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Investigating the persistence and “naturalization” potential of *Salmonella* in non-host environments using culture-based and molecular-based fingerprinting techniques

by

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Honours B.Sc. Biology, Wilfrid Laurier University, 2011

THESIS

Submitted to the Department of Biology

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Master of Science in Integrative Biology

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Abstract

Recent studies suggest the potential exists for enteric microorganisms to become “naturalized” to the complex natural environment through maintenance of their populations *via* replication and adaptation to the stress imposed by their external surroundings. The aim of this research was to better understand the potential for antimicrobial resistant (AMR) *Salmonella* to become “naturalized” and the physiological adaptations that allow for non-host survivability as reflected by AMR and community profiling. To better elucidate the prominent factors leading to a “naturalized” condition, two approaches were taken: 1) survey-based field research to gain perspective on the prevalence of AMR *Salmonella* isolated from variously impacted water sources; a local watershed, a constructed wetland for the treatment of human waste, and a wastewater treatment facility, and 2) bench-scale batch microcosm systems to monitor *Salmonella* survival in environmentally-relevant conditions including three temperatures 4°C, 12°C and 22°C. An integrative approach to phenotypic (culture-based) and genotypic (molecular-based) analysis was taken to profile test microorganisms at multiple levels. Specifically, quantitative PCR (qPCR) was used to enumerate pathogen removals from the environment and AMR analysis was conducted for resistance profiling against 12 antibiotics. The functional and structural fingerprints of the communities harbouring *Salmonella* were investigated by community-level physiological profiling (CLPP) and denaturing gradient gel electrophoresis (DGGE), respectively. The relative abundance of *Salmonella* was comparable to concentrations of *E. coli* within all impacted water sources (on average ranging from 10^3 to 10^5 gene copies/ 100 mL) and did not appear to be influenced by seasonality. The natural hardiness of environmental *Salmonella* was exemplified by high percent resistance occurrence levels (average between 20% and 40%) observed in these same waters. Microcosm study results indicated that *Salmonella* persistence was not dependent on temperature when interspecies diversity was a factor and that changes in *Salmonella* AMR levels in the absence of antimicrobial exposure were likely stress-induced. From both field study and microcosm results, functional and structural fingerprinting of microbial communities revealed that AMR *Salmonella* was harboured in both genetically and metabolically diverse communities, including communities demonstrating high metabolic versatility but low genetic diversity. Therefore, AMR levels of *Salmonella* derived from different community situations may be a consequence of both increased opportunities for the exchange of genetic material in healthy communities, and a stress response within low-integrity communities. Collectively, these findings suggest that the response of *Salmonella* to environmental stress not only dictates its ability to survive in non-host environments, but also impacts its phenotypic expression of AMR. This information is important with regard to the “naturalization” potential of *Salmonella* upon dissemination into the open environment following release from a host and the risk implications to public health.

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List of Abbreviations

AM	ampicillin
AMC	amoxicillin
AMR	antimicrobial resistance
APHA	American Public Health Association
AWCD	average well colour development
BHI	brain heart infusion
C	chloramphenicol
CAZ	ceftazidime
CFU	colony forming unit
CIP	ciprofloxacin
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
<i>C. perfringens</i>	<i>Clostridium perfringens</i>
CLPP	community-level physiological profiling
CLSI	Clinical and Laboratory Standards Institute
CRO	ceftriaxone
Ct	cycle threshold
CTX	cefotaxime
D	doxycycline
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
FIB	fecal indicator bacteria
G	sulfisoxazole
GC	guanine cytosine
HPC	heterotrophic plate count
LB	Luria-Bertani
LZD	linezolid
MAR	multiple antimicrobial resistance

MDR	multidrug resistance
N	number of isolates
NARMS	National Antimicrobial Resistance Monitoring System
OTU	operational taxonomic unit
(q)PCR	(quantitative) polymerase chain reaction
rDNA	ribosomal DNA
S	streptomycin
SXT	sulfamethoxazole-trimethoprim
TAE	tris-acetate-EDTA
TE	tetracycline
VA	vancomycin
VBNC	viable but non-culturable
WHO	World Health Organization

Chapter 1

Introduction

1.1 Overview

Zoonotic pathogens, disease-causing microorganisms transmissible between vertebrate animals and humans, account for 75% of infectious diseases described in humans (Bolin *et al.*, 2004). When zoonotic pathogens are shed from the intestinal tracts of warm-blooded hosts into waterways, their dissemination into the broader environment is readily enhanced. Upon release and the subsequent introduction of enteric bacteria to a secondary (non-host) environment, it was traditionally understood that persistence was limited to a brief time period primarily due to non-ideal conditions. Recently, new light has been shed on the ability of different enteric microorganisms, such as *Escherichia coli* and *Enterococcus* spp., to not only persist in non-host settings for a brief period of time after introduction, but to ultimately adapt to these non-host surroundings (Ishii and Sadowsky, 2008; Ferguson and Signoretto, 2011). However, less emphasis has been placed on the environmental survival of enteric pathogens such as *Salmonella*. In many related studies, the terms ‘persistence’ and ‘naturalization’ have been used interchangeably. For the purpose of this thesis, we will continue with Ferguson and Signoretto’s (2011) working definitions, where “persistence” refers to the ability of an organism to endure environmental conditions for a given length of time, while “naturalization” refers to the growth, replication and the adaptation of the organism to a secondary, non-host environment.

1.2 Background

1.2.1 Tolerance of Waste Associated Microorganisms to Environmental Stress

The open environment presents numerous abiotic and biotic challenges that oppose the survival of waste-associated microorganisms. Biotic pressures include predation and competition for resources, while low nutrient availability, osmotic pressure, exposure to UV and fluctuating pH and temperature are all examples of commonly encountered abiotic challenges. When microorganisms are shed into the open environment under relatively favourable conditions, the initial survivability of the organism may contribute to its physiological versatility later on (Ishii and Sadowsky, 2008).

Microorganisms have developed a number of survival strategies to combat unfavourable physiological responses to environmental pressures. Two of the most important survival mechanisms include biofilm development and the transition into a viable but non-culturable (VBNC) state (Winfield and Groisman, 2003; Ferguson and Signoreto, 2011; Halliday and Gast, 2011). The ability of bacteria to adhere to a variety of surfaces allows for the initiation of the complex biofilm process. Biofilms consist of microbial aggregates that function as a self-sufficient and protective community. Microorganisms isolated within the innermost regions of the biofilm benefit from the localized microenvironment and are less susceptible to the damaging effects of desiccation, UV and chemical exposure (Ferguson and Signoreto, 2011). Within biofilm communities, for example, bacteria have demonstrated up to 1000 times greater resistance to antibiotics than their suspended counterparts (Mah and O'Toole, 2001). In this way, biofilms harbour a significant potential for not only survival, but growth and maintenance of a population within the greater biofilm community. Moreover, microorganisms have demonstrated the ability to be induced into a VBNC state, whether within or external to a biofilm, while

experiencing stress. The VBNC state has been characterized by low metabolic activity but preservation of cellular integrity such that revival into a culturable state with the improvement of external conditions can occur. Fecal indicator bacteria (FIB) such as *E.coli* and *Enterococcus* spp. and enteric pathogens such as *Salmonella* are known biofilm producers and all have been described to enter a VBNC state (Oliver, 2005).

1.2.2 Aspects of Public Concern

It is currently understood that bacteria respond to antibiotics as another form of environmental stress. While environmental stressors may indirectly enhance antimicrobial tolerance, in turn antibiotic exposure has been shown to induce cross-protection against potentially damaging environmental conditions through regulating DNA repair systems (Hastings *et al.*, 2004; Poole, 2012). Consequences of imposed stresses, such as greater instances of recombination, increased rates of transient mutagenesis and genetic reorganization, all increase the heterogeneity of the affected population leading to overall greater survival (McMahon *et al.*, 2007). Accordingly, analysis of antibiotic resistance is a valuable means of measuring the diversity of microbial isolates by offering insight into their natural variability.

A major concern surrounding the persistence of enteric microorganisms in secondary environments is their potential to retain virulence factors. While some genes conferring pathogenicity may be readily lost due to the unnecessary energy costs associated with maintenance, other virulence factors may enhance the fitness of the microorganisms outside of the host (Winfield and Groisman, 2003; van Elsas *et al.*, 2011). For instance, *E. coli* strains able to cause disease share a collection of genes that augment their pathogenicity, including those that encode for colonization, biofilm and invasion factors, as well as toxin production. Such traits

may contribute to the environmental hardiness of *E. coli*, such as the colonizing factors that allow for attachment to plants or inanimate objects aiding in the beginning stages of biofilm development (van Elsas *et al.*, 2011). It is not easy to predict whether pathogens of enteric origin are hazardous to public health in the open environment, as the expression of virulence factors may depend on a number of surrounding conditions. More studies differentiating between isolates from fecal and non-fecal sources are necessary to determine what differentiates a pathogenic entity from an innocuous microorganism.

1.2.3 “Naturalization” of Fecal Indicator Bacteria

Agricultural livestock waste has been identified as a primary source of bacterial contamination in soil and water (Jamieson *et al.*, 2002). Drinking water, ground water and recreational waters are deemed unsanitary if the detection of fecal indicator bacteria exceeds water quality standards. As natural residents of the intestinal tracts of warm-blooded animals, the presence of indicator organisms, such as *E. coli* and *Enterococcus* spp., in water signifies the risk of fecal contamination and the potential presence of gastrointestinal pathogens such as *Salmonella* (Halliday and Gast, 2011). Disease-causing organisms are not directly enumerated as they are difficult to isolate and quantify due to their uneven dispersion and tendency to be present in relatively low numbers. While not commonly considered pathogens themselves, indicator bacteria have the following additional characteristics according to Health Canada (2010): 1) can be quantified accurately and efficiently; 2) presence connected exclusively with the occurrence of pathogens found in the fecal waste of humans and animals; 3) present in higher concentrations than the pathogens; 4) able to persist in the environment for a longer duration than the pathogen without being able to replicate and survive in the environment.

Recent studies are yielding increasing evidence that some microorganisms previously considered reliable fecal indicators are surviving in non-enteric habitats despite environmental stresses, such as fluctuating temperatures, unfavourable water/soil chemistry, exposure to solar radiation, limited moisture and organic matter availability, competition for resources and predation (Ishii and Sadowsky, 2008; Figure 1.1). “Naturalized” strains of *E. coli* have been isolated from soil, water and algae from both tropical and temperate environments. For example, Byappanahalli *et al.* (2006) provided evidence that *E. coli* was capable of long-term survival in the soils of a temperate forest in close proximity to a Great Lakes watershed. Topp *et al.* (2003) found that *E. coli* strains were often viable months after being left in nutrient-rich and moist environments of stockpiled manure. Lastly, Avery *et al.* (2008) identified the long-term prevalence of *E. coli* in temperate lakes and rivers of the United Kingdom.

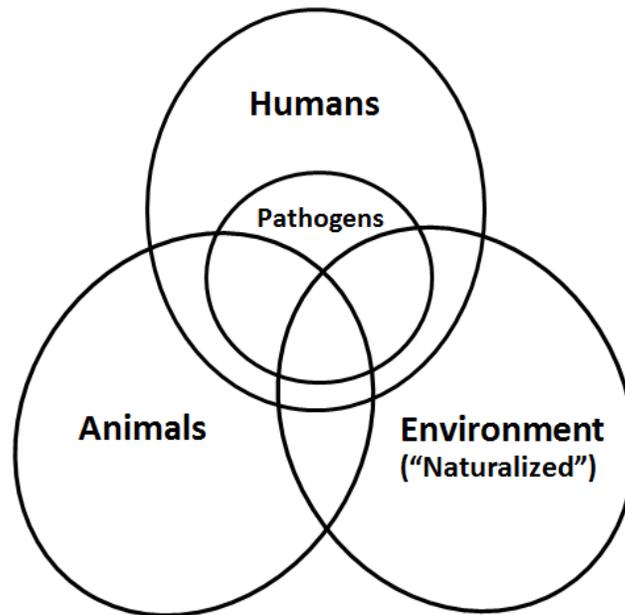


Figure 1.1. A schematic representation of the distribution of *E. coli* among humans, animals and environmental reservoirs. While certain *E. coli* strains show a high degree of host-specificity, other strains are able to survive in more than one host environment (modified from Ishii and Sadowsky (2008)).

Due to the relevance of *E. coli* as a fecal indicator and its overall use as a model microorganism, *E. coli* has been the focus of the majority of survival studies to date. The survivability of *E. coli* in a broad range of niches can be attributed to a number of factors that all contribute to the physiological robustness and adaptability of the microorganism. For instance, *E. coli* is a facultative anaerobe and by definition is able to survive in environments with varying amounts of oxygen. While the optimal growth temperature of *E. coli* is 37°C, this microorganism is capable of growth at a broader temperature range of 7.5°C to 49°C. The flexible means of energy acquisition also allows *E. coli* to adjust to fluctuating nutrient concentrations available in the environment. The nutritional requirements of *E. coli* consist of simple carbon and nitrogen sources, in addition to minor amounts of sulfur, phosphorous and other trace elements (Ishii and Sadowsky, 2008). At the genetic level, *E. coli* strains are largely distinguishable based on three types of surface antigens; somatic (O) antigens, capsular (K) antigens, and flagellar (H) antigens. Over 150 O antigens have been described for *E. coli*, as well as numerous K and H antigens, where a particular strain may display one of any number of possible antigen combinations. Thus, *E. coli* is an incredibly diverse species (Ryan *et al.*, 2010).

For this reason, our traditional understanding that *E. coli* is strictly an enteric microorganism of warm-blooded hosts, communicable to susceptible animal hosts through a fecal-oral route, and not able to survive in the environment long-term, needs to be re-evaluated (Halliday and Gast, 2011; Luo *et al.*, 2011). There is a great deal of evidence to support that following the release of enteric bacteria from their primary hosts through defecation, most of these bacteria expire due to harsh environmental conditions, both abiotic and biotic. However, feces-associated microorganisms may attach to sediment or vegetative surfaces and persist in the environment long enough to adapt to the posed selective pressures of the environment and

overcome challenges related to growth and replication. These microorganisms are considered “naturalized” and are distinct in that they can survive the fluctuating conditions of a complex, open environment and ultimately maintain their populations through the transfer of survival-promoting traits to future generations. The survival of *E. coli* and *Enterococcus* spp. in the environment may lead to the re-inoculation of animal hosts who come into contact with contaminated sources of food or water (Ishii and Sadowsky, 2008; Figure 1.2). The rising evidence for the potential survivability of fecal indicator bacteria in non-host habitats is revealing that it is becoming necessary to redefine the characteristics of reliable fecal pollution indicators.

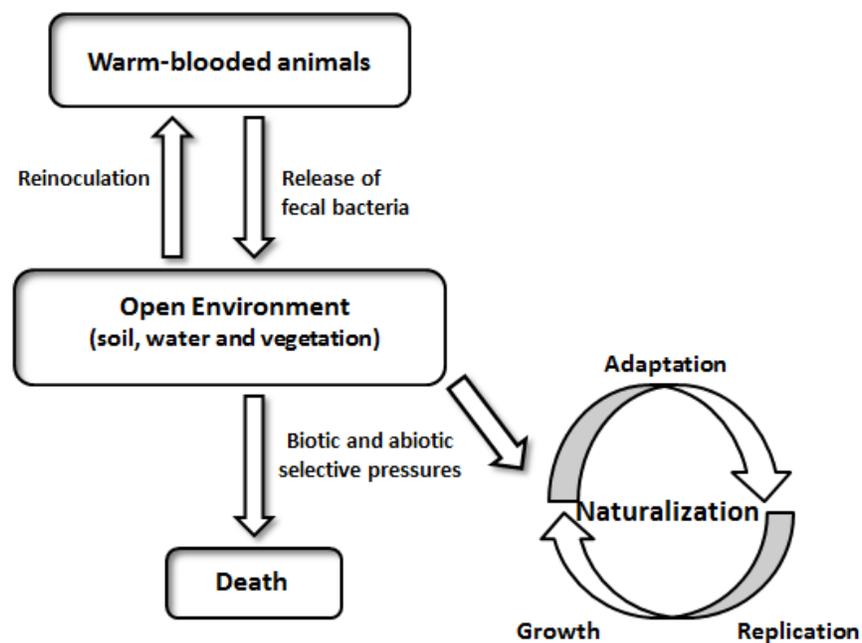


Figure 1.2. A depiction of the possible fates of fecal indicator bacteria, such as *E. coli* and *Enterococcus* spp., upon their introduction into the environment (image adapted from Ishii and Sadowsky, 2008).

It has been suggested that the genome of fecal indicator bacteria (FIB) such as *E. coli* can be largely shaped by selective pressures for enhanced adaptability to the environmental

conditions of non-host habitats. For instance, Topp *et al.* in their 2003 study noted that *E. coli* isolated from the gastrointestinal tract were genetically distinct from *E. coli* isolated from the environment. Such genetic variability may allow environmental strains to withstand starvation, desiccation, freezing and microbial competition.

1.2.4 Environmental Persistence of *Salmonella*

While findings on *E. coli* persistence in a secondary environment should not be generalized, *Salmonella* shares several physiological traits which similarly play an essential role in its ability to occupy a wide range of niches exterior to a host (e.g. the ability to grow within a broad temperature range, to utilize simple organic substrates and adjust its metabolism to accommodate aerobic or anaerobic conditions as necessary). In fact, a number of studies have shown that non-typhoidal *Salmonella* more readily survives in the broader terrestrial and aquatic environments than does *E. coli* (e.g. Himathongkham *et al.*, 1999 and Semenov *et al.*, 2007). The introduction of *Salmonella* into the environment occurs by the same means as *E. coli*; through infected animal hosts including humans, livestock, wildlife and pets (Baudart *et al.*, 2000). When released into waterways, *Salmonella* has demonstrated an enhanced ability to thrive in aquatic environments compared to *E. coli* and even more notably has been shown to outlive notorious waterborne pathogens such as *Staphylococcus aureus* and *Vibrio cholera* in ground water and streams. Furthermore, *Salmonella* is generally more resistant to biotic pressures than *E. coli* such as predation and competition for resources (Winfield and Groisman, 2003). Accordingly, *Salmonella* is considered to be one of the most highly tolerant pathogens of enteric origin to non-host surroundings (Winfield and Groisman, 2003), which in turn improves the possibility of inoculation into the next host. Therefore, it may be necessary to place attention on how the

enteric pathogens themselves, not just those microorganisms used for pathogenic detection, harbour the potential to adapt, grow and replicate in a non-host environment.

Due to its significance as one of the most commonly reported foodborne pathogens in developed countries, salmonellosis is primarily considered an infection contracted through the consumption of contaminated food (WHO, 2005; Hur *et al.*, 2010). Consequently, *Salmonella* has been excluded from the majority of water quality monitoring systems, as few drinking water outbreaks have been attributed specifically to this pathogen. However, some studies such as that conducted by Denno *et al.* (2009) have proposed that waterborne exposure may become just as important as foodborne exposure in contracting salmonellosis. While its presence is not currently regulated by the United States Environmental Protection Agency (USEPA), non-typhoidal *Salmonella* is included on the 2012 Contaminant Candidate List 3 for safe drinking water (USEPA, 2012).

Given that only a very small fraction of cases are clinically recognized, it is estimated that *Salmonella* serovars cause 1.2 million illnesses each year in the United States alone. Among the foodborne pathogens, *Salmonella* infections are the number one cause of hospitalizations and death according to the Foodborne Disease Active Surveillance Network (FoodNet) (Walnder *et al.*, 2012). Infection often leads to self-limited gastroenteritis where use of antimicrobial therapy can be avoided. However, highly invasive infections may prove life threatening in the young, elderly and immunocompromised individuals. With these more severe cases, hospitalization and antimicrobial administration are necessary (Hur *et al.*, 2010). First choice antimicrobial agents include fluoroquinolones for adult treatment and third generation cephalosporins for treatment in children (Quinn *et al.*, 2006).

1.2.5 The Role of Microbial Communities in Potential *Salmonella* “Naturalization”

It is widely known that the majority of bacteria in the natural environment exist in complex communities consisting of hundreds to thousands of distinct bacterial species, each occupying a unique niche within the microenvironment (Mah and O’Toole, 2001; Fierer and Lennon, 2011). Accordingly, the importance of community impact on the “naturalization” potential of enteric microorganisms should not be disregarded. Minimal research has investigated the influence of microbial community health on antimicrobial resistance within its constituent pathogenic populations. However, one such study conducted by Perron *et al.* (2012) demonstrated that within functionally diverse communities, incidences of bacterial recombination through the uptake of foreign genetic materials and horizontal gene transfer rapidly propagated the evolution of antimicrobial resistances. Furthermore, their findings indicated that resistance to single or multiple drugs could be developed in as little as a few generations through the exchange of resistance genes among diverse bacterial strains (Perron *et al.*, 2012). In this way, horizontal gene transfer within a community can confer fitness advantages to its populations more efficiently than through evolution by natural selection, resulting in enhanced environmental persistence and adaptation (Smets and Barkay, 2005; Perron *et al.*, 2012).

1.3 Research Need and Objectives

Upon evacuation from a host, *Salmonella* are most often deposited into aquatic environments such as municipal sewage systems or runoff from agricultural fields (Spector and Kenyon, 2012). While known to persist in a variety of waste effluents rich in organic content, little is understood about the ability of *Salmonella* to survive in nutrient-diluted open environments while migrating away from the contamination source. As such, more research

needs to be conducted with regard to the persistence and potential “naturalization” of *Salmonella* downstream of urban and agricultural runoff, in order to gain insight into the ability of *Salmonella* to manage pressures posed by the complex environment upon dissemination. This is of importance, as it is becoming increasingly evident that resistance levels reported in clinical cases are related to those found in environmental bacteria (Foley *et al.*, 2006; Tatavarthy *et al.*, 2006). Consequently, the environmental presence of drug resistant pathogens like *Salmonella* poses a serious public health risk. Given the evidence provided in this chapter, it was hypothesized that the pathogen *Salmonella* is able to become adapted to the environment to the point of being “naturalized”.

The overarching objectives of this research were to: 1) characterize the persistence profile of *Salmonella* upon migration into the broader environment, following release from a host, discharge from a wastewater treatment plant or runoff from agricultural practices; and 2) better understand the physiological potential that allows for non-host survivability and ultimately “naturalization” in *Salmonella* within the environmental microbial community. This information is important in relation to the ongoing development of regulatory guidelines for managing the risk of contamination of ground water, surface water and soil.

To address the overarching research objectives, the following supporting objectives were investigated:

- 1) Characterize the occurrence and spatial variability of antimicrobial resistant (AMR) *Salmonella* in association with its corresponding community in waters impacted by varying degrees of urban and agricultural runoff using complementary phenotypic and genotypic analysis.

- 2) Determine the antibiotic resistance profiles of environmental *Salmonella* spp. and the fecal indicator bacteria *E. coli* and enterococci against a panel of 12 clinically-relevant antibiotics using phenotypic techniques.
- 3) Enumerate selected pathogen removals present in different water sources using quantitative-polymerase chain reaction (qPCR). Specifically, to compare the presence of target fecal indicator bacteria (e.g. *E. coli* and *Enterococcus* spp.) to *Salmonella*, as well as non-target waterborne pathogens such as *Clostridium* spp. in impacted waters including an ecologically important watershed, a constructed wetland system and a pilot-scale wastewater treatment plant.
- 4) Investigate the impact of microbial community integrity on the occurrence of antimicrobial resistance within its constituent *Salmonella* populations, as determined through functional and structural profiles using community-level physiological profiling (CLPP) and denaturing gradient gel electrophoresis (DGGE), respectively.
- 5) Assess the effect of three environmentally-relevant temperatures (4°C, 12°C and 22°C) on the survivability of *Salmonella* from variable sources with respect to its associated community profile using bench-scale microcosms designed to simulate the natural environment.

1.4 References

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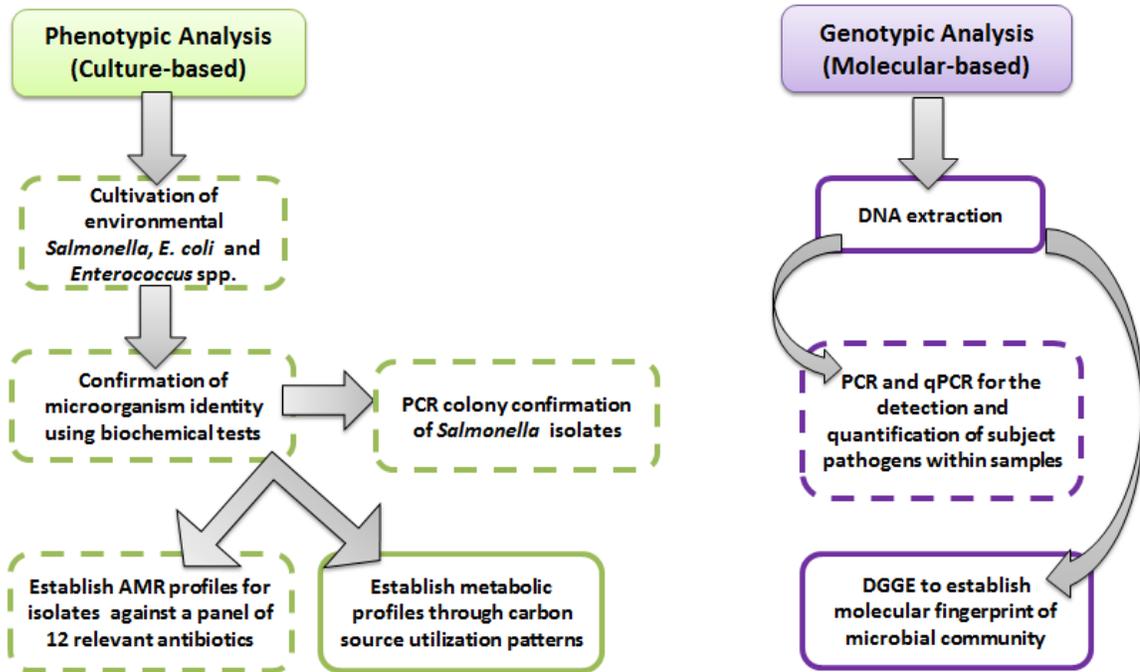
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Chapter 2

Experimental Design and Methodology

2.1 Overview of Experimental Approach

The integrative nature of this work is demonstrated in part by the interdisciplinary approach taken in the experimental methodology. The techniques used incorporate phenotypic (culture-based) and genotypic (molecular-based) analysis, for the profiling of test microorganisms at the level of the microbial genome, individual organism and community levels from which the sample was derived (Figure 2.1). This multi-technique approach was necessary in order to better elucidate the prominent factors which impose stress responses in *Salmonella* ultimately leading to a “naturalized” condition. In turn, the integrative approach allowed for the generation of a broader perspective concerning the significance of environmental adaptation of enteric microorganisms with pathogenic potential.



Legend

- AMR- antimicrobial resistance
- (q)PCR- (quantitative) polymerase chain reaction
- DGGE- denaturing gradient gel electrophoresis
- - - - - phenotypic analysis of individual isolates
- - - - - genotypic analysis of individual isolates
- phenotypic analysis of microbial communities
- genotypic analysis of microbial communities

Figure 2.1. A flow-chart depicting the experimental approach for the initial phase of the project.

2.2 Environmental Recovery of *Salmonella* and Fecal Indicator Bacteria

2.2.1 *Salmonella* Isolation

To recover sub-lethally damaged *Salmonella* from the collected environmental samples, a series of non-selective and selective enrichment media were required to compensate for the typical uneven distribution and low concentration of *Salmonella* in the environment (Ryan *et al.*, 2010). The differentiating ingredients of the media promoted the growth of *Salmonella* while

reducing the levels of competing background flora. Consequently, *Salmonella* cannot be accurately quantified using culture-based methods (BDTM, 2011).

Upon arrival in the lab, samples were immediately processed to isolate *Salmonella* following a procedure similar to that described by Rybolt *et al.* (2004). All media used in this study was provided by BD DifcoTM from Mississauga, ON, Canada unless otherwise stated. First, 90 mL of sample was inoculated into 10 mL of 10x buffered peptone water (BPW) to create a 1:10 dilution. The use of BPW broth allows for the non-selective recovery of sublethally damaged *Salmonella* through its buffering capacity and nutrient-rich content (BDTMa, 2011). Following this inoculation, samples were incubated in the lab-line incubator-shaker (Fisher Scientific, Whitby, ON, Canada) at 37°C for 24hrs at 140 rpm.

This recovery period was followed by the use of the selective enrichment medium tetrathionate broth (TB) with 2% (v/v) iodine solution for the inhibition of competing coliforms. The growth of *Salmonella* is promoted by its ability to reduce tetrathionate, while the presence of oxgall within the medium further inhibits the growth of coliforms (BDTMa, 2003). From the sample-BPW solution, 1 mL was inoculated into 9 mL of TB containing 2% (v/v) iodine. As a negative control, 1 mL of *E. coli* (ATCC® 12292, Inverness Medical Inc., Ottawa, ON, Canada) grown in nutrient broth (NB) overnight was also added to 9 mL of TB. In the same way, *Salmonella enterica* ser. *typhimurium* (ATCC® 13311, Inverness Medial Inc., Ottawa, ON, Canada) was used as a positive control. These controls were carried through the remainder of the isolation process in the same manner as the samples. The isolation step was performed in duplicate, and samples were incubated in the lab-line incubator-shaker (Fisher Scientific, Whitby, ON, Canada) at 37°C for 24 hrs at 170 rpm.

Next, 100 μ L of the TB-sample mix was inoculated onto the center of modified semisolid Rappaport-Vassiliadis (MSRV) medium for the detection of motile *Salmonella*, thereby distinguishing this organism from the closely related non-motile *Shigella* (BDTMb, 2011). Plates were incubated at 44°C for 24 hrs. Motile samples produced a halo of growth extending out from the original point of inoculation. This step was done in triplicate.

The third selective medium used in the isolation of *Salmonella* was MacConkey (MAC) agar. This medium is useful in differentiating lactose fermenting and non-fermenting organisms, such as *Salmonella* (BDTMc, 2011). A pre-sterilized tooth pick was used to take up a small portion of culture from the outer edge of the halo of growth on the MSRV to perform the first quadrant of streaking on MAC. A flame-sterilized loop was then used to complete streaking of the plate. This step was performed in duplicate and all plates were then incubated at $35 \pm 0.5^\circ\text{C}$ for 24 hrs. Clear, colourless colonies on MAC, indicating non-lactose fermenters, were then individually transferred to Luria-Bertani agar (LB; Bioshop Canada Inc., Burlington, ON) through streak plating and stored at 4°C until biochemical testing was performed.

2.2.1.1 Biochemical Testing

As a preliminary screening of the *Salmonella* isolates, three biochemical tests were performed in parallel. Environmental isolates yielding characteristic results for *Salmonella* were considered presumptive *Salmonella*. Two of these three tests involved inoculating a single colony from LB into triple sugar iron (TSI) agar and lysine iron (LIA) agar slants using an inoculating needle. The stab and streak method and double stab method were used for TSI and LIA, respectively. A positive result for *Salmonella* in TSI resulted in the production of hydrogen sulfide (H_2S) precipitate, hydrogen gas, and dextrose fermentation (BDTMb, 2003). Alternatively, H_2S production and the presence of lysine decarboxylase were indicative of a

positive *Salmonella* outcome for LIA (BD™, 2011). The third biochemical test involved inoculating a colony from LB into 2 to 3 mL of filter-sterilized urea broth. Because *Salmonella* does not contain the urease enzyme, a negative urea broth test result (an absence of a colour change) is positive for the identification of *Salmonella*. All inoculated test media underwent incubation for 24 to 48 hours at 37°C. A positive *Salmonella* control was used for each of the tests, and in the case of the urea broth test, a urease positive control was used (*Klebsiella pneumonia* ATCC® 700603, Inverness Medical Inc., Ottawa, ON, Canada).

2.2.1.2 PCR Colony Confirmation

The identity of the presumptive *Salmonella* isolates was confirmed using colony polymerase chain reaction (PCR). The genus-specific primers used in this reaction were the Sal-F (5' CGTTTCCTGCGGTACTGTAA 3') and Sal-R (5' AGACGGCTGGTACTGATCGAT 3') (Sigma-Aldrich) primer set described by Lee *et al.* (2006) for the detection of the *invA* gene; a highly conserved gene in nearly all serotypes of *Salmonella* (Garcia *et al.*, 2010). The PCR mastermix was prepared based on the protocol outlined by Löfström *et al.* (2004), where each 25 µL reaction consisted of 1x Go-Taq™ Flexi (Promega) Green PCR Buffer, 1.5 µM MgCl₂, 0.5 µM of each primer, 1.5 U Go-Taq™ Flexi (Promega), 200 µM dNTP (Promega) and 11.1 µL of Milli-Q water. PCR amplification was performed using a BioRad™ I-cycler iQ PCR machine (Bio-Rad Laboratories; Mississauga, ON, Canada). The reaction conditions involved an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 20 sec and 65°C for 1 min, with an extension step of 72°C for 7 min. At the conclusion of the reaction, the PCR products were held at 4°C until being stored at -20°C. *S. enterica* ser. *typhimurium* (ATCC® 13311, Inverness Medical Inc., Ottawa, ON, Canada) and *E. coli* (ATCC® 12292) were used as the positive and negative controls, respectively.

PCR products (10 μ L) were then loaded into a 1.8% (w/v) agarose gel in 1x tris-acetate-EDTA (TAE) buffer and run at 100 V for 60 min. The gels were stained in ethidium bromide for 40 min and imaged using a BioRad™ GelDoc™ XR (Bio-Rad Laboratories; Mississauga, ON, Canada) with amber filter for the detection of an 82 bp band confirming the presence of *Salmonella* (Figure 2.2).

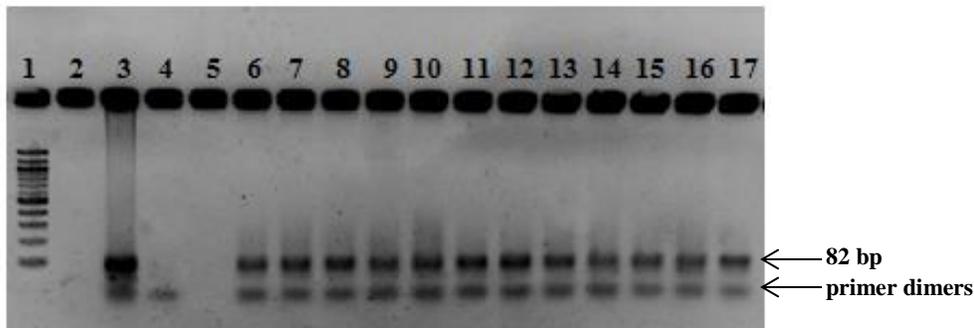


Figure 2.2. An example of an agarose gel containing products of *S. enterica* PCR colony confirmation. Lane 1 contains the 100 bp ladder, lanes 2 and 5 are blank, lane 3 contains *S. enterica* ATCC® 13311 (positive control), lane 4 contains *E. coli* ATCC® 12292 (negative control), and lanes 6 to 17 are PCR confirmed *Salmonella* isolates derived from Clair Lake in Waterloo, ON as established by the detection of an 82 bp band within each lane.

2.2.2 *E. coli* and *Enterococcus* spp. Isolation

E. coli and *Enterococcus* spp. were isolated from all water samples using the standard membrane filtration methods outlined by the American Public Health Association (APHA, 1998). Dilutions of 10^{-1} and 10^{-2} , as well as 1 mL and 10 mL of the samples were filtered through a 0.45- μ m, 47mm mixed cellulose ester filters (Difco, Fisher Scientific; Ottawa, ON, Canada) and then placed onto the suitable selective agar plates; mFC-BCIG agar and mEnterococcus agar for the quantitative recovery of *E. coli* and *Enterococcus* spp., respectively (Difco, Fisher Scientific). In addition, 100 μ L of *E. coli* (ATCC® 12292) and *Enterococcus faecalis* (ATCC® 49532) were also filtered as positive and negative controls regarding colony

presentation on the media. mFC-BCIG agar was made by supplementing mFC basal agar with 100 µg/L of 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexyl ammonium salt (Medox Diagnostics; Ottawa, ON, Canada) for the chromogenic differentiation of *E. coli* from other fecal coliforms in the sample (due to the cleaving of the BCIG substrate by the β-glucuronidase enzymatic activity of *E. coli*) (Kon *et al.*, 2007). mFC-BCIG agar plates were incubated at 44±0.5°C for 24 hrs in a hot water bath and mEnterococcus agar plates were incubated at 35±2°C for 48 hrs. Colonies obtained from the mEnterococcus agar plates were confirmed by transferring the filter to a pre-warmed bile esculin agar (Difco, Fisher Scientific) and incubating at 44°C for 2hrs. Dark brown colonies on BEA were considered presumptive *Enterococcus* spp. and blue colonies on mFC-BCIG agar plates were considered presumptive *E. coli*. Samples at each dilution were processed in duplicate and only plates containing between 20-200 colony counts were considered for enumerative analysis (Schraft and Watterworth, 2005). Individual colonies of *Enterococcus* spp. and *E. coli* were then plated on brain heart infusion (BHI) and LB agar respectively for storage at 4°C.

2.3 Characterization of Environmental Isolates and Microbial Communities from Impacted Water Sources

2.3.1 Culture-Dependent Methods

2.3.1.1 Antimicrobial Resistance Analysis

Confirmed *Salmonella* isolates and presumptive *E. coli* and *Enterococcus* isolates were tested for antimicrobial susceptibilities against a panel of twelve antimicrobials shown in Table A1. The use of these antibiotics represents a variety of antibiotic classes, including those currently used for the treatment of salmonellosis in children and adults. Resistance patterns were determined using the Kirby-Bauer disk-diffusion method as described by the Clinical and

Laboratory Standards Institute (CLSI, 2007). Isolates were inoculated into 5 mL of BHI broth and grown to 0.5McFarland standard turbidity, whereafter they were swabbed onto Mueller-Hinton (MH) agar. Antibiotics were then introduced with the use of a BD Sensi-Disc™ Designer Dispenser System. Following an incubation period of 16-18 hrs at $35 \pm 0.5^\circ\text{C}$, the resulting zones of inhibition were used to categorize the isolates as susceptible, intermediately resistant or resistant according to the CLSI interpretive chart (CLSI, 2007). Intermediate sensitivities were considered as resistant for the purposes of analysis. Figure 2.3 represents simplified results for classifying an organism as resistant or susceptible to particular antibiotics.

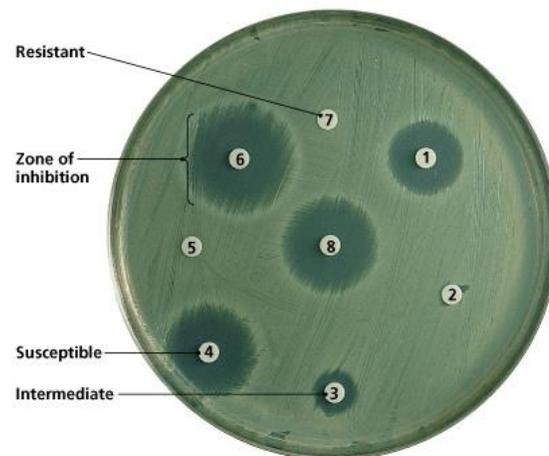


Figure 2.3. Differentiating between the phenotypic expression of antimicrobial resistance and susceptibility of an organism. Numbered discs represent different drugs. A zone of inhibition is indicative of the effectiveness of an antibiotic against the bacteria (discs 1, 3, 4, 6 and 8) while bacterial growth up to the antibiotic disc indicates resistance (discs 2, 5 and 7). Image adapted from Cassandra Helt (2012).

2.3.1.2 Community-Level Physiological Profiling

The ability of microorganisms to alter their means of energy acquisition when faced with low nutrient levels has been described as one of the most important physiological dynamics in the successful survival of microorganisms in the open environment (van Elsas *et al.*, 2011). Biolog MicroPlate™ technology is an important culture-based tool for monitoring the temporal

and spatial changes of microbial communities through the use of the EcoPlate™ derived from their metabolic capabilities. This method of community-level physiological profiling (CLPP) was originally established by Garland and Mills (1991) and has been continually developed to provide in-depth analysis based on carbon source utilization patterns (CSUP). The specific protocol used in this project follows the procedure outlined by Weber and Legge (2010).

The EcoPlate™ consists of 31 different sources of carbon important for community analysis and a blank all repeated in triplicate (Figure A1). Within each of the wells, a tetrazolium dye is present which becomes reduced upon cellular respiration. As a result, the dye undergoes a purple colour transformation signifying microbial growth (Garland and Mills, 1991).

Prior to inoculation of the specialized plates, environmental samples were spectrophotometrically analyzed using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, CA) at a wavelength of 420 nm to assess background carbon levels (Weber and Legge, 2010). If the optical density (OD) reading of the sample at 420 nm was greater than 0.2, then the sample was diluted one order of magnitude until the OD measurements were equal or less than 0.2. Using a multichannel pipette, 150 µL of sample was then inoculated into each of the wells while being careful to avoid cross-contamination of the carbon sources. Microbial growth of the inoculated sample was then measured every 24 hours over a period of 120 hours set to a wavelength of 590 nm, and plates stored in the dark at room temperature (approximately 22°C) between readings. If the microbial community was able to utilize a specific carbon source, then the tetrazolium dye present in that well was reduced to form a purple colouration that best absorbs light at around 590 nm (Garland and Mills, 1991) (Figure 2.4).

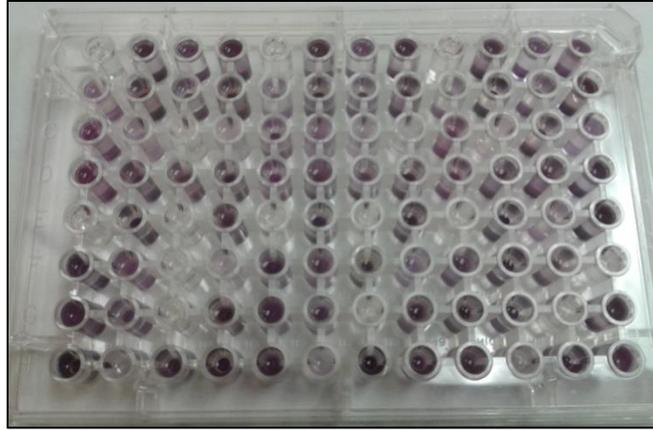


Figure 2.4. An example of an EcoPlate™ inoculated with an environmental water sample after 96 hours of incubation, where the development of a purple colouration is indicative of the utilization of the particular carbon substrate present in that well. Triplicate blanks are within wells 1, 5 and 9 of the top row.

CLPP is considered a relatively low-cost, reproducible and uncomplicated technique to execute in comparison to many other culture-based characterization methods. Aside from possible dilutions to lower background carbon levels, no laborious sample processing (e.g. isolation, enrichment, amplification, etc.) is required prior to plate inoculation, and the data generated offers detailed insight into microbial community shifts with regard to metabolic capacities (Calbrix *et al.*, 2005). The major limitation of CLPP, however, is the inconclusive nature of the methodology. For instance, it cannot be determined whether the utilization of a carbon substrate is due to a single species or microbial cooperation within a particular well. Alternatively, a lack of colour response reflecting metabolic activity within a well may be due to the inability of the present microbes to use the available carbon substrate or a result of competition (Garland *et al.*, 1997). However, Garland *et al.* (1997) has suggested that this limitation can also be viewed as a discerning advantage, as this method of characterization is somewhat unique in taking into account microbial interactions. A further disadvantage is the amount of time necessary for the development of functional profiles, which may range from 2 to

7 days. In this time, constraints may be placed on the communities within the well environments resulting in compositional alterations such as overgrowth and death of some organisms. Despite these shortcomings, CLPP is regarded as an insightful tool in characterizing microbial communities from a variety of sources through the establishment of functional fingerprints.

Statistical Analysis

Reaction profiles generated by the differential CSUPs of the inoculated sample were then translated into several functional diversity indices as first proposed by Zak *et al.* (1994). A time point of 96 hrs following incubation was chosen for analysis, following the rationale provided by Weber *et al.* (2008). Calculated statistical parameters included metabolic diversity, metabolic richness and average well colour development (AWCD) as a means to measure functional shifts in the community over space and time to generate a community fingerprint. Information obtained from these indices is significant in demonstrating the physiological versatility and stability of the communities upon extraction from the broader environment.

Shannon Diversity Index (H')

While traditionally the Shannon Diversity Index (H'; Shannon, 1948) was intended for computing species diversity and not substrate utilization diversity, it has since become one of the most commonly recounted indices in literature pertaining to CSUP data. It can be calculated as follows:

$$H'_{CLPP} = -\sum p_i \ln(p_i)$$

Where:

- H'_{CLPP} is substrate diversity
- p_i is the ratio of activity of a particular substrate to the sum of activity of all substrates

The maximum Shannon (H') value that can be generated for a BIOLOG EcoPlate™ is 3.434 which is the natural logarithm for the total number of substrates available; 31. This value occurs when all carbon sources within each of the wells are metabolized equally. Values between 1 and 2 are considered low diversity, and values equal to or greater than 3 are considered high diversity (Weber and Legge, 2010).

