

**Occurrence, Fate, and Mobility of Antibiotic Resistant Bacteria and Antibiotic  
Resistance Genes among Microbial Communities Exposed to Alternative Wastewater  
Treatment Systems**

by

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A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Doctor of Philosophy

in

Biology

Waterloo, Ontario, Canada, 2012

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## Abstract

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The ubiquitous nature of antibiotic resistance and antibiotic resistance genes (ARGs) among environmental pathogens from a variety of wastewater effluents, suggests that the aquatic environment, and specifically alternative wastewater treatment systems, may act as reservoirs for drug resistant bacteria and ARGs, thereby contributing to the widespread dissemination of antibiotic resistance. More research is necessary to contribute to our understanding of the occurrence, fate, and mobility of antibiotic resistance and ARGs among bacterial indicators of faecal contamination as well as pathogenic bacteria within Canadian wastewater treatment systems. The primary objective of this research was to determine the prevalence, fate, and potential transfer of bacterial resistance and ARGs among selected environmental pathogens exposed to alternative wastewater treatment systems, while considering the impact of treatment strategies on the expression of antibiotic resistance. A detailed analysis was initially conducted with respect to the characterization and quantification of microbial populations (including antibiotic resistant bacteria) in a variety of treatment systems and waste effluent sources. Traditional culture-based screening techniques in combination with molecular characterization (through colony or multiplex PCR), and molecular quantification using real-time quantitative PCR were utilized in order to help establish a preliminary environmental assessment of selected pathogens (*Escherichia coli*, *Enterococcus* spp., *Salmonella* spp.) and ARGs (*tetA*, *blaSHV*, & *ampC*) within a variety of wastewater treatment systems (lab-scale mesocosms, constructed wetland, constructed lagoon system, and pilot-scale biological nutrient removal (BNR) system).

Overall, the level of multiple antibiotic resistance (MAR) among culturable indicator (*E. coli* & *Enterococcus* spp.) and environmental bacteria was high (reaching 100% in several instances) within different types of wastewater treatment systems and effluent sources (poultry waste effluent, municipal wastewater, aquaculture wastewater). Common antibiotic resistance profiles among *E. coli* isolates included simultaneous resistance to between three and five antimicrobials, whereas common MAR profiles among *Enterococcus* spp. isolates showed resistance to ten or more antibiotics. Real time quantitative PCR was used to determine the concentration of three bacterial pathogens; *E. coli*, *Enterococcus faecalis*, and *Salmonella* spp., and three ARGs; *tetA*, *ampC*, and *blaSHV*, within a variety of wastewater samples. Based on the results, it was concluded that high concentrations of ARGs were present in the treated effluent ( $10^4$  -  $10^6$  target gene copies/100 mL), regardless of system type (i.e. constructed lagoon, pilot-scale BNR, or constructed wetland), which may ultimately serve as a potential route for entry of ARGs and antibiotic resistant bacteria into the natural environment.

Water is considered an important medium for transfer of resistance genes and resistant bacteria to the broader environment. Few studies have examined the transferability via conjugation of ARGs in *E. coli* and *Salmonella* spp. isolated from wastewater. Identification of three resistance determinants (*tetA*, *strA*, *strB*) conferring resistance to tetracycline and streptomycin was performed on selected multi-drug resistant *Salmonella* spp. and *E. coli* isolates. The potential for transfer of tetracycline and streptomycin resistance genes was demonstrated through broth conjugation experiments using multi-drug resistant *Salmonella* spp. and *E. coli* isolates as donors, and *E. coli* K12 as the recipient. Conjugation was

successfully observed in 75% (9/12) of donor isolates, occurring in both *Salmonella* spp. and *E. coli* isolates. Six strains (50%) were capable of transferring their *tetA*, *strA*, and *strB* genes to the recipient strain, resulting in 58.5% (38/65) of total transconjugant strains acquiring all three resistance determinants. The results confirm the role of environmental bacteria (isolated from wastewater treatment utilities) as a reservoir of antibiotic resistance and ARGs, containing mobile genetic elements, which are capable of disseminating and transferring ARGs. As concerns about water quality and environmental contamination by human and agricultural effluents have increased, it has become increasingly more important to consider the prevalence and transferability of ARGs to opportunistic and human pathogens.

As observed in this research, the ubiquitous nature of multi-drug resistant bacteria in water and wastewater effluents, the presence of diverse ARGs of human and veterinary health significance, as well as the transfer of resistance determinants through conjugative plasmids to recipient bacteria, suggests that environmental exposure through contact or consumption with contaminated water is probable. However, a lack of critical information still exists regarding the movement of resistance genes within and between microbial populations in the environment. In addition, the extent of human exposure to ARGs and antibiotic resistant bacteria is still not well understood, and future studies on human exposure to these resistant contaminants are necessary.

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## Acknowledgements

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First and foremost, I would like to express my sincere and utmost gratitude to my supervisors, mentors and friends, Drs. Robin Slawson (Wilfrid Laurier University) and Raymond Legge (University of Waterloo). Robin – I am so very grateful for your immeasurable encouragement, guidance and emotional support throughout my graduate career and life in general. Thank you for motivating me to my absolute limits of intellectual development and never allowing me to settle for anything less than my utmost best. I appreciate your unrestricted time and energy, including our lengthy, yet always enjoyable meetings. Ray – I would like to thank you for your valuable contributions to my research, and enthusiastic attitude during my graduate experience. I am so grateful for your time and patience; educating a microbiologist with no prior knowledge of engineering must have been a little trying at the best of times. My graduate career has been one of the most pleasurable and exciting periods of my life, and for this I am thankful to both Robin's and Ray's excellent supervision.

I would like to thank my committee members Dr. Wayne Parker, Dr. Josh Neufeld, and Dr. Barbara Butler, for your constructive and beneficial input on my research and for your encouraging words during our meetings over the years.

I would also like to thank the Natural Sciences and Engineering Research Council (NSERC) and the Ontario Graduate Scholarship Program (OGS) for their financial support in the form of graduate scholarships. In addition, this research was supported by financial assistance

provided by the Centre for Control of Emerging Contaminants (CCEC), and the Centre for Alternative Wastewater Treatment (CAWT) through collaborators at Fleming College.

I would like to acknowledge Dr. Kela Weber who was directly involved with a portion of the laboratory work presented in Chapter 2 of this thesis. I am grateful for his guidance, support and positive encouragement throughout my graduate career.

Thank you to the staff in the Department of Biology at Wilfrid Laurier University for allowing me to have an office space and a couch to rest on during those late nights and extensive hours in the lab. I am also grateful for the assistance of many undergraduate, graduate, and post-doctoral students for providing me with a remarkable working environment and for making day-to-day life in the lab enjoyable; thank you Jessica Morrison, Dr. Patrick Duriez, Mike Mitzel, and Robyn Morrison.

Most importantly, I would like to thank my family and friends for your love, encouragement, and endless patience along the way. I would especially like to acknowledge my parents, in which their hard-work, dedication, academic foundation, and general love of life have given me a continuous example of how to maintain balance, while enjoying and excelling at any endeavor. My accomplishments are a direct result of their unconditional love, support and encouragement.

## Table of Contents

Author's Declaration.....	ii
Abstract .....	iii
Acknowledgements .....	vi
List of Figures.....	xii
List of Tables.....	xvi
List of Abbreviations.....	xviii
<b>Chapter 1: Introduction.....</b>	<b>1</b>
1.1 Background.....	1
1.1.1 Antibiotics and antibiotic resistance.....	1
1.1.2 Antibiotic resistance in alternative wastewater treatment systems.....	8
1.1.3 Antibiotic resistance genes (ARGs) as emerging contaminants.....	12
1.1.4 Detection methods for waterborne pathogens and antibiotic resistance genes (ARGs).....	14
1.1.5 Antimicrobial resistance among indicator organisms and environmental pathogens.....	17
1.1.6 Fate and transmission of environmental pathogens and antibiotic resistance genes.....	22
1.2 Thesis Objectives.....	26
1.3 Organization of the Thesis.....	28
<b>Chapter 2: Antibiotic resistance profiles of wetland bacteria and faecal indicators following ciprofloxacin exposure in lab-scale constructed mesocosms.....</b>	<b>33</b>
2.1 Introduction.....	33
2.2 Research Needs & Objectives.....	37
2.3 Materials & Methods.....	39
2.3.1 Wetland mesocosms.....	39
2.3.2 Antibiotic addition.....	41
2.3.3 Water sampling.....	41
2.3.4 Isolation and enumeration of faecal indicator bacteria.....	42
2.3.5 Molecular confirmation of <i>Enterococcus</i> spp. and <i>E. coli</i> .....	43
2.3.6 Total heterotrophic community plate counts.....	45
2.3.7 Antimicrobial susceptibility testing.....	46
2.3.8 Statistical analysis.....	47
2.4 Results and Discussion.....	48
2.4.1 Enumeration of indicator organisms in mesocosms.....	48
2.4.2 Qualitative PCR to detect and characterize <i>Enterococcus</i> spp. and <i>E. coli</i> .....	51
2.4.3 Antibiotic resistance of interstitial bacterial community.....	53
2.4.4 Antibiotic resistance of <i>E. coli</i> .....	55
2.4.5 Antibiotic resistance of <i>Enterococcus</i> spp.....	60
2.5 Conclusions.....	64
2.6 Recommendations & Future Research Needs.....	64



<b>Chapter 3: Monitoring the occurrence, distribution, and frequency of selected bacterial pathogens and antibiotic resistance genes in treatment lagoons receiving poultry waste effluent.....</b>	<b>66</b>
3.1 Introduction.....	66
3.2 Research Needs & Objectives.....	69
3.3 Materials & Methods.....	71
3.3.1 Lagoon sampling.....	71
3.3.2 Isolation and enumeration of faecal bacteria.....	73
3.3.3 Molecular confirmation of <i>Enterococcus</i> spp. and <i>E. coli</i> .....	74
3.3.4 Detecting genes associated with antibiotic resistance by PCR.....	76
3.3.5 Antimicrobial susceptibility testing.....	77
3.3.6 Selection of PCR primers and TaqMan® probes.....	78
3.3.7 DNA extraction.....	79
3.3.8 Amplification of standards for real-time qPCR assays.....	79
3.3.9 Quantitative Polymerase Chain Reaction.....	81
3.3.10 Statistical analysis.....	82
3.4 Results & Discussion.....	82
3.4.1 Qualitative PCR to detect and characterize <i>Enterococcus</i> spp. and <i>E. coli</i> .....	82
3.4.2 Prevalence of antibiotic resistance among <i>E. coli</i> and <i>Enterococcus</i> spp.....	85
3.4.3 Analysis of antimicrobial resistance genes (ARGs).....	106
3.4.4 Quantitative detection of bacterial pathogens and ARGs in a treatment lagoon system.....	109
3.4.5 Antimicrobial removal efficiency of wastewater treatment in a constructed lagoon system.....	113
3.5 Conclusions.....	118
3.6 Recommendations & Future Research Needs.....	119
 <b>Chapter 4: Prevalence of antimicrobial resistance among <i>Aeromonas</i>, <i>Yersinia</i>, and <i>Flavobacterium</i> populations and selected ARGs during winter operation of a constructed wetland receiving fish hatchery effluent.....</b>	 <b>121</b>
4.1 Introduction.....	121
4.2 Research Needs & Objectives.....	126
4.3 Materials & Methods.....	128
4.3.1 Sub-surface flow constructed wetland.....	128
4.3.2 Antibiotic addition.....	130
4.3.3 Water sampling.....	130
4.3.4 Isolation and enumeration of selected environmental pathogens.....	131
4.3.5 Molecular confirmation of <i>Yersinia</i> spp. and <i>Aeromonas</i> spp.....	133
4.3.6 Antimicrobial susceptibility testing.....	134
4.3.7 DNA extraction.....	135
4.3.8 Selection of PCR primers and TaqMan probes.....	136
4.3.9 Quantitative Polymerase Chain Reaction.....	138
4.3.10 Statistical analysis.....	139
4.4 Results & Discussion.....	139

4.4.1	Enumeration of selected fish pathogens in CW.....	139
4.4.2	Qualitative PCR to detect and characterize <i>Aeromonas</i> spp. and <i>Yersinia</i> spp.....	147
4.4.3	Frequency and distribution of antibiotic resistance among <i>Aeromonas</i> spp., <i>Yersinia</i> spp., and <i>Flavobacterium</i> spp.....	150
4.4.4	Quantitative detection of ARGs in a sub-surface flow constructed wetland.....	164
4.5	Conclusions.....	169
4.6	Recommendations & Future Research Needs.....	171
<b>Chapter 5: Distribution, quantification, and potential dissemination of selected pathogens and antibiotic resistance genes in a pilot-scale BNR system receiving wastewater from a full-scale WWTP.....</b>		<b>172</b>
5.1	Introduction.....	172
5.2	Research Needs & Objectives.....	177
5.3	Materials & Methods.....	179
5.3.1	Pilot-scale BNR system and sampling.....	179
5.3.2	DNA extraction.....	181
5.3.3	Isolation and enumeration of faecal bacteria.....	182
5.3.4	Antimicrobial susceptibility testing.....	183
5.3.5	Selection of PCR primers and TaqMan® probes.....	184
5.3.6	Amplification of standards for real-time qPCR assays.....	185
5.3.7	Quantitative Polymerase Chain Reaction.....	185
5.3.8	Statistical analysis.....	187
5.4	Results and Discussion.....	187
5.4.1	Prevalence of antibiotic resistance among faecal indicator organisms.....	187
5.4.2	Quantitative detection of bacterial pathogens and ARGs in a pilot-scale BNR treatment system.....	200
5.4.3	Removal efficiency of bacteria and ARGs in a pilot-scale BNR treatment system.....	207
5.5	Conclusions.....	209
5.6	Recommendations & Future Research Needs.....	210
<b>Chapter 6: Prevalence and transferability of tetracycline and streptomycin determinants in <i>E. coli</i> and <i>Salmonella</i> spp. isolated from wastewater effluents.....</b>		<b>212</b>
6.1	Introduction.....	212
6.2	Research Needs & Objectives.....	218
6.3	Materials & Methods.....	219
6.3.1	Bacterial strains.....	219
6.3.2	Detection of tetracycline and streptomycin resistance genes.....	220
6.3.3	Antimicrobial susceptibility testing.....	221
6.3.4	Plasmid extraction.....	222
6.3.5	Conjugation experiments.....	222
6.3.6	Statistical analysis.....	223
6.4	Results and Discussion.....	224
6.4.1	Prevalence of antibiotic resistance among <i>Salmonella</i> spp. and <i>E. coli</i> .....	224
6.4.2	Distribution of tetracycline and streptomycin resistance genes.....	229

6.4.3	Transfer of tetracycline and/or streptomycin resistance genes by conjugation.....	231
6.5	Conclusions.....	239
6.6	Recommendations & Future Research Needs.....	241
<b>Chapter 7: Thesis Conclusions &amp; Recommendations .....</b>		<b>243</b>
7.1	Thesis Summary & Principle Contributions.....	243
7.1.1	Antibiotic resistance profiles of wetland bacteria and faecal indicators following ciprofloxacin exposure in lab-scale constructed mesocosms (Objectives A-B & E)....	245
7.1.2	Prevalence of antimicrobial resistance among <i>Aeromonas</i> , <i>Yersinia</i> , and <i>Flavobacterium</i> populations and selected ARGs during winter operation of a constructed wetland receiving fish hatchery effluent (Objectives A-C & E).....	246
7.1.3	Monitoring the occurrence, distribution, and frequency of selected bacterial pathogens and antibiotic resistance genes in treatment lagoons receiving poultry waste effluent (Objectives A-C).....	248
7.1.4	Distribution, quantification, and potential dissemination of selected pathogens and antibiotic resistance genes in a pilot-scale BNR system receiving wastewater from a full-scale WWTP (Objectives A-C).....	250
7.1.5	Prevalence and transferability of tetracycline and streptomycin determinants in <i>E. coli</i> and <i>Salmonella</i> spp. isolated from wastewater effluents (Objectives C & D).....	251
7.2	Thesis Recommendations.....	255
<b>References.....</b>		<b>257</b>
<b>Appendix A - Quantitative PCR results for selected pathogens and ARGs collected during the summer (July) 2010 from a constructed lagoon system.....</b>		<b>298</b>

## List of Figures

<b>Figure 1.1</b> - Hypothetical representation of removal mechanisms and release of antibiotics (ABs), antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARGs) in a typical wastewater treatment plant using the activated sludge process.....	6
<b>Figure 1.2</b> - Sources and movement of antibiotic resistance genes in the environment contributing to the widespread dissemination of antibiotic resistance.....	25
<b>Figure 2.1</b> - Group 1 genus and species multiplex PCR of enterococci.....	45
<b>Figure 2.2</b> - Mean values of total heterotrophs (A), <i>E. coli</i> (B), and <i>Enterococcus</i> spp. (C) as colony-forming units (CFUs) per 100 mL over three sampling dates in constructed mesocosms.....	49
<b>Figure 2.3</b> - Distribution and molecular confirmation of <i>Enterococcus</i> spp. (n=115) and <i>E. coli</i> (n=168) among screened isolates in interstitial water samples among the four mesocosms.....	52
<b>Figure 2.4</b> - Average Minimum Inhibitory Concentration (MIC) of total heterotrophic community to ciprofloxacin in two control mesocosms (CON1 & CON2) and two mesocosms exposed to a 2 mg L <sup>-1</sup> ciprofloxacin pulse (CIP1 & CIP2).....	54
<b>Figure 2.5</b> - Prevalence of antibiotic resistance (%) among <i>E. coli</i> for CIP1 mesocosm (A) and CIP2 mesocosm (B) before and after 2 mg L <sup>-1</sup> ciprofloxacin exposure.....	56
<b>Figure 2.6</b> - Prevalence of antibiotic resistance (%) among <i>E. coli</i> in mesocosms without ciprofloxacin exposure (CON1 & CON2) and for initial sludge seed at Day 0.....	59
<b>Figure 2.7</b> - Prevalence of antibiotic resistance (%) among <i>Enterococcus</i> spp. for control mesocosms, CON1 & CON2 (A) and ciprofloxacin exposed mesocosms, CIP1 & CIP2 (B).....	62
<b>Figure 3.1</b> - A general schematic depicting overset hydrology and sampling points in a constructed lagoon facility.....	73
<b>Figure 3.2</b> - An example of real-time PCR results showing the relationship between gene copy numbers/reaction (or DNA amount) and cycle threshold (CT).....	80
<b>Figure 3.3</b> - Distribution of <i>Enterococcus</i> spp. among screened isolates (n=93) in the constructed lagoon system.....	83
<b>Figure 3.4</b> - Prevalence of antibiotic resistance (%) among <i>E. coli</i> isolates collected during two sampling events (summer and fall) in a constructed lagoon system.....	88
<b>Figure 3.5</b> - Prevalence of multiple antibiotic resistance (MAR) profiles (%) among <i>E. coli</i> isolates collected during two sampling events (summer and fall) in a constructed lagoon system.....	89

<b>Figure 3.6</b> - Frequency (%) of antimicrobial resistance profiles among <i>E. coli</i> isolates (n=140) collected during two sampling events (summer and fall) in a constructed lagoon system.....	92
<b>Figure 3.7</b> - Prevalence of antibiotic resistance (%) among <i>Enterococcus</i> spp. isolates collected during two sampling events (summer and fall) in a constructed lagoon system...	100
<b>Figure 3.8</b> - Prevalence of multiple antibiotic resistance (MAR) profiles (%) among <i>Enterococcus</i> spp. isolates collected during two sampling events (summer and fall) in a constructed lagoon system.....	101
<b>Figure 3.9</b> - Frequency (%) of antimicrobial resistance profiles among <i>Enterococcus</i> spp. isolates (n=175) collected during two sampling events (summer and fall) in a constructed lagoon system.....	103
<b>Figure 3.10</b> - Proportion of total <i>E. coli</i> isolates carrying antibiotic resistance genes.....	108
<b>Figure 3.11</b> - Relative abundance (target gene copies/100 mL) of bacterial pathogens ( <i>E. coli</i> , <i>Salmonella</i> spp., and <i>E. faecalis</i> ) at different sampling points in the treatment lagoon system.....	110
<b>Figure 3.12</b> - Relative abundance (target gene copies/100 mL) of ARGs ( <i>blaSHV</i> , <i>ampC</i> , and <i>tetA</i> ) at different sampling points in the treatment lagoon system.....	112
<b>Figure 3.13</b> - Average log reduction of target gene copies/100 mL from pre-treatment to final lagoon (lagoon#3) samples among bacterial pathogens and ARG's.....	114
<b>Figure 4.1</b> - Schematic representation of a horizontal sub-surface flow constructed wetland.....	125
<b>Figure 4.2</b> - Schematic and photograph of the sub-surface flow CW.....	131
<b>Figure 4.3</b> - Mean values of <i>Aeromonas</i> spp. (A) and <i>Yersinia</i> spp. (B) as colony-forming units (CFUs) per 100 mL from various locations in a sub-surface flow constructed wetland system collected over five sampling dates.....	142
<b>Figure 4.4</b> - Mean values of <i>Flavobacterium</i> spp. as colony-forming units (CFUs) per 100 mL from various locations in a sub-surface flow constructed wetland system collected over three sampling dates.....	145
<b>Figure 4.5</b> - Prevalence of antibiotic resistance (%) among <i>Aeromonas</i> spp. to amoxicillin, doxycycline, streptomycin, and oxytetracycline in various sampling locations in the constructed wetland before and after (6, 20, 48 days) the oxytetracycline exposure.....	152
<b>Figure 4.6</b> - Prevalence of antibiotic resistance (%) among <i>Yersinia</i> spp. to chloramphenicol, streptomycin, doxycycline, oxytetracycline, ceftriaxone, and amoxicillin in	

various sampling locations in the constructed wetland before and after (6, 20, 48 days) the oxytetracycline exposure.....	156
<b>Figure 4.7</b> - Prevalence of antibiotic resistance (%) among <i>Flavobacterium</i> spp. isolated after the oxytetracycline exposure in the 'Septic Out' sample.....	161
<b>Figure 4.8</b> - Relative abundance (target gene copies/100 mL) of <i>tetA</i> (A), <i>ampC</i> (B), and <i>blaSHV</i> (C) at different sampling points in the sub-surface flow constructed wetland.....	166
<b>Figure 5.1</b> - A general schematic depicting the treatment processes in the pilot scale BNR system.....	180
<b>Figure 5.2</b> - Prevalence of antibiotic resistance (%) among <i>Enterococcus</i> spp. isolates from raw wastewater and treated effluent samples.....	190
<b>Figure 5.3</b> - Prevalence of antibiotic resistance (%) among <i>E. coli</i> isolates from raw wastewater and treated effluent samples.....	191
<b>Figure 5.4</b> - Prevalence of multiple antimicrobial resistance (MAR) profiles (%) among <i>E. coli</i> and <i>Enterococcus</i> spp. isolates collected from raw wastewater and treated effluent samples in pilot-scale treatment system.....	193
<b>Figure 5.5</b> - Resistance profiles among <i>Enterococcus</i> spp. isolated from wastewater samples in the pilot-scale BNR system (n=92).....	197
<b>Figure 5.6</b> - Resistance profiles among <i>E. coli</i> isolated from wastewater samples in the pilot-scale BNR system (n=99).....	199
<b>Figure 5.7</b> - Relative abundance (target gene copies/100 mL) of <i>E. coli</i> (A), <i>Salmonella</i> spp. (B), and <i>E. faecalis</i> (C) in wastewater samples from the BNR pilot-scale system.....	201
<b>Figure 5.8</b> - Relative abundance (target gene copies/100 mL) of <i>blaSHV</i> (A), <i>ampC</i> (B), and <i>tetA</i> (C) genes in wastewater samples from the BNR pilot-scale system.....	205
<b>Figure 5.9</b> - Average log reduction of target gene copies/100 mL for all three sampling events from raw wastewater to treated effluent samples among bacterial pathogens and ARG's.....	208
<b>Figure 6.1</b> - Prevalence of multiple antimicrobial resistance (MAR) profiles (%) among waterborne <i>Salmonella</i> spp. and <i>E. coli</i> isolates.....	228
<b>Figure 6.3</b> - Visualization of representative transconjugant isolates (n=48) carrying the <i>strB</i> gene (509 bp) conferring resistance to streptomycin.....	235

<b>Figure 6.4</b> - Visualization of PCR products amplified from transconjugant plasmid DNA isolates (n=85) carrying the <i>strA</i> gene (548 bp) conferring resistance to streptomycin.....	236
<b>Figure 6.5</b> - Visualization of PCR products amplified from transconjugant plasmid DNA isolates (n=85) carrying the <i>tetA</i> gene (210 bp) conferring resistance to tetracycline.....	238
<b>Figure A1</b> - Relative abundance (target gene copies/100 mL) of pathogens ( <i>E. coli</i> , <i>Salmonella</i> spp., <i>E. faecalis</i> ) and ARGs ( <i>blaSHV</i> , <i>ampC</i> , and <i>tetA</i> ) at different sampling points in the treatment lagoon system (July 2010).....	298

## List of Tables

<b>Table 1.1</b> - Modes of action and resistance mechanisms of commonly used antibiotics.....	3
<b>Table 2.1</b> - PCR primers, products, and reference strains for <i>Enterococcus</i> spp., and <i>E. coli</i> .....	44
<b>Table 3.1</b> - PCR primers, products, and reference strains for genotypic confirmation of <i>Enterococcus</i> spp., and <i>E. coli</i> collected from constructed lagoon wastewater samples.....	76
<b>Table 3.2</b> - PCR primers used for the detection of antibiotic resistance genes conferring resistance to streptomycin, sulfonamides, and tetracycline in poultry lagoon samples.....	77
<b>Table 3.3</b> - Primers and probes used for the detection of pathogens and antibiotic resistance genes among constructed lagoon wastewater samples using real-time qPCR.....	78
<b>Table 3.4</b> - Percentage of resistance per sampling location and overall resistance in <i>E. coli</i> isolates collected from a poultry slaughterhouse treatment lagoon system.....	86
<b>Table 3.5</b> - Percentage of resistance per sampling location and overall resistance in <i>Enterococcus</i> spp. isolates collected from a poultry slaughterhouse treatment lagoon system.....	97
<b>Table 4.1</b> - PCR primers, products, and reference strains for <i>Aeromonas</i> spp., and <i>Yersinia</i> spp. among sub-surface flow constructed wetland samples receiving fish hatchery effluent.....	134
<b>Table 4.2</b> - Primers and probes used for the detection of antibiotic resistance genes via qPCR in a sub-surface flow constructed wetland.....	137
<b>Table 4.3</b> - Molecular confirmation of presumptive <i>Aeromonas</i> spp. isolates and distribution of <i>Y. ruckeri</i> and <i>Y. enterocolitica</i> among screened isolates in the constructed wetland.....	147
<b>Table 5.1</b> - Primers and probes for real-time qPCR to detect pathogens and antibiotic resistance genes.....	184
<b>Table 6.1</b> - PCR primers used for the detection of <i>E. coli</i> , <i>Salmonella</i> spp., and antibiotic resistance genes.....	220
<b>Table 6.2</b> - Phenotype distribution of resistance among waterborne <i>Salmonella</i> spp. and <i>E. coli</i> .....	225
<b>Table 6.3</b> - Phenotypic and genotypic resistance profiles of <i>E. coli</i> and <i>Salmonella</i> spp. donor and recipient strains.....	230



<b>Table 6.4</b> - Antimicrobial resistance patterns of selected MDR <i>E. coli</i> and <i>Salmonella</i> spp. donor isolates and transconjugants.....	233
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## **List of Abbreviations**

