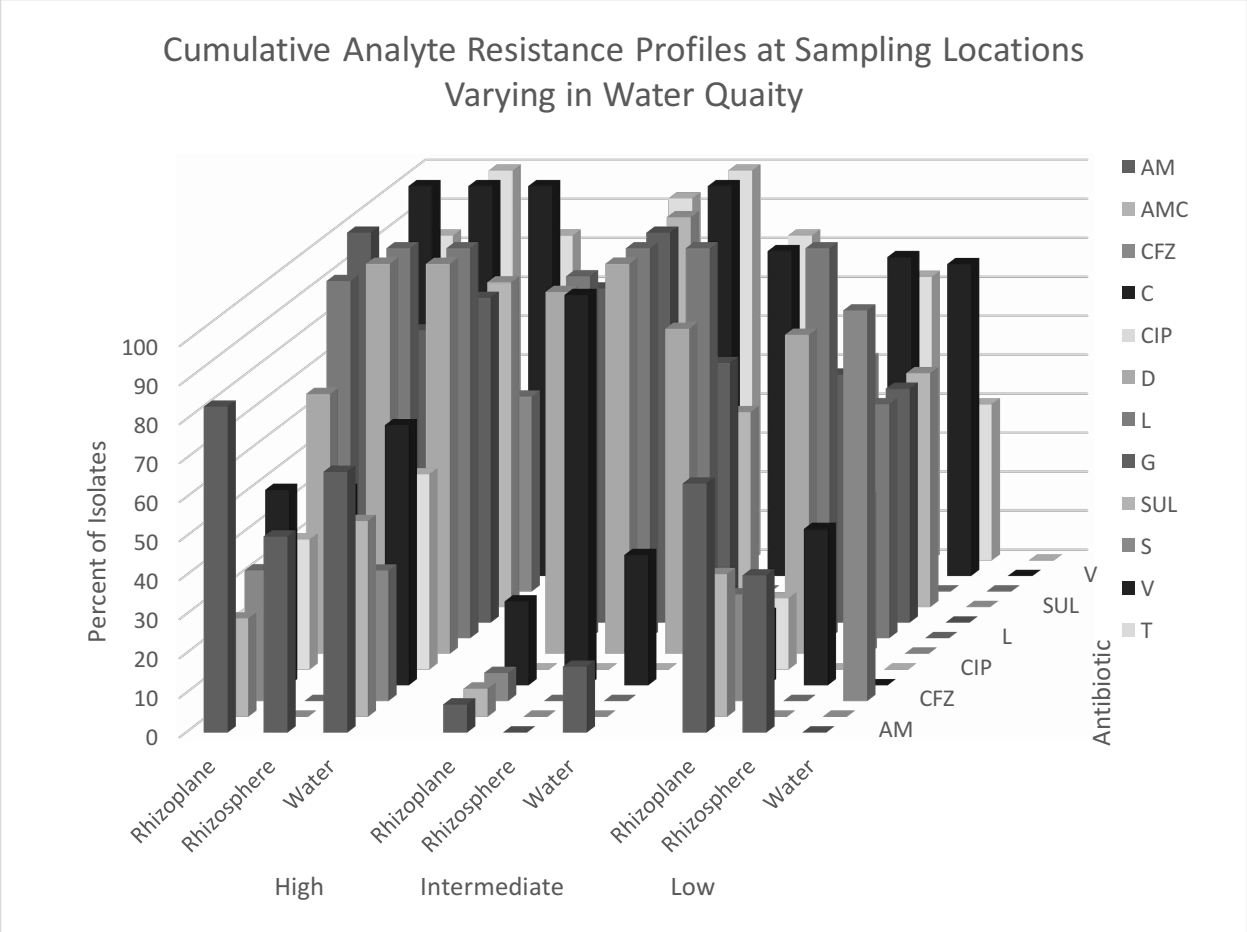
vancomycin, sulfadiazine, sulfasoxazole, linezolid, and ampicillin. Enterococci isolates were 80% or greater resistance to linezolid, doxycycline, and vancomycin. *E. coli* isolated from high water quality impacted areas showed 50% or greater resistance to ceftazidime, amoxicillin and ampicillin, which were highly effective against other microorganisms and at varying temperature. All three groups of pathogens showed resistance to at least 75% or more of the antibiotics introduced when incubated at 28^{o}C (Fig 3.6).

Isolates grown from intermediate water quality sites at 28^{o}C were completely susceptible to ciprofloxacin. *E. coli* displayed 100% resistance, to 66% (8/12) of antibiotics measured against, specifically tetracycline, vancomycin, sulfasoxazole, sulfadiazine, linezolid, doxycycline and ampicillin. Enterococci isolates showed partial to strong resistance against tetracycline, vancomycin, sulfasoxazole, sulfadiazine, linezolid and doxycycline. *Salmonella* spp. strongly resisted tetracycline (80%), vancomycin (78%), sulfadiazine (65%), sulfasoxazole (78%), linezolid (90%) and doxycycline (90%). Both enterococci and *Salmonella* spp. were susceptible (<40% resistance) to ciprofloxacin, chloramphenicol, ceftazidime, ampicillin and amoxicillin. *E. coli* was least resistant to ciprofloxacin, ceftazidime and ampicillin (all less than 20%), however 100% resistant against amoxicillin and clavulanic acid.

Microorganisms originating from sampled zones at low water quality sites and exposed to antibiotics at 28^{o}C again demonstrated that sulfadiazine, sulfasoxazole, linezolid and doxycycline were least effective at inhibiting microbial growth using this assay (60% or greater). Enterococci were most susceptible (>50%) to ciprofloxacin, chloramphenicol, ceftazidime, ampicillin and streptomycin, however most resistant to vancomycin and linezolid (>70%). All *Salmonella* spp.

were highly susceptible to all antibiotics except amoxicillin with clavulanic acid (100% resistance), whereas *E. coli* was 100% susceptible to ceftazidime and 60% susceptible to ciprofloxacin. Enterococcal isolates showed uniform levels of resistance amongst varying water quality, with 70% or greater resistance to doxycycline, tetracycline, vancomycin, sulfadiazine and sulfasoxazole.

Figure 3.7 illustrates antibiotic resistance categorized by analyte type pathogens were retrieved from, incubated at 28^{o}C, where all isolates from the rhizosphere, rhizoplane and water were 100% resistant to vancomycin. Microorganisms obtained from rhizospheric soil were 100% resistant to tetracycline, linezolid and doxycycline, and greater than 50% of isolates showing resistance to ampicillin, chloramphenicol, sulfadiazine and sulfasoxazole. Root-isolated bacteria showed 50% or greater resistance to ampicillin, chloramphenicol, doxycycline, linezolid, sulfadiazine, sulfasoxazole and chloramphenicol, and less than 50% of isolates showed resistance to ciprofloxacin, amoxicillin, ceftazidime and streptomycin. Further, organisms isolated from the water at high quality impacted sampling locations were resistant to 80% or more measured antibiotics, but least resistant (<50%) to ceftazidime.



<u>Figure 3.7.</u> Percentage of opportunist resistance during antibiotic exposure at 28^{o}C. Kirby-Bauer disc diffusion results of microorganisms isolated from the roots, soil and water of riparian zones sampled at high quality wetlands are shown (left), intermediate water quality impacted sites (center) and poor water quality impacted sites (right).

Soil-borne organisms isolated at medium water quality sites (Fig 3.7, center) were 100% resistant to tetracycline, vancomycin, sulfadiazine, sulfasoxazole, linezolid, doxycycline and chloramphenicol. These organisms were also 100% susceptible to ampicillin, amoxicillin, ceftazidime, ciprofloxacin, and streptomycin. Additionally, less than 20% of organisms found both in water and root samples were susceptible to ciprofloxacin, ceftazidime, ciprofloxacin, ampicillin and amoxicillin. Root-isolated bacteria were least susceptible (<50%) to tetracycline, vancomycin,

streptomycin, sulfadiazine, sulfasoxazole, linezolid, and doxycycline. Similarly, water-borne pathogens were best able to grow (50%) in the presence of doxycycline, linezolid, sulfadiazine, tetracycline and vancomycin as opposed to other antibiotics.

Further, at low water quality sites (Fig 3.7) microorganisms isolated from root samples had highest levels of multidrug resistance. Overall, greater than 50% of the total isolates were again resistant to tetracycline, vancomycin, sulfadiazine, sulfasoxazole, linezolid, doxycycline and, unlike higher quality sites, ampicillin. Streptomycin, ciprofloxacin, chloramphenicol, ceftazidime and amoxicillin. Organisms originating from water samples were only resistant to ceftazidime whilst greater than 50% of soil-isolated pathogens were not inhibited by vancomycin, sulfadiazine, sulfasoxazole, and linezolid. Greater than 60% of these organisms were susceptible to ampicillin, chloramphenicol, and doxycycline. Resistance to chloramphenicol, ampicillin and ceftazidime was surprising when compared to conditions of 37^{o}C (Figure 3.4), where isolated opportunists were most inhibited by these pharmaceuticals.

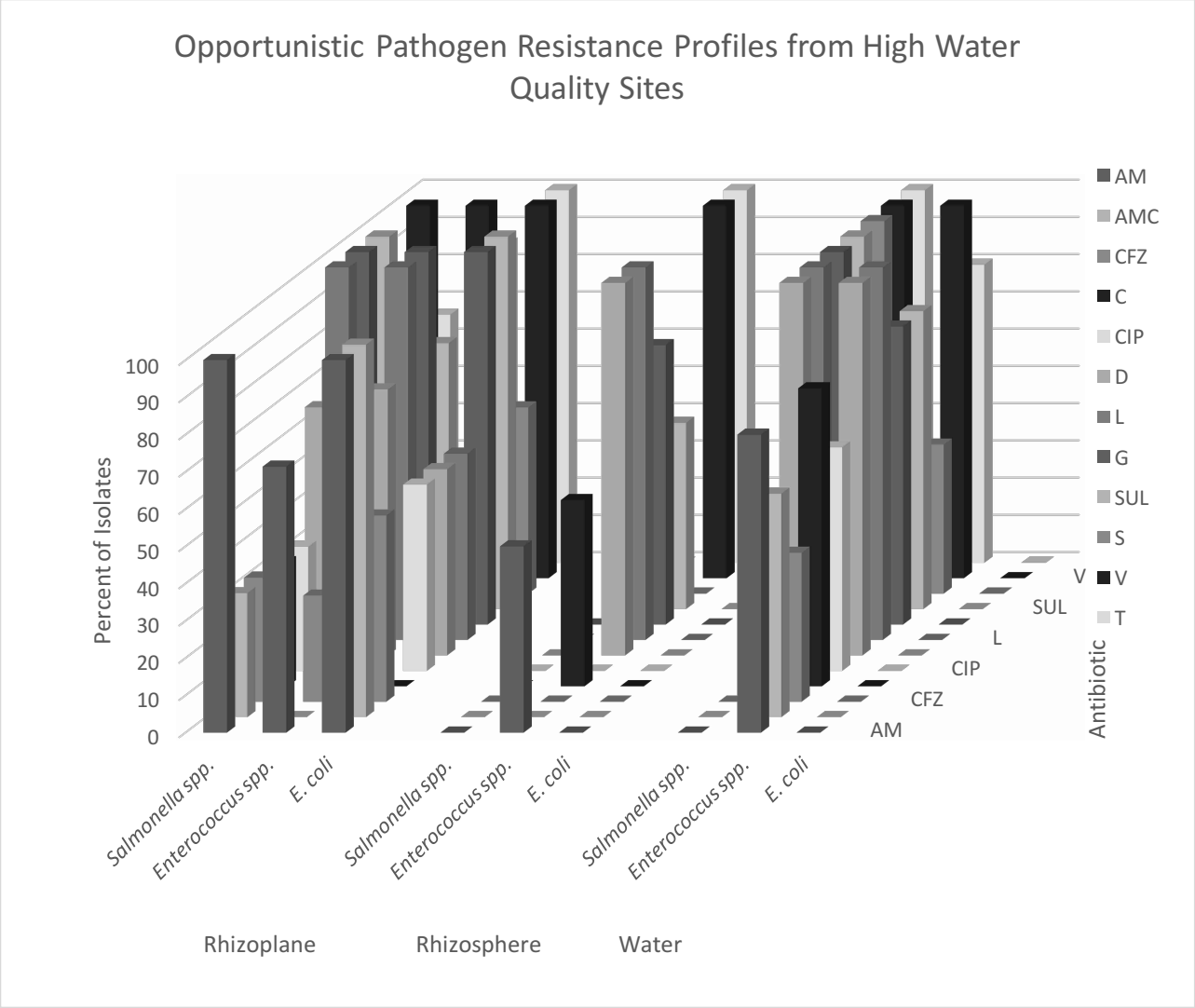


Figure 3.8. Percentage of resistant opportunistic pathogens isolated from low water quality areas exposed to antibiotics at 28°C. Organisms isolated from the rhizoplane (left), rhizosphere (center) and water (right).

Pathogen resistance profiles categorized by environmental sample type (rhizosphere, rhizoplane and water) were isolated from intermediate water quality wetlands then exposed at 28°C to the antibiotic regime. In figure 3.8, it is apparent that *E. coli* were most likely to be isolated at the rhizoplane, followed by *Enterococcus* spp. No *Salmonella* spp. isolates from the rhizosphere were resistant. All *E. coli* isolates displayed multidrug resistant behavior to tetracycline, vancomycin,

sulfasoxazole, sulfadiazine, streptomycin, linezolid, doxycycline and amoxicillin, however were susceptible to ciprofloxacin, ceftazidime, chloramphenicol and ampicillin. Enterococcal isolates obtained that were more affected by antibiotics other pathogens resisted. However, greater than 50% of isolates displayed resistance to tetracycline, vancomycin, sulfadiazine, sulfasoxazole, linezolid and doxycycline. Complete susceptibility (100% of isolsats) occurred when exposed to ciprofloxacin and amoxicillin, and less than 40% of pathogens were successful [growth] when exposed to ampicillin, chloramphenicol, streptomycin and ceftazidime.

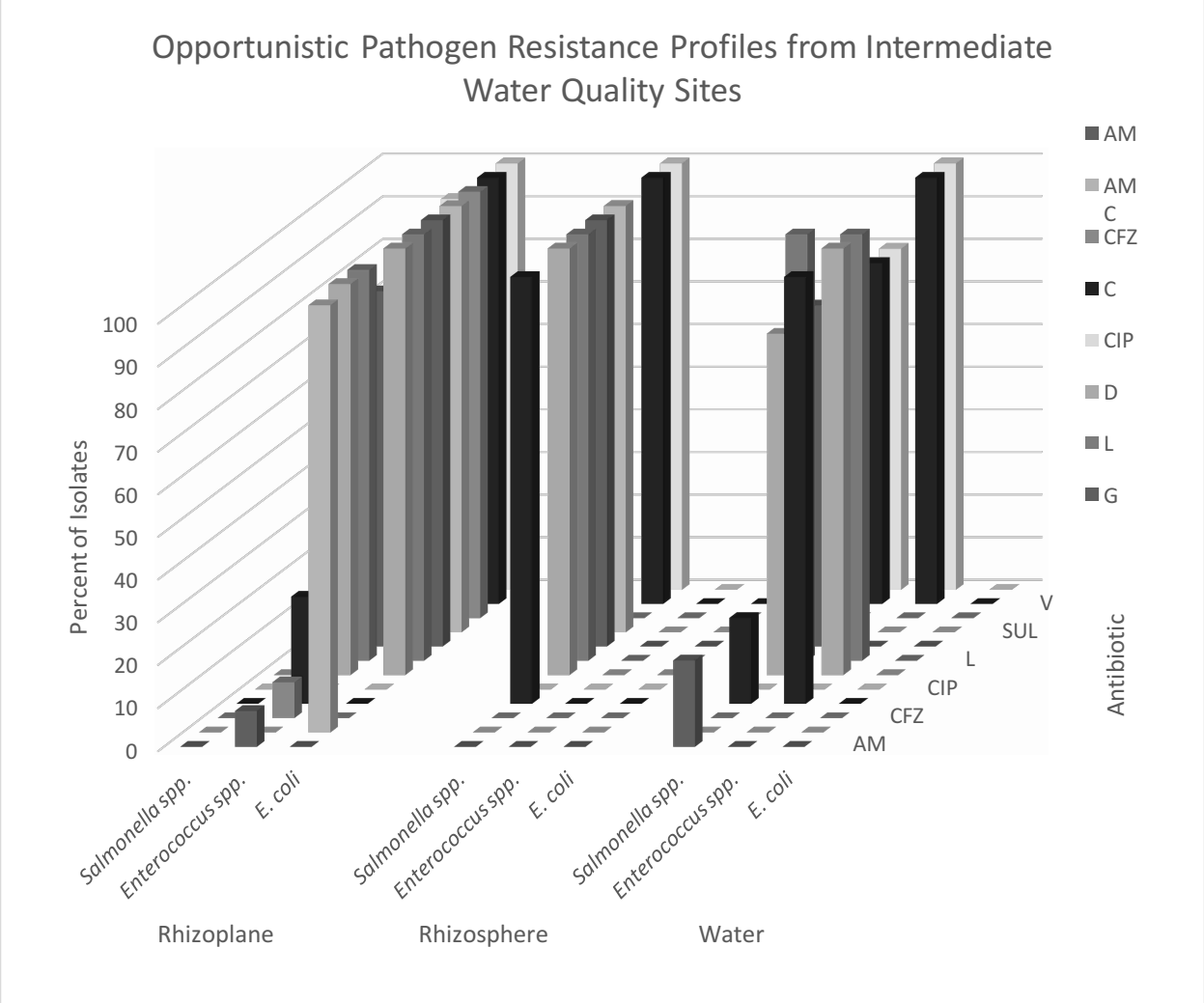


Figure 3.9. Percentage of resistant opportunistic pathogens isolated from low water quality areas exposed to antibiotics at 28°C. Organisms isolated from the rhizoplane (left), rhizosphere (center) and water (right).

Notably, within the rhizosphere samples (center), only *Salmonella* spp. was able to resist antimicrobials. All were resistant to tetracycline, vancomycin, sulfadiazine, sulfasoxazole, linezolid, doxycycline and chloramphenicol, whereas 100% isolate susceptibility was observed when exposed to streptomycin, ciprofloxacin, ceftazidime, ampicillin and amoxicillin with clavulanic acid.

All *Enterococcus* spp. resisted tetracycline, vancomycin, linezolid, doxycycline and chloramphenicol. More than half of these isolates were susceptible to all other drugs tested. *Salmonella* spp. did not show full isolate resistance (100%) to any antibiotic with the exception of linezolid, however, greater than 50% of isolates were resistant to tetracycline, sulfasoxazole, sulfadiazine and doxycycline. Less than 20% of these organisms were resistant to ampicillin and chloramphenicol.

Figure 3.10 displays that *Enterococcus* spp. and *E. coli* were the only pathogens isolated within the rhizosphere which showed antimicrobial-resistant behavior. The latter showed 100% resistance to doxycycline, linezolid, sulfadiazine, sulfasoxazole and vancomycin with complete susceptibility to all other antibiotics. *Enterococcus* spp. isolates were completely resistant to only linezolid, but greater than 50% of isolated pathogens still demonstrated resistance to tetracycline, vancomycin, sulfadiazine, sulfasoxazole and amoxicillin. More than 60% of all pathogens were susceptible (30% or less) to amoxicillin, ceftazidime, chloramphenicol, ciprofloxacin and streptomycin.

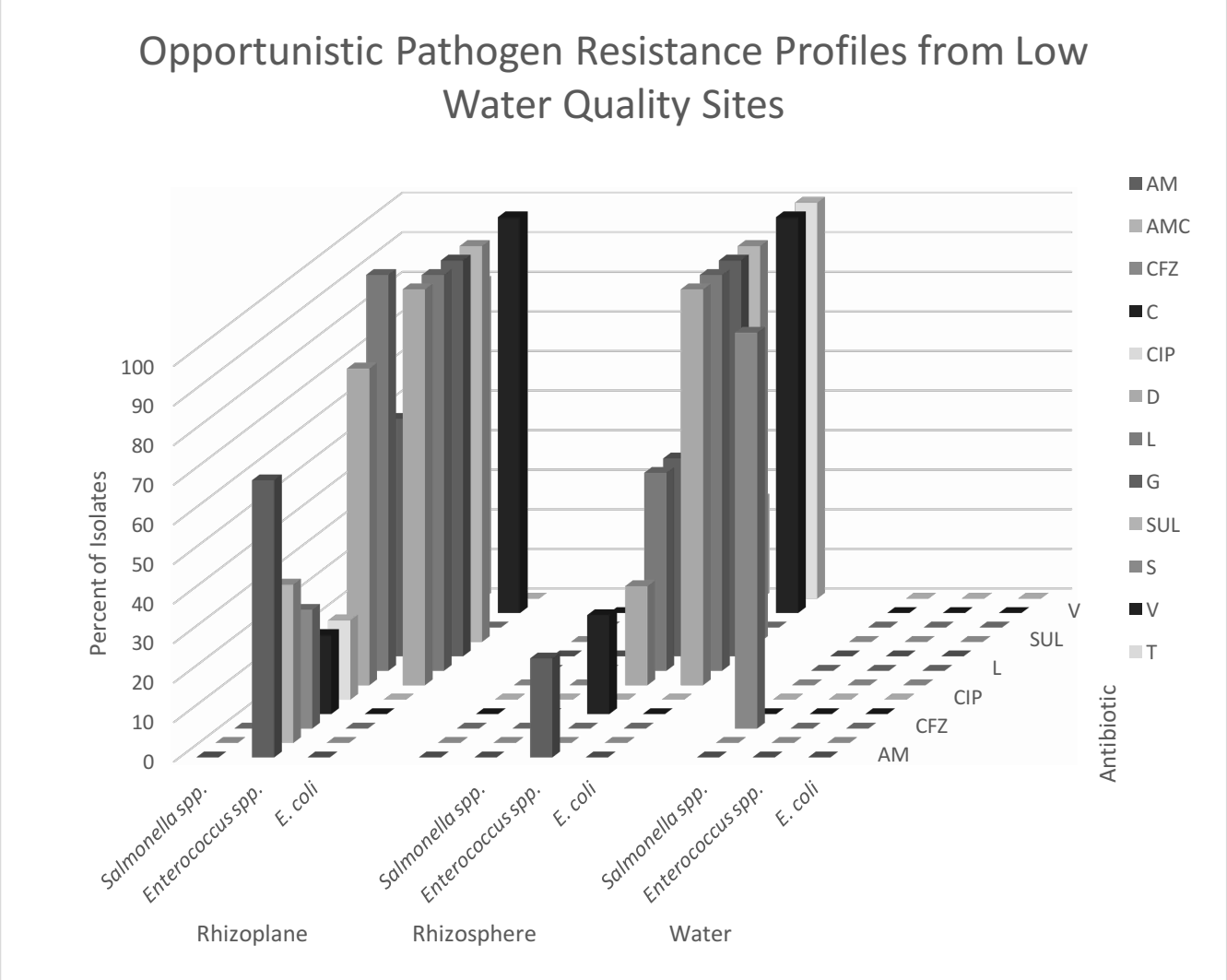


Figure 3.10. Percentage of resistant opportunistic pathogens isolated from low water quality areas exposed to antibiotics at 28°C. Organisms isolated from the rhizoplane (left), rhizosphere (center) and water (right).

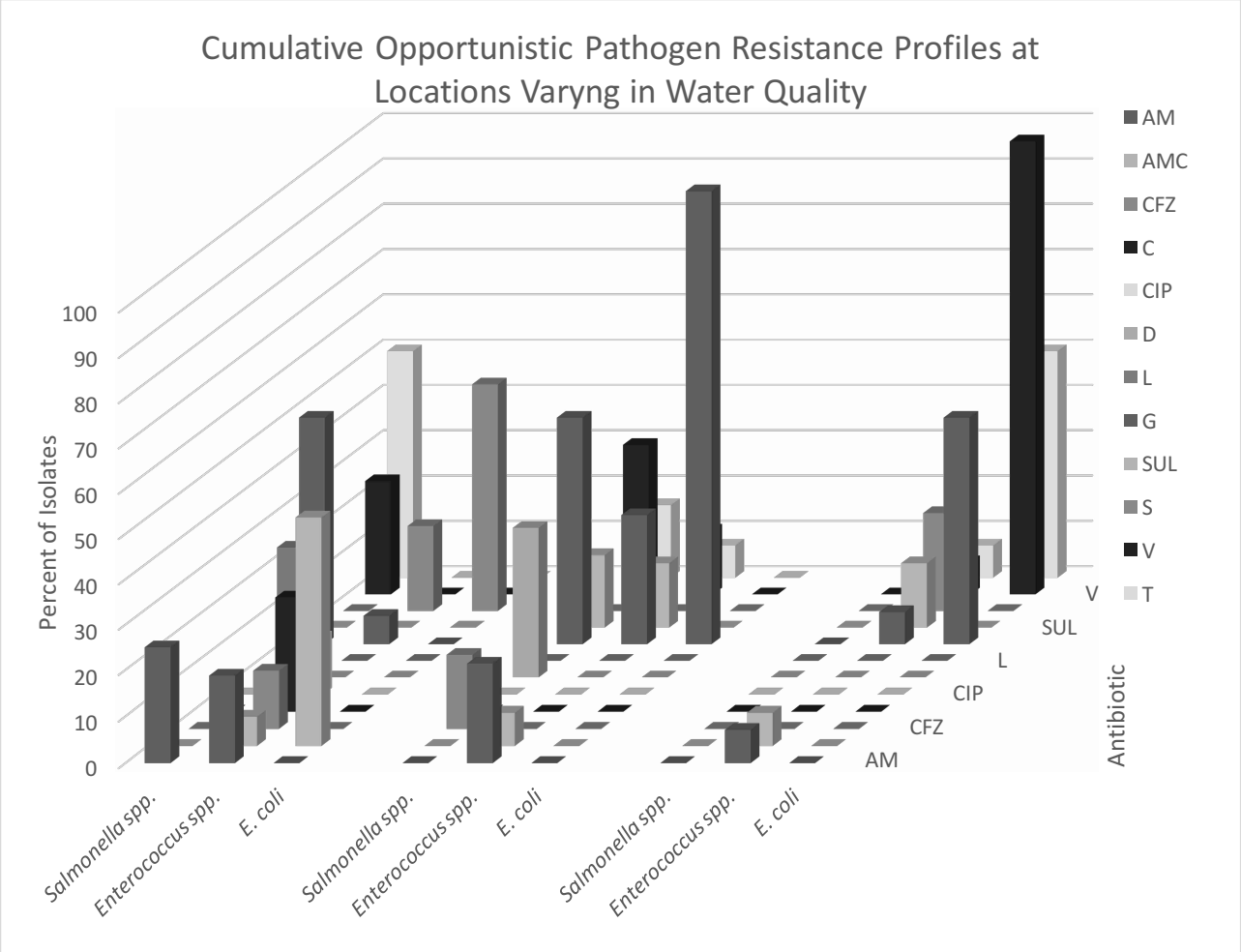
Similar trends are seen in pathogen responses to antibiotic exposure; particularly *E. coli* and *Enterococcus* spp. isolation. Complete resistance was observed by *E. coli* to tetracycline, vancomycin, sulfadiazine, sulfasoxazole, linezolid and doxycycline. Complete susceptibility was seen against streptomycin, ciprofloxacin, chloramphenicol, ceftazidime, ampicillin and

amoxicillin. Enterococcal isolates from the rhizoplane were not 100% resistant to any pharmaceutical, however greater than 60% showed resistance to linezolid, sulfadiazine, sulfasoxazole, and vancomycin. Less than 40% resistance was seen when exposed to ampicillin, chloramphenicol, and tetracycline, and were completely susceptible to streptomycin, ciprofloxacin, amoxicillin and ceftazidime. Lastly in Figure 3.10 (right), *Salmonella* was the only water-isolated pathogen that grew in the presence of antibiotics, albeit only ceftazidime.