Substrate Richness

Substrate or metabolic richness (R) is a parameter closely correlated with substrate diversity. In this case, richness is defined as the number of carbon sources oxidized by a microbial community, where an OD value of 0.25 at an absorbance of 590nm is used as a threshold for a positive response (Weber and Legge, 2010).

$$R = \sum (\text{carbon sources} > 0.25 \text{ at } 590\text{nm})$$

Average Well Colour Development

Average well colour development is considered an overall estimate of the metabolic rate of the inoculated microbial community (Garland *et al.*, 1997) and was calculated by dividing the sum of the corrected absorbance readings by the total number of carbon sources available, as shown below:

$$AWCD = \sum (C - \text{Ref}) / 31 \text{ Carbon Sources}$$

Where:

- “C” is the average colour response for each of the wells containing a carbon substrate
- “Ref” is the average colour response of the blank or reference wells

2.3.2 Molecular-Based Techniques

2.3.2.1 Quantitative Polymerase Chain Reaction

It has long been known that sublethally damaged microorganisms have been under-represented when performing culture-based colony enumerations. A number of studies have shown that an absence of microbial growth on solid media is not necessarily indicative of a lack of genetic or metabolic activity, or overall cellular integrity (McMahon *et al.*, 2007).

Real-time or quantitative PCR (qPCR) is a highly sensitive molecular technique used for the detection and quantification of specific target DNA. As such, qPCR is an important diagnostic method with a variety of uses including pathogen detection, genotyping and measurement of gene expression (Smith and Osborn, 2009). This molecular technique was employed in the project to accurately enumerate the copy numbers of genes per reaction pertaining to subject pathogens within collected environmental samples. Specifically, qPCR was used to quantify the following enteric microorganisms from the total DNA extracted from environmental water samples: *Salmonella* sp., *E. coli*, *Enterococcus faecalis*, and *Clostridium perfringens*. This was particularly important for the detection of pathogens from the environment able to persist in a viable but non-culturable (VBNC) state which could not otherwise be quantified using culture-dependent methods. A major limitation of qPCR, however, is that it cannot distinguish between living and dead cells such that quantitative results may be an overestimation of gene copies within viable cells (Taskin *et al.*, 2011).

The reaction is prepared similarly to standard PCR, with the addition of a fluorescent dye-labeled probe that detects only the template DNA containing the probe sequence. With each amplification cycle, the amplicon concentration increases and the intensity of the fluorescence proportionally increases with the amount of amplicon produced. The fluorescence data is

captured and analyzed during the exponential growth phase by the qPCR instrument to determine relative gene expression within the sample. This is done by referencing the amplified DNA to a standard curve dilution (10^0 - 10^{-7}) in order to determine the number of gene copies within each reaction. The results are represented by a 'fluorescence (corrected: baseline subtracted) versus cycle number' plot and a fluorescence threshold level set above the background but remaining within the linear phase of the amplification cycle. The cycle threshold (C_T) of each reaction, representative of the corrected starting concentrations of template DNA within the samples, occurs when emitted fluorescence exceeds threshold values (Figure 2.5).

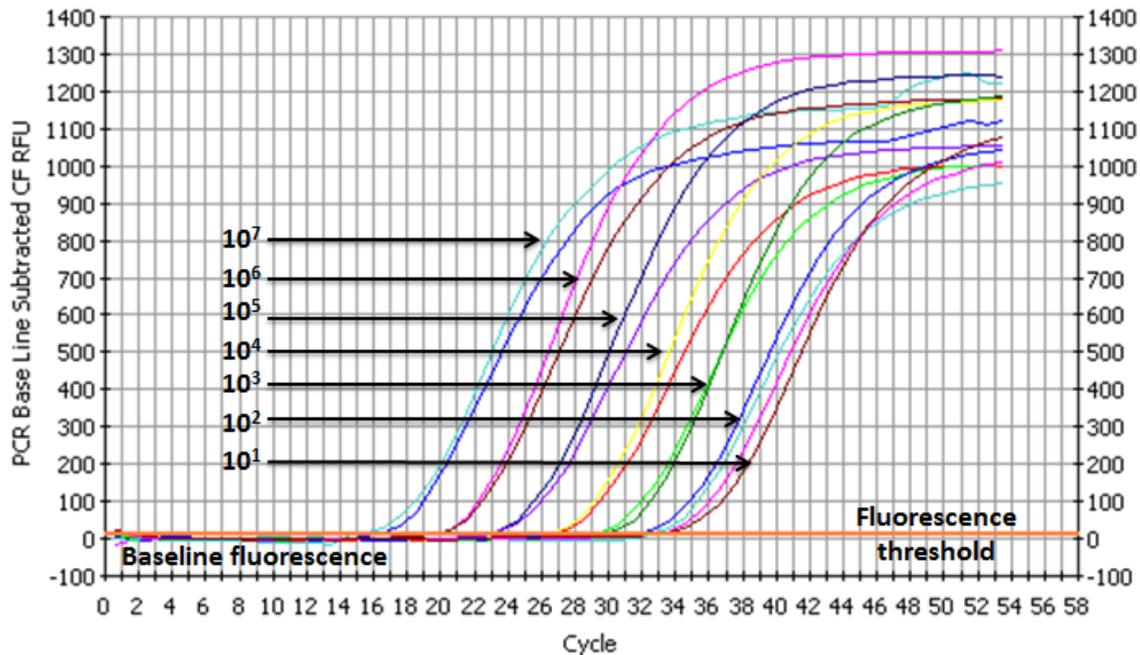


Figure 2.5. An example of real-time PCR results depicting the relationship between gene copy numbers/reaction and cycle threshold (C_T). Shown in this example is *E. coli* genomic DNA which has been serially diluted over 7 orders of magnitude and subjected to TaqMan real-time PCR.

Environmental DNA Extraction and Running Conditions

Within 3 hrs of collection, 250 mL of each of the original water samples were filtered using a 22 mm, 0.22 µm polycarbonate filter (Millipore™, Fisher Scientific, Whitby, ON, Canada). Each filter was then placed into bead tubes provided in the PowerSoil™ DNA isolation kit (Mo Bio Laboratories Inc., CA, USA) whereafter DNA extractions were carried out according to the protocol of the manufacturer.

qPCR was performed with the use of an iCycler and iQ Real-Time PCR system (BioRad Laboratories, Mississauga, ON, Canada), where individual 25 µL reactions consisted of genomic DNA, primers and a TaqMan® probe added to DyNAmo™ Probe qPCR Mastermix (New England Biolabs, Pickering, ON, Canada) for a final concentration of 250 nM TaqMan® probe and 1 µM for each primer. Included in Table 2.1 are the qPCR primers and probes used in this project. A volume of 5 µL of extracted sample DNA was used as template for each reaction. The PCR program used for the quantification of the subject pathogens included a three-step temperature profile with slight modifications from the protocol described by Shannon *et al.* (2007): (i) A uracil-DNA glycosylase step at 50°C for 2 min, (ii) a step at 95°C for 10 min to initiate DNA denaturation and activate *Taq* polymerase and (iii) 55 cycles of 95°C for 15 sec and 60°C for 1 min (Shannon *et al.*, 2007; Bockelmann *et al.*, 2009). A tenfold dilution series of respective standards for each gene of known concentration were run alongside sample DNA for the relative quantification of the genes within the unknown sample (described in section below). For each qPCR run, unknown samples were run in triplicate and a negative control in which template DNA was replaced with nuclease-free water was also included.

Table 2.1. Primers and probes used within this study for qPCR analysis.

Microorganism	Primers and Probes	5'→3' Sequence	Target Gene	Function	Reference
<i>Clostridium perfringens</i>	Clper-F Clper-R Clper-PR	GCATGAGTCATAGTTGGGATGATT CCTGCTGTTCCCTTTTTGAGAGTTAG TGCAGCAAAGGTA ACTT	<i>plc</i>	Alpha toxin	Shannon <i>et al.</i> , 2007
<i>Escherichia coli</i>	Eco-F Eco-R Eco-PR	GTCCAAAGCGGCGATTTG CAGGCCAGAAGTTCTTTTTCCA ACGGCAGAGAAGGTA	<i>uidA</i>	Glucuronidase	Shannon <i>et al.</i> , 2007
<i>Enterococcus faecalis</i>	Efaecal-F Efaecal-R Efaecal-PR	TGTGGCAACAGGGATCAAGA TTCAGCGATTTGACGGATTG TCGTTTCGTGCATTAGA	<i>groES</i>	Heat-shock protein	Shannon <i>et al.</i> , 2007
<i>Salmonella</i> spp.	Sal-F Sal-R Sal-PR	CGTTTCCTGCGGTACTGTTAATT AGACGGCTGGTACTGATCGATAA CCACGCTCTTTCGTCT	<i>invA</i>	Invasion protein	Shannon <i>et al.</i> , 2007

- F- forward, R- reverse, PR- probe

DNA Extraction from Control Organisms and the Creation of a Standard Curve

In order to extract DNA from control strains for the purpose of creating a standard curve, the microorganisms were first individually grown in 5-10 mL of BHI broth at $35 \pm 2^\circ\text{C}$ for 16-18 hrs. Following incubation, the broth tubes underwent centrifugation at 13,000 xg for 10 mins using a 5702 Centrifuge (Eppendorf, Mississauga, ON, Canada). The supernatant was decanted, and the pellet was resuspended in the buffer provided in the bead tubes of the PowerSoil™ DNA isolation kit (Mo Bio Laboratories Inc., CA, USA). The buffer containing the bacterial contents was then pipetted back into the bead tube, whereafter the DNA extractions were carried out according to the protocol of the manufacturer. The resulting DNA was then spectrophotometrically analysed using the Biodrop Duo (Montreal Biotech Inc., Montreal, QC, Canada) for quantity (at 260 nm) and purity (A_{260}/A_{280}). All DNA was then stored at -20°C for downstream applications.

Knowing the concentration of extracted DNA, the following equation was then used to calculate the number of gene copies present within the 10^0 sample:

$$\text{Copy number/ reaction} = \frac{6.02 \times 10^{23} \times [\text{DNA}] \text{ g}/\mu\text{L} \times 5 \mu\text{L}}{(\text{size of genome in bp})(660 \text{ g/mol})(\text{number of copies in genome})}$$

Where:

- 6.02×10^{23} is Avogadro's number
- [DNA] is the concentration of DNA obtained from pure isolate
- 5 μL is the amount of template DNA added in each qPCR reaction
- 660 g/mol is the mol weight of a single base pair of double stranded DNA

(Ritalahti *et al.*, 2006)

A tenfold dilution series (10^0 - 10^{-7}) for each of the control organisms was then created using nuclease-free water. In this way, it was possible to plot the standard curves based on the amplification profiles of the known concentrations for the accurate enumeration of the respective gene copies per reaction within the unknown samples.

2.3.2.2 Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting technique based on the separation of same-sized PCR products due to differences in their nucleic acid sequences and resultant denaturing profiles. For instance, sequences of DNA from diverse microbial populations within a community yield double-stranded PCR products that vary in their readiness to become denatured. As the PCR product runs through the polyacrylamide gel at a constantly applied temperature (60°C) and voltage (70V), the product moves through the gel towards the positive electrode through electrophoresis while being exposed to an increasing chemical denaturant concentration of urea/formamide. When the PCR product encounters its threshold denaturant concentration, the DNA unwinds which initially slows and ultimately

arrests its movement through the gel. DNA migration is considered to be largely an outcome of the guanine and cytosine (G+C) content of the DNA segment, where G+C rich segments travel further down the gel than G+C poor segments (Muyzer and Smalla, 1998). The use of a denaturant-resistant GC clamp attached to the 5' end of the forward primer for PCR amplification prevents the now single-stranded DNA fragments from dissociating during electrophoresis, thereby securing the position of the DNA within the gel (Sanz and Köchling, 2007). The gel is then stained in SYBR gold and imaged with a transilluminator to yield a gel of band patterns where a single band is hypothetically representative of the dominant bacterial populations within the sample community. However, due to the potential co-migration of distinct sequences, heteroduplex formations or the presence of single stranded DNA, bands are more accurately referred to as operational taxonomic units (OTUs) rather than distinct species (Fromin *et al.*, 2002), although both terms are found in the literature. Furthermore, the structural diversity of the microbial community based on DGGE-generated banding patterns may result in an overestimation of community diversity, as multiple bands can be derived from a single, pure organism as a result of the heterogeneity of 16S rDNA gene sequences (Nübel *et al.*, 2006; Rettedal *et al.*, 2010).

This method has been described as an important tool for monitoring the dynamic changes of microbial communities from a variety of environments and serves as a reflection of the genetic biodiversity of a given habitat (Sanz and Köchling, 2007). In this way, the molecular procedure of DGGE and culture-based procedure of CLPP are complementary, generating a structural fingerprint and functional fingerprint respectively for a more comprehensive community snapshot. Among the other advantages of DGGE, are the relative low costs and ability to process a large number of samples concurrently compared to other profiling methods

such as 16S rRNA clone libraries and restriction enzyme-based fingerprints (Sanz and Köchling, 2007; Malik *et al.*, 2008). However, a number of limitations are also associated with performing DGGE. Due to the necessary use of PCR to amplify the relatively low concentration of DNA within the environmental sample, PCR bias does exist (Muyzer *et al.*, 1993). Consequently, this may alter community richness and diversity with regards to analysis, since rare populations may be excluded despite their potential to be active contributors to the overall health and functionality of the community. Furthermore, due to the nature of working with extracted genetic materials, it is not possible to distinguish between viable and inactive members (Fromin *et al.*, 2002). Nevertheless, DGGE is considered the choice method for obtaining precise data corresponding to dominant species members within the microbial community (Sanz and Köchling, 2007; Malik *et al.*, 2008).

PCR for DGGE

The 50 μ L PCR reaction was performed using the primers 357f (5'-CCTACGGGAGGCAGCAG-3') with a GC-clamp added to the 5' end (5'-CGCCCGCCGCGCCCCGCG-CCCGTCCCGCCGCCCCCGCCCG-3') and 518r (5'-ATTACCGCGGCTGCTGG-3') (Sigma Aldrich; Oakville, ON, Canada). This universal primer set which targets the variable V3 region of bacterial 16S rDNA was originally designed by Ogino *et al.* (2001). The mastermix for this PCR reaction was prepared using 1x Go-TaqTM Flexi (Promega) Green PCR Buffer, 1.5 μ M MgCl₂, 0.5 μ M of each primer, 200 μ M dNTP, 1.5 U Go-TaqTM Flexi (Promega) and 21.3 μ L of Milli-Q (Millipore) water. The template used for this PCR reaction was 5 μ L of extracted sample DNA (see environmental DNA extraction protocol under qPCR section). A BioRadTM I-cycler iQ PCR machine (BioRad Laboratories, Mississauga, ON, Canada) was used to meet the touchdown PCR conditions as previously described by

Muyzer *et al.* (1993). The PCR conditions involved an initial denaturation step of 94°C for 5 min, followed by 20 cycles of 94°C, 65°C and 72°C for 1 min each. The annealing temperature of 65°C was decreased every 2 cycles by 1°C to a temperature of 56°C on the 20th cycle. This was followed by 10 additional cycles of 94°C, 55°C and 72°C for 1 min each. The final extension step consisted of 7 min at 72°C whereafter the final PCR product was held at 4°C until storage at -20°C.

To verify the success of the reaction, 10 µL of PCR product was loaded into a 1.5% (w/v) agarose gel in 1x TAE buffer and run at 100 V for 60 min. The gels were then stained using ethidium bromide for 20 min and imaged using a BioRad™ GelDoc™ XR (Bio-Rad Laboratories) with amber filter. A positive reaction involved the presence of a 233 bp band and an absence of a band in the blank which was run using 5 µL of Milli-Q water in place of the DNA template.

Running Conditions and Image Acquisition

DGGE was carried out following an adjusted procedure outlined by Green *et al.* (2009). 8% (w/v) acrylamide gels were used comprising a linear denaturant gradient ranging from 40 to 65%, where 100% denaturant is defined as a solution of 7M urea and 40% (v/v) formamide. Gels were run for 17 hrs at 70 V (1190 V·h) using a CBS Scientific™ DGGE-2401 machine (CBS Scientific Inc., Del Mar, CA) set to a temperature of 60°C. To each lane, 15 µL of sample PCR product was added allowing free lanes for the DGGE ladder which was loaded into the centre and outside lanes.

DGGE gels were then stained using a solution of 1X SYBR Gold (diluted from a 10,000x stock (Invitrogen, Burlington, ON, Canada) made in 1X TAE) for one to two hrs. After this time, gels were then carefully transported with the aid of wax paper to the BioRad™ GelDoc™

XR (Bio-Rad Laboratories, Mississauga, ON, Canada) where it was photographed using a BioRad™ SYBR Gold filter (Bio-Rad Laboratories, Mississauga, ON, Canada). Quantity One® software was used to capture the image which could then be exported as an 8-bit .tiff file, excluding overlays and saved at the scan resolution of 2879dpi at a size of 1360 x 1024.

DGGE Ladder Creation

The DGGE ladder was created using DNA isolated from the following twelve lab strain controls: *Bacillus subtilis* (ATCC® 11774), *Yersinia enterocolitica* (ATCC® 9610), *Enterococcus faecalis* (ATCC® 49532), *Pseudomonas aeruginosa* (Ward's Science Plus), *S. enterica* ser. *typhimurium* (ATCC® 13311), *E. coli* (ATCC® 12292), *Proteus mirabilis* (Ward's Science Plus), *Alcaligenes faecalis* (ATCC® 33950), *C. perfringens* (NCTC® 8237), *Arthrobacter aureus* (ATCC® 13344), *Bacillus thuringiensis* (Ward's Science Plus) and *Enterobacter cloacae* (ATCC® 35030). All ATCC® and NCTC® strains were purchased from Inverness Medial Inc. in Ottawa, ON, Canada, while the remaining strains were purchased from Ward's Science Plus in Niagara Falls, ON, Canada. The extraction of DNA from control microorganisms was consistent with the protocol outlined for qPCR. The selection of these bacterial species was based on their known prevalence in the environment, as well as those resulting in bands which migrated distinctly and consistently through the polyacrylamide gel.

The extracted and purified DNA was then individually amplified through PCR using the 357-GC and 518R primer set and running conditions previously described. A volume of 10 µL of each of the twelve PCR products was loaded into a 1.5% (w/v) agarose gel in 1x TAE buffer and run at 100 V for 60 min to confirm the success of the PCR reactions. The remaining 40 µL of each post-PCR reaction was then pooled and diluted with a matched volume of 480 µL of Tris-

HCl (pH 8; Sigma Aldrich; Oakville, ON, Canada) for a total DGGE ladder volume of 960 μ L. When performing DGGE, 7 μ L of the ladder was added to a central lane and both outside lanes.

Statistical Analysis

Genetic diversity and richness

Statistical indices used to interpret CLPP data can be similarly used for the analysis of DGGE banding pattern data sets. Whereas richness and diversity were previously described to interpret substrate utilization in CLPP analysis, these same parameters can be used to convey the genetic integrity and composition of a community through DGGE-generated banding patterns. With regards to DGGE, richness is translated into the number of bands, evenness corresponds to the variation in band intensities and diversity refers to both the richness and evenness of the bands all within a single lane. Again, the Shannon Index (H') (Shannon, 1948) is one of the most commonly documented indices in the literature for this analysis.

H' can be calculated as:

$$H'_{\text{DGGE}} = -\sum p_i \ln(p_i)$$

Where:

- H'_{DGGE} is genetic diversity
- p_i is the ratio of the intensity of a specific band to the sum of bands in a given lane

Averaged banding patterns

To further characterize the structural dynamics of the microbial communities, averaged banding patterns were generated based on the relative intensities of the bands and their respective migratory distances through the DGGE gel. Bands were categorized into 'band movement

groups' according to their final positioning within the gel, serving as an indication of the G+C content of the OTUs. Given that G+C composition dictates sequence stability due to the presence of a third hydrogen bond present between G-C base pairs that is absent between A-T base pairs, a higher denaturant concentration must be encountered before the melting of G+C rich DNA sequences (Madigan *et al.*, 2009). Accordingly, G+C rich OTUs would be assigned a higher band movement group signifying further movement in the gel than G+C poor OTUs. In this way, changes in the abundances of the observed OTUs can be visualized. The combination of OTUs with similar G+C composition in ribosomal DNA into the same band movement groups represents a source of error in DGGE analysis based on band migration and may mislead the interpretation of community structure, but does account for the fact that taxa with similar G+C content are phylogenetically similar (Wayne *et al.*, 1987).

2.4 Advantages and Limitations of Culture-based and Molecular-based Techniques

Culture-based methods are considered classic microbiological approaches to understanding the physiological profiles of cultivable organisms. When assessing microbial communities, however, a substantial bias exists in that standard methods have the capacity to target less than 1% of bacterial species in any given sample. Consequently, countless functional species are overlooked while many environmentally dormant species become active when placed under favourable laboratory conditions (Hugenholtz, 2002; Rastogi and Sani, 2011).

Much of the progress made in the field of microbial ecology over the past two decades can be accredited to advancements in molecular-based methods. These methods are considered significantly more inclusive when attempting to better elucidate the biodiversity within environmental samples, including the VBNC fraction untapped by standard methods (Rastogi and Sani, 2011). Additionally, genetic fingerprinting methods are often preferred as the

processing of multiple samples can be done simultaneously with relative ease. Molecular approaches to community analysis typically include PCR-based methods in which environmentally extracted DNA is used as the template for microbial characterization. In theory, isolated DNA should serve as a representation of the assortment of genes present in the environment, but in actuality biases occur due to factors such as the uneven dispersal of microbial organisms which may in turn distort the community structure. PCR generally involves the amplification of highly conserved genes (i.e. 16S rRNA) which are ubiquitous in the environment (Hugenholtz, 2002).

Both culture-dependent and independent methods have their benefits and limitations, as summarized in Table 2.2. It is through employing multi-technique strategies that more comprehensive observational insight can be obtained at the genomic, phenotypic and community levels.

Table 2.2. A summary of the advantages and limitations of culture-based and molecular-based techniques used within this study.

Experimental Approach	Advantages	Disadvantages
Culture-dependent Methods (Plate counts, CLPP, AMR analysis)	<ul style="list-style-type: none"> • Possible to gain information regarding the physiological or functional properties of detected organisms 	<ul style="list-style-type: none"> • Only culturable organisms are included • Fast growing organisms are favoured over fastidious organisms
Molecular Fingerprinting Techniques (qPCR, DGGE)	<ul style="list-style-type: none"> • Non-culturable organisms can also be detected • Numerous samples can be analyzed simultaneously • Circumvent the need for laborious isolation and cultivation steps 	<ul style="list-style-type: none"> • Bias exists due to PCR, differences in DNA extraction efficiency, etc. • Bands or peaks can represent several species in case of DGGE • Not possible to distinguish between DNA from living or dead cells resulting in false-positives (DGGE and qPCR)

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Chapter 3

Monitoring the Occurrence and Spatial Variability of Antimicrobial Resistant (AMR) *Salmonella* in Impacted Waters

3.1 Introduction

In developed countries, antimicrobial resistant (AMR) enteric pathogens originate from a variety of anthropogenic sources including hospital, municipal and agricultural effluents (Lu *et al.*, 2010), in addition to wildlife waste (Baquero *et al.*, 2008). However, the emergence of AMR bacteria is considered to be primarily a consequence of antibiotic overuse and improper use in food animals (Threlfall, 2002; Economou *et al.*, 2012). The mass administration of antibiotics in livestock as growth promoters and for disease control and prevention often involves low-potency doses over long durations, which consequently promotes the evolution of drug resistance in impacted microbial communities by giving resistant bacteria a survival advantage (Furuya *et al.*, 2006). Each year in the United States alone, an estimated 30 million pounds of antibiotics are used in the agricultural industry compared to an approximate 3 million pounds for human therapy (Mellon *et al.*, 2001). The increasing prevalence of AMR *Salmonella* in aquatic environments has concerning implications for public health, by challenging and limiting treatment strategies to combat bacterial infection (Furuya *et al.*, 2006; WHO, 2007). The contamination of waterways with AMR *Salmonella* is particularly problematic, as it leads to the

enhanced dissemination of the pathogen into the broader environment where it may become “naturalized”.

Accordingly, surveillance programs such as the National Antimicrobial Resistance Monitoring System (NARMS) in the United States and the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) in Canada have been put in place to closely monitor the emergence of drug resistance in enteric pathogens. Furthermore, Health Canada has categorized antimicrobials on a scale of I to IV based on their importance to human medicine as a means to prevent overlap in antibiotic classes administered to humans and animals which may drive cross-resistance in zoonotic pathogens (Health Canada, 2011). Examples of category I (very high importance) drugs include 3rd generation cephalosporins and chloramphenicol, currently used for the treatment of salmonellosis in adults and children, respectively (Health Canada, 2011). See Table A1 for further classifications of drug classes according to their importance as treatment options for humans.

On the cellular level, multiple drug resistance is a consequence of mutations (such as insertions, deletions, duplications and point mutations) or bacterial recombination by means of horizontal gene transfer (including transduction, transformation and conjugation) (Perron *et al.*, 2012). While typically considered a direct byproduct of drug exposure, the exchange of genetic material conferring resistance can occur in the presence or the absence of antibiotics and their selective pressures (Furuya *et al.*, 2006; Whitehead *et al.*, 2011). Bacterial determinants may be maintained in pathogen populations to facilitate colonization and ultimately survival in the broader environment. Due to the tendency of bacteria to exist in communities containing hundreds to thousands of bacterial taxa (Fierer and Lennon, 2011), microbial diversity and richness are likely to play a significant role in the rates of genetic recombination leading to the

evolution of antimicrobial resistance and the propagation of resistance genes in bacterial strains even without prior antibiotic exposure (Perron *et al.*, 2012).

3.2 Research Need and Objectives

In this study, *Salmonella* and the classical fecal indicator bacteria *E. coli* and *Enterococcus* spp. were isolated from four water sources: a local watershed, a retention area of the watershed, a constructed wetland designed for the treatment of human waste, and a wastewater treatment facility. The “naturalization” potential of *Salmonella* was investigated in part through antimicrobial resistance (AMR) analysis, providing a valuable measurement of the phenotypic diversity and natural hardiness of microbial isolates for which antibiotics may impose a form of environmental stress. Furthermore, little is known about the influence of microbial community integrity on the evolution of antimicrobial resistance within its constituent populations. Therefore, the overarching objective of this research was to characterize the occurrence and spatial variability of AMR *Salmonella* and its associated microbial community from water sources impacted by varying degrees of urban and agricultural runoff. In turn, this will enhance our understanding of the potential for *Salmonella* to adapt and ultimately become “naturalized” in a non-enteric environment along with the physiological potential that allows for non-host survivability.

The specific sub-objectives designed to support the overall objective of this chapter were to:

- Determine the prevalence of AMR levels and frequency of multiple antimicrobial resistance (MAR) among culturable *Salmonella*, *E. coli* and *Enterococcus* spp. derived from urban and rural impacted waters using the disk-diffusion assay.

- Characterize the occurrence and spatial variability of antimicrobial resistant (AMR) *Salmonella* between differing sampling dates (e.g. summer and fall) from the same site location.
- Quantitatively compare the presence of target fecal indicator bacteria (e.g. *E. coli* and *Enterococcus* spp.) to *Salmonella* as well as non-target waterborne pathogens such as *Clostridium perfringens* from different aquatic environments using qPCR.
- Characterize the temporal and spatial changes in the metabolic capabilities of microbial communities from which the pathogens were derived through community-level physiological profiling (CLPP).
- Establish a genetic fingerprint of the microbial communities within which the pathogens were derived or associated with through denaturing gradient gel electrophoresis (DGGE).

3.3 Materials and Methods

A detailed overview of the methodological approach taken in this study is provided in Chapter 2. Information included in this section is limited to aspects unique to this portion of the research.

3.3.1 Sample Collection

In order to better understand how effluent source influences the potential “naturalization” capabilities of *Salmonella*, it was important to isolate *Salmonella* from a variety of impacted sources. Sample sites were selected primarily according to effluent/runoff type and accessibility. All water samples collected were taken 5-10cm below the water surface. See Table

A3 in Appendix A for a summary of each of the sampling sources listed below, including location, site description, and type of samples collected

i) Grand River Watershed, Waterloo, ON

Coursing through various urban and rural landscapes of southwestern Ontario, the Grand River watershed is one of the most ecologically influential rivers of this region. Flowing a distance of over 300 km and covering an area of approximately 6,800 km² with its numerous tributaries, the Grand River spans from Dufferin County to Port Maitland on Lake Erie. As such, the Grand River is heavily impacted by natural, municipal, recreational and agricultural activities.

Because its waters are impacted by various sources of runoff, the Grand River watershed is an excellent model for examining the dissemination of potential waterborne pathogens. It is also convenient to study due to its easy access and nearness to the laboratory for efficient transport of samples. Furthermore, the hydrology, precipitation, temperature and wind speeds of the watershed are well documented according to date and readily available through the Grand River Conservation Authority (GRCA). See Figures A2 and A3 for specific sampling locations. Provided in Table A2 are measured environmental parameters for the second Grand River sampling event on July 3rd and 4th, 2012.

ii) Clair Lake, Waterloo, ON

Clair Lake is a reservoir of the Laurel Creek tributary which is part of the greater Grand River watershed. It was chosen as a sample source as it was undergoing the process of being dredged as part of its rehabilitation. As such, sampling from the lake material presented a unique opportunity to investigate the potential long-term survivability of *Salmonella* and classical fecal indicator bacteria exposed to prolonged environmental conditions. Samples were collected from

Clair Lake during dredging in winter, rehabilitation in the spring and following its recovery near the end of summer in 2013. See Figure A4 for the sample site location.

iii) Center for Alternative Wastewater Treatment (CAWT) Constructed Wetland; Fleming College, Lindsay, ON

Domestic wastewater effluent from the Frost Campus of Fleming College is treated using an on-site constructed wetland fashioned to optimize the natural biological, chemical and physical processes employed by natural wetlands for the efficient removal of pollutants. The on-site system consists of four test cells to simulate and integrate natural wetland progressions, and a final polishing pond able to treat approximately 3, 000 liters of human waste daily. The final discharge is either recycled back into the wetland system or released into the municipal sewer. A schematic representation of the system is depicted in Figure A5.

iv) Wastewater Treatment Center (WTC); Burlington, ON

The Wastewater Treatment Center (WTC) is a pilot plant receiving raw wastewater effluent from the Skyway Wastewater Treatment Plant which serves Burlington and its environs, including Joseph Brant Memorial Hospital. This specialized research facility is designed to enable the evaluation of varying treatment technologies and disposal alternatives for industrial and municipal wastewaters.

3.4 Results

In the present study, *Salmonella* and the traditional fecal indicator bacteria *E. coli* and *Enterococcus* spp. were extracted from four impacted water sources for comparative culture-based characterization. Table 3.1 provides a collective summary of the number of each bacteria species successfully isolated including the source and season from which it was cultivated.

Table 3.1. A summary of the total number of *Salmonella*, *E. coli* and *Enterococcus* spp. isolates derived from the environment according to sampling site and date.

Sampling Site	Sampling Date	Total Number of Environmental Isolates		
		<i>Salmonella</i>	<i>E. coli</i>	<i>Enterococcus</i> spp.
Grand River Waterloo, ON	April 27, 2012	51	70	70
	July 3 and 4, 2012	50	96	120
	TOTAL	101	166	190
Clair Lake Waterloo, ON (GR sub watershed)	November 19, 2012	74	21	42
	April 27, 2013	20	- ^a	-
	June 6, 2013	20	-	-
	TOTAL	114	21	42
CAWT Lindsay, ON	February 22, 2012	39	48	46
	June 26, 2012	26	28	54
	August 24, 2012	47	84	84
	TOTAL	112	160	184
WTC Burlington, ON	June 18, 2012	44	72	72
	TOTAL	44	72	72

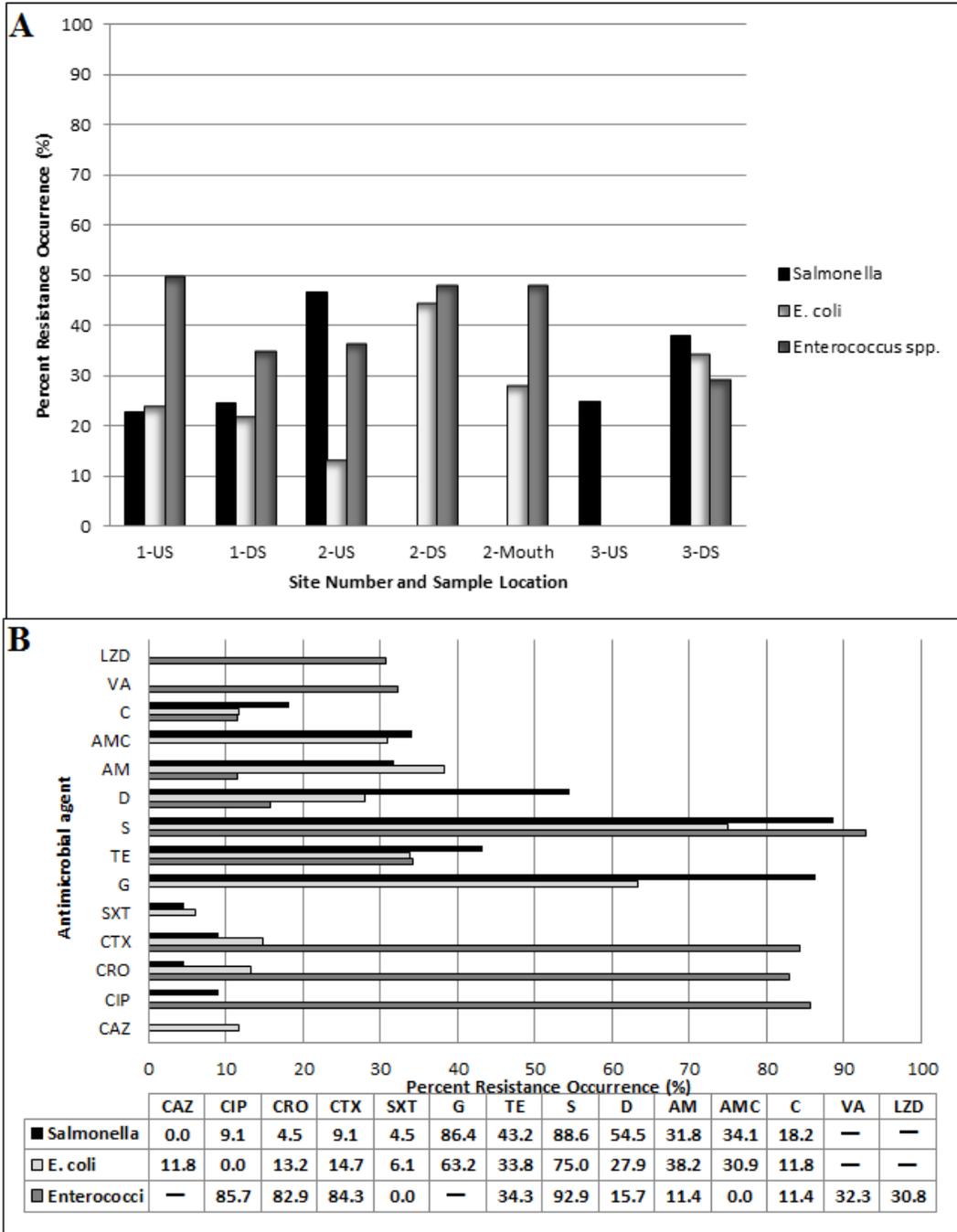
^a-Not analysed.

It is important to note that sample collection from the Grand River varied in location for the two recorded events (see Figure A2 and A3). However, the multiple sampling events at Clair Lake and the CAWT were consistent in site location.

3.4.1 Prevalence of Antibiotic Resistance among Waterborne *Salmonella*, *E. coli* and *Enterococcus* spp.

Grand River Watershed

As illustrated in Figure 3.1A, the highest resistance occurrence was observed in *Salmonella* isolated from the upstream location of site 2 and downstream sampling location of site 3 at approximately 46% and 38%, respectively. Overall, the resistance trends among isolated *Salmonella* and *E. coli* were similar, although a peak of resistance was observed in *E. coli* from the downstream sample of site 2 at approximately 44%. Generally, the greatest resistance occurrence was observed in *Enterococcus* spp. across all three sites, with the highest levels detected at the upstream sample of site 1 and downstream and mouth samples of site 2 at just under 50% in each case. The following pathogens were not detected by culture-based methods at the specified sites: *Salmonella* at sites 2-DS (downstream) and 2-Mouth; *E. coli* and *Enterococcus* spp. at site 3-US (upstream).



US-upstream, DS-downstream, Mouth-river opening

- Antibiotics not tested against pathogens due to constraint of testing a maximum of twelve antibiotics at a time.

CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; SXT, sulfamethoxazole-trimethoprim; G, sulfisoxazole; TE, tetracycline; S, streptomycin; D, doxycycline AM, ampicillin, AMC, amoxicillin; C, chloramphenicol; VA, vancomycin; LZD, linezolid.

Note: vancomycin (VA) and linezolid (LZD) are commonly utilized antibiotics for testing resistant enterococci.

Figure 3.1. (A) Overall percent resistance occurrence based on sample location and (B) combined percentage of resistance according to antimicrobials for Grand River pathogens collected from all three sites on April 27th, 2012 (*Salmonella* n=50, *E. coli* n=68, *Enterococcus* spp. n=82).

Overall resistance patterns were generated based on the specific antimicrobial agent tested by combining AMR profiles for each of the Grand River pathogen types isolated across all three sites sampled on April 27th, 2012 (Figure 3.1B). It was observed that all three pathogen types were most susceptible to the effects of sulfamethoxazole-trimethoprim (SXT) and chloramphenicol (C). Specific cases in which total antibiotic efficacy was observed include ceftazidime (CAZ) against *Salmonella*, ciprofloxacin (CIP) against *E. coli*, and ampicillin (AM) and SXT against enterococci isolates. Conversely, the actions of sulfisoxazole (G) and streptomycin (S) did little to deter the growth of these same pathogens.

Observations were also made regarding the frequency of multiple antimicrobial resistances (MAR), defined in this study as resistance to three or more of the tested antibiotics, for select Grand River pathogens derived from each of the three sites (not depicted). It was found that 50% (15/30) of *Salmonella* isolates, 45.8% (11/24) of *E. coli* isolates and 95.8% (23/24) of enterococci isolates exhibited MAR from site 1 impacted by the upstream wastewater treatment plant. At site 2, moderately impacted by an agricultural landscape, MAR rates were 83.3% (5/6) for *Salmonella*, 38.2% (13/34) for *E. coli* and 91.7% (33/36) for *Enterococcus* spp.. The highest frequency of MAR in *Salmonella* was observed from the agriculturally-impacted site 3 location at 92.9% (13/14). Additionally, 60% (6/10) of *E. coli* and 72.7% (16/22) of enterococci also demonstrated MAR at site 3.

The highest percent resistance occurrence across all July 3rd and 4th 2012 sampling sites from which the pathogen was successfully derived was observed in *Enterococcus* spp., followed by *Salmonella* and *E. coli* respectively (Figure 3.2). Overall, resistance in *Salmonella* was highest at sites 2 and 11 at approximately 37%. *E. coli* at sites 2 and 8 exhibited resistance

occurrence at approximately 20%, while over 64% resistance occurrence was observed in enterococci isolated from site 4. The following pathogens were not detected by culture-based methods at the specified sites: *Salmonella* at sites 3, 4, 5 and 6; *E. coli* at sites 4, 7 and 10; *Enterococcus* spp. at site 1 (Figure A3).

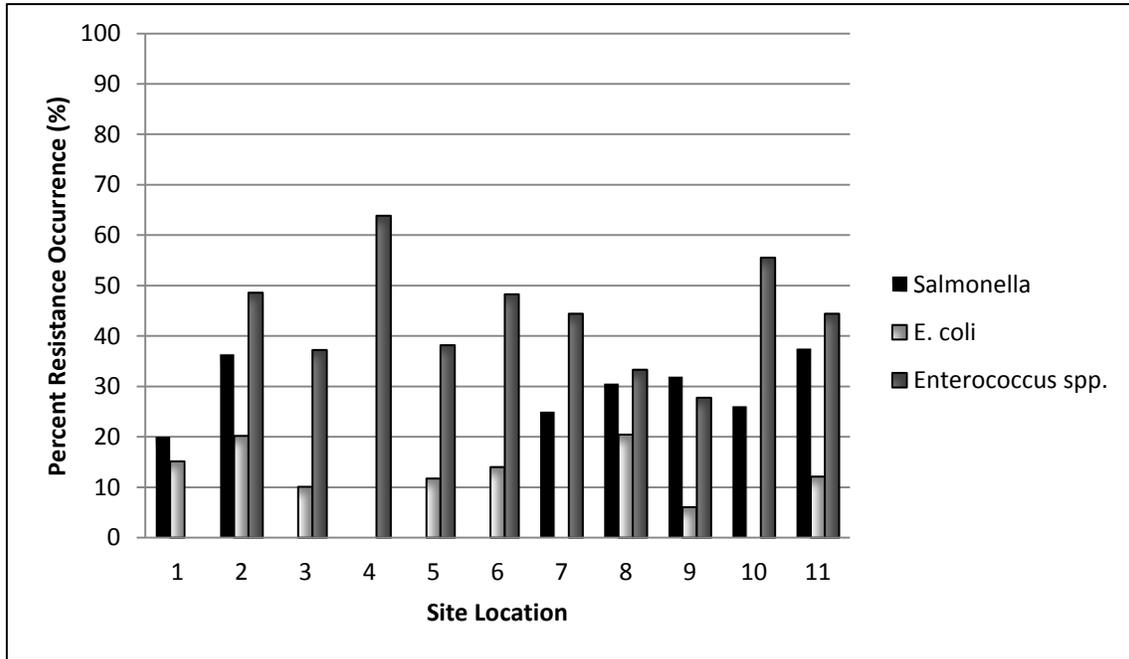


Figure 3.2. Total percent resistance occurrence in select pathogens isolated from eleven sites along the Grand River on July 3rd and 4th, 2012.

Low levels of antibiotic resistance were observed in Grand River *Salmonella* and *E. coli* to the 3rd generation cephalosporins tested (CAZ, CRO and CTX) as well as chloramphenicol (C), all of which are considered category 1 drugs by Health Canada for their importance in human treatment (Table 3.2). Alternatively, sulfamethoxazole-trimethoprim (SXT), doxycycline (D) and chloramphenicol (C) appeared to be the most effective against *Enterococcus* spp.. Medium to high resistance trends were generally observed towards ampicillin (AM) and amoxicillin (AMC) for all three waterborne pathogens isolated from the Grand River.

Table 3.2. Percentage of resistance according to the antibiotic tested and frequency of multiple antimicrobial resistances (MAR) in *Salmonella*, *E. coli* and *Enterococcus* spp. isolates collected across eleven sites of the Grand River on July 3rd and 4th, 2012.