A/A/O	– Anaerobic, Anoxic, and Oxic
ADA	– Ampicillin Dextrin Agar
AGP	– Antimicrobial Growth Promoter
AM	– Ampicillin
AMC	– Amoxicillin
AMD	– Acid Mine Drainage
AMR	– Antimicrobial Resistance
ARB	– Antibiotic Resistant Bacteria
ARGs	– Antibiotic Resistance Genes
AS	– Activated Sludge
BHI	– Brain Heart Infusion
BNR	– Biological Nutrient removal
BOD	– Biological Oxygen Demand
C	– Chloramphenicol
CAS	– Conventional Activated Sludge
CAZ	– Ceftazidime
cBOD	– Carbonaceous Biochemical Oxygen Demand
CFU	– Colony Forming Unit
CIP	– Ciprofloxacin
CIPARS	– Canadian Integrated Program for Antimicrobial Resistance Surveillance
CLSI	– Clinical and Laboratory Standards Institute
CN	– Cefsulodin-Novobiocin
COD	– Chemical Oxygen Demand
CRO	– Ceftriaxone
C <sub>t</sub>	– Cycle Threshold
CTX	– Cefotaxime
CW	– Constructed Wetland
D	– Doxycycline
DNA	– Deoxyribonucleic acid

EAF	– Electric Arc Furnace
EB	– Ethidium Bromide
ERM	– Enteric Redmouth
G	– Sulfisoxazole
HF	- Horizontal Flow
HFCW	– Horizontal Flow Constructed Wetland
HGT	– Horizontal Gene Transfer
HHOA	– Haliburton Highlands Outdoors Association
HPC	– Heterotrophic Plate Count
HRT	– Hydraulic Retention Time
LB	– Luria-Bertani
LZD	– Linezolid
MAR	– Multiple Antimicrobial Resistance
MBR	– Membrane Biological Reactor
MDR	– Multiple Drug Resistance
MI	– Minocycline
MIC	– Minimum Inhibitory Concentration
MRSA	– Methicillin Resistant <i>Staphylococcus aureus</i>
N	– Number of isolates
NAL	– Nalidixic acid
NARMS	– National Antimicrobial Resistance Monitoring System
ORP	– Oxidative Reduction Potential
OTC	- Oxytetracycline
PCR	– Polymerase Chain Reaction
PVC	– Polyvinylchloride
qPCR	– Quantitative Polymerase Chain Reaction
RFLP	– Restriction Fragment Length Polymorphism
S	– Streptomycin
SAF	- Submerged Aerated Filter
SB	– Sodium Borate
<i>sodA</i>	– superoxide dismutase

SRT	– Solids Retention Time
STP	– Sewage Treatment Plant
SXT	– Sulfamethoxazole-trimethoprim
TE	– Tetracycline
TF	- Trickling Filter
TN	– Total Nitrogen
TP	–Total Phosphorus
TSS	– Total Suspended Solids
UV	– Ultra Violet
VA	– Vancomycin
VBNC	– Viable but Non-Culturable
VRE	– Vancomycin Resistant Enterococci
WHO	– World Health Organization
WWTP	– Wastewater Treatment Plant
YSA	– Yersinia Selective Agar

# 1

## **Background & thesis objectives**

### **1.1 Background**

#### **1.1.1 Antibiotics and antibiotic resistance**

The term "antibiotic" can be defined as any class of organic molecule that causes microbial cell death or inhibits growth due to specific interactions with bacterial targets. For antimicrobials, resistance is usually quantified as the minimum concentration required to assert a definable effect (e.g. growth inhibition) on a population of cells (Kümmerer, 2004). An organism is referred to as resistant when a change in susceptibility renders the antibiotic ineffective against this organism. Many organisms are capable of being insensitive to and are thereby considered intrinsically resistant to a particular agent by nature of their physiology

or biochemistry. An extensive range of biochemical and physiological mechanisms may be responsible for antimicrobial resistance. Five major antibiotic targets exist; the bacterial cell wall, the cell membrane, protein synthesis, DNA and RNA synthesis, and folic acid (vitamin B9) metabolism (Wright, 2010). A collection of commonly used antibiotics, modes of action, and resistance mechanisms are shown in Table 1 (adapted from Davies and Davies, 2010). Resistance to antibiotics occurs through four general mechanisms: target modification, efflux, immunity and bypass, and enzyme-catalyzed destruction. Target modification can occur through mutation of the targets themselves or by production of enzymes that modify the antibiotic targets. For example, vancomycin resistance is a version of target modification where new biosynthetic machinery is engaged to alter cell-wall structure (Wright, 2010). Efflux occurs through a large family of protein pumps that eject antibiotics from inside the cell. In immunity, antibiotics or their targets are bound by proteins that prevent the antibiotic binding to its target. However, the most specific and evolved mechanisms of antimicrobial resistance are enzymes that recognize antibiotics and modify them in such a way as to eliminate the functional characteristics that enable them to interact with their targets. For example,  $\beta$ -lactamases hydrolytically cleave the core  $\beta$ -lactam ring that is characteristic of the class and crucial to antibiotic action (Wright, 2010).

**Table 1.1. Modes of action and resistance mechanisms of commonly used antibiotics (adapted from Davies and Davies, 2010).**

<b>Antimicrobial Class</b>	<b>Antibiotic Used</b>	<b>Spectrum</b>	<b>Target</b>	<b>Resistance mechanisms</b>
<b>Cephalosporin</b>	Cefotaxime (CTX30) Ceftriaxone (CRO30) Ceftazidime (CAZ30)	Broad-spectrum activity against Gram positive & Gram negative bacteria	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
<b>Penicillin</b>	Ampicillin (AM10) Amoxicillin (AMC30)	Activity against Gram positive & some Gram negative bacteria	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
<b>Glycopeptide</b>	Vancomycin (VA30)	Activity against Gram positive bacteria	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
<b>Quinolones</b>	Ciprofloxacin (CIP5)	Broad-spectrum activity against Gram positive & Gram negative bacteria	DNA replication	Acetylation, efflux, altered target
<b>Sulfonamide</b>	Sulfisoxazole (G.25) Trimethoprim-Sulfamethoxazole (SXT) Sulfadiazine (SD.25)	Activity against a wide range of Gram negative & Gram positive bacteria	C <sub>1</sub> metabolism	Efflux, altered target
<b>Oxazolidinone</b>	Linezolid (LZD30)	Active against most Gram positive bacteria	Translation	Efflux, altered target
<b>Aminoglycoside</b>	Streptomycin (S10)	Activity against Gram positive & Gram negative bacteria	Translation	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
<b>Tetracycline</b>	Oxytetracycline (T30) Doxycycline (D30) Tetracycline (TE30) Minocycline (MI30)	Broad-spectrum activity against Gram positive & Gram negative bacteria	Translation	Monooxygenation, efflux, altered target
<b>Phenicol</b>	Chloramphenicol (C30)	Activity against a wide variety of Gram negative and Gram positive bacteria	Translation	Acetylation, efflux, altered target

The predominant role of human activities in the generation of environmental reservoirs of antibiotic resistance cannot be disputed. Since the 1940s, escalating amounts of antibiotics designated for human applications have been manufactured, used clinically, released into the

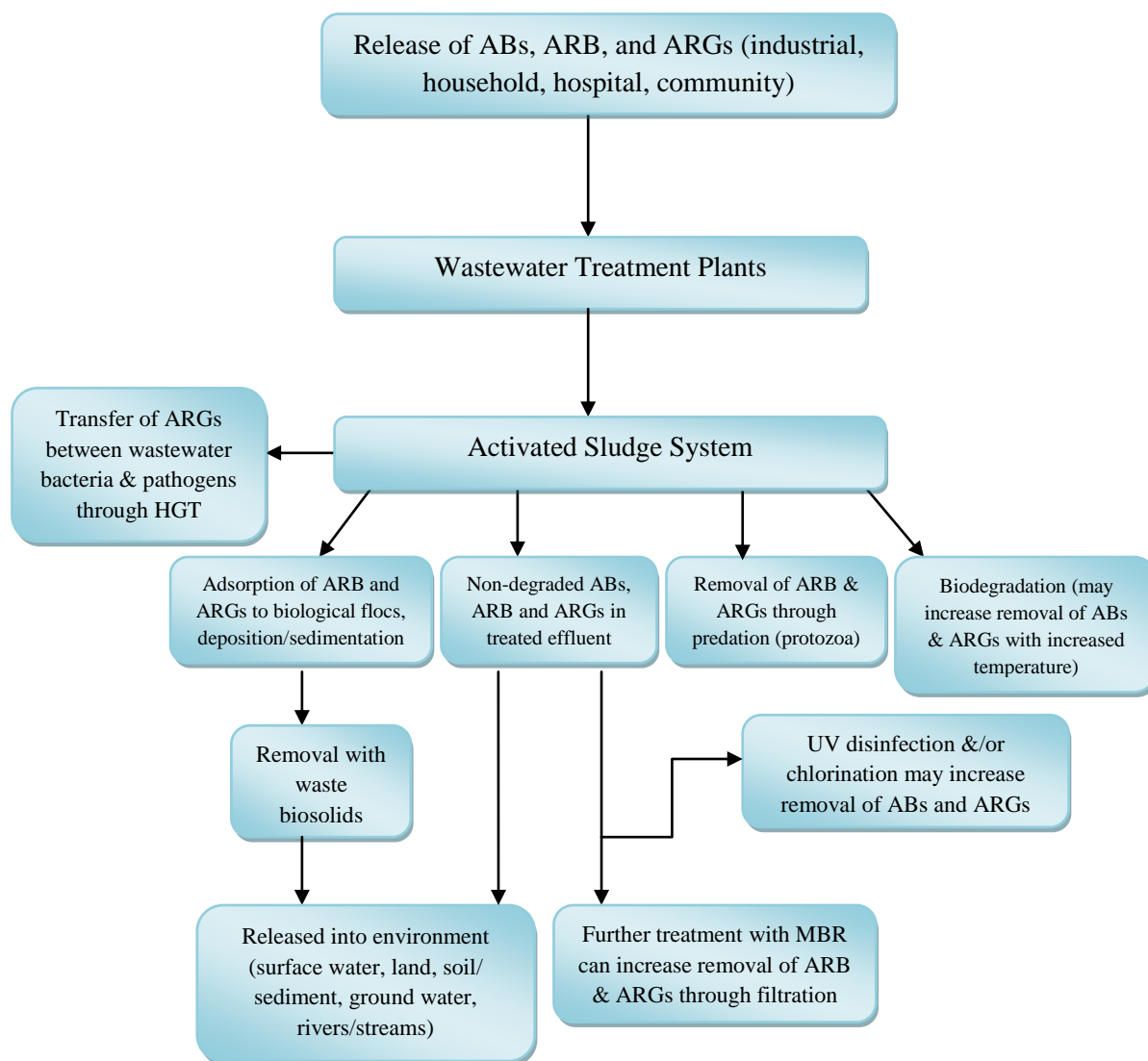
environment, and widely disseminated, thus providing continuous selection and maintenance pressure for populations of resistance strains in all environments (Davies and Davies, 2010). Since the only available evidence suggests that a very small proportion of antibiotics is contributed by naturally occurring antibiotic-producing strains in their native environments (Gottlieb, 1976), it is assumed that commercial production provides the vast bulk of the antibiotics found in the biosphere (Davies and Davies, 2010). The following alternative uses of antimicrobial agents play an important role in the development and spread of antimicrobial resistance: growth promotion/prophylactic use in animals; therapeutic/prophylactic use in humans; therapeutic/prophylactic use in aquaculture; therapeutic/prophylactic in household pets; pest control for agriculture; use as biocides in toiletries and household cleaning products; and culture sterility, cloning, and selection in research and industry (Davies and Davies, 2010). It is important to note that therapeutic use in humans accounts for less than half of all applications of antibiotics produced commercially.

Several antibiotics are natural compounds that have been in contact with environmental microbiota for millions of years and are thus biodegradable, and may even serve as a food resource for several microorganisms (Dantas *et al.*, 2008). Synthetic antibiotics (e.g. quinolones) can be more refractory to biodegradation; however, they are still degraded at different rates in natural environments. Recent work has shown that the binding of quinolones to soil and sediments delays their biodegradation, although wastewater treatment of quinolone-polluted waters efficiently removes these antibiotics through biodegradation and photodegradation (Sukul and Spiteller, 2007). While antibiotics are degraded in natural



ecosystems, that does not suggest they are not relevant pollutants. For example, the degradation process is slow at low temperatures in winter (Dolliver and Gupta, 2008), and the composition and moisture of the soil clearly impacts antibiotic degradation (Stobb *et al.*, 2007). More importantly, some environments are exposed to a constant release of antibiotics (e.g. hospital effluents, farm residues), in which they are continuously polluted irrespective of antibiotic degradation. Water chlorination helps to degrade antibiotics such as beta-lactams or trimethoprim (Li *et al.*, 2008) and wastewater treatment might eliminate 80% of fluoroquinolones or tetracyclines, with the removal of macrolides less efficient (Shellie *et al.*, 2003; Sukul and Spiteller, 2007). Several techniques such as coagulation and activated carbon filtration are effective for the removal of different antibiotics (Choi *et al.*, 2008), however, in all cases a variable percentage of the antibiotics usually remain after wastewater treatment (Brown *et al.*, 2006) and can challenge bacterial populations downstream the wastewater treatment processing plant (Watkinson *et al.*, 2007a). A hypothetical representation of the removal mechanisms and release of antibiotics (ABs), antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in a conventional wastewater treatment plant (WWTP) is depicted in Figure 1.1. As mentioned above, antibiotics are naturally degraded in the environment by processes that include photodegradation, chemical degradation and biodegradation. However, these processes are dependent on temperature, moisture, chemical composition of the environment (e.g. pH, ionic strength) and the microbiota that can contribute to biodegradation (Martinez *et al.*, 2009). Therefore, different habitats will render different paths of antibiotic degradation. For example, binding of antibiotics to clay and sediments delays their degradation, but simultaneously removes antibiotics from water, in such a way that particulate matter present

in rivers may reduce antibiotic pollution in waters at long distances from waste drainage, at the cost of increasing local antibiotic concentration in sediments (Baquero *et al.*, 2008). The goal of wastewater treatment is to reduce the concentration of dissolved organic carbon, nitrogen, phosphorous and eliminate viable pathogens from the liquid effluent (Zhang *et al.*, 2010). Physical (e.g. filtration), chemical (e.g. disinfection) and microbial activities are used to effectively remediate waste entering the treatment plant (Zhang *et al.*, 2010).



**Figure 1.1. Hypothetical representation of removal mechanisms and release of antibiotics (ABs), antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARGs) in a typical wastewater treatment plant using the activated sludge process. MBR - membrane biological reactor; HGT – horizontal gene transfer; UV - Ultra Violet.**

The evolution of bacterial antibiotic resistances, and its spread and emergence, represent one of the most threatening health care problems with worldwide consequences (Hawkey, 2008). The rise of multi-drug resistance urgently requires a better understanding of the factors and hot spots involved in its diffusion and development. Horizontal gene transfer events are responsible for the acquisition of resistance mechanisms among species and from antibiotic producers to commensal and pathogenic bacteria. Hospitals, human community, farms, aquacultures, and agriculture are reactors where the usage of antibiotics selects for resistant bacteria and promotes gene exchange (Lupo *et al.*, 2012). Recently, much more attention has focused on the role of the environment and of connected ecological habitats, water bodies such as rivers, streams, wastewater effluents, and lakes, that have been recommended as important in facilitating the transport and transfer of ARGs (Aminov and Mackie, 2007; Baquero *et al.*, 2008). ARGs that are naturally present in the chromosomes of environmental bacteria (Wright, 2007; Farjardo *et al.*, 2008) are now present in plasmids that can be transferred to human pathogens. It has been highlighted that the interaction of bacteria from human-associated microbiota with environmental microorganisms in sewage plants or in natural ecosystems is an important feature to understand the emergence of novel mechanisms of resistance in human pathogens (Baquero *et al.*, 2008). In strong contrast to the clinical environment, there are no data available on the epidemiology of antibiotic resistance in the environment, especially for geographically based data. This makes it extremely difficult to make any predictions on the risk of spread and emergence of new antibiotic resistances (Lupo *et al.*, 2012). Therefore, more knowledge on the environmental reservoir of antimicrobial resistance is fundamental to help predict the emergence of new resistances of clinical concern.

### **1.1.2 Antibiotic resistance in alternative wastewater treatment systems**

Alternative wastewater treatment systems, including constructed wetlands and constructed lagoon systems, are a popular treatment option for the improvement of water quality. They are used in the treatment of effluents from industry and agriculture, acid mine drainage, landfill leachate, road and airport runoff, as well as primary, secondary and tertiary treatment of sewage effluent (Hodgson *et al.*, 2004). Constructed wetlands (CWs) are engineered systems, which are designed and constructed to utilize natural processes involving wetland vegetation, solids and associated microbial assemblages to assist in treating wastewaters (Vymazal, 2005a). CWs are designed to take advantage of many processes that occur in natural wetlands, but with a more controlled approach, offering relatively low investment and operation costs, while producing high quality effluent with less dissipation of energy (Song *et al.*, 2008). A variety of chemical and biochemical processes, such as microbial degradation, assimilation, precipitation, and adsorption to soil particles operate in the wetland environment, contributing to improved water quality. Wetland vegetation enhances microbial activity by maintaining an extensive root and leaf substrate for bacterial biofilms, and by providing an additional source of dissolved oxygen (Reimold and McBrien, 1997). Recently, attention has focused on the ability of constructed wetlands to reduce pathogenic bacteria loads in wastewater (Karim *et al.*, 2004; Weber and Legge, 2008). The removal of microorganisms in constructed wetlands has shown varying degrees of effectiveness; however, several studies illustrate the improvement of microbial water quality using constructed wetlands (Vymazal, 2005b; Song *et al.*, 2006). Constructed wetlands have gained acceptance in North America as a cost-effective, efficient treatment alternative due to their natural ability to attenuate contaminant flows, conserve natural resources, reduce flood

hazard and erosion, and create wetland habitat. Because of these benefits, they are being used increasingly to treat a variety of wastewaters (Kennedy and Mayer, 2002). Currently, there are over 6000 constructed wetlands in Europe and the United Kingdom (Knight and Kadlec, 2000), consisting predominantly of reed beds (*Phragmites australis*). In comparison, North America operates over 1000 treatment wetlands (Knight and Kadlec, 2000), most of which are located in the United States and are implemented for large-scale treatment of municipal wastewater (Cole, 1998). They are seen as part of the sustainable development approach to waste management, offering both a low environmental impact and an appropriate ecological option. Relative to Europe and the United States, Canada has been slow to embrace constructed wetland technology as a viable treatment approach (Kennedy and Mayer, 2002). According to a 1994 survey, a total of 67 natural and constructed wetlands have been developed in Canada to treat a variety of waste streams, not including acid mine drainage (Pries, 1996). Agricultural wastewater treatment is an emerging application of treatment wetlands. To date, nine systems have been built in Ontario to treat livestock wastewater (Cole, 1998) and over 30 systems have been constructed in Canada and the U.S. to treat milkhouse wastewater (Serodes and Normand, 1999).

Constructed lagoon systems represent a common and effective method to purify communal sewage in rural areas, with low costs and high purification rates (Steinmann *et al.*, 2003). In lagoon systems, a high growth rate of phytoplankton from spring to fall is caused by the influence of light and continuous nutrient inflow. Such algal growth can contribute to the treatment of wastewater via the transformation of nutrients into particle aggregates (biomass), an effect which is occasionally utilized under special conditions (Picot *et al.*,

1993). Treatment lagoon systems are commonly used in temperate climates across the United States, as they are relatively easy to manage and very effective in reducing organic matter and nutrients when designed and operated properly (Graves *et al.*, 2011). Lagoons, however, were not designed to control pathogens, remove antibiotics or metabolites from waste. Previous studies have shown the persistence of pathogens in swine lagoon liquid and sludge, in manure piles, and in waste litter (Plym-Forsell, 1995; Radtke and Gist, 1989). Pathogens are more liable to persist in liquid or moist waste and in sludge or lagoon treatments because the temperatures achieved are not high enough to kill them (Kudva *et al.*, 1998).

Resistance to antibiotics has become a serious problem among pathogenic bacteria, which has led to an increased concern surrounding environmental risks and potential spread of antibiotic resistance among microorganisms. Resistance is typically common where antibiotics are heavily used (hospitals, long term care centers, and large livestock operations), although antibiotic resistant bacteria are also shown to be present in wastewater, ground water, sediments and soils, and increasingly in surface aquatic environments (Baquero *et al.*, 2008; Martinez, 2008; Zhang *et al.*, 2009a). Due to high amounts of microbial biomass and the abundance of nutrients, wastewater treatment plants serve as potential "hot spots" for horizontal gene transfer, frequently involving the passage of plasmids and transposons encoding antibiotic resistance (Guardabassi *et al.*, 2002). The combination of municipal sewage and hospital waste effluents, with surface waters, may facilitate the spread of antibiotics, ARGs, and ARB within wastewater treatment systems (Schluter *et al.*, 2007; Zhang *et al.*, 2009b). The widespread use of antibiotics in animal

husbandry and agriculture for treating or preventing infection and promoting growth, can also play an important role in the development and spread of antibiotic resistance (Witte, 2001). It is known, for example, that long-term exposure to low doses of an antibiotic is likely to contribute to the selection of resistant bacteria (Gellin *et al.*, 1989; Baquero *et al.*, 1997; Negri *et al.*, 2000; Gullberg *et al.*, 2011). Most often the main source of antibiotic resistant bacteria is from the discharge points of municipal WWTPs (Kim and Aga, 2007). However, seepage and run-off of lagoon wastewater from animal feeding operations and farm application of the lagoon sediments also leads to contamination of water sources with antibiotics and antibiotic-resistant bacteria. Ultimately, the amount of antibiotic-resistant bacteria that end up in the water supplies depends on the disinfection process employed during water and wastewater treatment and whether disinfection is employed seasonally or year-round (Kim and Aga, 2007).

Little attention has been given to the occurrence, distribution, and transfer of antimicrobial resistance and resistance genes between bacterial populations within Canadian wastewater treatment systems and alternative treatment systems. This information is essential for effective microbial source tracking and identification of public health risk and treatment options. Before learning about the fate and transport of ARGs in wastewater treatment systems, it is necessary to characterize the occurrence, and identify major habitats of environmental ARGs. The enumeration of ARB and ARGs within various stages of alternative wastewater treatment systems will prove helpful in understanding both the baseline level and potential movement of antibiotic resistance, which may correspond to the potential dissemination of ARGs into different ecosystems.

### **1.1.3 Antibiotic resistance genes (ARGs) as emerging contaminants**

Bacterial resistance to antibiotics and their ARGs has become a serious problem among pathogenic bacteria, and is becoming a major global health issue (Levy, 2002; Chee-Sanford *et al.*, 2001). The World Health Organization (WHO) recently released a report focusing on antibiotic resistance as one of the most critical human health challenges of the next century, announcing the need for "a global strategy to contain resistance" (WHO, 2000). It is estimated that more than two million Americans are infected each year with resistant pathogens, specifically 440,000 new cases of multidrug-resistant tuberculosis (MDR-TB) emerge annually, causing at least 150,000 deaths (WHO, 2011). Emerging contaminants, or contaminants of emerging concern are characterized as chemicals or microbiological constituents previously undefined or not recognized as being of concern to human health or the environment associated with their presence (Petrovic *et al.*, 2004). Increased attention has focused on pharmaceuticals, antibiotics, microorganisms, and ARGs, all of which can be exceedingly challenging to identify, remove or inactivate in water.

Although direct evidence surrounding the transfer of ARGs from the environment to humans is unavailable, some studies highlight the fact that ARGs can spread and be exchanged among environmental microorganisms of different genera (Agersø and Petersen, 2007). ARGs can enter into aquatic environments by direct discharging of untreated wastewater, or through sewage treatment plant (STP) effluents and discharged sludge (Auerbach *et al.*, 2007). Many ARGs, such as, *vanA* and *vanB*, are not completely removed by the activated sludge process widely used in STPs, with genes detected in both influent and effluent water



(Iversen *et al.*, 2002; Caplin *et al.*, 2008). ARGs enter into other water bodies with effluent water and can be transferred horizontally to the native bacteria in these aquatic environments (Schwartz *et al.*, 2003). Using real-time quantitative PCR (qPCR), Auerbach *et al.* (2007) investigated *tet* genes in Germany STPs and found that *tetQ* concentrations were highest in influent water while *tetG* concentrations were highest in activated sludge, and UV disinfection had no effect on the amount of detectable *tet* genes in wastewater effluent. A number of physicochemical factors can influence the dissemination of ARGs in aquatic environments. The first factor contributing to the horizontal transfer of ARGs is the selective pressure from increased antibiotic use and production for the treatment of disease and growth promotion. High selective pressure can facilitate the acquisition of ARGs, which may lead to increased fitness among certain bacteria, allowing for rapid emergence and dissemination (Enne *et al.*, 2004; Luo *et al.*, 2005). In addition, the presence of antibiotics at low subinhibitory concentrations can accelerate horizontal transfer and dissemination of environmental ARGs (Kümmerer, 2004). Genetic mechanisms involved in horizontal transfer of ARGs among environmental bacteria may include the following: (1) conjugative transfer by mobile genetic elements including plasmids, transposons, and integrons on plasmids or transposons; (2) transformation by naked DNA, in the case of a naturally competent state of some bacteria, or an environmentally induced competence such as the presence of calcium; and (3) transduction by bacteriophage (Zhang *et al.*, 2009a). Antibiotic resistance in most environmental bacteria is due to the acquisition of new genes, often associated with mobile elements. Studies have shown that even if cells carrying ARGs have been killed, the DNA released into the environment is able to persist, protected from DNase, especially by certain soil/clay compositions, and can eventually be transformed into

other cells (Blum *et al.*, 1997; Crecchio *et al.*, 2005; Hill *et al.*, 1998). Due to the broadening prevalence of resistant organisms, resistance-conferring genes themselves are now considered emerging contaminants, especially genes selected in one location (e.g., the gut of an animal) and spread to the environment via surface and groundwater flow (Pruden *et al.*, 2006; Koike *et al.*, 2007).

Little information is available regarding the fate of antibiotics in the environment and their link to the emergence of resistant genotypes found there. Environmental reservoirs and pathways of antibiotic resistance are acquiring increased attention (Allen *et al.*, 2010; Martinez, 2008; Pruden *et al.*, 2006) as new strategies beyond the hospital setting are being explored to attenuate antibiotic resistance and prolong the lifespan of antibiotics. One promising approach towards better understanding the distribution and transport of antibiotic resistance in the environment is to consider ARGs as the principle contaminants of interest rather than their bacterial hosts (McKinney *et al.*, 2010; Pruden *et al.*, 2006).

#### **1.1.4 Detection methods for waterborne pathogens and antibiotic resistance genes (ARGs)**

A traditional approach in water and wastewater microbiology has been the monitoring of water quality by detection and enumeration of microbial indicator organisms, rather than of specific pathogens. The total-coliform group is a broad group with several members of faecal and nonfaecal origin, and is conventionally used as the primary bacterial indicator of water suitability for domestic, industrial, or other uses (APHA *et al.*, 1999). These indicators

typically consist of coliform bacteria, faecal enterococci and *Escherichia coli* (NRC, 2004). Indicators of the presence of pathogens are predominantly utilized because of the inability to measure all microbial pathogens that may be present in environmental samples in a timely and cost-effective manner (Wade *et al.*, 2003; Yates, 2007). Coliform group density has been recognized as an important criterion of the degree of pollution and sanitary quality of water and wastewater. The significance of the tests and the interpretation of results are well authenticated and have been used as a basis for standards of bacteriological quality of water supplies (APHA *et al.*, 1999). However, more recently, a variety of reasons suggest that *E. coli* and other faecal indicator bacteria may not always be effective indicators for the presence of bacterial, viral, or parasitic pathogens. These organisms vary significantly with respect to a variety of factors that may influence their fate and transport in the environment, including the size of the microorganism, abundance in feces, environmental fitness, and nature of hydrological processes that transport organisms to and within the aquatic environment (Ogden *et al.*, 2001; Sadeghi and Arnold, 2002; Olyphant *et al.*, 2003; Anderson *et al.*, 2005; Yates, 2007). For example, in still waters, *Cryptosporidium* oocyst and bacterial concentrations decline at different rates partially due to variable settling rates (Brookes *et al.*, 2005). Commensal *E. coli* may acclimatize and proliferate in secondary habitats, whereas some pathogens may not (Byappanahalli *et al.*, 2006). Evidently, it can be expected that the relationships between indicator bacteria and pathogens in fecally contaminated waters will be complex and variable (Payment *et al.*, 2003; Yates, 2007).