3.3 Effectivity of Antibiotic Exposure at 11^{o}C

The final set of conditions to which pathogenic isolates were exposed involved the same group of antibiotics which were used in the previous trials. A temperature of 11^{o}C was chosen, as this condition was expected to be less ideal, introducing more stressful conditions to the isolates.

Shown in Figure 3.11 are similar trends to that observed previously at higher temperatures; whereby high water quality wetlands appear to contain the most resistant microorganisms. Microorganisms isolated from intermediate water quality impacted wetlands showed the second greatest level of resistance, and low water quality wetland isolates yielded the least amount of growth and antibiotic resistance during the assay.



<u>Figure 3.11.</u> An overview of pathogenic isolates when grown at a cold temperature, 11°C, in the presence of antibiotic stressors. The left row represents organisms from high quality sampling sites, middle intermediate quality areas and the right row, isolates from poor water quality impacted areas. AM= ampicillin, AMC = amoxicillin and clavulanic acid, CFZ = ceftazidime, C = chloramphenicol, CIP = ciprofloxacin, D = doxycycline, L= linezolid, G= sulfadiazine, SUL= sulfasoxazole, S= streptomycin, V= vancomycin, and T= tetracycline.

Sulfadiazine showed least effectivity, less than 20%, when exposed to isolated from all wetlands regardless of water quality type, whereas amoxicillin, ceftazidime, chloramphenicol, ciprofloxacin and linezolid were pharmaceuticals isolates were most susceptible. *Enterococcus* spp. isolates from high water quality areas had more diverse resistance patterns where a greater range of pharmaceuticals were resisted, followed by *Salmonella* spp. and lastly *E. coli.* These isolates

showed the greatest resistance to amoxicillin and streptomycin, whereas, *Salmonella* spp. and *Enterococcus* spp. were 100% susceptible. Isolates from intermediate quality sites were most resistant to sulfadiazine (*Enterococcus* spp. 25%, *Salmonella* spp. 40% and *E. coli* 90%). Notably, isolates found at the poor water quality sites were most susceptible to a broader range of antibiotics, although *E. coli* proved resistant against sulfadiazine, vancomycin and tetracycline (40%, 60% and 90%).

Represented in Figure 3.12 is an overview of the total resistance profiles demonstrated respectively after exposure to all genera from rhizospheric soil, rhizoplane and water samples. The first row of data represents sampling zones from high water quality areas, followed by intermediate quality in the middle and then low water quality. Isolates from high water quality areas proved to have more diverse levels of resistance, meaning that a wider range of antimicrobials were resisted when compared to isolates from lower water quality impacted areas.

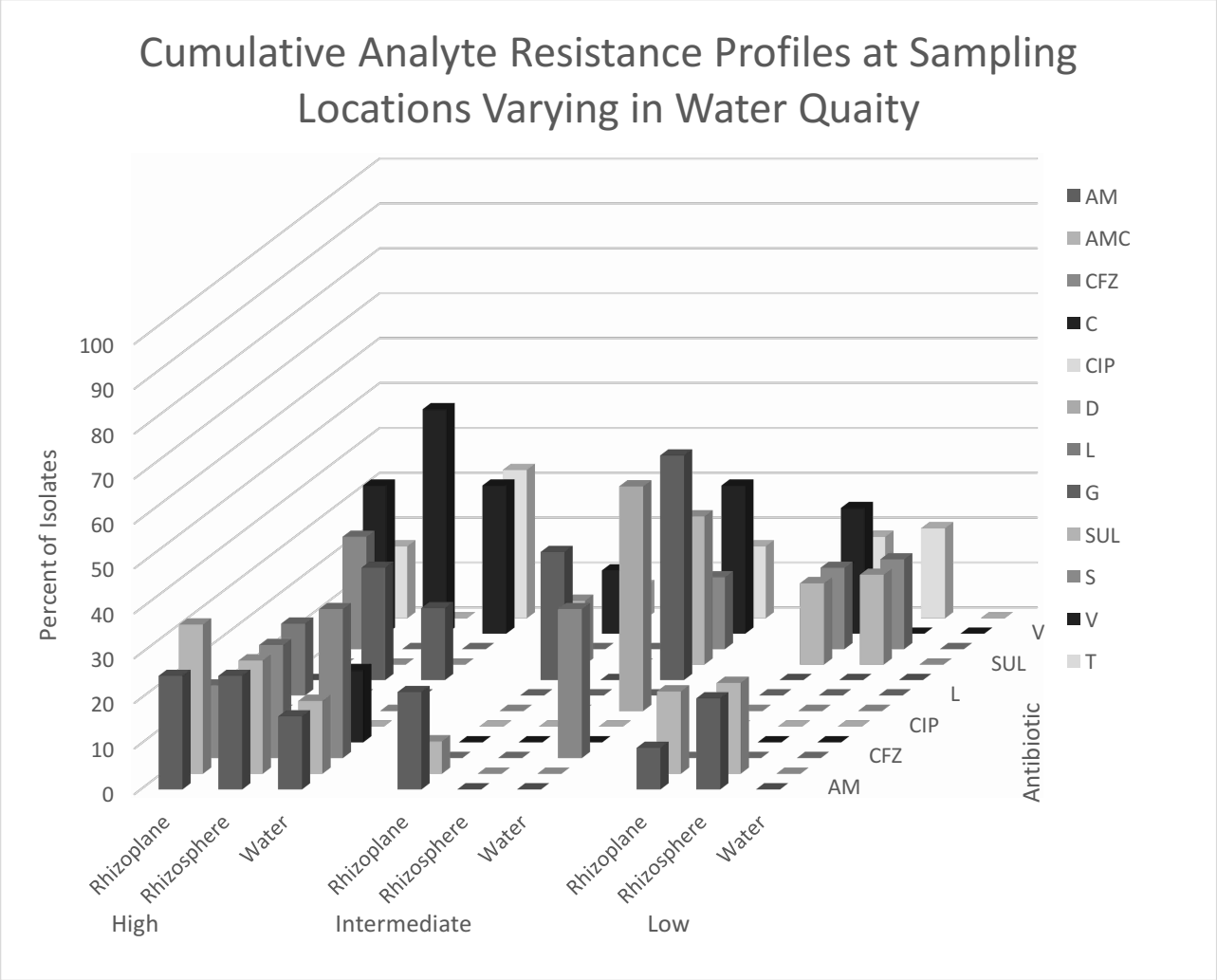


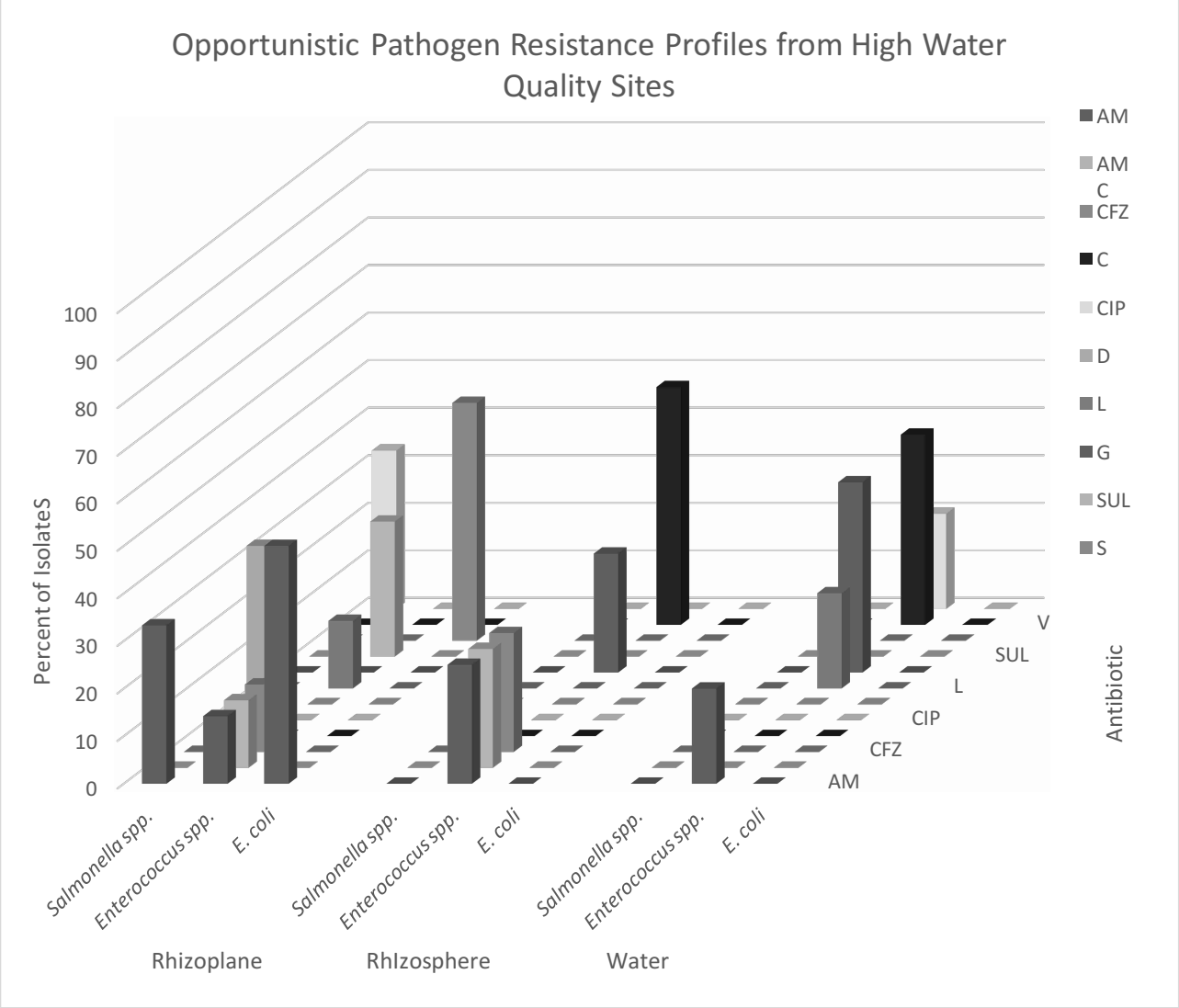
Figure 3.12. At 11°C, resistance profiles of organisms isolated from high, intermediate and low water quality wetlands are shown. On the left, resistance profiles for root, soil and water samples taken from high quality wetlands is plotted. In the middle rows, organisms isolated from intermediate water quality sampling areas and corresponding resistance profiles are shown, followed by low water quality sites on the right.

The success of microbial growth when exposed to two thirds of antibiotics is evident in pathogens isolated from all sample types at high water quality areas. Although most genera isolated proved to be less than 50% resistant to all antimicrobials, the largest diversity of resistance is found here. Root and soil-borne microbes were more tolerant than those found in the water, having shown

resistance against amoxicillin, ampicillin, ceftazidime, vancomycin and tetracycline. Vancomycin, as seen at higher water quality sites, had the largest number (20-50%) of resistant microorganisms. Moreover, microbes found in river water samples at the intermediate quality sites showed the highest levels of resistance (30-50% isolate resistance to 5 or more antimicrobials) in comparison to those isolated from roots and soil (maximum 20% isolate resistance for sulfadiazine), successfully growing (greater than 50 % of total isolates) when exposed to ceftazidime, doxycycline, linezolid, streptomycin, vancomycin and tetracycline at intermediate water quality sites. Soil isolates had the least amount of resistance to the 12 drugs used for exposure, while root-isolated microbes were less susceptible to ampicillin, sulfadiazine, and vancomycin (10%, 20% and 10% resistant). Lastly, at low water quality sites, water-originating isolates were susceptible. Root and soil microbes showed near identical resistance profiles, as each set of isolates were only partially resistant to ampicillin, amoxicillin, sulfadiazine, streptomycin and tetracycline.

At high water quality impacted locations, up to 30% of isolates obtained from the rhizoplane, rhizosphere and water, resisted ampicillin, amoxicillin and ceftazidime. Also, similar levels of pathogen resistance were identified for each analyte type, from all sampling locations, for streptomycin, vancomycin and tetracycline (20%-70%).

In Figure 3.13, comparisons highlight impacts of sample type on each pathogen for high water quality impacted isolates (*Enterococcus* spp., *Salmonella* spp. and *E. coli*). Specifically, trends seen in *Enterococcus* spp, *Salmonella* spp. and *E. coli* isolated from rhizospheric soil, rhizoplane and water can be seen.

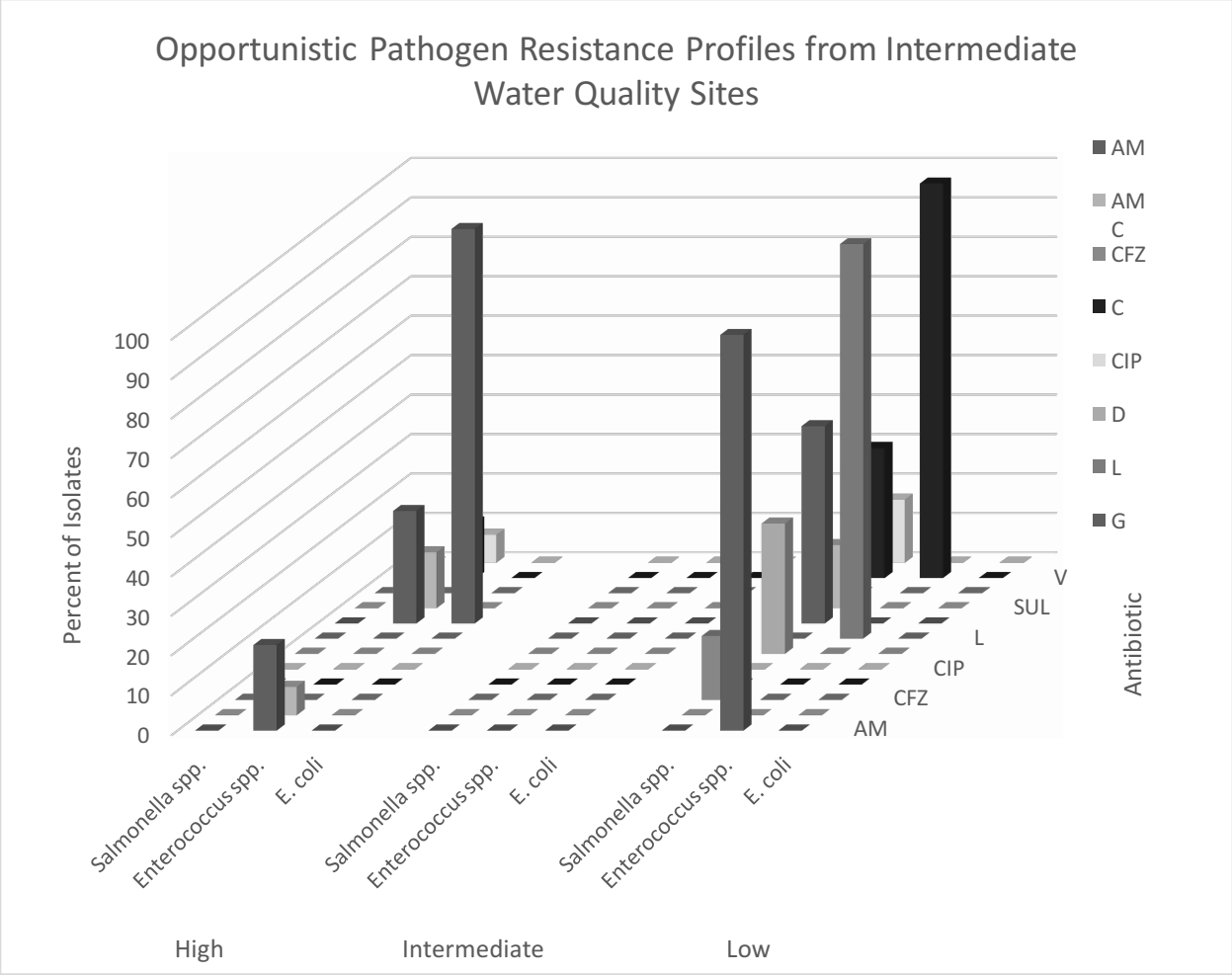


<u>Figure 3.13.</u> Three target pathogens are measured against various antibiotics at 11°C. Root isolates are found in the left rows of the bar graph, soil originating isolates in the center and organisms found in the water on the right.

Pathogens isolated from root samples showed highest resistance profiles when compared to soil and water originating isolates. Although *Salmonella* spp. showed the 30% isolate resistance to multiple antibiotics (ampicillin, ciprofloxacin and tetracycline) at 11°C, *Enterococcus* spp. were

more diverse resistance, growing when exposed to ampicillin, amoxicillin, ceftazidime, linezolid and sulfadiazine. *E. coli* was most resistant to ampicillin and streptomycin (40% isolate resistance). *Enterococcus* spp. isolates found in soil samples at high water quality impacted sites were the only resistant microbes, with 20-50% of isolates growing in the presence of ampicillin, amoxicillin, ceftazidime, sulfadiazine and vancomycin. Similarly, enterococci isolated from water at low water quality impacted sites shared familiar patterns of resistance, with 40% or less of the isolates able to grow when exposed to ampicillin, linezolid, sulfadiazine, vancomycin and tetracycline.

Intermediate water quality impacted isolate response is depicted in Figure 3.14 and shows similar trends as observed in Figure 3.13.



<u>Figure 3.14.</u> All genera of pathogens that were isolated from intermediate water quality sites were grown in the presence of antibiotics at 11°C. Bacterial isolates originating from rhizospheric soil, roots and water were processed and exposed to the 12 antibiotics.

When grown at 11°C, organisms which were isolated from intermediate water quality sites showed higher antibiotic susceptibility than organisms from higher water quality impacted sites. All microorganisms from these sites which were isolated from rhizosphere soil were 100% susceptible to all antimicrobials to which they were exposed. *Enterococcus* spp. showed low (less than 10% of isolates) levels of resistance to ampicillin, sulfadiazine, sulfasoxazole and tetracycline, whereas 80% of *E. coli* resisted sulfadiazine. Overall, *Salmonella* spp. and *Enterococcus* spp. showed the

best ability to grown during exposure to these pharmaceuticals, as 90% of enterococcal isolates grew in the presence of ampicillin, linezolid, and vancomycin. 10-30% of *Salmonella* spp. isolates were also able to grow when exposed to ceftazidime, doxycycline, sulfadiazine, sulfasoxazole, vancomycin and tetracycline.

Lastly, Figure 3.15 depicts the final series of data obtained throughout the exposure trials, comparing the 3 genera of pathogens retrieved from poor water quality impacted areas. It is noted that there was minimal resistance, as well as minimal growth at 11^{o}C. Overall, microbial growth on Mueller-Hinton agar proved greater inhibition had occurred than in trials at higher temperatures (28^{o}C and 37^{o}C).

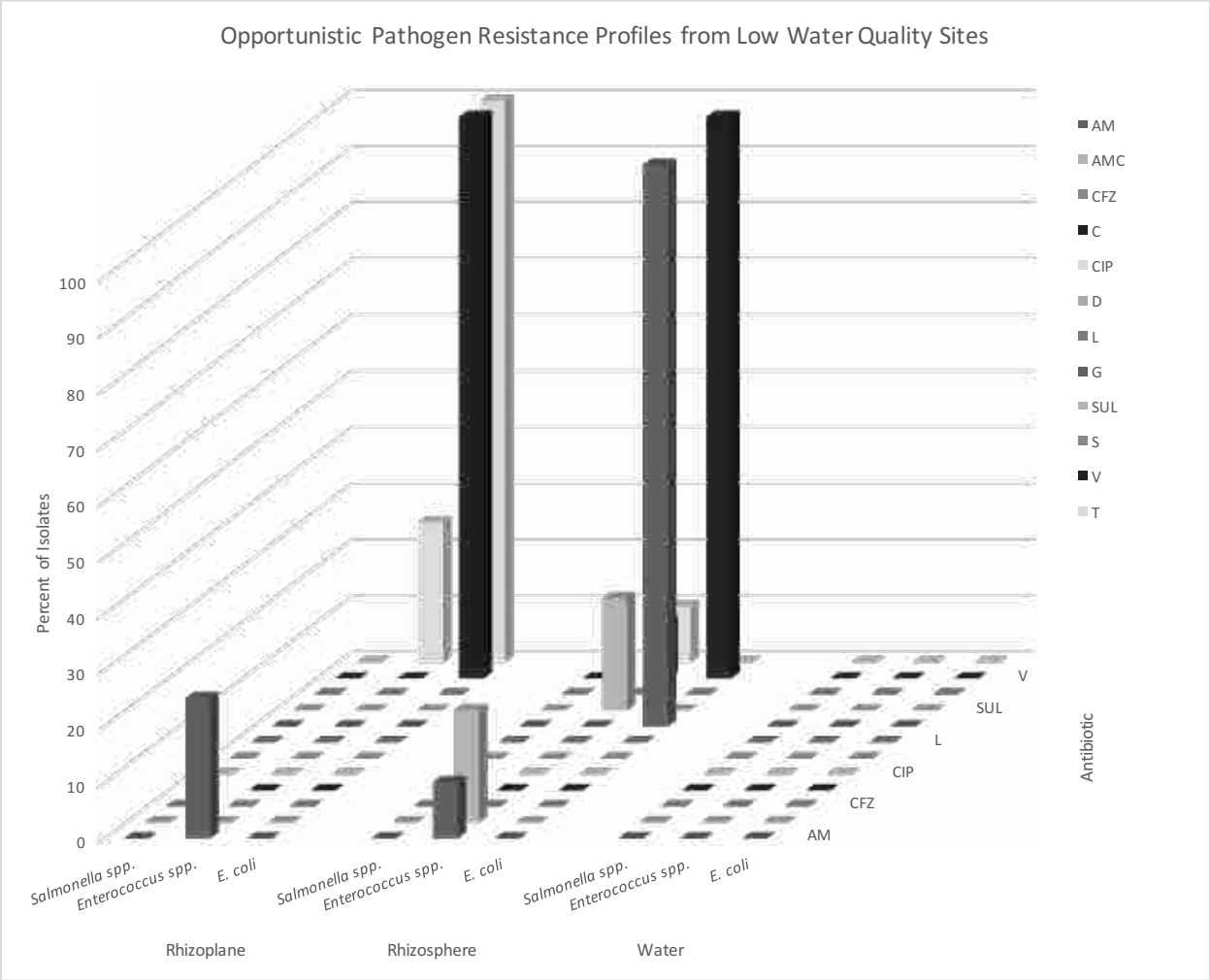


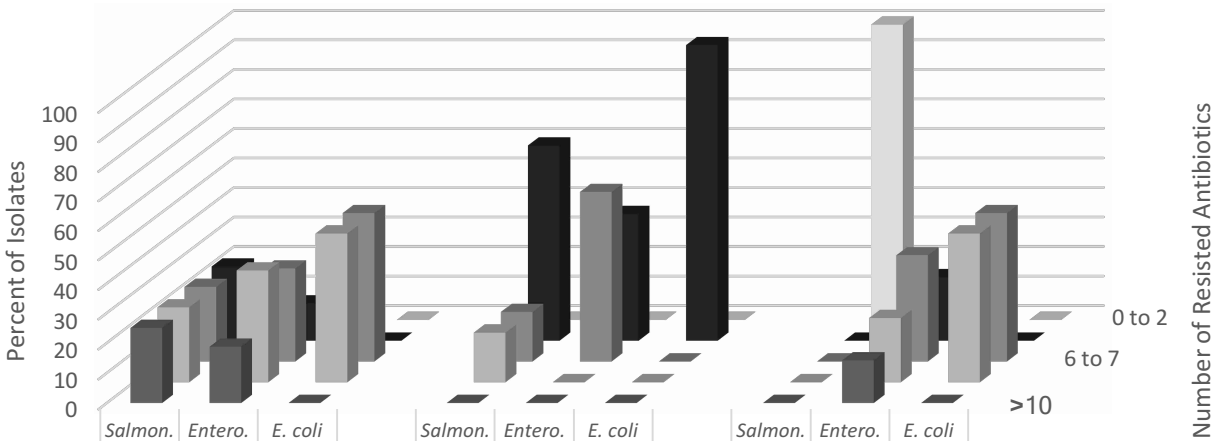
Figure 3.15. Resistance profiles of all genera of bacteria that were isolated from low water quality sites were grown in the presence of antibiotics at 11°C. Rhizoplane, rhizospheric soil and water isolates are shown.

Sites which were impacted by poor water quality yielded the lowest profiles of antibiotic resistance out of all sampling areas. All organisms which were isolated from water samples were susceptible at 11°C to all antibiotics. In root samples, *Enterococcus* spp. showed 10-20% isolates resistance to ampicillin and tetracycline, whereas, 100% of *E. coli* fully resisted vancomycin and tetracycline. Similarly, less than 10% of enterococcal isolates from soil samples showed resistance to

ampicillin, amoxicillin, sulfasoxazole and tetracycline, while 90% of *E. coli* isolates were resistant to vancomycin and sulfadiazine. *Salmonella* spp. isolates from all three zones (rhizoplane, rhizosphere, and water) were highly susceptible to all antibiotics.

3.4. Multi-Drug Resistance under Varying Growth Conditions of Pathogens Isolated

This next section is a brief summary of multi-drug resistance (MDR) found throughout the project. MDR was determined by grouping pathogen isolates together that exhibited resistance to 0-2, 3-5, 6-7, and 10 or more antimicrobials. Figure 3.16 shows differences in resistance profiles amongst all genera when grown at host temperature over 24 h.

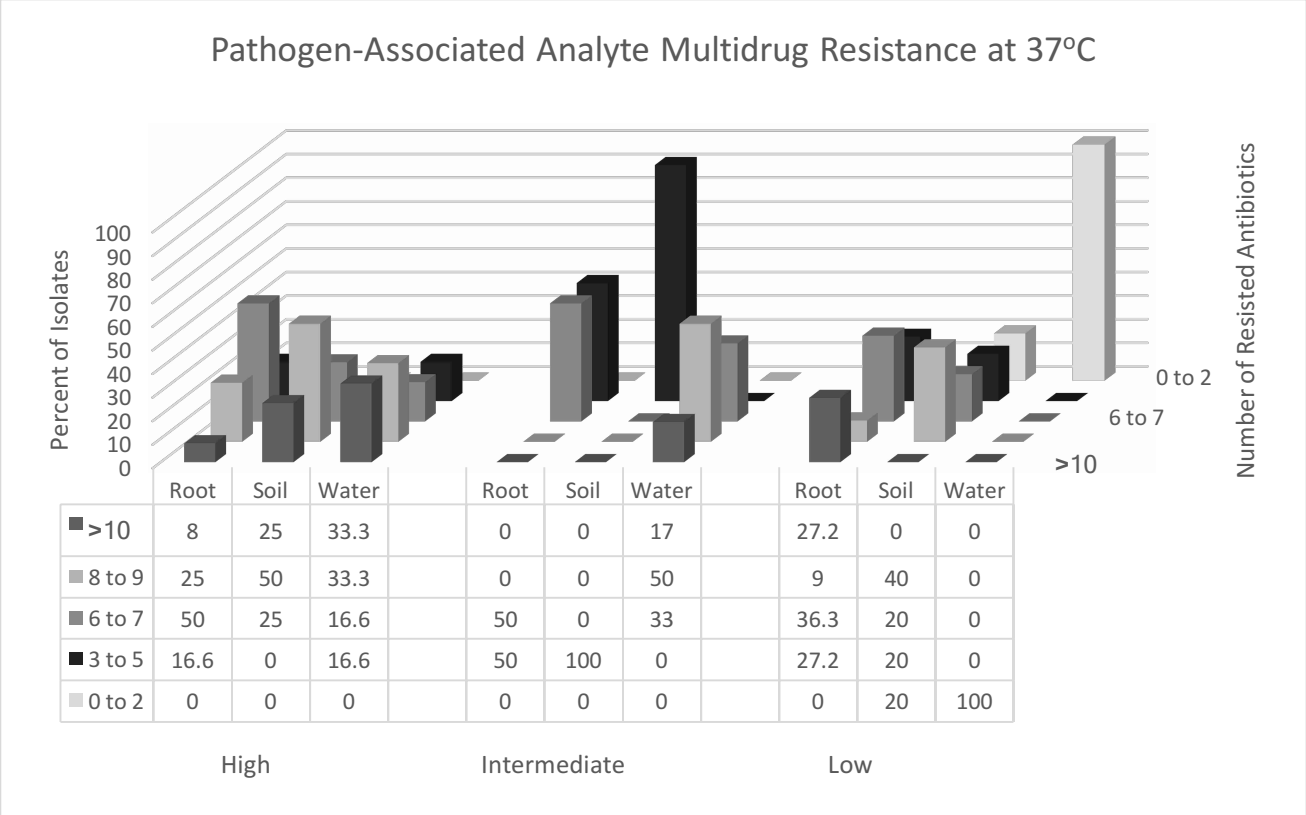


	High			Intermediate			Low		
	Salmon.	*Entero.*	*E. coli*	*Salmon.*	*Entero.*	*E. coli*	*Salmon.*	*Entero.*	*E. coli*
>10	25	18.75	0	0	0	0	0	14.2	0
8 to 9	25	37.5	50	16.5	0	0	0	21.4	50
6 to 7	25	31.25	50	16.5	57.14	0	0	35.7	50
3 to 5	25	12.5	0	66	42.8	100	0	21.4	0
0 to 2	0	0	0	0	0	0	100	7	0

Figure 3.16. Multidrug resistance was measured and compared among pathogens isolated from each water quality impacted site.