<i>Salmonella</i>											
	Site Number										
	1	2	3	4	5	6	7	8	9	10	11
No. of isolates	N=8	N=10	ND ^a	ND ^a	ND ^a	ND ^a	N=6	N=6	N=6	N=8	N=6
Antibiotic	%	%	%	%	%	%	%	%	%	%	%
CAZ	0.0	0.0	- ^b	-	-	-	0.0	0.0	0.0	0.0	0.0
CIP	0.0	0.0	-	-	-	-	0.0	0.0	0.0	0.0	0.0
CRO	0.0	0.0	-	-	-	-	0.0	0.0	0.0	0.0	0.0
CTX	0.0	0.0	-	-	-	-	0.0	0.0	0.0	0.0	0.0
SXT	0.0	0.0	-	-	-	-	0.0	0.0	0.0	0.0	0.0
G	-	-	-	-	-	-	16.7	0.0	66.7	12.5	50.0
TE	25.0	30.0	-	-	-	-	33.3	33.3	33.3	12.5	50.0
S	25.0	80.0	-	-	-	-	33.3	83.3	66.7	37.5	83.3
D	12.5	70.0	-	-	-	-	33.3	50.0	33.3	50.0	66.7
AM	100.0	100.0	-	-	-	-	100.0	100.0	66.7	100.0	100.0
AMC	100.0	100.0	-	-	-	-	83.3	100.0	100.0	100.0	100.0
C	12.5	20.0	-	-	-	-	0.00	0.00	16.7	0.0	0.0
MAR^c	50.0	100.0	-	-	-	-	33.3	66.7	83.3	62.5	100.0
<i>E. coli</i>											
	Site Number										
	1	2	3	4	5	6	7	8	9	10	11
No. of isolates	N=6	N=9	N=9	ND ^a	N=24	N=24	ND ^a	N=12	N=6	ND ^a	N=6
Antibiotic	%	%	%	%	%	%	%	%	%	%	%
CAZ	0.0	0.0	0.0	-	4.2	4.2	-	16.7	0.0	-	0.0
CIP	0.0	0.0	0.0	-	0.0	4.2	-	0.0	0.0	-	0.0
CRO	0.0	0.0	0.0	-	0.0	4.2	-	16.7	0.0	-	0.0
CTX	0.0	0.0	0.0	-	0.0	4.2	-	16.7	0.0	-	0.0
SXT	0.0	0.0	0.0	-	0.0	8.3	-	8.3	0.0	-	0.0
SD	50.0	44.4	100.0	-	91.7	70.8	-	100.0	100.0	-	66.7
TE	0.0	0.0	0.0	-	0.0	4.2	-	8.3	0.0	-	0.0
S	100.0	77.8	88.9	-	100.0	75.0	-	91.2	66.7	-	100.0
D	0.0	0.0	0.0	-	0.0	4.2	-	0.0	0.0	-	0.0
AM	33.3	77.8	12.5	-	12.5	20.0	-	41.7	0.0	-	33.3
AMC	33.3	66.7	12.5	-	12.5	20.0	-	25.0	0.0	-	0.0
C	0.0	0.0	0.0	-	0.0	8.3	-	0.0	0.0	-	0.0
MAR^c	33.3	55.6	11.1	-	16.7	20.8	-	41.7	0.0	-	16.7

<i>Enterococcus</i> spp.	Site Number										
	1	2	3	4	5	6	7	8	9	10	11
No. of isolates	ND ^a	N=18	N=18	N=6	N=24	N=24	N=6	N=6	N=6	N=6	N=6
Antibiotic	%	%	%	%	%	%	%	%	%	%	%
CIP	-	88.9	77.8	100.0	83.3	95.8	66.7	100.0	100.0	100.0	100.0
CRO	-	100.0	66.7	100.0	87.5	91.7	100.0	83.3	33.3	100.0	100.0
CTX	-	55.6	55.6	100.0	75.0	100.0	100.0	66.7	16.7	16.7	66.7
SXT	-	0.0	0.0	0.0	0.0	4.2	0.0	16.7	0.0	0.0	0.0
TE	-	94.4	50.0	66.7	8.3	45.8	33.3	0.0	0.0	66.7	0.0
S	-	100.0	94.4	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
D	-	0.0	0.0	0.0	0.0	29.2	0.0	0.0	0.0	0.0	0.0
AM	-	22.2	5.6	33.3	12.5	4.2	0.0	16.7	0.0	66.7	33.3
AMC	-	22.2	16.7	33.3	12.5	8.3	33.3	16.7	0.0	66.7	33.3
C	-	0.0	0.0	0.0	0.0	8.3	0.0	0.0	0.0	16.7	0.0
VA	-	38.9	33.3	66.7	41.7	33.3	66.7	66.7	66.7	66.7	66.7
LZD	-	38.9	22.2	66.7	33.3	33.3	33.3	33.3	66.7	66.7	66.7
MAR^c	-	100.0	83.3	100.0	95.8	100.0	100.0	100.0	100.0	100.0	100.0

CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; SXT, sulfamethoxazole-trimethoprim; G, sulfisoxazole; TE, tetracycline; S, streptomycin; D, doxycycline AM, ampicillin, AMC, amoxicillin; C, chloramphenicol; VA, vancomycin; LZD, linezolid.

^aND, Isolates not detected at site.

^b-, Not analysed.

^cMAR, Isolates displaying multiple antimicrobial resistance patterns to ≥ 3 antimicrobials

Multiple antimicrobial resistances were observed in *Salmonella* and enterococci isolated across all sites of the Grand River from which the isolates were successfully cultured. Conversely, not all *E. coli* isolates can be considered MAR strains, as Site 9 *E. coli* did not demonstrate resistance to more than three antibiotic agents (Table 3.2). The highest overall resistance was exhibited by *Enterococcus* spp. with an average MAR rate of 97.9% (approximately 117/120) across all ten sites from which it was cultivated. Grand River *Salmonella* exhibited an average MAR rate of 70.8% (approximately 37/52) and the lowest MAR rate was observed in *E. coli* at 24.5% (approximately 24/96).

Clair Lake

Of the total *Salmonella* isolates, 86.5% (64/74) were considered MAR strains by displaying resistance to between three to five antibiotics (Figure 3.3). The greatest overall MAR levels, however, were observed in enterococci with 59.5% of total isolates (25/42) demonstrating resistance to three to five antibiotics, 31.0% isolates (13/42) resistant to six to seven antibiotics and just less than 5.0% of isolates (2/42) exhibiting resistance to between eight and nine antimicrobial agents. MAR rates observed in *E. coli* were much less frequent, with only 28.6% of isolates (6/21) displaying resistance to three to five isolates.

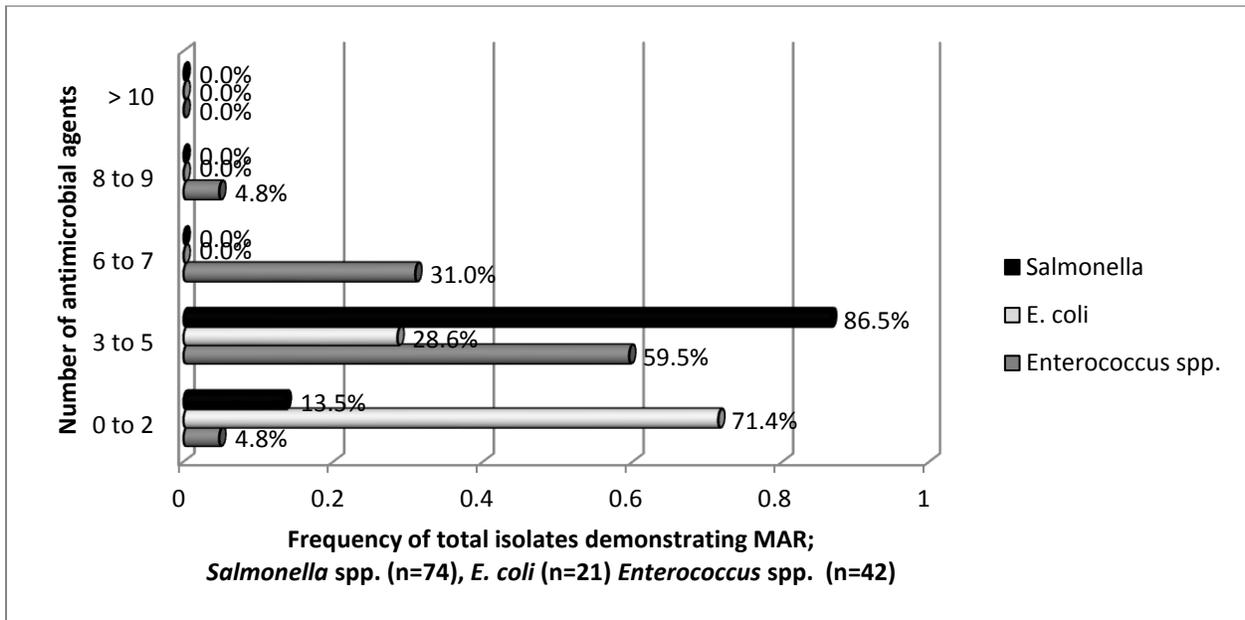


Figure 3.3. Prevalence of multiple antibiotic resistance (MAR) rates (%) among *Salmonella*, *E. coli* and *Enterococcus* spp. isolates collected from the dredged Clair Lake on November 19th, 2012.

Seasonal trends for AMR profiles observed in *Salmonella* isolated from Clair Lake in winter, spring and summer were recorded and are summarized in Table 3.3.

Table 3.3. Frequency (%) of antimicrobial resistance profiles and multiple antimicrobial resistances (MAR) observed in *Salmonella* isolated from the dredged Clair Lake over three seasons.

	Sampling Date		
	Nov. 19'12 (Winter)	April 26'13 (Spring)	June 6'13 (Summer)
No. of isolates	N=74	N=20	N=20
Observed resistance profiles	%	%	%
G, TE, D, AM, AMC	8.1	0.0	0.0
G, TE, AM, AMC	9.5	0.0	0.0
TE, D, AM, AMC	2.7	0.0	0.0
G, D, AM, AMC	8.1	0.0	0.0
G, AM, AMC	39.2	70.0	0.0
SXT, G, AM, AMC	2.7	0.0	0.0
SXT, G, TE, AM, AMC	1.4	0.0	0.0
G, S, AM, AMC	2.7	0.0	0.0
D, AM, AMC	12.2	5.0	0.0
G,S	4.1	0.0	90.0
AM, AMC	9.5	25.0	0.0
G	0.0	0.0	10.0
MAR^a	86.5	70.0	0.0

G, sulfisoxazole; TE, tetracycline; AM, ampicillin, AMC, amoxicillin; D, doxycycline; SXT, sulfamethoxazole-trimethoprim; S, streptomycin.

^aMAR, Isolates displaying multiple antimicrobial resistance patterns to ≥ 3 antimicrobials

In total, eleven different resistance profiles were observed in *Salmonella* isolated from Clair Lake in the winter season, followed by three different resistance profiles in spring *Salmonella* and two different resistance profiles in summer *Salmonella*. There was a decrease in MAR rates throughout the seasons, with 86.5% observed in the winter isolates, 70% in the spring isolates and no MAR *Salmonella* isolated during the summer sampling date. The most frequent resistance profile for winter and spring *Salmonella* was GAmAmC accounting for 39.2% and 70% of all isolates, respectively. Alternatively, a GS resistance profile was most commonly observed in summer *Salmonella* when the Clair Lake could be considered rehabilitated.

CAWT

The highest percent resistance occurrence across the CAWT system from which the pathogens were successfully derived was generally observed in *Enterococcus* spp., followed by *Salmonella* and *E. coli* respectively (Figure 3.4). Little variation in total resistance occurrence was seen across the constructed wetland vaults and three seasons sampled. The following pathogens were not detected by culture-based methods at the specified sites: *Salmonella* in vault 1 in winter and mid-summer, vault 3 in mid-summer, and vault 4 at the end of summer; *E. coli* in vault 3 in mid-summer and vault 4 in early and late summer; *Enterococcus* spp. in vault 4 in mid-summer.

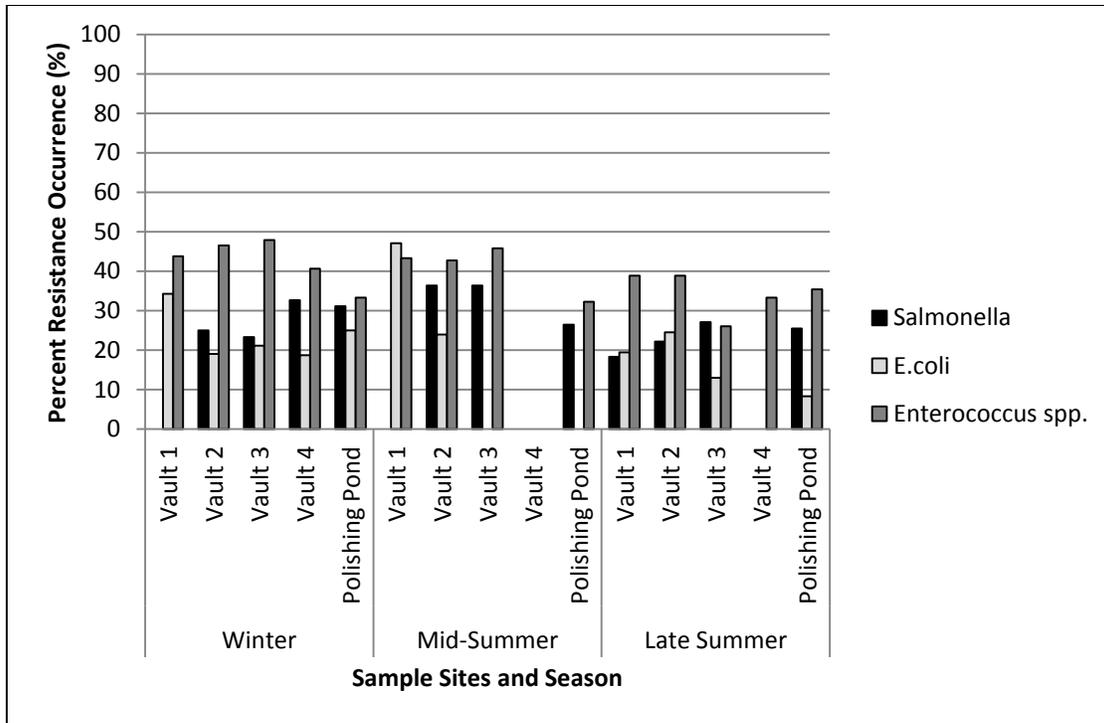
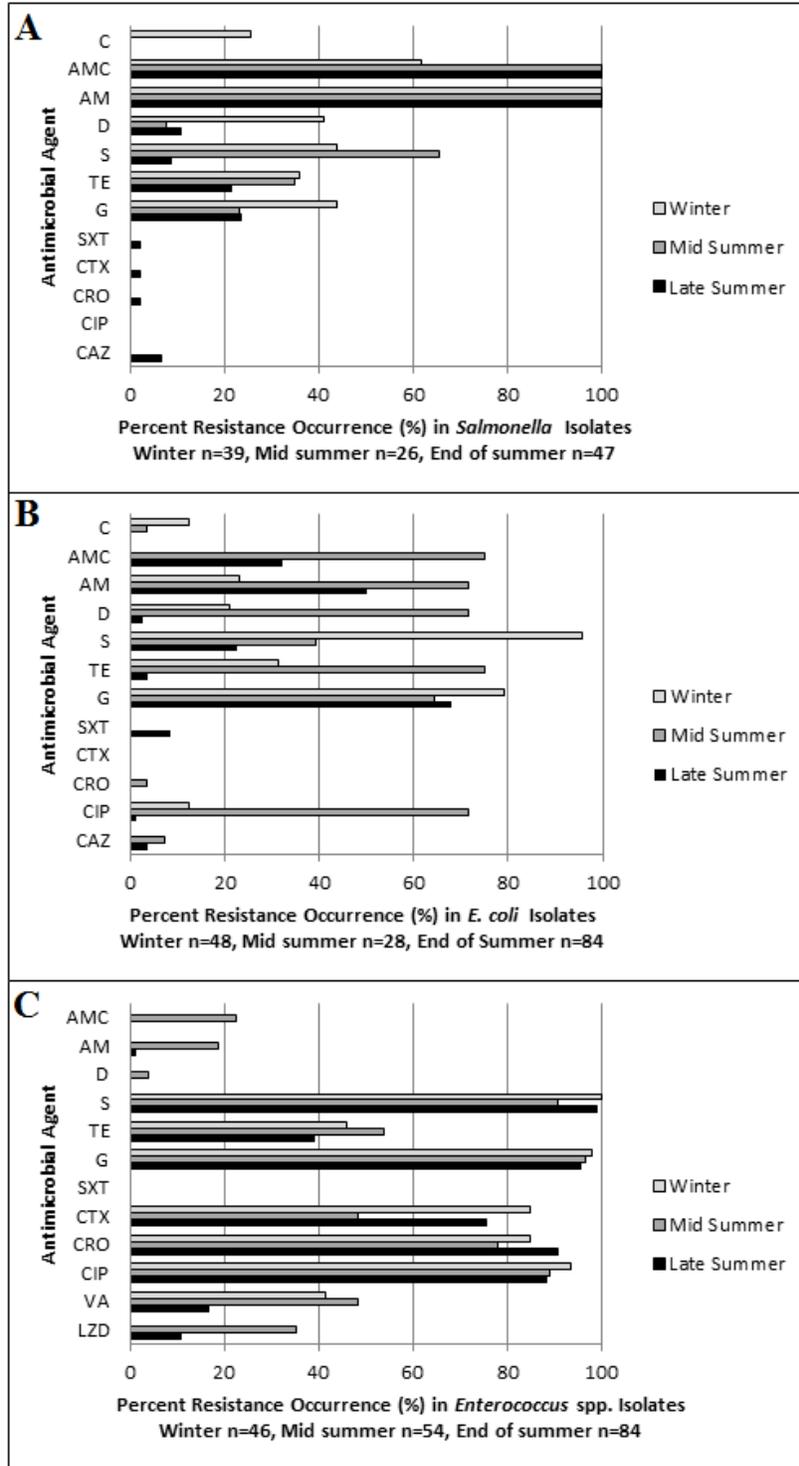


Figure 3.4. Total percent resistance occurrence in select pathogens isolated from the CAWT system over three seasons. Winter samples were collected on February 22nd, 2012, mid-summer samples were obtained on June 26th, 2012 and end of summer samples were collected on August 24th, 2012.

Seasonal resistance trends were more apparent when comparing CAWT pathogen susceptibilities to specific antibiotics (Figure 3.5). For all three pathogenic bacteria studied, winter and mid-summer isolates demonstrated overall greater resistance to the tested antibiotics compared to end of summer isolates. While minimal differences were observed in *Salmonella* from all seasons to the penicillins (AM and AMC), resistance towards chloramphenicol (C), doxycycline (D), streptomycin (S), tetracycline (TE) and sulfisoxazole (G) were typically elevated in winter and mid-summer *Salmonella*. Similar patterns were observed for *E. coli* and enterococci although mid-summer isolates tended to exhibit the highest overall antibiotic resistance followed by winter and then end of summer isolates. For *E. coli* specifically, resistance towards doxycycline (D), streptomycin (S), tetracycline (TE) and ciprofloxacin (CIP)

was markedly higher in winter and mid-summer as compared to end of summer isolates. Generally resistance levels in *Enterococcus* spp. isolates were less distinct on a per antibiotic basis among the three sampling seasons, however, winter and mid-summer isolates displayed a higher resistance to the penicillins (AM and AMC), vancomycin (VA) and linezolid (LZD) than end of summer isolates.



CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; SXT, sulfamethoxazole-trimethoprim; G, sulfisoxazole; TE, tetracycline; S, streptomycin; D, doxycycline AM, ampicillin, AMC, amoxicillin; C, chloramphenicol; VA, vancomycin; LZD, linezolid.

Figure 3.5. Percentage of resistance in CAWT isolates to a range of antimicrobials over three seasons (A) *Salmonella*, (B) *E. coli* and (C) *Enterococcus* spp..

MAR frequencies within *Salmonella* and fecal indicator bacteria derived from the constructed wetland system were also determined for further phenotypic-based resistance characterization of the isolates. Table 3.4 summarizes MAR trends both as the pathogens progressed through the treatment system and across the three seasons in which sampling took place.

Table 3.4. Frequency (%) of multiple antimicrobial resistance (MAR) in *Salmonella*, *E. coli* and *Enterococcus* spp. isolated from the test vaults and polishing pond of the CAWT constructed wetland system during three seasons. Winter samples were collected on February 22nd, 2012, mid-summer samples were obtained on June 26th, 2012 and end of summer samples were collected on August 24th, 2012.

MAR frequency (No. of MAR isolates/ total isolates) for each location sampled							
	Season Sampled	Vault 1	Vault 2	Vault 3	Vault 4	Polishing Pond	Total % MAR^a
<i>Salmonella</i>	Winter	ND ^a	50.0% (1/2)	60.0% (6/10)	83.3% (10/12)	93.3% (14/15)	79.5% (31/39)
	Mid summer	ND	100.0% (2/2)	100.0% (2/2)	ND	81.8% (18/22)	84.6% (22/26)
	Late summer	20% (2/10)	33.3% (5/15)	50% (2/4)	ND	72.2% (13/18)	46.8% (22/47)
<i>E. coli</i>	Winter	66.7% (6/9)	21.4% (3/14)	33.3% (5/15)	25.0% (2/8)	100.0% (2/2)	37.5% (18/48)
	Mid summer	100% (16/16)	58.3% (7/12)	ND	ND	ND	82.1% (23/28)
	Late summer	41.7% (10/24)	66.7% (12/18)	17.0% (3/18)	ND	4.0% (1/24)	31.0% (26/84)
<i>Enterococcus</i> spp.	Winter	100% (12/12)	91.7% (11/12)	91.7% (11/12)	87.5% (7/8)	100% (2/2)	93.5% (43/46)
	Mid summer	100% (15/15)	100% (8/8)	100% (8/8)	ND	78.3% (18/23)	90.7% (49/54)
	Late summer	95.8% (23/24)	100% (18/18)	85.7% (12/14)	100% (4/4)	100% (24/24)	96.4% (81/84)

^aMAR, Isolates displaying multiple antimicrobial resistance patterns to ≥ 3 antimicrobials

^aND, Isolates not detected at site.

As CAWT *Salmonella* travelled through the system, the observed MAR rates either increased (in the case of winter and end of summer isolated) or were maintained (as in the case

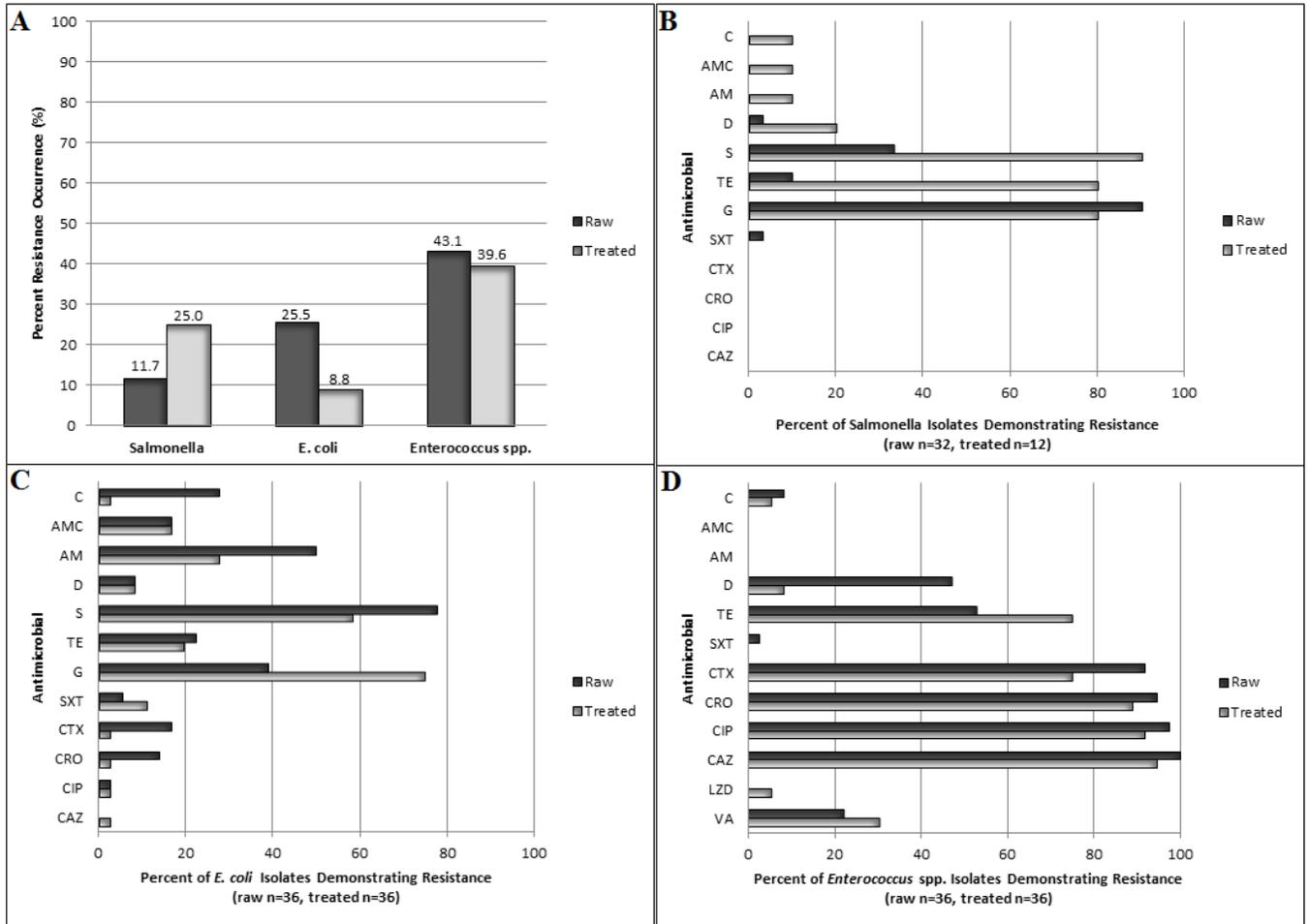
of mid-summer isolates). For instance, end of summer *Salmonella* collected from the polishing pond demonstrated a 52.2% higher MAR frequency than did *Salmonella* collected from vault 1 in the same season. Alternatively, a decrease in MAR rates of *E. coli* was typically observed as it progressed through the system, with the biggest decline observed in end of summer *E. coli* at 37.7%. MAR rates in *Enterococcus* spp. isolates were comparably consistent, whereby high MAR rates were maintained across all sampling locations and seasons. However, it should be noted that low sample sizes collected for some isolates (e.g. N=2) from specific wetland locations may bias these trends.

As presented in Table 3.4, the greatest total MAR rate for *Salmonella* was observed in mid-summer samples at 84.6% (22/26), followed by winter isolates at 79.5% (31/39), with the lowest instances of MAR seen in *Salmonella* obtained at the end of summer at 46.8% (22/47). Similarly, the highest MAR frequency for *E. coli* was exhibited in mid-summer at 82.1% (23/28), although the pathogen was only isolated from the first and second test vault. A slightly higher MAR rate was observed in winter *E. coli* over *E. coli* isolated at the end of the summer at 37.5% (18/48) and 31.0% (26/84), respectively. The highest overall frequency of MAR was exhibited by *Enterococcus* spp. isolates across all three seasons, ranging from 90.7% (49/54) in mid-summer isolates to 96.4% (81/84) in isolates obtained at the end of summer.

WTC

The percent resistance occurrence in select pathogens isolated from the raw and treated samples of the pilot-scale wastewater treatment plant was established and is depicted in Figure 3.6A. In the cases of *E. coli* and enterococci, it was observed that resistance occurrence was higher in the ‘raw’ isolates as compared to the ‘treated’ isolates, with the greatest difference

(approximately 17%) being observed in *E. coli*. The opposite trend was observed in *Salmonella* isolates, such that treated isolates exhibited a greater resistance occurrence than did raw isolates by just over 13%.



CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; SXT, sulfamethoxazole-trimethoprim; G, sulfisoxazole; TE, tetracycline; S, streptomycin; D, doxycycline AM, ampicillin, AMC, amoxicillin; C, chloramphenicol; VA, vancomycin; LZD, linezolid.

Figure 3.6. (A) Percent resistance occurrence in select pathogens isolated from raw and treated WTC Samples and (B) percentage of resistance in *Salmonella* (C) *E. coli* and (D) *Enterococcus spp.* isolates from raw and treated samples to a range of antimicrobials.

This trend was more explicitly demonstrated with the establishment of individual resistance profiles for each of three isolated pathogens (Figure 3.6B-D). On average, *E. coli* and *Enterococcus spp.* isolates displayed moderate to high levels of resistance against the tested

antimicrobials, with the raw isolates showing overall greater resistance than the treated isolates. The exception to these generalized findings include a higher resistance observed in treated *E. coli* to sulfisoxazole (G) and sulfamethoxazole-trimethoprim (SXT) than raw *E. coli*, and enhanced tolerance of treated *Enterococcus* spp. to tetracycline (TE) and vancomycin (VA) compared to raw *Enterococcus* spp. isolates. Conversely, *Salmonella* isolated from treated samples on average demonstrated not only greater resistance to the tested antibiotics as compared to *Salmonella* from raw samples, but an overall resistance to a greater total number of antibiotics. For instance, it was observed that treated *Salmonella* demonstrated resistance to chloramphenicol (C), amoxicillin (AMC) and ampicillin (AM), whereby raw *Salmonella* were completely susceptible to these drugs.

Of the total WTC *Salmonella*, 12.5% (4/32) raw isolates and 75% (9/12) of treated isolates were considered MAR strains by displaying resistance to three or more antibiotics (Figure 3.7A). In contrast, 47.2% (17/36) of raw *E. coli*, 27.8% (10/36) of treated *E. coli* and approximately 94.5% (34/36) of both raw and treated enterococci isolates demonstrated MAR by the same criteria (Figure 3.7B and C).

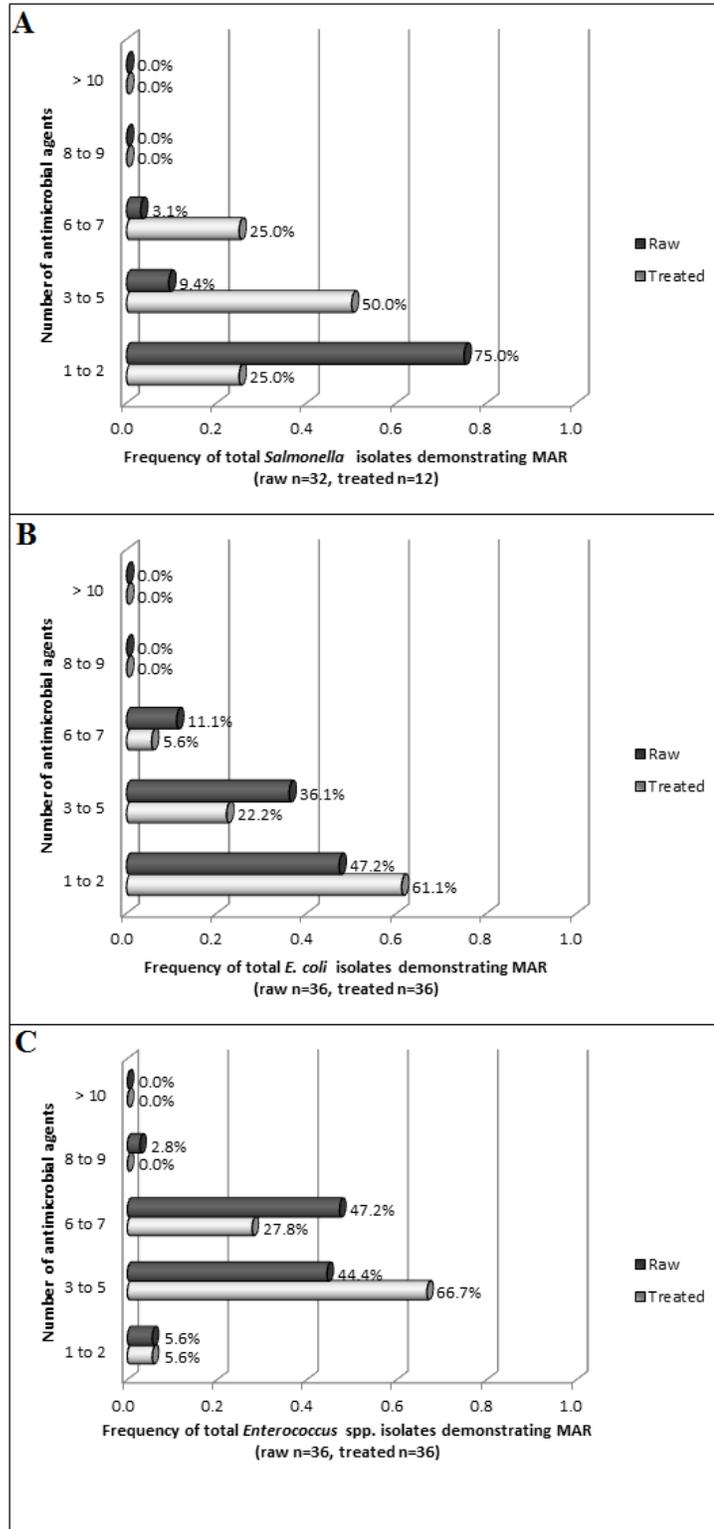


Figure 3.7. Prevalence of multiple antibiotic resistance (MAR) rates (%) among (A) *Salmonella*, (B) *E. coli* and (C) *Enterococcus* spp. isolates collected from raw and treated samples of the WTC.

Specifically, 50% (6/12) of treated *Salmonella* demonstrated resistance to 3 to 5 antimicrobials and 25% (4/12) of treated *Salmonella* exhibited resistance to 6 to 7 antimicrobials, compared to 9.4% (3/32) and 3.1% (1/32) of raw *Salmonella*, respectively. The highest levels of multiple resistances was observed in raw enterococci with 44.4% of isolates (16/36) demonstrating resistance to three to five antibiotics, 47.2% of isolates (17/36) resistant to six to seven antibiotics and just less than 3.0% of isolates (1/36) exhibiting resistance to between eight and nine antimicrobial agents. MAR rates observed in *E. coli* were less frequent, with only 11.1% (4/36) of raw isolates and 5.6% (2/36) of treated isolates displaying resistance to more than five antimicrobials.

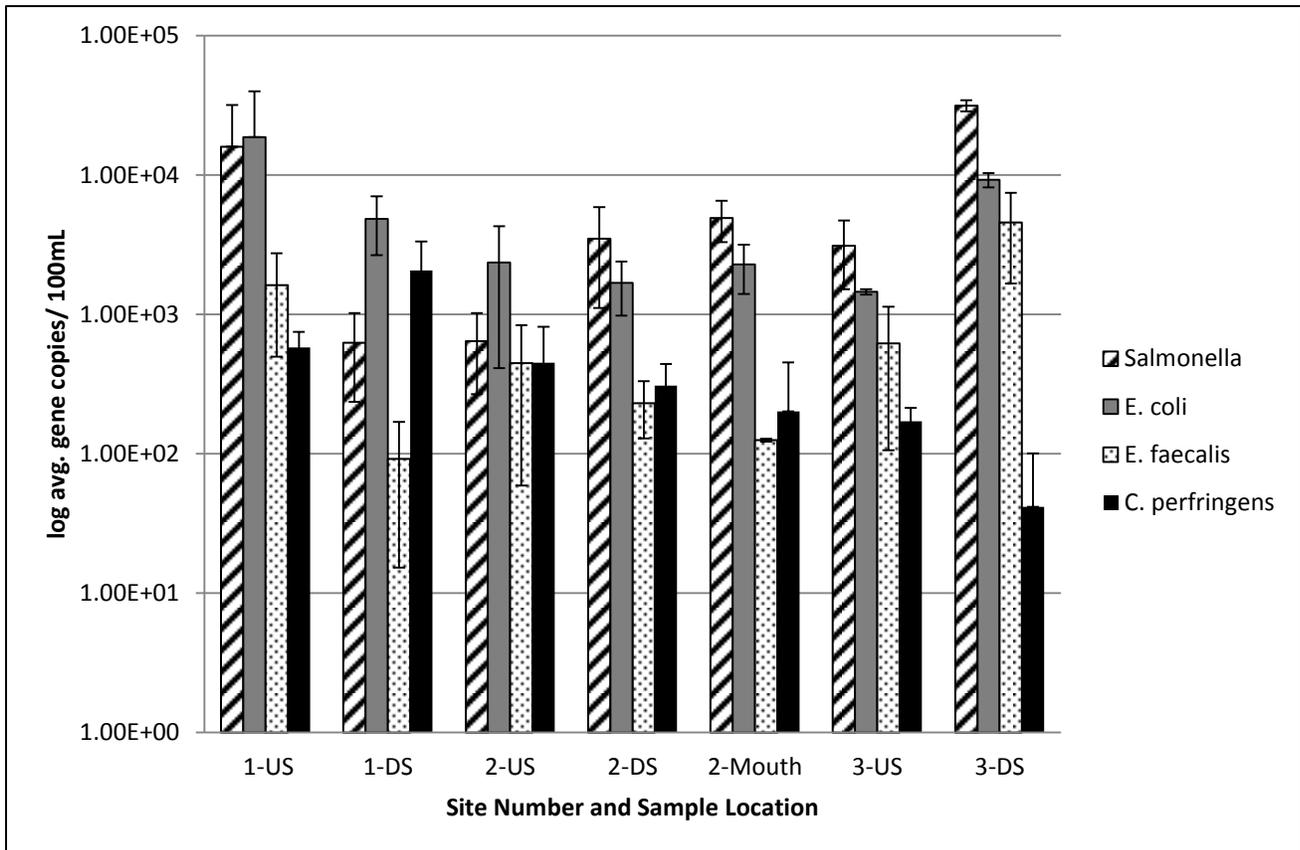
3.4.2 Quantitative Detection of Bacterial Pathogens from Impacted Water Sources using qPCR

With the use of qPCR, the enumeration of conserved genes within *Salmonella*, *E. coli*, *Enterococcus faecalis* and *C. perfringens* was successfully accomplished to detect concentration levels of the respective pathogens from the various test sources. In the absence of genus-specific primers for enterococci detection, primers were used for the detection of *E. faecalis* which is considered the most widespread enterococci species in the environment (Castillo-Rojas *et al.*, 2013). See Table 2.1 for primers and probes used for qPCR.

Grand River Watershed

Figure 3.8 illustrates the relative abundance in target gene copies per 100 mL of select bacterial pathogens across three variously impacted sites of the Grand River sampled in April, 2012 following the snowmelt. Pathogen concentrations were relatively consistent among all sample sites, with slight elevations in bacterial levels observed in site 1 locations impacted by an

upstream wastewater treatment plant and site 3 locations which were heavily impacted by agricultural activity. The highest overall values were obtained for *Salmonella* and *E. coli* which were typically one to two log orders of magnitude higher than *E. faecalis* and *C. perfringens* concentrations.



US-upstream, DS-downstream, Mouth-river opening

Figure 3.8. Mean values (target gene copies/100 mL) of select bacterial pathogens across three regions of the Grand River Watershed samples on April 27th, 2012 including an area less than 5 km downstream of the Waterloo wastewater treatment plant (site 1), an area downstream of agricultural land (site 2) and an area heavily impacted by agricultural practices (site 3).

Salmonella, *E. coli*, *E. faecalis* and *C. perfringens* were detected at all eleven Grand River sites sampled on July 3rd and 4th, 2012 with the use of qPCR. Again, the relative summer abundance of *Salmonella* and *E. coli* were typically higher than *E. faecalis* and *C. perfringens* by

one to two log orders of magnitude. Little to no variation was observed between the concentrations of the respective bacteria with the greatest overall site-dependent variation observed in *Enterococcus* spp..

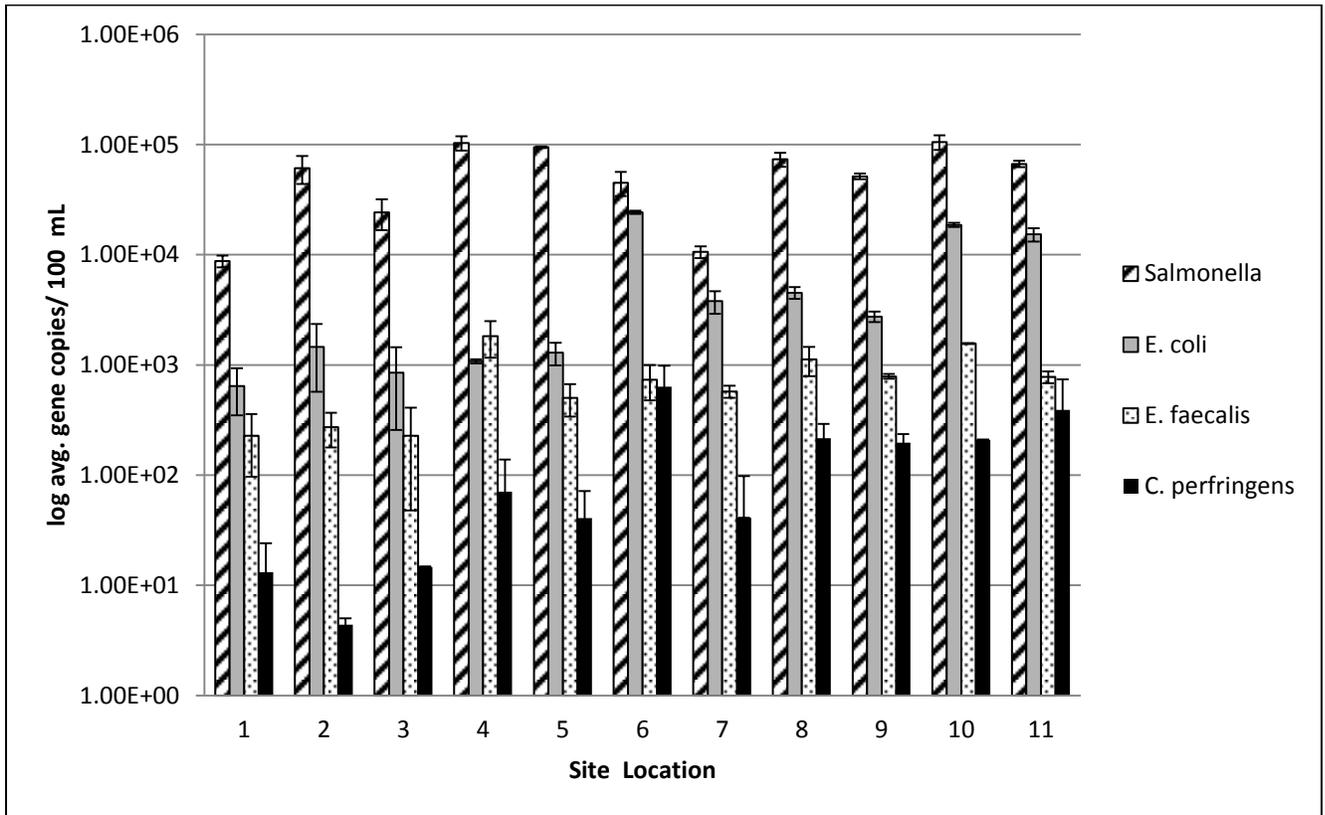


Figure 3.9. Relative abundance (target gene copies/100 mL) of select bacterial pathogens across eleven sites sampled along the Grand River on July 3rd and 4th, 2012.

Clair Lake

Few apparent changes were observed in the concentration of the tested pathogens (*Salmonella*, *E. coli*, *E. faecalis* and *C. perfringens*) in Clair Lake across seasons and throughout the four sampling events as shown in Figure 3.10. During dredging in November, the relative abundance of *Salmonella* and *E. coli* were highest at approximately 10⁴ gene copies per 100 mL. During the late stages of rehabilitation in April, and in June and August of 2013 when Clair Lake

could be considered fully rehabilitated, concentrations of the four bacterial pathogens were approximately equal at 10^3 gene copies per 100 mL.

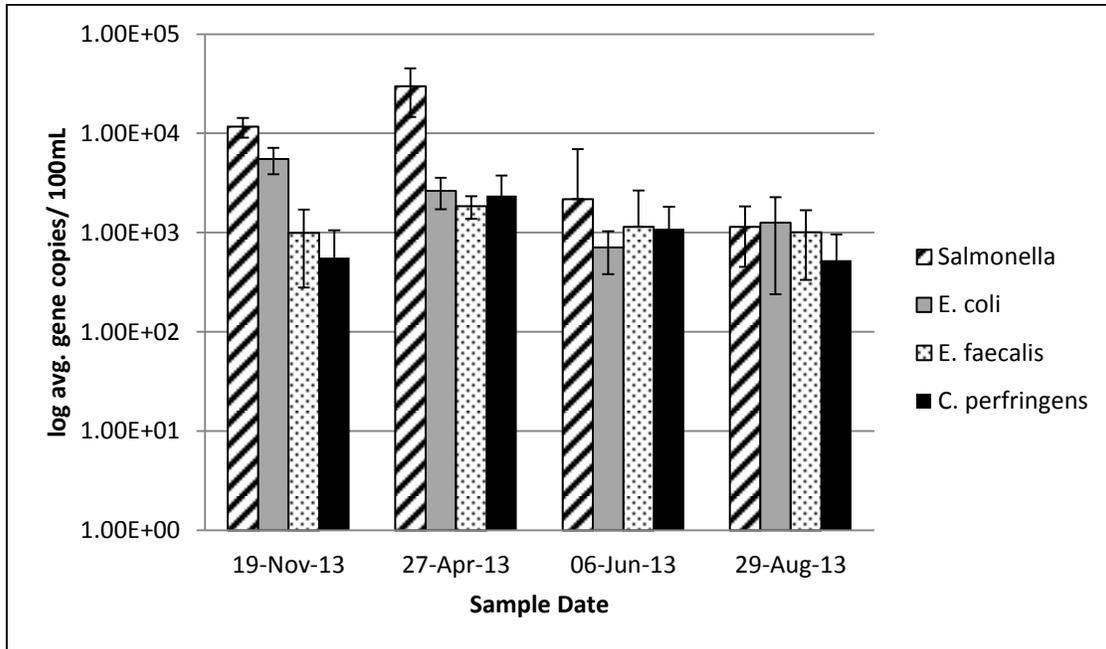


Figure 3.10. Quantification (target gene copies/100 mL) of select bacterial pathogens from Clair Lake over four sampling dates.