Although traditional or culture-based tests are relatively inexpensive and reproducible, they are known to underestimate the total number of bacteria by up to several orders of

magnitude (Amann *et al.*, 1995; Sartory and Watkins, 1999), even with prolonged incubation times and temperature variations (Elzanfaly *et al.*, 1998). It has long been documented that artificial culture media lead to only a very small fraction (0.01–1%) of the total viable bacteria present in any given sample (Watkins and Xiangrong, 1997). Furthermore, introduced bacteria can increasingly deteriorate in aqueous environments, with some initially able to grow on selective media, then only on non-selective media (stressed cells), and finally becoming non-culturable (viable but non-culturable (VBNC) if still capable of causing infection) (McFeters, 1990; Colwell *et al.*, 1996; Cervantes *et al.*, 1997; Alexandrino *et al.*, 2004). Therefore, even the application of selective agents in any culture-based method, including those for pathogens, is expected to produce a considerable underestimation of the actual or "true" numbers of potentially infective bacteria present (Ashbolt, 2005). Additionally, the cultivation and analysis of indicator microorganisms is labour-intensive, time consuming and difficult (Lemarchand *et al.*, 2005). Consequently, rapid, accurate and culture-independent alternatives are being investigated to facilitate monitoring of pathogens in water and wastewater (Straub and Chandler, 2003).

Advances in molecular biology over the past 20 years have resulted in a number of new detection methods that depend on the recognition of specific gene sequences. Such methods have resolved some of the problems encountered using conventional methods, and are usually more rapid and tailored to detect specific strains or groups of organisms. A molecular method, such as the polymerase chain reaction (PCR) has high specificity, speed and sensitivity in pathogen detection, with a detection limit of <10 copies of a specific gene present in a mixed sample (Call *et al.*, 2001). Despite this, there are problems with PCR-

based detection, including low throughput, quantification, differentiation between live and dead cells, and the decrease in sensitivity and specificity caused by post-PCR analysis (Shannon *et al.*, 2007). Some of the difficulties associated with PCR-based pathogen detection have been eliminated by real-time quantitative PCR (qPCR) technology. This technology is sensitive, specific, and yields accurate quantitative results (Guy *et al.*, 2003; MacKay, 2004; Shannon *et al.*, 2007; Böckelmann *et al.*, 2009) with rapid detection of microorganisms and ARGs in water samples. The amplified PCR product is quantified using lasers to detect a DNA-specific probe throughout the PCR cycling process, thereby eliminating any post-PCR processing, and providing a much lower detection which is ideal for quantification (Guy *et al.*, 2003). In order to monitor the efficiency of alternative wastewater treatment systems and ensure adequate removal concentrations of indicator organisms and bacterial pathogens/ARGs from the final effluent to receiving water bodies, the number of associated pathogenic bacteria and ARGs should be measured during various stages of treatment.

#### **1.1.5 Antimicrobial resistance among indicator organisms and environmental pathogens**

Faecal coliform bacteria are indicators of faecal contamination and of the potential presence of pathogens associated with wastewater or sewage sludge. Indicator organisms are typically used to demonstrate the potential presence or absence of groups of pathogens (Kator and Rhodes, 2003). *E. coli* is a useful enteric bacterium for the study of waterborne transfer of antibiotic resistance. It is adapted to human and other warm-blooded animal gastrointestinal tracts, and is exposed to a variety of medical and veterinary antibiotic treatments (Edge and

Hill, 2005). *E. coli* and *Enterococcus* spp. are two organisms that have been used as indicators of faecal contamination (Kator and Rhodes, 2003), yet some pathogenic *E. coli* strains are able to transfer or receive genes from other organisms, enhancing their resistance (Garcia *et al.*, 2007). *Enterococcus* spp. have become a common cause of nosocomial infections and also represents an important factor in the emergence of vancomycin resistant strains (CDC NNIS System, 2003). Vancomycin-resistant enterococci (VRE) cause significant human infections, including those of the urinary tract, wounds, bloodstream, and endocardium (Jett *et al.*, 1994; Moellering, 1992; Shepard and Gilmore, 2002). An important characteristic of enterococci is their intrinsic resistance or their ability to acquire vancomycin resistance genes (Shaghghi *et al.*, 2007; Panesso *et al.*, 2002), of which *vanA* and *vanB* are the most common. Enterococci are also considered intrinsically “rugged” bacteria, and as such are able to survive under unusually wide ranges of temperature, pH, and salinity, as well as resisting the bactericidal effects of detergents such as bile salts and sodium dodecyl sulfate (Flahaut *et al.*, 1996). The most common agents of the vast majority of clinical enterococcal infections in humans are *Enterococcus faecalis* and *Enterococcus faecium* (Mundy *et al.*, 2000). The emergence of *E. faecalis* and *E. faecium* as leading problematic nosocomial pathogens has paralleled the appearance of strains within both species resistant to most antimicrobial drugs used to treat human infections. The range of antimicrobial agents to which enterococci have acquired resistance is quite broad and appears to be escalating at a rate that closely approximates the introduction of new agents to the pharmaceutical market (Gonzales *et al.*, 2001; Shepard and Gilmore, 2002).

Enteric *Salmonella* infection is a global problem both in humans and animals, and is considered to be the most important bacterial etiology for enteric infections worldwide (McCormick *et al.*, 1993). The ability of *Salmonella* spp. to survive in a variety of aquatic environments, including streams and rivers, further compounds the threat of *Salmonella* arising from untreated wastewater originating from agricultural operations and human population effluents (Spector, 1998). For example, the high prevalence of this pathogen in fresh poultry presents a potential threat to human health through contamination of water bodies from poorly treated or untreated poultry-processing water (Burr *et al.*, 1998). *Salmonella* spp. are also commonly found in wastewater and urban sludge, and have been studied mainly using culture-based methods (Gantzer *et al.*, 2001; Godfree and Farrell, 2005). Compared to other bacteria, *Salmonella* spp. have high survival rates in aquatic environments and are able to withstand a variety of stresses, including thermal and pH fluctuation, high osmolarity and low nutrient availability (Chao *et al.*, 1987; Winfield and Groisman, 2003). *Salmonella* spp. have also been described as being more resistant than *E. coli* to biotic factors (microbial predators or competing organisms) in sources of drinking water (Wright, 1989), perhaps due to a difference in adhesion to protective particles (Winfield and Groisman, 2003). Wéry *et al.* (2008) studied the behaviour of pathogenic and indicator bacteria during urban wastewater treatment and found *Salmonella* spp. had a greater capacity to survive biological treatment, compared to that of *E. coli*. A possible explanation could be due to the specific survival strategies that pathogenic bacteria have developed to survive when outside hosts or, more generally, to survive under stress (Wéry *et al.*, 2008).

Antimicrobial resistance (AMR) is recognized as a global problem in human and veterinary medicine. To aid in the estimation of the AMR crisis and to follow its evolution, surveillance programs have been established in many countries worldwide, including the National Antimicrobial Resistance Monitoring System (NARMS) in the United States (Tollefson *et al.*, 2008) and the Canadian Integrated Program for Antimicrobial Resistance Surveillance in Canada (CIPARS). Most surveillance programs focus on isolates acquired from infected humans, farm-animals and related food-products (Government of Canada, 2007; CDC, 2007), but do not include an assessment of environmentally derived isolates. Unfortunately, few surveillance programs include specific pathogens from animals, and most are dedicated to assessing resistance phenotypes only. Frequently, a particular resistance phenotype may originate from several different genotypes (Chopra *et al.*, 2001; Sköld *et al.*, 2001). A more promising approach looks at assessing the diversity and distribution of resistance genes in bacterial populations, which represents a more detailed and potentially useful tool for improving our understanding of AMR epidemiology (Boerlin, 2005).

Over the last several decades, the focus of research has expanded beyond clinical settings and nosocomial infections, following the realization that resistant organisms are widespread throughout the environment. Resistant bacteria have been isolated from rivers and streams worldwide (Ash *et al.*, 2002; Schmidt *et al.*, 2000, 2001; Rhodes *et al.*, 2000; Gordon *et al.*, 2007; Stachowiak *et al.*, 2009; Thomas, 2011). Ash *et al.* (2002) reported high frequencies of ampicillin resistance in bacteria isolated from water samples obtained from rivers across the USA. In addition, they found a relatively high proportion of these isolates (40%) carried



plasmids coding for resistance and thus were potentially capable of transferring resistance to other organisms.

Major environmental pathogens involved in fish farm infections include the Gram negative species *Flavobacterium psychrophilum*, *Yersinia ruckeri*, and *Aeromonas salmonicida*. They are the etiological agents of cold water disease, enteric red mouth disease, and furunculosis, respectively (del Cerro *et al.*, 2002). Freshwater fish farming has been shown to impact environmental *Aeromonas* spp., as indicated by an investigation of antimicrobial resistance at four Danish rainbow trout farms (Schmidt *et al.*, 2001). Members of the genus *Aeromonas* readily develop single or multiple antimicrobial resistance phenotypes (Goni-Urriza *et al.*, 2000; Guardabassi *et al.*, 2000), and resistance (R) plasmids are commonly found (Klein *et al.*, 1996; Adams *et al.*, 1998). Therefore, *Aeromonas* spp. represents an important environmental pathogen for monitoring the prevalence and distribution of antibiotic resistance, and for examining the potential conjugative spread of antibiotic resistance genes in the environment. In the field of aquaculture, both therapeutic and environmental problems have been addressed, as antimicrobial agents are released into the surrounding water during medical treatment of bacterial fish diseases (Aoki, 1992). The impact of these substances on the resident microflora is difficult to assess because of the complexity of the aquatic environment, while the resistance patterns of bacterial fish pathogens often reflect an intensive use of antimicrobial substances (Smith *et al.*, 1994; Bruun *et al.*, 2000). In order to improve our understanding of the dissemination of antibiotic resistance and ARGs among indicator organisms and environmental pathogens within aquatic environments, the study of

the occurrence, distribution, AMR profiles, and frequency of conjugal transfer amongst waterborne isolates is needed.

#### **1.1.6 Fate and transmission of environmental pathogens and antibiotic resistance genes (ARGs)**

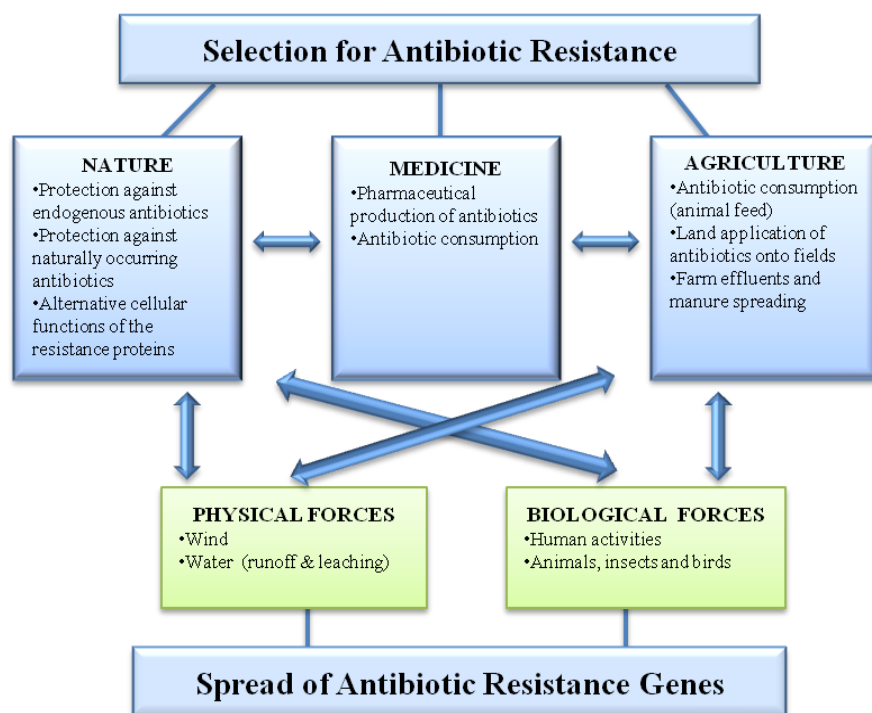
Outbreaks of waterborne disease via public water supplies continue to be reported in developed countries even though there is increased awareness of, and treatment for, pathogen contamination (Payment *et al.*, 1997; Gibson *et al.*, 1998; Howe *et al.*, 2002). In order to establish a realistic assessment of the overall risk of waterborne pathogens to human health, it is necessary to recognize and understand the important variables controlling the fate and distribution of pathogens and ARGs in each part of the source water and supply system, including wastewater treatment systems, constructed wetlands, lakes and reservoirs, water purification plants, and distribution systems. The predominant sources of waterborne pathogens are from excreted material and other animal waste products, specifically wastewater/biosolids from municipal and agricultural processes, feces, urine, carcasses, and abattoir waste (WHO, 2004). Intestinal pathogens use these materials as transport vehicles from the animal reservoir to the particular water environment, where their stability in that environment will influence the infectivity and thereby the risk to human health. Concern for waterborne disease is dominated primarily by pathogens transmitted by the faecal–oral route and by drinking water. However, waterborne transmission can also include diseases transmitted by faecal droplet inhalation (e.g. some adenoviruses) and exposure through contact (e.g., recreational and occupational) (WHO, 2004). Waterborne zoonotic pathogens cause both gastrointestinal diseases such as diarrhea and other illnesses including

leptospirosis and hepatitis. Up to 75% of emerging pathogens may be of zoonotic origin (e.g. *E. coli* O157:H7), while others have both human and zoonotic sources (e.g. *Cryptosporidium*) (WHO, 2004). However, defining the source of specific pathogens within a watershed is difficult, as enteric pathogens are transient in most host animals. Along with trends in animal populations, the prevalence of a given pathogen (e.g., *Salmonella*) among animal populations may vary, and the intensity of shedding may be influenced by a variety of factors, including seasonality as well as the age and type of animal (WHO, 2004).

Raw and treated wastewater has traditionally been considered a source of pathogenic microorganisms, given the fact that, at any given time, there is a level of endemic disease and infection in its service area (Crockett, 2007). One of the main factors affecting the considerable resistance of emerging pathogens to various treatment techniques and technologies is their natural propensity for survival in the environment. Survival of emerging pathogens can be affected by physical characteristics or structure, temperature, organic matter availability, moisture availability, sunlight exposure, microbial predation, and particle association (Crockett, 2007). Survival of zoonotic pathogens in water and the environment is a crucial factor in the transmission of waterborne zoonoses. Many zoonotic pathogens of concern (e.g., *Cryptosporidium*, *Giardia*, *E. coli* O157:H7) are able to survive for months in the environment under the appropriate conditions, leading to an increase in the probability of waterborne transmission. Emerging pathogens represent a significant challenge to control because of their ability to survive at significant concentrations for extensive periods of time in the environment (7 days), low infectious dose required for infection, and difficulty for removal and inactivation by water and wastewater treatment processes (Crockett, 2007).

Therefore, meeting the traditional regulatory standards based on indicator organisms may not be sufficient in some cases to mitigate emerging pathogen effects from wastewater discharges on downstream water supplies and recreational areas (Crockett, 2007).

The prevalence of horizontal gene transfer, the process by which bacteria acquire genes from the environment, has allowed for the wide dissemination of antibiotic resistance elements (i.e. ARGs) in pathogenic bacteria (Thomas and Nielsen, 2005). Many of the known ARGs are found on transposons, integrons or plasmids, which can be mobilized and transferred to other bacteria of the same or different species. The selection pressure applied by antimicrobial agents used in clinical and agricultural settings has supported the evolution and spread of genes that confer resistance, regardless of their origins (Allen *et al.*, 2010). The spread or transmission of ARGs can be enhanced by a variety of factors, including physical forces (i.e. wind and water), as well as biological forces (i.e. human activities, animals, and birds) (Figure 1.2). Resistance genes exist naturally in the environment due to a range of selective pressures in nature. Humans have applied additional selective pressures for ARGs because of the large quantities of antibiotics that are consumed, produced and applied in medicine and agriculture. Physical and biological forces also contribute to the widespread dissemination of ARGs throughout many environments.



**Figure 1.2. Sources and movement of antibiotic resistance genes in the environment (adapted from Allen *et al.*, 2010).**

Transfer of resistance genes from faecal organisms to indigenous soil and water bacteria may occur (Nielsen *et al.*, 2000; Daane *et al.*, 1996; DiGiovanni *et al.*, 1996; Lorenz and Wackernagel, 1994), and because native populations are usually better adapted for survival in aquatic or terrestrial ecosystems, persistence of resistance traits may be likely in natural environments once they are acquired (Chee-Sanford *et al.*, 2009). Once antibiotic resistant bacteria and their corresponding resistance genes enter the soil and/or water, the persistence and fate of the introduced determinant depends on the nature and viability of the host bacteria containing the determinant(s), as well as the segregation of free genetic material following cell lysis that may be subject to degradation, sorption, or uptake by new cells (Chee-Sanford *et al.*, 2009). As long as a resistance gene is present in the environment, the possibility for its transfer exists. Recently, studies have shown that horizontal transfer of

antibiotic resistance genes between bacteria of different genera and species occurs readily and frequently in natural systems such as soil and groundwater (Onan and LaPara, 2003; Chee-Sanford *et al.*, 2001; Salyers and Amábile-Cuevas, 1997).

Overall, the exact mechanisms contributing to antibiotic resistance gene acquisition and maintenance in natural environments are not yet well established; however, an increasing number of studies support lateral gene transfer events. Further research is needed in order to achieve accurate and meaningful information on the fate, persistence, and transmission of antibiotic resistance genes, which will allow for improved potential health risk and environmental quality assessments.

## **1.2 Thesis Objectives**

More research is necessary to understand the occurrence, fate, and mobility of antibiotic resistance and ARGs among bacterial indicators of faecal contamination as well as pathogenic bacteria within Canadian wastewater treatment systems. This information is essential for effective microbial source tracking and identification of public health risks and treatment options. Before learning about the fate and transport of ARGs in wastewater treatment systems, it is necessary to characterize the occurrence, and identify major habitats of environmental ARGs. The results of this research will contribute to further the understanding of the impact of wastewater treatment strategies on the expression of antibiotic resistance and ARGs in pathogenic bacteria, more specifically among *Enterococcus* spp., *Escherichia coli*, *Aeromonas* spp., *Yersinia* spp., and *Salmonella* spp..

Considering the growing evidence that clinical resistance is closely associated with environmental ARGs and bacteria (Tatavarthy *et al.*, 2006; Prabhu *et al.*, 2007; Abriouel *et al.*, 2008), additional research needs to be done to include nonpathogenic and environmental microorganisms. Further information is needed with respect to the transfer of bacterial resistance and environmental ARGs (via conjugation) both within and between bacterial populations in wastewater treatment systems. This information could lead to the modification or optimization of wastewater treatment processes, in order to target and/or enhance the removal efficiency of antibiotic resistant bacteria and/or resistance genes. Therefore, the primary objective of this research is to determine the prevalence, fate, and potential transfer of bacterial resistance and ARGs among selected environmental pathogens within bacterial communities of alternative wastewater treatment systems, while considering the impact of treatment strategies on the expression of antibiotic resistance in such pathogens. A detailed analysis will be conducted initially with respect to the characterization and quantification of the microbial populations (including antibiotic resistant bacteria) in a variety of treatment systems and waste effluent sources. To aid in the achievement of the overall objective, supporting objectives include:

- A) Confirmation and monitoring the prevalence of selected faecal-indicators (*E. coli*, *Enterococcus* spp.), and selected environmental pathogens (*Yersinia* spp., *Aeromonas* spp., *Flavobacterium* spp. & *Salmonella* spp.) through screening using culture-based phenotypic characterization within various types of alternative wastewater treatment systems (bench-scale mesocosms, sub-surface flow constructed wetland, constructed lagoon, pilot-scale biological nutrient removal (BNR) system). Quantification of

selected environmental pathogens using real-time quantitative PCR (qPCR) will also be performed;

- B) To determine antibiotic resistance profiles for 10-12 common antibiotics using representative isolates of *E. coli*, *Enterococcus* spp., *Salmonella* spp., *Yersinia* spp., *Aeromonas* spp., and *Flavobacterium* spp., using culture-based techniques;
- C) To quantify, track, and compare the presence of selected antibiotic resistance genes using real-time qPCR (*ampC*, *tetA*, and *blaSHV-5*) in different alternative wastewater treatment systems, including constructed wetlands, constructed lagoons, and pilot-scale WWTPs;
- D) To assess the ability to completely or partially transfer antibiotic resistance patterns and ARGs via conjugation experiments using multi-drug resistant isolates (*E. coli* and *Salmonella* spp.) as donors, and *E. coli* K12 as the recipient; and
- E) To better understand or assess the impact of changing conditions or environmental stress (i.e. antibiotic exposure) on the level of antibiotic resistance among representative faecal indicators (*E. coli* and *Enterococcus* spp.) and selected environmental pathogens (*Aeromonas* spp., *Yersinia* spp., & *Flavobacterium* spp.) in constructed wetland systems.

### **1.3 Organization of the Thesis**

The thesis contains five data chapters (Chapters 2-6), preceded by an introduction (Chapter 1) and followed by thesis conclusions and recommendations (Chapter 7). Each data chapter consists of a combination of the overall research objectives, while specifically focusing on a different type of alternative wastewater treatment system.



Chapter 2 presents data on antibiotic resistance profiles of wetland bacteria and faecal indicators following exposure to the antibiotic ciprofloxacin in lab-scale constructed mesocosm systems (Helt *et al.*, 2012). The overall objective of this study was to better understand the effect an influent antibiotic has on both the intrinsic microbial community and the resulting impact on the level of antibiotic resistance among representative faecal indicators (*E. coli* and *Enterococcus* spp.) in a model wetland system. Specifically, the prevalence of culturable *E. coli*, *Enterococcus* spp., and total heterotrophs were determined from both ciprofloxacin exposed and control mesocosms before and after the antibiotic exposure, including confirmation of *E. coli* and *Enterococcus* spp. isolates using molecular techniques (PCR) and to further characterize *Enterococcus* spp., distinguishing between three common strains using species-specific multiplex PCR. The agar dilution screening method was used to establish the minimum inhibitory concentrations (MIC) among the interstitial bacterial community. In addition, antimicrobial resistance profiles for *E. coli* and *Enterococcus* spp. isolates (using the disc-diffusion method) were established, including a comparison of the prevalence of resistance both before and after ciprofloxacin exposure.

Chapter 3 examines the occurrence, distribution and frequency of selected bacterial pathogens and ARGs, conferring resistance to three classes of antimicrobials (tetracyclines, penicillins, and  $\beta$ -lactam resistance), within multiple stages of a constructed lagoon treatment system receiving poultry slaughterhouse waste. The occurrence of antimicrobial resistance among *E. coli* and *Enterococcus* spp. was examined within different stages of the constructed lagoon system, while comparing any differences in the level of antibiotic resistance between different seasons, and stages of treatment. The frequency of multiple

antimicrobial resistance (MAR) and antimicrobial resistance profiles were established among culturable *E. coli* and *Enterococcus* spp. isolates. A genotypic assessment of selected ARGs, including *tetA*, *strA*, *strB*, *sulI*, and *sul2* (conferring resistance to tetracycline, streptomycin, and sulfonamides) was also performed on *E. coli* and *Enterococcus* spp. isolates using PCR analysis. In addition, real-time quantitative PCR was used to enumerate *E. coli*, *Enterococcus faecalis* and *Salmonella* spp., and antibiotic resistance genes (*ampC*, *tetA*, *blaSHV*) within various stages of the lagoon treatment system. The efficiency of the lagoon system in treating poultry slaughterhouse wastewater was also assessed by comparing the log removal of pathogens and ARGs in treated wastewater samples.

In Chapter 4, the overall objective was to better understand the frequency of selected bacterial fish pathogens, antibiotic resistance, and ARGs, within multiple stages of a subsurface flow constructed wetland treating fish hatchery waste during winter operation. Antibiotic resistance profiles were established among fish pathogens, *Yersinia* spp., *Aeromonas* spp., and *Flavobacterium* spp.. As well, the frequency of ARGs was determined in different stages of the constructed wetland, both before and after the addition of an oxytetracycline spike. Genotypic confirmation using qualitative PCR was also performed on culturable *Yersinia* spp. and *Aeromonas* spp. isolates, with further distinction between two common strains; *Y. ruckeri* and *Y. enterocolitica* by species-specific PCR.

Chapter 5 presents data on the distribution, quantification, and dissemination of *E. coli*, *E. faecalis*, and *Salmonella* spp. and ARGs (*tetA*, *ampC*, *blaSHV*) in samples of raw wastewater, activated sludge, and final effluent from a pilot-scale biological nutrient

removal (BNR) system receiving full-scale WWTP effluent. The prevalence of antibiotic resistance among culturable *E. coli* and *Enterococcus* spp. isolates was studied using the disc-diffusion method, while comparing the difference in levels of resistance between raw wastewater, activated sludge, and treated effluent. Multiple antimicrobial resistance rates and resistance profiles were further established among culturable faecal indicators (*E. coli* and *Enterococcus* spp.). Lastly, the efficiency of the pilot-scale BNR system was assessed by determining the total log removals of pathogens and ARGs in treated wastewater samples.

Chapter 6 examines waterborne *Salmonella* spp. and *E. coli* isolates collected from different raw and/or treated wastewater effluents, and determines the prevalence of antimicrobial resistance using both phenotypic and genotypic methods. Genotypic evaluation of drug resistance in *Salmonella* spp. and *E. coli* strains occurring naturally in the environment is limited. In order to have a better understanding of the extent to which wastewater effluents are associated with the spread and dissemination of antimicrobial resistance (AMR), knowledge of the prevalence, diversity, distribution, and conjugal transfer of resistance genes in waterborne isolates is needed. Therefore, the distribution and diversity of ARGs including, *tetA*, *strA*, *strB* conferring resistance to tetracycline and/or streptomycin among waterborne *E. coli* and *Salmonella* spp. was investigated. Conjugation experiments ascertained the transferability of tetracycline (*tetA*) and/or streptomycin (*strA* and *strB*) resistance determinants and antibiotic resistance patterns using multi-drug resistant *E. coli* and *Salmonella* spp. isolates as donors and *E. coli* K12 as the recipient. Molecular

techniques were also utilized to confirm plasmid-mediated conjugal transfer of ARGs through PCR detection among transconjugant isolates.

Chapter 7 presents thesis conclusions and recommendations for future research.

Appendix A illustrates additional quantitative PCR data for selected pathogens and ARGs collected during the summer (July 2010) sampling event within the constructed lagoon treatment system receiving poultry slaughterhouse waste.