It is observed that in poor water quality impacted areas, *Enterococcus* spp. had a broader range of MDR, containing isolates falling in each category of MDR. *Salmonella* was resistant to the fewest pharmaceuticals in these areas, followed by *E. coli.* As water quality increases (intermediate), *Salmonella* isolates obtained here are more prevalent to resist 3-5 antimicrobials (66%), then 6-7 or 8-9 (both 15%), whereas *Enterococcus* spp. were more likely to show MDR to between 6-7 antimicrobials (57%). *E. coli* was 100% resistant to 3-5 antibiotics. As seen at poor water quality, *Enterococcus* spp. showed a range of MDR, however, appeared more likely to resist 6-7 (31%) and 8-9 (38%) of the antibiotics. *Salmonella* showed an even distribution of MDR, as each category from 3-5 to greater than 10 had equal likelihood of displaying MDR (25%).

Figure 3.17 measures MDR found in pathogens again isolated from all water quality sampling sites, with a respect to sample type origins. The goal of this was to show that there may be different levels of MDR depending on isolate location (ie. rhizospheric soil, rhizoplane/root, or water). The bars towards the back of the chart represent lesser levels of MDR, whereas the bars foremost correspond with high MDR levels.



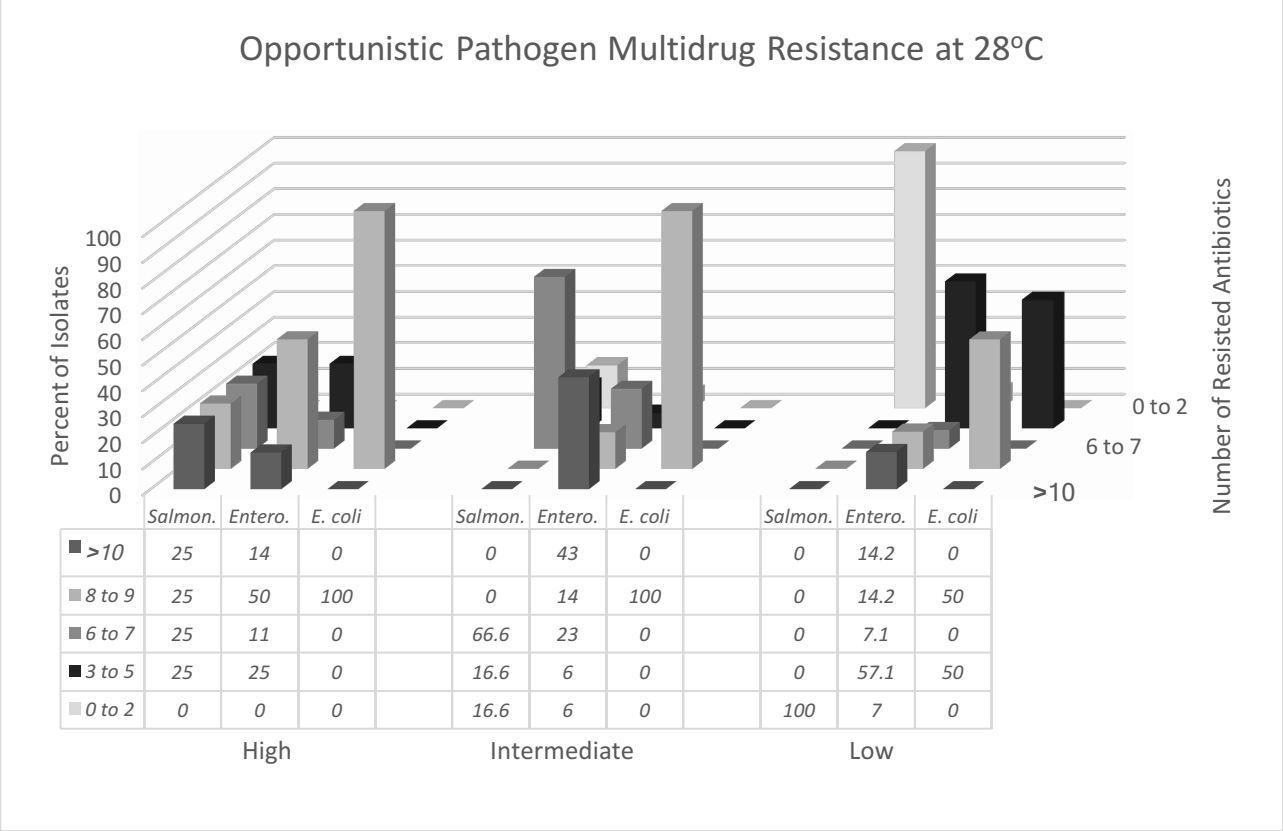
	Root	Soil	Water		Root	Soil	Water		Root	Soil	Water
>10	8	25	33.3		0	0	17		27.2	0	0
8 to 9	25	50	33.3		0	0	50		9	40	0
6 to 7	50	25	16.6		50	0	33		36.3	20	0
3 to 5	16.6	0	16.6		50	100	0		27.2	20	0
0 to 2	0	0	0		0	0	0		0	20	100

Figure 3.17. Multidrug resistance was measured and compared in organisms isolated from each water quality impacted sites and grouped by analyte type they were isolated from.

Notably, poor and high water quality impacted areas show the most variation in MDR. In the rhizospheric soil, isolates were equally resistant (20% of isolates) to o-2, 3-5 and 6-7 of the antimicrobials, and 40% resistance to 8-9. Rhizoplane isolates were more likely to show MDR to 6-7 (37%) than >10 (27%) or 3-5 (27%). At intermediate quality sites, 50% of waterborne isolates were resistant to 8-9 antibiotics, followed by the next highest MDR in rhizoplane areas where 50% of isolates showed MDR to 3-7 antimicrobials.

Figure 3.18 depicts MDR observed in pathogens isolated from all water quality sampling sites.. The goal of this was to show that there may be different levels of MDR depending on isolate genus

when exposed to antibiotics at 28°C. The bars towards the back of the chart represent lesser levels of MDR, whereas the bars foremost correspond with high MDR.



	Salmon.	Entero.	E. coli	Salmon.	Entero.	E. coli	Salmon.	Entero.	E. coli
>10	25	14	0	0	43	0	0	14.2	0
8 to 9	25	50	100	0	14	100	0	14.2	50
6 to 7	25	11	0	66.6	23	0	0	7.1	0
3 to 5	25	25	0	16.6	6	0	0	57.1	50
0 to 2	0	0	0	16.6	6	0	100	7	0

Figure 3.18. Multidrug resistance was measured and compared among pathogens isolated from each water quality impacted site. Incubation occurred for 48h at 28°C, then zones of inhibition were measured.

Shown above, 100% of *Salmonella* spp. isolated from low water quality impacted sites were resistant to 2 or less antibiotics. Enterococcal isolates were more likely to be resistant to 3-5 antimicrobials (57%) in comparison to MDR of 6-7 (7%), 8-9 (14%) or 10 or more (14%). *E. coli* isolates from low water quality impacted sites were either resistant to 3-5 or 8-9 (50% each). At intermediate water quality impacted areas, 100% of *E. coli* isolates were resistant to 8-9 pharmaceuticals, whereas, 84% of enterococci isolated showed MDR to either 6-7 and or 10 or

more antibiotics, while 14% resisted 8-9. 66% of *Salmonella* spp. isolated at intermediate water quality impacted areas resisted 6-7 antimicrobials, followed by 17% of isolates resistant to 3-5 and another 17% resisting 0-2. Lastly, at high water quality impacted areas, resistance was uniform amongst *Salmonella* spp. as MDR was 25% for exposure to 3-5, 6-7, 8-9 and 10 or more pharmaceuticals. All *E. coli* isolates and 50% of enterococcal isolates were resistant to 8-9 pharmaceuticals. Further, 12.5% of *Enterococcus* spp. at high water quality impacted wetlands showed MDR to 10 or more antibiotics, another 12.5% resistant to 6-7 and lastly the final 25% of isolates resistant to 3-5.

Figure 3.19 depicts MDR displayed by pathogens when grown at 28^{o}C based on where the pathogen originated. The goal of this was to show that there may be different levels of MDR depending on isolate location (ie. rhizospheric soil, rhizoplane or water), and how conditions like temperature (28^{o}C) affect resistance or susceptibility. The bars towards the back of the chart represent lesser levels of MDR, whereas the bars foremost correspond with high MDR.

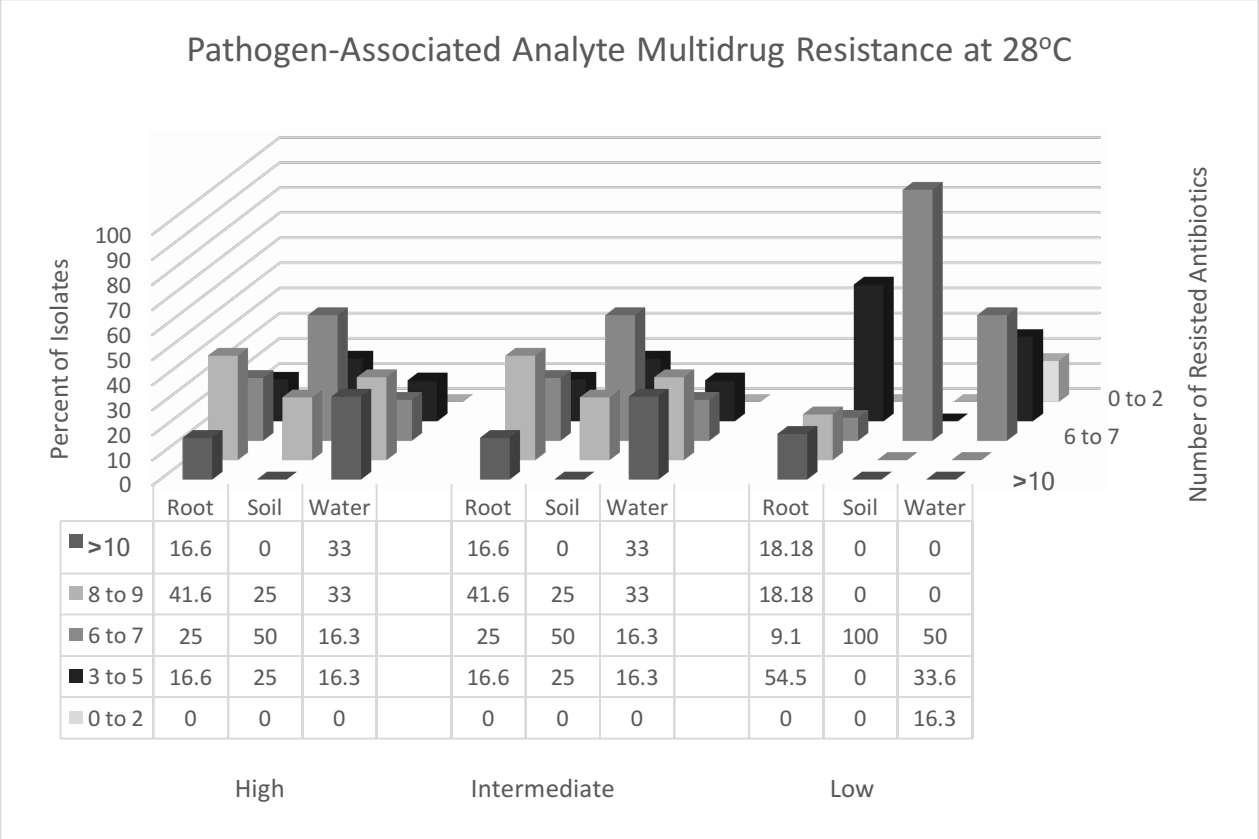
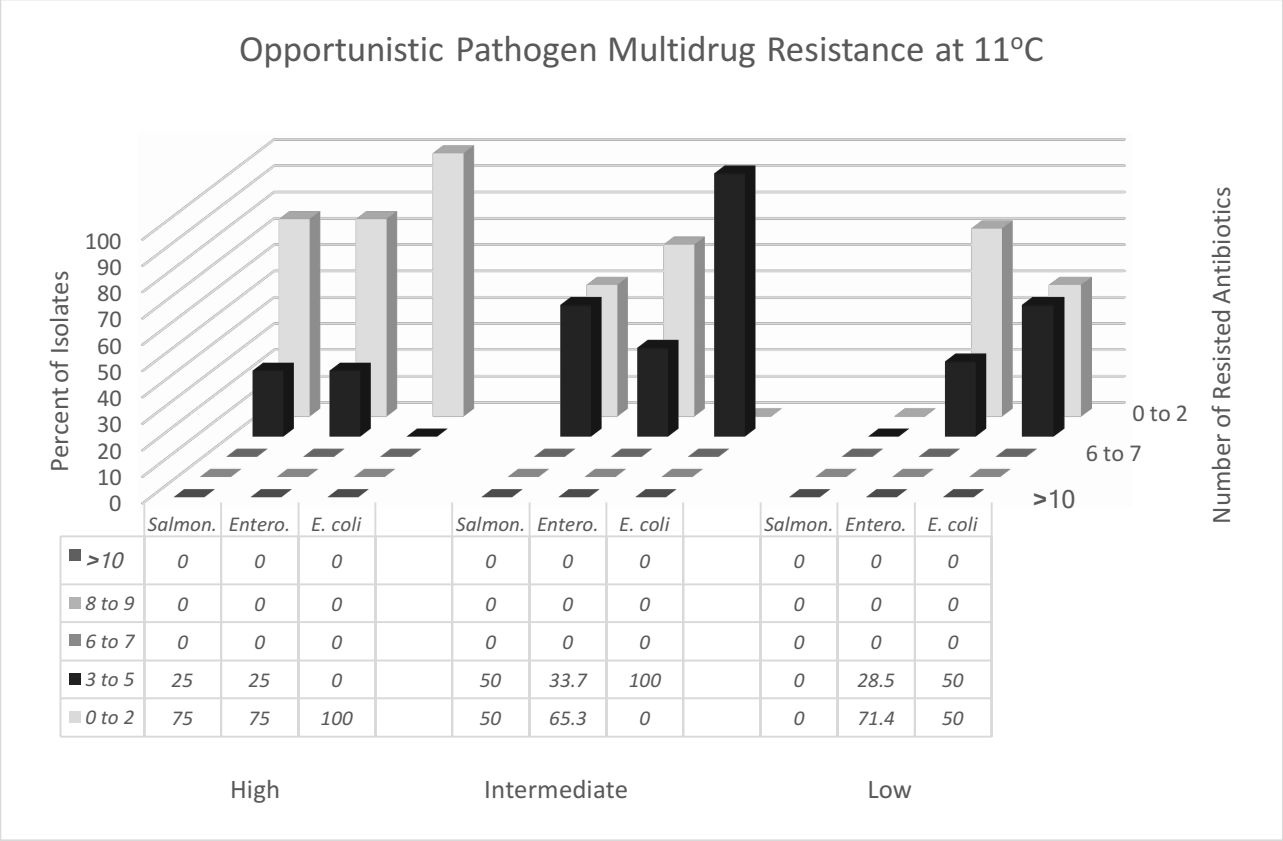


Figure 3.19. Multidrug resistance was measured and compared in organisms isolated from each water quality impacted sites and grouped by analyte type they were isolated from. They were incubated at 28°C for 48 h, then zones of inhibition were measured for resistance.

At low water quality impacted sampling areas, 100% of waterborne isolates were resistant to 3-5 drugs but showed no MDR to more than 5. More than half of soil isolates (60%) displayed MDR to 3-5 drugs, while 20% resisted 0-2 and another 20% of isolates resisted 8-9. Root isolates ranged in MDR, with 19% resisting 10 or greater, 19% showing MDR to 8-9 followed by 10% of isolates in the 6-7 range and finally over half (54.5%) exhibiting MDR to 3-5. At intermediate water quality impacted wetlands, waterborne isolates were more likely to resist 6-7 drugs (50%) followed by 3-5 (34%) and 0-2 (16%). Root-associated [rhizoplane] isolates were most likely to resist 6-7 pharmaceuticals (48%) followed by 36% within the 3-5 MDR range and 21% within 7-8. All

rhizospheric soil isolates were resistant to 6-7 pharmaceuticals. Lastly, waterborne pathogens at high water quality impacted sites resulted in 33% of isolates resistant to 10 or more antibiotics, another 33% resisting 8-9, and then the remaining 32% resistant to 7 or fewer. Rhizospheric soil isolates were more likely to show MDR to 6-7 antibiotics (50%), followed by 25% in the 8-9 and the final 25% in the 3-5 MDR range. Lastly, rhizoplane root samples yielded 42% of isolates resistant to 8-9 drugs followed by 25% showing MDR to 6-7, 17% with MDR to 10 or more and the final 17% in the 3-5 range.

Figure 3.20 depicts MDR demonstrated by pathogens isolated from varying water quality sampling sites, based on where the pathogen originated. The goal of this was to show that there may be different levels of MDR depending on isolate location (ie. rhizospheric soil, rhizoplane or water), and how less ideal conditions like temperature ($11^{o}C$) affect resistance or susceptibility. The bars towards the back of the chart represent lesser levels of MDR, whereas the bars foremost correspond with high MDR.



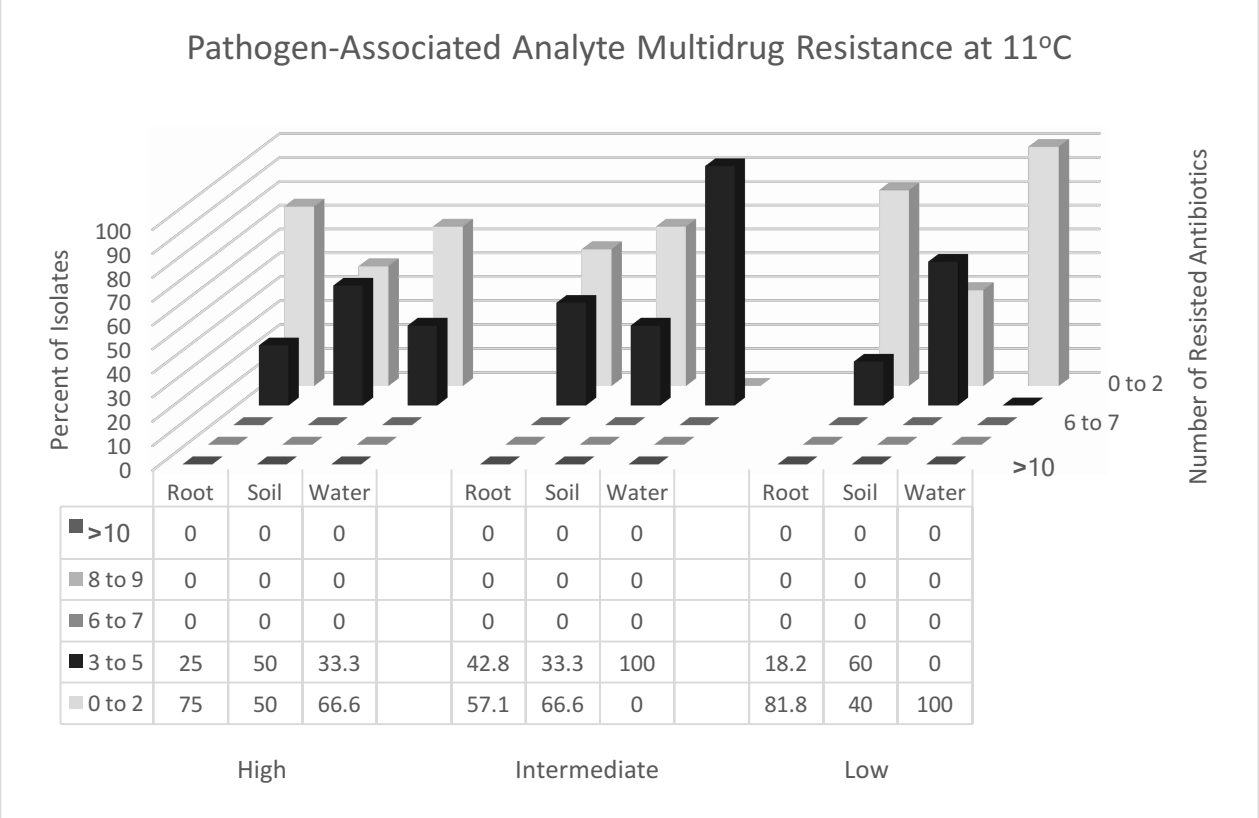
	Salmon.	Entero.	E. coli		Salmon.	Entero.	E. coli		Salmon.	Entero.	E. coli
>10	0	0	0		0	0	0		0	0	0
8 to 9	0	0	0		0	0	0		0	0	0
6 to 7	0	0	0		0	0	0		0	0	0
3 to 5	25	25	0		50	33.7	100		0	28.5	50
0 to 2	75	75	100		50	65.3	0		0	71.4	50

Figure 3.20. Multidrug resistance was measured and compared among pathogens isolated from each water quality impacted site. Incubation occurred for 96h at 11°C, then zones of inhibition were measured.

Unlike in Figures 3.16 to Figure 3.19, the range of MDR for all environmental samples ranges between 0-2 or 3-5, but no more. As seen in rhizoplane, rhizosphere and water samples at low water quality impacted sites, the greatest level of MDR was found in 60% of rhizospheric soil isolates (3-5 antibiotics resisted). The majority of isolates from both rhizoplane (82%) and water (100%) had MDR to 0-2 antibiotics. At intermediate water quality impacted areas, 100% of waterborne isolates resisted 3-5 drugs, followed 42% of rhizoplane and 33% of rhizosphere isolates. Lastly, at high water quality impacted wetlands, 75% of rhizoplane isolates were within the 0-2 MDR range, followed by 67% of waterborne and 50% rhizospheric soil isolates. Multidrug

resistance was highest in soil-isolated organisms at high water quality sites for this site type, as 50% resisted 3-5 antimicrobials.

Figure 3.21 depicts MDR found in different genera of pathogens again isolated from all sampling sites, however focus' on where the pathogen genus. Exposure took place at 11°C. The goal of this was to show that there may be different levels of the degree of growth and MDR depending on isolate genera. The bars towards the back of the chart represent lesser levels of MDR, whereas the bars foremost correspond with high MDR.



	High			Intermediate			Low		
	Root	Soil	Water	Root	Soil	Water	Root	Soil	Water
>10	0	0	0	0	0	0	0	0	0
8 to 9	0	0	0	0	0	0	0	0	0
6 to 7	0	0	0	0	0	0	0	0	0
3 to 5	25	50	33.3	42.8	33.3	100	18.2	60	0
0 to 2	75	50	66.6	57.1	66.6	0	81.8	40	100

<u>Figure 3.21.</u> Multidrug resistance was measured and compared in organisms isolated from each water quality impacted sites and grouped by analyte type they were isolated from. They were incubated at 28°C for 48 h, then zones of inhibition were measured for resistance.

Intermediate water quality wetlands showed the highest percentages of isolates with MDR range of 3-5. All (100%) of *E.* coli, 50% of *Salmonella* spp., 36% of *Enterococcus* spp. resisted 3-5 pharmaceuticals. At low water quality impacted wetlands, 50% of *E. coli* isolates resisted 3-5 antibiotics followed by 28.5% of *Salmonella* spp. isolates. At areas of high water quality impact, 25% of both *Salmonella* spp. and *Enterococcus* spp. showed MDR to a maximum of 3-5 pharmaceuticals. All remaining isolates at all water quality areas were within the 0-2 MDR range.

3.5 Measuring the Effects of Metal Exposure on Pathogenic Isolates

Contaminant response is a critical aspect of understanding the behavior of bacteria acclimating to a new environment. Particularly, the ability for fecal organisms like *Enterococcus faecalis* to grow in the presence of contaminants, such as the metals copper and silver alone and combined with an antimicrobial (chloramphenicol), is measured. This section aims to help gather an understanding of behavior of these organisms in riparian wetlands, when exposed to multiple stressors. An isolate from poor [low] water quality impacted wetlands was chosen to test the hypothesis of organism's located in these areas being better equipped to respond to contaminant stress.

Although there are less abiotic factors being influenced here (ie. only temperature and contaminant levels [nm]), direct influence of stressors are observed. *Enterococcus faecalis* was selected for this series of exposure trials due to the relative abundance the organism was found based on sample site water quality and environmental sample type. In Figures 3.22 through 3.26, an *E. faecalis* isolate from a low water quality impacted wetland was grown in the presence of two metals, copper and silver, and one antibiotic, chloramphenicol. This enterococcal isolate originated from the rhizosphere and displayed antimicrobial resistance to most (75%) of the tested antibiotics,

including chloramphenicol. The metals and antibiotic chosen were environmentally relevant, as per data obtained from the MOECC. Additionally, combinations of copper and chloramphenicol and then silver and chloramphenicol were used as exposure settings. Using results obtained from the antimicrobial susceptibility trials, 28^{o}C was chosen as this was a condition invoking resistant behavior. This assay was done in triplicate and the average absorbance for each trial used to generate logarithmic growth curves.

Assessing the Ability of Environmentally Isolated *Enterococcus faecalis* to Respond to Metal Contaminants Alone and in Combination with an Antibiotic

In Figure 3.22, the growth of *E. faecalis* was measured every 2 h throughout a 24 h exposure trial. Four concentrations of copper were used (10 mM-0.25 mM). Absorbance readings were taken at 450 nm. Standard error of the mean was calculated and used to generate error bars. Each condition was done in triplicate.