CAWT

The relative abundance of test pathogens as they progressed through the CAWT system was measured by real-time quantitative PCR during three seasons; winter, mid-summer and the end of summer. These findings are summarized in Figure 3.11. High levels of *Salmonella* and *E. coli* were most frequently detected in the different locations throughout the constructed wetland across all three sampled seasons ranging in magnitude from 10^2 to greater than 10^5 . Concentrations of *E. faecalis* and *C. perfringens* were typically lower ranging from approximately 10^1 to 10^5 , and no detection (in vaults 2 and 4 as well as the polishing pond in February) to approximately 10^4 , for the two pathogens respectively. Overall, the highest concentrations of all four bacteria types were detected in late August, followed by June, with the

lowest frequencies observed in February. For all sampling events, it was generally observed that the relative concentrations of the detected pathogens were highest in vaults 1 and 2, with a slight reduction in vaults 3 and 4 and a recovery of pathogen levels in the final polishing pond.

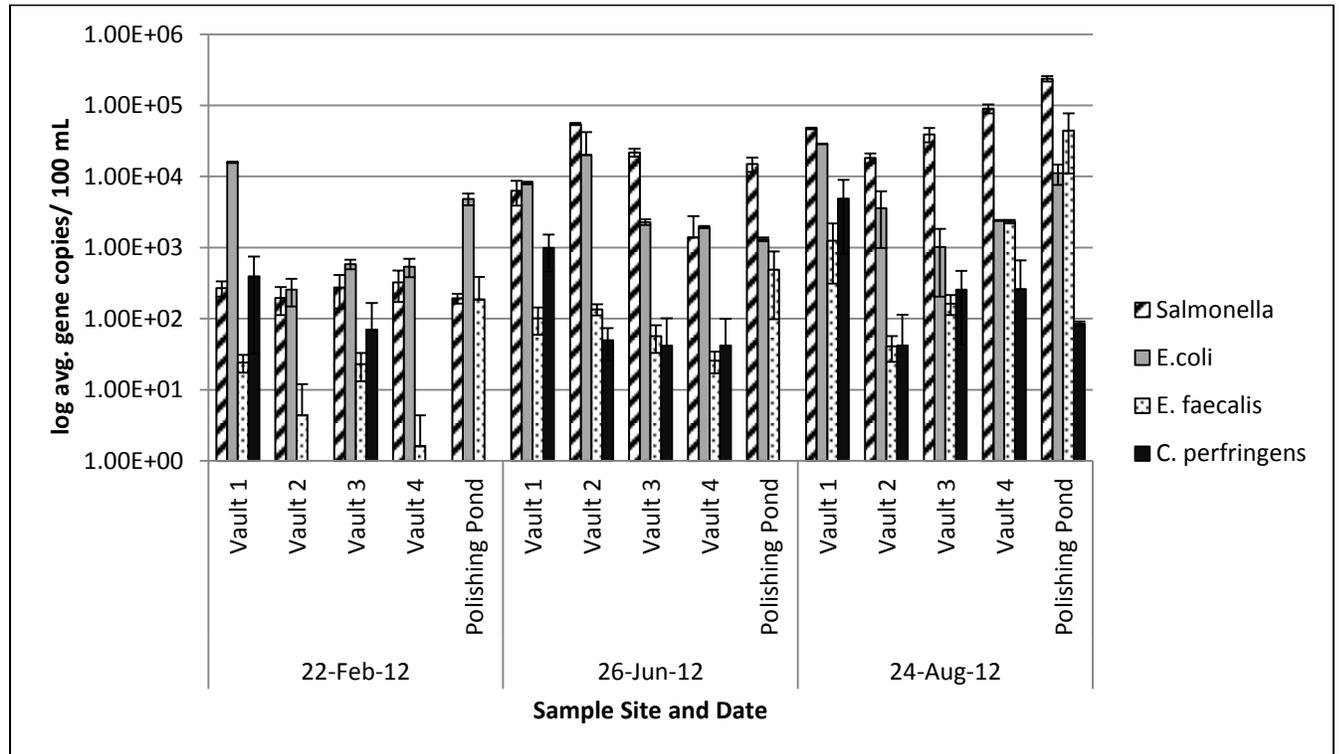


Figure 3.11. Enumeration (target gene copies/100 mL) of select bacterial pathogens isolated from the various locations of the CAWT constructed wetland system over three seasons.

WTC

Real-time qPCR was used to determine the concentration of four bacterial pathogens in raw and treated wastewater samples from the pilot-scale WTC, as illustrated in Figure 3.12. In all cases, concentrations were higher in raw samples than treated samples for the respective pathogens, indicating at least slight removal of the bacteria following treatment. The highest values from the raw samples were obtained for *Salmonella* at just under 10^5 target gene copies/100 mL, while similar concentrations were found for *E. coli*, *E. faecalis* and *C. perfringens* at

approximately 10^4 target gene copies/100mL. The greatest pathogen removal following treatment was observed in *E. faecalis*, with an approximately two log reduction of the pathogen in the treated sample as compared to the raw sample. Alternatively, *Salmonella*, *E. coli* and *C. perfringens* concentrations were reduced by nearly one order of magnitude.

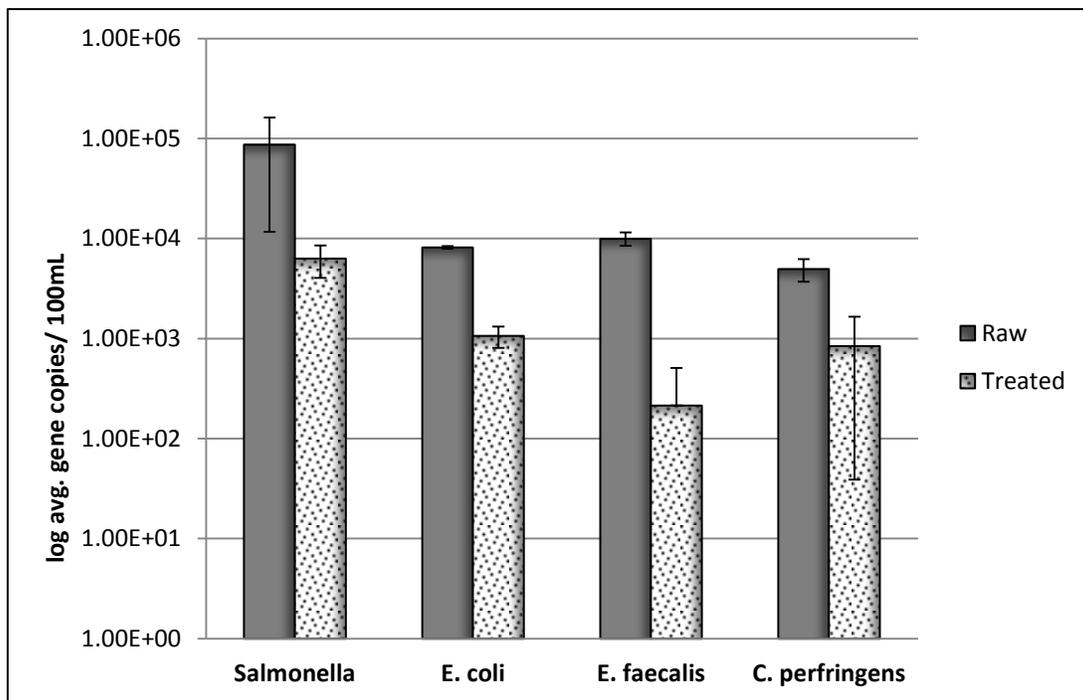


Figure 3.12. Relative abundance (target gene copies/100 mL) of select bacterial pathogens within raw and treated wastewater samples of the WTC.

3.4.3 Community-Level Physiological Profiling of Communities from which Waterborne Pathogens were Extracted

The use of CLPP technology was not introduced in the study until after the Grand River watershed sampling events and CAWT sampling which occurred on February 22nd, 2012.

Clair Lake

AWCD trends indicate that there were variations in the metabolic rates of the Clair Lake microbial communities depending on the sampling season (Figure 3.13A). Specifically, samples

obtained in the winter appeared to be the slowest to metabolize the available carbon sources with an OD_{590nm} reading of less than 1 following 96 hrs of incubation. A gradual increase in metabolic rate was observed from November to April with a peak in metabolic rate observed in the June community at an absorbance of approximately 1.5. A slight decline to an OD_{590nm} reading of 1.3 was thereafter observed in the microbial community sampled in August. This pattern was similarly reflected with regard to the metabolic diversity of the communities calculated according to the Shannon Index (H') (Figure 3.13B). Again, the microbial community extracted from Clair Lake in November demonstrated the lowest diversity in carbon source utilization at a value of just over 3.2, while the highest metabolic diversity was seen in the June community at just below 3.4. Correspondingly, a reduction in the metabolic diversity was apparent in the August Clair Lake community with a measured value below 3.3. Minimal variation was observed between the Clair Lake communities in terms of metabolic richness, or the total number of carbon sources metabolized resulting in an absorbance reading of 0.25 or higher, across all four sampling events (Figure 3.13C). While the November microbial community was only able to utilize 28 of the 31 substrates made available in the EcoPlate™, all communities sampled in April, June and August expressed the capacity to utilize all 31 carbon sources over an incubation period of 96 hrs.

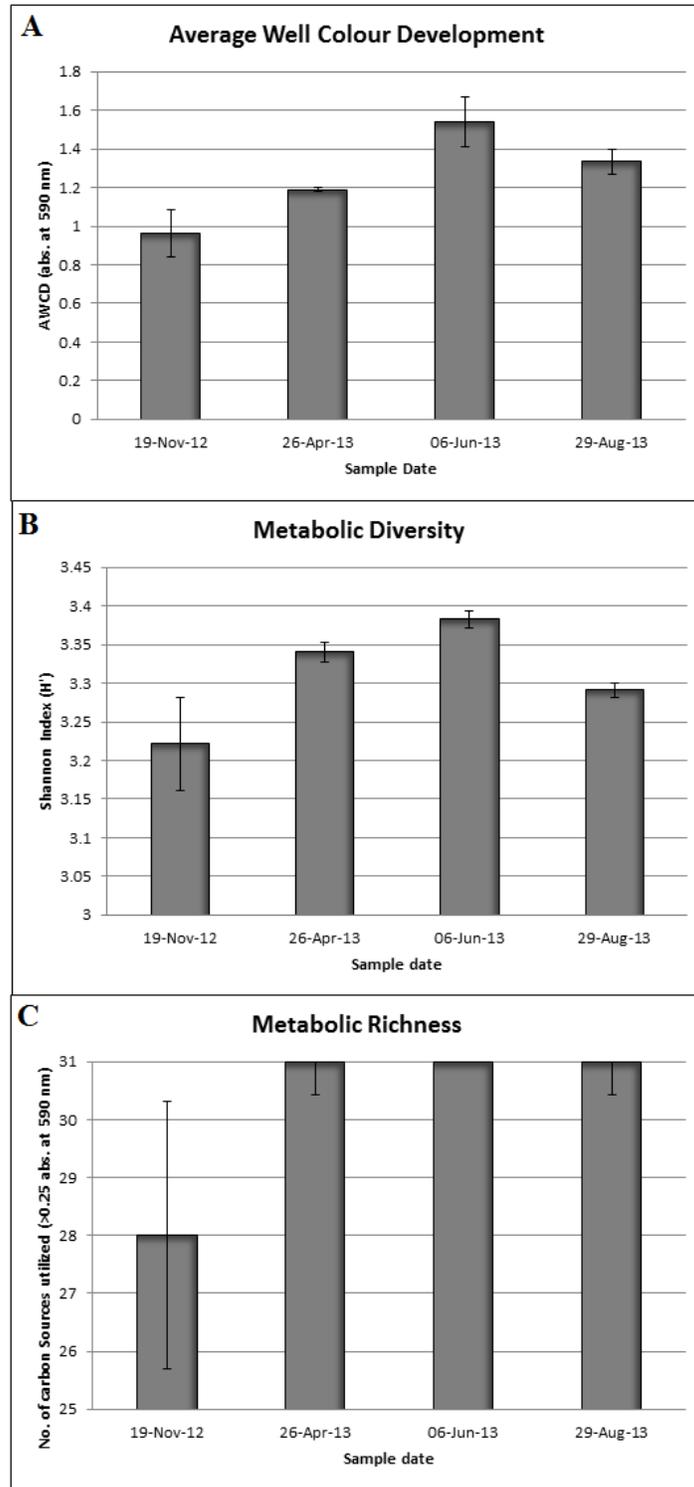


Figure 3.13. Community-level physiological profiles of bacterial communities from Clair Lake from which test pathogens were derived across four seasons based on (A) average well colour development, (B) metabolic diversity and (C) metabolic richness following 96 hrs of incubation.

CAWT

Across all the stages of the CAWT system, the August microbial community more readily metabolized the available nutrient substrates than did the June community based on AWCD trends (Figure 3.14A). While similar metabolic rates were observed in communities extracted from the first and second test vaults, a reduction in metabolic capabilities were observed in the third and fourth vaults followed by a recovery in functionality in the final polishing pond for both June and August communities. A similar pattern was again observed in the August community with regard to metabolic diversity, whereby a loss of substrate diversity was observed in vaults 3 and 4 and recovered by the final polishing pond (Figure 3.14B). Alternatively, the June community displayed a gradual decline in functional diversity from vault 1 with an H' value of approximately 3.4 to the polishing pond with an H' value of approximately 3.28. Overall, the measured metabolic richness of both the June and August communities across the CAWT system reached maximal potential by utilizing all available substrates following 96 hrs of incubation (Figure 3.14C).

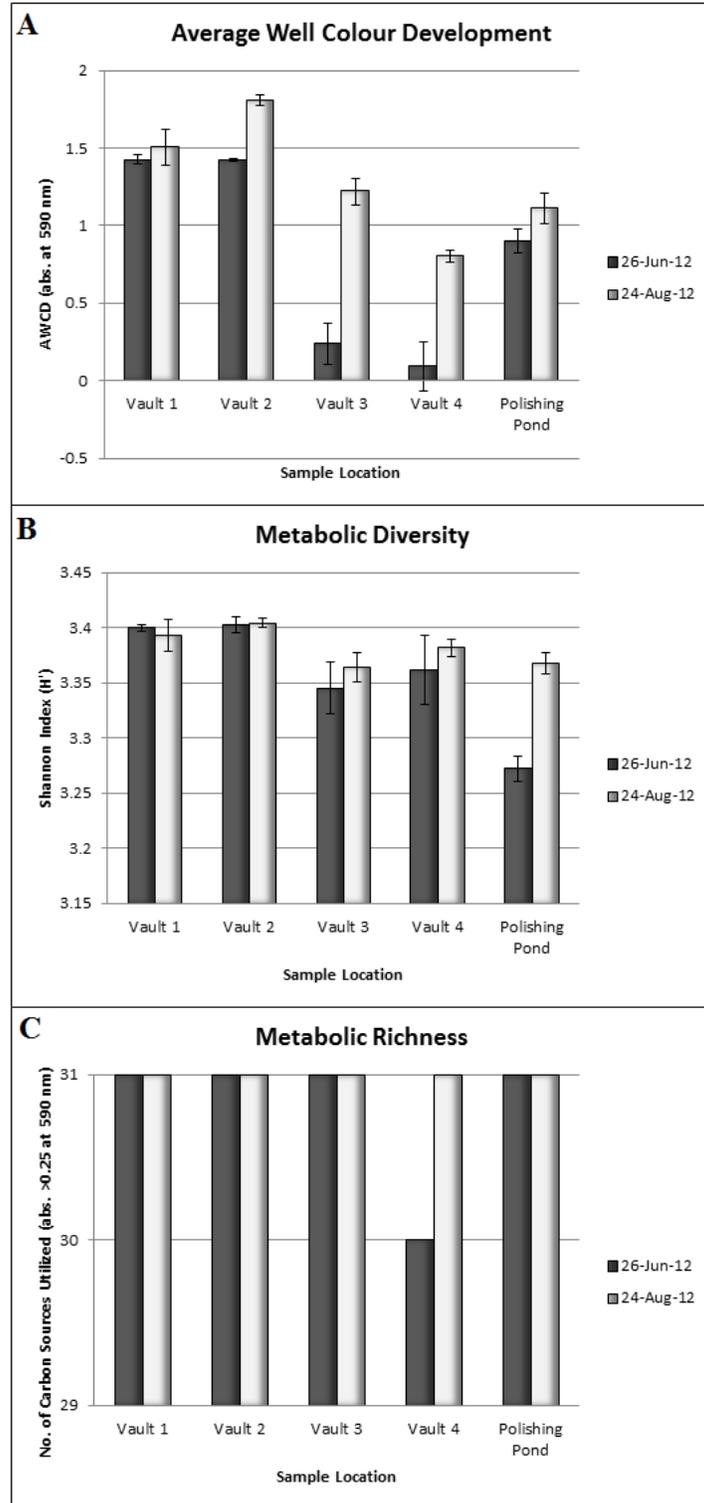


Figure 3.14. Community-level physiological profiles of bacterial communities from the CAWT system from which test pathogens were derived based on (A) average well colour development, (B) metabolic diversity and (C) metabolic richness following 96 hrs of incubation.

WTC

Based on AWCD trends, the microbial communities from raw and treated WTC samples initially showed different capacities to metabolize the 31 available carbon sources, where raw communities demonstrated higher versatility than treated communities (Figure 3.15A). By 72 hours of incubation, carbon substrate consumption between the communities stabilized at an absorbance value of approximately 1.1 at 590 nm. Metabolic diversity analysis based on the Shannon Index (H') revealed that the metabolic diversity of the raw microbial community was overall greater than the treated microbial community for an incubation period of 0 hr to 96 hr (Figure 3.15B). Following 114 hrs of incubation, however, diversity measurements equalized between the two community types. Alternatively, little difference was observed between the raw and treated communities in terms of metabolic richness (Figure 3.15C). By 96 hrs, both communities were able to use all 31 carbon sources supplied in the EcoPlate™.

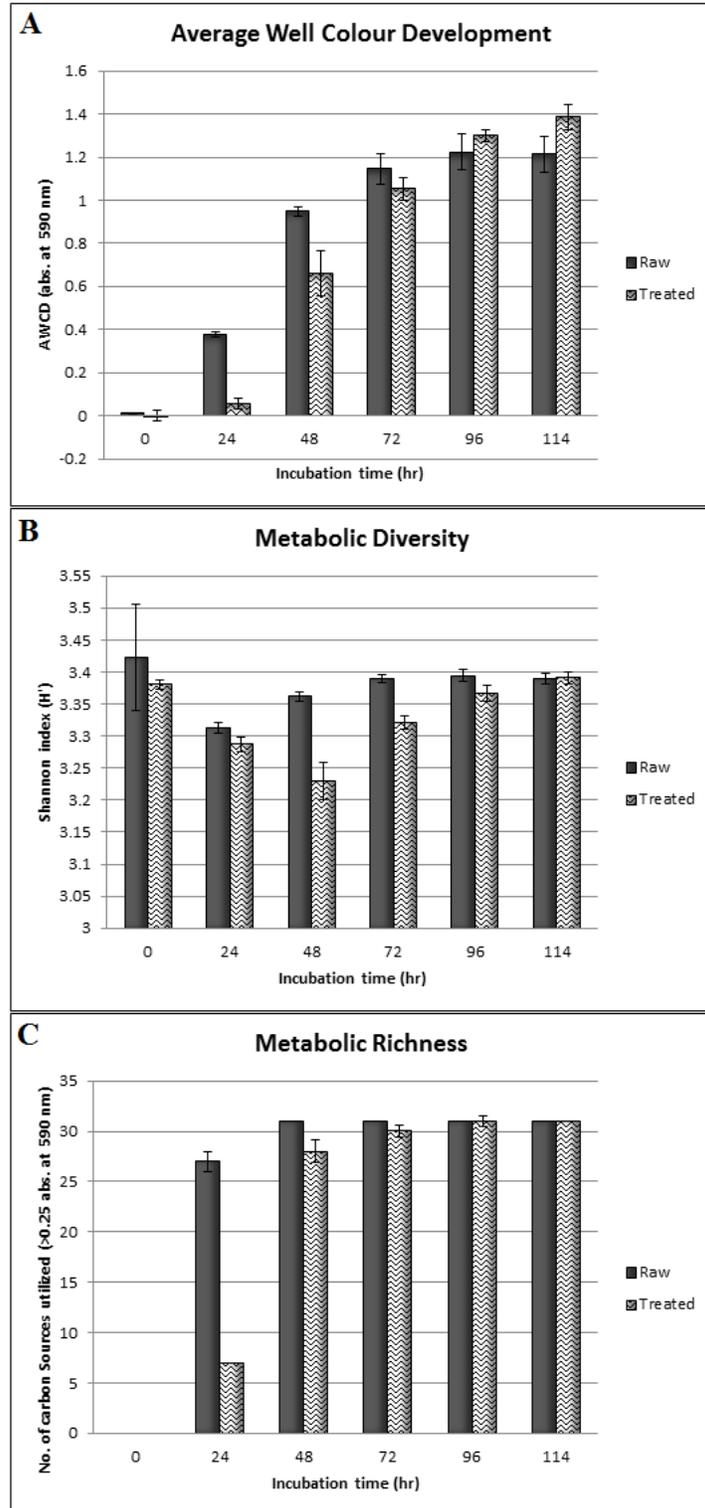
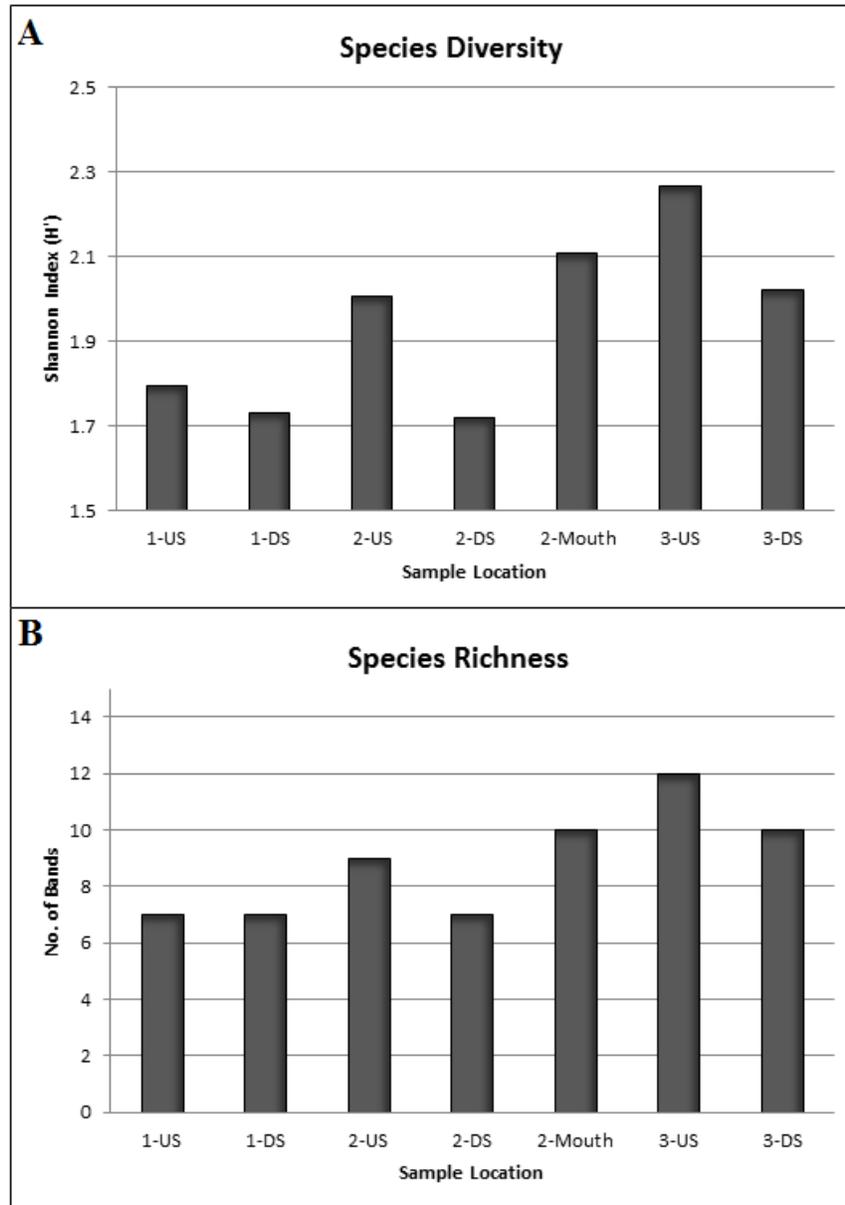


Figure 3.15. Community-level physiological profiles of raw and treated WTC bacterial communities from which test pathogens were derived based on (A) average well colour development, (B) metabolic diversity and (C) metabolic richness.

3.4.4 Structural Fingerprinting of Communities through DGGE

Grand River Watershed

Species diversity, a proportional measure of the richness and evenness of a sample defined by the Shannon Index (H'), and species richness, defined as the number of bands above background representing operational taxonomic units (OTUs), were calculated for April 27th, 2012 Grand River samples and these trends are displayed in Figure 3.16. An increase in community structural diversity was observed in the downstream sample of site 2, as well as both sample locations of site 3 where the impact of agricultural practices is greatest. A similar trend was apparent when considering community richness, whereby OTU counts for microbial communities from these same sample locations were slightly elevated as compared to the more urban impacted sites.



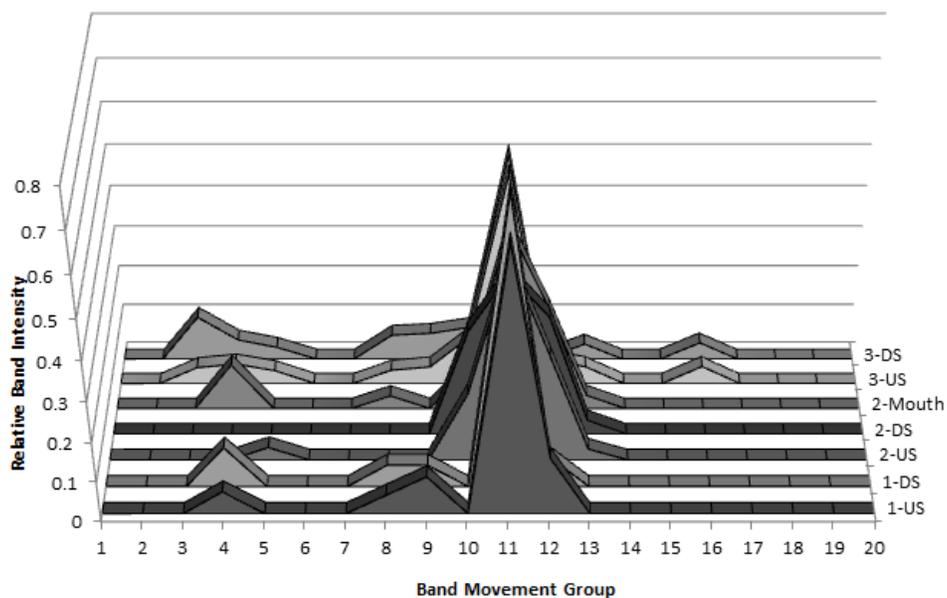
US-upstream, DS-downstream, Mouth-river opening

Figure 3.16. DGGE-based community analysis of Grand River samples collected from sites 1, 2 and 3 on April 27th, 2012 including (A) species diversity calculated using the Shannon index (H') and (B) species richness as interpreted by the band number representing operational taxonomic units (OTUs) within the sample.

To further characterize the structural dynamics of the microbial communities, average banding patterns were generated based on the relative intensities of the bands and their respective migratory distances through the DGGE gel. Bands were categorized into ‘band movement

groups' according to their final positioning within the gel, serving as an indication of the G+C content of the OTUs such that G+C rich groups would be assigned a higher band movement group than G+C poor OTUs.

According to the average banding plot shown in Figure 3.17, all sampling locations within the three sites were dominated by a single large peak between the 10th and 13th band movement group. In each case, the collective relative intensities of the bands ranged from 60% (in the downstream sample of Site 3) to 99% (in the downstream sample of Site 2) of the lane intensity for the sample community. Moderate peaks were observed approximately between the 2nd and 5th movement group as well as between the 7th and 10th group indicating low G+C content OTUs at Site 1 and 3 locations. These peaks accounted for an average of 12% and 17% of relative band intensities, respectively. Unique to the Site 3 locations, was a peak of high G+C content OTUs at the 16th movement group comprising 4.47% of the relative band intensity for the upstream community and 4.27% relative intensity for the downstream community.



US-upstream, DS-downstream, Mouth-river opening

Figure 3.17. DGGE-based averaged banding patterns for site-specific Grand River samples collected April 27th, 2012.

Comparable to findings from the Grand River sampling event in spring, the species diversity observed in microbial communities from sites impacted by an agricultural landscape (sites 7 to 11) was generally higher than urban impacted sites (sites 1 through 6) from Grand River samples obtained in the summer (Figure 3.18A). Of the urban sites, a peak in H' value was observed at site 6 which is the location of a discharge pipe from the upstream wastewater treatment plant. However, the highest overall genetic diversity was apparent in water sources collected through agricultural landscapes of the Grand River (sites 7 through 11). Similarly, samples containing the greatest diversity also consisted of the greatest species richness, while site 1 community seemed to deviate the most from this correlation (Figure 3.18B).

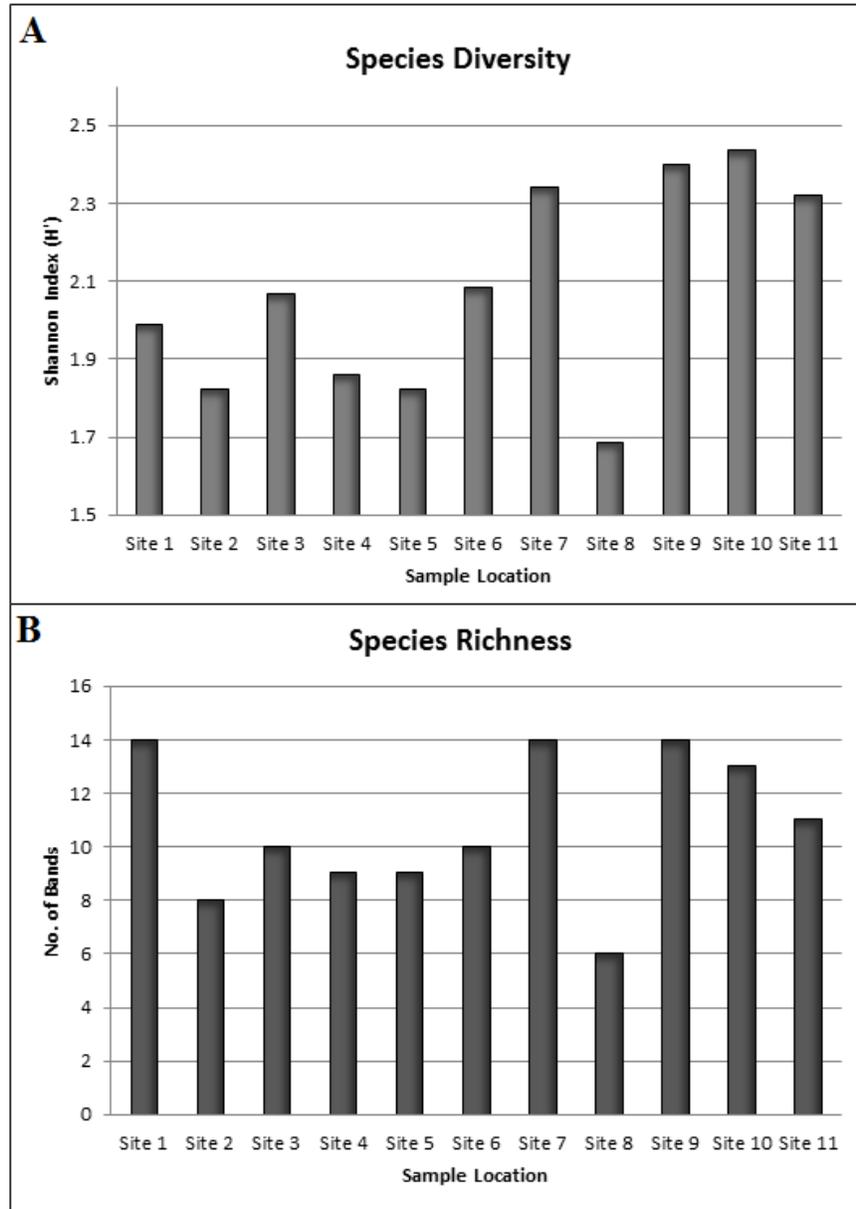


Figure 3.18. DGGE-based community analysis of Grand River samples collected July 3rd and 4th, 2012 including (A) species diversity calculated using the Shannon index (H') and (B) species richness as interpreted by the band number within the sample above background levels.

A very similar genetic profile based on banding patterns was observed across all eleven sites sampled on July 3rd and 4th, 2012 as seen in Figure 3.19. Profiles were dominated by moderate G+C content OTUs, generally with one distinct peak between approximately the 9th and 11th band movement group, and three to four peaks with a lesser intensity. On average the distinct peak accounted for anywhere from 55% to 85% of the total OTUs for individual communities collected from each of the sample sites. There was no apparent trend between the DGGE-generated banding profiles for communities extracted from urban-impacted sites versus rural-impacted sites for this sample set.

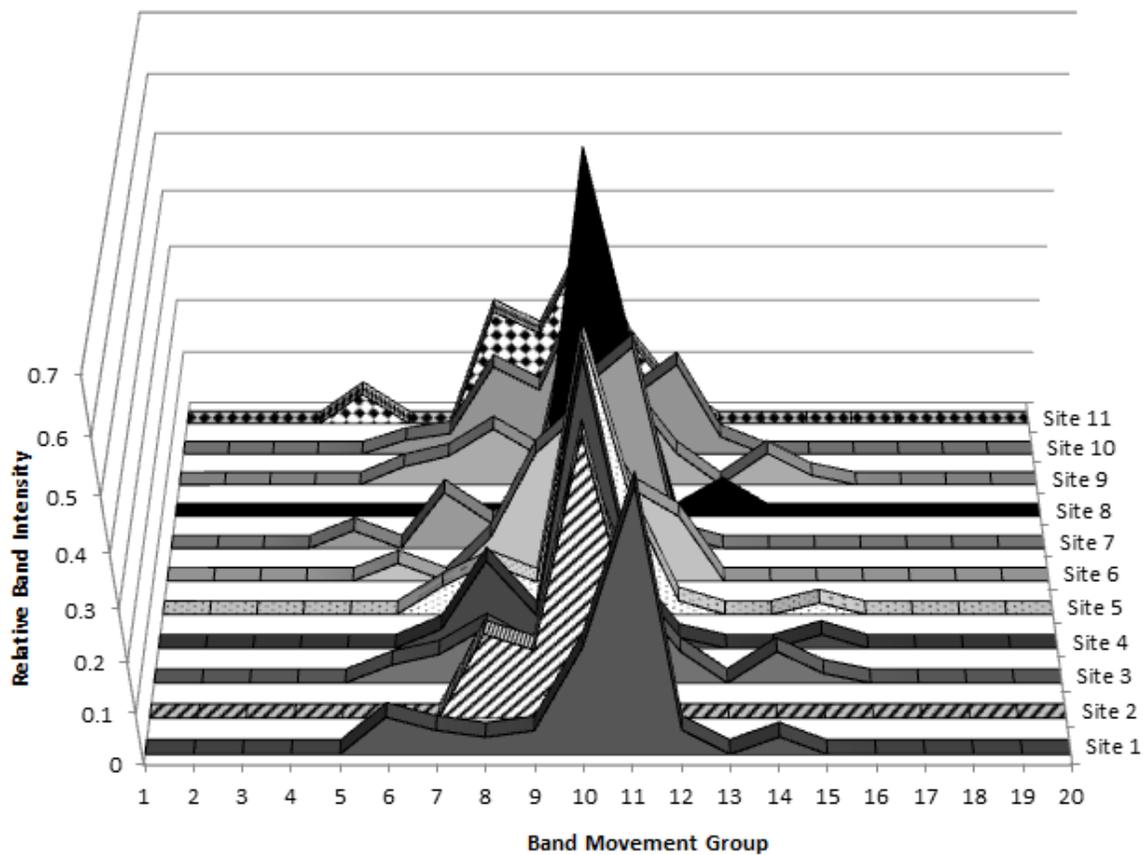


Figure 3.19. DGGE-based averaged banding patterns for site-specific Grand River samples collected July 3rd and 4th, 2012.

Clair Lake

The lowest genetic diversity was observed in the water sample collected in November when Clair Lake was newly dredged, with an H' value of less than 1.9 (Figure 3.20A). A steep rise in genetic diversity was observed in the April 27th sample followed by a very slight and gradual increase in June 6th and August 29th, such that the restoration progress of the lake correlated with a proliferation in the structural diversity of the microbial community. The peak in species diversity was observed in August with an H' value of approximately 2.5. This general trend was again reflected in the structural richness of the community across the sampling dates (Figure 3.20B). Species richness measured in the August sample was twice as high as that in the November sample, at eighteen and nine OTUs, respectively.

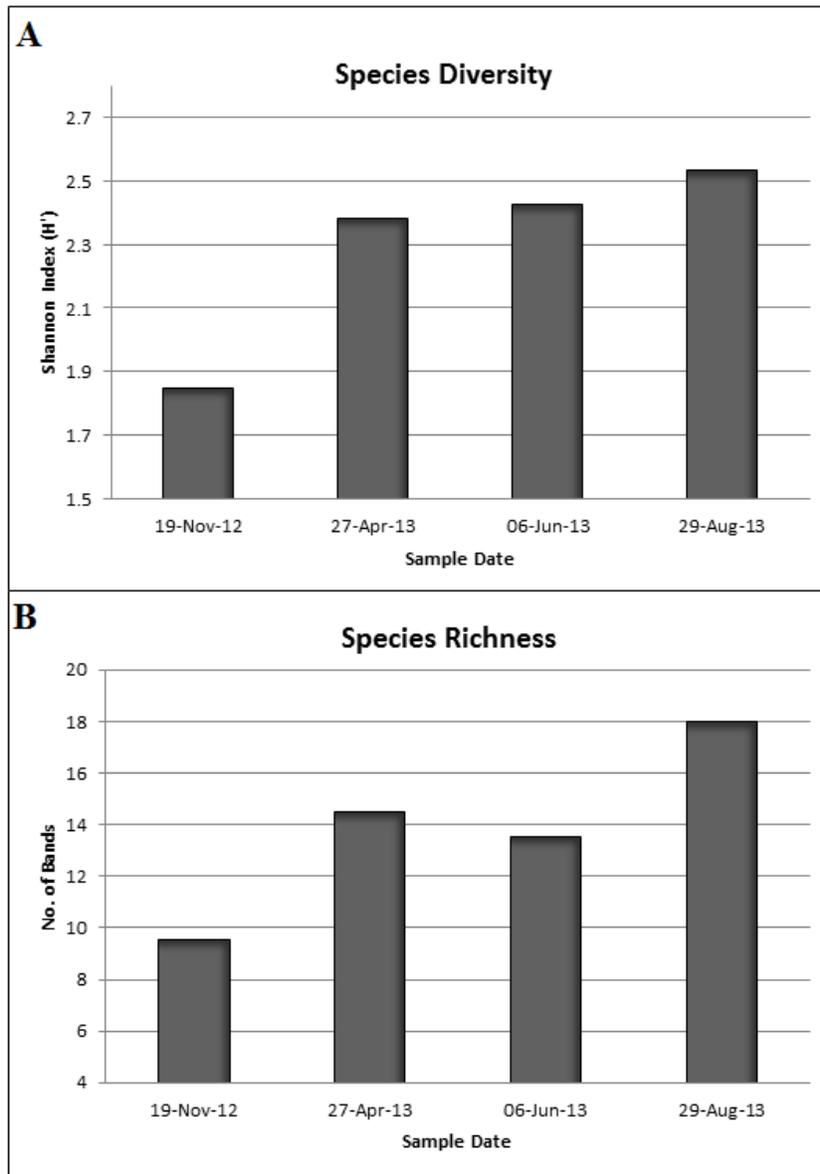


Figure 3.20. DGGE-based community analysis of Clair Lake samples collected over four sampling dates including (A) species diversity calculated using the Shannon index (H') and (B) species richness as interpreted by the band number within the sample.

Despite seasonal changes at Clair Lake (sampled over winter, spring and summer), dominant OTUs remained within similar movement groups for all collected water communities as shown in Figure 3.21. The November 19th community was dominated by two peaks of OTUs with moderate G+C content at the 6th to 9th group and 10th to 14th group, collectively comprising

100% of the relative band intensity within the sample. A similar high intensity peak was again observed in the remaining three samples at between approximately the 10th to 14th movement group with an average relative intensity of 70% of the individual samples. June and August communities displayed the most expansive genetic banding profiles, with slightly richer G+C OTUs unique to the summer samples observed.

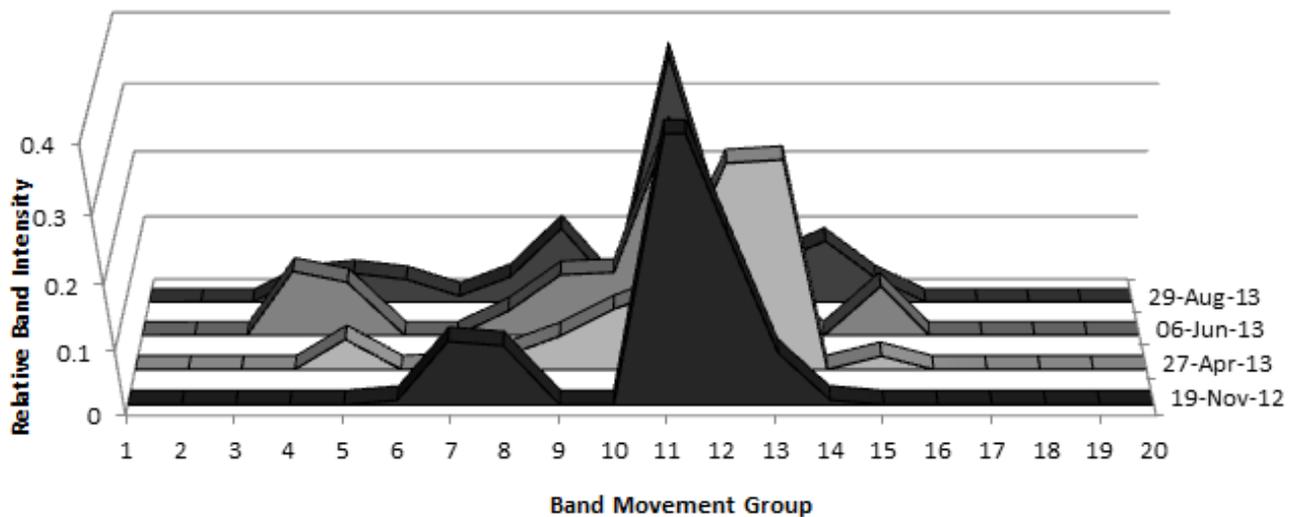


Figure 3.21. DGGE-based average banding patterns of Clair Lake samples collected over four dates.

CAWT

The observed genetic diversity was highest in the first two test vaults of the CAWT constructed wetland system (Figure 3.22A). This was followed by an apparent loss of diversity in the third and fourth vaults and a final recovery in the compositional diversity of the communities extracted from the polishing pond. Similarly, microbial communities within vaults 1 and 2 displayed the highest instances of genetic richness, while the distribution of richness across vault 3, vault 4 and the polishing pond were approximately equal (Figure 3.22B). The overall seasonal trends regarding both genetic diversity and richness are inconclusive due to variances in these

measured parameters throughout the constructed wetland system on a per season basis. However, by the time the community had reached the final polishing pond, both species diversity and richness were approximately even for communities extracted in winter, mid-summer and late summer seasons.