# 2

## **Antibiotic resistance profiles of representative wetland bacteria and faecal indicators following ciprofloxacin exposure in lab-scale constructed mesocosms<sup>1</sup>**

### **2.1 Introduction**

Bacterial resistance to antibiotics has become a serious problem among pathogenic bacteria, which has led to increased concern surrounding environmental risks and potential spread of antibiotic resistance among microorganisms. Resistance is typically common where antibiotics are heavily used (hospitals, long term care centers, and large livestock operations), although antibiotic resistant bacteria are also shown to be present in wastewater,

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<sup>1</sup> *This chapter has been published as an invited paper in “Ecological Engineering” (Helt, C.D., Weber K.P., Legge R.L., Slawson R.M. 2012. Antibiotic resistance profiles of representative wetland bacteria and faecal indicators following ciprofloxacin exposure in lab-scale constructed mesocosms. Ecol. Eng. 39: 113-122).*

surface water, groundwater, sediments and soils, and increasingly in surface aquatic environments (Baquero *et al.*, 2008; Martinez, 2008; Zhang *et al.*, 2009a). Due to the high amounts of microbial biomass and the abundance of nutrients, wastewater treatment plants serve as potential "hot spots" for horizontal gene transfer, frequently involving the passage of plasmids and transposons encoding antibiotic resistance (Guardabassi *et al.*, 2002). The combination of municipal sewage and hospital waste effluents, with surface waters, may facilitate the spread of antibiotics, ARGs, and antibiotic resistant bacteria within wastewater treatment systems (Schluter *et al.*, 2007; Zhang *et al.*, 2009b). The widespread use of antibiotics in animal husbandry and agriculture for treating or preventing infection and promoting growth, can also play an important role in the development and spread of antibiotic resistance (Witte, 2001). It is known, for example, that long-term exposure to low doses of an antibiotic over time is likely to contribute to the selection of resistant bacteria (Gellin *et al.*, 1989; Negri *et al.*, 2000; Gullberg *et al.*, 2011).

Faecal indicator organisms are typically used to demonstrate the potential presence or absence of groups of pathogens associated with wastewater or sewage sludge. *Escherichia coli* and *Enterococcus* spp. are two representative organisms that have been used as indicators for faecal contamination (Kator and Rhodes, 2003). *E. coli* is a useful enteric bacterium for the study of waterborne transfer of antibiotic resistance because it has adapted to human and other warm-blooded animal gastrointestinal tracts, and is exposed to a variety of medical and veterinary antibiotic treatments (Edge and Hill, 2005). Due to their intrinsic ability to acquire vancomycin resistance genes (Panesso *et al.*, 2002; Shaghaghi *et al.*, 2007), *Enterococcus* spp. have become a common cause of nosocomial infections and also

represent an important factor in the emergence of vancomycin resistant strains (CDC NNIS System, 2003). The most common causes of the vast majority of clinical enterococcal infections in humans are with *Enterococcus faecalis* and *Enterococcus faecium* (Mundy *et al.*, 2000). The emergence of *E. faecalis* and *E. faecium* as leading nosocomial pathogens has paralleled the appearance of resistant strains within both species to most antimicrobial drugs used to treat human infections. The range of antimicrobial agents to which enterococci have acquired resistance is quite broad and appears to be escalating at a rate that closely approximates the introduction of new agents to the pharmaceutical market (Gonzales *et al.*, 2001; Shepard and Gilmore, 2002).

The occurrence of antibiotics in aquatic environments is of ecotoxicological concern because of potential ecosystem alteration. Antibiotic residues have been detected in the final effluents of wastewater treatment plants (WWTPs) worldwide (Batt and Aga, 2005; Carballa *et al.*, 2004, Costanzo *et al.*, 2005). Contamination of surface waters by antibiotics and other pharmaceutical compounds has been reported in recent studies through discharge from domestic sewer systems (Metcalf *et al.*, 2003). Antibiotics used in human treatment have the potential to enter the environment by excretion or by disposal of surplus drugs into sewage systems, which are eventually released into the local aquatic surroundings from the effluent of WWTPs (Jorgensen and Halling-Sorensen, 2000). Ciprofloxacin is a second generation fluoroquinolone antibiotic, and is commonly used to treat bacterial infections. Although quinolones are prescribed less often than macrolides, these compounds are still the fourth most prescribed class of antimicrobials in Canada (Miao *et al.*, 2004). In particular, ciprofloxacin has dominated the Canadian and global quinolone markets since its entry in

the late 1980s. Ciprofloxacin was also chosen as the antibiotic of choice because it is a broad-spectrum antibiotic, effective against both Gram-negative (e.g. *E. coli*) and Gram-positive (e.g. *Enterococcus* spp.) bacteria.

Constructed wetlands (CWs) are engineered systems that are designed and constructed to utilize natural processes involving wetland vegetation, solids and associated microbial assemblages to assist in treating wastewaters (Vymazal, 2005a). CWs are designed to take advantage of many processes that occur in natural wetlands, but with a more controlled approach offering relatively low investment and operation costs, while producing high quality effluent with less dissipation of energy (Song *et al.*, 2008). Recently, attention has focused on the ability of constructed wetlands to reduce pathogenic bacterial loads in wastewater (Karim *et al.*, 2004; Weber and Legge, 2008). The removal rate of microorganisms in constructed wetlands has shown varying degrees of effectiveness; however, several studies illustrate the improvement of microbial water quality using constructed wetlands (Vymazal, 2005b; Song *et al.*, 2006). Several mesocosm studies have recently been used for undertaking a quantitative approach to the study of constructed wetlands (CW) (Stein *et al.*, 2006; Werker *et al.*, 2004; Weber *et al.*, 2008; Weber *et al.*, 2010). The mesocosm approach has been shown to suitably represent interactions between microorganisms, differing substrates, and contaminants within a complex rhizosphere system (Stein *et al.*, 2006; Werker *et al.*, 2007; Weissner *et al.*, 2008). CW mesocosms cannot be said to completely represent full-scale CWs, as full-scale CWs can contain many different hydrological, biological and geochemical sub-environments (or “units”) within the same system. CW systems are complex ecosystems where any number of chemical,



biological and physical transformations can be taking place in a seemingly random or ordered fashion. There is a considerable lack of attention given to the comparative quantification of the specific mechanisms of pathogen treatment and antibiotic resistance profiles among faecal indicators within CWs. Small-scale controllable CW systems are considered favourable when trying to conduct well-designed, controlled experiments where fundamental mechanisms and variables, such as those involved in pathogen and/or antibiotic resistance removal, can be comparatively quantified. It has been suggested that if the fundamental mechanisms and variables affecting pathogen removal and antibiotic resistance levels among indicator bacteria in CWs are better understood and quantified, the large performance variations reported for similarly designed treatment wetland systems can be better explained, engineered and controlled (Weber and Legge, 2008).

## **2.2 Research Needs & Objectives**

Little information is available on the prevalence of antibiotic resistance among indicator and pathogenic microorganisms in wetlands designed for wastewater treatment or in natural wetland systems. Some researchers have used mesocosms to study the effects of antibiotics on removal of faecal indicators (Atoyan *et al.*, 2007) or on the development of antibiotic resistance (Yu *et al.*, 2009). However, to the best of our knowledge, this is the first study that examines and monitors antibiotic resistance profiles among both faecal indicators (*E. coli* and *Enterococcus* spp.) and total heterotrophs before and after the addition of an antibiotic exposure, within a bench-scale system. The overall objective of this work was to better understand the effect an influent antibiotic has on both the intrinsic microbial community

and the resulting impact on the level of antibiotic resistance among representative faecal indicators in a model wetland system. This information will prove useful when examining antibiotic resistance levels and profiles among other alternative wastewater treatment systems, including full-scale constructed wetlands and lagoons.

The specific objectives were to:

1. Determine the prevalence of culturable *Escherichia coli*, *Enterococcus* spp., and total heterotrophic bacteria from both control and ciprofloxacin exposed mesocosms before and after the antibiotic exposure;
2. Confirm presumptive *E. coli* and *Enterococcus* spp. isolates using molecular techniques (qualitative PCR), and further characterize *Enterococcus* spp. between three common strains using multiplex species-specific PCR;
3. Determine the level of resistance to various concentrations of ciprofloxacin (establish minimum inhibitory concentrations) using the agar dilution screening method among the interstitial bacterial community (total culturable heterotrophs);
4. Establish antimicrobial resistance profiles using the disc-diffusion method for *E. coli* isolates, and compare the prevalence of antibiotic resistance before and after the ciprofloxacin exposure; and,
5. Determine antimicrobial resistance profiles for *Enterococcus* spp. isolates using the disc- diffusion method, and examine the occurrence of antibiotic resistance before and after the ciprofloxacin exposure.

## 2.3 Materials and Methods

### 2.3.1 Wetland mesocosms

Four mesocosm wetlands used in this study (labelled as CON1, CON2, CIP1, & CIP2) were planted with *Phragmites australis* and seeded with activated sludge from a wastewater treatment plant in south-western Ontario. The mesocosms in this study were set up in parallel duplicates, and are of the same design as those used previously by Weber and Legge (2010) and Weber *et al.* (2011). Water entered approximately 65 cm from the bottom of the mesocosm resulting in an oxidative reduction potential (ORP) of ~-125 mV at the bottom. Each mesocosm was comprised of schedule 80 (wall thickness of 1.5 cm), polyvinylchloride (PVC) columns (90 cm by 25 cm diameter) filled to ~ 80 cm with pea gravel (average equivalent spherical diameter of 2 cm, 80% limestone) and operated to 70 cm with tap water. Water was circulated using a 1/200 HP, 3200 rpm, March (Glenview, Illinois) series 1 (1A-MD 1/2) centrifugal pump. The water inlet was situated about 5 cm below the water level. Bacterial community seeding was completed by adding fresh limestone gravel alternating with ~160 mL of inoculum at depths of 10 cm, ~40 cm and ~65 cm. The *Phragmites australis* was collected from a local marsh, cultured in pots with peat moss and transferred to the mesocosms with a small amount of peat moss in the top section. Three small plants (~30 cm high) were used initially for each planted mesocosm. Initial root depth was ~20 cm in all cases. The *Phragmites australis* used was of the North American native non-invasive type.

The mesocosms were maintained under laboratory conditions with a relative humidity and temperature of 40-60% and 26-28°C, respectively. All mesocosms were exposed to artificial

illumination (14,000 lumens) with a 15 hr photoperiod. Plants were sprayed daily with tap water to avoid drying. The mesocosms were completely drained once per week. After draining, the mesocosms were then refilled with a simulated wastewater solution based on the descriptions of Droste (1996) and solutions used by Kargi and Karapinar (1995) and Wang *et al.* (2008). As a plant supplement, a nutrient solution according to Hoagland and Arnon (1938) was also added to the simulated wastewater. The nutrient solution was mixed in regular tap water and fed to the wetlands resulting in interstitial concentrations of 28.75 mg L<sup>-1</sup> NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>; 151.5 mg L<sup>-1</sup> KNO<sub>3</sub>; 236 mg L<sup>-1</sup> Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O; 123.25 mg L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O; 9.175 mg L<sup>-1</sup> FeNaEDTA; 0.715 mg/L H<sub>3</sub>BO<sub>3</sub>; 0.4525 mg/L MnCl<sub>2</sub>·4H<sub>2</sub>O; 0.055 mg/L ZnSO<sub>4</sub>·7H<sub>2</sub>O; 0.0125 mg L<sup>-1</sup> CuSO<sub>4</sub> and 0.005 mg L<sup>-1</sup> (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O (Weber *et al.*, 2010; Weber *et al.*, 2011). The simulated wastewater consisted of ~1 g L<sup>-1</sup> molasses, 0.049 g L<sup>-1</sup> urea, 0.0185 g L<sup>-1</sup> NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, yielding a glucose concentration of ~0.588 g L<sup>-1</sup> from the molasses, a COD of ~500 mg L<sup>-1</sup> and a COD:N:P ratio of ~100:5:1.

The mesocosms were operated in a constant recycle mode where water was circulated at approximately 2.4 L min<sup>-1</sup> resulting in an average cyclic hydraulic retention time of approximately 4-5 min. Each week the mesocosms were filled with the simulated wastewater and operated under constant recycle for 1 week which is similar to hydraulic retention times reported for full-scale CW systems. After 1 week the mesocosm was completely drained before being again refilled with the simulated wastewater for the following week's operation.

### **2.3.2 Antibiotic addition**

Two of the four mesocosms (CIP1 & CIP2) were exposed to a one-time ciprofloxacin (Mediatech Inc., Manassas, VA, USA) addition at a final concentration of 2 mg L<sup>-1</sup> after a 1 week operational period (inoculated on Day 7). Water was circulated to provide continuous mixing of the antibiotic stock solution within the mesocosms for a period of 5 days (before drainage).

### **2.3.3 Water sampling**

Interstitial bacterial communities were assessed in this study. In order to study the attached biofilm communities, the mesocosms would need to be disassembled. Due to the interest in the temporal nature of the response, disassembling and thus discontinuing mesocosm development and operation was not an option. Due to the design, sloughed biofilm with this mesocosm set-up is retained, so assessment of the interstitial water communities likely provides a reasonable representation of the outermost attached biofilm communities over time (Weber *et al.*, 2011). Samples of interstitial water were collected from the four mesocosms 0, 7, 14, 28, 49, and 63 days following start-up. Duplicate samples from each mesocosm were collected in sterile, 50-mL screw-capped polypropylene containers. Samples were transported on ice ( $\approx 4^{\circ}\text{C}$ ) to the microbiology laboratory and analyzed within 6-12 hours of collection.

### 2.3.4 Isolation and enumeration of faecal indicator bacteria

*Enterococcus* spp. and *E. coli* were isolated from each interstitial water sample using standard membrane filtration methods (American Public Health Association; APHA, 1999). Dilutions were filtered through 0.45- $\mu$ m, 47-mm mixed cellulose ester membrane filters (Difco, Fisher Scientific, Ottawa, Ontario, Canada), and then placed onto appropriate selective agar plates. mEnterococcus agar (Difco, Fisher Scientific) was used for the detection and enumeration of *Enterococcus* spp., followed by confirmation with bile esculin agar (Difco, Fisher Scientific). mFC-BCIG agar was used for the detection and isolation of *E. coli* (Kon *et al.*, 2007), made with mFC basal agar (Difco, Fisher Scientific) and 100  $\mu$ g 5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronide cyclohexyl ammonium salt (Medox Diagnostics, Ottawa, Ontario, Canada) per liter. Incubation conditions for the agar plates were as follows: mEnterococcus plates,  $35 \pm 2^\circ\text{C}$  for 48 hr and mFC-BCIG plates,  $44.5 \pm 0.5^\circ\text{C}$  for 24 hrs (hot waterbath). After 48 hr, membrane filters from mEnterococcus agar were placed onto pre-warmed bile esculin agar plates and incubated at  $44 \pm 0.5^\circ\text{C}$  for 2 hr. Positive colony characteristics of *Enterococcus* spp. ranged from pink to dark red on mEnterococcus agar and produced a brown to black precipitate on bile esculin agar. Blue colonies arising on mFC-BCIG agar plates were considered positive for *E. coli* (indicative of a positive reaction for  $\beta$ -glucuronidase). Samples were prepared in duplicate, and enumeration counts between 20 and 200 (Schraft and Watterworth, 2005) colonies per plate were considered for analysis. Characteristic colonies (confirmed using multiplex and/or colony PCR) were recorded (and calculated in CFUs per 100 mL filtered sample) and further streaked for purity onto Brain Heart Infusion (BHI) agar (Difco, Fisher Scientific) for

*Enterococcus* spp. or Luria-Bertani (LB) agar (Bioshop Canada Inc., Burlington, Ontario) for *E. coli* and incubated at  $35 \pm 2^{\circ}\text{C}$  for 24 hr.

### **2.3.5 Molecular confirmation of *Enterococcus* spp. and *E. coli***

*Enterococcus* spp. isolates were identified using genus- and species-specific multiplex PCR in order to distinguish three common species of *Enterococcus*, including; *E. faecalis*, *E. faecium*, and *E. durans*. Genus-specific PCR primers to 16S rRNA genes were designed previously and used in each reaction to confirm the genus *Enterococci* (Deasy *et al.*, 2000). For species-specific identification, the enterococcal superoxide dismutase (*sodA*) gene sequences were used. A previous report identified the manganese-dependent superoxide dismutase gene *sodA* as an ideal gene for species identification of enterococci (Poyart *et al.*, 2000). Four sets of PCR primers (Table 2.1) were used as previously published (Deasy *et al.*, 2000; Jackson *et al.*, 2004) and synthesized by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). The PCR master mix consisted of 1.25  $\mu\text{L}$  of genus and species-specific primers (16  $\mu\text{M}$ ) for *E. durans*, and *E. faecium*, with the exception of *E. faecalis* (FL1, FL2 primers), in which 2.5  $\mu\text{L}$  was added to the base mix, consisting of 3 mM  $\text{MgCl}_2$ , 0.2 mM deoxynucleoside triphosphate mix, 5 X GoTaq Flexi Buffer (4.5  $\mu\text{L}$ ), and 2.5 U of GoTaq Flexi DNA Polymerase (Promega, Fisher Science). PCR was performed using a BioRad™ I-cycler iQ PCR machine. The PCR mixtures were performed in a final volume of 22.5  $\mu\text{L}$  consisting of 20  $\mu\text{L}$  of master mix and 2.5  $\mu\text{L}$  of template (or a single isolated colony). Following an initial denaturation at  $95^{\circ}\text{C}$  for 4 min, products were amplified by 30 cycles of denaturation at  $95^{\circ}\text{C}$  for 30 s, annealing at  $55^{\circ}$  for 1 min, and elongation at  $72^{\circ}\text{C}$  for 1 min.

Amplification was followed by a final extension at 72°C for 7 min. Ten microliters of PCR product was electrophoresed on a 1.6% 1X sodium borate agarose gel and confirmed under UV light after ethidium bromide (EB) staining.

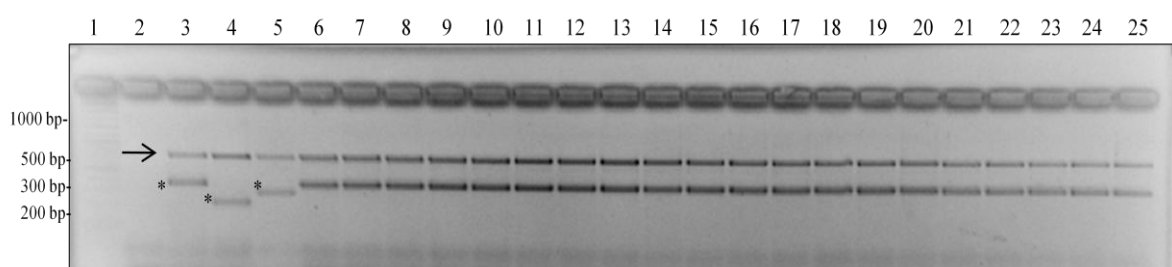
Similarly, *E. coli* isolates were confirmed using species-specific PCR primers as previously developed by Lee *et al.*, (2006) (Table 2.1). PCR mixture (20 µL) contained 5 X GoTaq Flexi Buffer (4.0 µL), 2.5 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleoside triphosphate mix, 1.0 U of GoTaq Flexi DNA Polymerase (Promega), and 2.0 µL of each primer (10 µM). The PCR conditions were 95°C for 5 min for pre-denaturing, followed by 35 cycles at 95°C for 20 s, 60°C for 1 min, and a final extension at 72°C for 10 min. PCR products were examined on 1.6% 1X sodium borate agarose gels and confirmed under UV light after ethidium bromide (EB) staining.

**Table 2.1. PCR primers, products, and reference strains for *Enterococcus* spp., and *E. coli***

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



All *Enterococcus* spp. isolates reacted with the enterococcal genus primer, indicating that they were members of *Enterococcus* (Figure 2.1). An example of a typical agarose gel amplifying 20 representative enterococcal isolates from CON1 is shown in Figure 2.1. All amplicons produced were required to be the same size as the genus and species PCR product in order to be identified as an *Enterococcus* species.



**Figure 2.1. Group 1 genus and species multiplex PCR of enterococci. Genus-specific bands are indicated by the arrow, and species-specific bands are indicated by asterisks. Species positive controls are in lanes 3 to 5 as follows: *E. faecalis* (360 bp), *E. faecium* (215 bp), and *E. durans* (295 bp). Negative control in lane 2 contained no DNA; lane 1 contained the DNA standard.**

### 2.3.6 Total heterotrophic community plate counts

Conventional culture-based methods were used to examine the general heterotrophic population within the interstitial water. Enumeration of colony-forming units (CFU) was performed using serial dilutions of interstitial water by inoculating 0.1 mL of each dilution onto R2A agar (Difco, Fisher Scientific). Incubation was performed according to Standard Methods using R2A agar incubated at  $35 \pm 2^\circ\text{C}$  for 48 hrs (APHA, 1999) after which bacterial colonies were counted. Samples were prepared in duplicate, and enumeration counts between 20 and 200 colonies per plate were considered for further calculation. R2A agar (Massa *et al.*, 1998) was used to capture a population pertaining to a large number of

species within the mixed microbial population of the mesocosms. The oxidation-reduction potential (ORP) fluctuated within each mesocosm system, with a value of -125mV near the bottom, approaching 200-400mV (dependent on the day and temperature) towards the top of the system. The heterotrophic plate count (HPC) represents the culturable aerobic and facultative anaerobic bacteria within the interstitial water. At ORP values greater than +50mV, free molecular oxygen is available in the water and may be used by aerobes and facultative anaerobes for the degradation of organic compounds (Gerardi, 2003).

### **2.3.7 Antimicrobial susceptibility testing**

Antimicrobial resistance patterns were determined using the disc-diffusion method as set by the Clinical and Laboratory Standards Institute (CLSI, 2007). *Enterococcus* spp. and *E. coli* isolates were inoculated into Brain Heart Infusion (BHI) broth (Difco, Fisher Scientific) and grown to a 0.5 McFarland turbidity standard (4-6 hrs) and swabbed onto Mueller Hinton (Difco, Fisher Scientific) agar plates. The following antimicrobial agents were selected as important representatives of different antibiotic classes, with abbreviations and disc concentrations shown in brackets: streptomycin (S 10 µg), cefotaxime (CTX 30µg), vancomycin (VA 30 µg), ciprofloxacin (CIP 5 µg), chloramphenicol (C 30 µg), ceftriaxone (CRO 30 µg), amoxicillin (AMC 30 µg), ceftazidime (CAZ 30 µg), doxycycline (D 30 µg), ampicillin (AM 10 µg), linezolid (LZD 30 µg), sulfisoxazole (G 0.25 mg) and sulfamethoxazole-trimethoprim (SXT 23.75/1.25 µg). After incubation for 16-18 hours at 35±2°C, the isolates were classified as susceptible, intermediate or resistant by comparing the diameters of inhibition zones with the breakpoints according to CLSI recommendations

(CLSI, 2007). Organisms considered intermediate by the Kirby–Bauer disc-diffusion method were recorded as resistant for the purpose of this study.

Resistance to ciprofloxacin for the total heterotrophic community was also studied by the agar dilution screening method as described by the Clinical and Laboratory Standards Institute (CLSI, 2006b) using R2A agar supplemented with six different ciprofloxacin concentrations; 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0  $\mu\text{g mL}^{-1}$ . Aliquots (100  $\mu\text{L}$ ) of interstitial water samples from each of the four mesocosms (CON1, CON2, CIP1, CIP2) were spread onto duplicate plates, and incubated at  $35 \pm 2^\circ\text{C}$  for 48 hrs. Minimum inhibitory concentrations (MICs) were determined using the following breakpoint criteria:  $\text{MIC} \leq 1 \mu\text{g mL}^{-1}$  was considered susceptible to the antibiotic;  $\text{MIC} = 2 \mu\text{g mL}^{-1}$  was considered intermediately susceptible and  $\text{MIC} \geq 4 \mu\text{g mL}^{-1}$  was considered resistant to ciprofloxacin.

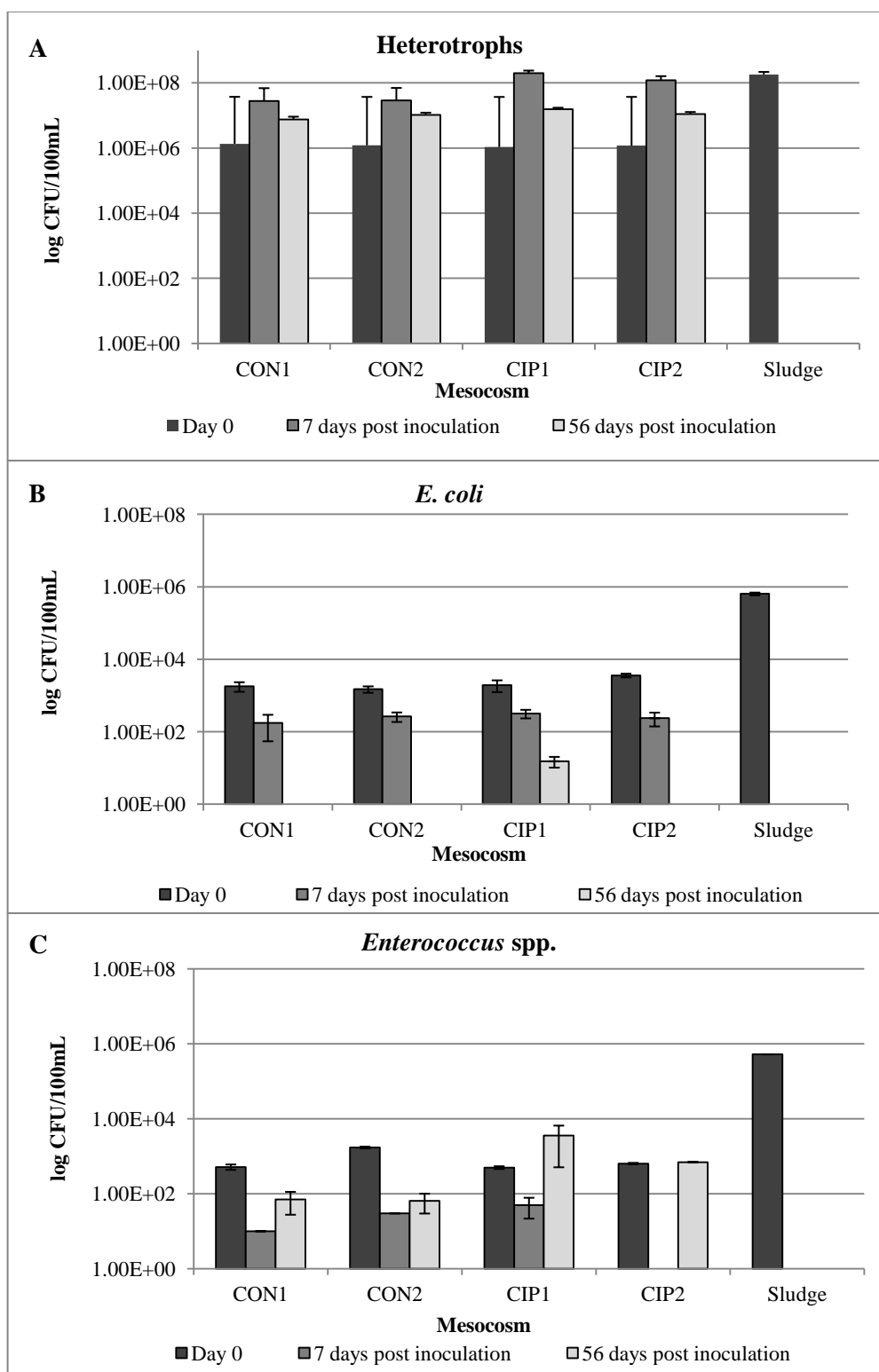
### **2.3.8 Statistical analysis**

The chi-squared test was used to compare the prevalence of antibiotic resistance phenotypes among the isolates both before and after ciprofloxacin exposure for each of the four mesocosms (CON1, CON2, CIP1, CIP2). All statistical computations were performed using Microsoft Excel version 2007 for Windows. A  $p$  value of  $<0.05$  was considered statistically significant.