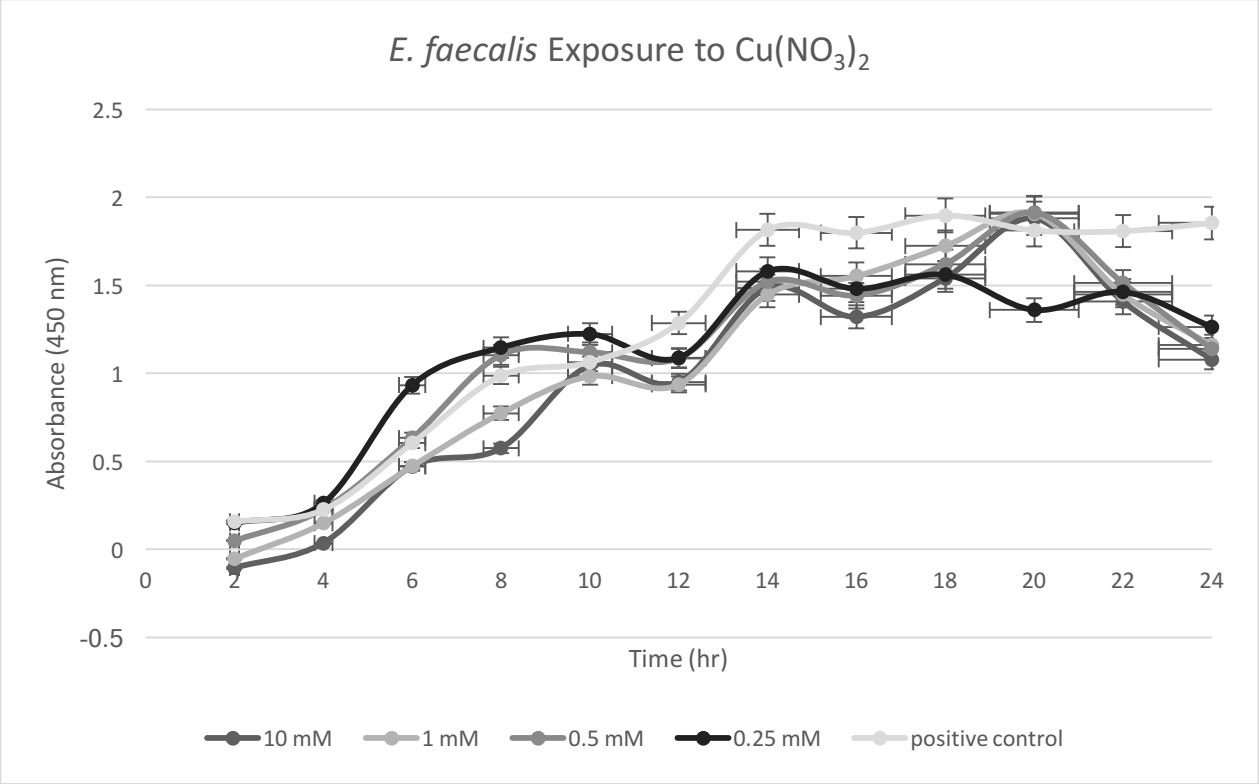


Figure 3.22. A gradient of molar concentrations of copper nitrate were used to measure the growth and response of an *Enterococcus faecalis* isolate at 28°C. *E. faecalis* was isolated from a low water quality impacted wetland in the Grand River watershed. This was done in triplicate and the average absorbance plotted.

Over the course of a 24 h exposure to all conditions, growth was not inhibited by the presence of copper (Figure 3.22). The absorbance began to increase immediately, with turbidity in the flasks increasing after 2 h. At 10 h, it appears exponential phase had completed and stationary phase began, then entering death phase after 12-13 h. At earlier time points, conditions with lower concentrations of copper exceeded growth than that of the positive (no copper) control.

In Figure 3.23, the growth of *E. faecalis* was measured over 24 h, at 28°C when exposed to silver. Four concentrations of silver were used (0.5 mM-0.0625 mM). Absorbance readings were taken every 2 h, at 450 nm.

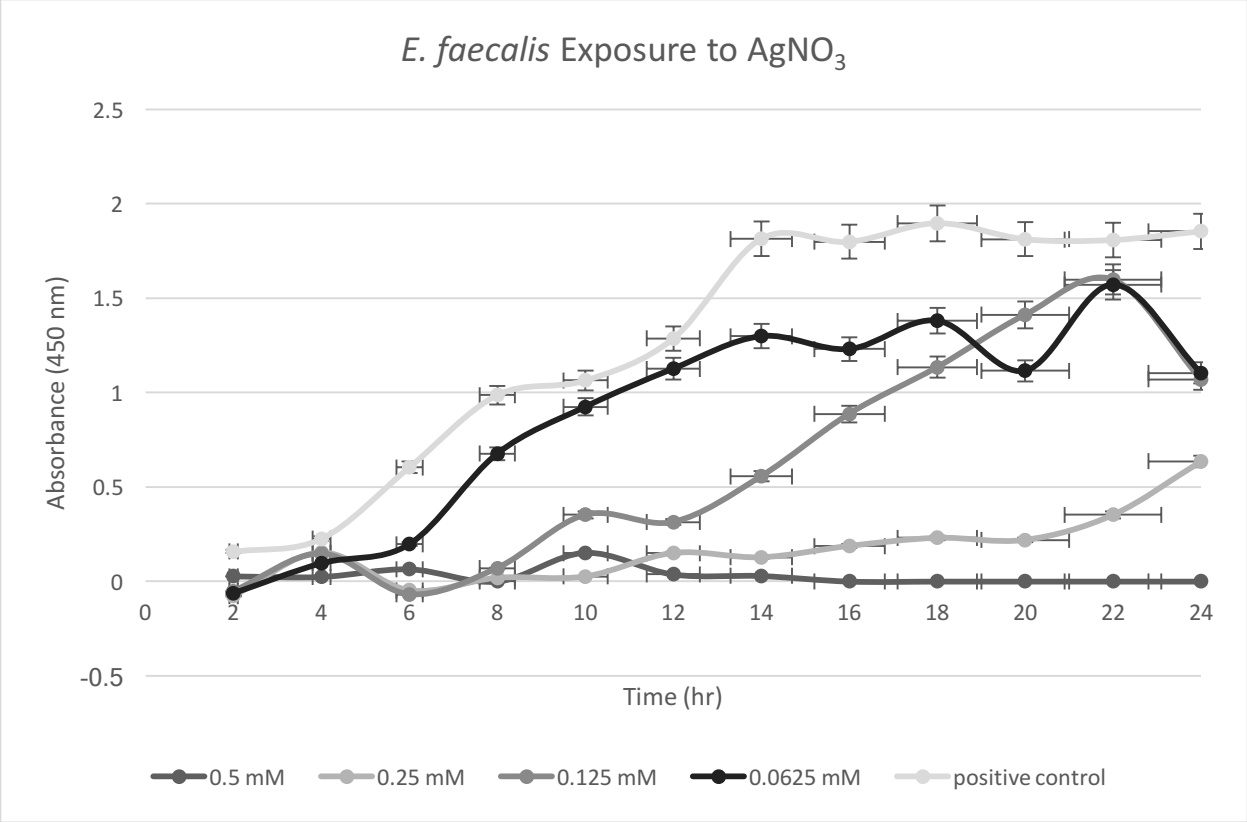
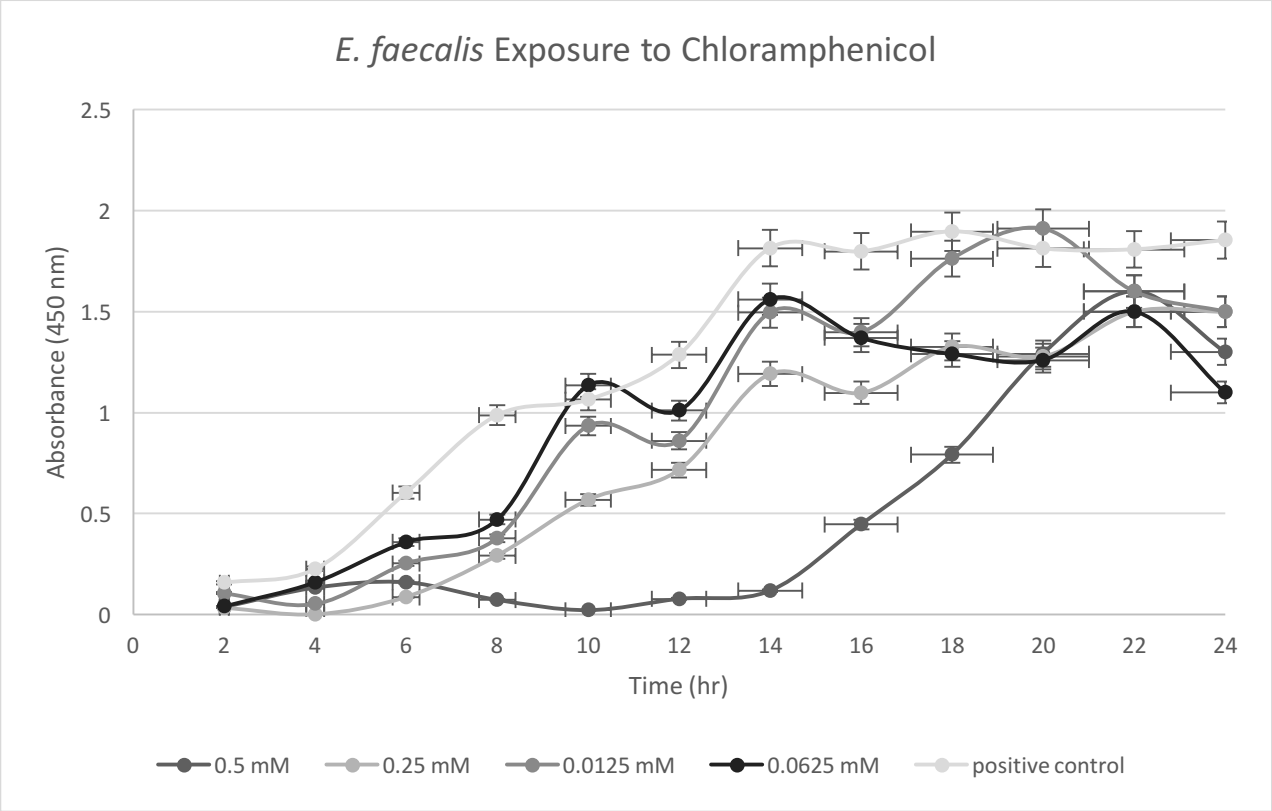


Figure 3.23. A gradient of molar concentrations of silver nitrate were used to measure the growth and response of an enterococcal isolate from a low water quality impacted wetland in the Grand River watershed at 28°C. This was done in triplicate and the average absorbance plotted.

In Figure 3.23, higher concentrations of silver had more noticeable inhibitory effects on the microbial growth. After about 6 h, *E. faecalis* appears to leave the lag phase and enters log phase when exposed to 0.5 mM and 0.0625 mM of silver, whereas, at higher concentrations took more time (6-10 h), for a positive growth response to be seen.

In Figure 3.24, the growth of *E. faecalis* was measured over 24 h, at 28^{o}C when exposed to chloramphenicol. Four concentrations of chloramphenicol were used (0.5 mM-0.0625 mM). Absorbance readings were taken every 2 h, for 24 h using a spectrophotometer at 450 nm.



<u>Figure 3.24.</u> A gradient of molar concentrations of the antibiotic chloramphenicol were used to measure the growth and response of an enterococcal isolate from a low water quality impacted wetland in the Grand River watershed at 28^{o}C. This was done in triplicate and the average absorbance plotted.

In contrast to Figure 3.23, exposure to chloramphenicol (Figure 3.24) resulted in better adaptation and growth earlier in the time cycle. At much earlier time points (4-8 h), Although the time point varied for initiating lag phase, this isolate appeared to tolerate all exposure conditions concentrations.

In Figure 3.25, the growth of *E. faecalis* was measured over 24 h, at 28°C when exposed to both silver and chloramphenicol. Three combinations of chloramphenicol (0.2mM-0.1mM) and silver were used (0.2mM-0.1mM). Absorbance readings were taken every 2 h, for 24 h using a spectrophotometer.

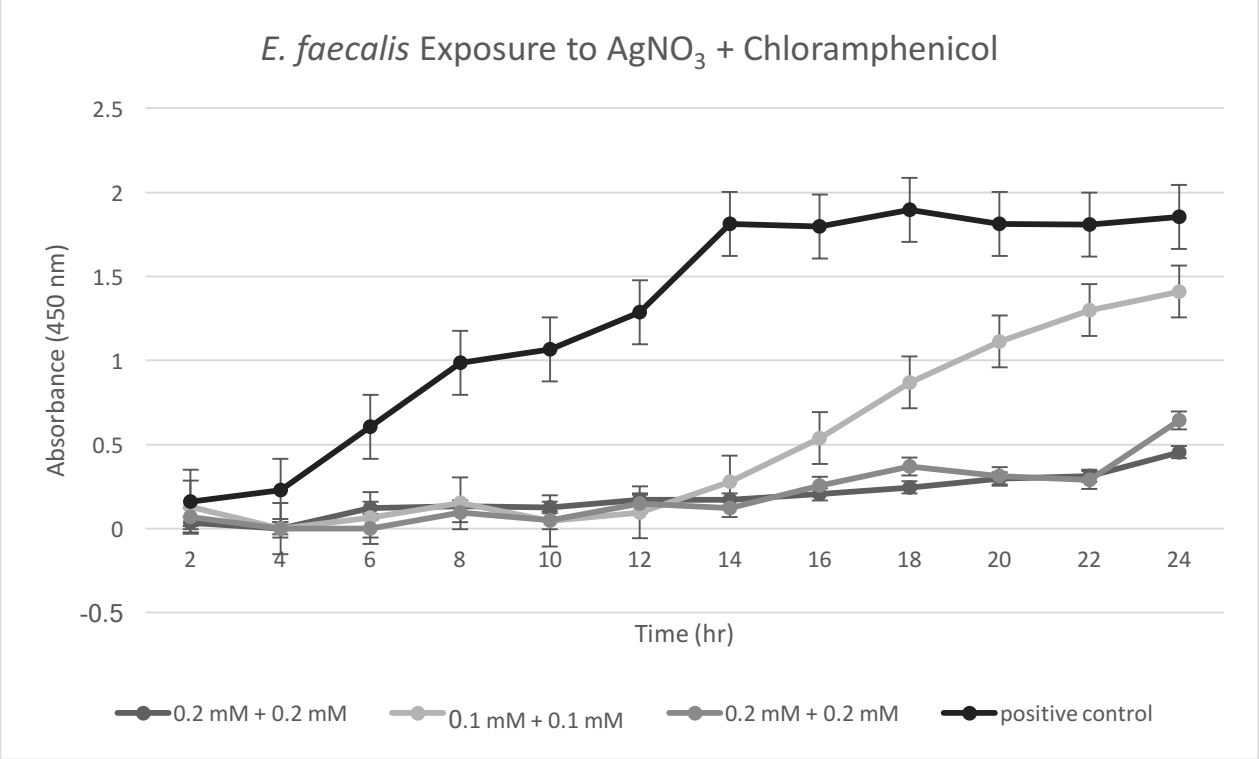
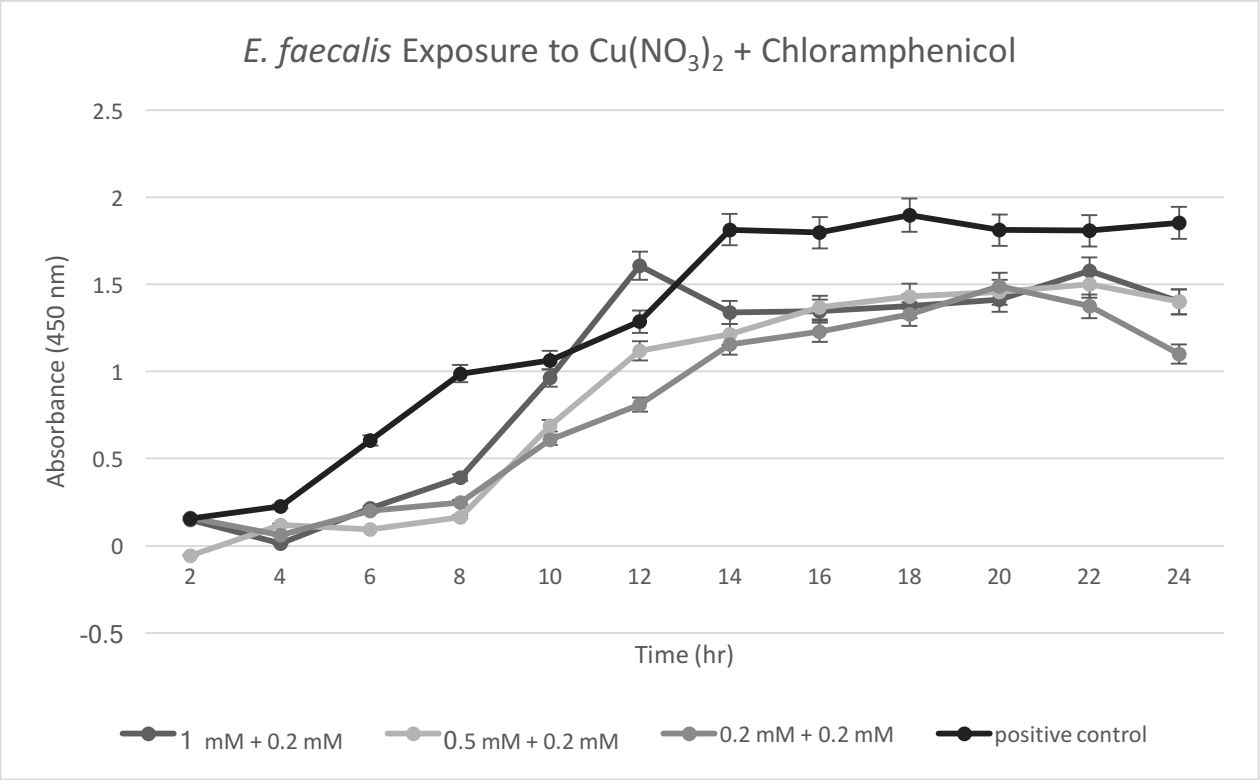


Figure 3.25. A gradient of molar concentrations of a mixture of silver nitrate and chloramphenicol were used to measure the growth and response of an enterococcal isolate from a low water quality impacted wetland in the Grand River watershed at 28°C. This was done in triplicate and the average absorbance plotted.

The combination of silver and chloramphenicol shown in Figure 3.25 (0.02 mM $AgNO_3$ + 0.02 mM chloramphenicol and 0.02 mM $AgNO_3$ + 0.001 mM chloramphenicol) shows that microbial growth was inhibited until 18 h, when absorbance began to increase. Further, the combination in

which 0.001 nM of silver and 0.02nM of chloramphenicol was used resulted in the highest degree of growth over 24 h at 28^{o}C.

In Figure 3.25, the growth of *E. faecalis* was measured over 24 h, at 28^{o}C when exposed to copper and chloramphenicol. Three combinations of chloramphenicol (0.02 mM-0.01 mM) and copper (1 mM-0.25 mM) were used. Absorbance readings were taken every 2 h, for 24 h using a spectrophotometer.



<u>Figure 3.26.</u> A gradient of molar concentrations of a mixture of copper nitrate and chloramphenicol were used to measure the growth and response of an enterooccal isolate from a low water quality impacted wetland in the Grand River watershed at 28^{o}C. This was done in triplicate and the average absorbance plotted.

Lastly, the exposure involving both chloramphenicol and copper (Figure 3.26) resulted in growth occurring at an earlier time point in comparison to other combinatory approaches, but was lesser (absorbance at time point) than when *E. faecalis* was exposed solely to copper or chloramphenicol alone. After 8 h of growth, log phase was initiated, which *E. faecalis* in the 0.1 nM $CuNO_3$ + 0.02 nM chloramphenicol conditions began exponential growth. All other conditions resulted in a similar acclimation at this time point where microbial growth occurs at a higher rate (absorbance increases). At 4-6 h, there is a delay in all exposure conditions before entering lag phase. Conditions with the highest levels of copper began replication before those with reduced levels.

Discussion

In this section, we looked to address the innate capabilities of isolated opportunistic pathogens to respond to various contaminant stress. Based on data collected by the GRCA, Ministry of Agriculture and Ministry of Environment and Climate Change, specific pharmaceutical metabolites and metals were chosen. The selected pharmaceuticals and metals were utilized for the purpose of providing an accurate reflection of what would be found at riparian wetlands varying from high to low water quality. At poor water quality impacted wetlands, metal and antimicrobial metabolites would be found in higher quantities in comparison to wetlands of higher water quality, thus predicted that microbial stress response would be more well adapted at these conditions. The objectives for this set of experiments were to, firstly, isolate opportunistic pathogens from rhizoplane, rhizosphere and water samples of riparian wetlands, secondly, evaluate the ability of these isolated microorganisms to respond to adverse conditions caused by contaminant stress, including antibiotics and metals, and lastly, quantify occurrences of

contaminant-resisting opportunistic pathogens from each sampling area to determine how water quality may influence resistance profiles.

The research hypothesis stated that exposure to stress-inducing conditions reflective of abiotic factors would result in possible acclimation. In response, opportunistic pathogen replication would not be halted. What was found was that, when exposure to varying classes of antibiotics at different temperature conditions, opportunistic pathogen isolates were better able to survive during exposure at 28^{o}C and above. Further, we posited that low water quality impacted sites would exhibit a higher efficacy of resistance to antibiotic stress. Analysis proved otherwise, where it was found that all isolates from high water quality impacted sites were more efficient resistors of pharmaceuticals. Interestingly, the *E. faecalis* strain obtained from a rhizospheric analyte at a low water quality impacted sampling site showed acclimating capabilities to toxic metals and chloramphenicol. It was expected that trials measuring exposure of the isolate to silver and chloramphenicol would inhibit growth. This proved to be false, as *E. faecalis*, which was initially inhibited, acclimated to the conditions, initiating replication.

By reviewing levels of contaminant exposure in the GRWS (Cooke *et al.*, 2014; Pileggio *et al.* 2016), antibiotic type and metal concentrations were selected. Water quality was determined using GRCA data providing information on Nitrogen and Phosphorous levels in the GRWS. Highly impacted areas were more likely to have higher levels of N and P due to direct introduction of contaminated waters by anthropogenic sources. Conversely, minimally impacted sampling locations were identified as having lesser levels of N and P present in surface waters. Intermediate quality impacted areas were regarded as possessing mid-range levels of N and P. The designation

of high, intermediate or low water quality impacted areas is utilized to represent levels of nutrient loading in waters, indicating contamination and pollution of a water source. Specifically, levels of organic N and P, whether free or bound (nitrates, nitrites, phosphates, orthophosphates) contribute to nutrient loading, and possible eutrophication (Greening and Janick, 2006; Tsiaras *et al.* 2014).

Additionally, information gathered by WHO (2017) globally essential antibiotics to combat bacterial infections (ie. enterococcal bacteremia, salmonellosis) provided relevance to the needs of this study. By isolating and assessing microbial responses to varying conditions, like antibiotic exposure at fluctuating temperature conditions, novel findings of the behavior of environmentally deposited opportunistic pathogens was found. Further, measuring the response of microbial pathogens to other contaminants, specifically various metals, aided in elucidating responses to unfavorable conditions presented by a different type of invoked stress. In particular, measuring responses between over-stimulating levels of copper (a micronutrient), and silver (antimicrobial properties) yielded surprising results.

Firstly, when examining the results gathered after exposure to the 12 selected antibiotics, multiple major trends were revealed. Initially, I hypothesized that isolates from high water quality impacted quality sites would have minimal efficacy when grown in the presence of antimicrobials (Leclerc and Moreau, 2002; Payment *et al.* 2000). Conversely, I thought isolates located from low water quality impacted areas would be more tolerant, and physiologically display a better response (ie. growth) (Teitzel and Parsek, 2003; Winfield and Groisman, 2003; Trebitz *et al.,* 2007). Intermediate water quality impacted sites were expected to have an average number of isolated opportunistic pathogens showing both susceptible and resistant behavior (Pandey *et al.*, 2014;

Fong and Lipp, 2005). Additionally, I hypothesized that at lower temperatures the isolated microorganisms would be more susceptible during exposure trials, increasing resistant behavior linearly with rising temperatures (28^{o}C, 37^{o}C).

Moreover, I expected that the rhizosphere would provide the most numbersome amount of resistant opportunistic pathogens isolated, as this environmental zone is highly accommodating to microbial communities (Karim *et al.,* 2004; Berg and Smalla, 2009; Brundrett, 2009). This was predicted as there are high levels of nutrients (root exudate), water, and organic matter, such as root and soil, which organisms can adhere to (Piceno and Lovell, 2000; Ferguson and Signoretto, 2011). Analyzed results (Table 2.1) proved that what was occurring in the GRWS was not what we had expected. At high water quality impacted areas, pathogens were more numbersome and likely to be recovered from environmental samples in comparison to analyte from lower water quality impacted areas. Perhaps this was due to increased naturalization and persisting behavior of opportunists at high water quality sites.

Of course, this could also be explained by decreased microbial competition that may be occurring at high water quality impacted areas, which theoretically should have a lesser load of opportunistic pathogens than areas defined as intermediate and poor water quality. At locations containing higher nutrient loads, pollution due to non-point sources would introduce higher loads of fecal run-off, thus naturally having higher loads of opportunistic pathogens present. The key difference in these settings is that at high water quality areas, nutrients are lesser than at low water quality areas, thus microbial competition is more prominent as they compete for resources. Conversely, areas with low water quality have such high nutrient levels that organisms do not have to adapt to

survive. This may also be a trait of pathogenic isolates from low water quality impacted areas and how their corresponding response to antibiotics is less efficient than those form higher water quality impacted zones.

Although the initial prediction of bacterial persistence and isolate recovery was proved to be opposite our hypothesis, what we did find in all locations was consistency regarding pathogen recovery. Rhizoplane and rhizosphere analytes yielded the highest amounts of recovered isolates, followed by lower numbers of isolates recovered from water samples (Table 2.2). Interestingly, *E. coli* and *Salmonella* spp. were isolated much less frequently than *Enterococcus* spp., which apparently is a very successful environmental persistor. It is likely that the rhizoplane and rhizospheric areas yielded high isolate recovery due to expansive root systems providing surfaces to adhere to (Teplitski *et al.,* 2000). Equally important are nutrient levels found in these regions, which are very high due to organic decomposition occurring in the upper soil horizons, as well as nutrients provided through this root exudate (Stanley and Lazazzera, 2004). In comparison, the ratio of isolates recovered from water were identified to be less numbersome (Table 2.2).

In research done by Janice Thomas (2012) out of Dr. Slawson's Applied and Environmental Microbiology lab, her experiments were highly successful in retrieving organisms like *Salmonella* spp. from water. The research done now identified water samples to be less reliable sources for retrieving pathogenic isolates. An important factor for the difference in recovered values could be explained by weather events, such as temperature fluctuations according to seasonality, and extreme weather events, including heavy winds and rain. The research presented here was

concerned with deposited organisms that may persist in the rhizosphere and rhizoplane in comparison to those more likely to be transient.

After isolating and purifying the opportunistic pathogens from all sampling locations, exposure trials revealed a very interesting trend. At low temperatures (11^{o}C), classified in this research as stress-inducing, responses to antibiotics were poor. When temperatures increased to a higher range including environmental (28^{o}C) and host conditions (37^{o}C), antimicrobial susceptibility decreased. As seen in Figure 3.1 and Figure 3.6, bacterial responses to 75% or greater types of antibiotics they were exposed to showed the ability to resist. In Figure 3.11, there is a much greater chance that 25% of isolates or more, from each genera were, susceptible to up to 100% of antibiotics they were exposed to.

During stress-inducing events, it has been shown that microorganisms enter a dormant phase, often in the form of sporulation, to best withstand adverse conditions (Seuntjens *et al.,* 2004; Yergeau *et al.*, 2014). During these period, these organisms are less metabolically active, thus would be expected to have a decreased ability to produce antibiotic resistance mechanisms as this requires a great amount of energy. Moreover, as conditions become more favorable and stressful stimuli is decreased, increases in resistance was identified. This is due to increased metabolically activity, resulting in more efficient nutrient acquisition to facilitate physiological events, like protein assembly and efflux transport systems (Bengoechea and Skurnik, 2000; Konkel and Tilly, 2000).

Additionally, at increased temperatures, rhizoplane and rhizospheric isolates were better able to adapt and grow during exposure (Figure 3.2, Figure 3.7) in comparison to waterborne isolates. As well, at 11^{o}C, antimicrobial susceptibility increased, however, the most resistant behavior was also

found in rhizosphere and rhizoplane samples (Figure 3.12). Root surface and soil charge often compliment those of antimicrobial metabolites and other pharmaceuticals, which may influence the resistance profiles of opportunists retained in these areas. This may enhance their capabilities to acclimate to stressful conditions as greater exposure to static levels over time could facilitate behavioral adaptation (Ramey *et al.,* 2004).