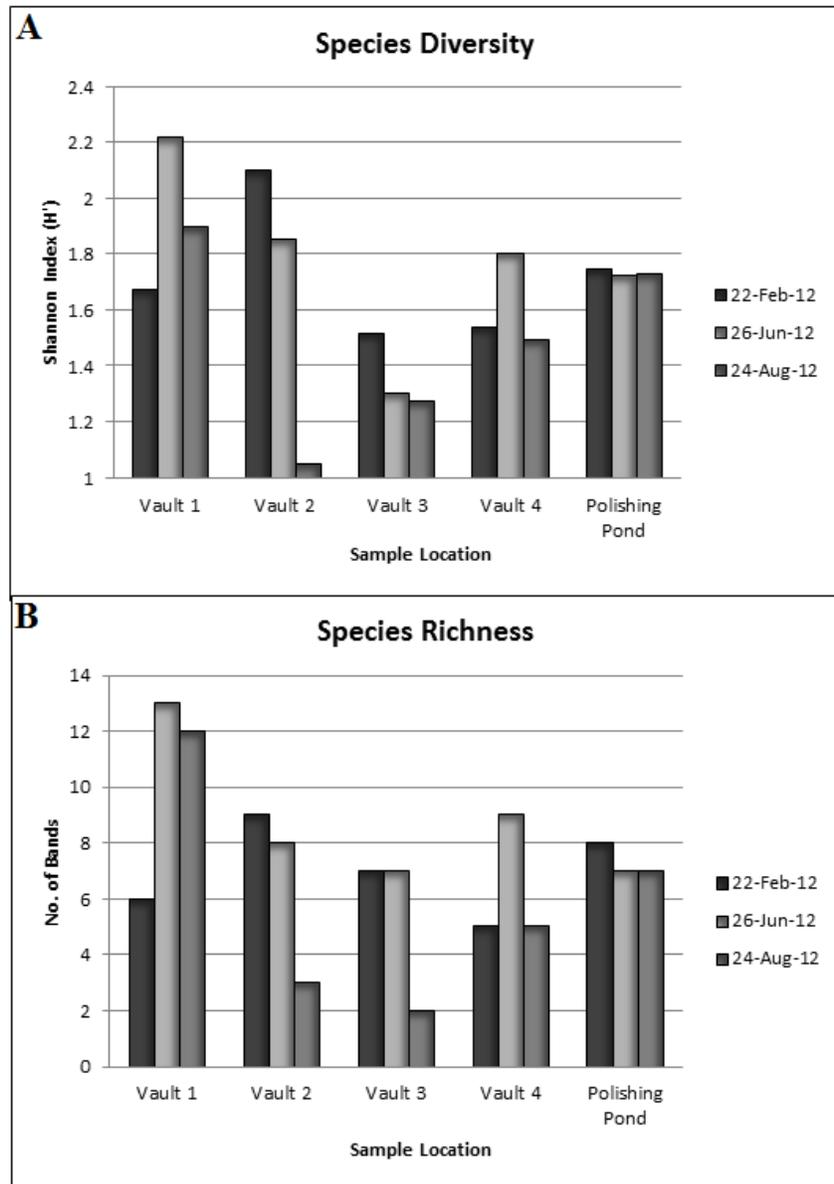


Figure 3.22. DGGE-based community analysis of CAWT samples collected according to location within the system and date including (A) species diversity calculated using the Shannon index (H') and (B) species richness as interpreted by the band number within the sample.

Changes in the abundances of observed OTUs across three seasons (winter, mid-summer and late summer) can be visualized in Figure 3.23 based on the average banding profiles of microbial communities extracted throughout the different stages of the CAWT system. While the total vault 1 community in February was contained within two peaks indicating moderate G+C richness, communities observed from June and August were more proportionally distributed across the band movement groups. Consequently, a slight increase in OTU diversity based on G+C content was observed in vault 1 during the summer months as compared to the winter month. Alternatively, the genetic structure of vault 2 remained fairly consistent throughout the three seasons resulting in a number of peaks all contributing relatively equal proportions to the community in vault 2. A slight shift in banding patterns were observed in June and August communities as compared to the February community in vault 2, indicating an increase in OTUs with greater G+C compositions during warmer months.

On August 24th, a novel and distinct G+C rich phylogenetic group was observed in vault 3 accounting for over 92% of the total community. This contrasted observations obtained in February and June, in which the communities consisted of a few moderate G+C OTUs, each contributing less than 50% of the community. Vault 4 was dominated by moderate G+C OTUs during the three sample seasons somewhat resembling the structure and distribution observed in vault 1 communities. Slight seasonal variations in the vault 4 community include a less than 10% increase in G+C rich OTUs in the June and August communities. The opposite general trend was observed in the final polishing pond communities, whereby slightly higher G+C rich OTUs were observed in the winter months compared to the summer months. However, polishing pond communities were dominated by a number of moderate G+C OTUs across all sampled seasons.

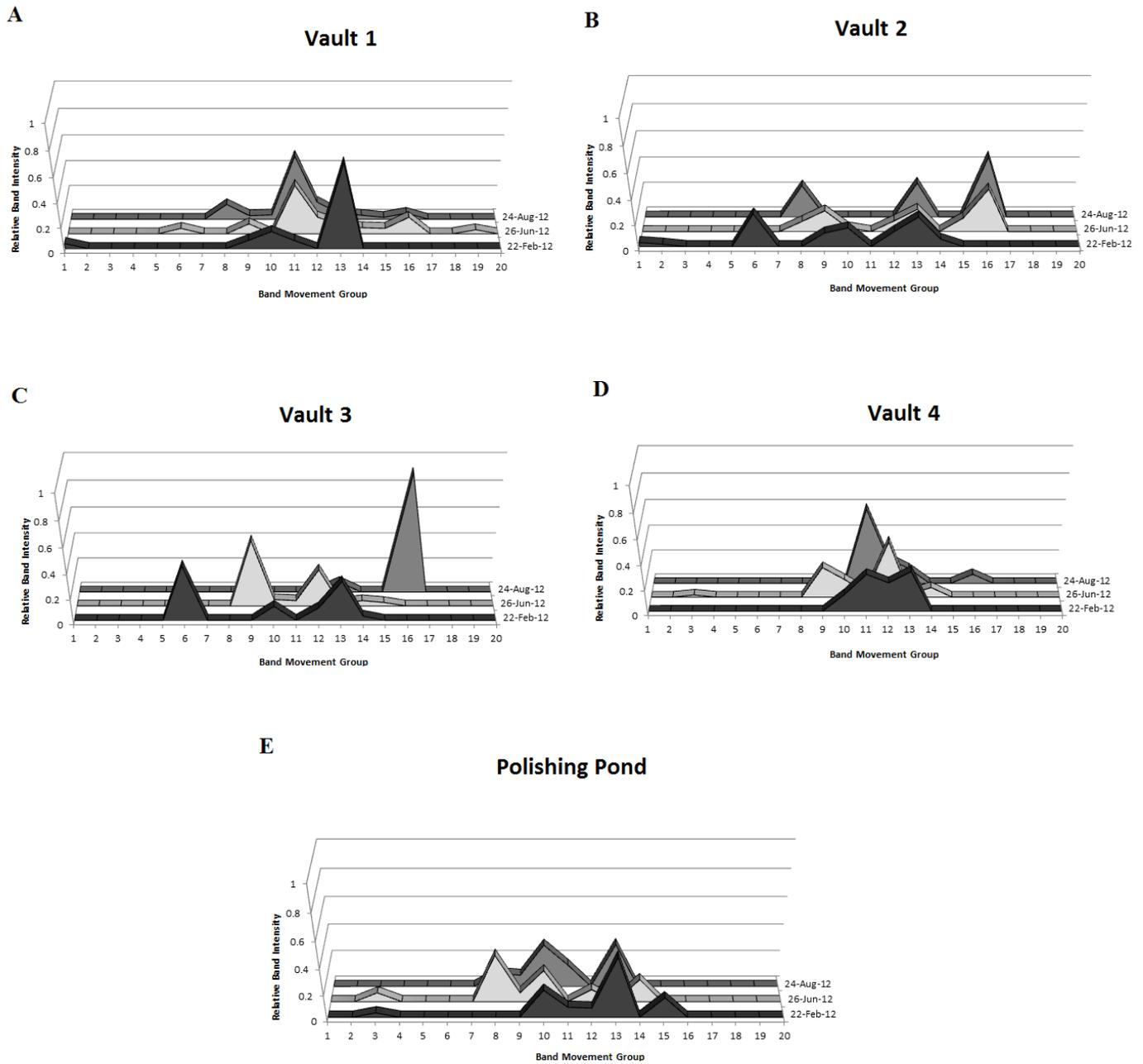


Figure 3.23. DGGE-based averaged banding patterns for the various locations of the CAWT system and sampling dates including (A) vault 1, (B) vault 2, (C) vault 3, (D) vault 4 and (E) polishing pond.

WTC

The structural composition of the microbial community extracted from the raw wastewater WTC sample was shown to be much more genetically diverse than the community from the treated sample (Figure 3.24A). Specifically, the calculated H' value for the raw community was approximately 1.5 times more than what was observed for the treated community. As illustrated in Figure 3.24B, the raw bacterial community also contained more than twice the species richness as compared to the treated community based on the presence of OTUs within the collected samples.

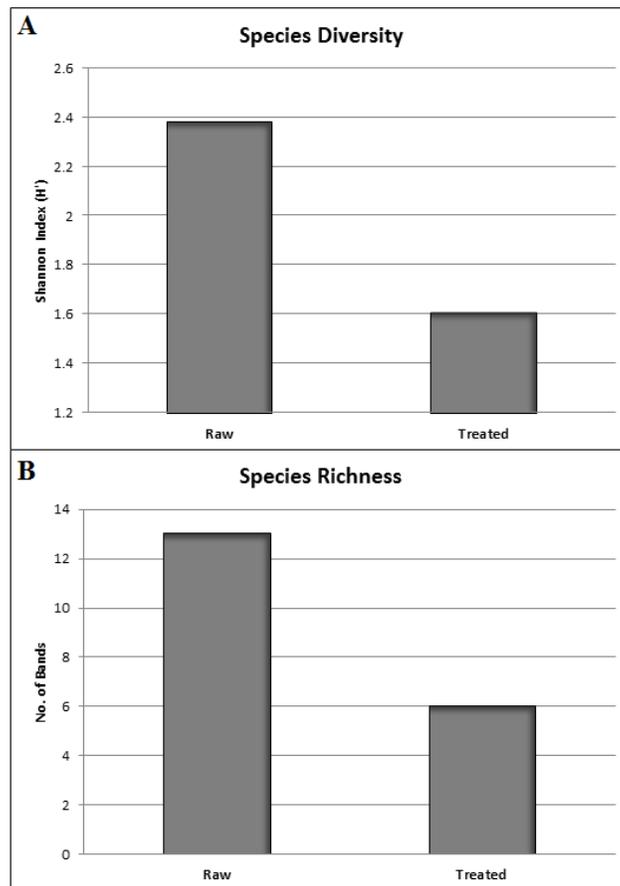


Figure 3.24. DGGE-based community analysis of raw and treated WTC samples including (A) species diversity calculated using the Shannon index (H') and (B) species richness as interpreted by the band number within the sample.

Based on the average banding patterns shown in Figure 3.25, both the raw and treated communities were dominated by two distinct peaks. Interestingly, the community exposed to the wastewater treatment process contained OTUs with both the poorest and richest G+C composition, whereas the raw wastewater community contained OTUs with moderate G+C content. The low G+C OTUs of the treated sample at the 6th band movement group and the high G+C content OTUs at the 13th group were shown to contribute 32% and 35% respectively to the treated community composition. The peak between the 7th and 9th movement group comprised approximately 53% of the raw microbial community, while the peak between the 11th and 12th group accounted for 42%.

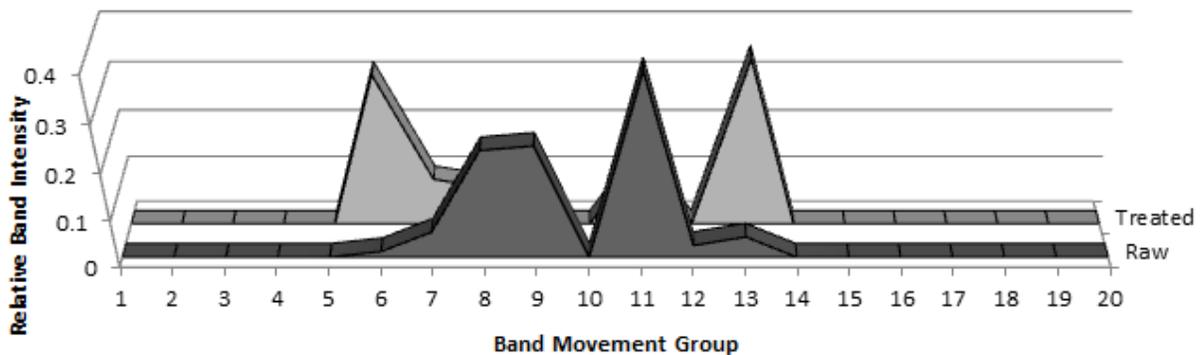


Figure 3.25. DGGE-based averaged banding patterns of raw and treated WTC samples.

3.5 Discussion

3.5.1 Spatial and Temporal Occurrence of AMR in *Salmonella* Derived from Impacted Waters

In recent years, the occurrence of resistant strains of *Salmonella* to commonly used antibiotics has become increasingly widespread in developed countries (Threlfall, 2002). In this study, AMR levels in *Salmonella* isolated from four water sources; a local watershed, a retention

area of the watershed, a constructed wetland designed for the treatment of human waste, and a wastewater treatment facility, were characterized. Due to the extensive use of antibiotics in human treatment and agriculture for purposes such as therapy and growth promotion and the resulting selective pressures imposed on microorganisms (Furuya *et al.*, 2006), it was expected that high instances of AMR would be observed in *Salmonella* isolated from heavily impacted water sources.

Approximately 68.5% (148/216) of total *Salmonella* cultured from the Grand River watershed (including Clair Lake, considered a small retention area of the Laurel Creek sub-watershed of the Grand River) demonstrated resistance to three or more of the antimicrobial drugs used in this study. The overall resistance occurrences of these isolates ranged from 20% to 50%, with the highest amount observed in *Salmonella* isolated from regions of the Grand River running through rural landscapes rather than urban landscapes, as depicted in Figure 3.1A and Figure 3.2. Similar findings were reported by Patchanee *et al.* (2010), whereby Californian watersheds contaminated by runoff from livestock productions were highly associated with resistant *Salmonella* as compared to urban rivers of the same watershed.

This trend was reinforced by the rate of MAR frequencies observed in *Salmonella* isolated from different regions of the Grand River. For example, *Salmonella* obtained from the agriculturally-impacted site 3 on April 27th, 2012 had a 92.9% (13/14) MAR frequency compared to 50% (15/30) at site 1 impacted by the upstream wastewater treatment plant and 38.2% (13/34) at site 2 downstream of farmland and an area enjoyed recreationally. Based on the sampling event on July 3rd and 4th, 2012, *Salmonella* collected from the agriculturally-impacted sites 7 through 11 exhibited MAR rates of 33.3% to 100% (Table 3.3). However, a distinct association between MAR rates in urban versus rural areas of the watershed cannot be made

from this case, as *Salmonella* was not successfully cultured from the majority of urban sites sampled (sites 3 to 6). The inability to extract *Salmonella* from these urban sites, nevertheless, may be indicative of the natural prevalence of healthy *Salmonella* in water sources contaminated by agricultural practices. Conflictingly, Thomas *et al.* (2012) experienced a higher success rate when isolating *Salmonella* from urban-impacted streams of the Grand River rather than agriculturally-impacted streams at 89% (56/63) and 48% (12/25), respectively. However, the seasonality and resulting differences in the physiochemical environment during sample collection may account for these discrepancies. Variations in temperature, hydrology and water activity, light exposure, and availability of organic substrates are all examples of characteristic seasonal and temporal changes known to influence the composition of microbial communities within streams (Hullar *et al.*, 2006).

Salmonella collection from Clair Lake in Waterloo, ON during and after the dredging process provided a unique opportunity to investigate the survival of *Salmonella* in deposited materials that have been sheltered from open environmental conditions (such as sunlight and UV inactivation, temperature fluctuations, etc.) for an extended amount of time. Interestingly, substantial differences in MAR frequencies were observed in *Salmonella* from dredged sediment as compared to the months following in which the lake was reconstructed (Figure 3.3, Table 3.4). Specifically, *Salmonella* extracted from dredged material on November 19th, 2012 displayed an MAR rate of 86.5% (64/74), while MAR in *Salmonella* collected on April 26th, 2013 decreased to 70% (14/20). No *Salmonella* (0/20) demonstrated resistance to more than two antibiotics on June 6th, 2013 when the lake could be considered restored, although seasonal effects may also play a role in these trends. Limited information is documented regarding antimicrobial resistance levels in dredged materials in freshwater lakes.

Aside from potential antimicrobial exposure, an alternative explanation for the high resistance levels observed in *Salmonella* from the watershed is that environmental pressures have been shown to alter innate drug susceptibilities in some bacteria. A number of studies have demonstrated how SOS responses induced by common environmental stresses may indirectly work to cross-protect against antimicrobial susceptibilities through regulating DNA repair systems (Hastings *et al.*, 2004; Poole, 2012; Rodríguez-Verdugo *et al.*, 2013). Consequences of imposed stresses such as greater instances of recombination, increased rates of transient mutagenesis and genetic reorganization all increase the heterogeneity of the affected population leading to overall greater survival (McMahon *et al.*, 2007). However, the idea of cross-protection is inconsistently reported in the literature and is likely stress-dependent. For instance, a 2007 study conducted by McMahon *et al.* reported greater antimicrobial susceptibility in *S. enterica* and *E. coli* following temperature stresses, while resistance to the same tested antibiotics was enhanced nearly fourfold when subjected to sub-lethal pH and salt stresses. Interestingly, baseline drug tolerance was restored in *S. enterica* upon removal of the pH and salt stresses, while elevated antibiotic tolerance was maintained in *E. coli* (McMahon *et al.*, 2007). This suggests that transiently induced stresses may lead to more permanent physiological amendments in waste-associated pathogens. However, studies typically fall short of being able to fully investigate the SOS response of bacteria to the myriad of complex abiotic and biotic stresses faced in the environment.

It was originally expected that CAWT and WTC *Salmonella* would exhibit higher AMR than *Salmonella* extracted from the Grand River watershed, as it was presumed that selective pressures resulting from antibiotic exposure would be more diluted in the watershed than that experienced by bacteria from concentrated waste sources. As *Salmonella* from the CAWT

constructed wetland travelled through the system and underwent increasingly intensive biological and physiochemical stresses, the observed MAR rates either increased or were maintained for all three seasons of sample collection (winter, mid-summer and late summer). For instance, late summer *Salmonella* collected from the polishing pond demonstrated a nearly 10% higher overall resistance occurrence and a 52.2% greater MAR frequency than did *Salmonella* collected from vault 1 in the same season (Figure 3.4 and Table 3.5). The highest overall MAR resistance was observed in the polishing pond during the winter sample collection period. However, it is probable that the resistance levels observed in pathogens extracted from the final polishing pond were affected by a neighbouring wetland system designed for the treatment of aquaculture waste which similarly makes use of the pond (see Figure A5). Nevertheless, since the final discharge of the constructed wetland system is either recycled back into the system or discharged to the municipal sewer, inadvertent transmission of resistance may be possible.

Comparable to findings regarding resistance levels in *Salmonella* within the final polishing pond of the CAWT system, *Salmonella* isolated from treated samples of the WTC were shown to have a higher percent resistance occurrence than raw isolates by over 13% (Figure 3.6A). Furthermore, treated *Salmonella* was 75% more likely to demonstrate MAR, with as much as 25% of MAR isolates demonstrating resistance to six or seven of the tested antibiotics (Figure 3.7A). Therefore, while fewer *Salmonella* isolates were successfully cultured from the treated wastewater sample, those isolates able to endure the treatment process and are thereafter released into the broader environment are likely more physiologically resilient and robust. Consequently, these organisms may prove problematic if they become inoculated into a susceptible host. Resistance findings from the CAWT and WTC treatment systems are supported by a Swiss study conducted in 2012, in which levels of multi-resistant bacteria discharged into

Lake Geneva were measured following the treatment of wastewater. While the studied wastewater treatment plant successfully reduced the bacterial loads from incoming wastewaters by 78%, Lake Geneva was identified as a reservoir of antimicrobial resistance genes and multidrug resistant pathogens as a result of its use as a receiving water body (Czekalski *et al.*, 2012).

Despite assumptions that greater overall resistance would be observed in *Salmonella* from the CAWT constructed wetland and the WTC, percent resistance occurrence and MAR rates in *Salmonella* extracted from the Grand River watershed and Clair Lake were generally higher. An explanation for this finding may be the contribution of wildlife and particularly waterfowl to the dissemination of AMR strains across the watershed originating from anthropogenically-impacted sources. While limited data is available specifically pertaining to the role of wild animals in the transmission of AMR *Salmonella*, a number of studies have investigated the occurrence of AMR *E. coli* in relation to wildlife and waterfowl activity (Sayah *et al.*, 2005; Allen *et al.*, 2010; Guenther *et al.*, 2011; Wellington *et al.*, 2013). For instance, transitory wildlife and birds which come into contact with sewage and animal manure can become carriers and spread AMR *E. coli* strains upon migration of these animals into low impacted areas (Sayah *et al.*, 2005; Wellington *et al.*, 2013). Comparably high resistance levels in Grand River *Salmonella* may also be a consequence of agricultural runoff and the overuse of antibiotics in food animals, which is believed by some researchers to be the most significant contributor to the environmental prevalence of AMR bacterial contamination due to the sheer quantity of its use in the industry (Davies and Davies, 2010). According to the Ontario Ministry of Agriculture and Food (OMAF) in 2005, antibiotic use was approved for prophylactic and treatment purposes, but not as feed additives for growth promotion. While enforced by the

Canadian Food Inspections Agency through the Feeds Act and Regulations, instances of unapproved imported drugs for “own use” and some usage of approved drugs without a proper prescription are not all together uncommon (OMAF, 2005). A final supporting explanation for the high frequency of resistance in Grand River *Salmonella* may be attributed to the watershed acting as a receiving waterway of effluent discharged from several wastewater treatment plants which also may experience the ramifications of system malfunctions and major sewage spills. As recently as October 7th, 2013, for example, a power failure at the Waterloo Wastewater Treatment Plant resulted in 860, 000 L of raw sewage being poured into the Grand River (CTV news, 2013).

Resistance Profiles of Waterborne Salmonella

Variability in resistance profiles of waterborne *Salmonella* appeared to be influenced by both source and season in this study. For instance, *Salmonella* extracted from urban and rural-impacted sites of the Grand River watershed on April 27th, 2012 demonstrated resistance to two of three 3rd generation cephalosporins tested (CTX and CRO, category I; Figure 3.1B), while *Salmonella* collected from different regions of the Grand River on July 3rd and 4th, 2012 demonstrated full susceptibility to these drugs (Table 3.2). Similarly, resistances to ciprofloxacin (CIP, category II) and sulfamethoxazole-trimethoprim (SXT, category III) were unique to Grand River *Salmonella* collected in the spring. This observed resistance has concerning implications, as third generation cephalosporins are considered category I antibiotics by Health Canada for their ‘very high importance’ in human treatment. It is the choice class of drugs for the management of salmonellosis in infected adults while fluoroquinolones such as ciprofloxacin are considered the next treatment alternative (CDC, 2013). Of additional concern, is the prevalence of chloramphenicol (C, category I) resistance observed in 18.2% of spring *Salmonella* and 8%

of summer *Salmonella* from the Grand River, as this is the choice drug for antibiotic therapy in children suffering from salmonellosis (CDC, 2013a). However, no *Salmonella* isolated from Clair Lake during all three sampling seasons demonstrated resistance to any of the 3rd generation cephalosporins, fluoroquinolones or chloramphenicols tested (Table 3.3). Further evidence of *Salmonella* resistance to 3rd generation cephalosporins was apparent in late summer CAWT isolates (Figure 3.5). Alternatively, over 20% of winter CAWT isolates and 8% of isolates from the treated WTC sample demonstrated reduced susceptibility to chloramphenicol (Figure 3.6B).

Both quinolone-resistance and 3rd generation-cephalosporin resistance in non-typhoidal *Salmonella* have been associated with the respective use of these drugs in food animals. After its introduction in the United States in 1995 as a growth-promoting additive in animal feed, the emergence of quinolone-resistant zoonotic *Salmonella* followed shortly thereafter. Accordingly, the World Health Organization (WHO) has since reserved the use of quinolones strictly for human treatment (Bager and Helmuth, 2001). Similarly, the recent detection of 3rd generation cephalosporin-resistant *Salmonella enterica* serovar Heidelberg in humans has been directly linked to the use of the drug class in Canada for the prophylactic treatment of *E. coli* infections in poultry and cattle (Furuya *et al.*, 2005; CDC, 2013b). Alternatively, the use of chloramphenicol in the agricultural industry is relatively low, whereby reduced susceptibility to the antibiotic is more likely a consequence of being encoded on a plasmid containing gene clusters conferring resistance to streptomycin, sulfanomides and tetracyclines. Thus the exposure to associated antibiotics through its widespread use in food animals, may act to indirectly drive chloramphenicol resistance (Rowe *et al.*, 1997).

Common resistances observed in *Salmonella* from all impacted water sources studied include resistances to sulfisoxazole (G), streptomycin (S), ampicillin (AM), amoxicillin (AMC),

tetracycline (TE) and doxycycline (D) (Figure 3.1B; Table 3.2; Table 3.3; Figure 3.5A; Figure 3.6B). The high resistance rates to these antibiotics are supported by findings from a Health Canada study conducted over a four year period (1994 to 1997) in which AMR trends were determined in *Salmonella* from animals, animal food products and the environments of the animals. It was found that across the test period, the highest instances of *Salmonella* resistance was observed against tetracycline, sulfisoxazole and streptomycin, with average resistances of 27.3%, 23.6% and 30.6%, respectively (Health Canada, 2001). While these resistances are relatively consistent with findings from the present study, discrepancies may be a result of differing sample environments (e.g. terrestrial environments rather than water sources), methodological approach (e.g. minimum inhibitory concentration agar dilutions rather than the disk-diffusion assay) and the passing of time in which increasing exposure and, therefore, developed resistances may have occurred.

While seasonality appeared to impact the diversity in *Salmonella* resistance profiles, trends were inconsistent between sample sites. As an example, *Salmonella* extracted from Clair Lake during dredging in November of 2012 exhibited a total of eleven different resistance profiles, compared to three resistance profiles observed in spring isolates and two in summer isolates (Table 3.3). In contrast, the greatest overall diversity in resistance to tested antibiotics was observed in CAWT *Salmonella* isolated from the system in late summer with resistance observed to ten of the twelve antimicrobial agents (Figure 3.5A). Winter CAWT *Salmonella* demonstrated the next highest resistance diversity with resistance observed to seven different antibiotics, while mid-summer isolates demonstrated resistance to six of the twelve antibiotics.

Further genetic analysis of the *Salmonella* isolates obtained in this study should be performed to generate insight into the inherent linkage between strain types and AMR profiles.

For example, Sojka *et al.* (1986) and Poppe *et al.* (1996) found that drug resistance was highly specific to *Salmonella* serovars, while Frost *et al.* (1989) made a connection between the AMR levels in particular serovars, such as *S. typhimurium* and *S. enteritidis*, and phage type.

Comparison of Antimicrobial Susceptibility in Waterborne Salmonella and the Classical Fecal Indicator Bacteria E. coli and Enterococcus spp.

For the purposes of this study, the drug susceptibilities of *E. coli* and *Enterococcus* spp. were additionally tested in order to compare levels of AMR in *Salmonella* to those found in traditional fecal indicator bacteria. It was expected to see comparable AMR levels in the enteric bacteria, since they occupy a similar ecological niche in the gut, with greater AMR resemblance observed between the genetically related *Salmonella* and *E. coli* (Poppe *et al.*, 2005).

General findings indicated that *E. coli* isolates across all studied water sources were more likely than *Salmonella* and *Enterococcus* spp. to demonstrate resistance to a single drug rather than resistance to several drugs. MAR frequencies in *E. coli* were typically less than one third of that observed in *Salmonella* isolates, and less than half observed in enterococci isolates. For instance, the average MAR rate in *E. coli* extracted from the Grand River on July 3rd and 4th, 2012 was 24.4%, compared to 70.8% of *Salmonella* and 97.9% of *Enterococcus* spp. (Table 3.2). Again, this trend was reflected in the pathogens extracted from the dredged Clair Lake material, in which only 28.6% of *E. coli* isolates could be considered multidrug resistant in comparison to 86.5% of *Salmonella* and 95.2% of enterococci (Figure 3.3). These results are in contrast to general documented findings, in which *E. coli* derived from agriculturally-impacted areas are thought to display two times the resistance seen in *Salmonella* (Government of Canada, 2007). However, results from this study are in relative agreement with those described by Jan Thomas in her 2011 PhD dissertation in which waterborne *Salmonella* isolates from urban and rural-

impacted regions of the Grand River watershed reportedly demonstrated twice the resistance observed in *E. coli*.

Overall, enterococci exhibited the greatest AMR levels from all study sites and across all sampled seasons, where resistance levels in enterococci appeared to be less dependent on season than resistances observed in *Salmonella* and *E. coli*. These high levels of AMR observed in enterococci from impacted waters are in strong agreement with literature findings (Mundy *et al.*, 2000; Hollenbeck and Rice, 2012; Castillo-Rojas *et al.*, 2013). Enterococci are typically characterized as an extensively resistant pathogen due to its vast intrinsic resistances and readily acquired resistances through sporadic mutations and uptake of foreign genetic materials. Consequently, enterococci have the potential to exhibit resistance to all clinically useful drugs, severely limiting standard treatment options (Hollenbeck and Rice, 2012). The increasing prevalence of enterococci infections based on recent surveillance data is therefore of great public concern, as it is now the third most common nosocomial pathogen in the United States accounting for 12% of hospital-acquired infections (Hollenbeck and Rice, 2012).

The relative ratio of MAR frequencies among the pathogens isolated from the CAWT constructed wetland system and the WTC for the treatment of domestic waste remained consistent, with the highest resistance occurrence observed in enterococci and the lowest in *E. coli* (Table 3.4; Figure 3.4; Figure 3.6A). Interestingly, *Salmonella* was the only pathogen to demonstrate enhanced resistance as it progressed through the treatment systems, whereby levels in *E. coli* tended to decrease and levels in *Enterococcus* spp. were relatively maintained. This pattern was especially apparent in the raw and treated samples of the WTC, where total resistance in *Salmonella* was 13.3% higher in treated samples than raw samples, compared to a 16.7% and 3.5% loss of resistance in treated *E. coli* and enterococci, respectively (Figure 3.6).

The resistance levels observed in treated *Salmonella* isolates can likely be linked to the natural hardiness of the microorganism as exemplified through its ability to manage stress (Poole, 2012).

The greatest similarity in antibiotic-specific resistances was typically observed in *Salmonella* and *E. coli*. Discrepancies in resistance profiles include slightly elevated incidences of 3rd generation cephalosporin-resistance and fluoroquinolone-resistance in *E. coli*, where increasing cases of *E. coli* resistance to 3rd generation cephalosporins have been reported in Ontario (Boerlin *et al.*, 2005; Government of Canada, 2006). Alternatively, a greater diversity in specific resistances was observed between enterococci and *Salmonella*, largely due to the vast intrinsic resistances of enterococci to a variety of drugs including 3rd generation cephalosporins, and aminoglycosides (Hollenback and Rice, 2012). Therefore, based on this study neither *E. coli* nor enterococci could accurately serve as surrogates to estimate the AMR of *Salmonella* from the same aquatic environment. While *E. coli* more accurately reflects the types of resistances observed in *Salmonella* than enterococci, the frequency of resistances would be considerably underrepresented through *E. coli* monitoring.

Quantitative Comparison of the Presence of Salmonella to the Classical Fecal Indicator Bacteria E. coli and Enterococcus spp., and Non-target Pathogen Clostridium perfringens using Quantitative Real-time PCR.

The use of the molecular technique of real-time qPCR in this study allowed for the enumeration of subject pathogens within the collected environmental water samples. Specifically, qPCR was used to quantify the following enteric microorganisms from the total DNA extracted from environmental water samples: *Salmonella* spp., *E. coli*, *Enterococcus faecalis* (considered the most environmentally-prevalent *Enterococcus* spp. (Castillo-Rojas *et al.*, 2013)), and *Clostridium perfringens*, a commonly isolated pathogen from the intestinal tract of

humans and animals as well as the environment (PHAC, 2013). This was particularly important for the detection of pathogens from the environment able to persist in a viable but non-culturable (VBNC) state which could not otherwise be quantified using culture-dependent methods. With the exception of *C. perfringens*, all pathogen types were successfully detected at every sample location.

Quantitative PCR results indicate that across all samples collected from the Grand River watershed on April 27th and July 3rd and 4th of 2012, the relative abundance of *Salmonella* and *E. coli* were typically higher than enterococci and *C. perfringens* by one or two orders of magnitude (Figure 3.8; Figure 3.9). The high prevalence of *Salmonella* was unanticipated due to the tendency of the pathogen to be present in relatively low numbers and unevenly distributed in the environment (Ryan *et al.*, 2010) and challenges the reliability of currently used fecal indicator bacteria. However, it is important to note that the relative abundance of enterococci enumerated from the environment is underrepresented in this study due to the obligatory use of species-specific primers, such that only *E. faecalis* of the many enterococci species was measured (Figure 3.10). Peaks in the abundance of *Salmonella*, *E. coli* and *E. faecalis* were observed in sites impacted by discharge from an upstream wastewater treatment plant (e.g. upstream sample of site 1 in April and site 6 in July) as well as agriculturally-impacted sites (e.g. site 3 sample locations in April and sites 7 to 11 in July), whereas this trend was less apparent with *C. perfringens*. In contrast, results obtained for Clair Lake showed that while *Salmonella* and *E. coli* concentrations were approximately one order of magnitude higher than *E. faecalis* and *C. perfringens* during dredging in November, the relative abundances of all tested pathogens were approximately equal at 10^3 gene copies per 100 mL by the time the retention area could be considered fully rehabilitated (Figure 3.10). Clair Lake is an area heavily populated with a

variety of waterfowl which are known to adversely affect the bacteriological quality of small water bodies. Higher incidences of fecal indicator bacteria, *Salmonella* and to a lesser extent *C. perfringens* have been reported in ponds with ducks and geese as opposed to those without prominent waterfowl residents (Abulreesh *et al.*, 2004).

Consistent with previously mentioned qPCR findings, high levels of *Salmonella* and *E. coli* were most frequently detected throughout the various stages of the CAWT system and across the three seasons sampled, with the highest overall abundance observed in late summer samples. These findings may be reflective of increase system usage (i.e. by returning students to campus) and/or the favourable conditions (i.e. warm temperatures) of late summer. Typically, the relative concentrations of the pathogens were highest in vaults 1 and 3, slightly reduced in vaults 3 and 4, followed by a recovery in pathogen levels in the final polishing pond (while no *C. perfringens* was detected in vault 2, 4 or the polishing pond in February) (Figure 3.11). The elevated pathogen loads detected in the final polishing pond, however, may be in part due to the treated aquaculture waste from the adjacent constructed wetland being released into the same polishing pond (Figure A5). Quantitative PCR data from the WTC revealed that for all pathogen types, concentrations were higher in raw samples than treated samples indicating at least slight removal of the bacteria following treatment (Figure 3.12). However, relative concentrations were only reduced by a single order of magnitude in *Salmonella*, *E. coli* and *C. perfringens*, and two orders of magnitude in *E. faecalis*, such that relative abundances of 10^2 to 10^4 gene copies per 100 mL are still being potentially discharged into the environment. Therefore, the high pathogen levels detected by qPCR in the polishing pond and treated sample of the WTC may give rise to the potential dissemination of these bacteria into receiving water bodies. A major limitation of traditional qPCR, however, is that it does not distinguish between DNA within viable cells

versus free DNA in the environment (Taskin *et al.*, 2011), such that an apparent lack of reduction in pathogen loads does not directly correlate with the prevalence of active pathogens being discharged.

3.5.2 Relationship between AMR levels in Waterborne *Salmonella* and the Functional and Genetic Profile of the Microbial Community from which it was Derived

Few studies to date have investigated the influence of microbial community health on drug resistance within its constituent populations. However, one such study conducted by Perron *et al.* (2012) demonstrated that incidences of bacterial recombination through the uptake of foreign genetic materials was enhanced within functionally diverse communities, such that the evolution of antimicrobial resistance in a population was driven at a more rapid pace than that resulting from a product of mutation alone. Furthermore, their findings indicated that resistance to single or multiple drugs could be developed in as little as a few generations through the exchange of resistance genes among diverse bacterial strains (Perron *et al.*, 2012). Since the majority of bacteria exist within communities consisting of hundreds to thousands of bacterial species (Fierer and Lennon, 2011), the importance of community influence on antimicrobial resistance should not be overlooked. In the present research, AMR levels in *Salmonella* were measured in relation to the health of the community (based on complementary functional and structural profiles) from which it was extracted. Functional profiles were generated using CLPP technology to determine average metabolic rates, diversity and richness, while DGGE-generated structural profiles provided insight with regard to the genetic diversity and richness of the sampled communities, in addition to their averaged banding patterns based on G+C composition.

It is important to note that the same environmentally extracted DNA was used for both qPCR and DGGE, such that it is confidently known that *Salmonella* was a contributing

constituent to the structural community profiles generated from the variously impacted water sources in this study. While G+C content has been described as an important parameter of sequence variation within bacterial genomes relaying important taxonomic information, some evolutionists have suggested that G+C composition can vary considerably in response to environmental selective pressures (Muto and Osawa, 1987; Hildebrand *et al.*, 2010; Raghavan *et al.*, 2012). For instance, stresses within specific environments may lead to genomic mutations that shape G+C composition, thereby conferring a certain level of fitness of the organism (Hildebrand *et al.*, 2010). However, available knowledge in this area is severely limited and largely theory-based. Therefore, it is impossible to identify which OTU(s) within the DGGE-generated profile are *Salmonella* without excising bands and sending them for sequencing. Nevertheless, taxonomically *Enterobacteriaceae* such as *Salmonella* and *E. coli* are considered relatively G+C rich (Desai *et al.*, 2013).

While functional profiles for Grand River samples were not obtained within this study due to the introduction of BiologTM technology following the onset of the project, DGGE was still performed to obtain a genetic fingerprint of the communities. A greater community structural diversity was observed in agriculturally-impacted regions of the Grand River, including upstream and downstream sample locations of site 3 of the April sampling event and sites 7 through 11 from the July sampling event (Figure 3.16A; Figure 3.18A) where resistance levels in *Salmonella* were highest. Due to the interconnected relationship between diversity and richness, this trend was similarly reflected by the species richness within the communities (Figure 3.16B; Figure 3.18B). Comparisons between community diversity among differing sites was reinforced by averaged banding patterns, which revealed greater variation and band movement group distribution in the phylotypes (represented by bands or OTUs) constituting

communities from rural landscapes as compared to urban landscapes (Figure 3.17; Figure 3.19). From these findings, it was apparent that rurally-impacted regions of the Grand River harboured more phylogenetically distinct taxa than urban areas, likely due to the availability of organic nutrients within agricultural wastes which supports diverse microbial growth (Burkholder *et al.*, 2007). The amalgamation of OTUs with similar G+C composition in ribosomal DNA into the same band movement groups is somewhat misleading with regard to the interpretation of community structure, but does account for the fact that taxa with similar G+C content are phylogenetically similar (Wayne *et al.*, 1987).

Shifts in the functional and structural profiles of Clair Lake microbial communities were apparent across samples collected during different seasons and different stages of lake rehabilitation. In the midst of dredging in November of 2012, functional trends were similar to those in communities sampled from the restored lake in spring and summer months. While slower to metabolize the available carbon sources as expressed by AWCD trends, the microbial activity of the community within the dredged material demonstrated utilization of 90% of the available carbon sources after 96 hours (Figure 3.13A and C). Although metabolic diversity of the November community was lower than in other sampled months, catabolic diversity within the community can still be considered high with an H' value of greater than 3 (Figure 3.13B). Conversely, structural-related data revealed that the genetic integrity of the dredged sample community was much lower than in other months, with markedly greater diversity and twice the species richness observed in the August community, for example (Figure 3.20). Community averaged banding patterns similarly displayed a more expansive genetic profile in the spring and summer months compared to the November community (Figure 3.21). Therefore, it is likely that observed catabolic activity was linked to an increased physiological stress response in the select

microbial species able to endure unfavourable environmental conditions for long durations (Campbell *et al.*, 2001). Moreover, 86.5% of *Salmonella* isolated from dredged samples expressed resistance to three or more antimicrobials, compared to no incidences of multiple drug resistance in *Salmonella* isolated from Clair Lake in June. This is an indication of the natural hardiness of organisms able to persist long-term despite severe environmental conditions and further evidence towards the association between resistance and tolerance mechanisms as previously discussed.

The metabolic and genetic community profiles extracted from the various stages of the CAWT constructed wetland system were more in sync than that observed in Clair Lake samples (note: CLPP data is absent for the February sampling event). The same general trend was observed across all seasons sampled, whereby bacterial communities within the first and second vaults appeared to have the greatest integrity as expressed by physiological and structural diversity and richness (Figure 3.9; Figure 3.22). A loss in community fitness was observed in vaults 3 and 4, but was restored within the final polishing pond communities. Thus, these findings suggest that bacterial communities were able to functionally and structurally recover before being discharged into the municipal sewer or recycled back into the wetland system; a trend consistent with observed AMR levels in *Salmonella* (Figure 3.4). However, community dynamics were impacted by a neighbouring wetland system designed for the treatment of aquaculture waste which similarly makes use of the polishing pond (see Figure A5). Due to the variation in bacterial types associated with human and fish wastes, both community functionality and structure were likely to be enhanced. Seasonality effects were most explicit when considering the averaged banding patterns generated by communities from the different locations within the constructed wetland (Figure 3.23). With the exception of the polishing pond, an

increase in phylogenetic groups with rich G+C compositions was apparent in warmer months, while overall the number of dominant taxa remained consistent. This indicates that while the diversity of phylotypes with distinct sequence identities was maintained in the various stages of the CAWT system over the three seasons sampled (as reflected by band movement group assignments), the conditions of the wetland in the warmer months likely favoured more genetically stable (i.e. G+C rich) groups than those extracted in February. Given that *Salmonella* is typically considered G+C rich in genomic content (Desai *et al.*, 2013), it can loosely be deduced that *Salmonella* persistence in the CAWT system in June and August was more likely than in February.

In the final studied aquatic environment, the functional profiles of the bacterial communities from raw and treated WTC samples initially differed in their capacity to metabolize the available nutrient sources, whereby the community from the raw waste sample demonstrated greater functional versatility than the community from the treated sample (Figure 3.10). By 96 hours of incubation, however, the functional abilities of the communities extracted from the two sample types equalized, and by 114 hours the treated community surpassed the raw community in terms of metabolic rate. The catabolic resiliency of the microorganisms within the treated sample comparably emulates resistance levels observed in *Salmonella* isolates, where the total percent resistance occurrence within *Salmonella* from the treated sample was over 13% higher than *Salmonella* extracted from the raw sample (Figure 3.6A). Again, this evidence strengthens the idea that microorganisms able to endure stresses imposed by wastewater treatment may lead to the dissemination of hardy and resistant microorganisms into the broader environment. Since DGGE provides a structural snap shot of the community at the time of DNA extraction, it was expected that a greater fitness would be observed in the raw community as compared to the

treated community. Accordingly, the resulting genetic profiles revealed 1.5 times greater diversity and 2 times the richness in the raw community over the treatment community (Figure 3.24). Interestingly, the banding profile generated from the treated community contained phylogenetic members with both the lowest and highest G+C content observed (Figure 3.25), perhaps signifying that taxa with moderate G+C content are more likely to be eliminated during the disinfectant process. Results from this study, therefore, suggest that there is a relationship between AMR in *Salmonella* and microbial community health.

3.6 Conclusions

In the present study, the physiological tolerance and natural hardiness of *Salmonella* was exemplified by high antimicrobial resistance levels observed in Clair Lake samples during dredging, the polishing pond of the CAWT system, and treated samples of the WTC, all of which are exposed to physiochemical and biological stresses beyond typical environmental pressures. It was found that resistance profiles expressed by environmental *Salmonella* were more comparable to *E. coli* than enterococci, while the frequency of resistance observed in *Salmonella* was typically much higher than that of *E. coli*. Furthermore, the temporal influence on AMR levels was inconsistent amongst sampling locations and microorganisms, which may be a result of the physiological response to the unique combination of stresses imposed within each impacted water source. Based on quantitative real-time PCR, the relative abundance of *Salmonella* and *E. coli* were typically one to two orders of magnitude higher than *E. faecalis* and *C. perfringens* across all study sites. Lastly, functional and structural fingerprinting of microbial communities revealed that AMR *Salmonella* was harboured in both genetically and metabolically diverse communities, as well as communities demonstrating high metabolic resiliency but low genetic diversity. Therefore, AMR levels of *Salmonella* derived from different

community situations may be a consequence of both increased opportunities for the exchange of genetic material in healthy communities, and a physiological stress response to low-integrity communities.

Mounting evidence in the literature, and results from this study, suggest that aquatic environments may be an important reservoir of AMR *Salmonella* which dually serves as a vehicle for the transmission of the zoonotic pathogen into the broader environment (Winfield and Groisman, 2003; Spector and Kenyon, 2012). The contamination of waterways with *Salmonella* demonstrating reduced susceptibilities to commonly used antibiotics (such as 3rd generation cephalosporins, fluoroquinolones and chloramphenicol) has considerable implications for public health and may result in treatment failures upon infection of a host. Despite legislation instated for the regulation of antibiotic usage and surveillance programs like NARMS and CIPARIS for monitoring resistant levels in enteric pathogens, incidences of multidrug resistance are being increasingly documented in *Salmonella* (Threlfall, 2002). Continued surveillance will aid in defining the association of AMR levels in human and food-producing animal pathogens, while new focus should be emphasized on characterizing AMR levels in zoonotic bacteria in non-host environments.

3.7 Recommendations and Future Research Needs

- Year-round samples should be collected in particular from the Grand River regions to better elucidate the seasonality of *Salmonella* including persistence and AMR levels from urban and rural-impacted waterways.
- *Salmonella* isolates obtained in this study should undergo further genetic analysis, including serotyping and phage-typing, to generate insight into the inherent linkage between strain types and AMR profiles.