## 2.4 Results and Discussion

### 2.4.1 Enumeration of indicator organisms in mesocosms

Culturable *E. coli*, *Enterococcus* spp., and total heterotrophic counts from four mesocosms and the initial sludge seed inoculum, were enumerated over the course of 10 weeks to assess the persistence of faecal indicator organisms and total heterotrophic populations during the start-up phase. Initial start-up period is the first week after construction where the bacterial communities are adapting and attaching to the mesocosm media, with development continuing throughout the start-up period (Weber and Legge, 2010). Total heterotrophic counts for each mesocosm are shown in Figure 2.2A, including bacterial counts on mEnterococcus agar for *Enterococcus* spp. (Figure 2.2B), and mFC-BCIG agar for the enumeration of *E. coli* (Figure 2.2C). Ciprofloxacin was added to two mesocosms (CIP1 & CIP2) one week following initial start-up (Nov. 23<sup>rd</sup>) and allowed to circulate for 5 days before drainage (Nov. 27<sup>th</sup>). The average concentration for total heterotrophs within the interstitial water on Day 0 was in the order of  $10^6$  CFU 100 mL<sup>-1</sup> among all four mesocosms, whereas *E. coli* and *Enterococcus* spp. were present in lower concentrations, at  $10^2$  -  $10^3$  CFU 100 mL<sup>-1</sup>. For the first sampling date (Day 0) faecal indicators were present in similar concentrations for the four mesocosms, with the *E. coli* concentrations slightly higher than the *Enterococcus* spp.. The initial concentration of total heterotrophs and faecal indicators (*E. coli* and *Enterococcus* spp.) within the sludge seed inoculum was in the order of  $10^8$  and  $10^5$  CFU 100 mL<sup>-1</sup>, respectively, 2-3 orders of magnitude greater than interstitial water samples from the four mesocosms.



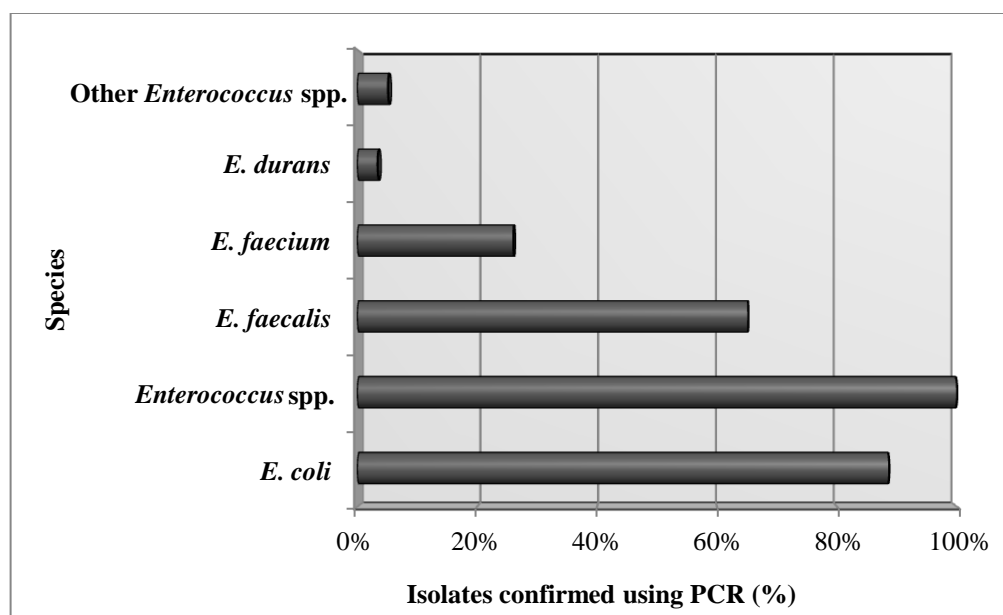
**Figure 2.2.** Mean values of total heterotrophs (A), *E. coli* (B), and *Enterococcus spp.* (C) as colony-forming units (CFUs) per 100 mL over three sampling dates within constructed mesocosms. Error bars indicate 1 standard deviation.

The number of *Enterococcus* spp. decreased to below statistically valid detection limits (reported as estimates) by the second (Day 14) and third sampling events (Day 63). *E. coli* concentrations were found to decrease throughout the system, falling to non-detectable limits for all mesocosms, including the controls (with the exception of CIP1 reported as an estimate) by the third sampling date (Day 63). Possible explanations may be that *E. coli* is less capable of adapting to the ciprofloxacin addition (within the exposed mesocosms) compared to other enteric microorganisms (*Enterococcus* spp.), *E. coli* could have an increased die-off rate (decay) compared to *Enterococcus* spp., or *E. coli* are more readily incorporated into biofilms and sediment more easily, which could explain the decrease observed among the control mesocosms. Studies using small-scale constructed wetlands (mesocosms) have shown a reduction by more than two orders of magnitude among faecal coliforms and enterococci (Hench *et al.*, 2003). Enterococci are generally considered to be more robust bacteria than *E. coli* and have the capability of surviving under extreme conditions (Mims *et al.*, 1993), which could account for the increased counts observed at day 63. The general decline in both *Enterococcus* spp. and *E. coli* at day 14 is likely due to both an initial adjustment, and incorporation into the attached phase. Both of the mesocosms exposed to ciprofloxacin showed an increase in heterotrophic counts by more than 2 orders of magnitude over the first 7 days. This increase in HPCs may be attributed to an alteration in cell surface properties, which could lead to either the detachment of the fixed microbial community or the impaired attachment ability within the mesocosms after exposure to ciprofloxacin. Weber *et al.* (2008) observed similar behaviour for total interstitial bacterial communities exposed to acid mine drainage (AMD), with increased heterotrophic counts following AMD treatment. In general, the control mesocosms (CON1 & CON2) showed

comparable concentrations of HPCs throughout the duration of the study (63 days), whereas *E. coli* and *Enterococcus* spp. concentrations decreased to below the statistically valid range by Day 63. Heterotrophs remained culturable within the interstitial water over 63 days ( $10^6$  -  $10^7$  CFU 100 mL<sup>-1</sup>), while *E. coli* and *Enterococcus* spp. decreased to very low concentrations, suggesting incorporation into the fixed microbial biofilm community or loss of viability resulting in low measurability within the interstitial water.

#### **2.4.2 Qualitative PCR to detect and characterize *Enterococcus* spp. and *E. coli***

Genotypic confirmation using qualitative PCR was performed on a total of 115 representative *Enterococcus* spp. isolates and 149 presumptive *E. coli* isolates, collected from various interstitial water samples. Among *Enterococcus* spp. isolates, 100% were confirmed as *Enterococcus* spp. using genus and species-specific multiplex PCR (previously developed by Deasy *et al.*, 2000; Jackson *et al.*, 2004). All isolates reacted with the enterococcal genus primer, indicating that they were members of *Enterococcus* (Figure 2.3). Similarly, 149/168 (89%) presumptive *E. coli* isolates were confirmed as positive with molecular confirmation using species-specific PCR primers as previously developed by Lee *et al.* (2006). Multiplex species-specific PCR was used to differentiate between three common species of *Enterococcus* spp., with 65.2% of total isolates identified as *E. faecalis* (75/115), 26.1% as *E. faecium* (30/115), 3.5% (4/115) as *E. durans*, and 5.2% (6/115) identified as "other" *Enterococcus* spp., reacting only with the enterococcal genus primer (requires more discriminate analysis) (Figure 2.3).



**Figure 2.3. Distribution and molecular confirmation of *Enterococcus* spp. (n=115) and *E. coli* (n=168) among screened isolates within interstitial water samples within the four mesocosms.**

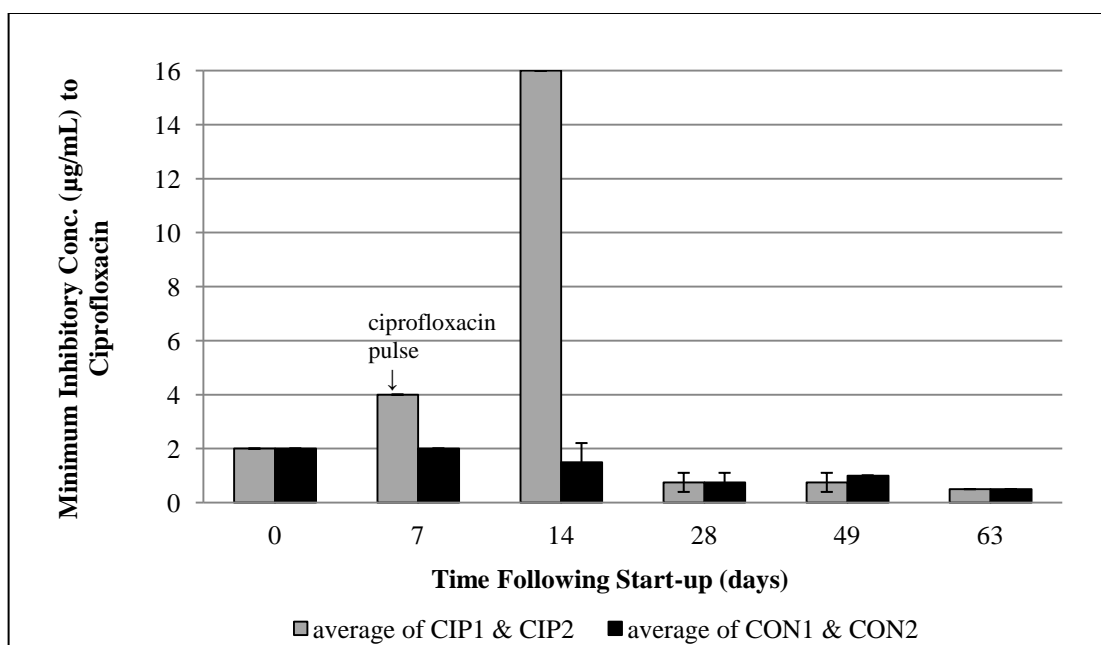
In total, over 90% of representative *Enterococcus* spp. isolates were confirmed to be either *E. faecalis* or *E. faecium*. These two strains of *Enterococcus* have presented serious challenges clinically, and are the third leading cause of nosocomial infections in intensive care units in the United States, becoming increasingly resistant to treatment with antimicrobials (CDC, 2001). The findings of *E. faecalis* as the predominant *Enterococcus* spp. in this study is similar to that previously reported in other parts of the world (Molitoris *et al.*, 1986; Welton *et al.*, 1998; Butaye *et al.*, 2001; Aarestrup *et al.*, 2000; Jensen *et al.*, 2002). Hayes *et al.* (2004) examined multiple antibiotic resistances among *Enterococcus* spp., and found similar results when characterizing *Enterococcus* spp. isolates. A total of 541 *Enterococcus* spp. isolates were recovered and identified to the species level, with *E. faecalis* being the most predominant species (53.2%), followed by *E. faecium* (31.4%), *E. gallinarum* (6.0%), *E. durans* (1.5%), and *E. casseliflavus* (1.2%) (Hayes *et al.*, 2004). The



results here are in agreement with those of Hayes *et al.* (2004), where approximately 85% of tested *Enterococcus* spp. isolates in their study and 90% of isolates presented here were characterized as either *E. faecalis* or *E. faecium*.

### **2.4.3 Antibiotic resistance of interstitial bacterial community**

Minimum Inhibitory Concentrations (MICs) were determined for the interstitial bacterial community (total culturable heterotrophs) for each of the four mesocosms using the following ciprofloxacin concentrations: 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0  $\mu\text{g mL}^{-1}$ . Figure 2.4 illustrates the average MIC for the control (CON1/CON2) and ciprofloxacin exposed (CIP1/CIP2) mesocosms, both before and after the ciprofloxacin spike (Day 7). All four mesocosms had the same MIC of 2  $\mu\text{g mL}^{-1}$  on Day 0 and Day 7 (with the exception of CIP1/CIP2), followed by a large increase for CIP1 and CIP2 on Day 14, reaching a maximum MIC of 16  $\mu\text{g mL}^{-1}$ . The resistance levels of interstitial heterotrophic counts determined by the agar dilution method peaked 7 days following ciprofloxacin exposure and decreased thereafter.



**Figure 2.4.** Average Minimum Inhibitory Concentration (MIC) of the total heterotrophic community to ciprofloxacin in two control mesocosms (CON1 & CON2) and two mesocosms exposed to a 2 mg L<sup>-1</sup> ciprofloxacin pulse (CIP1 & CIP2). Error bars indicate 1 standard deviation (range between 0-0.71).

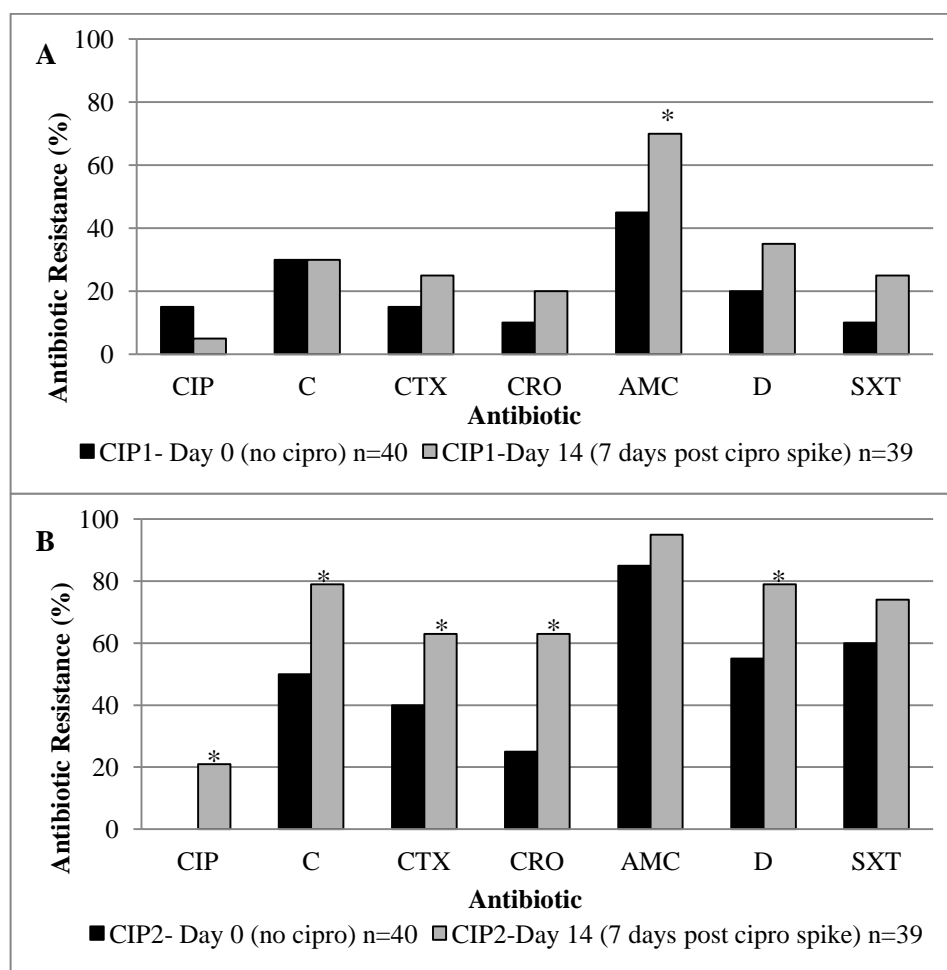
The results demonstrate a natural level of intermediate resistance among bacteria within all four mesocosms before ciprofloxacin exposure (MIC  $\geq 4 \mu\text{g mL}^{-1}$  resistant; MIC =  $2.0 \mu\text{g mL}^{-1}$  intermediate susceptible; MIC  $\leq 1.0 \mu\text{g mL}^{-1}$  susceptible), which can become more pronounced following the selective pressure from antimicrobial drug exposure. The development of drug-resistant microorganisms is attributed to the maintenance of selective pressure in the environment which can either permit the development of resistant strains, or eliminate drug-sensitive strains, allowing for the proliferation of resistant microbes until resistance becomes dominant (Cloutier, 1995). This adaptation and competition may be responsible for the increased proportion of bacteria with a higher level of resistance soon after ciprofloxacin exposure. Yu *et al.* (2009) found a similar trend in the ciprofloxacin MIC

value following drug administration in *Enterococcus faecalis*, with high-level resistance developing and peaking three days after exposure to the antibiotic. Exposure of bacteria to antimicrobial concentrations is thought to increase the speed with which resistant bacterial strains are selected, e.g. if antibiotics are used as growth promoters (Witte *et al.*, 1999; Khachatourians, 1998) or by improper use in veterinary and/or human medicine (Salyers, 2002; Teubner, 1999). Overall, the introduction of an antibiotic can have a considerable effect on the total level of bacterial resistance within an aquatic system.

#### **2.4.5 Antibiotic resistance of *E. coli***

Antimicrobial resistance (AMR) to twelve common antibiotics was determined by the disc diffusion method, according to the standardized guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2007). The prevalence of antibiotic resistance among *E. coli* isolates before and after ciprofloxacin exposure for the two mesocosms, CIP1 and CIP2 are shown in Figure 2.5. The resistance level among all antibiotics for the two mesocosms, CIP1 and CIP2 demonstrated an increase 7 days following ciprofloxacin exposure (with the exception of ciprofloxacin resistance slightly decreasing and chloramphenicol resistance remaining the same in CIP1). An increase in antimicrobial resistance was observed to the following antibiotics, revealing a significant increase ( $p < 0.05$ ) in resistance 7 days following exposure to ciprofloxacin within mesocosm CIP2: ciprofloxacin (CIP), chloramphenicol (C), cefotaxime (CTX), ceftriaxone (CRO), and doxycycline (D), as shown in Figure 2.5B. Amoxicillin (AMC) was the only antibiotic that demonstrated a significant increase ( $p < 0.05$ ) in resistance 7 days following exposure to ciprofloxacin within

mesocosm CIP1, as shown in Figure 2.5A. The initial resistance among CIP1 *E. coli* isolates on Day 0 was 45%, increasing to 70% on Day 14 (7 days post ciprofloxacin). The overall comparison between replicate mesocosms (CIP1 and CIP2) revealed apparent differences in levels of antibiotic resistance among *E. coli* isolates, both before and after ciprofloxacin exposure. The differences observed between the four mesocosms are most likely due to the ecological complexity or the temporal and spatial variations in wetland processes occurring in the individual mesocosm systems.

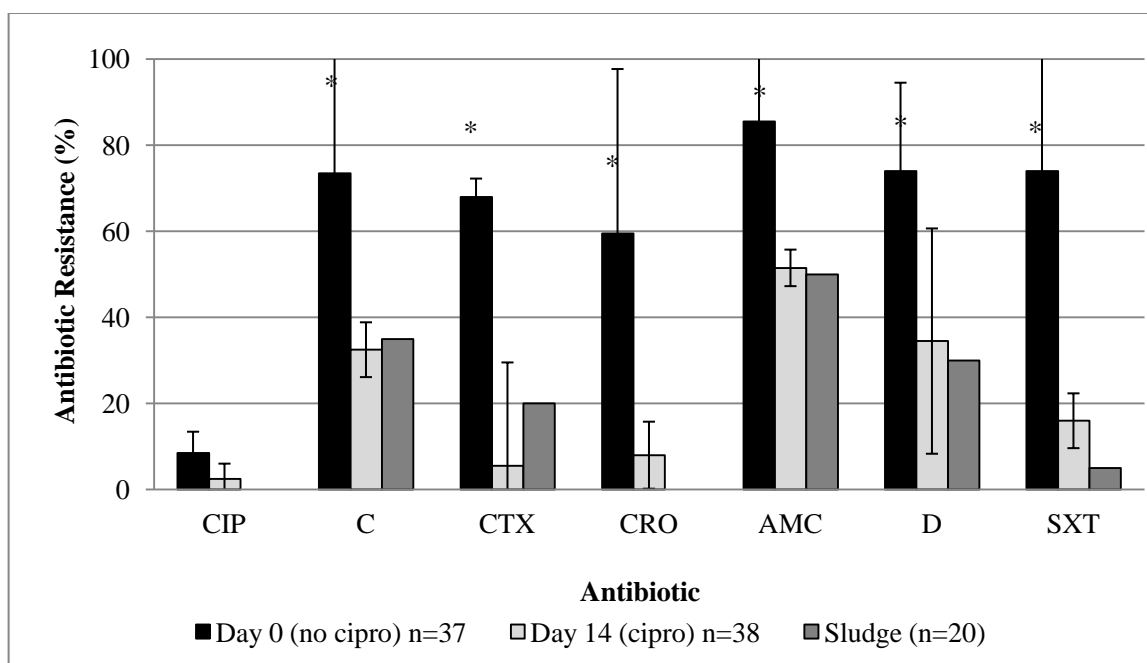


**Figure 2.5.** Prevalence of antibiotic resistance (%) among *E. coli* for CIP1 mesocosm (A) and CIP2 mesocosm (B) before and after 2 mg L<sup>-1</sup> ciprofloxacin exposure. The following antimicrobial agents were selected as important representatives of different antibiotic classes (abbreviations are shown in brackets); ciprofloxacin (CIP), chloramphenicol (C), cefotaxime (CTX), ceftriaxone (CRO), amoxicillin (AMC), doxycycline (D), and sulfamethoxazole-trimethoprim (SXT). An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n = number of isolates.

The results for *E. coli* illustrate the potential for a single antibiotic to create a statistically significant increase in antibiotic resistance, not only to the antibiotic itself, but to additional antibiotics as well. In *E. coli*, a putative multidrug resistance pump, YhcQ, was reported to be involved in antibiotic resistance of biofilms (Lynch *et al.*, 2007; Zhang and Mah, 2008), and Mar, Sox, and AcrAB-TolC efflux pump-encoded genes have been found to be upregulated under stress conditions, such as a stationary growth condition, growth in biofilms, and exposure to several antimicrobial agents (Bailey *et al.*, 2006; Domain *et al.*, 2007). Quinolone resistance resulting from the multiple antimicrobial resistance (MAR) phenotype is often associated with cross-resistance to many other structurally unrelated antimicrobials such as beta-lactams, puromycin, tetracyclines, nalidixic acid and chloramphenicol (George and Levy, 1983). Therefore, it is possible that the increase in antibiotic resistance among *E. coli* from Day 0 to Day 14 in CIP2 was attributed to the upregulation of certain efflux pump-encoded genes after the 5-day ciprofloxacin exposure. Another possible explanation for the increased resistance among several antibiotics could be the acquisition of a multi-drug resistance plasmid. Plasmid-mediated transmission is the most common mechanism of horizontal gene transfer (HGT) (Norman *et al.*, 2009), with subinhibitory concentrations of antibiotics facilitating the process of antibiotic resistance development (Davies *et al.*, 2006). For example, antimicrobials have been shown to enhance gene transfer and recombination (Couce and Blazquez, 2009), partially through the activation of the SOS system (Guerin *et al.*, 2010); in addition, antimicrobials have been shown to induce phage production from lysogens. Such factors may play an important role in the proliferation of gene exchange within aquatic environments. These results are in contrast to those of Atoyan *et al.* (2007) who studied the effects of tetracycline on antibiotic

resistance and removal among faecal indicators in aerated and unaerated leachfield mesocosms (antibiotic exposure for 10 days; monitored for 52 days). Their results suggest that the presence of environmentally relevant tetracycline concentrations ( $5 \text{ mg L}^{-1}$ ) in domestic wastewater is likely to have minimal consequences on pathogen removal and the development of antibiotic resistance among pathogenic bacteria. The differences seen here could be due to the different type of system and different conditions studied, including differences in the resistance mechanisms and means of drug transmission, length of antibiotic exposure, duration of monitoring period, as well as differences in the behaviour, fate, and transport of ciprofloxacin compared to tetracycline.

The prevalence of antibiotic resistance among *E. coli* in mesocosms not administered the ciprofloxacin (CON1 & CON2) and the initial sludge seed is shown in Figure 2.6. The same antibiotic resistance profiles that increased in CIP2 following ciprofloxacin addition were found to decrease significantly two weeks following start-up (Day 14). The following antibiotics revealed a significant decrease ( $p < 0.05$ ) in resistance 7 days following exposure to ciprofloxacin within the two control mesocosms (CON1/CON2): chloramphenicol (C), cefotaxime (CTX), ceftriaxone (CRO), amoxicillin (AMC), doxycycline (D), and sulfamethoxazole-trimethoprim (SXT). The average decrease in antibiotic resistance from Day 0 to Day 14 was 6%, 41%, 62.5%, 51.5%, 34%, 39.5%, and 58%, for ciprofloxacin, chloramphenicol, cefotaxime, ceftriaxone, amoxicillin, doxycycline, and sulfamethoxazole-trimethoprim, respectively.



**Figure 2.6. Prevalence of antibiotic resistance (%) among *E. coli* within mesocosms without ciprofloxacin exposure (CON1 & CON2) and for initial sludge seed isolates from Day 0. The following antimicrobial agents were selected as important representatives of different antibiotic classes (abbreviations are shown in brackets); ciprofloxacin (CIP), chloramphenicol (C), cefotaxime (CTX), ceftriaxone (CRO), amoxicillin (AMC), doxycycline (D), and sulfamethoxazole-trimethoprim (SXT). An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n= number of isolates. Error bars indicate 1 standard deviation.**

The results suggest that after a period of time with no antibiotic exposure a decrease in antibiotic resistance may be observed among *E. coli* isolates. Different studies have shown that it is possible at least to some degree to limit the occurrence of resistance by removing the selective pressure, or antimicrobial agent (Klare *et al.*, 1999; Pantosti *et al.*, 1999; Aarestrup *et al.*, 2001). Boerlin *et al.* (2001) suggest that the complete ban of antimicrobial growth promoters (AGP) applied in Switzerland had a relatively rapid effect on antimicrobial resistance among enterococci. Resistance to macrolides and lincosamides drastically decreased shortly after the ban enforcement. However, it is not clear whether the occurrence of resistance will ever reach the same low level as before the antimicrobials were

introduced. For example, despite the avoparcin ban in 1997, vancomycin resistant enterococci (VRE) were still present at a significant level in Swiss pigs more than 2 years after the discontinuation of avoparcin use (Aarestrup *et al.*, 2001).

Resistance to several antibiotics as seen here could be due to a single physiological stress response/adaption. Exposure of bacteria to sub therapeutic levels of antimicrobial concentrations is thought to increase the occurrence of antimicrobial resistance. Langlois *et al.* (1986) found that in a pig herd where the animals were continuously exposed to antibiotics, both as feed additives and therapeutic agents, almost 100% of the faecal coliform bacteria were tetracycline resistant. Similarly, Westen (1996) concluded that the use of one antimicrobial agent can lead to increased levels of resistance not only to a specific drug but to many others, including those which use different modes of antibacterial action (cross resistance). The results obtained for *E. coli* isolates support this concept, in which the introduction of one antibiotic can lead to an increase in resistance, not only to ciprofloxacin (quinolone class), but to other classes of antimicrobials as well (cephalosporins, penicillins, tetracyclines, and sulfonamides).