Not only did the rhizosphere yield the highest numbers of opportunistic pathogens isolated, having approximately 60% more isolates found than any other sample type (Table 2.1), *Enterococcus faecalis* and *Enterococcus faeceium* were the most prominent species. These organisms were confirmed using multiplex PCR (Table 2.5, Figure 2.2). *Salmonella* spp. and *E. coli* were found in surprisingly low amounts (30-70% less likely than *Enterococcus* spp.), possibly due to the time of year sampling occurred or due to constraints on mimicking environmental conditions in the laboratory, resulting in viable but not detectable (VBNC) organisms. Berg and colleagues (2005) as well as Patel *et. al* (2009) demonstrated that the rhizosphere is an ideal location for pathogenic retention, as both teams isolated organisms like *Staphylococcus.* Patel and associates (2009) displayed using *Stenotrophomonas* and enterohemorrhagic *E. coli* from this zone, the importance of electrostatic charge, nutrient and water availability on pathogen persistence.

Retention of pharmaceuticals in the rhizoplane and rhizosphere occurs by binding of complimentary-charged compounds (Liu *et al.*, 2016). In particular, exposure of environmentally persisting microbial pathogens to retained pharmaceutical metabolites and metallic ions could influence contaminant-resisting behavior (Malchi *et al.*, 2014). Effects of absorbed contaminants would be expected to affect both the water quality and microorganismal behavior. Freshwater

wetlands innately act as biological filtration systems, often providing a tertiary level of water purification (Ji *et al.*, 2002). As polluted water flows through riparian wetlands, present contaminants would be deposited in soil, likely leaching into the various soil horizons (Verhoevena and Meulemanb, 1999).

It was evident during exposure at 37^{o}C that *Enterococcus* spp. and *Salmonella* spp. isolates from high water quality impacted sites were exceptionally resistant to more drugs than at any other water quality or temperature (Figure 3.16). It would appear as though more *Salmonella* spp. isolates were resistant to 10 or more pharmaceuticals when compared against *Enterococcus spp.,* however this may actually be a limitation, as there were four times more *Enterococcus* spp. isolates. This is reinforced in the category of resistance to 6 to 7, and 8 to 9 antimicrobials. Additionally, it should be noted that in a healthcare setting, enterococcal infections have proved extremely troublesome and difficult to eradicate, such as vancomycin-resistant strains, reinforcing the validity of results found in this study. Similar to multi-drug resistance found at high water quality impacted areas involving *Salmonella* spp., *E. coli* isolates showed to be divided evenly in multiple drug resistance, with 50% resisting 6-7, and 50% resisting 8-9. This trend would possibly change as more isolates were collected and analyzed, as it is unlikely that 100% of *E. coli* isolates should display resistance to drug classes they are not commonly treated against.

At high water quality impacted locations, antibiotic resistance may be higher as the organisms in these riparian areas may have the ability of less microbial turnover. Specifically, in areas that are less prone to contaminant introduction by urbanized or agricultural effluent, it would be expected that survivability would be high in the microbes present at these locations (Moriarty, 1999). More

specifically, the ability for nutrient acquisition, competition and increased, specialized functions of metabolic processes would be prevalent in these areas (Macler and Merkle, 2000). Conversely, at areas of low water quality, there are such frequent levels of high contaminant and nutrient cycling that microorganisms deposited there never have an opportunity to adapt, and acclimate to these settings. As seen in the multidrug resistance trials, pathogens at these high water quality sampling areas were much more efficient at resisting seven or more antibiotic in comparison to isolates located from intermediate and poor water quality areas.

Further, at these locations, rhizoplane and rhizospheric-originating isolates were best able to survive during exposure to wider ranges of drug class. This would reinforce the notion that not only are higher water quality impacted wetlands more hospitable for opportunistic pathogen success, yet also that these zones are much more desirable for colonization as opposed to the water column. At these specific zones, the ability for establishment is promoted due to high nutrient sources and large surface areas for attachment, resulting in robust and dynamic communities. Due to this, these areas see the highest degree of opportunist replication, where genetic transfer and mutations are occurring at higher rates, additionally impacting the ability to tolerate and even resist contaminants. Although multi-drug resistance to 75% or greater classes of antibiotics was most likely found at high water quality areas, some *Enterococcus* spp. isolates were able to resist multiple antibiotics at all wetlands assessed.

Throughout the GRWS, isolates obtained performed poorly when exposed to ciprofloxacin (Figure 3.16-3.20), which increases DNA supercoiling by decreasing gyrase activity. Alternatively, when exposed to cell-wall inhibitors such as vancomycin, resistance was high. This suggests that

inherent capabilities may be present in these environmental strains, correlating to findings in hospital settings about secondary acquired infection, specifically vancomycin-resistant enterococci. However, antimicrobials which affected protein synthesis, such as chloramphenicol, resulted in mixed levels of resistance, yielding some susceptible and others resistant when exposed.

I selected an *E. faecalis* isolate from a low water quality impacted sampling location for exposure to copper, silver and chloramphenicol. This isolate performed well during chlorapmehnicol exposure, and was selected to determine effects of environmentally relevant levels of the above contaminants. As seen in Figure 3.22, copper had not elicited a lethal effect, as growth occurred in all concentration ranges. This was surprising as copper, known to be a micronutrient, did not prove toxic when levels were high in the system, suggesting possible increases in efflux mechanisms to reduce toxicity, as noted earlier (Macler and Merkle, 2000). When silver was introduced to *E. faecalis,* minimal growth was noted in all concentrations, which were markedly lower than those of copper (Figure 3.23). Silver is inherently antimicrobial, inhibiting signal transduction as well as releases reactive oxygen species, disrupting microbial cells. It was seen that during silver exposure trials, lethal effects were seen in higher concentrations, however possible acclimation may have occurred as amounts of silver decreased (Figure 3.23). Interestingly, in a dynamic system which this assay was conducted. As time increased, so did *E. faecalis'* capability to adapt to the presence of chloramphenicol. This suggests that in comparison to a closed system, opportunistic pathogens may perform better during exposure to certain contaminants, increasing resistant behavior (Figure 3.24) as nutrient availability is constant.

Notably, when combinatory trials of either metal plus chloramphenicol were undertaken, growth effectively occurred in both conditions. However, as Figure 3.25 would show, acclimation to copper and chloramphenicol occurred more quickly than in the trials containing silver and chloramphenicol (Figure 3.26). This may be due to, again, increased activity of efflux mechanisms reducing internal microbial contaminant accumulation, or perhaps mutations occurring as replication occurred over time, resulting in acquired resistance. This may shed light on how opportunistic pathogen retention and performance varies when regarding water quality. There is data to suggest that processes are occurring at high water quality areas that reinforce resistant behaviour, however also evidence to suggest acclimation and resistance can occur at areas of low water quality. Additionally, the ability for retention in the rhizosphere and rhizoplane at these riparian zones enables opportunistic retention, which also reinforces contaminant resistance due to adherence of metals and pharmaceuticals in these zones. Further work is required to better elucidate resistant behavior of *Salmonella* spp., *Enterococcus* spp., and *E. coli*, as well as other known opportunistic pathogens that may pose the risk of environmental naturalization.

4

Effects of Water Quality on Bacterial Pathogen Biofilm Formation

The ability of environmentally isolated microbial pathogens to synthesize adhesion mechanisms and generate biofilms was assessed. Isolates from high, intermediate and poor water quality areas found at riparian zones along the Grand River Watershed were obtained from root, soil and water samples. The following sections will present findings of how enteric opportunistic pathogens such as *Salmonella* spp., *Escherichia coli,* and *Enterococcus* spp., colonize environmental settings. Exposure to a range of temperatures, including 11^{o}C, 28^{o}C and 37^{o}C, were utilized to help understand biofilm-producing capabilities of isolated pathogens, under static and dynamic conditions.

This chapter addresses the research question of how temperature, water quality and environmental zones (rhizosphere, rhizoplane and water) influences microbial behavior. At each water quality site, rhizospheric soil, roots (rhizoplane) and water samples were collected, processed and analyzed. The objectives investigated in this chapter were:

1) Measure the ability of environmentally isolated opportunistic pathogens, *Salmonella* spp., *Enterococcus* spp., and *Escherichia coli,* to produce attachment structures.
2) Evaluate the biofilm-forming capabilities of the above opportunistic pathogens and determine how this may be affected by water quality and temperature.
3) Assess how static versus dynamic conditions influence biofilm-forming capabilities in opportunistic pathogens isolated from the rhizosphere, rhizoplane and water, to determine how location in riparian wetlands influences persistence.

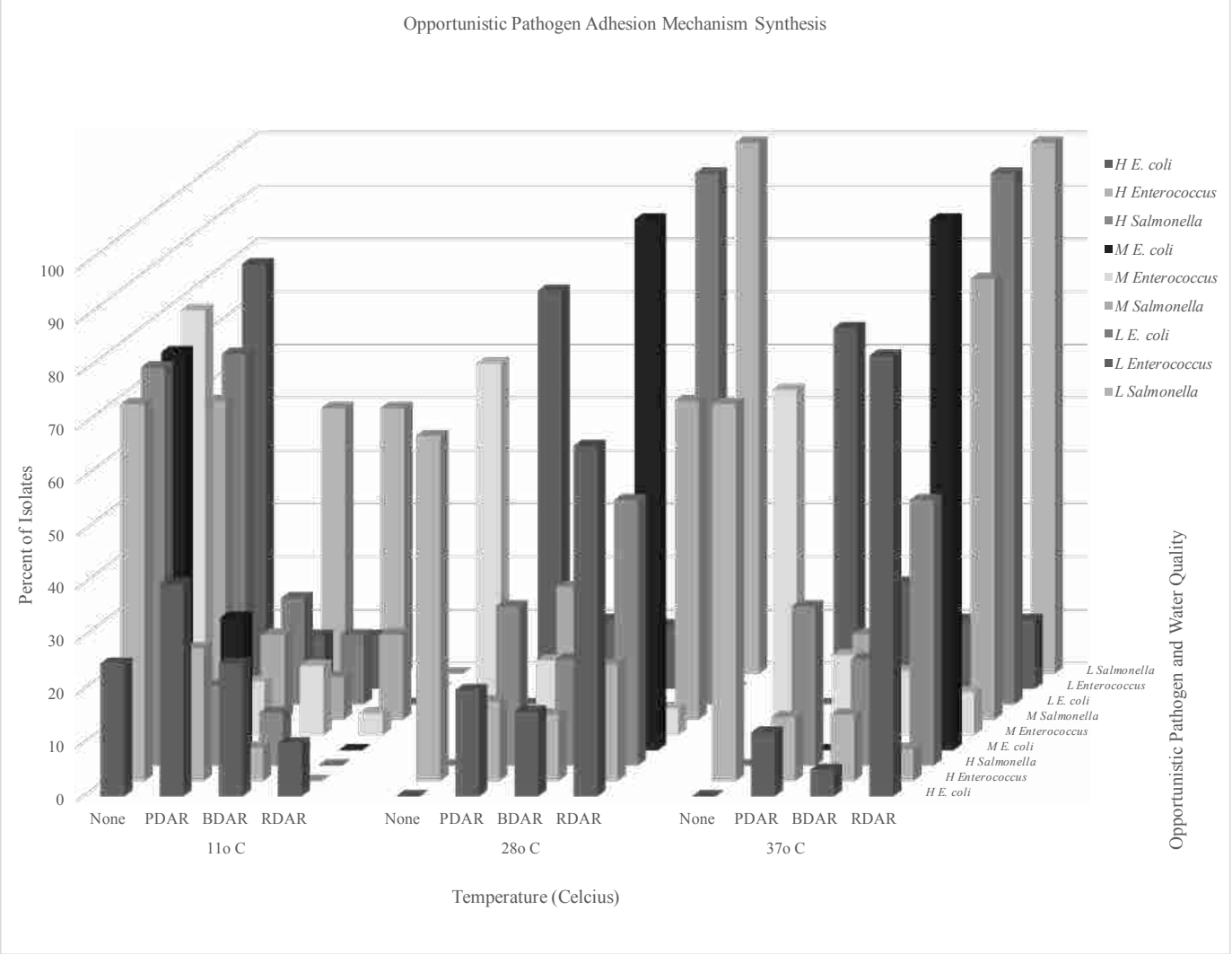
Similar to the contaminant-response trials, temperature parameters were kept constant (11ºC, 28ºC and 37ºC). Static conditions involved inoculation in 96 well microtiter dishes or on dye-infused agars. Dynamic conditions provided a force by means of an introduced flow rate (0.2 dyne), to help mimic natural settings isolates were derived from. Each assay is sub-divided to best reflect trends of biofilm and adhesion mechanism profiles amongst isolated pathogens and the environmental analyte (rhizoplane, rhizosphere, water) these organisms were isolated from.

4.1. Opportunistic Pathogen Proteinaceous and Carbohydrate Attachment Mechanisms

Initial attachment is imperative for planktonic cells to initiate biofilm formation. A proteinaceous structure, curli fimbriae, is produced to help this initial attachment and was evaluated. Also, a polysaccharide substance, cellulose, is generated to assist in attachment. Using Congo Red infused media, microbial efficacy of synthesizing these structures was assessed. Known morphotypes (Romling, 2003) were used as phenotypic morphotype references. Brown, dry and rough morphology (bdar) corresponded with proteinaceous growth, such as curli fimbriae or pili. Pink, dry and rough morphology (pdar) characterized carbohydrate-rich adhesion structures, such

as cellulose or glycoasminoglycans. Red, dry and rough morphotypes (rdar) represents both proteinaceous and carbohydrate production. Each assay was conducted in triplicate.

Figure 4.1 provides a representation of the abilities of *Salmonella* spp., *E. coli* and *Enterococcus* spp. isolates from ranging water quality impacted wetlands to produce adhesion structures. This was measured using Congo Red infused LB Agar. Incubation occurred at 11°C for 96 h, 28°C for 36-48 h, and 37°C for 24 h.



<u>Figure 4.1.</u> Expressed adhesion morphotypes of isolated *Salmonella* spp., *Enterococcus* spp., and *E. coli* isolates from high, intermediate and low water quality wetlands, at 11°C, 28°C, and 37°C. The sample types listed include pathogen type and water quality that group of isolates was obtained from. For example, H *E. coli* represents *E. coli* from high water quality impacted sites, M, medium water quality impacted areas, and L, low water quality impacted sites.

At 11°C (left), 16% of *Salmonella* spp. from intermediate water quality impacted sites generated both mechanisms, in comparison to high and low water quality impacted areas. *Salmonella* spp. isolates from low water quality impacted areas were more efficient at producing either extracellular polysaccharides (50%) or proteins (50%). Near 15% of *Salmoenlla* spp. isolated from intermediate and low water quality impacted sites were capable of producing cellulose, while approximately 10% produced curli. All remaining isolates did not express either. The majority of *Enterococcus* spp. were unable to produce either appendage, as high (71%), intermediate (80%) and low (80%) water quality impacted isolates lacked either. However, isolates from each location (25%, 10%, and 10%) extracellularly produced proteinaceous components, possibly indicative of pili. Regarding *E. coli*, 10% of isolates from high water quality impacted areas produced both curli and cellulose, however 40% (H), 25% (I) and 20% (L) of isolates singularly expressed carbohydrate synthesis. Around 25%(H) and 13% (L) exuded highly proteinaceous substances.

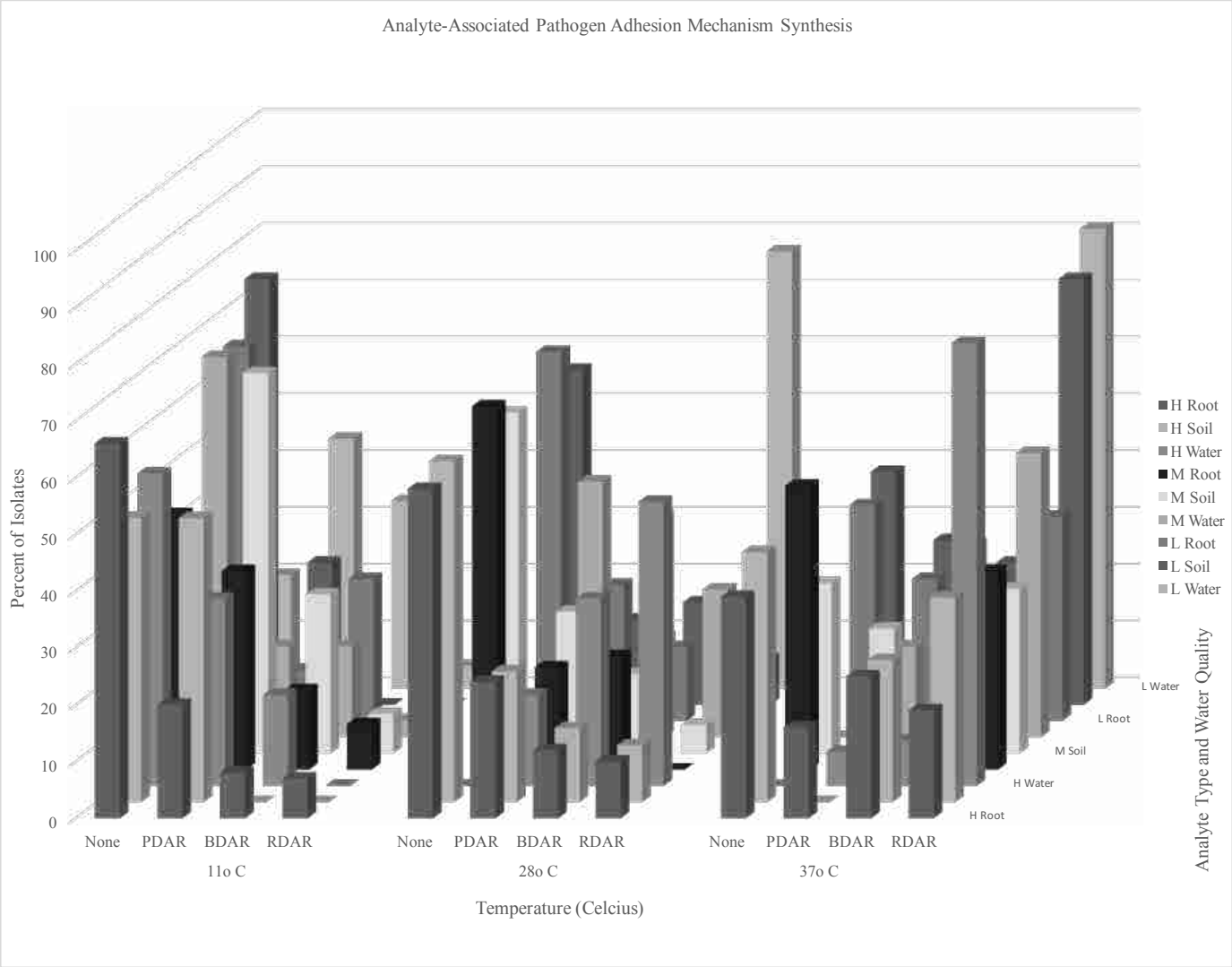
At 28°C (center), increases in expression of protein and carbohydrate combinations were observed. *Salmonella* spp. isolates (100%) from low water quality impacted areas displayed this, whereas about 70% form high and 60% from intermediate water quality sites synthesized both. Regarding curli fimbriae, 20% (H), 15% (I) and 0% (L) of *Salmonella* spp. displayed this growth. The remaining, 20% (H), and 25% (I) of these isolates seemingly showed morphotypes for cellulose. Approximately 65% (H), 70% (I) and 75% (L) of *Enterococcus* spp. isolates were unable to create

any either. Interestingly, 22% (H) exhibited the ability to manufacture both proteins and carbohydrates. Similar to *Salmonella* spp. isolates, 66% (H), and 100% (I,L) *E. coli* displayed rdar morphotype, indicating combinatory generation. At high water quality impacted areas, between 15-20% of *E. coli* constructed either curli fimbriae or cellulose.

Incubation at 37^{o}C resulted in 83% (H), 100% (I) and 100% of *E. coli* expressing a rdar morphotype, an increase of nearly 20% at high water quality impacted sites. As well, 100% of *Salmonella* spp. low water quality impacted areas proved efficient curli and cellulose producers, while 50% (H) and 83% (I) displayed this behaviour. A maximum of 13% of *Enterococcus* spp. were capable of protein or carbohydrate construction. Similar trends were seen in enterococcal isolates form all sites, as the majority (60% or greater) produced neither.

4.2. Analyte-Specific Pathogen Protein and Carbohydrate Adhesion Expression

Figure 4.2 examines variation in adhesion mechanisms categorized by type of analyte environmental pathogens were retrieved from. In correlation with Figure 4.1, lower temperatures yielded minimal expression. In analyte which *Salmonella* spp. and *E. coli* was recovered from, combinations of protein and carbohydrate synthesis appears common. Analyte for which *Enterococcus* spp. was derived from was least likely to produce either biological compound.



<u>Figure 4.2.</u> Adhesion mechanism profiles of isolated opportunistic pathogens purified from rhizoplane (root), rhizosphere (soil) and water samples from high, intermediate and low water quality wetlands, at 11°C, 28°C, and 37°C.

Similar to Figure 4.1, most isolates perform poorly during growth at this temperature. At each sampling area, pathogens isolated from the rhizoplane or rhizosphere performed better than those which were extracted from water. In the rhizoplane, 63% (H), 45% (I) and 66% (L) either did not grow or have a distinct morphotype. Nearly 20% (H), 35% (I) and 9% (L) of rhizoplane isolates

generated extracellular polysaccharides, whereas 8% (H) 14% (I) and 25% (L) expressed some protein growth. Isolates originating from the rhizosphere were more likely to generate protein than either a carbohydrate, both or neither, as 50% (H), 67% (I) and 44% (L) expressed a phenotypes conventional to cellulose or other external carbohydrates. Waterborne isolates were moderately successful, as

When temperature increases from 11^{o}C to 28^{o}C, expression of curli fimbriae and cellulose is noticed in water isolates (100%). As noted in Table 2.1 and Table 2.2, *E. coli* and *Salmonella* spp. were predominantly isolated from water. In the rhizoplane (68%, 60%, 63%) and rhizosphere (58% 63% and 69%), it was observed that many isolates were unable to generate either mechanism, corresponding with isolated *Enterococcus* spp. Moreover, up to25% of all isolates taken from rhizosphere and rhizoplane samples were capable of producing curli fimbriae and up to 15% capable of generating cellulose. Additionally, it was noticed that rhizosphere samples from all locations retained organisms capable of producing both mechanisms (6%, 3%, 10%). Similar trends continued as temperature rose to 37^{o}C, where increases in expression of both extracellular modifications were enhanced.

4.3. Opportunistic Pathogen Extra Polymeric Substance (EPS) Production

In relation to generating physiological adhesion appendages, EPS production is critical to the establishment and maturation of biofilms. In this section, isolated opportunistic pathogens from all sample sites assessed affinities for EPS production. All organisms were grown overnight (18-24 h) and cultivated on LB agar containing Calcofluor (w/v%), which fluoresced under UV light if EPS was produced. EPS is a poly-saccharide biopolymer exuded from bacterial biofilms,

promoting attachment and adsorption of environmental substances. Each assay was carried out in triplicate.

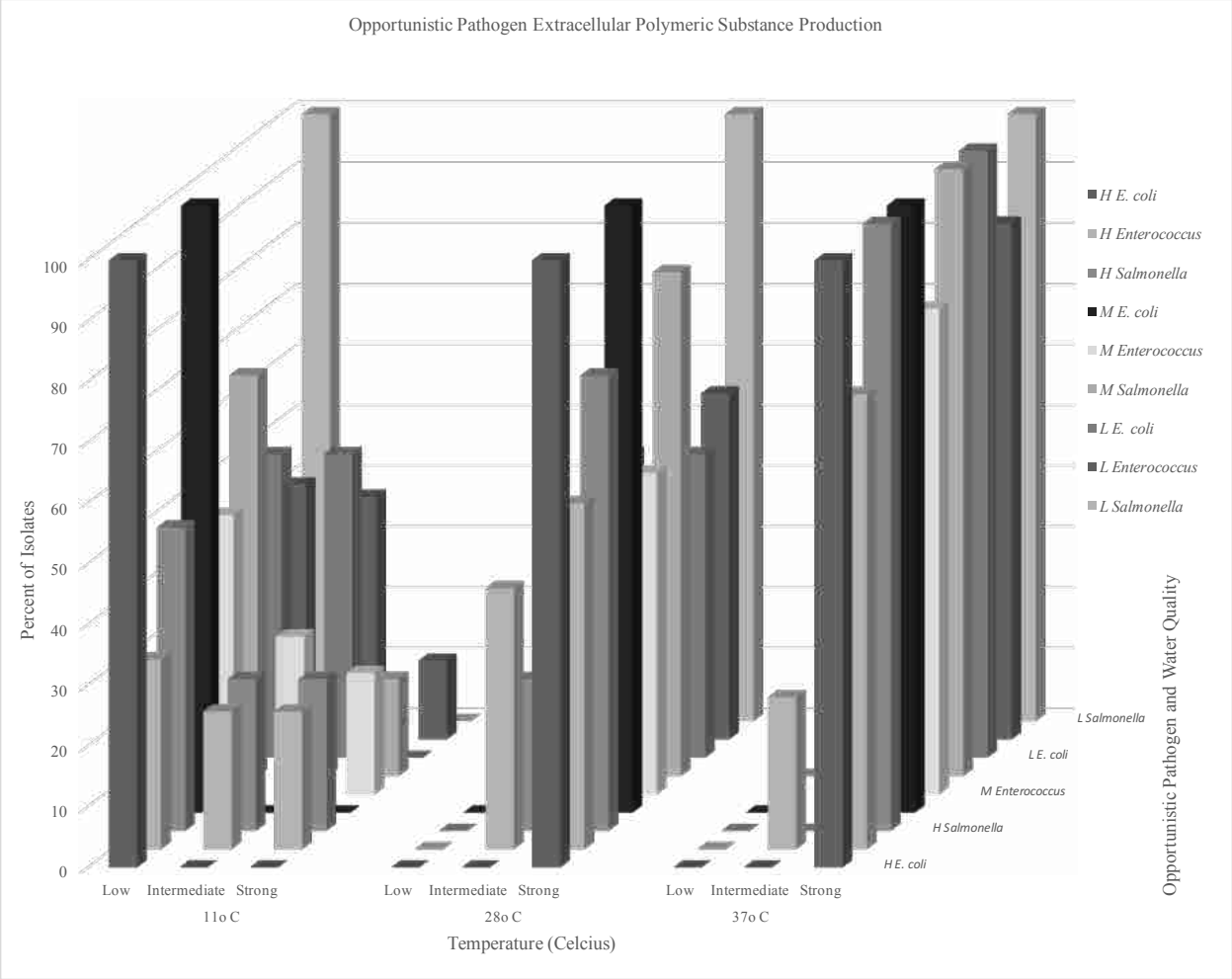


Figure 4.3. Extracellular Polymeric Substance profiles of the isolated opportunistic pathogens *Salmonella* spp., *Enterococcus* spp., and *E. coli* from high, intermediate and low water quality wetlands, at 11°C, 28°C, and 37°C. The pathogens were categorized as low (poor), intermediate, or strong EPS producers. The sample types listed include pathogen type and water quality that group of isolates was obtained from. For example, H *E. coli* represents *E. coli* from high water quality impacted sites, M**, medium water quality impacted areas, and L, low water quality impacted sites.**

As with expression and assembly of proteinaceous and carbohydrate-rich extracellular structures, temperature significantly impacted EPS growth. At 11^{o}C, the majority of isolates from all water quality sites were limited in their ability to produce EPS. High water quality impacted wetland isolates (25%) were most likely be strong producers of EPS in comparison to isolates from lower water quality impacted sites. Enterococcal isolates from high, intermediate and poor water quality impacted areas were able to produce strong amounts of EPS. The majority of *E. coli* isolated produces EPS in minimal amounts, with isolates from low quality sites demonstrating intermediate at best production. *Salmonella* spp. and *Enterococcus* spp. isolates at high water quality impacted sites are best able to generate EPS at low temperatures, as each have 20-40% of isolates capable. Increased water quality does show an increasing trend in EPS production in these two organisms, whereas *E. coli* from lesser water quality areas may be more efficient in EPS.