- To bypass the limitations of qPCR, the quantification of mRNA should be performed for the analysis of genes being actively expressed to serve as an indicator of viable *Salmonella*. Furthermore, the expression of additional genes of interest should be measured including those associated with *Salmonella* pathogenicity and biofilm formation.
- Pulsed-field gel electrophoresis should be performed to aid in the identification of the dominant sources of AMR *Salmonella* contamination, to reveal the genetic relatedness of *Salmonella* isolates from this study, and to better elucidate the overall epidemiology of the enteric pathogen.
- Beyond phenotypic antimicrobial resistance profiling, transmission of resistance genes on the cellular level should be investigated to better understand the context in which resistance is mediated in enteric pathogens such as *Salmonella*.
- Additional studies should be performed that centers on the impact of microbial community integrity on antimicrobial resistance levels in its constituent pathogenic populations. In this way, the ecology of antibiotic resistance, including fate, transport and occurrence in microbial communities will be better understood.

3.8 References

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Chapter 4

Investigating the “Naturalization” Potential of Environmentally-isolated *Salmonella*: A Microcosm Study

4.1 Introduction

Health and economic-related consequences of *Salmonella* contamination, particularly with regard to food products and waterways, has motivated an increasing number of studies centred on the survival and transmission of this enteric pathogen outside of a host (Winfield and Groisman, 2003; Carbral, 2010). While the primary niche of *Salmonella* is thought to be within animal hosts, the ability of *Salmonella* to successfully colonize the natural environment indicates that the zoonotic pathogen is readily capable of non-host specific adaptations. Furthermore, *Salmonella* has proven to be a resilient pathogen genetically endowed to withstand stresses present not only within microenvironments of the host, but also the natural environment despite the myriad of abiotic and biotic pressures that oppose its survival (Spector and Kenyon, 2012). This is of particular importance, as the prospect of reinoculation into its next host is correlated with its long-term persistence ability in the broader environment. In turn, the response of *Salmonella* to environmental stresses not only dictates its growth and survival, but also impacts virulence and expression of antimicrobial resistances (McMahon *et al.*, 2007; Spector and Kenyon, 2012). As previously discussed in Chapter 3, antimicrobial resistance profiles can

provide a phenotypic marker for “naturalization” due to the generalized response of *Salmonella* for stress management resulting in cross-resistances (Spector and Kenyon, 2012).

Soil and aquatic microcosms designed to simulate natural environmental conditions have been widely used in the field of microbial ecology as a means to investigate the influence of abiotic and biotic parameters on pathogen persistence in relation to community dynamics (Sjogren, 1993; Garcia *et al.*, 2010). While it is difficult to emulate the interconnected biological, chemical and physical factors existing within the complex environment, the use of microcosm experiments allows for the manipulation of selected conditions within a controlled setting to generate reproducible results with low variability between replicates as compared to field studies (Hill and Top, 1998). In combination with culture-based and molecular-based fingerprinting techniques, microcosms can be prepared to establish functional and structural community profiles to monitor physiological responses leading to pathogen survival in the open environment (Hill and Top, 1998; Garcia *et al.*, 2010).

4.2 Research Need and Objectives

Due to its relevance as a waste-associated microorganism, the majority of *Salmonella* survival studies to date have been conducted in association with sludge or manure-amended soil, where the nutrient rich environment has been shown to support *Salmonella* longevity external to a host (Hill and Top, 1998; Garcia *et al.*, 2010; Rogers *et al.*, 2011). More research needs to be conducted with regard to *Salmonella* persistence in nutrient-diluted environments, such as downstream of urban and agricultural runoff, in order to get a true sense of the ability of *Salmonella* to manage broader environmental pressures upon dissemination. In this study, microcosms were used to assess the physiological response of *Salmonella* derived from different sources to imposed “naturalization”-simulated conditions, as reflected by shifts in antimicrobial

resistance profiles, population dynamics, and the functional and structural profiles of the microcosm communities.

The specific objectives of this chapter were to:

- Assess and compare the effects of three temperatures (4°C, 12°C and room temperature) on the survival and potential “naturalization” of various *Salmonella* strains using batch system microcosms designed to simulate a retention area environment.
- Measure differences in *Salmonella* survivability through *invA* gene quantification using qPCR and stress response through AMR analysis.
- Characterize the temporal changes in the functional and structural profiles of microbial communities within the various microcosm types over the duration of the experiment through heterotrophic plate counts (HPCs), community-level physiological profiling (CLPP) and denaturing gradient gel electrophoresis (DGGE).

4.3 Materials and Methods

A detailed overview of the methodological approach taken in this study is provided in Chapter 2. Information included in this section is limited to aspects unique to this portion of the study.

4.3.1 Microcosm Design and Experimental Parameters

Triplicate microcosms containing sediment and water from the Clair Lake retention area were prepared in sterile 250 mL glass flasks sealed with rubber stoppers covered in parafilm to prevent leaching of rubber chemicals. Prior to inoculation, 75 g (wet weight) of soil was placed in each flask whereafter Clair Lake water was added to the 150 mL mark on the flask. The flasks were then covered in tin foil as a means to eliminate light exposure as a source of experimental variability, and left for 24 hrs at room temperature to allow for acclimation.

The experimental design involved four microcosm test conditions each prepared in triplicate and incubated at 4°C, 12°C and room temperature (approximately $21 \pm 2^\circ\text{C}$, hereafter denoted as 22°C for simplicity) for a total of thirty-six microcosms. One such microcosm test condition labelled the “sterile control” (SC) flask was prepared by first autoclaving the flask and its Clair Lake contents at 121°C for 45 mins to remove as much as possible of the background microbial community and thereby reduce biotic interactions. Heterotrophic plate counts (HPC, methodology explained below) performed post-sterilization revealed the persistence of some bacteria, although growth was reduced by 3 to 4 log orders of magnitude (final counts of approximately 10^2 - 10^3 CFU/ mL from initial 10^5 - 10^6 CFU/ mL in Clair Lake background levels). While some studies have shown that sterilization of media through autoclaving may alter the physiochemical composition of the soil post-sterilization (Razavi and Lakzian, 2007), the effects are considered relatively inconsequential and was considered as such for the purposes of this study. Following sterilization, SC microcosms were then spiked with a lab strain of *Salmonella enterica* ser. *typhimurium* (ATCC 13311®, Inverness Medical Inc., Ottawa, ON, Canada). The flask contents of the remaining three microcosm types did not undergo sterilization such that the biotic pressures of predation and competition for available resources was a factor throughout the duration of the 58 day experiment. The “biological control” (BC) microcosm was inoculated with a lab strain of *Salmonella enterica* ser. *typhimurium* (ATCC 13311®, Inverness Medical Inc., Ottawa, ON, Canada) and *E. coli* (ATCC 12292®, Inverness Medical Inc., Ottawa ON, Canada), the “Clair Lake” (CL) microcosm was spiked with a multidrug resistant (MDR) strain of *Salmonella* isolated from dredged material of Clair Lake (resistance profile AmAmcGTeS), and the “Water Technology Centre” (WTC) microcosm was inoculated with a

MDR strain of *Salmonella* isolated from treated wastewater (resistance profile AmDGS). See Table 4.1 for a summary of each of the four test conditions.

Table 4.1. An overview of the four microcosm test conditions of this study.

Test Condition Label	Water and sediment	Inoculum
Sterile control (SC)	sterile	lab strain of <i>Salmonella</i> (ATCC® 13311)
Biological control (BC)	unaltered	lab strains of <i>Salmonella</i> (ATCC® 13311) and <i>E. coli</i> (ATCC® 12292)
Clair Lake (CL)	unaltered	strain of MDR <i>Salmonella</i> derived from Clair Lake dredge samples in Waterloo, ON
Water technology Centre (WTC)	unaltered	strain of MDR <i>Salmonella</i> derived from the treated sample of the Water Technology Centre in Burlington, ON

The microcosms were subjected to a series of culture-dependent and independent analyses to measure the stress response of both the community and *Salmonella*. Shown below in Figure 4.1 is an overview of the experimental design.

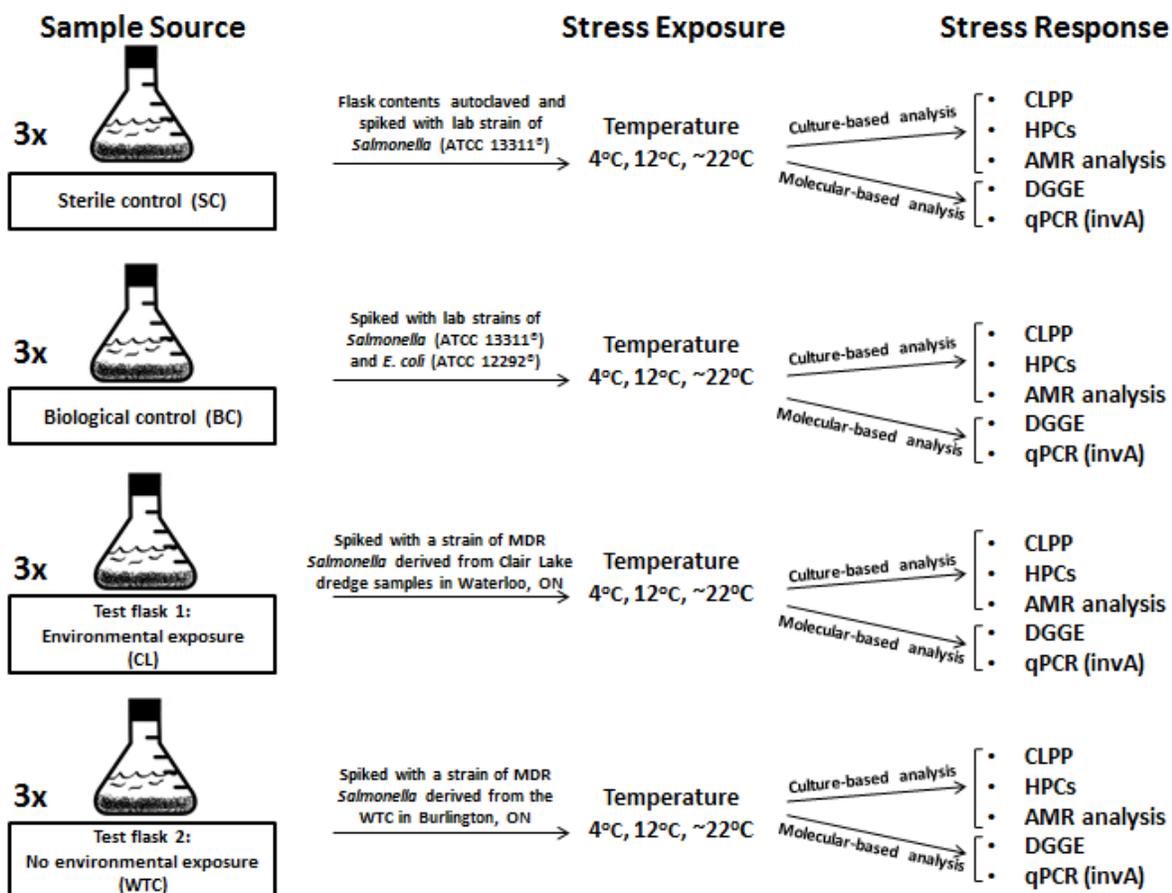


Figure 4.1. A summary representation of the experimental approach taken to investigate the potential for *Salmonella* “naturalization” using a series of microcosms. The three test temperatures were selected as characteristic average temperatures of winter, fall/spring and summer seasons.

Each isolate used to inoculate the microcosms was taken from frozen (-80°C) cultures and grown in 10 mL of LB broth at 37°C for at least one hour until an OD_{600nm} reading of approximately 0.1 was attained. Subsequently, the culture was centrifuged (10,000 xg, 10 mins) and the cells were resuspended and washed twice in sodium-free phosphate buffer. The total inoculum volume for each microcosm was 1% (v/w) of the soil weight with a density of approximately 3.0 to 4.0E+05 CFU/ mL based on plate counts on LB agar. Throughout the experiment, microcosms were continually shaken at 100 rpm on an orbital shaker to simulate the gentle movement of the Clair Lake environment, promoting opportunity for microbiological interactions and preventing the settling out of organisms. Following 5 seconds of intensive flask

swirling, 800 μL of microcosm materials were obtained with which to perform a DNA extraction. This volume was selected due to the capacity constraints of equipment provided in the PowerSoil™ DNA isolation kit (Mo Bio Laboratories Inc., CA). The running conditions for the experiment are summarized in Table 4.2.

Table 4.2. An overview of the experimental parameters of the microcosm setup.

Experimental Parameters	
Incubation temperature ($^{\circ}\text{C}$)	4, 12 and 21 ± 2
Inoculum density (CFU/mL), Inoculum volume	3-4.0E+05, 1% volume to soil weight
Total run time	58 days at each of the three temperatures (4 $^{\circ}\text{C}$, 12 $^{\circ}\text{C}$ and ~22 $^{\circ}\text{C}$)
Orbital speed (rpm)	100
Sample days (HPCs, DNA extraction, DGGE, qPCR)	0, 1, 2, 4, 7, 11, 16, 19, 24, 30, 44, 58
Sample days (CLPP, AMR analysis)	0, 4, 8, 16, 32, 45, 58*
Amount of soil/water for DNA extraction	800 μL

*AMR analysis not performed on day 58.

As previously mentioned, each of the test conditions were prepared in triplicate. Heterotrophic plate counts (explained below) were performed for all replicates on the specified sample days to monitor background levels of the microcosm communities over the course of the experiment. For all other analyses, a single replicate of each of the test conditions was selected at random at each of the incubation temperatures due to cost and time constraints.

4.3.2 Heterotrophic Plate Counts

Heterotrophic plate counts (HPCs), also commonly referred to as standard plate counts, are considered a classical microbiological technique used to measure the microbial flora within water or other media types requiring an external source of organic nutrients for growth

(organisms typically termed heterotrophs) (Allen *et al.*, 2004). Specifically, HPCs are traditionally used to measure the bacteriological quality of a water source based on colony forming units (CFU). In this study, the HPC method was employed to monitor the overall health and die-off of microorganisms within the microcosms over the duration of the experiment.

The HPC procedure was performed according to the standard method described by APHA (1998). Following the creation of a 10^0 to 10^7 dilution series using 9 mL of sodium-free phosphate buffer, 100 μ L from each dilution was spread plated in duplicate onto R2A agar (BD Difco, Fisher Scientific, Whitby, ON). HPCs were then counted after 5 to 7 days of incubation at room temperature, where plates with less than 30 or more than 300 colonies were considered statistically invalid and excluded from CFU per mL calculations.

The primary limitation of the HPC method is that only a very small fraction or subpopulation of the total bacterial community present within a given sample is successfully enumerated on the agar plate (approximately 1%). This procedural drawback is largely a consequence of the complex nutritional requirements of certain heterotrophs as well as the presence of bacteria able to persist in a viable but non-culturable (VBNC) state (Allen *et al.*, 2004). To investigate HPC bias, a comparison was made between DNA extracted from plating 1 gram of Clair Lake soil according to standard methods (APHA, 1998) and DNA extracted directly from 1 gram of Clair Lake soil. The DNA was then subject to PCR-DGGE to generate a banding profile from which the diversity and richness of the samples were calculated (see Figure B1 for results). See Figure B2 for DGGE image.

4.4 Results

4.4.1 Bacteriological Quality of Microcosms Based on HPCs

In order to determine an appropriate concentration of the *Salmonella* inoculum with which to spike the microcosms, HPCs were performed to establish the background heterotrophic level within Clair Lake water and sediment (Figure 4.2). Given that the resulting counts between the media types ranged from approximately $4.0E+4$ to $7.0E+05$ CFU/ mL in the water sample and soil sample, respectively, an inoculum concentration within the same range of approximately 3.0 to $4.0E+05$ CFU/ mL was used. This was considered a suitable density that would not be expected to overwhelm the microcosm community yet still be environmentally-relevant in the case of waste contamination.

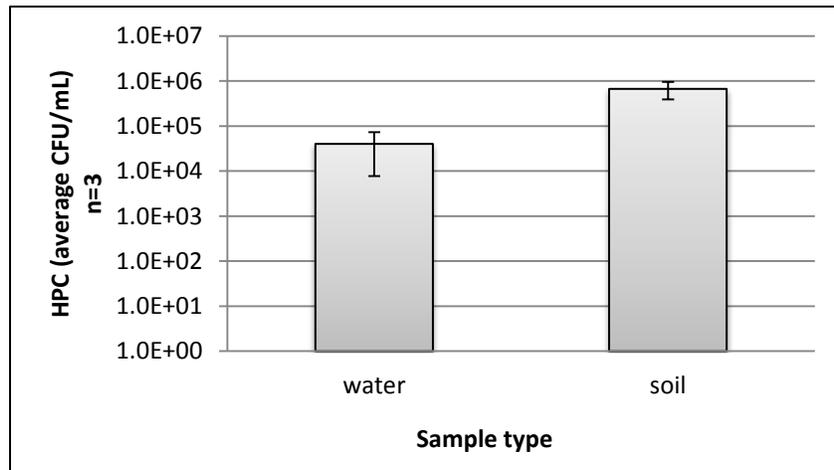


Figure 4.2. A graph depicting heterotrophic plate counts from Clair Lake water and soil samples at point of collection of microcosm materials on June 6th, 2013. Counts were read on R2A plates following 7 days of incubation at room temperature (approximately 22°C). HPCs were performed in triplicate.

Throughout the 58 day duration of the experiment, HPCs were performed to monitor the overall bacteriological quality of the four microcosm types incubated at each of the three test temperatures. In this way, the sustainability of the culturable heterotrophic portion of the

microcosm community could be measured to give an estimation of the overall microbial health of the microcosms and die-off of the heterotrophic population. Figure 4.3 provides a summary of these findings.

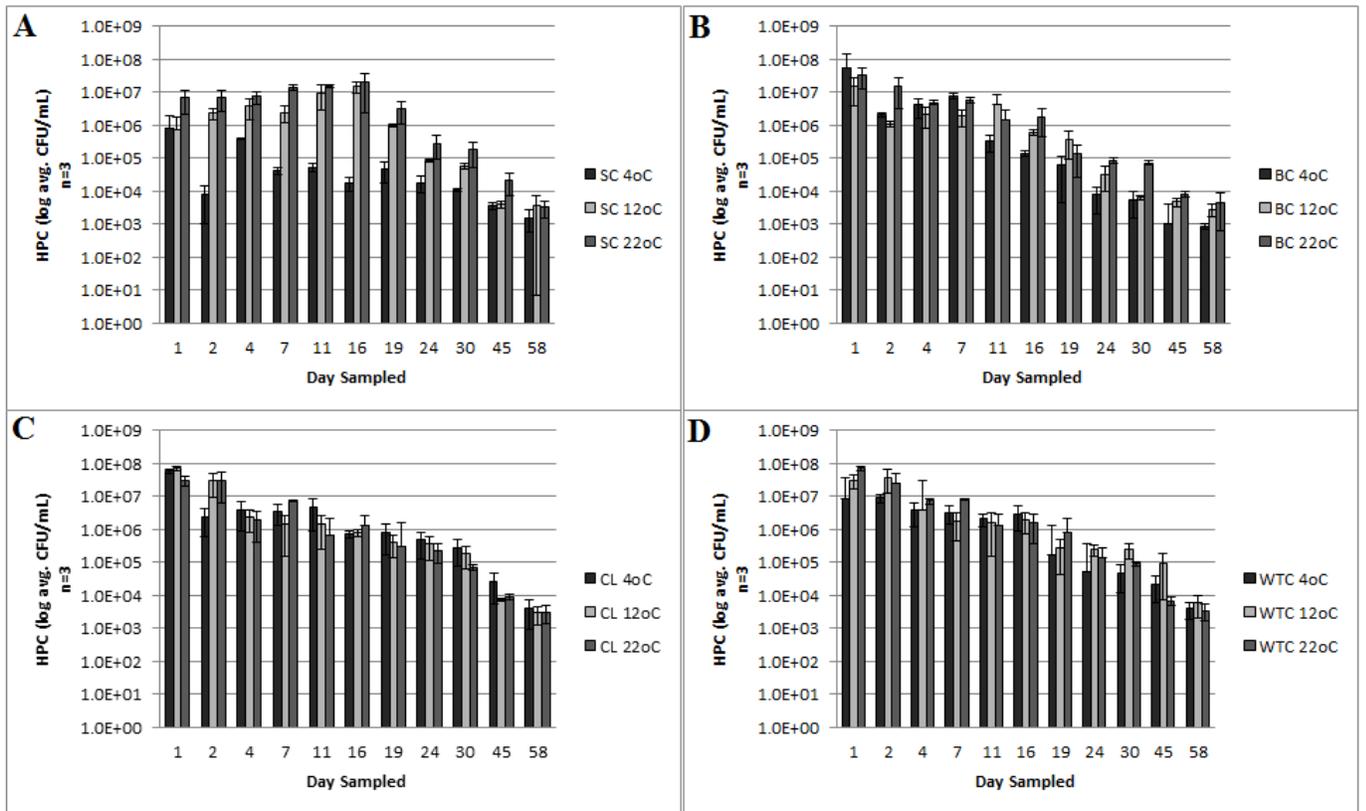


Figure 4.3. Graphical representation of heterotrophic plate counts including (A) sterile control (SC), (B) biological control (BC), (C) Clair Lake (CL) and (D) Water Technology Center (WTC) test flasks, each incubated in triplicate at 4°C, 12°C, and 22°C.

Incubation temperature appeared to have the greatest influence on heterotrophic growth in SC microcosms, whereby plate counts from microcosms incubated at 12°C and 22°C were typically one to three orders of magnitude higher than counts collected from SC microcosms incubated at 4°C across the experimental test period (Figure 4.3A). Unique to results obtained for SC microcosms at 12°C and 22°C, was a gradual increase in count numbers up until day 16 peaking at approximately 10⁷ CFU/ mL, followed by a steady decline to a final concentration of

just under 10^4 CFU/ mL on day 58. Alternatively, a slow two log reduction was observed from SC microcosms incubated at 4°C over the 58 days. A similar trend was observed for the BC, CL and WTC microcosms, such that a gradual loss in heterotrophic counts was observed from day 1 to day 58, all with an approximately equal final concentration of about 10^3 to 10^4 CFU/ mL (Figure 4.3B-D). While HPC results from the CL and WTC microcosms demonstrated inconsistent variation in relative abundances at the three different incubation temperatures, BC microcosms expressed slightly elevated counts at the warmer test temperatures.

4.4.2 Quantitative Detection of *Salmonella* using qPCR

Figure 4.4 illustrates the relative abundance of target *invA* gene copies per 100 mL indicating the presence of *Salmonella* spp. within each of the four microcosm types (see Table 2.1 for the primers and probe used for *invA* detection). Similar to HPC result findings, *Salmonella* concentrations most notably varied in response to temperature in SC microcosms, where levels of the enteric pathogen were generally one to two orders of magnitude higher when incubated at 12°C and 22°C as compared to 4°C . Conversely, incubation temperature did not appear to impact the relative abundance of *Salmonella* within BC, CL and WTC microcosms. A peak in the abundance of *Salmonella* was observed within the SC microcosms between days 1 to 4 at approximately 10^9 gene copies/ 100 mL. In all microcosm varieties, *Salmonella* DNA levels were consistently maintained over the duration of the 58 days at levels of approximately 10^7 gene copies/ 100 mL in SC microcosms and 10^5 gene copies/ 100 mL in BC, CL and WTC microcosms.

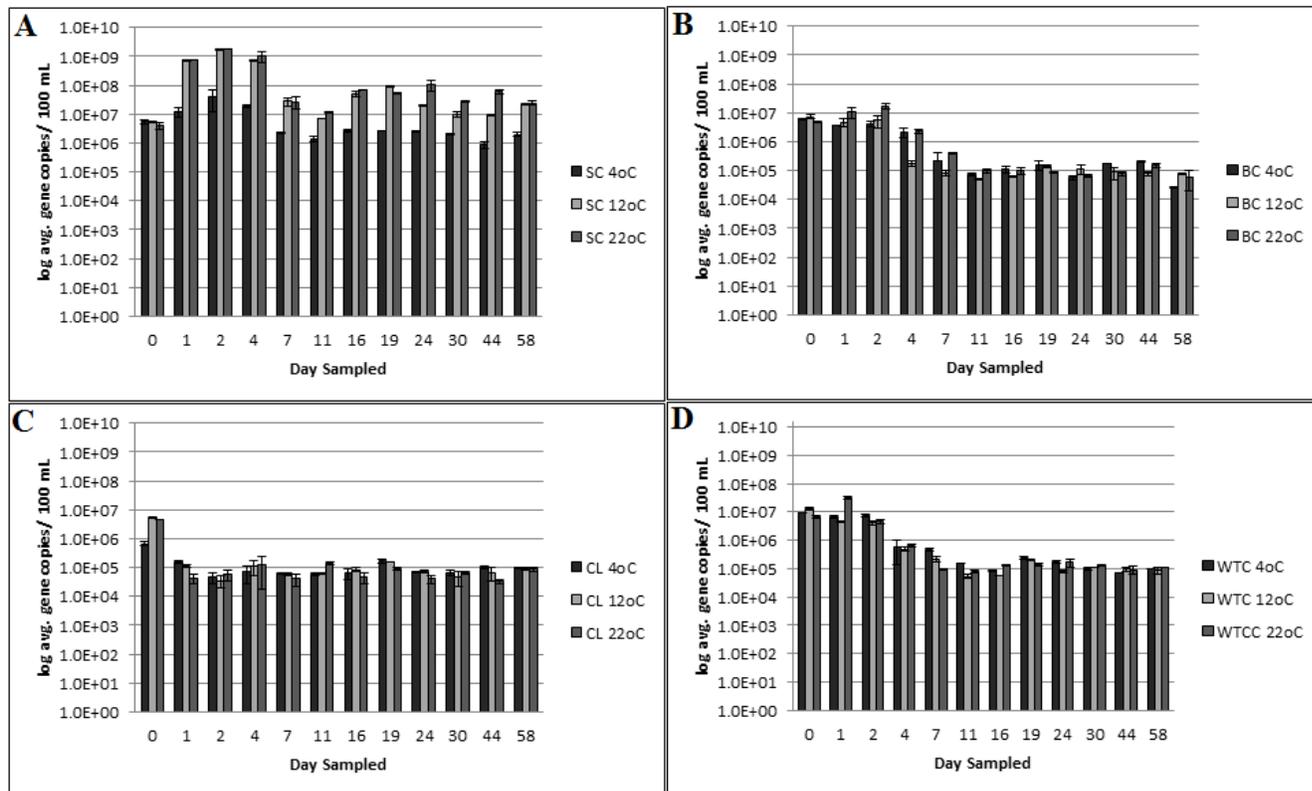


Figure 4.4. Relative abundance (target gene copies/100 mL) of *invA* gene for the quantification of *Salmonella* over the duration of 58 days within (A) sterile control (SC), (B) biological control (BC), (C) Clair Lake (CL) and (D) Water Technology Center (WTC) test flasks, incubated at 4°C, 12°C, and 22°C. DNA extractions from a single test condition replicate at each incubation temperature were examined in triplicate for qPCR.

4.4.3 Antimicrobial Resistance Profiles of *Salmonella* within Test Microcosms

The resiliency of *Salmonella* within the four microcosm types was in part assessed through the relative differences in percent resistance occurrence over the course of the experiment as compared to results from an initial resistance screening upon inoculation of the microcosms (illustrated in Figure 4.5). No observable trends were apparent in SC and BC microcosms spiked with a lab strain of *Salmonella*, such that relative increases or reductions in antibiotic susceptibilities were seemingly sporadic over the 45 days. Following the 45 days of incubation, however, the highest resistance occurrence observed in *Salmonella* recovered from

SC and BC microcosms was apparent in those incubated at 4°C, with a relative increase of 8.33% and 2.08% in overall antibiotic resistance, respectively.

Alternatively, resistance levels in *Salmonella* isolated from CL and WTC microcosms generally expressed reduced drug susceptibilities as the study period progressed. In environmental *Salmonella* extracted from CL microcosms, different trends were observed for each of the three incubation temperatures. For instance, an overall increase in resistance was observed at 22°C until Day 45 where initial resistance levels were restored. Similarly, a gradual increase in resistance was observed in 12°C microcosms, peaking after the 45 days with a 20.83% higher resistance than levels observed in *Salmonella* on day 0. Interestingly, *Salmonella* extracted from CL microcosms stored at 4°C demonstrated a 12.50% increase in resistance on day 45, where only slightly elevated resistance levels were detected on the other tested days. Overall, resistance levels within *Salmonella* from the WTC microcosms incubated at each of the three incubation temperatures demonstrated a gradual increase in resistance to the tested antibiotics as compared to day 0 levels. The final percent resistance levels recorded on day 45 were 11.11%, 10.42% and 6.94% higher than on the first day for microcosms at 4°C, 12°C and 22°C, respectively. Therefore, in all cases, an overall increase in resistance towards the tested antibiotics was apparent in *Salmonella* isolated from each the four microcosm types incubated at 4°C.

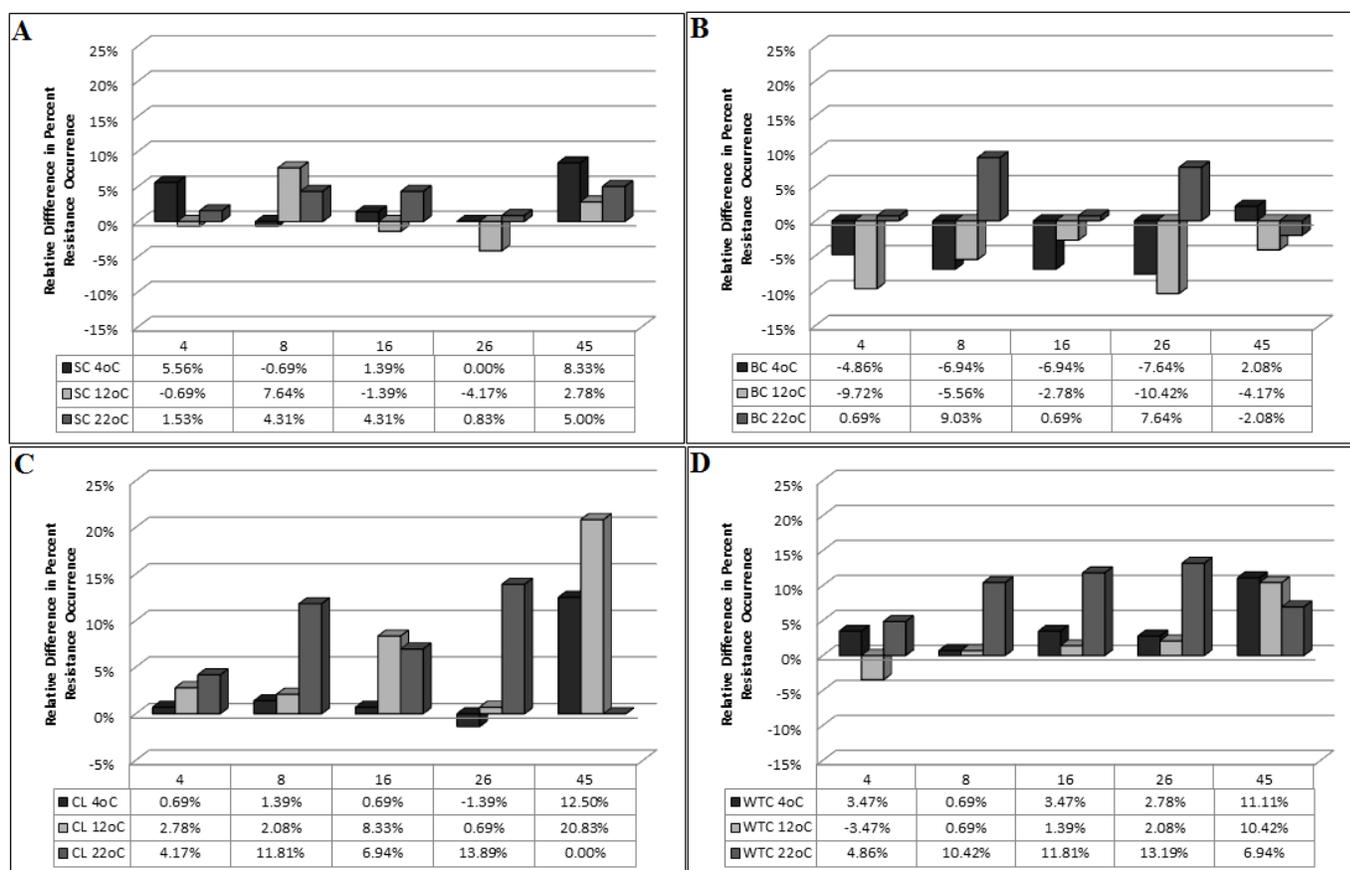


Figure 4.5. Relative difference in the percent resistance occurrence of *Salmonella* over the duration of 45 days within (A) sterile control (SC), (B) biological control (BC), (C) Clair Lake (CL) and (D) Water Technology Center (WTC) test flasks, incubated at 4°C, 12°C, and 22°C. Trends are based on 12 representative *Salmonella* isolates extracted from a single replicate of each of the 12 test conditions.

The overall frequency of multiple antimicrobial resistance (MAR), defined as resistance to three or more of the tested antibiotics, for *Salmonella* isolated from each of the four microcosm types over 45 days is summarized in Table 4.3. The highest overall MAR frequencies were observed in *Salmonella* derived from the WTC microcosms at each of the three incubation temperatures, followed by *Salmonella* isolated from CL, BC and SC microcosms, respectively. As was observed with relative differences in percent resistance occurrence, MAR rates seemed to

vary somewhat inconsistently across the test period. On average, however, relatively high MAR rates were observed within all microcosm types on day 45.

Table 4.3. Frequency (%) of MAR in *Salmonella* isolated from the four test microcosms at 4°C, 12°C, and 22°C over the duration of 45 days. Trends are based on 12 representative *Salmonella* isolates extracted from a single replicate of each of the 12 test conditions.

MAR frequency (No. of MAR isolates/ total isolates) for each day sampled								
Microcosm Type	Incubation Temp. (°C)	Day 0	Day 4	Day 8	Day 16	Day 26	Day 45	Total % MAR^a
SC	4	8.33% (1/12)	58.3% (7/12)	0.0% (0/12)	0.0% (0/12)	0.0% (0/12)	75.0% (9/12)	23.6% (17/72)
	12	8.33% (1/12)	41.7% (5/12)	41.7% (5/12)	16.7% (2/12)	0.0% (0/12)	83.3% (10/12)	31.9% (23/72)
	22	0.0% (0/12)	8.33% (1/12)	41.7% (5/12)	25.0% (3/12)	8.33% (1/12)	50.0% (6/12)	22.2% (16/72)
BC	4	25.0% (3/12)	33.3% (4/12)	0.0% (0/12)	16.7% (2/12)	0.0% (0/12)	100% (12/12)	29.2% (21/72)
	12	41.7% (5/12)	8.33% (1/12)	50.0% (6/12)	75.0% (9/12)	0.0% (0/12)	66.7% (8/12)	40.3% (29/72)
	22	0.0% (0/12)	8.33% (1/12)	91.7% (11/12)	8.33% (1/12)	50.0% (6/12)	0.0% (0/12)	26.4% (19/72)
CL	4	33.3% (4/12)	33.3% (4/12)	41.7% (5/12)	25.0% (3/12)	8.33% (1/12)	100% (12/12)	40.3% (29/72)
	12	33.3% (4/12)	33.3% (4/12)	25.0% (3/12)	66.7% (8/12)	8.33% (1/12)	100% (12/12)	44.4% (32/72)
	22	58.3% (7/12)	41.7% (5/12)	100% (12/12)	66.7% (8/12)	75.0% (9/12)	0.0% (0/5)	63.1% (41/65)
WTC	4	33.3% (4/12)	83.3% (10/12)	33.3% (4/12)	75.0% (9/12)	58.3% (7/12)	100% (12/12)	63.9% (46/72)
	12	41.7% (5/12)	8.33% (1/12)	41.7% (5/12)	75.0% (9/12)	50.0% (6/12)	100% (12/12)	52.8% (38/72)
	22	58.3% (7/12)	41.7% (5/12)	91.7% (11/12)	91.7% (11/12)	91.7% (11/12)	83.3% (10/12)	76.4% (55/72)

^aMAR, Isolates displaying multiple antimicrobial resistance patterns to ≥ 3 antimicrobial compounds.

4.4.4 Functional Fingerprinting of Microcosm Communities through CLPP

Average well colour development (AWCD) trends indicate that there were variations in the metabolic rates of the microbial communities within the four microcosm types incubated at 4°C, 12°C and 22°C (Figure 4.6). Of the SC microcosms, overall those incubated at 22°C demonstrated the greatest metabolic rate, followed by communities at 12°C and 4°C. Alternatively, BC, CL and WTC microcosms, all containing background communities in addition to the specified inoculum, revealed the reverse trend, whereby rates of carbon metabolism were highest in microcosms incubated at 4°C and lowest at 22°C. Over the duration of 58 days, a gradual decline in metabolic efficiencies was observed in BC, CL and WTC microcosm types at each of the incubated temperatures. This differed from microbial communities within the SC microcosms where a steep decline in metabolic rates was observed within the first ten days followed by a levelling out in efficiencies for the duration of the test period. The microbial community within the SC microcosms expressed the lowest overall capacity for metabolizing the available carbon sources, while similar rates of substrate metabolism were observed in the remaining three microcosm test conditions.

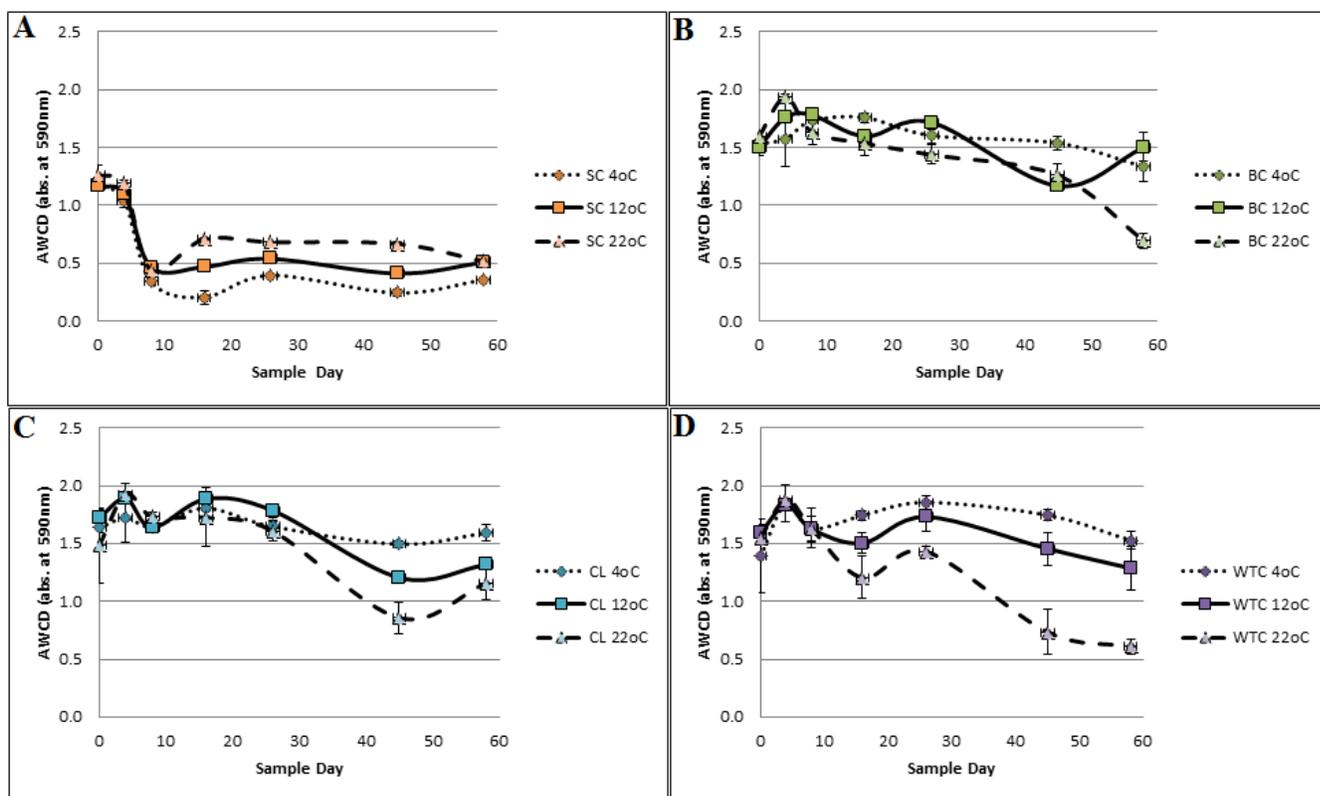


Figure 4.6. CLPP-based average well colour development of microbial communities within (A) sterile control (SC), (B) biological control (BC), (C) Clair Lake (CL) and (D) Water Technology Center (WTC) test flasks, incubated at 4°C, 12°C, and 22°C. Trends are based on microcosm samples extracted from a single replicate of each of the 12 test conditions.

Based on the Shannon Index (H'), the functional diversity of the tested microbial communities reflected a similar pattern to that established by AWCD rates. For instance, the highest functional diversity within BC, CL and WTC microcosms was observed at 4°C and the lowest at 22°C (Figure 4.7), where again the opposite was true for the metabolic diversity expressed by SC microcosms. Therefore, as the incubation temperature decreased, the metabolic diversity of microcosm communities containing interspecies biological interactions appeared to increase. However, variation according to temperature was less apparent in terms of metabolic diversity than metabolic rate. For instance, at each of the three incubation temperatures, comparable degrees of metabolic diversity were expressed by communities in the three

microcosms containing Clair Lake background flora. The opposite tendency was again observed in the SC microcosm in which the background flora was largely removed, such that the functional diversity at 22°C was much lower than that observed in microcosms stored at 4°C and 12°C.

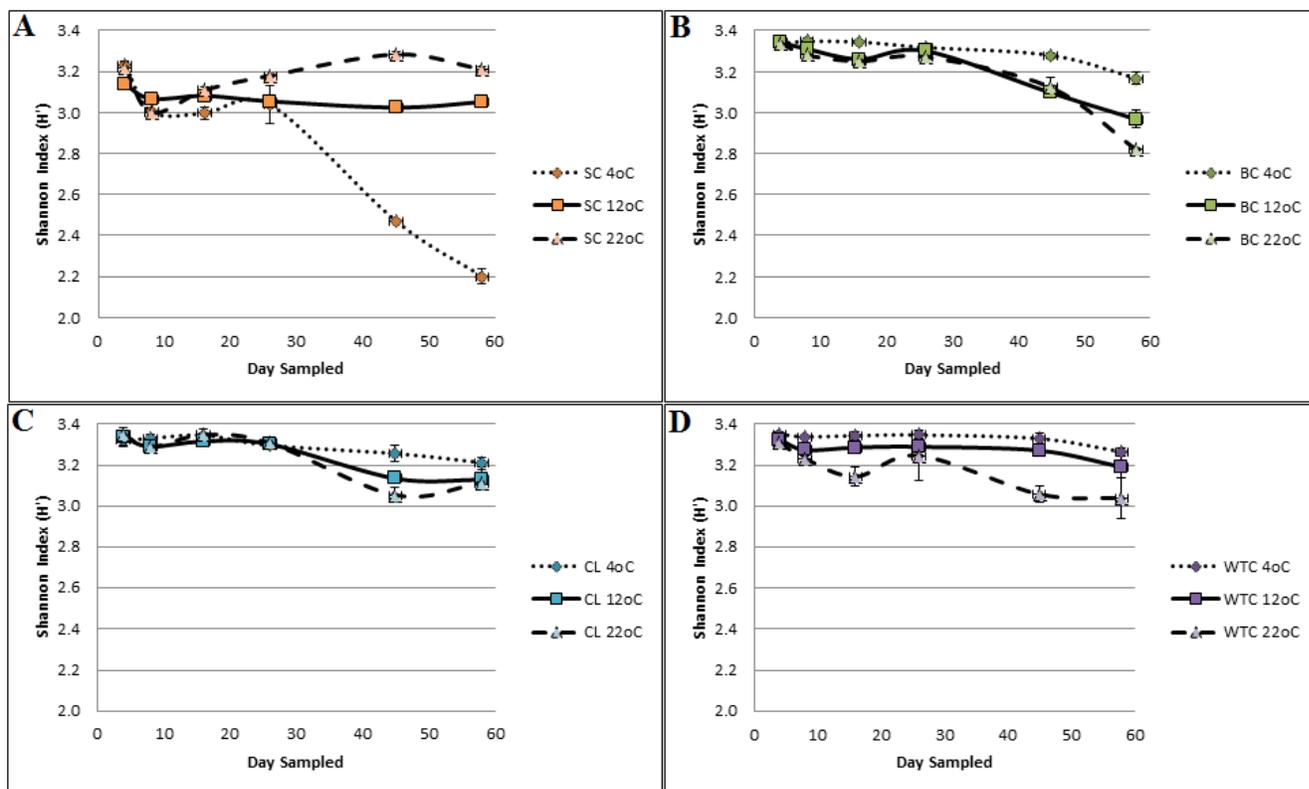


Figure 4.7. CLPP-based community metabolic diversity calculated using the Shannon Index (H') within each of the test microcosms: (A) sterile control (SC), (B) biological control (BC), (C) Clair Lake (CL) and (D) Water Technology Center (WTC) test flasks, incubated at 4°C, 12°C, and 22°C. Trends are based on microcosm samples extracted from a single replicate of each of the 12 test conditions.