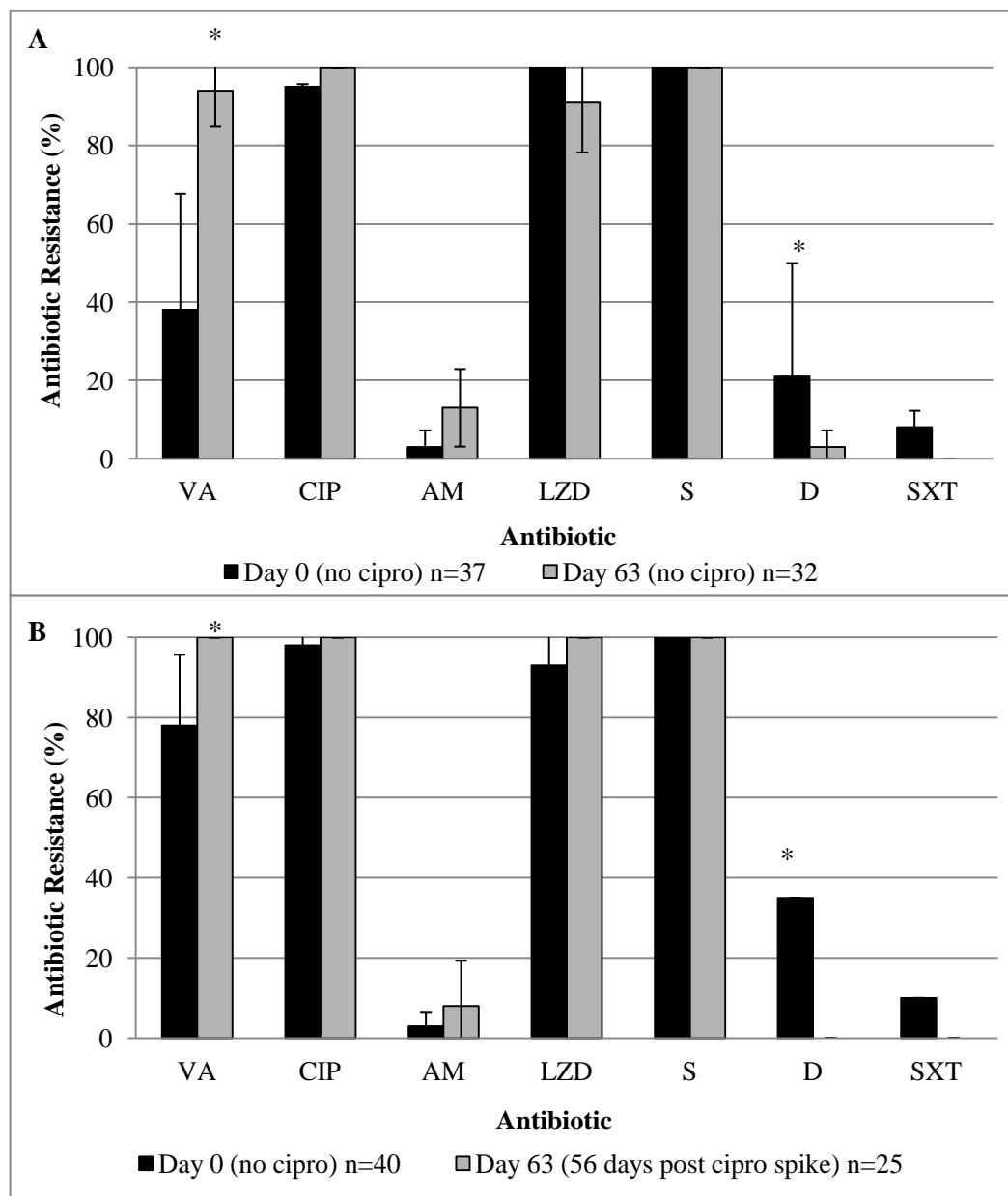
#### **2.4.6 Antibiotic resistance of *Enterococcus* spp.**

The prevalence of antibiotic resistance (%) among *Enterococcus* spp. isolates to seven common antibiotics before and after the addition of ciprofloxacin is shown in Figure 2.7. It is important to note that the antimicrobial resistance profiles of *Enterococcus* spp. isolates were tested on Day 63 (as opposed to Day 14), which was due to the limited number of



isolates recovered on Day 14 (refer to Figure 2.2). The initial background level of ciprofloxacin resistance observed on Day 0 was significantly higher ( $p < 0.05$ ) than observed among *E. coli*, with *Enterococcus* spp. exhibiting a combined average among all four mesocosms of 96%, 96%, and 100% resistance to ciprofloxacin (CIP), linezolid (LZD), and streptomycin (S), respectively. Ampicillin (AM), doxycycline (D), and sulfamethoxazole-trimethoprim (SXT), seemed to be highly active against *Enterococcus* spp.; isolates displaying fairly low levels of antibiotic resistance to these compounds (Figure 2.7). Similar studies assessing the level of antibiotic resistance among *Enterococcus* spp. isolated from wastewater treatment plants have also shown low levels of resistance to ampicillin (Martins da Costa *et al.*, 2006), and sulfamethoxazole-trimethoprim (Ferreira da Silva *et al.*, 2006). A significant decrease ( $p < 0.05$ ) in doxycycline resistance was observed among both control (Figure 2.7A) and ciprofloxacin exposed (Figure 2.7B) mesocosms on Day 63. The reason for a decrease in doxycycline resistance (tetracycline class) following exposure to ciprofloxacin (fluoroquinolone class) is unknown, as studies have shown the opposite to be true. For example, Fung-Tomc *et al.* (1993), studied the exposure of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* to subinhibitory concentrations of ciprofloxacin, resulting in the development of low-level resistance to structurally unrelated antimicrobial agents (including tetracycline antimicrobials). Following the addition of ciprofloxacin, *Enterococcus* spp. maintained their high levels of resistance to ciprofloxacin, linezolid, and streptomycin, with a significant increase ( $p < 0.05$ ) in resistance to vancomycin (VA), reaching as high as 100% on Day 63. Overall, the ciprofloxacin spike did not seem to have a significant effect on the antibiotic resistance profiles of *Enterococcus*

spp., with isolates maintaining their level of resistance (either high or low) before and after the antibiotic exposure.



**Figure 2.7. Prevalence of antibiotic resistance (%) among *Enterococcus* spp. for control mesocosms, CON1 & CON2 (A) and ciprofloxacin exposed mesocosms, CIP1 & CIP2 (B). The following antimicrobial agents were selected as important representatives of different antibiotic classes (abbreviations are shown in brackets); vancomycin (VA), ciprofloxacin (CIP), ampicillin (AM), linezolid (LZD), streptomycin (S), doxycycline (D), and sulfamethoxazole-trimethoprim (SXT). An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n= number of isolates. Error bars indicate 1 standard deviation.**

*Enterococcus* spp. have been recently identified as a major concern, as their role in nosocomial infections has increased due to their ability to acquire high-level resistance to antibiotics, making them difficult to treat (Linden and Miller, 1999). The level of resistance to both vancomycin and linezolid after the ciprofloxacin addition reached 100% in both CIP1 and CIP2. One explanation for the high prevalence of VRE in the present study could be from the initial activated sludge inoculum used to seed the mesocosms. Iversen *et al.* (2002) reported a high prevalence of VRE among untreated sewage samples (60%), and concluded the possible origin may be from both healthy individuals as well as individuals in hospitals. Similarly, Sahlström *et al.* (2009) studied VRE in Swedish sewage sludge and found that *E. faecium*, among other species of enterococci isolated from sewage sludge, were found to have high MICs to vancomycin. One isolate of *E. durans* carried a high level vancomycin resistance (MIC >128 µg mL<sup>-1</sup>) and harboured the *vanA* gene (Sahlström *et al.*, 2009). Overall, the high level of vancomycin and linezolid observed in this study is likely attributed to the activated sewage sludge used to seed the mesocosms. Sewage constitutes a favourable environment, consisting of variable mixtures of bacteria, nutrients, and antimicrobial agents (Hirsch *et al.*, 1999) that might promote growth of resistant bacteria or gene transfer between bacteria in the sewage or in the biofilm lining the pipelines (Iversen *et al.*, 2002). The average increase in VRE from Day 0 (58%) to Day 63 (100%) among ciprofloxacin exposed mesocosms (CIP1/CIP2) and control mesocosms (CON1/CON2) could be attributed to the horizontal gene transfer of antibiotic resistant genes. Silva *et al.* (2006) reported that a high percentage (89%) of multi-drug resistant coliforms isolated from a sewage treatment plant could partially or completely transfer their resistance patterns to the recipient strain.

## 2.5 Conclusions

The results obtained for the level of antibiotic resistance among *E. coli* isolates supports the idea that introduction of one antibiotic (i.e. ciprofloxacin) to an aquatic system can lead to an increase in resistance, not only to ciprofloxacin (quinolone class), but to other classes of antibiotics as well (cephalosporins, penicillins, tetracyclines, and sulfonamides). These results expand on the observations of others (Aubert *et al.*, 1992; Fung-Tomc *et al.*, 1993) that suggest that ciprofloxacin can promote resistance to drugs with unrelated modes of action. The data support the notion that the transient presence of ciprofloxacin could have an impact on the development of antibiotic resistance among faecal indicators in constructed wetland treatment systems (specifically in *E. coli*). Future studies should investigate the effect of ciprofloxacin, in addition to other classes of antibiotics, on antimicrobial resistance within already operational and developed wetland systems.

## 2.6 Recommendations and Future Research Needs

- Future studies evaluating the development of antimicrobial resistance among faecal indicators exposed to other classes of antimicrobials is needed, while considering different concentrations, exposure times, and persistence of such antibiotics in the aquatic environment.
- A better understanding is needed regarding the impact of sub-therapeutic levels of an antibiotic, and prevalence of antibiotic resistance within already operational and developed wetland systems (full-scale). This should include an assessment as to

whether or not the addition of an antibiotic affects the removal of indicator organisms and pathogens.

- Advancement beyond phenotypic (culture-based) antimicrobial evaluation and understanding how drug resistance is mediated within these isolates (chromosomally or plasmid mediated) may provide further insight into the spread of antimicrobial resistance among environmental bacteria.
- Further scientific understanding of the effects antimicrobials have on wetland systems could be discerned through direct observation and characterization of biofilm materials, in addition to examining and comparing the levels of antimicrobial resistance within biofilm bacteria and the total interstitial bacterial community. This knowledge would help both microbiologists and water treatment engineers gain a greater understanding for the effects antibiotics have on wetland systems and overall antibiotic resistance levels.

# 3

## **Monitoring the occurrence, distribution, and frequency of selected bacterial pathogens and antibiotic resistance genes in treatment lagoons receiving poultry waste effluent<sup>2</sup>**

### **3.1 Introduction**

The escalating concern surrounding antibiotic resistance of pathogenic bacteria and their antibiotic resistant genes is becoming a major global health issue (Levy, 2002; Chee-Sanford *et al.*, 2001). The widespread use of antibiotics in animal husbandry and agriculture for treating or preventing infection and promoting growth, can play an important role in the

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<sup>2</sup>Helt, C.D., Legge, R.L., Slawson, R.M. *Monitoring the Occurrence, Distribution, and Frequency of Selected Bacterial Pathogens and Antibiotic Resistance Genes in Treatment Lagoons Receiving Poultry Waste Effluent*, in preparation.

development and spread of antibiotic resistance. Significant amounts of antibiotics are excreted unaltered or as metabolites (up to 75%), which presents a major source of antibiotic input to the environment (Böckelmann *et al.*, 2009). Many of these compounds are easily detected in water resources (Kolpin *et al.*, 2002; Lindsey *et al.*, 2001) leading to increasing concerns with regard to their contribution to the abundance and persistence of antibiotic resistance in populations of pathogenic, commensal, and non-pathogenic microorganisms (Böckelmann *et al.*, 2009; Auerbach *et al.*, 2007; Pauwels and Verstraete, 2006). These concerns may arise from the lack of critical information with respect to the transfer of ARGs within and between bacterial populations in the environment (Böckelmann *et al.*, 2009; Isaacson and Torrence, 2002). Little information is available regarding the fate of antibiotics in the environment and their link to the emergence of resistant genotypes found there. The annual production of livestock and poultry waste in the United States is nearly 180 million tons (dry weight) (Haapapuro *et al.*, 1997; Hagedorn *et al.*, 1999), and coupled with antibiotic usage, this waste is a potentially large source of both antibiotics and antibiotic-resistant bacteria released into the environment. Environmental reservoirs and pathways of antibiotic resistance are acquiring increased attention (Allen *et al.*, 2010; Martinez, 2008; Pruden *et al.*, 2006) as new strategies beyond the hospital setting are being explored to attenuate antibiotic resistance and prolong the lifespan of antibiotics. One promising approach towards better understanding the occurrence, fate, and transport of antibiotic resistance in the environment is to consider ARGs as the principle contaminants of interest rather than their bacterial hosts (McKinney *et al.*, 2010; Pruden *et al.*, 2006). Livestock operations are known to harbour elevated concentrations of antibiotics and ARGs compared to surrounding environments (Peak *et al.*, 2007; Koike *et al.*, 2007) with the potential

incorporation of these genes by indigenous environmental bacteria via horizontal gene transfer. However, the extent of the environmental impact of resistant bacteria is not yet known. Treatment lagoon systems are commonly used in temperate climates across the United States as they are simple to manage and very effective in reducing organic matter and nutrients when designed and operated properly (Graves *et al.*, 2011). Lagoons, however, were not designed to control pathogens, remove antibiotics or antibiotic residues from waste. Previous studies have shown the persistence of pathogens in swine lagoon liquid and sludge, in manure piles, and in waste litter (Plym-Forsell 1995; Radtke and Gist 1989). Pathogens are more likely to persist in liquid or moist waste and in sludge or lagoon treatments because temperatures are not high enough to kill them (Kudva *et al.*, 1998).

Few studies have examined both the occurrence and fate of antibiotic resistance among environmental bacterial pathogens and different classes of ARGs using a combined approach of traditional culture-based screening and molecular quantification through real-time PCR. Previous experimental gene-fate studies have primarily focused on cattle or swine wastes (Engemann *et al.*, 2006, 2008; Knapp *et al.*, 2010; Chee-Sanford *et al.*, 2001), and it was desired to examine wastes from other agricultural operations, such as poultry slaughterhouse operations, which may ultimately contain a different composition of genes and bacterial hosts. Therefore, the following study was conducted to investigate the presence and behaviour of the following ARGs: *ampC* (ampicillin resistance), *blaSHV* (extended  $\beta$ -lactam resistance conferring broad resistance to penicillins and cephalosporins), *tetA* (tetracycline resistance) and selected bacterial pathogens (*E. coli*, *Enterococcus faecalis*, *Salmonella* spp.) in a constructed treatment lagoon system receiving poultry slaughterhouse waste. The



resistance genes were selected due to their increasing abundance in the environment (Böckelmann *et al.*, 2009; Auerbach *et al.*, 2007; Ferreira da Silva *et al.*, 2006; Kümmerer, 2004). To monitor the efficiency of waste treatment lagoons and ensure adequate removal concentrations of indicator organisms and bacterial pathogens/ARGs from the final effluent to receiving water bodies, the number of pathogenic bacteria and ARGs has to be measured during various stages of treatment. However, traditional detection methods for pathogenic bacteria possess many drawbacks with regard to very special culture requirements and long cultivation time. Consequently, rapid, accurate and culture-independent alternatives are being investigated to enable monitoring of pathogens in water and wastewater (Straub and Chandler, 2003). Real-time quantitative PCR (qPCR) technology is sensitive, specific, and yields accurate quantitative results (MacKay, 2004; Shannon *et al.*, 2007), while eliminating post-PCR processing, including gel electrophoresis. For this purpose, quantitative polymerase chain reaction (qPCR) molecular assays for three ARGs (*ampC*, *blaSHV*, *tetA*) and three bacterial pathogens (*E. coli*, *E. faecalis*, *Salmonella* spp.) were utilized. In addition, traditional culture-based screening was performed on *E. coli* and *Enterococcus* spp. isolates in order to evaluate the effects of wastewater treatment on the presence of antimicrobial resistance patterns among culturable indicator bacteria of wastewater origin.

### **3.2 Research Needs & Objectives**

The enumeration of antibiotic-resistant bacteria and ARGs in various locations of an alternative wastewater treatment system (i.e. constructed lagoons) will prove helpful in understanding both the baseline and potential movement of antibiotic resistance, and possible dissemination of ARGs into different ecosystems. Few studies have examined both

the occurrence and fate of environmental pathogens and different classes of ARGs using a combined approach of traditional culture-based screening and molecular quantification using real-time PCR. Previous studies have focused on the quantification of tetracycline resistance genes within agricultural environments; however, other classes of antibiotics including penicillins, and  $\beta$ -lactams are also used in agriculture, and must be examined. Furthermore, the results of this study will establish a preliminary environmental assessment of lagoon treatment systems receiving poultry slaughterhouse waste. The overall objective of the research reported in this chapter was to better understand the occurrence, distribution and frequency of selected bacterial pathogens and ARGs, conferring resistance to three classes of antimicrobials; tetracyclines, penicillins, and  $\beta$ -lactam resistance (conferring broad resistance to penicillins and cephalosporins), within multiple stages of a constructed treatment lagoon system receiving poultry slaughterhouse waste.

The specific objectives were to:

1. Confirm presumptive *E. coli* and *Enterococcus* spp. isolates using molecular techniques (qualitative PCR), and further differentiate between five common species of *Enterococcus* spp. using multiplex species-specific PCR;
2. Determine the prevalence of antibiotic resistance among culturable *E. coli* using the disc- diffusion method, while comparing any differences in the level of antibiotic resistance between different seasons (summer vs. fall), and various stages of treatment;
3. Establish the frequency of multiple antimicrobial resistance (MAR) and antimicrobial resistance profiles among *E. coli* isolates;

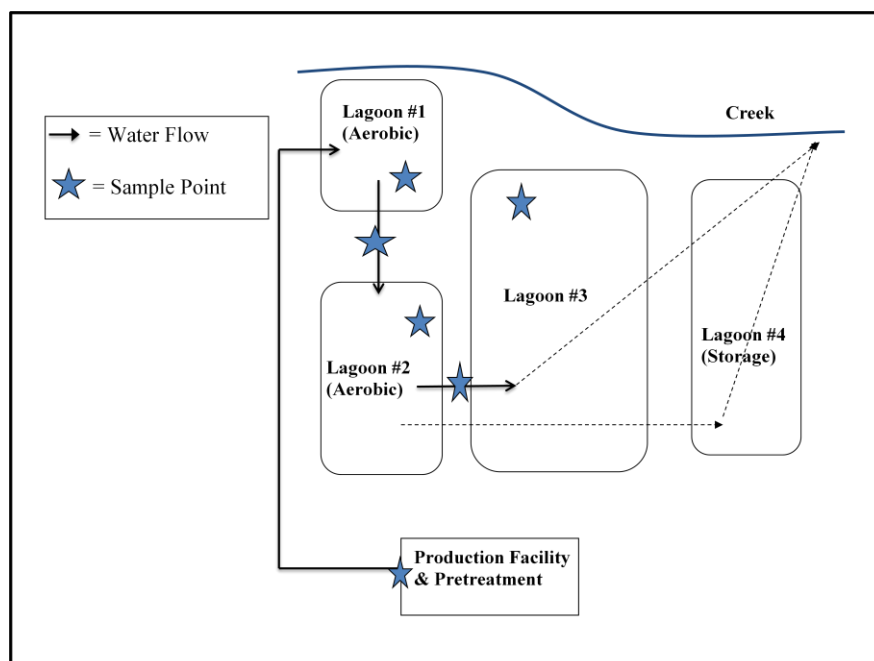
4. Determine the occurrence of antimicrobial resistance among culturable *Enterococcus* spp. isolates using the disc-diffusion method, and compare any differences between the frequency of antibiotic resistance among seasons (summer vs. fall), and various stages of treatment;
5. Assess the frequency of multiple antimicrobial resistance (MAR) and antimicrobial resistance profiles among *Enterococcus* spp. isolates;
6. Identify selected ARGs, including *tetA*, *strA*, *strB*, *sul1*, and *sul2* (conferring resistance to tetracycline, streptomycin, and sulfonamides) within culturable isolates of *E. coli* and *Enterococcus* spp. using PCR analysis;
7. Quantify selected bacterial pathogens (*E. coli*, *Salmonella*, *E. faecalis*) and ARGs (*ampC*, *tetA*, *blaSHV*) within various stages of the lagoon treatment system using real-time quantitative PCR; and
8. Determine the efficiency of wastewater treatment in the constructed lagoon system receiving poultry slaughterhouse effluent through the comparison of pathogen and ARG log removals.

### **3.3 Materials and Methods**

#### **3.3.1 Lagoon sampling**

A constructed lagoon system managing waste from a poultry processing facility (slaughterhouse) in Southern Ontario was selected for study. The lagoon system consists of

three serial lagoons (lagoons 1, 2 & 3; Figure 3.1) with an overflow/storage lagoon (lagoon 4; Figure 3.1). The first lagoon receives aerated post-flocculated effluent composed of primary factory effluent, sanitary waste and rain water from the plant over 8 hours per day at a flow rate of approximately 400-500 gallons/min. Lagoon 1 flows by gravity into the second lagoon, which is aerated. The retention times for water in the first two lagoons range from 8 to 20 days. The third and fourth lagoons are discharged late fall/early winter into a creek. Sampling was carried out between June 2009 and September 2010, with a total of five sampling events during this period. One-litre wastewater samples were collected at various locations throughout different stages of the lagoon treatment system (total of six sampling points), allowing for representative samples. Samples were stored on ice and transported to the laboratory for immediate processing. The bacterial biomass in a 500 mL water sample was concentrated by centrifugation at  $11,000 \times g$  for 20 min at 4 °C followed by storage of the pellets (water solids) at -20°C until DNA extraction was performed for molecular analysis.



**Figure 3.1.** A general schematic depicting overset hydrology and sampling points for a constructed lagoon facility. Dashed lines indicate path of the overflow water depending on the amount of water in the third lagoon.

### 3.3.2 Isolation and enumeration of faecal bacteria

*Enterococcus* spp. and *E. coli* were isolated from each water sample using standard membrane filtration methods (American Public Health Association; APHA, 1999). Diluted samples were filtered through 0.45- $\mu$ m, 47-mm mixed cellulose ester membrane filters (Difco, Fisher Scientific, Ottawa, Ontario, Canada) and then placed onto appropriate selective agar plates. mEnterococcus agar (Difco, Fisher Scientific) was used for the detection and enumeration of *Enterococcus* spp. followed by confirmation with bile esculin agar (Difco, Fisher Scientific). mFC-BCIG agar was used for the detection and isolation of *E. coli* (Kon *et al.*, 2007) made with mFC basal agar (Difco, Fisher Scientific) and 100  $\mu$ g 5-bromo-4-chloro-3-indolyl  $\beta$ -d-glucuronide cyclohexyl ammonium salt (Medox Diagnostics, Ottawa, Ontario, Canada) per liter. Incubation conditions for the agar plates were as follows:

mEnterococcus plates,  $35 \pm 2^{\circ}\text{C}$  for 48 hr and mFC-BCIG plates at  $44.5 \pm 0.5^{\circ}\text{C}$  for 24 hrs (hot waterbath). After 48 hr, membrane filters from mEnterococcus agar were placed onto pre-warmed bile esculin agar plates and incubated at  $44 \pm 0.5^{\circ}\text{C}$  for 2 hrs. Positive colony characteristics of *Enterococcus* spp. ranged from pink to dark red on mEnterococcus agar and produced a brown to black precipitate on bile esculin agar. Blue colonies arising on mFC-BCIG agar plates were considered positive for *E. coli* (indicative of a positive reaction for  $\beta$ -glucuronidase). Samples were prepared in duplicate, and counts between 20 and 200 (Schraft and Watterworth, 2005) colonies per plate were considered for analysis. Characteristic colonies (confirmed using multiplex and/or colony PCR) were recorded (and calculated in CFUs per 100 mL filtered sample) and further streaked for purity onto Brain Heart Infusion (BHI) agar (Difco, Fisher Scientific) for *Enterococcus* spp. or Luria-Bertani (LB) agar (Bioshop Canada Inc., Burlington, Ontario) for *E. coli* and incubated at  $35 \pm 2^{\circ}\text{C}$  for 24hrs.

### **3.3.3 Molecular confirmation of *Enterococcus* spp. and *E. coli***

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

identification of enterococci (Poyart *et al.*, 2000). Six sets of PCR primers (Table 3.1) were used as previously published (Deasy *et al.*, 2000; Jackson *et al.*, 2004) and synthesized by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Two PCR master mixes consisting of different primer sets were prepared. Group 1 included *E. durans*, *E. faecalis*, and *E. faecium*; and Group 2 consisted of *E. casseliflavus* and *E. gallinarum*. The base master mix was comprised of 3 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleoside triphosphate mix, 5 X GoTaq Flexi Buffer (4.5 µL), 2.5 U of GoTaq Flexi DNA Polymerase (Promega, Fisher Science), and 1.25 µL of genus and species-specific primers (16 µM). With the exception of *E. faecalis* (FL1, FL2 primers), and *E. gallinarum* (GA1, GA2 primers), 2.5 µL of each species primer was added to the base mix. PCR was performed using a BioRad™ I-cycler iQ PCR machine. The PCR reactions were performed in a final volume of 22.5 µL consisting of 20 µL of master mix and 2.5 µL of template (or a single isolated colony). Following an initial denaturation at 95°C for 4 min, products were amplified by 30 cycles of denaturation at 95°C for 30 s, annealing at 55° for 1 min, and elongation at 72°C for 1 min. Amplification was followed by a final extension at 72°C for 7 min. Ten microliters of PCR product was electrophoresed on a 1.6% 1X sodium borate agarose gel and confirmed under UV light after ethidium bromide (EB) staining.

Similarly, *E. coli* isolates were confirmed using species-specific PCR primers as previously developed by Lee *et al.*, (2006) (Table 3.1). PCR mixture (20 µL) contained 5 X GoTaq Flexi Buffer (4.0 µL), 2.5 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleoside triphosphate mix, 1.0 U of GoTaq Flexi DNA Polymerase (Promega), and 2.0 µL of each primer (10 µM). The PCR conditions were 95°C for 5 min for pre-denaturing, followed by 35 cycles at 95°C for 20 s,

60°C for 1 min, and a final extension at 72°C for 10 min. PCR products were examined on 1.6% 1X sodium borate agarose gel and confirmed under UV light after ethidium bromide (EB) staining.

**Table 3.1. PCR primers, products, and reference strains for genotypic confirmation of *Enterococcus* spp., and *E. coli* collected from constructed lagoon wastewater samples**

Strain	Primer	Nucleotide Sequence (5'-3')	Product size (bp)	Multiplex Group	Reference
<i>Enterococcus</i> spp.	E1 E2	TCAACCGGGGAGGGT ATTACTAGCGATTCCGG	733	1 & 2	Deasy <i>et al.</i> (2000)
<i>E. casseliflavus</i>	CA1 CA2	TCCTGAATTAGGTGAAAAAAC GCTAGTTTACCGTCTTTAACG	288	2	Jackson <i>et al.</i> (2004)
<i>E. durans</i>	DU1 DU2	CCTACTGATATTAAGACAGCG TAATCCTAAGATAGGTGTTTG	295	1	Jackson <i>et al.</i> (2004)
<i>E. faecalis</i>	FL1 FL2	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGTTTGG	360	1	Jackson <i>et al.</i> (2004)
<i>E. faecium</i>	FM1 FM2	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATCTTCTTTA	215	1	Jackson <i>et al.</i> (2004)
<i>E. gallinarum</i>	GA1 GA2	TTACTTGCTGATTTTGATTCTG TGAATTCTTCTTTGAAATCAG	173	2	Jackson <i>et al.</i> (2004)
<i>Escherichia coli</i>	Eco-F Eco-R	GTCCAAAGCGGCGATTG CAGGCCAGAAGTCTTTTCCA	<100		Lee <i>et al.</i> (2006)

### 3.3.4 Detecting genes associated with antibiotic resistance by PCR

PCR was used to detect the following antimicrobial resistance genes (*tetA*, *strA*, *strB*, *sul1*, and *sul2*), as previously described (Faldynova *et al.*, 2003; Levings *et al.*, 2005). These five antimicrobial resistance genes confer resistance to three categories of antimicrobials, including tetracyclines, sulfonamides, and aminoglycosides. Table 3.2 outlines the sequences and predicted sizes of the amplified products.