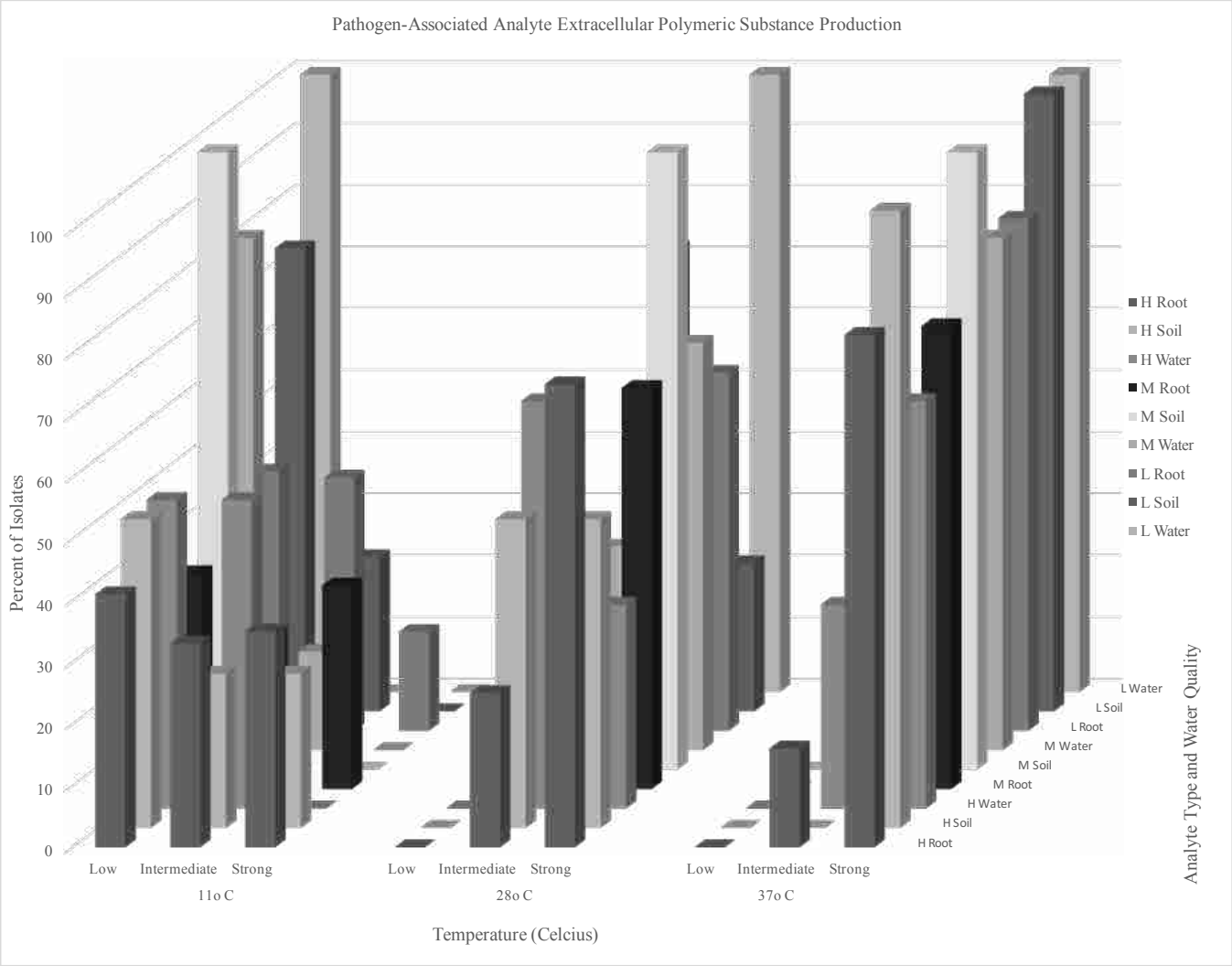
As temperature increased to 28^{o}C, all obtained isolates from each sampled site showed at least intermediate EPS production values (40% of enterococcal isolates, 45% of *E. coli*.). Poor water quality sites housed the highest isolate number of intermediate EPS production, whereas high quality wetland isolates had the highest percentage of strong EPS production. At host temperature, a shift to strong EPS production for isolates at all sites was observed, whilst minimal numbers of isolates still generated intermediate amounts of EPS.

Additionally, as the temperature increases so does the ability for each genus to produce EPS. At 37^{o}C, the ability for EPS production greatly increases. All isolated pathogens from all varying water quality wetlands show strong EPS production, with high water quality areas having the strongest total profile (all 3 microorganisms' ability to produce). *E. coli* and *Salmonella* spp. show

better capabilities to produce this EPS, whereas *Enterococcus* spp. isolates range from intermediate to strong generative abilities.

4.4. Pathogen-Associated Analyte EPS Production

Figure 4.4 provides an overview of EPS production at varying water quality impacted sites and corresponding sample types (rhizoplane, rhizosphere, water), when grown at 11^{o}C. The first series of data (on the left) corresponds with the ability to produce low, intermediate and high amounts of EPS at high water quality impacted sites. This is followed by production capabilities at intermediate (middle) and poor water quality areas (right).



<u>Figure 4.4.</u> Extracellular Polymeric Substance profiles of isolated opportunistic pathogens purified from rhizoplane (root), rhizosphere (soil) and water samples from high, intermediate and low water quality wetlands, at 11°C, 28°C, and 37°C.

At the lowest temperature condition, low levels of EPS production were predominant, followed by intermediate and then strong levels. At high and intermediate water quality impacted areas, root and soil isolates were more likely (30-90%) to produce intermediate levels of EPS. Further, up to 40% of these isolates were strong EPS producers. As conditions warmed, a shift from low EPS producers, to intermediate at worst, was noticed. Rhizosphere and rhizoplane isolates from all

types of water quality improved to at least 65% strong EPS production, whereas the rest of isolates from these regions produced intermediate levels. Noticeably, waterborne isolates were weak performs regarding EPS production, as about 40% of these isolates from high and intermediate water quality sites were efficient EPS producers. Interestingly, low water quality impacted area isolates were highly efficient at EPS production. At the warmest temperature condition, all organisms display peak EPS production, as at least 70% of all isolates appeared to be strong generators. Again, rhizosphere and rhizoplane isolates performed well.

4.5. Environmental Pathogen Biofilm Formation

A static biofilm assay was undertaken (as described in chapter 2) using 96-well microtiter dishes to determine, based on absorption, how effective environmental isolates were at producing biofilm material. The biofilm adhering to the polystyrene surface of the wells was stained with crystal violet and dissolved using acetic acid. Absorbance was measured using a BioRad plate Xmark plate reader. This was not a specific assay, meaning the total amount of biofilm produced was measured as opposed to specific components, as seen in the previously. Depending on temperature and incubation time, comparisons between pathogens and environmental sample source (sample type, rhizospheric soil, rhizoplane or water) were again assessed. Each assay was carried out in quadruplicate.

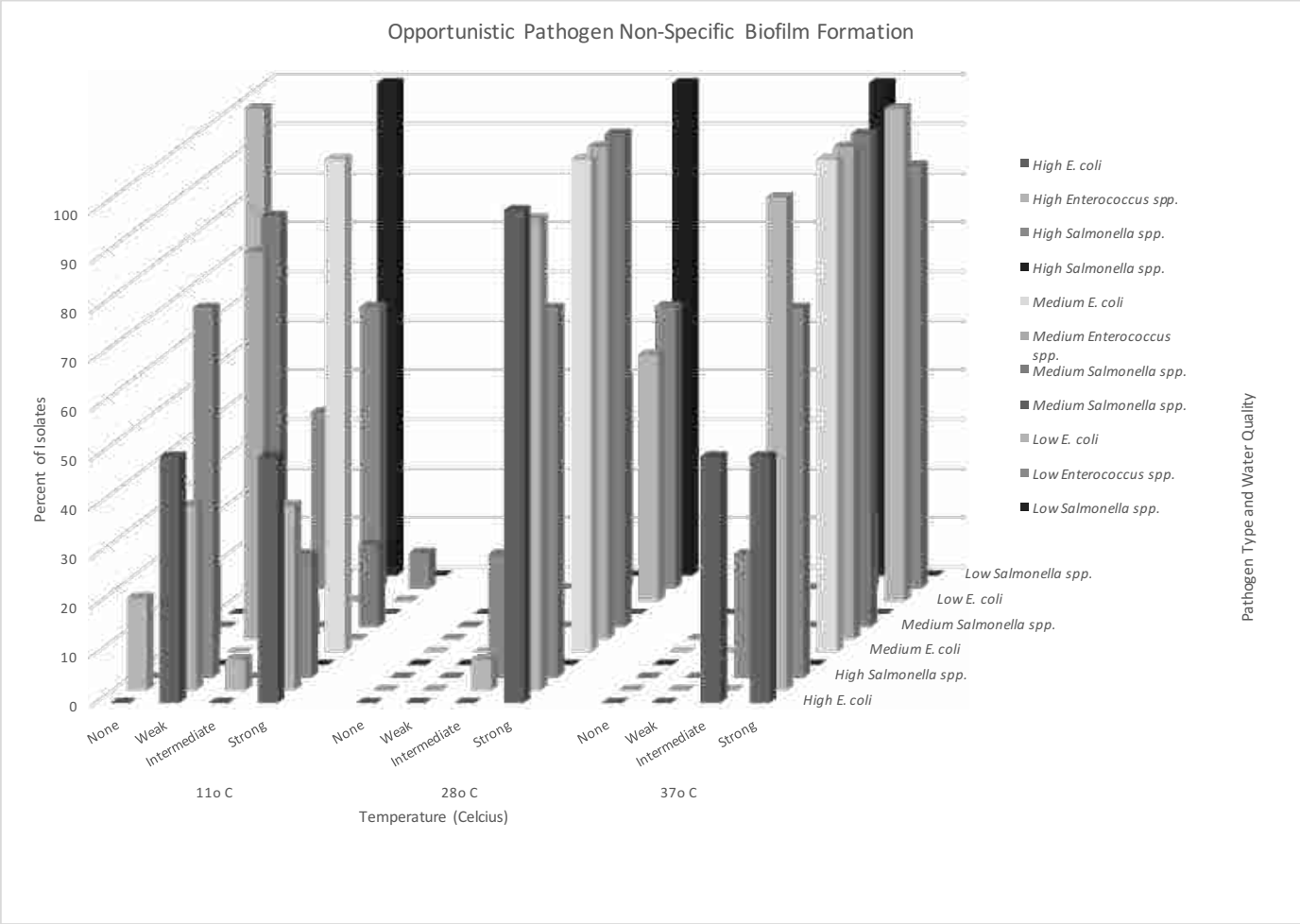


Figure. 4.5. A non-specific microtiter biofilm assay used to quantify the degree of biofilm-forming capabilities of *Salmonella* spp., *Enterococcus* spp., and *E. coli* from high, intermediate and low water quality wetlands, at 11°C, 28°C, and 37°C.

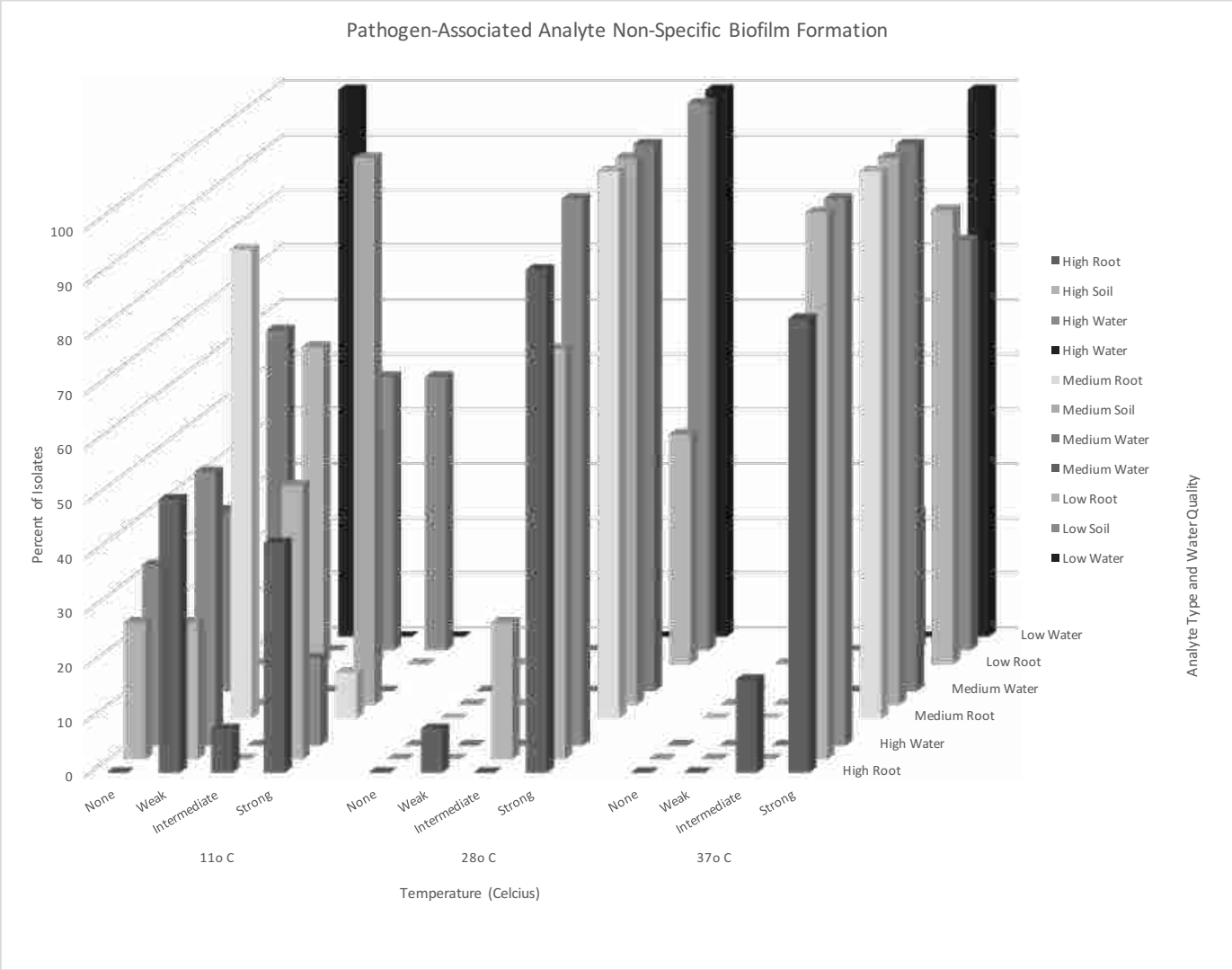


Figure 4.6. A non-specific microtiter biofilm assay used to quantify the degree of biofilm-forming capabilities of environmental pathogens associated with rhizoplane (root), rhizosphere (soil) and water analyte from high, intermediate and low water quality wetlands, at 11°C, 28°C, and 37°C.

The ability to form static biofilms without any external influence other than temperature was measured. In Figure 4.5, the total amount of biofilm production was compared at 11°C for the sampled areas. The majority of isolates were weak biofilm formers, however, each type of water quality site had isolates that were produced strong biofilms. Isolates from low water quality

impacted sites formed the least amount of biofilm, whereas isolates from intermediately water quality impacted sites made up the majority of intermediate biofilm formers, and lastly isolates from more pristine areas the strongest biofilm formers.

Figure 4.6 examines variation at low temperatures in biofilm production between genera that were isolated and purified. Low quality sites primarily provided weak to intermediate biofilm formers, however, 45% of enterococcal isolates form high water quality impacted sited proved to be strong biofilm formers under these static conditions. As site quality increased, again so did biofilm forming capabilities. *E. coli* and *Salmonella* isolates were stronger biofilm generators than *Enterococcus,* however these ratios became more even amongst organisms from high quality sites. All three genera had organisms that could form strong biofilms, however *Enterococcus* again had isolates that fell into all categories.

Figure 4.6 shows that in all sampled areas, water-isolated bacteria were least-best at generating strong biofilms. At low quality sites, bacteria from the soil produced similar amounts of intermediate and strong biofilms. Increasing water quality resulted in increased biofilm production for soil and root originating microbes, as medium quality sites yielded soil bacteria that were highly proficient in biofilm production. High quality sites provided organisms isolated from roots, soil and water that were most efficient at biofilm generation in comparison to lesser quality areas.

Non-specific biofilm formation at 28^{o}C shows a marked increase in biofilm production for all isolates. In comparison to those exposed at 11^{o}C, isolates from all water quality sites improved in biofilm production at a relatively higher temperature than that observed previously. Low quality

sites had even distributions of intermediate and strong biofilm formers, whereas medium water quality sites were all strong generators.

It is evident that all genera from medium quality sites are proficient biofilm formers, as they all generated strong biofilms (Figure 4.5). *Salmonella* spp. from poor water quality sites all formed strong biofilms, whereas, *E. coli* and *Enterococcus* were both comparable amongst intermediate and strong biofilm formers. At high quality sites, *E. coli* and *Enterococcus* isolates improved in biofilm production, as both exceeded 80% of total isolates forming strong biofilms. *Salmonella* spp. generated strong biofilms under all conditions.

Biofilm production based on sample type was assessed. All isolates from corresponding samples were grown at a 28^{o}C for 48 h. The data on the left represents isolates found in Rhizospheric soil, the rhizoplane and water from high water quality sites. The middle portion represents those from intermediate water quality, and the data series on the right corresponds with poor water quality.

All root, soil and water samples from medium quality sites displayed strong biofilm production. Rhizospheric soil and water-derived isolates from low water quality areas generated strong biofilms, while root samples had more intermediate than strong producers. This clearly changed as water quality improved, with majority of isolates from rhizosphere, rhizoplane and water samples at intermediate and high water quality impacted wetlands strong biofilm producers.

The final temperature used in assessing static biofilm growth was at 37^{o}C. An increase from 11^{o}C to 28^{o}C, 90% of isolates were highly proficient in biofilm generation (Figure 4.5). All organisms from high, medium and low impacted sites proved to be efficient biofilm formers, as again all

isolated from intermediate water quality impacted sites consistently showed strong biofilm formation.

At low water quality impacted locations, 100% *Salmonella* spp. isolates were intermediate biofilm producers. Conversely, *Salmonella* spp. isolated at low water quality areas shifted to intermediate biofilm formers, whereas enterococcal biofilm strength increased. At high quality sampling areas, *Enterococcus* generated the strongest biofilms, followed by *Salmonella* and *E. coli.* All environmental samples yielded bacteria capable of forming biofilms at high temperatures, as only soil and roots at low quality sites, and roots from high quality sites, retained organisms with intermediate biofilm-producing capabilities, at minimum.

At 37^{o}C, intermediate water quality impacted sites showed 100% of isolates were strong biofilm formers. At poor water quality impacted sites, 18-23% of rhizoplane and rhizosphere-isolated organisms produced intermediate levels of biofilm, while the remaining percentage were strong biofilm formers. Similarly, less than 20% of rhizoplane isolates from high water quality areas were intermediate biofilm producers whereas all other organisms isolated from rhizoplane, rhizosphere and water samples were strong biofilm producers.

4.6. Dynamic Biofilm Generation

The previous sections of Chapter 4 examined the capabilities of environmentally persisting opportunistic pathogens' efficacy at biofilm and adherence mechanism production under static conditions. With the help of Christopher Bartlett, the ability to measure biofilm during dynamic conditions was measured. Using the BioFlux 200 System (Fluxion Biosciences, Alameda, CA, USA) quantification of planktonic attachment and biofilm maturation under fluid conditions was

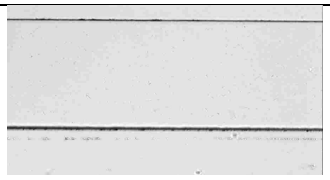
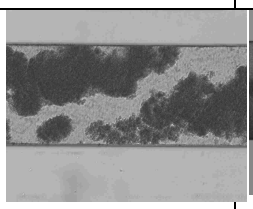
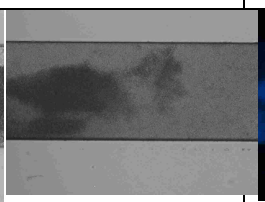
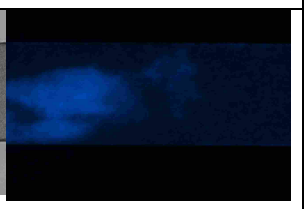
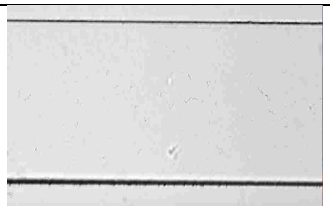
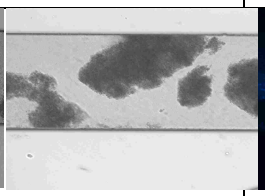
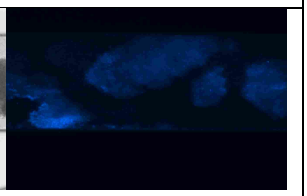
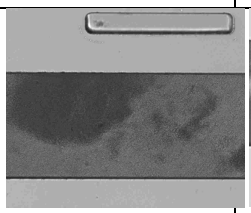
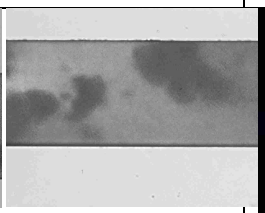
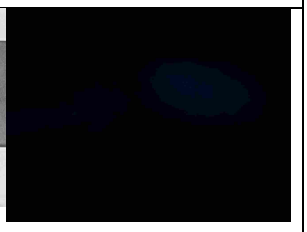
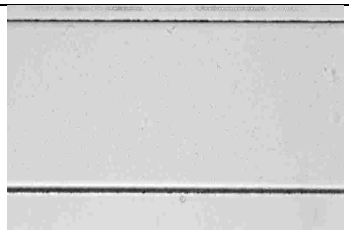
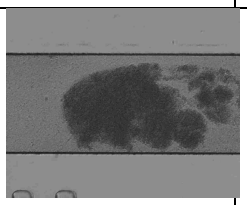
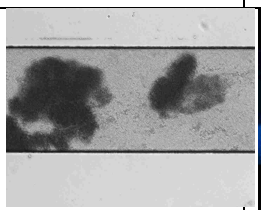
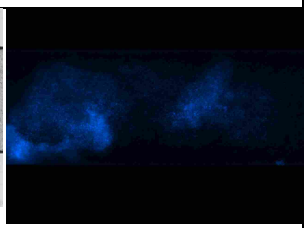
achieved (ie. flowrate in dynes). For this experiment, *E. faecalis* was chosen as the test organism due to high retrieval yields from high, intermediate and low water quality impacted wetlands.

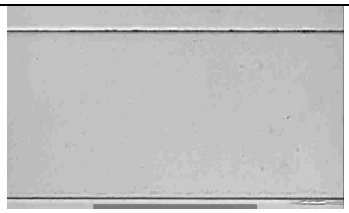
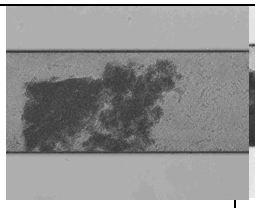
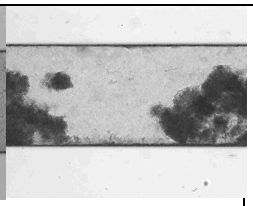
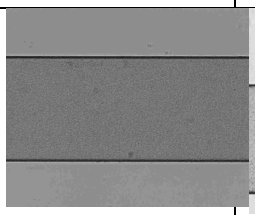
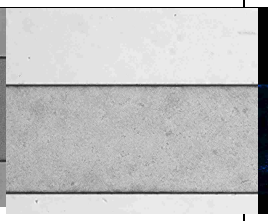
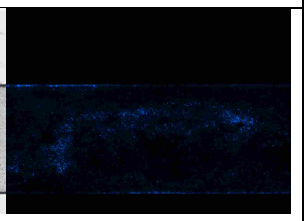
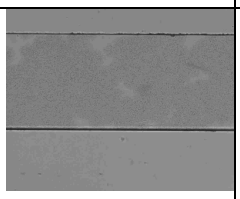
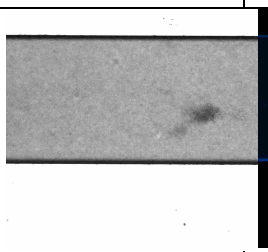
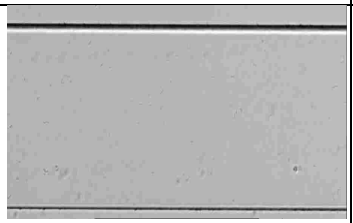
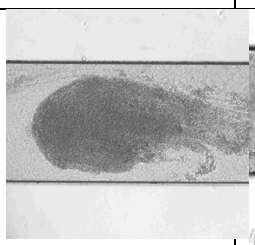
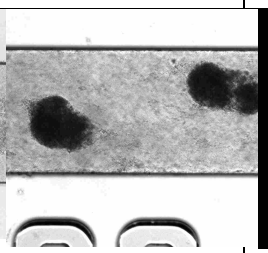
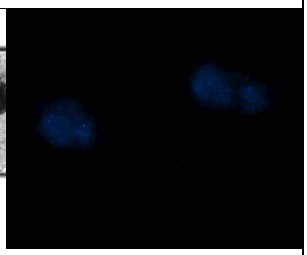
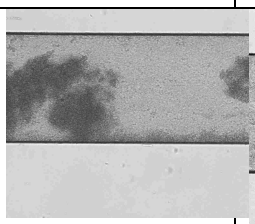
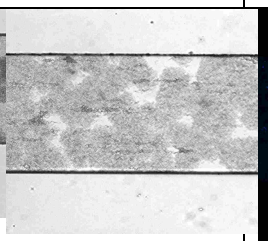
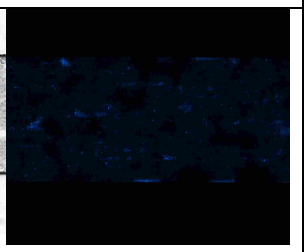
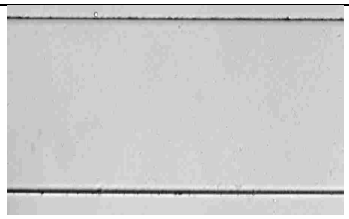
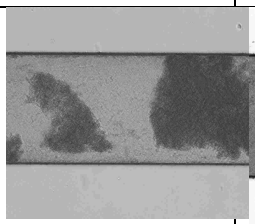
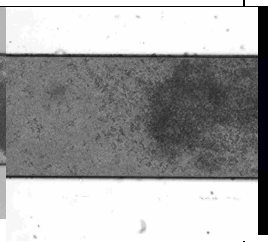
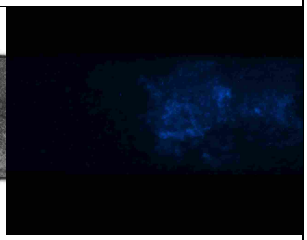
Dynamic biofilm formation over the span of 24 h is visually summarized in Figure 4.7. Each row corresponds with a specific *E. faecalis* isolate from either high, intermediate or low water quality impacted sampling areas. At maturation (24 hr), biofilms were stained with Calcofluor to show EPS production at 28^{o}C.