The functional profiles of the microcosm communities were also characterized according to their expressed metabolic richness, defined as the total number of carbon sources metabolized resulting in an OD_{590nm} absorbance reading of 0.25 or higher, and results are summarized in Figure 4.8. Again, microbial communities within the SC microcosms demonstrated the overall lowest capacity to utilize the available nutrient sources, with the lowest metabolic richness

observed in SC communities stored at 4°C. As was observed with calculated AWCDs, within the first ten days of incubation at each of the three test temperatures, the functionality of the SC community dropped and then stabilized for the duration of the experiment. At the conclusion of the experimental period, SC microcosms incubated at the warmer temperatures were able to utilize approximately 15 of the 31 available sources while those stored at 4°C metabolized approximately 5 sources. Trends in metabolic richness observed for BC, CL and WTC microcosms were comparable overall such that communities incubated at 4°C and 12°C initially demonstrated the ability to utilize all 31 carbon sources to approximately 25 carbon sources by the end of 58 days. Conversely, microcosms kept at 22°C expressed the capacity to metabolize 15 of the carbon sources within BC and WTC microcosms, and 20 carbon sources within CL microcosms by 58 days.

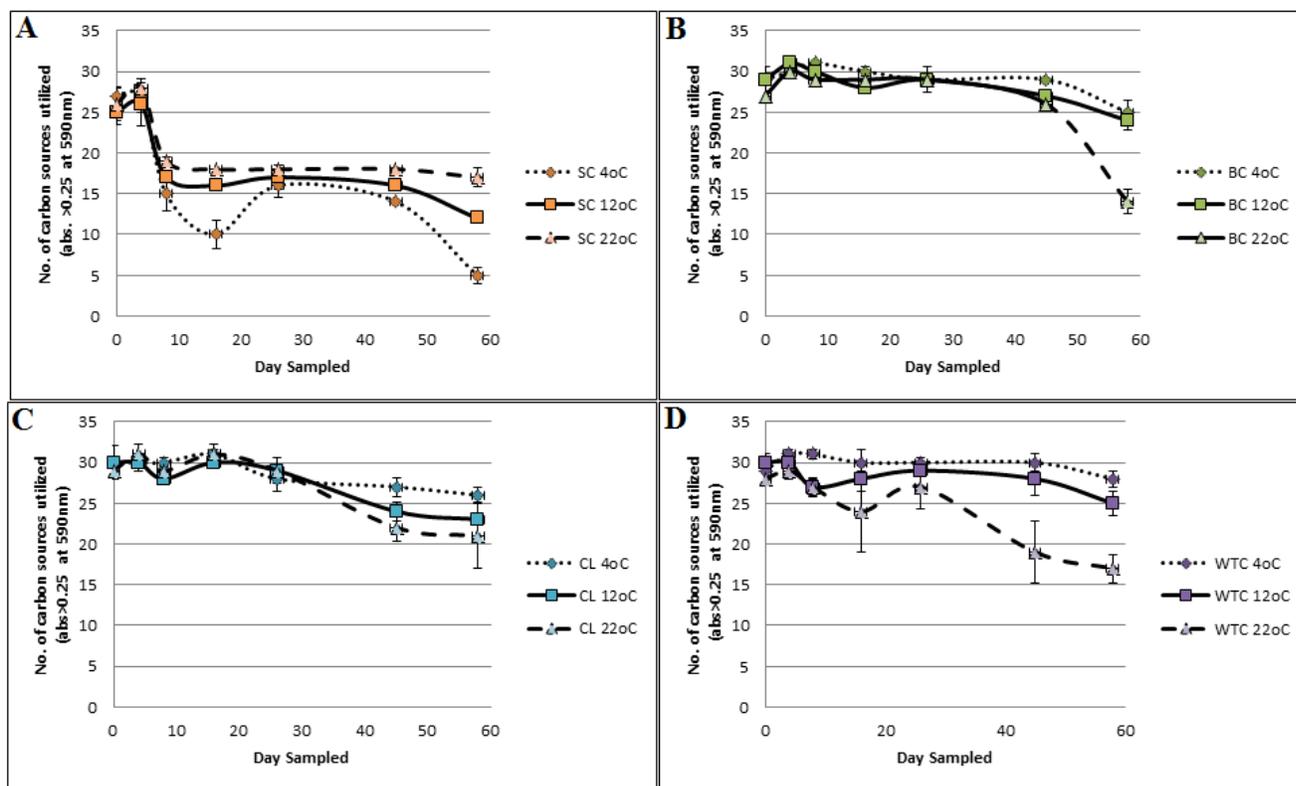


Figure 4.8. CLPP-based metabolic richness of microbial communities within (A) sterile control (SC), (B) biological control (BC), (C) Clair Lake (CL) and (D) Water Technology Center (WTC) test flasks, incubated at 4°C, 12°C, and 22°C. Trends are based on microcosm samples extracted from a single replicate of each of the 12 test conditions.

4.4.5 Structural Fingerprinting of Microcosm Communities through DGGE

Species diversity, a proportional measure of the richness and evenness of a sample defined by the Shannon Index (H'), was calculated for each of the microcosm types incubated at the three temperatures. These trends are displayed in Figure 4.9. Overall, the greatest community diversity within each of the respective microcosm varieties was observed in those kept at 12°C and 22°C. A structural decrease in community diversity was most notably observed in SC and BC 4°C microcosms over the duration of the experiment. Conversely, the structural diversity of CL and WTC communities were generally maintained at all three test temperatures with H' values ranging from approximately 1.5 to 2.5 at the conclusion of the experimental period.

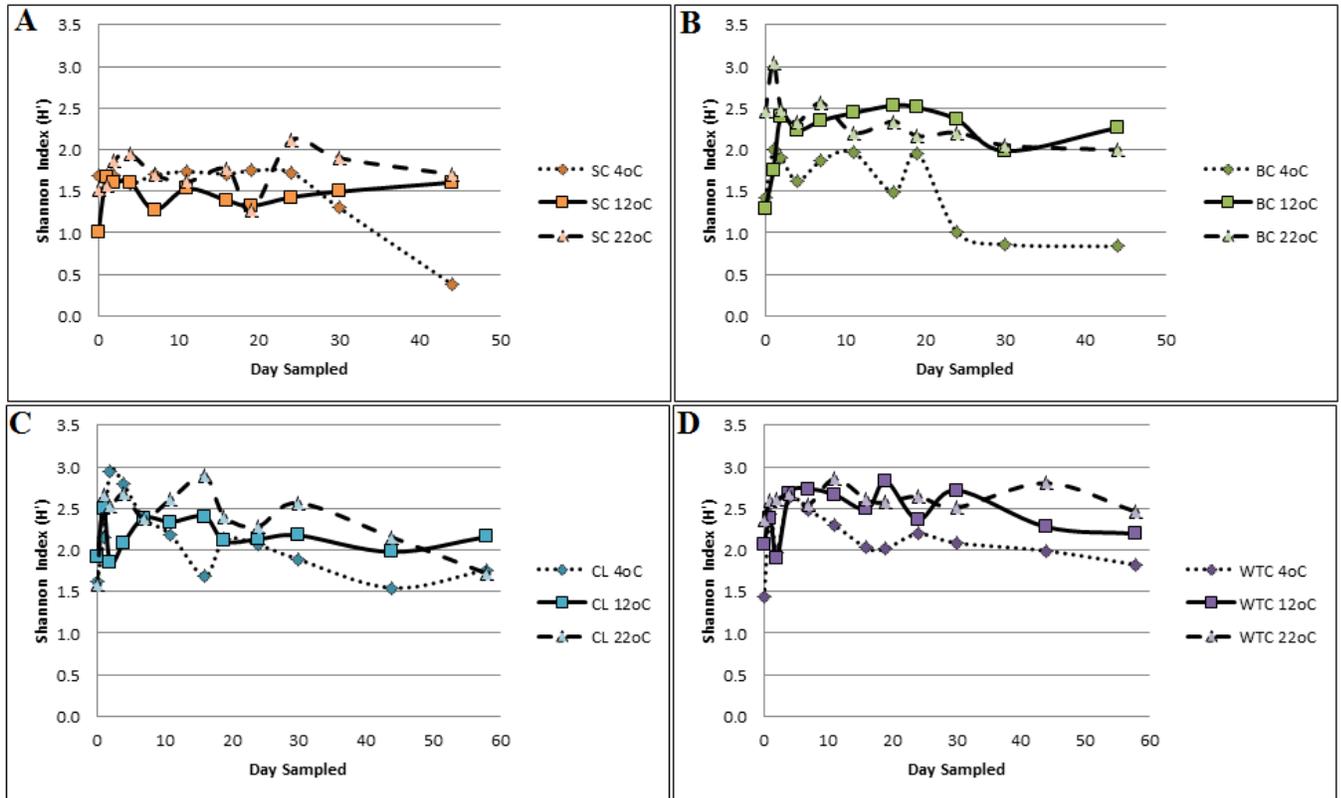


Figure 4.9. DGGE-based structural community diversity calculated using the Shannon Index (H') within each of the test flasks, incubated at 4°C, 12°C, and 22°C: (A) sterile control (SC), (B) biological control (BC), (C) Clair Lake (CL) and (D) Water Technology Center (WTC). DNA extractions from a single test condition replicate at each incubation temperature were examined.

A summary of species richness, defined as the number of bands above background representing operational taxonomic units (OTUs), within each of the microcosms is provided in Figure 4.10. General trends observed in genetic diversity were similarly reflected in terms of genetic richness when characterizing the microcosm communities. With the exception of the BC microcosms, genetic richness was typically highest in microcosms incubated at 22°C, followed by 12°C and 4°C. Overall, the greatest structural richness was observed in WTC microcosm communities, followed closely by CL and BC communities. While the lowest genetic richness

was observed in SC communities, there was no apparent loss in richness over the course of the test period in microcosms incubated at 12°C and 22°C.

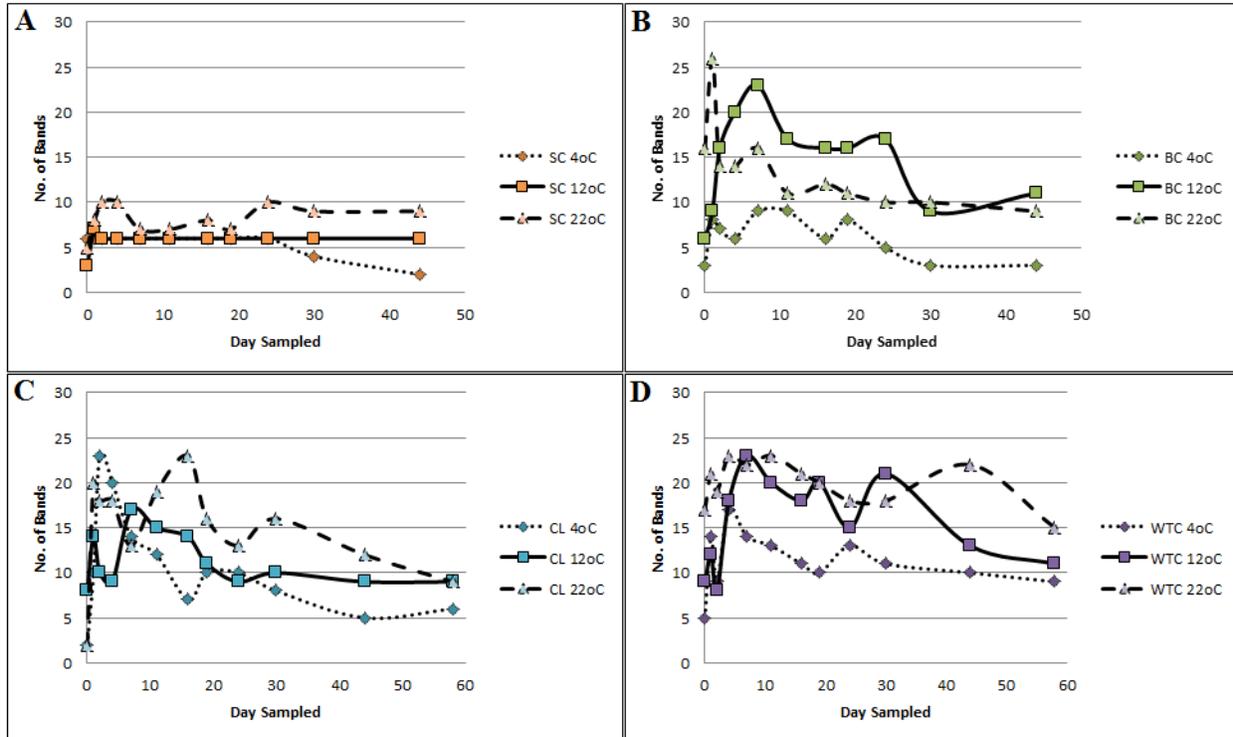


Figure 4.10. DGGE-based genetic richness of microbial communities within each of the test flasks, incubated at 4°C, 12°C, and 22°C: (A) sterile control (SC), (B) biological control (BC), (C) Clair Lake (CL) and (D) Water Technology Center (WTC). DNA extractions from a single test condition replicate at each incubation temperature were examined.

Average banding patterns were established based on the migratory distances of the DNA sequences throughout the gel and the relative intensities of each of the bands within a sample. As previously mentioned in Chapters 2 and 3, bands were assigned into ‘band movement groups’ based on their final position within the gel, which corresponds to the G+C content of the specific OTU sequence. G+C composition dictates sequence stability due to the three hydrogen bonds present between G-C base pairs compared to the two hydrogen bonds between A-T base pairs (Madigan *et al.*, 2009), and as a result requires a higher denaturant concentration before DNA

melting will occur. Accordingly, OTUs with a high G+C composition were assigned a higher band movement group signifying further movement through the gel than OTUs with low G+C content. For example, *Salmonella* is typically considered to have a rich G+C genomic composition (Desai *et al.*, 2013), such that it would likely be found within a high band movement group.

According to the average banding plot shown in Figure 4.11, all SC samples at 4°C, 12°C and 22°C were dominated by one to three peaks corresponding to moderate G+C content OTUs. These peaks accounted for on average between 5% and 90% of band intensities within samples extracted from the SC microcosms stored at the three test temperatures. Despite having the same inoculum and the background community flora of the microcosm materials removed, some variation was observed between SC flasks incubated at 4°C, 12°C and 22°C. Greater variation in banding placement throughout the duration of the experiment was observed in SC microcosms incubated at 4°C and 12°C as compared to 22°C, although overall, peaks were dispersed between similar ranges of banding groups (i.e. between the 7th and 16th band movement group). Following seven days of incubation at 22°C, SC banding profiles remained fairly consistent until the end of the experiment with peaks located at the 8th banding group, between the 10th and 12th banding group, and at the 14th banding group accounting for approximately 10%, 85% and 5% of the OTU composition, respectively.

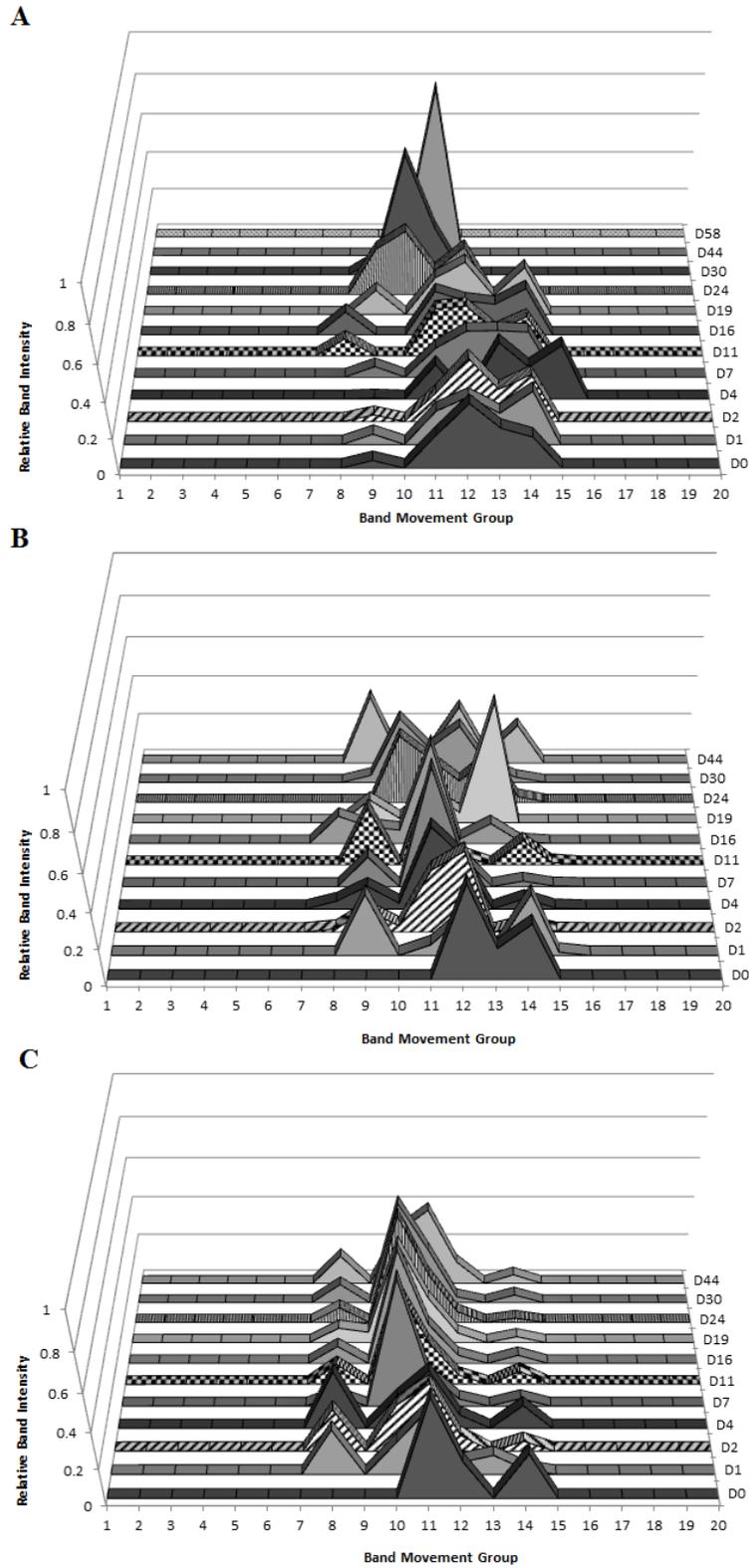


Figure 4.11. DGGE-based average banding patterns of SC test flasks over the duration of the experiment incubated at (A) 4°C, (B) 12°C, and (C) room temperature (approximately 22°C).

Similar genetic profiles based on banding patterns were observed across all samples extracted from the BC microcosm incubated at 4°C as seen in Figure 4.12A. Profiles typically consisted of one dominant peak contributing approximately 75% of the community composition and a lesser peak comprising the remaining 25%. Interestingly, the greatest diversity of OTUs based on the distribution of band movement groups was observed within samples collected from the BC microcosm incubated at 12°C, consisting primarily of two to three moderate peaks (Figure 4.12B). Of the BC communities, those stored at 12°C contained OTUs with the lowest G+C content, apparent within samples collected between test days 4 and 24. Following day 30 samples, however, the community structure reverted back to a profile similar to that observed at the onset of the experiment. Microcosm communities stored at 22°C were composed predominantly of moderate G+C content (Figure 4.12C). Typically, a dominant peak was observed between the 13th and 15th band movement group, corresponding to approximately 50 to 95% of the community composition throughout the duration of the experiment. OTUs consisting of the highest G+C content, as demonstrated by the highest band group assignments, were observed in communities within BC microcosms kept at 22°C.

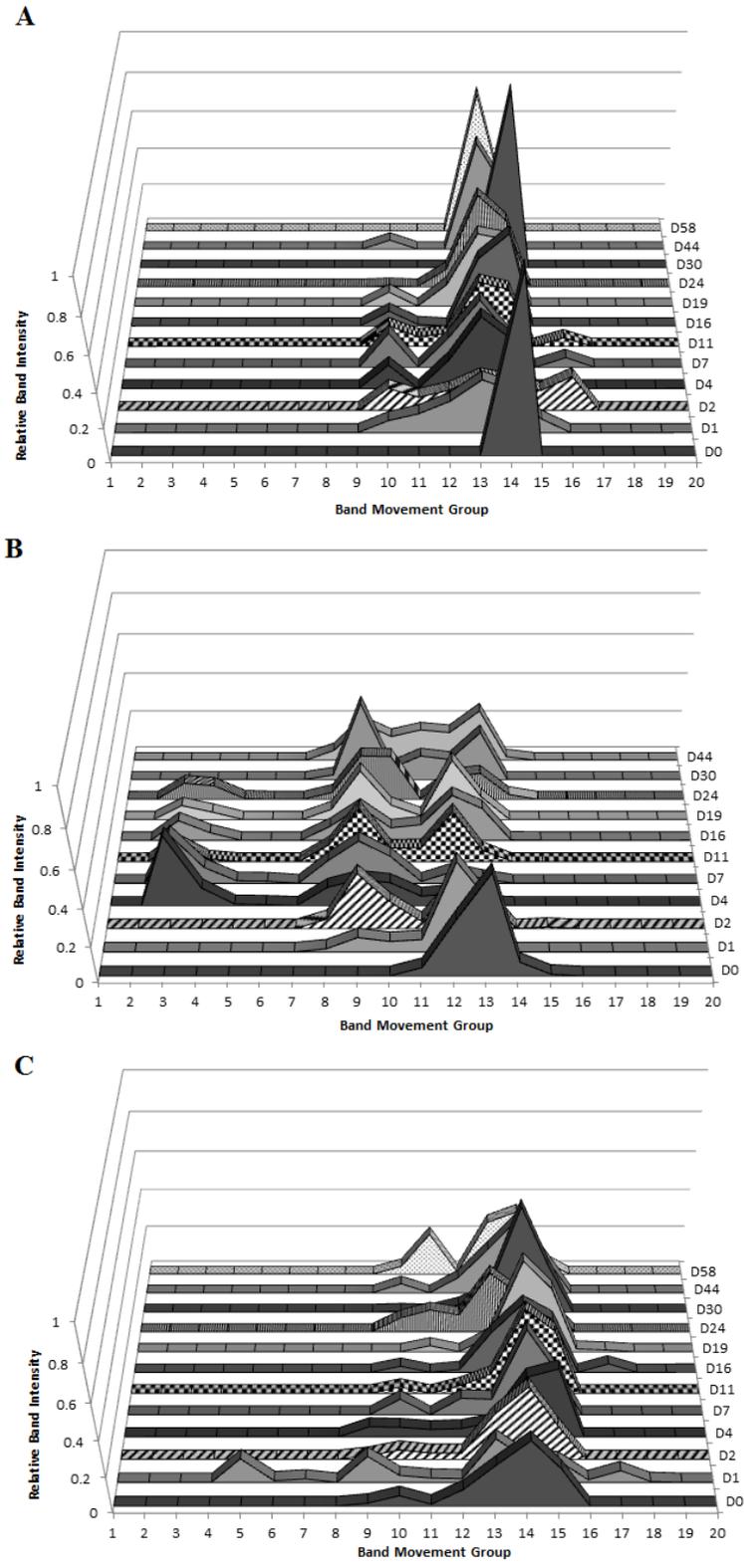


Figure 4.12. DGGE-based average banding patterns of BC test flasks over the duration of the experiment incubated at (A) 4°C, (B) 12°C, and (C) room temperature (approximately 22°C).

In comparison to microcosms inoculated with lab strain controls, the average banding profiles of CL microcosm communities exhibited greater variability both in terms of samples collected throughout the course of the experiment, as well as between profiles generated from microcosms incubated at each of the three different temperatures (Figure 4.13). The structures of CL communities at the different temperatures were typically dominated by two to three peaks contributing relatively equal proportions to the community. However, overall greater profile diversity was observed in CL microcosms kept at 22°C based on the distribution of OTUs across movement groups (Figure 4.13C). While 4°C and 12°C CL communities occupied similar band movement groups with OTUs of moderate G+C richness, the profiles of communities at 22°C consisted of profiles comprised of rich G+C OTUs. At each of the three incubation temperatures, the community structures of day 58 samples resembled profiles generated from day 0 samples, with the exception of a slight profile shift to the right such that OTU peaks occupied higher banding movement groups at the conclusion of the experiment.

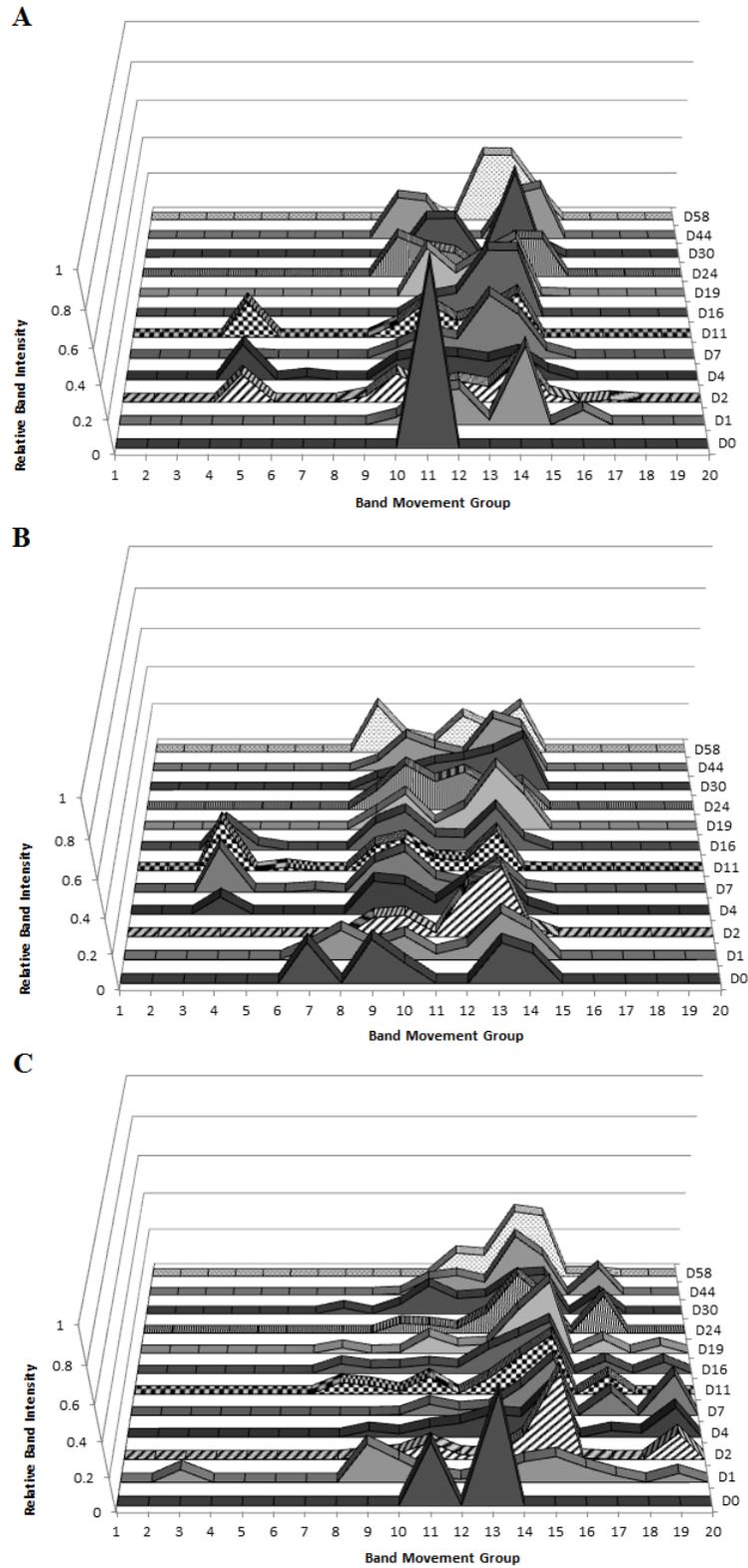


Figure 4.13. DGGE-based average banding patterns of CL test flasks over the duration of the experiment incubated at (A) 4°C, (B) 12°C, and (C) room temperature (approximately 22°C).

Throughout the duration of the experiment, moderate OTUs occupied similar band movement groups for WTC communities incubated at 4°C and 22°C (Figure 4.14). Unique to WTC microcosms, the greatest structure diversity was apparent in 12°C communities, such that these communities were comprised of OTUs with both the lowest and highest G+C content. While the composition of communities at 4°C and 22°C involved one to two dominant peaks, 12°C communities were made up of numerous, low-intensity peaks. By day 4, a shift was apparent in 12°C communities towards an increasing prevalence of low G+C content OTUs, while by day 44 detected OTUs were restored to moderate G+C content as was observed at the beginning of the experimental series.

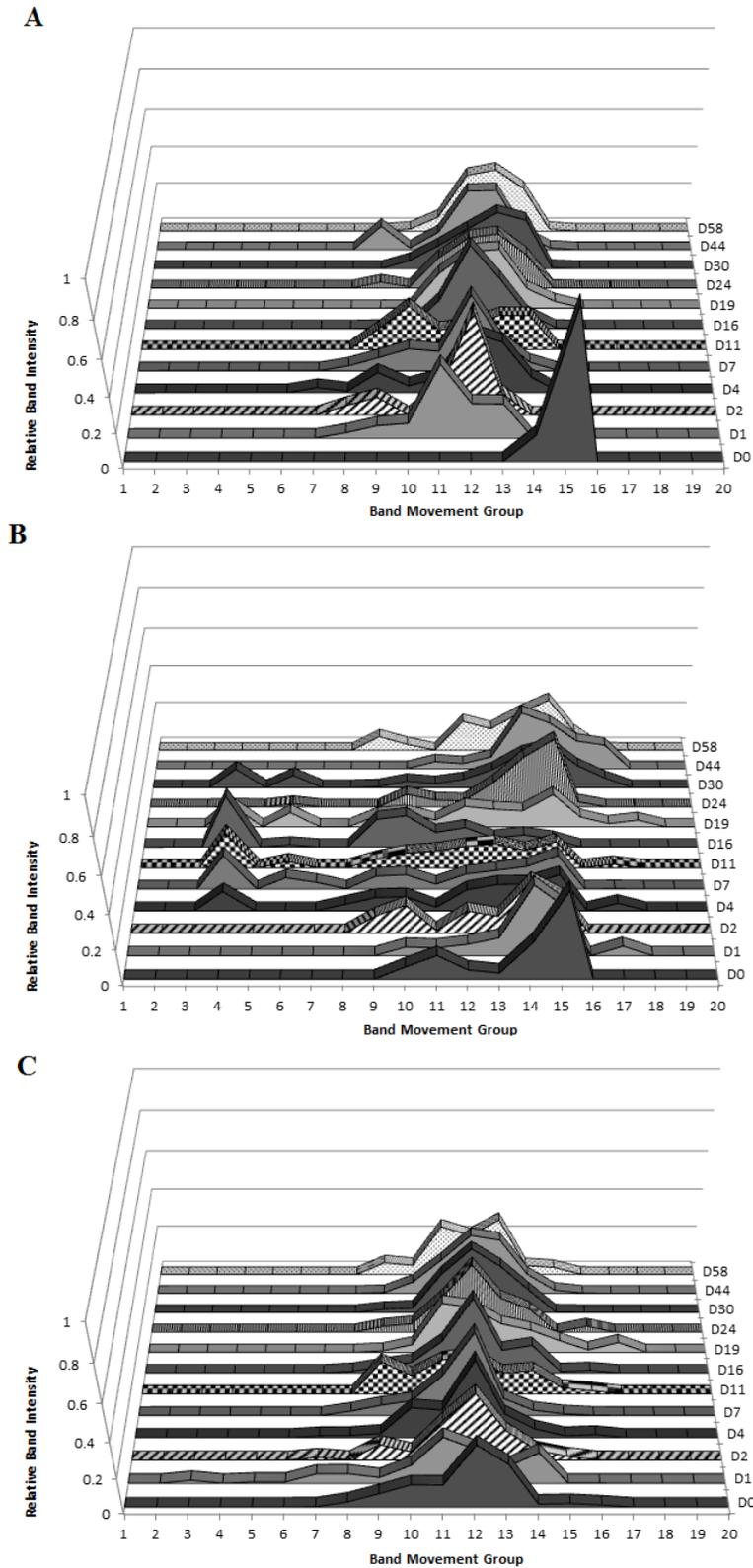


Figure 4.14. DGGE-based average banding patterns of WTC test flasks over the duration of the experiment incubated at (A) 4°C, (B) 12°C, and (C) room temperature (approximately 22°C).

4.5 Discussion

Before proceeding with the discussion pertaining to the information gained from this study, a few experimental limitations need mention. First, since complete kill was not accomplished in the background of Clair Lake microflora within SC microcosm test conditions prior to the onset of the experiment (as evident from initial HPC screenings), interpretation of findings are somewhat complicated. As mentioned in Chapter 2, community structural diversity based on DGGE-generated banding patterns may result in an overestimation of community diversity, as multiple bands can be derived from a single, pure organism as a result of the heterogeneity of 16S rDNA gene sequences (Nübel *et al.*, 2006; Rettedal *et al.*, 2010). Therefore, it cannot be confidently established which peaks correspond to residual background microflora and which can be attributed to the lab strain *Salmonella* inoculum. Secondly, beneficial information would come from the addition of microcosm test conditions in which the environmental materials were completely sterilized before being individually spiked with each of the environmental and lab strains of *Salmonella* used within this present research. While not possible to do so in this study given time and spatial constraints, information gained would contribute further insight into the role of microbial communities in *Salmonella* persistence in non-enteric and nutrient-poor environments. In spite of the stated experimental limitations, however, we were still able to assess shifts in the antimicrobial resistance profiles of *Salmonella*, population dynamics, and the functional and structural profiles of the microcosm communities.

4.5.1 The Ability of Batch Microcosm Systems to Maintain Microbial Communities and *Salmonella* Populations

As is evident from HPC results, the batch system microcosms were able to successfully sustain microbial life within the test microcosm conditions over the duration of the 58 day experiment, with final bacterial levels of approximately 10^4 CFU/ mL (Figure 4.3). As was expected, incubation temperature seemed to influence heterotrophic growth most noticeably within SC microcosms, inoculated with a lab strain of *Salmonella enterica* ser. *typhimurium* (ATCC® 13311) and the competitor microflora of Clair Lake primarily removed. Due to the elimination of interspecies diversity, and likely in part to a lack of previous environmental exposure, the growth of the lab-strain inoculum was most successful when incubated closer to optimal, mesophilic temperatures (ICMSF, 1996). Specifically, the growth of the lab strain inoculum averaged one to two orders of magnitude higher at 12°C and 22°C, than at 4°C. Alternatively, heterotrophic bacterial levels within BC, CL and WTC microcosms containing background Clair Lake microflora were able to withstand environmentally-relevant incubation temperatures without discrimination. This finding is most likely a consequence of the diversity of community populations within the experimental microcosms with their own specific temperature ranges for optimal growth. Furthermore, plate count concentrations did not appear to be impacted by inoculum type, suggesting that the largest contributor of the heterotrophic population was Clair Lake microflora. The gradual decline in heterotrophic levels over the experimental period may be attributed to common biotic stresses encountered in the natural environment including: predation in the case of BC, CL and WTC communities, a depletion of the available organic content leading to an increased competition for nutrients, and an accumulation of microbial wastes.

According to results obtained through qPCR, *Salmonella* populations within each of the microcosms designed to simulate natural conditions of an environmental retention area were preserved over the experimental test period (Figure 4.4). In this way, the opportunity for *Salmonella* naturalization was maintained, including both the specific *Salmonella* spike inoculum and environmental *Salmonella* within Clair Lake materials, contributing to the relative abundance of *Salmonella* detected in BC, CL and WTC microcosms. The sensitivity of qPCR allowed for the enumeration of *Salmonella* subpopulations subsisting in both culturable and non-culturable conditions (i.e. damaged, dormant or in a VBNC state). The latter is of particular importance, as the ability of *Salmonella* to exist in a VBNC state has been described as a primary survival strategy for the pathogen to endure harsh stresses imposed by the environment (Winfield and Groisman, 2003; Spector and Kenyon, 2012; Waldner *et al.*, 2012). The VBNC state is defined as a condition in which bacterial replication does not occur, while metabolic activity and virulence and antibiotic resistance traits are thought to be maintained. Furthermore, bacteria in this state are typically able to fully resuscitate following the removal of induced stresses and the return of favourable conditions (Lleo *et al.*, 2007; Waldner *et al.*, 2012). Consequently, a number of molecular studies in recent years have inferred that the VBNC state constitutes an important reservoir for pathogens in the natural environment (Rowan, 2004; Sardesai, 2005; Lleo *et al.*, 2007; Oliver, 2010). Therefore, non-culturable *Salmonella* are likely pathologically relevant entities and should not be ignored as a public health threat.

The validity of DNA detection through qPCR has been disputed based on its inability to discriminate between nucleic acid materials within viable and dead cells (Lleo *et al.*, 2005; Garcia *et al.*, 2010). While it has been reasoned that the half-life of DNA external to a bacterial cell is fairly short due to the presence of nucleases within the environment (Lleo *et al.*, 2005),

others have claimed that extracellular DNA can persist for several weeks in soil (Lorenze and Wackernagel, 1991; Nielsen *et al.*, 2007) and water (Bae and Wuertz, 2009). Despite these conflicting results, given the limited nutrient cycling within the closed batch microcosm systems of the present study, it is likely that any extracellular genetic material would be readily taken up as a rich source of amino acids by bacteria experiencing nutrient deprivation. Therefore, it was assumed that detected DNA through qPCR was more likely to be from live cells than free DNA. The metabolic flexibilities of the microcosm communities are later discussed in reference to CLPP data.

Previous studies have found temperature to be an important factor in the survival of *Salmonella* outside of a host (Mawdsley *et al.*, 1995; Garcia *et al.*, 2010). In the present study, however, results obtained through qPCR revealed that *Salmonella* abundance (denoted by the relative quantification of *invA* genes) was not influenced by temperature. These findings can be compared to a study conducted by Alonso *et al.* (1992), in which the survival of *Salmonella* in marine waters was shown to be independent of water temperature and did not fluctuate with seasonality. High levels of *Salmonella* remained present following the 58 days in all microcosm experiments at 4°C, 12°C and 22°C at approximately 10^7 and 10^5 *invA* gene copies per 100 mL in SC microcosms and WTC, CL and WTC microcosms, respectively. As with HPC counts, temperature again appeared to be most influential on SC microcosms inoculated with a lab strain of *Salmonella* without prior exposure to environmental-type conditions. With the elimination of the microbial community in the SC microcosm, the elevated presence of *Salmonella* compared to concentrations in the other microcosm types is likely a direct result of a lack of predation and competition for nutrients. Alternatively, the near constant levels of *Salmonella* detected in BC, CL and WTC microcosms (indicative of a lack of die-off or DNA degradation) is demonstrative

of the ability of *Salmonella* to successfully compete for resources and resist microbial predation (Winfield and Groisman, 2003).

Therefore, it is evident that the bacteriological quality of the microcosm communities was maintained over the duration of the 58 day experiment as demonstrated by the total culturable heterotrophic growth. Furthermore, based on qPCR results it can be deduced that the constituent *Salmonella* populations within the greater microcosm communities was similarly preserved over the test period, although the portion of *Salmonella* subpopulations existing in a culturable vs. non-culturable state cannot readily be ascertained. It was found, however, that *Salmonella* was readily cultivated in order to collect data pertaining to phenotypic AMR profiling of isolates as discussed below.

4.5.2 *Salmonella* Response Following Exposure to Antimicrobial Compounds

Relative differences in the levels of *Salmonella* susceptibilities to the tested antimicrobials appeared to vary inconsistently both in terms of incubation temperature and length (Figure 4.5). While levels of antibiotic susceptibilities were somewhat sporadic with regards to *Salmonella* extracted from SC and BC microcosms, an overall increase in resistance was observed within *Salmonella* isolated from all microcosms stored at 4°C on day 45. Similarly, *Salmonella* from CL and WTC microcosms incubated at 4°C expressed 12.50% and 11.11% higher drug tolerance on day 45 compared to levels screened at the onset of the experiment. However, the largest overall increase in antimicrobial resistance over the progression of the experiment was observed in *Salmonella* from CL microcosms incubated at 12°C with a nearly 21% elevation in resistance levels. These findings are in agreement with results obtained in Chapter 3, where *Salmonella* extracted from Clair Lake water samples in winter and spring months generally demonstrated higher profiles of antimicrobial resistance than those obtained in

summer months. Patterns generated by relative differences in percent resistance occurrence were similarly reflected by MAR frequencies expressed by the *Salmonella* isolates (Table 4.3). In nearly all cases, higher MAR rates were observed in *Salmonella* isolated from the various microcosm types incubated at each of the three temperatures on day 45 compared to day 0.

Since there was no increase in drug exposure to the microcosm communities throughout the course of the experiment within the microcosm environments, it can be assumed that changes in *Salmonella* drug susceptibilities and MAR frequencies were stress-response induced. In recent years, it has become increasingly understood that environmental type stresses can elicit protective responses in pathogens necessary for survival that can in turn impact resistance to antimicrobials even in the absence of drug-related selective pressures (Poole, 2012). Cellular mechanisms to evade the detrimental effects of growth-comprising stressors including unfavourable temperatures, limited nutrient availability, and cell envelope damage are similar to processes employed by Gram negative bacteria when challenged with antimicrobial exposure (McMahon *et al.*, 2007; Poole, 2012; Spector and Kenyon, 2012). For instance, in response to unfavourable environmental stimuli, the expression of an assortment of regulators (including sigma factors, two-component systems and transcriptional regulators) modified to counter one stress may lead to changes in the expression of a set of genes that overlap with other response mechanisms. These generalized, multi-defense stress strategies typical of *Salmonella* are particularly concerning, as a response evoked for the management of one stress may lead to protection against seemingly unrelated stresses through cross-resistance. In this way, the pathogen can inadvertently prepare for future stressful encounters leading to the resiliency of *Salmonella* and increasing its potential for survival in various non-host and host surroundings (Spector and Kenyon, 2012).

4.5.3 Community Integrity as Measured through Functional and Structural Fingerprinting and Corresponding AMR Levels in *Salmonella* Populations

The dynamic shifts in the microbial communities within each of the microcosm designs were monitored with regard to incubation temperature and duration. As previously described, functional profiles were generated using CLPP technology to determine average metabolic rates (based on the progression of average well colour development), substrate diversity (as calculated by the Shannon Index (H' ; Shannon, 1948)) and substrate richness (based on the number of available carbon sources utilized). DGGE-generated structural profiles provided complimentary information with regard to the genetic diversity (as calculated by the Shannon Index) and richness (number of OTUs) of the sampled communities, in addition to their averaged banding patterns (based on the relative intensities of the bands and their respective migratory distances through the DGGE gel). It is important to note that the same DNA extracted from each of the microcosm test conditions was used for both the detection of *Salmonella* through qPCR and genetic profiling of the communities based on DGGE, such that the *Salmonella* population within the microcosms contributed to the genetic fingerprinting of its corresponding communities.

It was evident that the metabolic capacities of the microbial communities within each of the microcosm types appeared to vary in connection with incubation temperature and the presence of interspecies diversity, according to the findings from this study. For instance, the greatest metabolic versatility of the SC communities composed primarily of a lab strain of *Salmonella* was observed when incubated at 22°C followed by those incubated at 12°C and 4°C, respectively (Figure 4.6A, Figure 4.7A, Figure 4.8A). These findings were anticipated, as 12°C and 22°C are temperatures closest to the mesophilic optimal growth temperature of *Salmonella*

(ICSMF, 1996). Conversely, BC, CL and WTC microcosms containing the background microflora of Clair Lake in addition to specific *Salmonella* inoculum were similarly able to metabolize the available carbon sources most readily at 4°C whereas communities at 22°C demonstrated the slowest metabolic rates (Figure 4.6B-D). These trends in functionality in accordance with incubation temperature were again reflected in terms of substrate diversity (Figure 4.7) and substrate richness (Figure 4.8). Specifically, the metabolic diversity and richness were highest in BC, CL and WTC microcosm communities incubated at 4°C, 12°C and 22°C, respectively. Due to the complementary nature of CLPP and DGGE techniques, the functional and genetic profiles of the communities need to be fully discussed before uncovering the significance of these findings.