**Table 3.2. PCR primers used for the detection of ARGs conferring resistance to streptomycin, sulfonamides, and tetracycline within poultry lagoon samples**

Primer	Nucleotide Sequence (5'–3')	Product (bp)	Accession no.	Reference
strA-F strA-R	CTTGGTGATAACGGCAATTC CCAATCGCAGATAGAAGGC	548	M95402	Levings <i>et al.</i> (2005)
strB-F strB-R	ATCGTCAAGGGATTGAAACC GGATCGTAGAACATATTGGC	509	M95402	Levings <i>et al.</i> (2005)
sul1-F sul1-R	ATGGTGACGGTGTTCGGCATTCTG GCTAGGCATGATCTAACCCCTCGG	841	NC011083.1	Faldynova <i>et al.</i> (2003)
sul2-F sul2-R	AGGGGGCAGATGTGATCGAC GCAGATGATTCGCCAATTG	249	AY524415.2	Faldynova <i>et al.</i> (2003)
tetA-F tetA-R	GCTACATCCTGCTTGCCCTTC CATAGATCGCCGTGAAGAGG	210	X61367	Faldynova <i>et al.</i> (2003)

### 3.3.5 Antimicrobial susceptibility testing

Antimicrobial resistance patterns were determined using the disc-diffusion method as set by the Clinical and Laboratory Standards Institute (CLSI, 2007). *Enterococcus* spp. and *E. coli* isolates were inoculated into Brain Heart Infusion (BHI) broth (Difco, Fisher Scientific) and grown to a 0.5 McFarland turbidity standard (4-6 hrs) and swabbed onto Mueller Hinton (Difco, Fisher Scientific) agar plates. The following antimicrobial agents were selected as important representatives of different antibiotic classes, with abbreviations and disc concentrations shown in brackets: streptomycin (S 10 µg), cefotaxime (CTX 30 µg), vancomycin (VA 30 µg), ciprofloxacin (CIP 5 µg), chloramphenicol (C 30 µg), ceftriaxone (CRO 30 µg), amoxicillin (AMC 30 µg), ceftazidime (CAZ 30 µg), doxycycline (D 30 µg), ampicillin (AM 10 µg), linezolid (LZD 30 µg), sulfisoxazole (G 0.25 mg) and oxytetracycline (T 30 µg). After incubation for 16-18 hours at 35±2°C, the isolates were classified as susceptible, intermediate or resistant by comparing the diameters of inhibition zones with the breakpoints according to CLSI recommendations (CLSI, 2007). Organisms considered intermediate by the Kirby–Bauer disc-diffusion method were recorded as resistant for the purpose of this study.

### 3.3.6 Selection of PCR primers and TaqMan® probes

All primers and probes (Table 3.3) were synthesized by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Six sets of real-time PCR primers and probes were previously designed (Ng *et al.*, 2001; Volkmann *et al.*, 2004; Lee *et al.*, 2006; Böckelmann *et al.*, 2009) to detect pathogens and/or ARGs in wastewater samples. TaqMan probes and primer sets were designed to detect the following organisms and ARGs: *E. coli*, *E. faecalis*, *Salmonella* spp., *ampC*, and *blaSHV* (Table 3.3), while *tetA* was quantified using the SYBR Green approach.

**Table 3.3. Primers and probes used for the detection of pathogens and ARGs among constructed lagoon wastewater samples using real-time qPCR**

Primer or Probe	Target gene	Sequence (5'–3')	Reference	GenBank accession no.
ampCF <sup>a</sup> ampCR <sup>b</sup> ampCP <sup>c</sup>	<i>ampC</i> <i>ampC</i> <i>ampC</i>	GGGAATGCTGGATGCACAA CATGACCCAGTTCGCCATATC TEXASRED- CCTATGGCGTGAAAACCAACGTGCA–BHQ-1 <sup>d</sup>	Volkmann <i>et al.</i> (2004)	AJ005633
SHVF SHVR SHVP	<i>blaSHV-5</i> <i>blaSHV-5</i> <i>blaSHV-5</i>	AACAGCTGGAGCGAAAAGATCCA TGTTTTTCGCTGACCGGCGAG FAM–TCCACCAGATCCTGCTGGCGATAG–BHQ-1	Böckelman <i>n et al.</i> (2009)	X55640
tetAF tetAR SYBR <sup>e</sup>	<i>tetA</i> <i>tetA</i>	GCTACATCCTGCTTGCCCTTC CATAGATCGCCGTGAAGAGG	Ng <i>et al.</i> (2001)	X61367
Efaecal-F ( <i>Enterococcus faecalis</i> ) Efaecal-R Efaecal-PR	<i>groES</i> (Heat-shock protein) <i>groES</i> <i>groES</i>	TGTGGCAACAGGGATCAAGA TTCAGCGATTTGACGGATTG FAM–TCGTTTCGTGCATTAGA–BHQ-1	Lee <i>et al.</i> (2006)	AF335185
Eco-F ( <i>Escherichia coli</i> ) Eco-R Eco-PR	<i>uidA</i> (Glucuronidase) <i>uidA</i> <i>uidA</i>	GTCCAAAGCGGCGATTTG CAGGCCAGAAGTTCTTTTTCCTCA HEX–ACGGCAGAGAAGGTA–BHQ-1	Lee <i>et al.</i> (2006)	S69414
Sal-F <i>Salmonella</i> spp. Sal-R Sal-probe	<i>invA</i> (Invasion protein) <i>invA</i> <i>invA</i>	CGTTTCCTGCGGTACTGTAAATT AGACGGCTGGTACTGATCGATAA HEX–CCACGCTCTTCGTCT–BHQ-1	Lee <i>et al.</i> (2006)	U43272

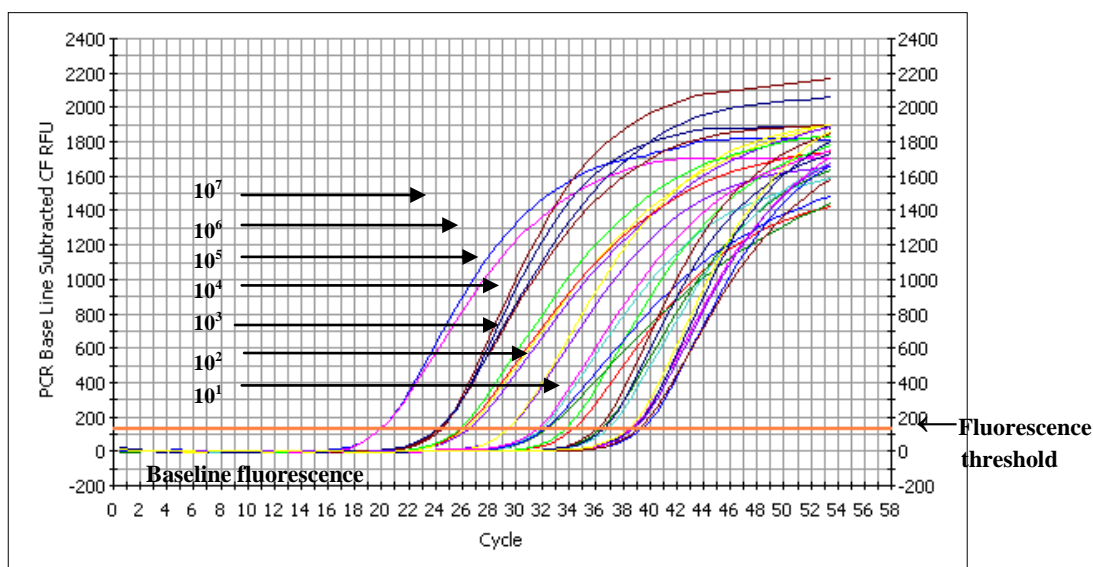
<sup>a</sup> Forward primer, <sup>b</sup> Reverse primer, <sup>c</sup> TaqMan probe, <sup>d</sup> BHQ-1, black hole quencher, <sup>e</sup> SYBR Green

### 3.3.7 DNA extraction

DNA was extracted from bacterial biomass collected from 500 mL of each sample and concentrated by centrifugation at  $11,000 \times g$  for 20 min at 4 °C. Genomic DNA was extracted from wastewater samples and bacterial cultures (control strains) using the Mo Bio PowerMax DNA Isolation Kit for soil (Mo Bio Laboratories Inc., Carlsbad, CA), following the manufacturer's protocol. The quantity and purity of the DNA extract was determined spectrophotometrically using a Nanophotometer (Montreal Biotech Inc., Montreal, Canada) at an absorbance of 260 nm, and  $A_{260}/A_{280}$ , respectively. The extracted DNA was stored in the freezer at -20°C for long-term storage with duplicate DNA samples stored at 4°C for short-term storage.

### 3.3.8 Amplification of standards for real-time qPCR assays

The abundance of six genes, three ARGs (*ampC*, *blaSHV*, *tetA*) and three bacterial genes (*E. coli*, *E. faecalis*, *Salmonella* sp.) were quantified against their respective standard using the appropriate real-time PCR primers and probes (Table 3.3) and assay conditions optimized as previously reported (Volkmann *et al.*, 2004; Lee *et al.*, 2006; Böckelmann *et al.*, 2009). Genomic DNA was extracted from the following strains and used as positive controls for qPCR; *E. coli* (ATCC#25922), *E. faecalis* (ATCC#49532), *Salmonella* sp. (ATCC#13311), *Enterobacter cloacae* (ATCC#BAA-1143) containing *ampC*, *Klebsiella pneumoniae* (ATCC#700603) containing *blaSHV*, and *E. coli* containing *tetA* (plasmid DNA). A representative TaqMan PCR standard curve of the *E. coli uidA* gene, consisting of serial dilutions of positive controls over 7 orders of magnitude is shown in Figure 3.2.



**Figure 3.2.** An example of real-time PCR results showing the relationship between gene copy numbers/reaction (or DNA amount) and cycle threshold ( $C_t$ ). In this example, *E. coli* genomic DNA was serially diluted over 7 orders of magnitude and subjected to TaqMan real-time PCR.

The signal intensity produced by the amplification process was plotted on a linear scale. A threshold value above the baseline fluorescence was chosen for the significant increase of the fluorescence signal, as indicated in the plot. Thus, the intercepts of the fluorescence signals with the threshold line determined the threshold cycle values  $C_t$  as a quantifiable measure of the abundance of targeted DNA in the sample (Invitrogen, 2008). By measuring the amount of amplified product during each stage of the PCR cycle, quantification is possible. If a particular DNA sequence is abundant in the sample, amplification is observed in earlier cycles, whereas, if the sequence is limited, amplification is observed in later cycles (Invitrogen, 2008). The serially diluted suspensions of the reference strains corresponded with the continuously increasing  $C_t$  values, which is essential for quantification of targets. The developed primer–probe systems were tested by duplicate amplification runs of the

serially diluted reference strains. Calibration curves were prepared for each gene with serial dilutions of positive controls over 7 orders of magnitude.

### 3.3.9 Quantitative Polymerase Chain Reaction

Real-time Polymerase Chain reaction for the quantification of *ampC*, *blaSHV-5*, and *tetA* genes and *E. faecalis*, *E. coli*, and *Salmonella* sp. organisms in total DNA extracted from water samples was performed with an iCycler & iQ Real-Time PCR System (Bio-Rad Laboratories; Mississauga, ON, Canada). Each 25  $\mu$ L PCR reaction contained template genomic DNA, primers and TaqMan® probes added to DyNAmo™ Probe qPCR Mastermix (New England Biolabs, Pickering, ON, Canada) to a final concentration of 250 nM TaqMan® probes, and 1  $\mu$ M for each primer. A volume of up to 5  $\mu$ L of DNA was added to each reaction. The following three-step PCR program was used for the quantification of pathogens and ARGs: (i) a uracil-DNA glycosylase step at 50°C for 2 min, (ii) an initial denaturation and *Taq* polymerase activation step at 95°C for 10 min, and (iii) 55 cycles of 95°C for 15 s and 60°C for 1 min (Shannon *et al.*, 2007; Böckelmann *et al.*, 2009). SYBR Green was used for the quantification of *tetA*, as TaqMan probe sequences have not yet been developed for the *tetA* gene. Previous studies have shown SYBR Green to be a reliable method for the quantification of *tet* genes detected in cattle farm waste (Yu *et al.* 2005), groundwater (Mackie *et al.* 2006), river sediments (Pei *et al.* 2006), and different water environments (Zhang *et al.* 2009). Reactions for *tetA* were conducted with a final volume of 20  $\mu$ L, including 10  $\mu$ L Power SYBR Green PCR Mastermix, plus 2  $\mu$ L each primer (1 $\mu$ M), and 5-8  $\mu$ L template DNA. The following protocol was used for the quantification of *tetA*:

94°C for 3 min, followed by 45 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s (Zhang *et al.*, 2009). Tenfold dilution series of the standards for the respective genes were run along with the unknown samples. Each sample was tested in duplicate for each run and two independent runs were performed. Quantification was achieved using standard curves obtained from the amplification profiles of known concentrations of the respective standard. For each probe and primer set tested against wastewater DNA using real-time PCR, a negative control was tested, which included primers, the TaqMan® probe, PCR mastermix, and sterile water in place of DNA template.

### **3.3.10 Statistical analysis**

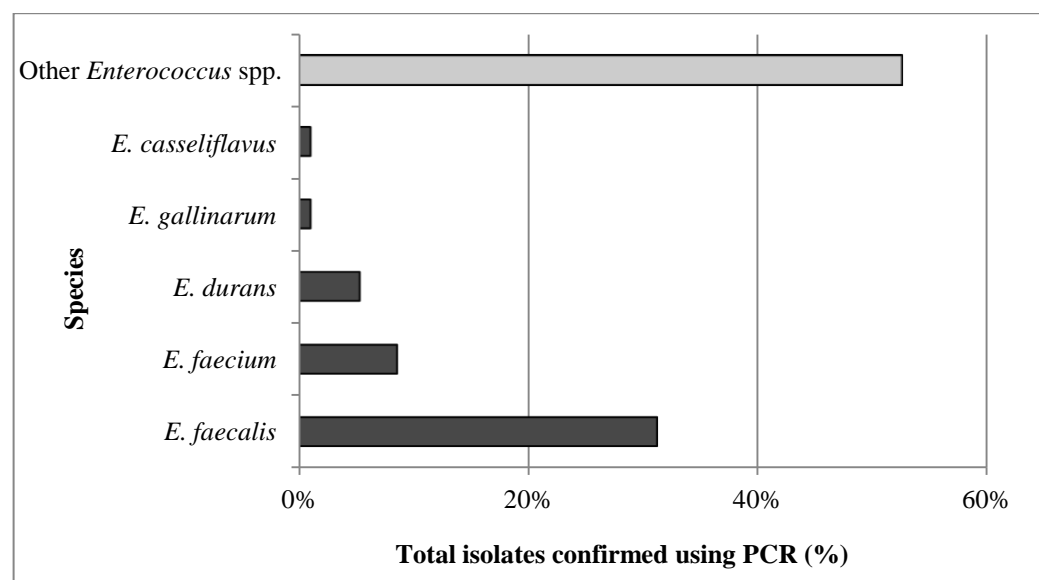
The chi-squared test and student t-test were used to conduct the statistical analysis of the results (i.e., for comparison of antibiotic resistance during different stages of treatment and for comparison of concentration means). The null hypothesis being the concentration of ARGs (or antibiotic resistance) was the same between different samples was rejected at a *p*-value less than or equal to 0.05. All statistical computations were performed using Microsoft Excel version 2007 for Windows.

## **3.4 Results and Discussion**

### **3.4.1 Qualitative PCR to detect and characterize *Enterococcus* spp. and *E. coli***

Among presumptive *E. coli* (n=100; June 2009) all isolates were identified and confirmed as *E. coli* with the use of qualitative PCR. In the case of enterococci, a total of 93 isolates (June

2009) were tested using qualitative PCR and 100% of isolates reacted with the enterococcal genus primer (16S rRNA), indicating they were members of the genus *Enterococcus* (data not shown). When coupled with genus and species-specific primers, the multiplex PCR provides an accurate and quick method for the identification of enterococci, without the need for extensive phenotypic tests. Multiplex species-specific PCR was used to differentiate between five common species of *Enterococcus* spp., with 31.2% of total isolates identified as *Enterococcus faecalis* (29/93), 8.6% as *E. faecium* (8/93), 5.4% (5/93) as *E. durans*, and 2.1% (2/93) as *E. casseliflavus/gallinarum* (Figure 3.3).



**Figure 3.3. Distribution of *Enterococcus* spp. among screened isolates (n=93) within the constructed lagoon system. Identified *Enterococcus* species are represented by the black bars, whereas the lighter grey bar represents *Enterococcus* isolates of another species.**

*Enterococcus* spp., particularly *E. faecalis* and *E. faecium*, have presented serious challenges clinically, as they are the third leading cause of nosocomial infections in intensive care units in the United States and are becoming increasingly resistant to treatment with antimicrobials (CDC, 2001). Hayes *et al.* (2004) examined multiple antibiotic resistance of *Enterococcus*

spp. isolated from commercial poultry production environments and found similar results when characterizing *Enterococcus* spp. isolates. A total of 541 *Enterococcus* spp. isolates were recovered and identified to the species level, with *E. faecalis* being the most predominant species (53.2%) followed by *E. faecium* (31.4%), *E. gallinarum* (6.0%), *E. durans* (1.5%) and *E. casseliflavus* (1.2%) (Hayes *et al.*, 2004). The results presented here are in accordance with those of Hayes *et al.* (2004), finding the two most prevalent *Enterococcus* spp. to be *E. faecalis* and *E. faecium*. The identification of enterococci isolated from the constructed lagoon system receiving poultry wastewater revealed that approximately 53% (49/93) of tested isolates require more discriminate analysis, reacting only with the enterococcal genus primer. *Enterococcus* species are part of the natural intestinal microflora of mammals and birds (Domig *et al.*, 2003) and therefore are commonly isolated from wastewater treatment plants of broiler slaughterhouses (including conventional systems and constructed lagoons) which receive faecal contents from a relatively large population. Enterococci from these environments are frequently exposed to measurable concentrations of antibiotics that could potentially encourage the selection of antibiotic resistance during the course of wastewater treatment processes (Kümmerer *et al.*, 2003). Finding *E. faecalis* predominance in this study was similar to that previously reported for poultry production environments in other parts of the world, including the United States (Molitoris *et al.*, 1986; Welton *et al.*, 1998), Belgium (Butaye *et al.*, 2001), the United Kingdom (Kaukas *et al.*, 1986), and Denmark (Aarestrup *et al.*, 2000). Studies from Japan (Yoshimura *et al.*, 2000) in contrast, suggested that *E. faecium* was the dominant enterococcal species of poultry faecal flora. The distinction observed with regards to species prevalence may reflect differences in isolation methodology (Butaye *et al.*, 1999), variation



in geographic location, or the effect of different medicated feed on the intestinal enterococcal microflora (Bager *et al.*, 1997; Molitoris *et al.*, 1986).

### **3.4.2 Prevalence of antibiotic resistance among *E. coli* and *Enterococcus* spp.**

In the current study, antimicrobial susceptibility results were obtained for 175 *Enterococcus* spp. isolates and 140 *E. coli* isolates from various stages within the constructed lagoon system. Resistance profiles per sampling location and overall resistance levels are shown in Tables 3.4 and 3.5 for *E. coli* and *Enterococcus* spp. isolates, respectively. Among *E. coli*, the most commonly observed resistance for both sampling events were to streptomycin, sulfisoxazole, oxytetracycline/doxycycline, ampicillin, and amoxicillin (Table 3.4). The finding that resistances to penicillins, tetracyclines and sulfonamides are more prevalent in *E. coli* than resistances to quinolones and third generation cephalosporins (CAZ, CTX, and CRO) is consistent with previous reports (Reinthalder *et al.*, 2003; Watkinson *et al.*, 2007b; Hu *et al.*, 2008; Servais and Passerat 2009). This is in good agreement with the extent to which these antimicrobial classes have been prescribed to date. Penicillins, tetracyclines and sulfonamides have been extensively used for decades, while the introduction of quinolones (and more particularly fluoroquinolones) and third generation cephalosporins into clinical use is more recent (Davies, 2007).

**Table 3.4. Percentage of resistance per sampling location and overall resistance in *E. coli* isolates collected from a poultry slaughterhouse treatment lagoon system**

Date: June '09 (Summer)	Pre-treatment	Lagoon#1	Flow through 1-2	Lagoon#2	Flow through 2-3	Lagoon#3	Total Resistance
# of isolates tested	N=20	N=20	N=20	N=20	N=20	ND <sup>a</sup>	N=100
Antibiotic	%	%	%	%	%	%	%
CTX	45.0	60.0	40.0	35.0	20.0	- <sup>b</sup>	40.0
CIP	20.0	40.0	10.0	15.0	20.0	-	21.0
C	25.0	45.0	15.0	15.0	40.0	-	28.0
AM	60.0	90.0	80.0	80.0	90.0	-	80.0
G	100	100	100	95.0	100	-	99.0
CRO	35.0	55.0	35.0	30.0	20.0	-	35.0
AMC	50.0	80.0	80.0	65.0	70.0	-	69.0
S	100	100	100	100	100	-	100
T	95.0	100	90.0	95.0	95.0	-	95.0
CAZ	45.0	45.0	30.0	20.0	15.0	-	31.0
<b>MAR<sup>c</sup></b>	<b>95</b>	<b>100</b>	<b>95</b>	<b>100</b>	<b>100</b>	<b>-</b>	<b>98.0</b>
Date: Sept '09 (Fall)	Pre-treatment	Lagoon#1	Flow through 1-2	Lagoon#2	Flow through 2-3	Lagoon#3	Total Resistance
# of isolates tested		N=10	N=10	N=10	N=10	ND	N=40
Antibiotic	%	%	%	%	%	%	%
CTX	-	40.0	60.0	40.0	30.0	-	42.5
CIP	-	0.0	0.0	0.0	0.0	-	0.0
C	-	0.0	0.0	0.0	20.0	-	5.0
AM	-	70.0	70.0	60.0	60.0	-	65.0
G	-	80.0	90.0	40.0	80.0	-	72.5
CRO	-	40.0	60.0	40.0	40.0	-	45.0
AMC	-	50.0	60.0	0.0	-	-	36.7
S	-	100	100	100.0	-	-	100
T	-	-	-	-	-	-	-
CAZ	-	40.0	60.0	40.0	30.0	-	42.5
D	-	50.0	50.0	70.0	70.0	-	60.0
<b>MAR</b>		<b>90.0</b>	<b>90.0</b>	<b>70.0</b>	<b>70.0</b>	<b>-</b>	<b>80.0</b>

CTX, cefotaxime; VA, vancomycin; CIP, ciprofloxacin; C, chloramphenicol; AM, ampicillin; G, sulfisoxazole; CRO, ceftriaxone; LZD, linezolid; AMC, amoxicillin; S, streptomycin; T, oxytetracycline; CAZ, ceftazidime; D, doxycycline.

<sup>a</sup>ND, Not detected.

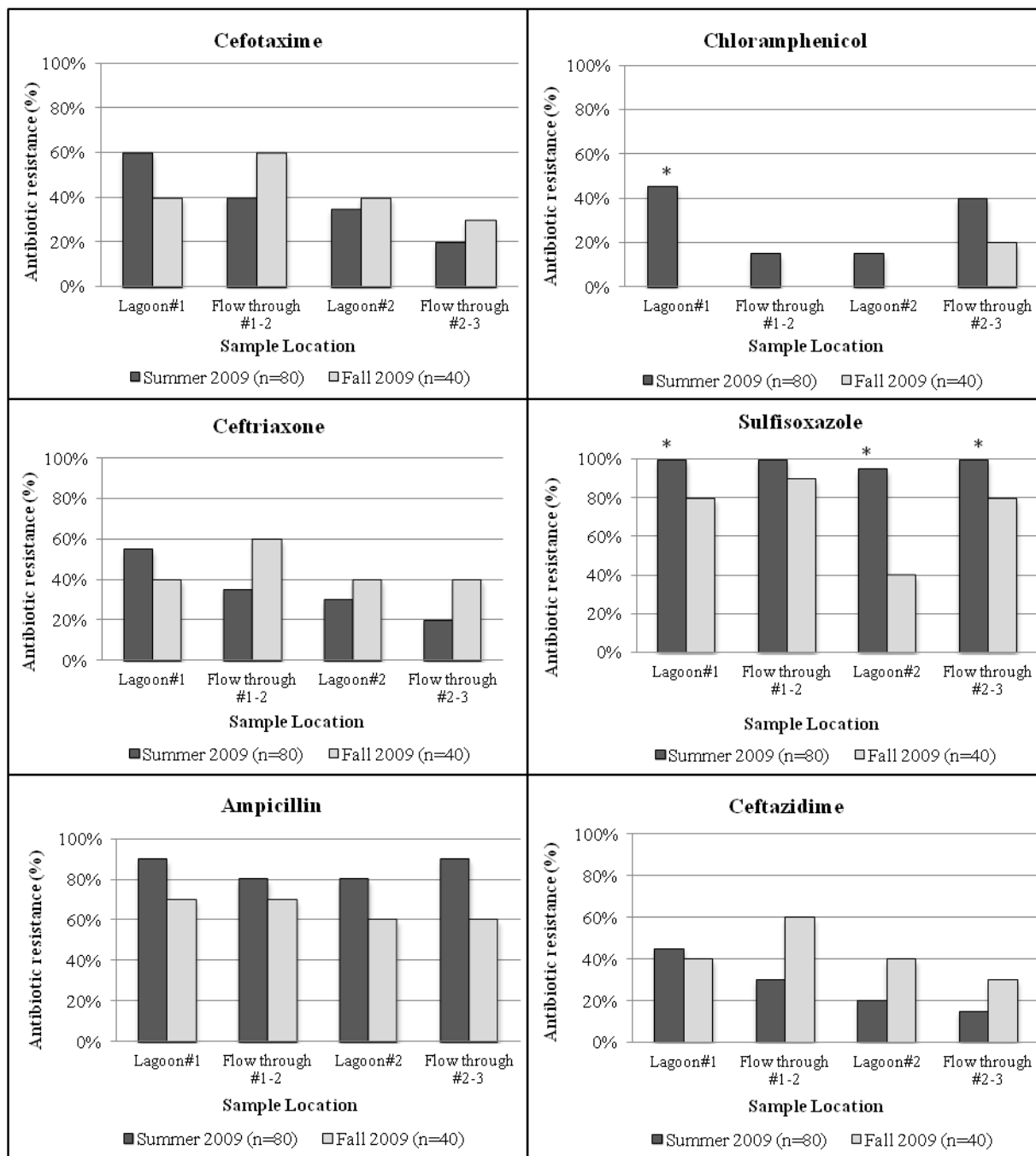
<sup>b</sup>-, Not analysed.