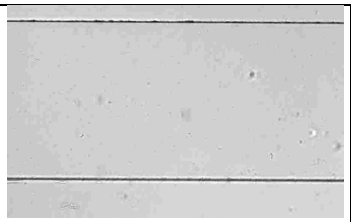
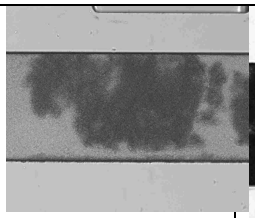
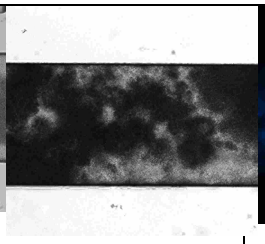
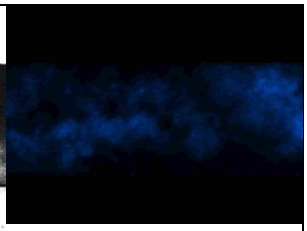
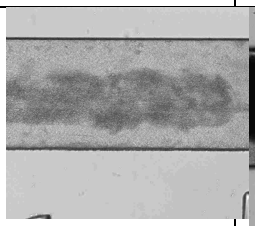
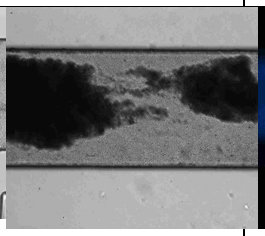
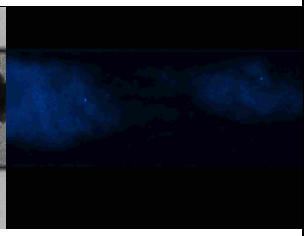
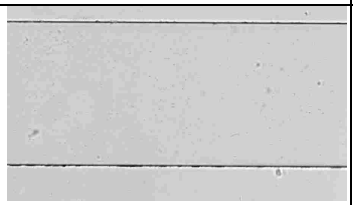
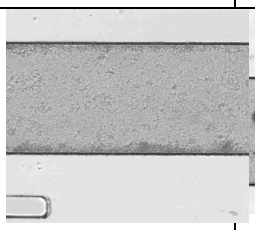
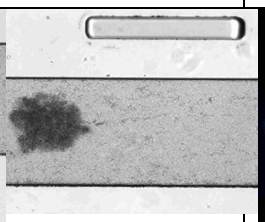
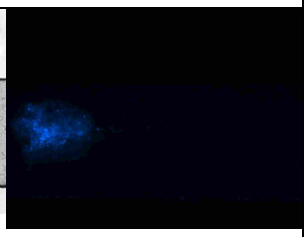
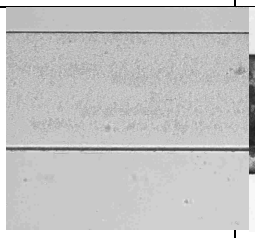
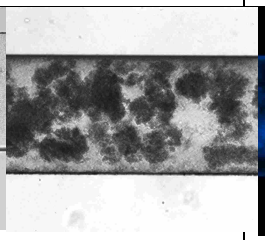
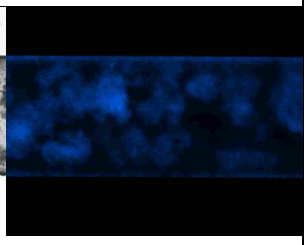
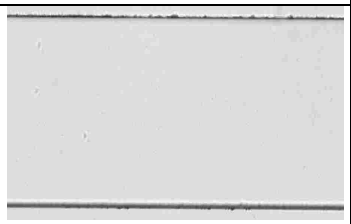
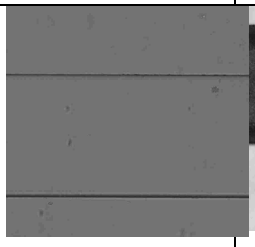
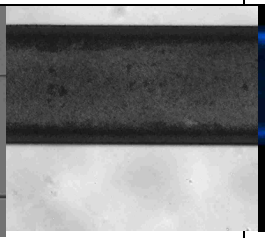
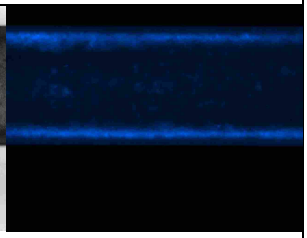
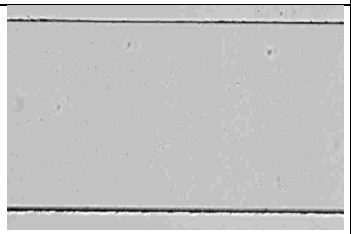
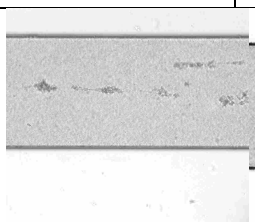
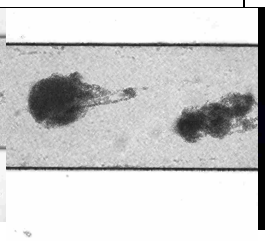
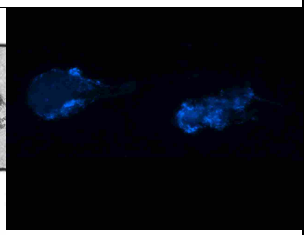
Table 4.1. *Enterococcus faecalis* isolate legend for pathogens assessed for determining biofilm production efficiency under dynamic conditions.

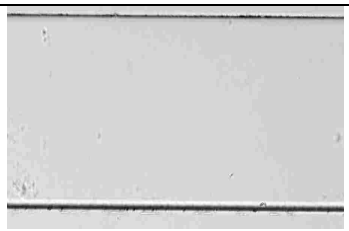
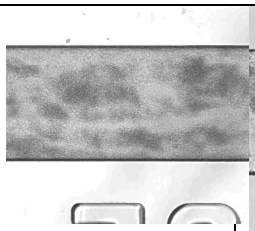
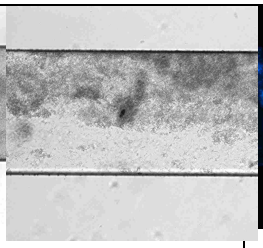
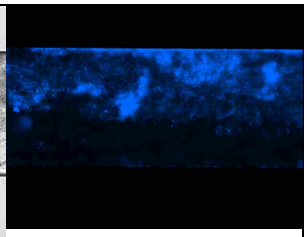
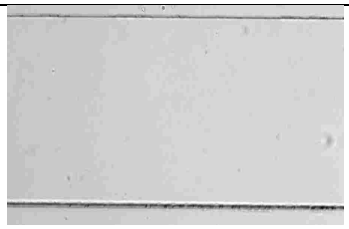
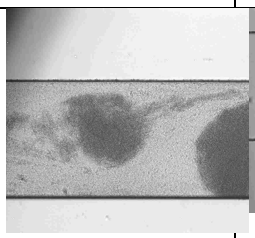
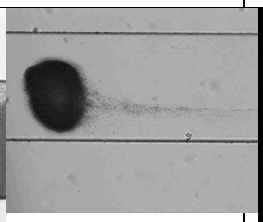
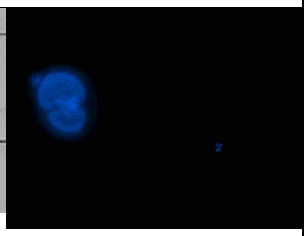
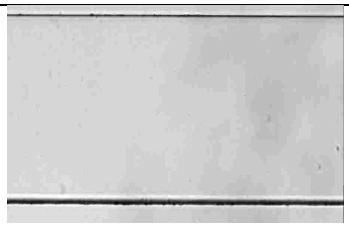
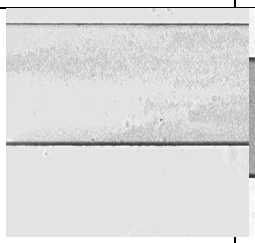
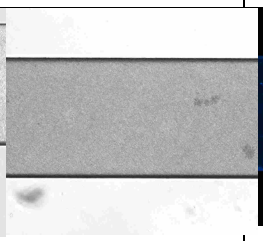
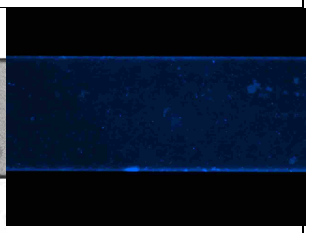
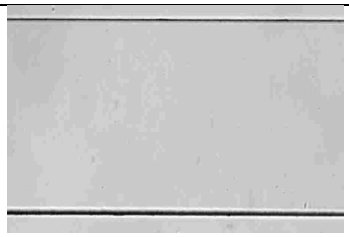
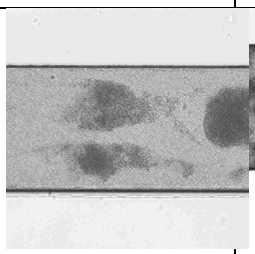
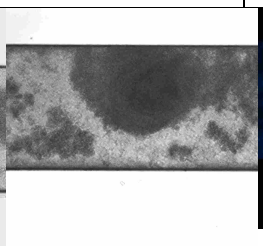
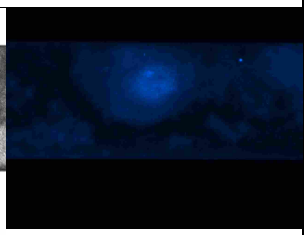
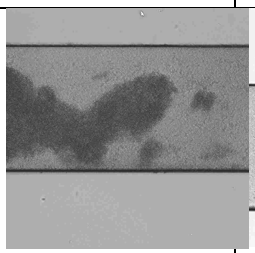
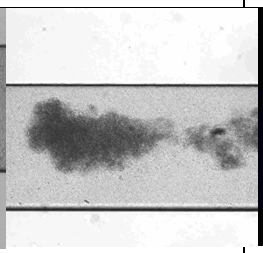
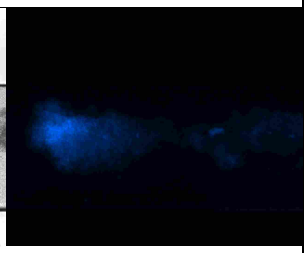
Isolate Name	Isolate Location	Organism
H1	**High Water Quality Site**	***E. faecalis***
H2	**High Water Quality Site**	***E. faecalis***
H3	**High Water Quality Site**	***E. faecalis***
H4	**High Water Quality Site**	***E. faecalis***
H5	**High Water Quality Site**	***E. faecalis***
H6	**High Water Quality Site**	***E. faecalis***
H7	**High Water Quality Site**	***E. faecalis***
H8	**High Water Quality Site**	***E. faecalis***
I1	**Intermediate Water Quality Site**	***E. faecalis***
I2	**Intermediate Water Quality Site**	***E. faecalis***
I3	**Intermediate Water Quality Site**	***E. faecalis***
I4	**Intermediate Water Quality Site**	***E. faecalis***
I5	**Intermediate Water Quality Site**	***E. faecalis***
I6	**Intermediate Water Quality Site**	***E. faecalis***
I7	**Intermediate Water Quality Site**	***E. faecalis***
I8	**Intermediate Water Quality Site**	***E. faecalis***

L1	**Poor Water Quality Site**	***E. faecalis***
L2	**Poor Water Quality Site**	***E. faecalis***
L3	**Poor Water Quality Site**	***E. faecalis***
L4	**Poor Water Quality Site**	***E. faecalis***
L5	**Poor Water Quality Site**	***E. faecalis***
L6	**Poor Water Quality Site**	***E. faecalis***
L7	**Poor Water Quality Site**	***E. faecalis***
L8	**Poor Water Quality Site**	***E. faecalis***

Isolate	**0 Hour**	**18 Hours**	**24 Hours**	**24 Hours DAPI**
HQ1				
HQ2				
HQ3				
HQ4				

HQ5				
HQ6				
HQ7				
HQ8				
IQ1				
IQ2				

IQ3				
IQ4				
IQ5				
IQ6				
IQ7				
IQ8				

LQ1				
LQ2				
LQ3				
LQ4				
LQ5				

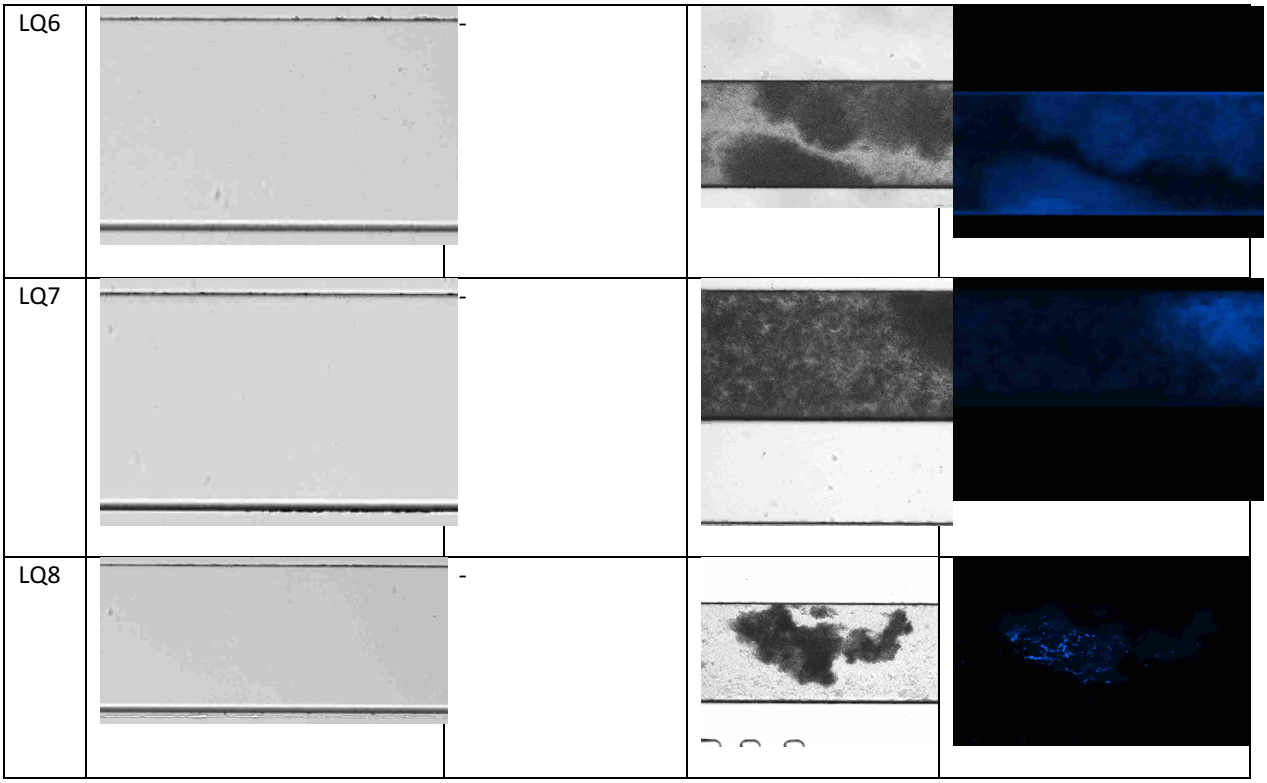


Figure 4.7. ***E. faecalis*** **from high, intermediate and low water quality impacted wetlands were grown under dynamic conditions using the Bioflux™ system. Eight isolates from each water quality site were chosen, and grown for 24 h at 28°C. Images were captured at 0 (2nd row), 18 (3nd row) and 24 h (4th panel), and stained with Calcofluor at the final time point (right). Images on the right showing increased blue color signifies heavy biofilm growth, whereas the same image in the 4th column appears as dark black.**

When examining the dynamic biofilm growth using the Bioflux™ system, various levels of biofilm production occurred over the 24 h growth period at 28°C (Figure 4.6). The first seven rows correspond with biofilm growth captured at 0 h, 18 h and 24 h. As seen for all isolates obtained from high water quality wetlands, the planktonic phase shows minimal adherence to the walls of the microtiter dish. After 18 hours, 75% of isolates in this trial appear to generate strong biofilms (H1-H5, H8), whilst two others (H6,7) are not as proficient. In H4 and H8, a comet-like appearance in biofilm structure is observed, signifying the formation of biofilms under flowing conditions. For the 75% of isolates forming strong biofilms at 18 h, similar trends are seen over the next six

hours. At 24 h, we see maturation of the biofilm (dark black), especially in H1,4 and 8, where they are extremely dense. When stained with Calcofluor, the noticeable interaction of the stain with EPS fluoresces using DAPI (blue). Strongly intense blue coloration represents increased EPS production. In H6 and H7, biofilm has covered the surface of the flow-through channels more uniformly than in any of the others,.

The intermediate water quality areas (I1-I8) resulted in almost 50% of *E. faecalis* at 18 hours between heavy and low biofilm producers. I1,4,5,6 7 and 8 appear to be slowly producing adhesive mechanisms, showing more even distributions around channel walls as opposed to attaching and increasing density at distinct locations as seen in the strong biofilm formers isolated from high water quality areas. As the incubation proceeds for 6 more hours and maturation continues, there is an apparent increase in biofilm production [density], again seen in the fourth column as dark black areas, and bright blue when stained with Calcofluor. At intermediate water quality locations, I6,7, and 8 show areas where biofilm production is more pronounced (uniform), while in all others the blue intensity is not as obvious.

Lastly, the isolates obtained from low water quality areas showed a poor ability to form biofilms, or sub-optimally binding Calcofluor. All isolates show the least proficient ability to generate biofilms after 18 hours when compared to isolates originating from higher water quality impacted areas, however I, 5-8 appear to have increased production at 24 h. When staining, it is seen that, although there are dense (dark) areas when looking at the microscopic images, Calcofluor may not have bound, resulting in ineffective DAPI imaging. Areas of thick biofilm and active replication are not as apparent here as in the higher water quality isolates.

Discussion

Assessing pathogen fate and persistence in the environment is a comprehensive task. This portion of the research project aimed to assess isolated pathogens that may have originated from mammalian fecal matter, that have been recovered from the environmental setting. Chapter 4 examines the capabilities of environmentally isolated pathogens (*Salmonella* spp., *E. coli, Enterococcus* spp.) to synthesize curli fimbriae, cellulose, and extracellular polymeric substances. Additionally, under static conditions net biofilm production was quantified to measure correlations between specific adhesion mechanisms, and non-specific complete biofilm production. Lastly, this section aimed to identify how variation of environmental conditions, specifically static versus dynamic conditions, affected microbial pathogen adherence and establishment.

In this chapter, the specific objectives measured were to, firstly, measure the ability of environmentally isolated opportunistic pathogens, *Salmonella* spp*., Enterococcus* spp*.,* and *Escherichia coli,* to produce attachment structures. Next, evaluate the biofilm-forming capabilities of the above opportunistic pathogens and determine how this may be affected by water quality and temperature. The final objective was to assess how static versus dynamic conditions influence biofilm-forming capabilities in opportunistic pathogens isolated from the rhizosphere, rhizoplane and water, to determine how location in riparian wetlands influences persistence.

Similar to contaminant exposure in Chapter 3, it was posited that at areas highly impacted by contaminants, urbanization and agriculture, opportunistic pathogens would be better able to produce adherence mechanisms, enabling them to acclimatize to these conditions (Cabral, 2010). Correspondingly, these pathogens would be capable of producing strong biofilm structures under

both static and dynamic conditions, producing robust biofilms (Dunny *et al.,* 2014). Conversely, at areas least impacted by anthropogenic influences, opportunistic pathogen isolates should be poorer biofilm producers (Kaiser *et al.,* 2013).

Further, it was thought that at these areas of low water quality, nutrient levels would be in abundance thus more favorable to colonize these regions when compared to high water quality areas, having lower nutrient availability. Comparably, the ability to form biofilm structures and persist were thought to influence contaminant resisting behavior, resulting in a higher degree of microbial succession at low water quality areas.

Regarding analyte sources for which microbial pathogens were retrieved, we anticipated that at all riparian wetlands sampled, biofilm-forming capabilities would be best displayed by rhizosphere and rhizoplane organisms. As mentioned in Chapter 1 and Chapter 3, the increased surface area and nutrient levels at these regions would be more favorable to establish, resulting in upregulation of adhesive mechanisms. Additionally, waterborne isolates were projected to be least efficient biofilm formers, as when in the water there is not nearly as much material for microbes to attach.

In the first series of assays, Congo Red-infused media was utilized to determine whether cellulose or curli fimbriae were produced by *Salmonella* spp., and *E. coli* isolates, whereas pili is produced by *Enterococcus* spp. (Chapter 2). What was found was that, similar to contaminant exposure trials at 11^{o}C (Figure 3.14), the efficiency of adhesion mechanism activity was lesser than at 28^{o}C and 37^{o}C. When evaluating levels of mechanistic expression, *E. coli* from high water quality areas and *Salmonella* spp. from intermediate water quality areas displayed the ability to produce both carbohydrate-rich and proteinaceous mechanisms (Figure 4.1).

Moreover, it appears as though *Enterococcus* spp. isolates performed poorly in this assay, which is not surprising as these Gram-positive bacteria are not known to synthesize either curli nor cellulose. Instead, they produce proteinaceous pilli (type IV), aggregative substances as well as enterococcal surface proteins. These physiological mechanisms assist in cell interactions with surfaces when initializing attachment, as seen in *E. faecalis* strains resulting in hospital-acquired infections. With this in mind, the data showing that cellulose and curli-like morphotypes identified for enterococcal isolates may be due to a decreased selectivity of Congo Red for binding proteins. Congo Red is an azo dye, binding organic substrates and resulting in pigmentation. As *Salmonella* spp. and *E. coli* are known producers of both structures, it was not surprising to see one or both mechanism produced. However, it is notable that at low temperatures, these mechanisms were not only generated by these organisms, but contaminant response also occurred (Figure 3.13). Although resistance to multiple pharmaceuticals was found to be a maximum of two at low temperatures (Figure 3.20), the data suggests that perhaps there is a correlation between adhesin synthesis and resistant behavior.

As temperature increased, so did the ability for curli fimbriae and cellulose synthesis. At 28^{o}C, *E. coli* and *Salmonella* spp. isolates improved drastically, where at high and low water quality impacted areas 100% of these isolates produced both. Not surprisingly, multi-drug resistance markedly increased in these organisms (Figure 3.18), further suggesting synergistic behavior relating biofilm structure and performance during contaminant exposure.

Also, *Enterococcus* spp. had apparent increases in both mechanisms (Figure 4.1), which is fascinating as this genus of microorganism does not synthesize these mechanisms, reinforcing the

idea of the level of affinity Congo Red has for proteinaceous compounds. Notably, resistance to contaminants also shows a linear increase with temperature and amyloid synthesis in this group of pathogens, again contributing to the possibility of biofilm synthesis facilitating contaminant response.

Regarding environment sample types these pathogens were isolated, rhizoplane samples from high water quality areas contributed one of the most diverse expression of extracellular proteins and carbohydrates, followed next by rhizospheric samples from intermediate (M) water quality impacted areas. All other analyte types from high, intermediate and low water quality impacted areas showed similar profiles in no mechanisms, or singular expression at 11^{o}C (Figure 4.5). Similarly, multidrug resistance was found to be low at these areas, however at high and low water quality sites, rhizoplane and rhizosphere isolates did perform better than waterborne pathogens (Figure 3.21).

An interesting level of resistance was found in waterborne isolates from intermediate water quality impacted sampling areas in waterborne pathogens, which may address the efficiency of curli and cellulose in initial establishment (Figure 4.5), or reveal differences in time of collection (seasonality) and sample size. This was expected, as at lower temperatures signal transduction and detoxifying systems are not as active compared to less stressful conditions (Wood *et al.* 2013).

When measurements of adhesion mechanisms were compared to contaminant response and resistance profiles at 28^{o}C, a positive correlation was found. Studies have shown that this is a cardinal temperature for adhesion activity, seeming that when organisms are cultivated at this temperature, optimal expression of these mechanisms results (Romling *et al.,* 2012; Romling *et*

al., 2003). Increasing temperature resulted in not only up-regulation of pathogen adhesion mechanisms, but also resistant behavior. As *E. coli* and *Salmonella* spp. improved to at least 80% curli and cellulose production (Figure 4.1), multidrug resistance also improved. When isolated from high water quality impacted areas, 75% or greater *Salmonella* spp. and 100% of *E. coli* isolates resisted at least 5 or more antibiotics. In relation to this, 66% of *Salmonella* spp. resisted 5 or more pharmaceuticals at intermediate water quality areas, however non proved resistant at low water quality areas (Figure 3.18). *E. coli* maintained resistant behavior, as 50% of isolates were not affected by 5 or more antibiotics.

Enterococcal isolates showed the greatest levels of pharmaceutical resistance, however appeared to express the least amount of adhesion mechanisms. Of these pathogens, 25% or greater resisted up to 5 antibiotics, and 50% or more resisted 8 to 12 (Figure 3.18). Although Congo Red is useful in identifying adhesion mechanisms in organisms producing curli fimbriae and cellulose, it may not be best for those utilizing other proteinaceous or carbohydrate-rich adhesins.

Further, when measuring amyloid presence, it was noted that again waterborne isolates proved most efficient, generating both structures. Surprisingly, areas denser in nutrients, containing large surfaces areas for attachment, provided isolates that did not express this behavior (Zhang *et al.* 2013). This, again, is interesting in comparison to the rhizosphere and rhizoplane isolates in this assay, however is not surprising as this group of samples was comprised of *E. coli* and *Salmonella* spp. (Toledo-Arana *et al.,* 2001; Bonafonte *et al.,* 2000). There was not a marked increase in adhesin behavior between 28^{o}C and 37^{o}C, further suggesting that the most impactful transition in adhesion expression occurs at or before 28^{o}C.

Regarding water quality, a similar trend appears throughout all organisms isolated. This was unexpected, as we predicted low water quality impacted areas to be more specialized in amyloid synthesis. Perhaps these findings reveal that, in a laboratory setting where variables are easily controlled, microorganisms can potentially revert to a specific behavior as their new environmental setting is not as taxing. There is no microbial competition, so the need to create biofilms for protection of nutrient acquisition is minimal.

Also, abiotic stressors, such as contaminate levels, nutrient levels, predation, pH and more, are less hostile in the laboratory setting. Further, the influence of plant assemblages at these water quality areas may correlate with adhesin expression. Throughout the GRWS, similar plant types were found at West Montrose and Shand Dam when measured with those from Doon and RARE. This would help explain the similar trends noticed at each wetland. Further, soil composition, moisture and charge were similar, however varied with seasonality. The influence of plants on microbial establishment is significant, as plants produce specific antimicrobials, invoking stressful conditions for grazing or attached bacteria (Maschner *et al.,* 2004; Bossio *et al.,* 1998). This may also contribute to drug resistance identified in Chapter 3.

Adhesion mechanisms like curli fimbriae and cellulose promote attachment of planktonic microorganisms to surfaces, where chemical signaling molecules like N-acyl-homoserine lactone and N-acetyl-glucosamine facilitate communal growth and well-developed biofilms (Teplitski *et al.,* 2000; Waters and Bassler, 2005). When planktonic microbes adhere to a surface and establish themselves, signaling to others results in attachment (Figure 4.7). The ability to quorum sense and produce a very mature, specialized biofilm enables microorganisms to not only remain in the

environmental setting, but also acquire nutrients. More importantly, biofilms are a source of contaminant response, resulting in upregulation of adhesion mechanisms resulting in enhanced biofilm production (White-Ziegler *et al.*, 2007). Channels formed in mature biofilms help transport metals, chemicals, nutrients and more, throughout the architecture of the community (Stanley and Lazazzera, 2004). Similar to efflux mechanisms in singular cells, biofilms may act as a highly efficient detoxifying mechanism (Sabater *et al.,*2002). Moreover, Diffusion of nutrients was more efficient in less-dense biofilms, as transport was more effective and the biofilm community was more effective in self-purification, and that micro colonies may be best able to survive in these areas (Stanley and Lazazzera, 2004).

Not only are these mechanisms important for environmental persistence and success, but also for establishing infection in hosts, which may also shed light on resistant behavior seen in Chapter 3. The notable abilities of these pathogens to illicit infection is largely attributed to being able to penetrate host enterocytes and colonize in mammals resulting in infection (Yu *et al.,* 2012). As well, similar mechanisms are required to adhere or penetrate plant structures, such as roots, to become established (Benakanakere and Kinane, 2012; Ma *et al.* 2017).

Assessing extracellular polymeric substance (EPS) in pathogenic isolates revealed near-identical trends as seen throughout Congo Red trials. The largest noticeable difference was that *Enterococcus* spp. isolates were much more efficient at producing this carbohydrate-rich substance compared to curli and cellulose. As identified by Cobo and colleagues (2008), *Enterococcus* are notable biofilm formers, particularly notorious for causing hospital-acquired (nosocomial) infection, it is not surprising they were isolated in high quantity. Further, their presence in the

environment suggest not only fecal contamination, but high levels of acclimatization du to biofilm production, retention in the rhizosphere and contaminant resistance (Baquero *et al.,* 2011).

There are several surface adhesion molecules which are innate in *E. faecalis* and *E. faecium,* which help reason why at low temperatures and varying water quality, high biofilm production was observed. Aggregative substances may enable cell-cell recognition thus resulting in the generation of thicker biofilms under sub-optimal conditions, when they become more favorable, are increasingly expressed in areas high in sugar content, such as plant roots (Clewe1l and Weaver, 1989; Creti *et al.* 2006). In addition, pili synthesis and Microbial Surface Components Recognzing Ahesive Matrix Molecules (MSCRAMMs) enable initial planktonic adherence to abiotic surfaces and secreting or containing extracellular matrix components (Sillanpää *et al.,* 2008; Sillanpää *et al.,* 2004).