In contrast to metabolically-derived community profiles, the greatest overall species diversity and richness within all microcosm communities was observed in those incubated at 12°C and 22°C (Figure 4.9, Figure 4.10). As was expected due to the removal of the Clair Lake microflora, the lowest structural diversity and richness was observed in the SC microcosms, while comparable genetic profiles were obtained for BC, CL and WTC microcosms. While averaged banding patterns provided a snapshot of the community distribution according to final positioning within the gel, banding profiles cannot be considered an absolute indication of community diversity or richness due to the clumping of phylogenetic members with similar G+C content in like band movement groups. An example of this bias is demonstrated when comparing the calculated diversity and richness for 22°C WTC communities (Figure 4.9D, Figure 4.10D), and its resulting banding pattern (Figure 4.14C). Specifically, while the highest H' values and band numbers were observed in WTC samples collected from microcosms kept at 22°C, the resulting average banding profiles appeared to be dominated by only one to two distinct

phylogenetic groups compared to the numerous phylotypes comprising the 12°C WTC communities. Within all microcosm test conditions, the greatest diversity in phylogenetic taxa in terms of G+C content and resulting band movement group distribution was observed in microcosm communities stored at 12°C and 22°C. This finding possibly suggests that warmer temperatures can support a greater diversity of phylotypes than 4°C, likely including members of *Enterobacteriaceae* such as *Salmonella* that are typically considered high G+C content organisms (Desai *et al.*, 2013).

Therefore, study findings indicate that while incubation temperatures nearest to the mesophilic range were able to support higher species diversity, it was also functionally taxing on the population constituents within the community. This is likely a direct consequence of increased bacterial activity at higher temperatures, leading to overall increased competition for available resources to sustain growth. This is demonstrated by the fact that an association was observed between genetic diversity and metabolic versatility in SC microcosms lacking interspecies competition, while a negative relationship was apparent in BC, CL and WTC microcosm communities. Furthermore, according to functional profiles, greater community resilience was observed at lower temperatures within communities exposed to more diverse biotic interactions. This suggests community support may be a possible mechanism for pathogen persistence in unfavourable conditions. The concept of community support has been well studied when considering biofilm formation as an adaptive mechanism for pathogen survival in animal and environmental reservoirs alike (Ferguson and Signoreto, 2011). Specifically, *Salmonella* is a known biofilm participant in the natural environment, whereby the enteric pathogen is even thought to rely on the protective biofilm community to support its existence in a VBNC state if necessary (Waldner *et al.*, 2012).

In conjunction with *Salmonella* AMR levels, as previously touched upon in section 4.5.2, study results demonstrated that communities classified as having a high functional capacity but low genetic integrity harboured the most resistant *Salmonella* for all microcosm test conditions studied. For instance, within all microcosm test conditions incubated at 4°C, an overall increase in resistance was observed in *Salmonella* isolated from the corresponding microcosm communities, with an average increase in drug tolerance by nearly 10% at the conclusion of the experiment (Figure 4.5). Similarly, MAR rates were generally highest in *Salmonella* extracted from 4°C microcosms on day 45 compared to comparable microcosms stored at 12°C or 22°C (Table 4.3). However, in nearly all cases, higher MAR rates were observed in *Salmonella* isolated from the various microcosm types incubated at each of the three temperatures on day 45 compared to day 0. Due to the novelty and exploratory nature of this work, many questions are left unanswered with regard to the specific influences of temperature and community integrity (structurally and functionally), in impacting AMR levels in *Salmonella*. Specifically, it remains unclear as to whether the higher incidences of drug resistance in microcosms stored at 4°C were a response to temperature stress or stress related to being in a community lacking genetic diversity.

4.6 Conclusions

Microcosms were successfully used in this study to assess the physiological response of *Salmonella* derived from different sources to imposed “naturalization”-simulated conditions. Specifically, the effects of three incubation temperatures (4°C, 12°C, 22°C) were monitored through shifts in antimicrobial resistance profiles, population dynamics, and the functional and structural profiles of the microcosm communities.

Based on HPC and qPCR results, the batch microcosm systems were effectively able to sustain microbial communities and the *Salmonella* population, such that the opportunity for “naturalization” was maintained over the duration of the experiment. Furthermore, a correlation was observed between community diversity and a lack of sensitivity to incubation temperature according to culturable heterotrophic growth. Similarly, the relative abundance of *Salmonella* persisting in culturable or non-culturable states was not influenced by temperature.

An overall increase in antimicrobial resistance was observed within *Salmonella* isolated from microcosms stored at 4°C compared to drug tolerance screenings performed at the onset of the experiment. Moreover, in nearly all cases, higher MAR rates were observed in *Salmonella* isolated from the various microcosm types incubated at each of the three temperatures on day 45 compared to day 0, suggesting cross-resistance between environmental stresses and antibiotic exposure. In microcosms containing interspecies communities, the greatest genetic diversity and richness were observed at 22°C and 12°C temperatures, whereas the highest metabolic versatility was observed at 4°C. Therefore, while incubation temperatures near the mesophilic range appeared to support a more structurally diverse community, these same temperatures also appeared taxing on the microbial community with respect to its metabolic versatility. This is likely due to increased microbial activity in diverse communities leading to an increase in competition for available nutrient sources. Consequently, greater community resilience was observed at lower temperatures according to the functional profiles of the microcosm communities. Furthermore, *Salmonella* with the most distinguishable antimicrobial tolerance were shown to be harboured in these same low diversity, high metabolically functioning communities.

Therefore, this study demonstrated the ability of *Salmonella* from a variety of sources to persist in a non-host environment despite opposition by abiotic and biotic stresses (such as temperature, a lack of nutrient cycling, predation and competition for available resources), and the role of stress response in the expression of antimicrobial resistance. The long-term survival of resistant *Salmonella* in the natural environment is of concern, as the transmission of the pathogen and likelihood of re-inoculation into a susceptible host is enhanced.

4.7 Future Recommendations

- More *Salmonella* survival studies need to be conducted in simulated aquatic and soil environments without nutrient-rich manure or sewage amendment. In this way, the persistence and transmission of *Salmonella* in nutrient-diluted environments, such as downstream of manure, sewage or storm runoff can be more thoroughly investigated.
- Future studies should include additional test microcosms with sterile environments and individually spiked with each of the environmental and lab strains of *Salmonella* in this current research to gain further insight into the role of microbial communities in *Salmonella* persistence and expression of AMR profiles external to a host.
- To circumvent the limitations of qPCR, the quantification of mRNA should be performed for the analysis of genes being actively expressed to serve as an indicator of viable *Salmonella* (i.e. more in-depth analysis of the influence of temperature on *Salmonella* activity). Furthermore, the expression of additional genes of interest including those associated with *Salmonella* pathogenicity and biofilm formation could similarly be measured.

- Future studies should focus on the role of biofilm formation in *Salmonella* “naturalization” in soil and aquatic environments. This information would offer insight into the importance of biofilm community support in the survival of *Salmonella* in non-host environments.

4.8 References

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Chapter 5

General Discussion and Research Summary

5.1 Overview of Research

Mounting evidence in the literature and results from this study suggest that aquatic environments may be an important reservoir of antimicrobial resistant (AMR) *Salmonella*. Furthermore, the near ubiquitous detection of *Salmonella* in both urban and rural impacted water sources in the present research suggests that waterways are significant vehicles in the transmission of the zoonotic pathogen between susceptible hosts. The overarching objectives of this study were to: 1) to characterize the persistence profile of *Salmonella* upon migration into the broader environment, following release from a host, discharge from a wastewater treatment plant or runoff from agricultural practices, and 2) to better understand the physiological potential that allows for non-host survivability and ultimately “naturalization” in *Salmonella* within the broader microbial community existing in the natural environment. This information is important with regards to the ongoing development of regulatory guidelines for managing the risk of contamination of nearby surface water, ground water and the surrounding environment.

In order to better elucidate the prominent factors leading to a “naturalized” condition, an integrative approach was taken to measure the response of *Salmonella* to imposed environmental stresses. Complementary phenotypic (culture-based) and genotypic (molecular-based) analyses

were incorporated in this study for the profiling of test microorganisms at the level of the microbial genome, individual organism and community levels from which the sample was derived. Chapter 3 includes survey-based field research conducted in order to gain perspective on the prevalence of AMR levels in *Salmonella* isolated from four variously impacted water sources; a local watershed, a retention area of the watershed, a constructed wetland designed for the treatment of human waste, and a wastewater treatment facility. Chapter 4 consists of data collected from bench-scale batch microcosm systems designed to simulate the natural environment of Clair Lake, to monitor *Salmonella* survival in the open environment despite abiotic and biotic challenges (i.e. temperature, limited nutrient availability, osmotic pressures, increased predation and competition for resources) on its own and with respect to the community.

Commonalities in techniques used in the two data chapters include:

- Kirby-Bauer disk-diffusion to establish phenotypic antimicrobial resistance profiles of *Salmonella* (Chapter 3 and 4) and the fecal indicator bacteria *E. coli* and enterococci (Chapter 3).
- Quantitative polymerase chain reaction (qPCR) to enumerate the presence of target fecal indicator such as *E. coli* and *Enterococcus* spp. (Chapter 3) to *Salmonella* (Chapter 3 and 4) as well as non-target waterborne pathogens such as *C. perfringens* from different aquatic environments (Chapter 3).
- Community-level physiological profiling (CLPP) to characterize the temporal and spatial changes in the metabolic capabilities of microbial communities from which the pathogens were derived (Chapter 3 and 4).

- Denaturing gradient gel electrophoresis (DGGE) to generate a genetic fingerprint of the microbial communities within which the pathogens were derived or associated with (Chapter 3 and 4).

5.2 Major Findings

As outlined in detail in the concluding sections of the two data chapters, the major findings from this research are as follows (refer to Chapter 1 for the supporting objectives of this research):

- 1) Of the total *Salmonella* cultured from the Grand River watershed (including Clair Lake, considered a retention area of the Grand River), the overall greatest resistance occurrences and frequencies of multiple antimicrobial resistance (MAR) were detected in *Salmonella* from agriculturally-impacted regions (Chapter 3; supporting objective 1). This supports literary evidence that the widespread use of antibiotics in food animals is largely responsible for the emergence of AMR bacteria (Threlfall, 2002; Economou *et al.*, 2012).
- 2) Resistance profiles expressed by environmental *Salmonella* were more comparable to *E. coli* than enterococci, while overall resistance occurrences and MAR frequencies observed in *Salmonella* were typically much higher than that of *E. coli*. Furthermore, the seasonal influence on AMR levels were inconsistent amongst sampling locations and microorganisms, which may be a result of the physiological response to the unique combination of stresses imposed within each impacted water source (Chapter 3; supporting objective 2).

- 3) As demonstrated by results from the WTC and CAWT systems, following treatment exposure *Salmonella* expressed enhanced tolerance to the tested antimicrobials and were more likely to exhibit MAR. While fewer *Salmonella* isolates were successfully cultured from the treated wastewater samples, those isolates able to endure the disinfectant/treatment process and are thereafter released into the broader environment are likely more physiologically resilient and robust (Chapter 3; supporting objectives 1 and 2).
- 4) The relative abundance of the fecal indicator bacteria *E. coli* and *Enterococcus faecalis* were found to be present in similar concentrations as *Salmonella* in the environment as detected through qPCR. This indicates that the reliability of these microorganisms as fecal indicators in aquatic environments may need to be reconsidered (Chapter 3; supporting objective 3).
- 5) AMR levels of *Salmonella* derived from different microbial community situations may be a consequence of both increased opportunities for the exchange of genetic material in healthy communities, and a physiological stress response to low-integrity communities (Chapter 3 and 4; supporting objective 4).
- 6) In the open environment, a direct relationship was observed between the genetic integrity of the microbial communities harbouring *Salmonella* and their corresponding functional profiles in terms of carbon source utilization (Chapter 3). Conversely, in the microcosm experiments where nutrient cycling was limited and available carbon sources were being gradually depleted, the opposite trend was observed (Chapter 4). This may be due to the increased incidences of predation and competition for resources (supporting objective 4 and 5).

- 7) In the absence of antimicrobial exposure, an overall increase in resistance was observed within *Salmonella* isolated from microcosms stored at 4°C such that changes in *Salmonella* drug susceptibilities and MAR frequencies were likely stress-response induced. Therefore, environmental type stresses can elicit generalized protective responses in *Salmonella* that may indirectly work to cross-protect against antimicrobial susceptibilities (concept introduced in Chapter 3 and investigated in Chapter 4; supporting objective 5).
- 8) Results obtained through qPCR revealed that *Salmonella* abundance (denoted by the relative quantification of *invA* genes) in microcosms containing interspecies diversity was not influenced by temperature suggesting that community support may be a factor in pathogen persistence in unfavourable environmental conditions (Chapter 4; supporting objective 5).

5.3 Future Directions and Recommendations

- 1) While wastewater treatment plants are routinely successful at reducing the pathogenic load of incoming wastewaters, strategies need to be implemented to prevent the discharge of resilient and highly resistant enteric pathogens, such as *Salmonella*, into the broader environment.
- 2) As touched upon throughout the thesis, the formation of biofilms may promote the “naturalization” of enteric organisms like *Salmonella* through its protective properties. A number of biofilm factors have been described for *Salmonella*, some of which serve a dual purpose associated with its pathogenicity. Therefore, the expression of biofilm-associated genes may serve as important biological markers for examining the ability of *Salmonella* to survive in the external environment and its potential to remain pathogenic.

- 3) The rising evidence for the abundance and potential long-term survivability of FIBs in non-enteric habitats signifies that it is necessary to reevaluate the characteristics of dependable fecal pollution indicators.
- 4) Additional studies should be performed that center on the impact of microbial community integrity on AMR levels in its constituent pathogenic populations. In this way, the ecology of antibiotic resistance, including fate, transport and occurrence in microbial communities will be better understood.
- 5) Continued surveillance by the National Antimicrobial Resistance Monitoring Systems (NARMS) in the United States and the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) in Canada will aid in defining the association of AMR levels in human and food-producing animal pathogens. However, new focus should be emphasized on characterizing AMR levels in zoonotic bacteria like *Salmonella* in non-host environments to better elucidate the epidemiology of AMR pathogens.

5.4 Concluding Remarks

It was evident from this study that the response of *Salmonella* to environmental stress not only dictates its ability to survive and become “naturalized” in non-host environments, but also impacts its phenotypic expression of antimicrobial resistance. Hospital, municipal and agricultural effluents as well as wildlife waste are all sources of AMR enteric bacterial contamination in the open environment (Baquero *et al.*, 2008; Lu *et al.*, 2010). Consequently, thoughtful action is necessary to prevent further improper use of antibiotics in humans and livestock such that the selective pressures imposed on enteric microorganisms can be reduced.

The research presented within this thesis adds context to the occurrence and “naturalization” potential of *Salmonella* in the broader environment. While enhanced

experimental rigor (e.g. the number of isolates analyzed in Chapter 3 and the number of replicates used in Chapter 4) would ultimately be required to support any policy amendments, this research along with similar “naturalization” studies have laid important groundwork upon which to consider modification or improvement of ongoing source water protection strategies. It is necessary to implement regulatory guidelines to manage the environmental presence of infectious *Salmonella* and thereby reduce its detrimental impact on public health. The association between the applications of this research to factors directly affecting society is one way in which the integrative nature of this research is illustrated.

This study can also be considered integrative with regard to the methodological approach taken, which generated perspective at the microbial genome, organism and community levels. The originality of the presented research lies primarily in the characterization of the occurrence of *Salmonella* as an individual organism and with respect to its associated non-host, environmental community. By taking a multi-technique approach, a more comprehensive understanding of the inherently complex concept of “naturalization”, and specifically as it relates to *Salmonella*, was gained. Therefore, the novelty and importance of this work is apparent in terms of both the research question investigated and the experimental design and approach.

5.5 References

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Appendix A- Supplementary Tables and Figures

Table A1. A summary of tested antibiotics by class, including category of importance as determined by Health Canada, common uses and mechanisms of action.

Class of Antibiotic (Health Canada Category of Importance)	Generic Name of Antibiotics Used (abbreviation, concentration)	Common Uses	General Mode of Action
Aminoglycoside (II: High importance)	Streptomycin (S, 10µg)	Used against infections caused by Gram negative bacteria	Binds to bacterial 30S ribosomal subunit to inhibit the translocation of the peptidyl-tRNA from the A-site to the P-site
3rd Generation Cephalosporins (I: Very high importance)	Cefotaxime (CTX, 30µg) Ceftazidime (CAZ, 30µg) Ceftriaxone (CRO, 30µg)	Coverage of many Gram negative bacteria excluding <i>Pseudomonas</i>	Disrupts the synthesis of peptidoglycan layer of bacterial cell walls
Chloramphenicol (I: Very high importance)	Chloramphenicol (C, 30µg)	Meningitis, MRSA	Inhibits bacterial protein synthesis by binding to the 50S subunit of the ribosome
Penicillins (II: High importance)	Ampicillin (AM, 10µg) Amoxicillin (AMC, 30µg)	Used against a broad range of infections including streptococcal infections, syphilis and Lyme Disease	Disrupts the synthesis of the peptidoglycan layer of bacterial cell walls
Quinolones (II: High Importance)	Ciprofloxacin (CIP, 5µg)	Used against urinary tract infections, bacterial prostatitis, bacterial diarrhea, mycoplasmal infections and gonorrhea	Inhibits DNA replication and transcription by inhibiting the bacterial DNA gyrase or topoisomerase IV enzyme
Tetracyclines (III: Medium importance)	Doxycycline (D, 30µg) Tetracycline (Te, 30µg)	Used against chlamydial infections, mycoplasmal infections, Lyme Disease and rickettsial infections	Inhibits the binding aminoacyl-tRNA to the mRNA-ribosome complex
Sulfonamides (III: Medium importance)	Sulfisoxazole (G, 0.25mg) Sulfamethoxazole-trimethoprim (SXT, 1.25µg)	Urinary tract infections, eye infections	Competitively inhibits the bacterial enzyme dihydropteroate synthetase, blocking the synthesis of folate which is necessary for nucleic acid synthesis

Table A2. Environmental parameters measured using an YSI meter (YSI Incorporated, Yellow Springs, Ohio, USA) for the eleven Grand River sites sampled in the summer of 2012. Sites 1 through 6 were sampled on July 3 while sites 7 through 11 were sampled on July 4 of 2012.

	Site Number										
Environmental Measurements	1	2	3	4	5	6	7	8	9	10	11
Temperature (°C)	24.2	24.4	23.6	24.3	22.0	23.1	25.6	26.5	27.5	26.0	26.0
DO%	-	-	-	-	104	77.7	113.4	116.6	111.3	113.7	107.68
DO (mg/L)	7.83	7.72	7.08	8.64	9.08	6.85	9.23	9.00	8.82	9.21	8.72
Conductivity	440	444	451	441	1530	1196	583	590	586	710	791
NH₄ (mg/L)	0.47	0.40	0.30	0.30	0.79	8.94	0.30	0.31	0.30	1.57	2.14
NH₃ (mg/L)	2.55	2.7	6.60	6.20	5.06	5.78	7.86	6.69	6.93	7.40	6.37
Pressure (mmHg)	-	-	-	-	732.5	731.9	733.0	732.8	732.9	733.1	733.1

Table A3. A summary of Chapter 3 sampling sources, including location, site description, and type of sample collected.

Sampling Source	Location	Description	Type of Sample Collected
Grand River	Waterloo, ON	<ul style="list-style-type: none"> •one of the most ecologically influential rivers of this region •courses over 300km and covers an area of approx. 6,800km² •heavily impacted by natural, municipal, recreational and agricultural activities 	<ul style="list-style-type: none"> •water samples
Clair Lake (GR Sub watershed)	Waterloo, ON	<ul style="list-style-type: none"> •reservoir of the Laurel Creek tributary, part of the greater GR watershed •last winter, underwent dredging, presenting a unique sampling opportunity to investigate long-term survival of <i>Salmonella</i> and classic fecal indicator bacteria (FIB) 	<ul style="list-style-type: none"> •water and soil samples were taken during dredging in winter, rehabilitation in the spring, and following its recovery in the summer
Centre for Alternative Wastewater Treatment (CAWT)	Lindsay, ON	<ul style="list-style-type: none"> •on-site constructed wetland for the treatment of domestic wastewater effluent from the Frost Campus of Fleming College •the system consists of four test cells to simulate and integrate natural wetland processes and a final polishing pond •final discharge either recycled back into wetland system or released into municipal sewer •able to treat ~3,000L of wastewater daily 	<ul style="list-style-type: none"> •water from vaults 1,2, 3 and 4, and final polishing pond
Water Treatment Centre (WTC)	Burlington, ON	<ul style="list-style-type: none"> •pilot scale plant receiving raw wastewater from the Skyway •Wastewater Treatment Plant which serves Burlington and its environs including Joseph Brant Memorial Hospital 	<ul style="list-style-type: none"> •raw influent and treated effluent

A1 Water	A2 β -Methyl-D-Glucoside	A3 D-Galactonic Acid γ -Lactone	A4 L-Arginine	A1 Water	A2 β -Methyl-D-Glucoside	A3 D-Galactonic Acid γ -Lactone	A4 L-Arginine	A1 Water	A2 β -Methyl-D-Glucoside	A3 D-Galactonic Acid γ -Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α -Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ -Hydroxybutyric Acid	E4 L-Threonine	E1 α -Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ -Hydroxybutyric Acid	E4 L-Threonine	E1 α -Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ -Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α -Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α -Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α -Ketobutyric Acid	G4 Phenylethyl-amine
H1 α -D-Lactose	H2 D,L- α -Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α -D-Lactose	H2 D,L- α -Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α -D-Lactose	H2 D,L- α -Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Figure A1. Representation of the BIOLOG EcoPlateTM format containing 31 carbon sources in triplicate. Each square box represents an individual well on the plate (image obtained from www.biolog.com)

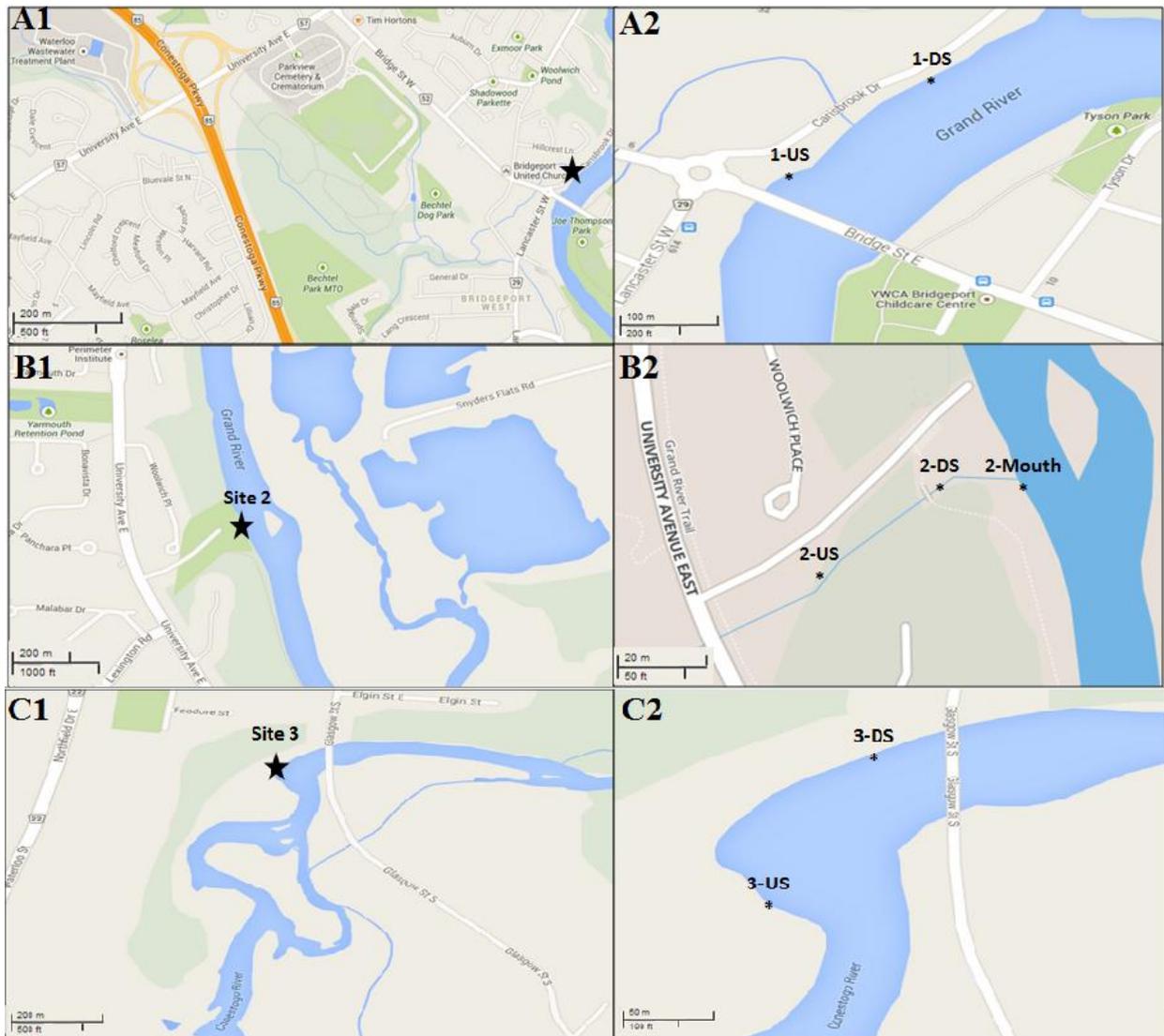


Figure A2. Maps of the Grand River site locations for samples collected on April 27, 2012. (A1) A zoomed out image of the Site 1 location (denoted by the star) near the intersection of Bridge St E. and Lancaster St W.. This site was located less than 5 km downstream of the Waterloo Wastewater Treatment Plant (top left of image). (A2) A closer look at upstream (1-US) and downstream (1-DS) sampling locations at Site 1. (B1) A zoomed out image of Site 2 (denoted by the star), at Kaufman flats off University Ave. E.. (B2) A zoomed in image of the specific sampling locations of Site 2, including an upstream sample (2-US), downstream sample (2-DS) and the mouth of the tributary (2-Mouth) which opens into the Grand River. (C1) A zoomed out image of Site 3 (denoted by the star) on the Conestoga River in an area heavily impacted by agricultural activity. (C2) A closer view of the upstream (3-US) and downstream (3-DS) sampling locations of Site 3 (images from maps.google.ca).

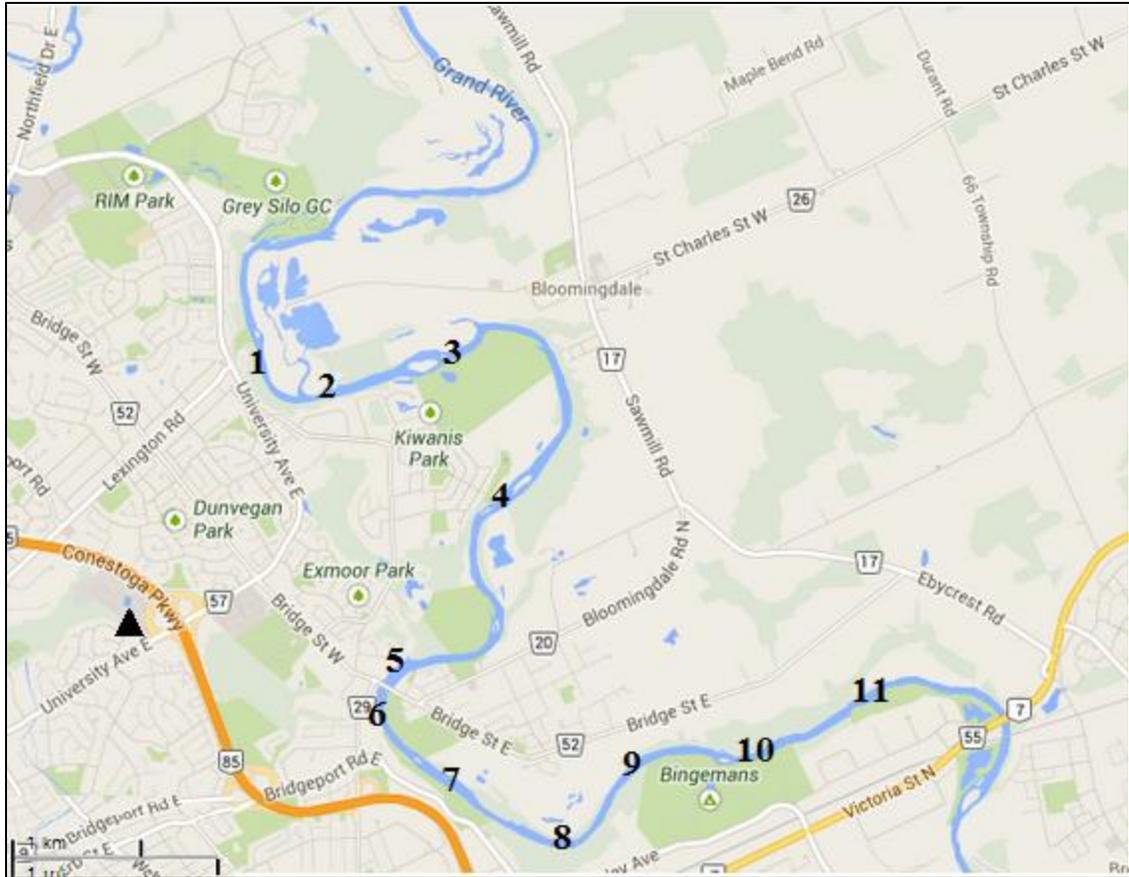


Figure A3. A map depicting the eleven Grand River sites sampled on July 3rd and 4th, 2012. Approximate sample site locations are numerically indicated and the black triangle denotes the location of the Waterloo Wastewater Treatment Plant (images from maps.google.ca).



Figure A4. A map depicting the location of Clair Lake from which samples were collected on November 19'12, April 26'13, June 6'13 and August 29'13 (images from maps.google.ca).

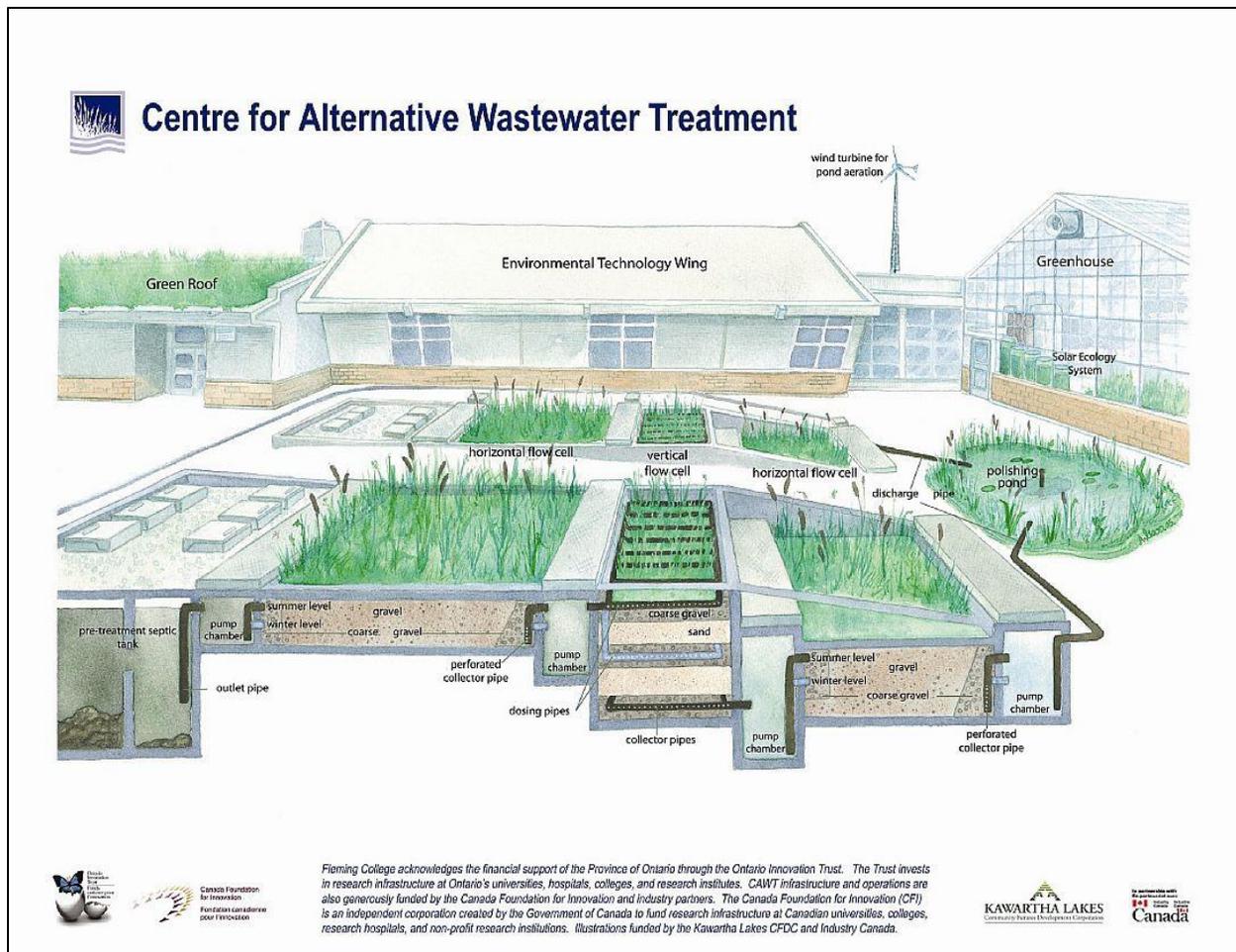


Figure A5. A schematic representation of the CAWT constructed wetland designed for the treatment of domestic waste Frost Campus of Fleming College. The constructed wetland consists of three test vaults in sequence and a final polishing pond, whereafter the treated wastewater is either discharged into the municipal sewer or recycled back into the system (image obtained from http://www.iees.ch/EcoEng071/EcoEng071_Wootton.html).

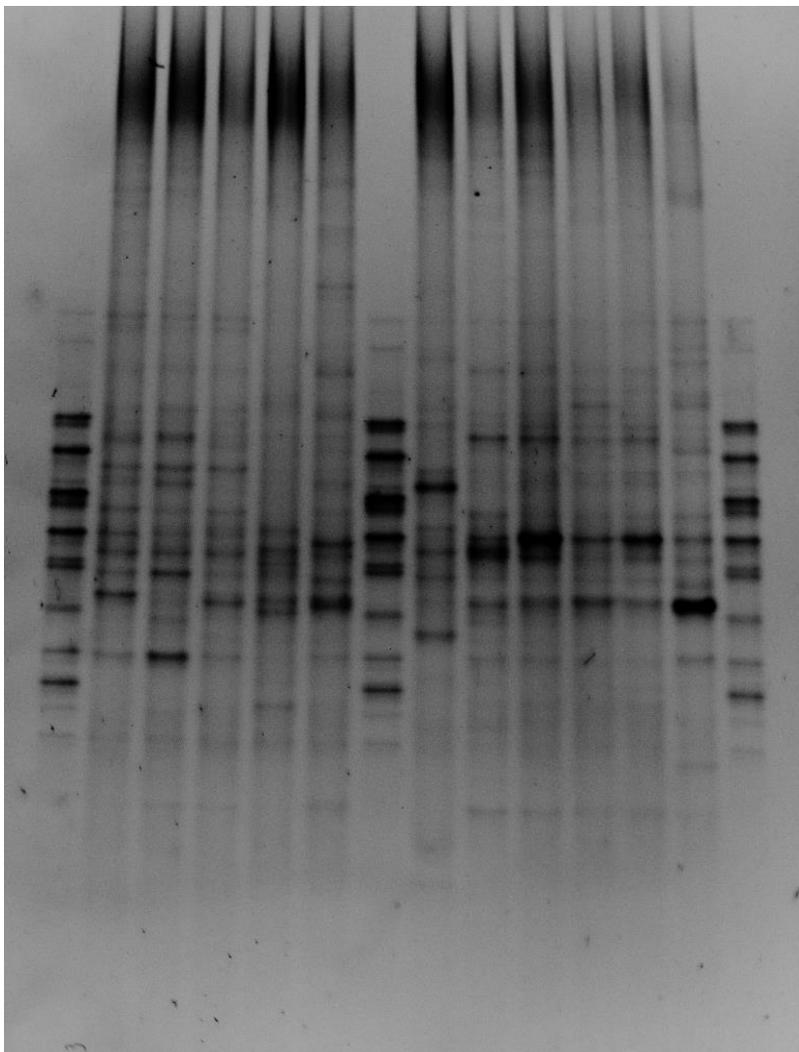


Figure A6. An example DGGE gel image from Grand River samples collected on July 3rd and 4th, 2012. Ladders are in lanes 1, 7 and 14. All other lanes are loaded with sample.

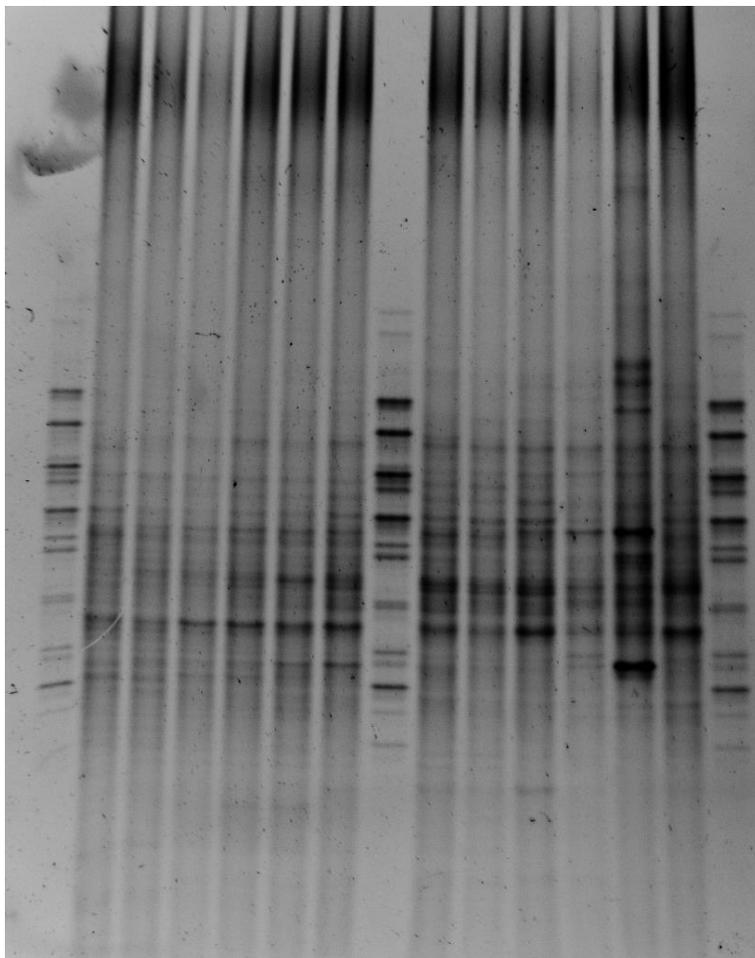


Figure A7. An example DGGE gel image displaying results from WTC microcosms stored at 22°C. Ladders are within lanes 1, 8 and 15. All remaining lanes are loaded with sample.

Appendix B- Investigating HPC bias

As illustrated in Figure B1, the subpopulation of bacteria able to grow under standard heterotrophic plate count conditions greatly underrepresents the microbial community present within a gram of soil as detected through direct DNA extraction. Based on DGGE profiling, species diversity as measured by the Shannon Index yielded an average H' value of over 0.5 higher as a result of direct DNA extraction compared to standard plate counts. Furthermore, nearly double the amount of bands representing OTUs were detected in DNA extracted directly from the gram of soil compared to DNA extracted from heterotrophic growth of organisms on R2A, a loss in genetic richness was also apparent through plate counts. Therefore, when investigating the genetic structure contained within a gram of Clair Lake soil, growth as a result of HPC methods represents a very small subpopulation of the total microbial community.

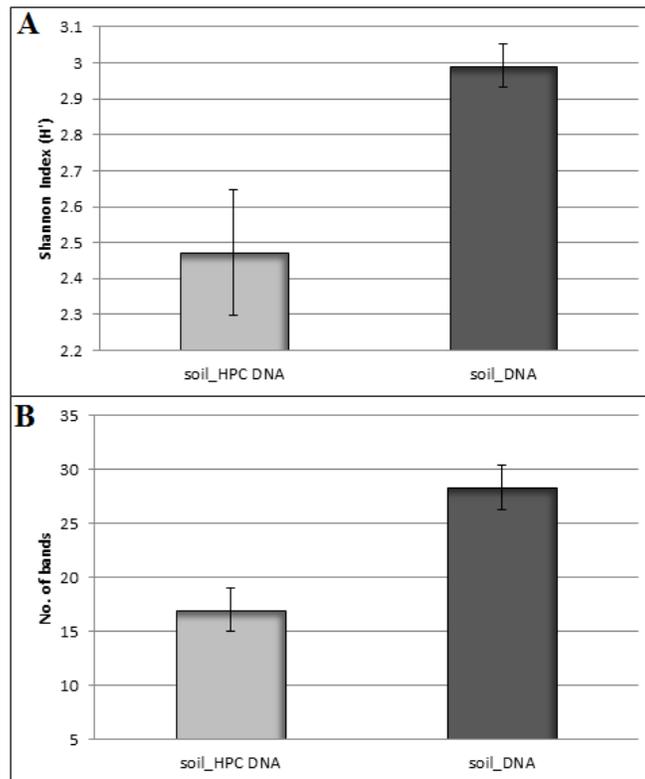


Figure B1. DGGE-based analysis of 1 gram of Clair Lake soil using DNA extracted from growth obtained on R2A agar following standard methods for HPCs vs. direct DNA extracted from 1 gram of soil in terms of (A) species diversity and (B) species richness.

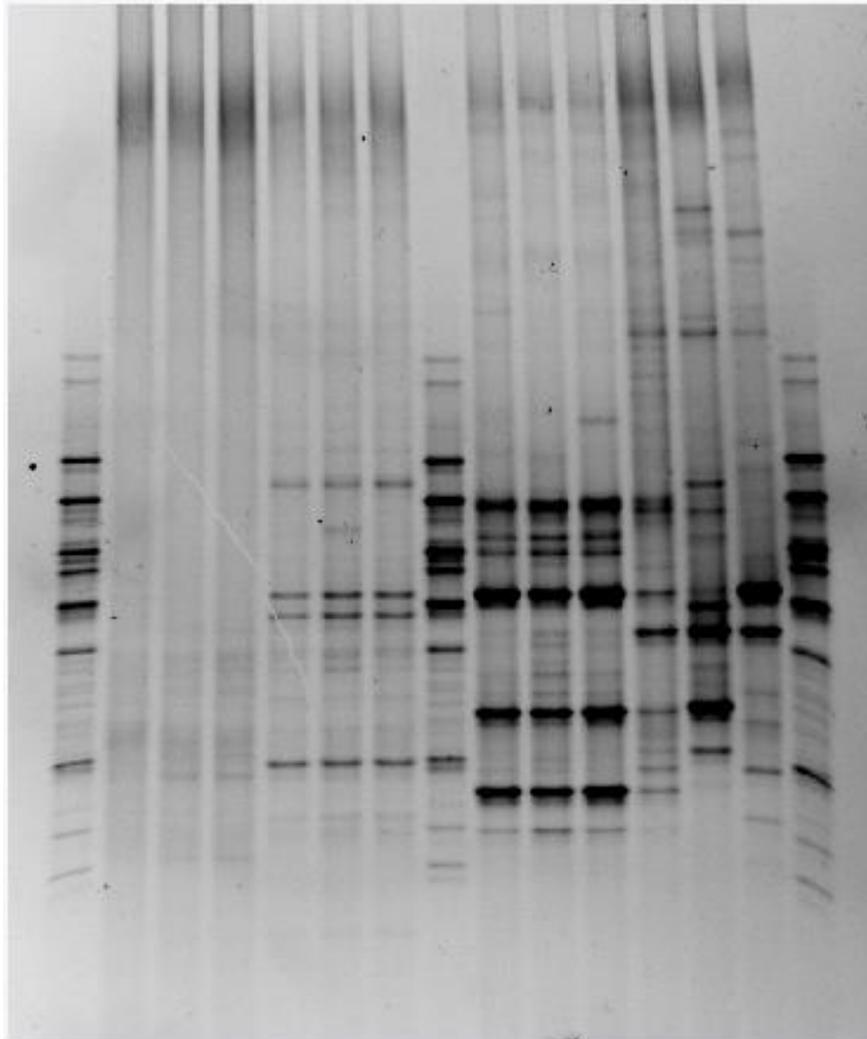


Figure B2. DGGE gel image demonstrating HPC bias. Ladders are in lanes 1, 8 and 15. Lanes 5, 6, 7 contain PCR products containing DNA extracted from growth resulting from 1 gram of soil plated under standard heterotrophic plate count conditions. Lanes 12, 13 and 14 are loaded with PCR product containing DNA extracted directly from 1 gram of soil. Lanes 2-3 and 9-11 contain DNA extracted from water samples through HPC and membrane filtration, respectively, but due to volume inconsistencies a direct comparison of banding profiles cannot be made.