It was seen that increasing temperature positively correlated with the number of EPS producers, with the two warmer (28^{o}C and 37^{o}C) temperatures resulting in highest levels of expression. Similarly, when comparing this to levels of resistant behavior (Figure 3.16-Figure 3.20), the ability to synthesize adhesion mechanisms and EPS result in at least 50% resistance to 5 or more drugs when exposure occurs at 28^{o}C or greater. High water quality sampling sites generated the most EPS, followed by low water quality impacted areas. Nguyen and Yuk (2013) observed similar trends when measuring pathogenic biofilm formation on abiotic surfaces, showing that *Salmonella* spp. formed stronger, more resistant biofilms at 37^{o}C, followed by the next highest biofilm production efficiency at 28^{o}C. Figures 4.5 and 4.6 show the ability of the pathogens of interest to generate non-specific biofilm. In a study done by Jones and Bradshaw (1996), *Salmonella* and *E.*

coli displayed the ability to generate EPS over a span of 72 h, noting efficiency of polysaccharide production resulting in biofilm adherence, which further improved throughout biofilm maturation.

Enterococcal biofilms utilize EPS as a way for protecting internal, active cells of the community as well as communication when enduring stressful stimuli (Santos *et al.*, 2008). The esp-encoded gene, enterococcal surface proteins, has bene shown to upregulate in expression at increased temperature (Johnston and Jaykus, 2004). Further, when comparing adhesion mechanisms identified in these environmental isolates to static biofilm formation, it was shown that whether *esp*-producing genes were active or not, biofilm formation still was initiated from planktonic (2h) to microcolony (8h) and lastly a complex biofilm structure after 24 h (Kristich *et al.*, 2004). To my knowledge, the calcofluor staining of EPS production of environmental *E. faecalis* isolates exposed to dynamic conditions is novel. The ability to assess in real-time the capabilities of pathogens to adhere to surfaces and replicate in high numbers is extremely important in beginning to understand environmental persistence. Although this assay was carried out in a singular trial, the biofilm and EPS levels imaged (Figure 4.24) can be reinforced by the data found in all other adhesion-specific and general biofilm measuring assays.

Further, within enterococcal biofilm matrices, the exchange of important transposable elements such as antibiotic resistance and biofilm growth is facilitated (George *et al.,* 2005). Confocal laser scanning microscopy was initially used to assess biofilm generation, however, since has been replaced with using atomic force microscopy (Morris *et al.*, 1999) and now in this study, a combination of brightfield-fluoresence microscopy utilizing Bioflux dynamic conditions enabled measuring of environmentally isolated pathogen biofilm formation.

In summary, when comparing results for adhesion mechanism formation, EPS production and biofilm formation under static conditions, temperature was a significant factor. As temperature increased from stressful conditions (11^{o}C) to adhesion mechanism-stimulating (28^{o}C) and host (37^{o}C) temperatures, curli fimbriae, cellulose and EPS was more actively synthesized in all pathogens, from all water quality sites. Additionally, the sample type, particularly root and soil organisms, were more efficient biofilm producers. Surprisingly, at areas of higher water quality, biofilm formation occurred better than at lower water quality impacted areas. The impact that temperature has on biofilm structure is significant. Next, organisms from soil samples were second highest for both biofilm production and resistance capabilities, lastly followed by those found in water samples. Further, at higher temperatures, rhizoplane and rhizospheric isolates were prominent biofilm synthesizers and contaminant resistors as hypothesized.

5

Summary, Conclusions and Future Directions

5.1. Summary

At the conclusion of this study:

- it was found that the Grand River Watershed and the sampled riparian zones were acceptable habitats for opportunistic pathogen retention. Microbes of mammalian [fecal] origin, namely *E. coli, Enterococcus* spp., and *Salmonella* spp. were often found in wetlands varying from high (good) to low (poor) water quality. *Enterococcus* spp. was most often to be isolated from all wetlands and water quality, followed by *Salmonella* spp. and lastly *E. coli*. Key abiotic factors, specifically temperature and contaminant levels, were determinants of microbial growth.
- It was found that at low (sub-optimal) temperatures like 11^0C, microbial resistance to antibiotics was low and often isolates were susceptible to multiple classes of antibiotics. Additionally, adhesion mechanisms like curli fimbriae and cellulose, extra polymeric

substances (EPS) and static biofilm formation occurred at the lowest reported rates. As temperature increased to 28^0C, antibiotic effects were reduced and isolates were able to better respond [grow] in the presence of the stressor. Amyloid structure and biofilm thickness were more efficiently produced. At host temperature, 37^0C, similar results were found as at 28^0C.

- Notably, most Enterococcal isolates from the GRWS appeared to be heavily resistant to vancomycin, a clinically relevant antibiotic often used to treat enterococcal infection. Enterococcal biofilm synthesis varied amongst sampling areas, with high water quality sampling areas resulting in the most efficient biofilm formers. As well, resistance profiles (least susceptible) were higher for all microorganisms at high water quality areas, followed by poor and intermediate quality sites.
- Organisms which were found isolated from the rhizoplane and rhizosoheric soil proved as the best biofilm formers and had highest levels of MDR, followed by waterborne pathogens that were isolated.
- At low to high levels of copper (mM) based on environmental values, *E. faecalis* appeared to adapt to stressful conditions as exposure time increased, and grew more successfully than at varying levels of silver and chloramphenicol. When exposed to combinatory additives of silver and chloramphenicol, as well as copper and chloramphenicol, acclimation was most likely to happen at low levels (0.2 mM) of chloramphenicol and copper (1mM-0.2mM. Under dynamic conditions, biofilm formation was observed to occur effectively over 24 h in isolated *E. faecalis* strains.

Those located at high water quality areas were proficient biofilm formers, retaining Calcofluor better than those isolated from poor and intermediate water quality areas.

5.2. Integrative Nature of Research

This research project incorporated many aspects of biological and chemical sciences. Firstly, ecology and environmental biology was utilized to help determine and choose sampling locations within the Grand River Watershed. Having knowledge of the river topography, areas of discharge from varying anthropogenic sources and understanding microbial ecology were critical to this project. Identifying microbial niches in roots, soil and water at varying riparian zones enabled the sampling process allowing for the beginning of pathogen isolation. Upon bringing samples to the lab, the processing and understanding of microbial metabolism was critical to isolation and cultivation of pathogens. Molecular biology aided in confirmation of isolated organisms, whether by species or genus, allowing for further assays to help gain a better understanding of environmental behaviour. The ability to use molecular techniques to answer questions regarding bacteria will help further the field of microbiology.

5.3. Future Directions

Obviously when beginning a project, it is nice to see it through to the end. In the nature of environmental research, specifically regarding bacteria, this is easier said than done. The time required to isolate and purify organism cultures, and run many replicates of multiple assays is tremendous. If time allowed, there are areas for which this research could be further bolstered, having more molecular biology and mesocosm scale experiments incorporated to add depth to

similar projects and fulfill many research needs. The following are all ideas which, if time were not a factor or this project were continued after I leave it, would be beneficial.

1) Compile a library of genes in addition to the *esp* (enterococcal surface protein) gene influencing enterococcal adherence, virulence and persistence. Additionally, investigating genes influencing environmental persistence and naturalization of *Salmonella* spp. and *E. coli* will further address current unanswered questions in applied microbiology.
2) Community level assessments of opportunistic pathogens and those with naturalizing potential using rapid sequencing, such as Illumina sequencing, would aid in the time required (at a cost of high price), but would provide great detail as to organisms which may be present in certain communities. To be specific, mammalian pathogens and fecal-indicating bacteria would in rhizosphere and rhizoplane samples, as well as in biofilm material they may form, would help elucidate environmental behaviour of these organisms.
3) Lastly, determining ways to limit the exchange of environmental resistance genes and reduce anthropogenic sources as a factor is key to the health of watersheds. Better practices to limit the use of antibiotics in agriculture, or limit exposure to water sheds, may help in lowering causes and effects of antibiotic resistance.

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Appendix A

Luria Bertani Broth (LB Broth)

Enzymatic Digest of Casein ..... 10 g

Yeast Extract..... 5 g

Sodium Chloride ..... 5 g

Final pH: 7.3 ± 0.2 at 25°C

Luria Bertani Agar (LB Agar)

Enzymatic Digest of Casein ..... 10 g

Yeast Extract..... 5 g

Sodium Chloride ..... 5 g

Agar ..... 12 g

Final pH: 7.3 ± 0.2 at 25°C

Xylose Lysine Deoxycholate (XLD) Agar

Yeast Extract..... 3 g

Lactose.....7.5g
Sucrose..... 7.5 g

Xylose ..... 3.5 g

L-Lysine..... 5 g

Ferric Ammonium Citrate..... 0.8 g

Phenol Red ..... .0.08 g

Sodium Chloride ..... 5 g

Sodium Deoxycholate ..... 2.5 g

Sodium Thiosulfate ..... 6.8 g

Agar ..... 13.5 g

Final pH: 7.4 ± 0.2 at 25°C

Hektoen Enteric (HE) Agar

Enzymatic Digest of Animal Tissue..... 16.5 g

Yeast Extract..... 3 g

Bile Salts Mixture ..... 4.5 g

Lactose.....12g
Sucrose.....12 g

Salicin ..... 2 g

Sodium Chloride ..... 5 g

Sodium Thiosulfate ..... 5 g

Ferric Ammonium Citrate..... 1.5 g

Bromthymol Blue..... 0.065 g

Acid Fuchsin ..... 0.1 g

Agar ..... 13.5 g

Final pH: 7.6 ± 0.2 at 25°C

MacConkey Agar

Enzymatic Digest of Gelatin..... 17 g

Enzymatic Digest of Casein ..... 1.5 g

Enzymatic Digest of Animal Tissue..... 1.5 g

Lactose ..... 10 g

Bile Salts Mixture ..... 1.5 g

Sodium Chloride ..... 5 g

Neutral Red.....0.03 g

Crystal Violet..... 0.001 g

Agar ..... 13.5 g

Final pH: 7.1 ± 0.2 at 25°C

Tetrathionate Broth

Enzymatic Digest of Casein..... 2.5 g

Enzymatic Digest of Animal Tissue ..... 2.5 g

Bile Salts ..... 1 g

Calcium Carbonate ..... 10 g

Sodium Thiosulfate ..... 30 g

Final pH: 8.4 ± 0.2 at 25°C

Rappaport Vassiliadis Enrichment Broth

Sodium Chloride.....8.0 g

Potassium Phosphate, monobasic .....0.60 g

Potassium Phosphate, dibasic .....0.40 g

Magnesium Chloride, anhydrous* .....13.58 g

Malachite Green .....0.036 g

Iodine……0.1g

Final pH: 5.2 ± 0.2 at 25°C

Rappaport Vassiliadis Modified Semisolid Medium

Enzymatic Digest of Casein ..... 4.59 g

Novobiocin…… 20 mg

Casein Acid Hydrolysate..... 4.59 g

Sodium Chloride ..... 7.34 g

Potassium Dihydrogen Phosphate..... 1.47 g

Magnesium Chloride, Anhydrous..... 10.93 g

Malachite Green Oxalate ..... 0.037 g

Agar ..... 2.7 g

Final pH: 5.6 ± 0.2 at 25°C

m-Enterococcus Agar

Enzymatic Digest of Casein ..... 15 g

Enzymatic Digest of Soybean Meal ..... 5 g

Yeast Extract.....5g

Dextrose..... 2 g

Dipotassium Phosphate..... 4 g

Sodium Azide..... 0.4 g

2,3,5-Triphenyl Tetrazolium Chloride..... 0.1 g

Agar ..... 10 g

Final pH: 7.2 ± 0.2 at 25°C

Bile Esculin Agar

Beef Extract ..... 11 g

Enzymatic Digest of Gelatin..... 34.5 g

Esculin.....1g
Oxbile..... 2 g

Ferric Ammonium Citrate..... 0.5 g

Agar ..... 15 g

Final pH: 6.6 ± 0.2 at 25°C

m-FC-BCIG Agar

Enzymatic Digest of Casein ..... 10.0 g

Enzymatic Digest of Animal Tissue..... 5.0 g

Yeast Extract..... 3.0 g

Sodium Chloride ..... 5.0 g

Lactose ..... 12.5 g

Bile Salts ..... 1.5 g

Aniline Blue ..... 0.1 g

BCIG…….0.2g

Agar ..... 15.0 g

Final pH: 7.4 ± 0.2 at 25°C

Triple Sugar Iron Agar

Enzymatic Digest of Casein ..... 5 g

Enzymatic Digest of Animal Tissue..... 5 g

Yeast Enriched Peptone.....10g

Dextrose..... 1 g

Lactose.....10g
Sucrose..... 10 g

Ferric Ammonium Citrate..... 0.2 g

Sodium Chloride ..... 5 g

Sodium Thiosulfate ..... 0.3 g

Phenol Red ..... 0.025 g

Agar ..... 13.5 g

Final pH: 7.3 ± 0.2 at 25°C

Lysine Iron Agar

Enzymatic Digest of Gelatin..... 5 g

Yeast Extract.....3g

Dextrose..... 1 g

L-Lysine..... 10 g

Ferric Ammonium Citrate..... 0.5 g

Sodium Thiosulfate ..... 0.04 g

Bromcresol Purple ..... 0.02 g

Agar .....*13.5 g

*10 -15 g according to gel strength Final pH: 6.7 ± 0.2 at 25°C

Urea Broth

Enzymatic Digest of Casein.....10g

Dextrose..... 1 g

Sodium Chloride ..... 5 g

Monopotassium Phosphate ..... 2 g

Urea ..... 20 g

Phenol Red ..... 0.012 g

Final pH: 6.8 ± 0.2 at 25°C

R Codes

```
#Aaron's analysis.
#Dec 2021
library(Hmisc)
```

```
## Loading required package: lattice
## Loading required package: survival
## Loading required package: Formula
## Loading required package: ggplot2
##
## Attaching package: 'Hmisc'
## The following objects are masked from 'package:base':
##
##     format.pval, round.POSIXt, trunc.POSIXt, units
```

```
rm(list=ls())
getwd()
```

```
## [1] "C:/Users/Dan Miles/Documents/WLU/Data/Aaron's Data"
```

```
#Bring in data

loc.r <- read.csv("location only resistance.csv")
head(loc.r)
```

```
##   temp.  rp anti location sample
## 1    37 high   AM     Root  58.30
## 2    37 high  AMC     Root  35.00
## 3    37 high  CHZ     Root   8.33
## 4    37 high    C     Root  50.00
## 5    37 high  CIP     Root  25.00
## 6    37 high    S     Root  75.00
```

```
attach(loc.r)

spec.r <- read.csv("Species only resistance.csv")
head(spec.r)
```

```
##   temp.  rp anti.   species sample
## 1    37 high   AM Salmonella     60
## 2    37 high  AMC Salmonella      0
## 3    37 high  CHZ Salmonella      0
## 4    37 high    C Salmonella      0
## 5    37 high  CIP Salmonella     25
## 6    37 high    S Salmonella     75
```

```
attach(spec.r)
```

```
## The following objects are masked from loc.r:
```

```
##
##     anti, rp, sample, temp
```

```
loc(spec.r <- read.csv("species and location resistance.csv")
head(spec.r)
```

```
##   temp   rp anti    species sample
## 1   37 high   AM Salmonella     BD
## 2   37 high  AMC Salmonella      C
## 3   37 high  CEC Salmonella      C
## 4   37 high    C Salmonella      C
## 5   37 high  CIP Salmonella     26
## 6   37 high    D Salmonella     75
```

```
attach(spec.r)
```

```
## The following objects are masked from spec.r (pos = 3):
##
##     anti, rp, sample, species, temp

## The following objects are masked from loc.r:
##
##     anti, rp, sample, temp
```

```
bisf.r <- read.csv("bacilli resistance.csv")
attach(bisf.r)
```

```
## The following objects are masked from spec.r (pos = 3):
##
##     sample, species, temp

## The following objects are masked from spec.r (pos = 4):
##
##     sample, species, temp

## The following objects are masked from loc.r:
##
##     sample, temp
```

```
head(bisf.r)
```

```
##   temp site.loc sample loc species resistance   sample
## 1   11        2      8   W      BD          1 0.107878
## 2   11        3      W   W      BD          1 0.868378
## 3   11        4      W   W      BD          1 0.078000
## 4   11        5      2   BD     BD          3 3.072878
## 5   11        8      2   NB     BD          1 0.079125
## 6   11        7      8   BD     BD          3 1.320250
```

```
#detach(bisf.r)

str(loc.r)
```

```
## 'data.frame':    304 obs. of  5 variables:
##  $ temp    : int  37 37 37 37 37 37 37 37 37 37 ...
##  $ rp      : Factor w/ 3 levels "high","low","medium": 1 1 1 1 1 1 1 1 1 1 ...
##  $ anti    : Factor w/ 12 levels "AM","AMC","C",..: 1 2 4 3 5 6 7 10 8 ...
##  $ location: Factor w/ 5 levels "Bank","east",..: 1 1 1 1 1 1 1 1 1 1 ...
```

```
##  $ sample  : num  68.3 25 8.33 50 25 ...
lr <- lm(sample ~ factor(temp) + rp + location + anti, data = loc_r)
summary(lr)

##
## Call:
## lm(formula = sample ~ factor(temp) + rp + location + anti, data = loc_r)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -60.369 -19.989   2.389  18.873  81.756
##
## Coefficients:
##                 Estimate Std. Error t value Pr(>|t|)
## (Intercept)       17.202      6.189   2.779 0.005794 **
## factor(temp)20    38.501      3.573  11.060  < 2e-16 ***
## factor(temp)37    42.155      3.573  11.797  < 2e-16 ***
## rplow            -25.318      3.573  -7.086 9.23e-12 ***
## rpmedium         -10.111      3.573  -2.830 0.004879 **
## locationmarsh     -4.130      3.573  -1.156 0.248304
## locationwater     -8.226      3.573  -2.302 0.022003 *
## antiAMC          -11.740      7.140  -1.644 0.101287
## antiC             -3.667      7.140  -0.373 0.710345
## antiCFX           -9.894      7.140  -1.386 0.167224
## antiCIP          -17.211      7.140  -2.456 0.016614 *
## antiD             33.059      7.140   3.934 0.001479 **
## antiP             24.125      7.140   3.378 0.000831 ***
## antiL             26.301      7.140   3.683 0.000278 ***
## antiS            -10.713      7.140  -1.499 0.134877
## antiSOL            9.880      7.140   1.383 0.167811
## antiT             24.667      7.140   3.454 0.000655 ***
## antiV             23.757      7.140   4.104 4.07e-05 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 29.28 on 306 degrees of freedom
## Multiple R-squared:  0.5372, Adjusted R-squared:  0.5051
## F-statistic: 20.39 on 17 and 306 DF,  p-value: < 2.2e-16
#species only

str(spec_r)

## 'data.frame':    324 obs. of  5 variables:
##  $ temp    : int  37 37 37 37 37 37 37 37 37 37 ...
##  $ rp      : Factor w/ 3 levels "high","low","medium": 1 1 1 1 1 1 1 1 1 1 ...
##  $ anti    : Factor w/ 12 levels "AM","AMC","C",..: 1 2 4 3 5 6 8 7 10 9 ...
##  $ species: Factor w/ 3 levels "ecoli","Enterococcus ",..: 3 3 3 3 3 3 3 3 3 3 ...
##  $ sample  : num  80 0 0 0 20 75 100 80 60 0 ...
sr <- lm(sample ~ factor(temp) + rp + species + anti, data = spec_r)
summary(sr)

##
## Call:
```

```
## lm(formula = sample ~ factor(temp) + rp + species + anti, data = spec.r)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -60.364 -21.410   1.347  19.475  98.529
##
## Coefficients:
##                      Estimate Std. Error t value Pr(>|t|)
## (Intercept)            14.672      6.687   2.194 0.031252 *
## factor(temp)28         61.665      3.861  15.740  < 2e-16 ***
## factor(temp)37         36.042      3.861   7.260 3.18e-12 ***
## rplow                 -17.424      3.861  -4.513 9.15e-06 ***
## rpmedium              -7.788       3.861  -2.017 0.044611 *
## speciesEnterococcus    0.387       3.861   0.100 0.920320
## speciesSalmonella     -8.941       3.861  -2.316 0.002714 *
## antiAMC               -6.888       7.722  -0.892 0.373079
## antiC                 -16.868      7.722  -2.184 0.029887 *
## antiCFZ               -13.015      7.722  -1.686 0.111779
## antiCIP               -15.078      7.722  -1.953 0.051777 .
## antiD                  22.387      7.722   2.899 0.004012 **
## antiG                  23.638      7.722   3.061 0.002403 **
## antiL                  24.104      7.722   3.122 0.001930 **
## antiN                  -8.491      7.722  -1.100 0.272389
## antiOXL                11.548      7.722   1.496 0.135648
## antiT                  22.105      7.722   2.863 0.004491 **
## anti7                  29.396      7.722   3.806 0.000292 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 28.37 on 306 degrees of freedom
## Multiple R-squared:  0.4515, Adjusted R-squared:  0.4316
## F-statistic: 18.63 on 17 and 306 DF,  p-value: < 2.2e-16
```

```
#Species and location

str(loc.spec.r)
```

```
## 'data.frame':    972 obs. of  7 variables:
##  $ temp    : int  37 37 37 37 37 37 37 37 37 37 ...
##  $ water   : Factor w/ 3 levels "high","low","medium": 1 1 1 1 1 1 1 1 1 1 ...
##  $ rp      : Factor w/ 3 levels "high","low","medium": 1 1 1 1 1 1 1 1 1 1 ...
##  $ anti    : Factor w/ 22 levels "AM","AMC","C",..: 1 2 4 5 6 8 7 10 9 ...
##  $ species : Factor w/ 3 levels "ecoli","Enterococcus",..: 3 3 3 3 3 3 3 3 3 3 ...
##  $ location: Factor w/ 3 levels "root","soil",..: 1 1 1 1 1 1 1 1 1 1 ...
##  $ sample  : num  66.6 33.3 0 33.3 33.3 66.6 100 100 100 0 ...
```

```
loc <- lm(sample ~ factor(temp) + water + location + anti + rp + species, data = loc.spec.r)
summary(loc)
```

```
##
## Call:
## lm(formula = sample ~ factor(temp) + water + location + anti +
##     rp + species, data = loc.spec.r)
##
## Residuals:
```

```
##      Min       1Q  Median       3Q      Max
## -76.832 -24.482   4.568  25.288  89.552
##
## Coefficients: (2 not defined because of singularities)
##                     Estimate Std. Error t value Pr(>|t|)
## (Intercept)           45.848     30.820   1.488  0.13744
## factor(temp)11       -32.062     31.159  -1.029  0.30419
## factor(temp)28        10.443     31.115   0.336  0.73720
## factor(temp)RT         9.928     31.054   0.319  0.74962
## waterlow             -17.812      3.340  -5.272 1.96e-07 ***
## watermedium           -8.728      3.190  -2.730  0.00668 **
## locationsoil           4.691      3.359   1.397  0.16306
## locationwater         -1.283      3.372  -0.362  0.71798
## antiAMC              -12.883      6.400  -1.982  0.04796 *
## antiC                 -7.642      6.400  -1.194  0.23332
## antiCFZ              -17.669      6.400  -2.767  0.00604 **
## antiCIP              -19.993      6.400  -3.113  0.00196 **
## antiD                 27.087      6.400   4.231 2.75e-05 ***
## antiE                 29.781      6.400   4.651 4.17e-06 ***
## antiL                 33.328      6.400   5.208 1.71e-07 ***
## antiS                -14.610      6.400  -2.282  0.02289 *
## antiSXL               15.124      6.400   2.362  0.01852 *
## antiT                 31.198      6.400   4.873 1.48e-06 ***
## antiV                 37.214      6.400   5.812 1.06e-08 ***
## rplow                     NA         NA      NA       NA
## rpmedium                  NA         NA      NA       NA
## speciesEnterococcus   -5.732      5.448  -1.052  0.27903
## speciesSalmonella    -12.549      4.181  -3.006  0.00268 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 30.53 on 531 degrees of freedom
##   (420 observations deleted due to missingness)
## Multiple R-squared:  0.4906, Adjusted R-squared:  0.4716
## F-statistic: 25.88 on 20 and 531 DF,  p-value: < 2.2e-16
```

```
#hist(hf)

hf <- lm(sample ~ factor(temp) + factor(resistance) + species + sample.loc, data = biof_r)
summary(hf)
```

```
##
## Call:
## lm(formula = sample ~ factor(temp) + factor(resistance) + species +
##     sample.loc, data = biof_r)
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -1.10275 -0.22641 -0.06362  0.14811  1.31871
##
## Coefficients:
##                  Estimate Std. Error t value Pr(>|t|)
## (Intercept)       0.62686    0.08292   8.006 1.52e-09 ***
## factor(temp)28   -0.53658    0.10684  -5.558 1.45e-08 ***
## factor(temp)RT   -0.81013    0.10838  -7.478 4.08e-12 ***
```

```
## factor(resistance)2  0.74387    0.15092   4.771 3.96e-06 ***
## factor(resistance)3  1.19575    0.11872   9.988  < 2e-16 ***
## species2             0.03888    0.15049   0.258 0.796511
## species3            -0.62610    0.09700  -6.187 4.84e-09 ***
## species4            -0.28299    0.14610  -1.923 0.070065 .
## species23           -0.96536    0.09930  -9.690 0.000000 ***
## species4M           -0.19122    0.09526  -2.000 0.041968 *
## sample.locS          0.06700    0.09584   0.704 0.482700
## sample.locW         -0.18642    0.08151  -2.287 0.023483 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.6283 on 168 degrees of freedom
## Multiple R-squared:  0.4943, Adjusted R-squared:  0.4813
## F-statistic: 14.03 on 11 and 168 DF,  p-value: < 2.2e-16
```

```
#Chisquare tests

#location tests
chiSquare(sample ~ factor(temp) + rp + location + anti, data = loc.r)
```

```
## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

##
## Pearson Chi-square Tests    Response variable:sample
##
##              chisquare  df chisquare-df      P   n
## factor(temp)    223.95  96       127.83 0.0000 324
## rp              264.60  96       168.60 0.0000 324
## location        238.99  96       143.99 0.0000 324
## anti            334.99 528         6.99 1.0000 324
```

```
#species test
chiSquare(sample ~ factor(temp) + rp + species + anti, data = spec.r)
```

```
## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

##
## Pearson Chi-square Tests    Response variable:sample
##
##              chisquare  df chisquare-df      P   n
## factor(temp)    163.52  78        74.52 0.0000 324
## rp              229.54  78       140.54 0.0000 324
## species         289.14  78       211.14 0.0000 324
## anti            468.00 429        36.00 0.1114 324
```

```
#species and location test
chiSquare(sample ~ factor(temp) + water + location + anti + rp + species, data = loc.spec.r)

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

##
## Pearson Chi-square Tests    Response variable:sample
##
##              chisquare  Df chisquare-Df     P  n
## factor(temp)    263.71 123       136.71 0.000 602
## water           187.81  62       106.81 0.000 602
## location        187.94  62       106.94 0.000 602
## anti            540.46 451        88.46 0.031 602
## rp              187.94  62       106.94 0.000 602
## species         215.01  62       139.01 0.000 602
#Profiling
chiSquare(sample ~ factor(temp) + factor(resistance) + species + sample.loc, data = bief.r)

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

## Warning in chisq.test(x, y): Chi-squared approximation may be incorrect

##
## Pearson Chi-square Tests    Response variable:sample
##
##                    chisquare  Df chisquare-Df      P   n
## factor(temp)          900.00 903         0.00 0.3728 100
## factor(resistance)    900.00 902         0.00 0.3728 100
## species               900.00 880        20.00 0.3124 100
## sample.loc            896.98 902         4.00 0.4175 100
```