<sup>c</sup>MAR, isolates displaying MAR patterns to  $\geq 3$  antimicrobials

Chi-square tests revealed that significant differences ( $p < 0.05$ ) existed between time of sampling event (summer vs. fall) for chloramphenicol and sulfisoxazole resistance (Figure

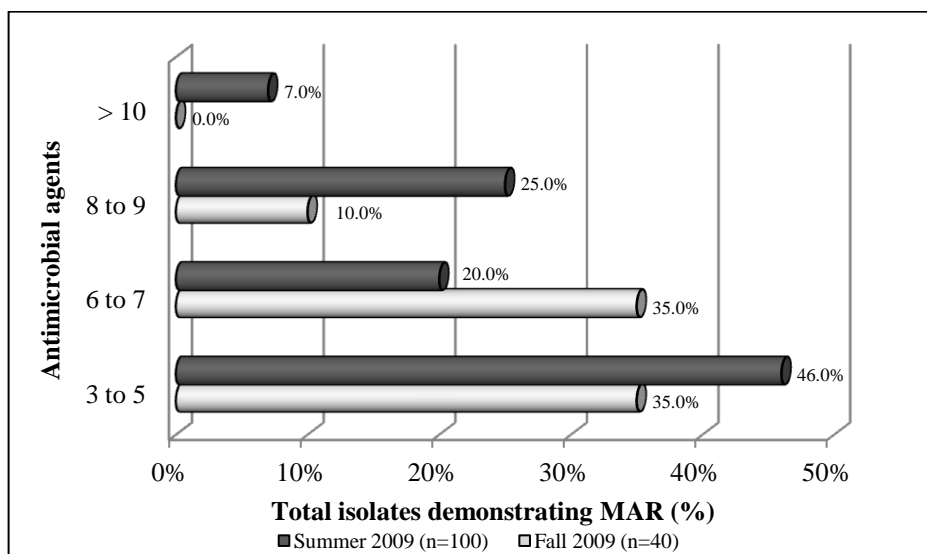
3.4). Chloramphenicol resistance in lagoon #1 significantly decreased among *E. coli* isolates, diminishing to 0% in the fall sampling event, compared to 45% observed during the summer sampling event. Similarly, sulfisoxazole resistance among *E. coli* isolates decreased significantly within lagoon #1, lagoon #2, and flow through between lagoons # 2 and #3 samples, with 100%, 95%, and 100% resistance in the summer, declining to 80%, 40%, and 80%, collected during the fall sampling event, respectively (Figure 3.4). In addition, a decrease in antibiotic resistance among *E. coli* was observed with other antimicrobial agents, although significant differences were not observed between individual sampling locations (i.e. lagoon #1), total ciprofloxacin resistance decreased from 21% to 0%, total ampicillin resistance declined from 80% to 65%, and total amoxicillin resistance decreased from 69% to 36.7%, when comparing summer and fall samples (Table 3.4). A possible explanation for the decrease in resistance observed among *E. coli* isolates during the fall season could be reflective of reduced metabolic activity, in which bacterial growth is reduced at cooler temperatures and therefore, the presence of antibiotic resistance may also be diminished. Alternatively, overall resistance levels among *E. coli* to third generation cephalosporins (CTX, CRO, CAZ) were found to increase from summer to fall samples; 40% to 42.5%, 35% to 45%, and 31% to 42.5% for cefotaxime, ceftriaxone, and ceftazidime, respectively (Table 3.4). The reason for an increase among third generation cephalosporin resistance in *E. coli* during the fall season is unknown, and further study would be essential to verify any seasonal trends/patterns. Comparison of antibiotic resistance between lagoon #1 and the flow through between lagoons #2 and #3 revealed significant differences ( $p < 0.05$ ) for cefotaxime, ceftriaxone, and ceftazidime (third generation cephalosporins) in the summer 2009 sampling

event. No significant differences were observed among resistance rate and sample origin (lagoon#1 to flow through between lagoons #2 and #3) with the fall 2009 sampling event.



**Figure 3.4. Prevalence of antibiotic resistance (%) among *E. coli* isolates collected during two sampling events (summer and fall) in a constructed lagoon system. An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n = number of isolates.**

Resistance was observed to all ten antimicrobial agents, and 92.9% of total *E. coli* isolates (n=140) expressed the phenotype of resistance to three or more antimicrobial agents (MAR) and MAR isolates were detected in all analysed sampling points (Table 3.4). Multiple antibiotic resistance profiles (%) among *E. coli* isolates for each sampling event are shown in Figure 3.5. Although differences were observed between the summer and fall *E. coli* isolates to the number of antimicrobial agents, no significant differences were detected. The most common resistance profile among *E. coli* isolates for both summer and fall samples were 46% and 35%, respectively, displaying simultaneous resistances to between three and five antibiotics (Figure 3.5).



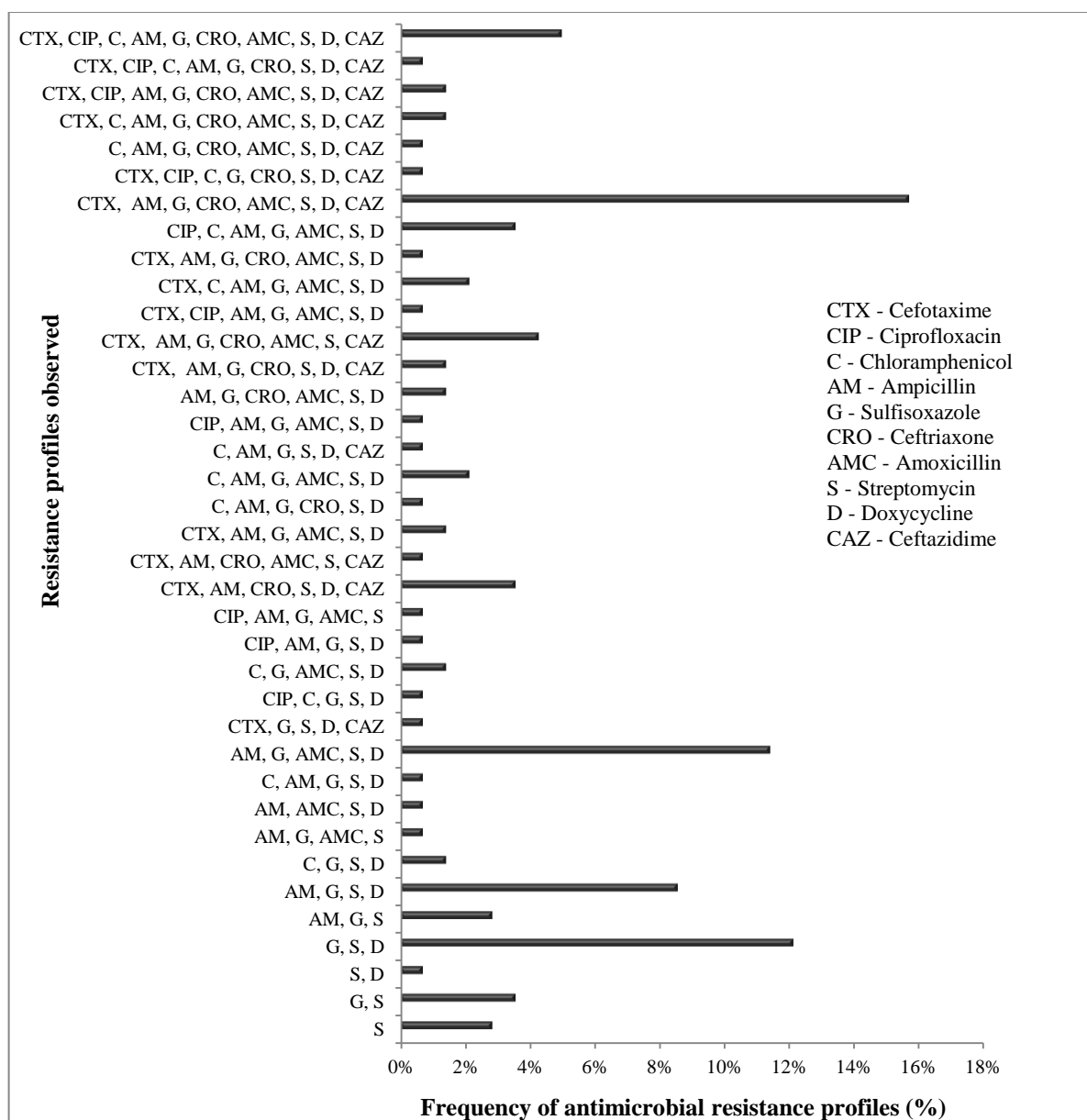
**Figure 3.5. Prevalence of multiple antibiotic resistance (MAR) profiles (%) among *E. coli* isolates collected during two sampling events (summer and fall) in a constructed lagoon system. n = number of isolates.**

Overall, 25% of summer *E. coli* isolates were resistant to eight or nine antimicrobials, followed by 20% resistant to between six and seven antibiotics, and only 7% of total isolates

(7/100) displayed resistance to ten or more antimicrobials tested. The second most common resistance profile among fall *E. coli* isolates was between six and seven antibiotics with 35% (n=14), followed by 10% (n=4) displaying simultaneous resistance to eight or nine antimicrobials. None of the isolates tested during the fall sampling displayed resistance to ten or more of the tested antibiotics (Figure 3.5). Garcia *et al.* (2007) reported ampicillin resistance was common among multiple drug resistant (MDR) strains of *E. coli*, present in 77% of the MDR strains from a wastewater treatment facility. These results are in agreement with Garcia *et al.* (2007), in that ampicillin resistance among *E. coli* MDR isolates was prevalent, reaching 76% (106/140). Genes for  $\beta$ -lactamases can reside in the chromosome (Davies, 1994) or in plasmids (Goni-Urriza *et al.*, 2000). Horizontal gene transfer can lead to a variety of resistance patterns, suggesting that the multi-drug resistance observed may have been due to more than one plasmid containing genes for ampicillin resistance. Servais and Passerat (2009) evaluated the presence of antimicrobial resistance and multiple antimicrobial resistance (MAR) of faecal bacteria in waters of the Seine river watershed in France (highly anthropogenically impacted, due to the high population density, intense industrial activities and intensive agriculture). In their study they found 42% of *E. coli* isolates from the watershed were antimicrobial resistant, and 35% were MAR (Servais and Passerat, 2009). The MAR results presented here are considerably higher than those reported by Servais and Passerat (2009) reaching nearly 93% among *E. coli* isolates expressing the phenotype of resistance to three or more antimicrobial agents.

Overall, 37 different resistance profiles were observed among *E. coli* isolates collected from various locations within the constructed lagoon system. In total, 92.9% (130/140) of resistant

isolates demonstrated multiple-drug resistance (MDR) to three or more antimicrobial agents. The most commonly observed resistance profile was octa-drug resistance to cefotaxime, ampicillin, sulfisoxazole, ceftriaxone, amoxicillin, streptomycin, doxycycline, and ceftazidime (CtxAmGCroAmcSDCaz), representing 16% (22/140) of the resistant isolates observed (Figure 3.6). The second most predominate profile was resistance to sulfisoxazole, streptomycin, and doxycycline (GSD) followed by resistance to ampicillin, sulfisoxazole, amoxicillin, streptomycin, and doxycycline (AmGAmcSD) and to ampicillin, sulfisoxazole, streptomycin, and doxycycline (AmGSD). These four different profiles accounted for almost half (48%) of the total drug resistant *E. coli* isolates. Resistance to only one or two antimicrobial agents was observed in 7.1% (10/140) of the isolates in response to sulfisoxazole, streptomycin, and/or doxycycline. In total, 62/140 isolates displayed resistance to one or more of the third-generation cephalosporins (CTX, CRO, and/or CAZ) with 79% (49/62) demonstrating simultaneous resistance to two or three cephalosporins (Figure 3.6). In addition, streptomycin (aminoglycoside class) resistance and doxycycline (tetracycline class) resistance were very prominent among MDR *E. coli* profiles, at 100% (140/140) and 84.3% (118/140), respectively. The high levels of tetracycline and cephalosporin resistance in *E. coli* agrees with findings from previous studies on the antimicrobial resistance among *E. coli* from a variety of different sources throughout the world (Erskine *et al.*, 2002; Klein and Bulte, 2003; Schroeder *et al.*, 2002; Sayah *et al.*, 2005).



**Figure 3.6. Frequency (%) of antimicrobial resistance profiles among *E. coli* isolates (n=140) collected during two sampling events (summer and fall) in a constructed lagoon system; cefotaxime (CTX), ciprofloxacin (CIP), chloramphenicol (C), ampicillin (AM), sulfisoxazole (G), ceftriaxone (CRO), amoxicillin (AMC), streptomycin (S), doxycycline (D), ceftazidime (CAZ).**

The patterns of resistance to the antimicrobial agents may be due to widespread and lengthy use of tetracycline, streptomycin, and cephalosporins (Piddock, 1996; van den Bogaard *et*



*al.*, 2001). Since tetracycline, streptomycin and cephalosporins are naturally derived compounds, bacteria can be exposed to these agents in nature and outside any human use for disease treatment, for prophylaxis or for livestock growth promotion (Sayah *et al.*, 2005). Tetracycline is a commonly used first line antibiotic for many domestic animals and is often used before the antibiotic resistance profile of a pathogen has been determined. Resistance to tetracycline is plasmid mediated, with a wide variety of genetic determinants, while resistance to many of the cephalosporins is often the result of stable mutations (Prescott *et al.*, 2000). Plasmid-mediated acquired resistance to third-generation cephalosporins has also been reported (Payne and Aymes, 1991). The large number of genetic determinants for tetracycline resistance makes it more possible for a susceptible bacterium to acquire resistance factors than if only a few determinants were available. The stable mutations which confer resistance to cephalosporins are easily retained by bacteria, even in the absence of selective pressure to maintain resistance (Sayah *et al.*, 2005). Chandran *et al.* (2008) studied the prevalence of multiple drug resistant *E. coli* serotypes, revealing more than 95% of *E. coli* were MAR, very similar to the prevalence found in this study (93%). A much lower prevalence of resistance, ranging from 31 to 75%, has been previously reported for *E. coli* isolates from various aquatic environments (Gomathinayagam *et al.*, 1994; Park *et al.*, 2003). It has been well documented that plasmid exchange readily occurs between *E. coli* and other coliform bacteria in stagnant areas of wastewater systems (Grabow *et al.*, 1976). Recent studies have also shown that antibiotics can accumulate in the environment, and even persist for up to a year (Zuccato *et al.*, 2000), suggesting a possible explanation for the increase among MAR bacteria. It has also been suggested that MAR microorganisms are fit or more robust than their nonresistant counterparts, and are therefore able to withstand or

survive under harsh conditions (McKeon *et al.*, 1995). The general rise in frequency of drug resistant isolates supports the view that widespread use of antibiotics results in the selection of resistant strains carrying plasmid encoding resistance.

In the case of enterococci, percentage of resistance and overall resistance (%) per sampling location are depicted in Table 3.5. The most commonly observed resistance profiles for both sampling events were to streptomycin, sulfisoxazole, third generation cephalosporins (CAZ, CRO, and CTX) and ciprofloxacin (Table 3.5), reaching 100% in most cases. Although enterococci are commensal organisms in the guts of most animals, their intrinsic resistance to many antimicrobial agents and their ability to efficiently acquire antibiotic resistance determinants makes them a major concern, as their role in nosocomial infections has increased due to their ability to acquire high-level resistance to antibiotics, making them difficult to treat (Linden and Miller, 1999). Widespread use of vancomycin and extended-spectrum cephalosporins in U.S. hospitals has likely contributed to the emergence and dramatic increase of vancomycin-resistant enterococci (VRE) over the past 20 years (Edmond *et al.*, 1995; KIRST *et al.*, 1998). Vancomycin-resistant *Enterococcus* spp. (VRE) that are resistant to high levels of vancomycin have been readily isolated from the feces of domestic animals in Europe (Aarestrup *et al.*, 1998; Devriese *et al.*, 1996) and from humans with no prior exposure to hospitals (Gambartotto *et al.*, 2000; Stobberingh *et al.*, 1999). To my knowledge, this is the first report of vancomycin and linezolid resistant *Enterococcus* spp. isolated from poultry wastewater within Ontario, Canada. A significant difference was observed ( $p < 0.05$ ) among vancomycin resistance between the summer and fall sampling events. In total, 60% ( $n=48$ ) of *Enterococcus* spp. isolates collected during the summer

sampling event displayed resistance to vancomycin, whereas 75.8% (n=72) of *Enterococcus* spp. isolates collected during the fall demonstrated vancomycin resistance. A possible explanation accounting for the increased vancomycin resistance among *Enterococcus* spp. isolates collected during the fall sampling event could be due to exposure of additional environmental stressors (i.e. decreased temperature) during the fall season, possibly contributing to an increase in antibiotic resistance. Resistance to linezolid (LZD) was observed among 127/175 (72.6%) *Enterococcus* spp. isolates. VRE that are intrinsically resistant to low levels of vancomycin, such as *E. gallinarum*, *E. casseliflavus*, and *E. flavescens*, have been isolated from bird feces (Sellin *et al.*, 2000), and *E. gallinarum* containing the *vanC* gene has been isolated from chickens and farm lagoons (Coque *et al.*, 1996) within the United States. Sahlström *et al.* (2009) studied vancomycin resistant enterococci in Swedish sewage sludge and found that *E. faecium*, among other species of enterococci isolated from sewage sludge were found to have high MICs for vancomycin. One isolate of *E. durans* carried a high level vancomycin resistance (MIC >128 µg mL<sup>-1</sup>) and harboured the *vanA* gene (Sahlström *et al.*, 2009). Similarly, Helt *et al.* (2012) found high levels of resistance to both vancomycin and linezolid (reaching 100%) among *Enterococcus* spp., in lab-scale constructed mesocosms seeded with activated sludge from a wastewater treatment plant. Overall, the high levels of vancomycin and linezolid resistance observed among *Enterococcus* spp. isolates in this study may be attributed to the dissemination potential of antimicrobial resistance among enterococci. *Enterococcus* spp. are unique in their ability to disseminate antibiotic resistance through a variety of routes, since the *vanA* gene cluster is located on a mobile genetic element, Tn1546, horizontal transmission of vancomycin resistance between different enterococcal strains occurs

frequently (Donabedian *et al.*, 2010). Many studies have established the transmission of Tn1546 between animals and humans (Aarestrup *et al.*, 1996; Jensen, 1998; Willems *et al.*, 1999; Woodford *et al.*, 1998). Insertion sequences (IS) are commonly found in bacterial genomes and may also facilitate transfer of resistance genes. IS1216V is commonly found in enterococci from both human and animal sources and can sometimes be present in multiple copies (Hammerum *et al.*, 2000). Silva *et al.* (2006) reported that a high percentage (89%) of multi-antimicrobial resistant coliforms isolated from a sewage treatment plant could partially or completely transfer their resistance patterns to a recipient strain. Another possible explanation for the high level of resistance observed among both faecal indicators (*E. coli* and *Enterococcus* spp.) could be simply due to the type of sample being analysed. Sewage constitutes a favourable environment, consisting of variable mixtures of bacteria, nutrients, and antimicrobial agents (Hirsch *et al.*, 1999) that might promote growth of resistant bacteria or the transfer of genetic material between bacteria (Iversen *et al.*, 2002).

*Enterococcus* spp. displayed fairly low levels of resistance to ampicillin (AM) and amoxicillin (AMC) (Table 3.5). Similar studies assessing the level of antimicrobial resistance among wastewater enterococcal strains have also shown low levels of resistance to ampicillin and amoxicillin (Martins da Costa *et al.*, 2006; Ferreira da Silva *et al.*, 2006, Łuczkiewicz *et al.*, 2010) with results presented here confirming high susceptibility to penicillin-based antibiotics.

**Table 3.5. Percentage of resistance per sampling location and overall resistance in *Enterococcus* spp. isolates collected from a poultry slaughterhouse treatment lagoon system**

Date: June '09 (Summer)	Pre-treatment	Lagoon#1	Flow through 1-2	Lagoon#2	Flow through 2-3	Lagoon#3	Total Resistance
# of isolates tested	N= 19	N= 18	N= 9	N= 3	N= 17	N= 14	N= 80
Antibiotic	%	%	%	%	%	%	%
CTX	94.7	94.4	100	100	100	78.6	93.4
VA	78.9	61.1	22.2	33.3	58.8	64.3	60.0
CIP	84.2	94.4	77.8	100	94.1	100	91.3
C	84.2	94.4	22.2	33.3	88.2	71.4	76.3
AM	10.5	0.0	33.3	0.0	0.0	0.0	6.3
G	100	100	100	100	100	100	100
CRO	94.7	94.4	100	100	100	57.1	90.0
LZD	84.2	83.3	11.1	0.0	82.3	50.0	66.3
AMC	5.3	0.0	11.1	0.0	0.0	0.0	2.5
S	100	100	100	100	100	100	100
T	89.5	100	100	100	94.1	71.4	91.3
CAZ	100	100	100	100	100	100	100
<b>MAR<sup>c</sup></b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>
Date: Sept '09 (Fall)	Pre-treatment	Lagoon#1	Flow through 1-2	Lagoon#2	Flow through 2-3	Lagoon#3	Total Resistance
# of isolates tested	ND <sup>a</sup>	N=20	N=20	N=20	N=19	N=16	N= 95
Antibiotic	%	%	%	%	%	%	%
CTX	- <sup>b</sup>	100	100	100	100	87.5	97.9
VA	-	100	100	60.0	57.9	56.3	75.8
CIP	-	100	100	90.0	78.9	93.8	92.6
C	-	100	100	90.0	73.7	62.5	86.3
AM	-	0.0	0.0	0.0	0.0	0.0	0.0
G	-	100	100	100	100	100	100
CRO	-	100	100	100	100	87.5	97.9
LZD	-	90.0	95.0	85.0	73.7	37.5	77.9
AMC	-	0.0	0.0	0.0	0.0	0.0	0.0
S	-	100	100	100	100	100	100
T	-	-	-	-	-	-	-
CAZ	-	100	100	100	100	100	100
D	-	100	100	75.0	84.2	25.0	78.9
<b>MAR</b>	<b>-</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>

CTX, cefotaxime; VA, vancomycin; CIP, ciprofloxacin; C, chloramphenicol; AM, ampicillin; G, sulfisoxazole; CRO, ceftriaxone; LZD, linezolid; AMC, amoxicillin; S, streptomycin; T, oxytetracycline; CAZ, ceftazidime; D, doxycycline.

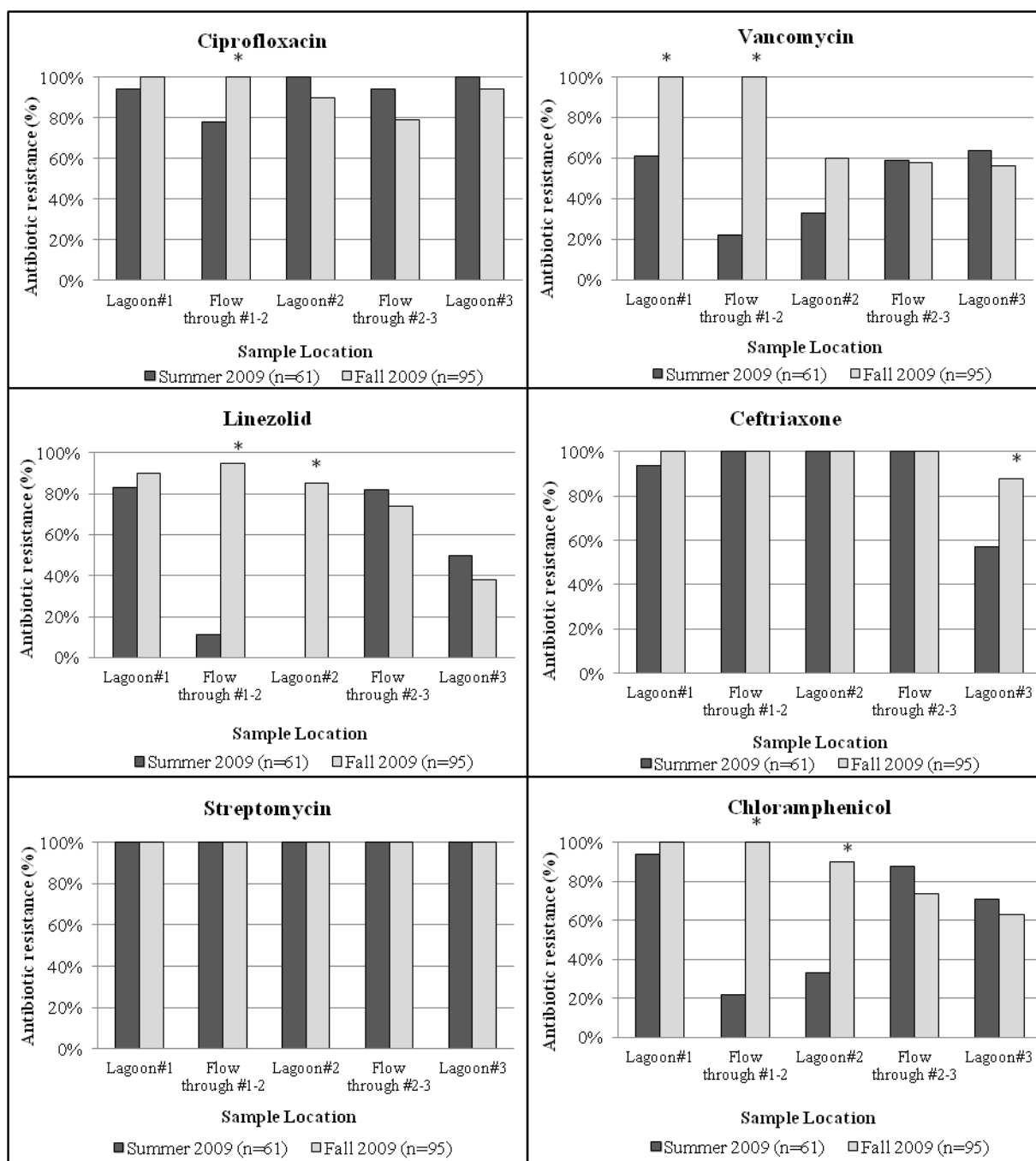
<sup>a</sup>ND, Not detected.

<sup>b</sup>-, Not analysed.

<sup>c</sup>MAR, isolates displaying MAR patterns to  $\geq 3$  antimicrobials

Chi-squared tests revealed that significant differences ( $p < 0.05$ ) existed between the time of sampling event (summer vs. fall) for ciprofloxacin, vancomycin, chloramphenicol, ceftriaxone, and linezolid resistance (Figure 3.7), with significantly increased resistance in the fall. Therefore, the results of *Enterococcus* spp. contrasted the results obtained for *E. coli*, where an increase in antibiotic resistance levels was observed among most antimicrobial agents (with the exception of cephalosporins) during the warmer, summer sampling event. An increase in antibiotic resistance levels during the fall season among *Enterococcus* spp. isolates could be due to the physiological differences between bacteria, as enterococci are generally considered more resilient, compared to *E. coli* and have the capability of surviving under extreme conditions (Mims *et al.*, 1993). Outside of a host organism, enterococci can tolerate a wide variety of growth conditions, including temperatures of 10°C to 45°C, and hypotonic, hypertonic, acidic, or alkaline environments (Huycke *et al.*, 1998), as well as resisting the bactericidal effects of detergents such as bile salts and sodium dodecyl sulfate (Flahaut *et al.*, 1996). Environmental stresses have been demonstrated to induce transient, generally mutagenic pathways (Galhardo *et al.*, 2007), and therefore, common environmental stressors, such as starvation, have been postulated to stimulate mutagenesis leading to antibiotic resistance (Martinez and Baquero, 2000). For example, Petrosino *et al.* (2009) demonstrated that starvation and stress responses can provoke *ampD*  $\beta$ -lactam resistance mutagenesis in *E. coli*. As a result, the increased antibiotic resistance observed during the fall season among *Enterococcus* spp. could be due to environmental stressors, thereby stimulating mutagenesis and promoting enhanced resistance levels. Comparison of antibiotic resistance profiles between lagoon #1 and lagoon #3 (final lagoon) for the summer sampling event revealed a significant decrease ( $p < 0.05$ )

among ceftriaxone, linezolid, and chloramphenicol resistance levels (Figure 3.7), whereas a significant decrease was observed from lagoon #1 to lagoon#3 for linezolid, vancomycin, and chloramphenicol resistance levels during the fall sampling event. Overall, *Enterococcus* spp. isolates maintained a high degree of resistance to ciprofloxacin, ceftriaxone, and streptomycin throughout the treatment process, with very little difference observed between lagoon #1 and lagoon #3 (Figure 3.7).

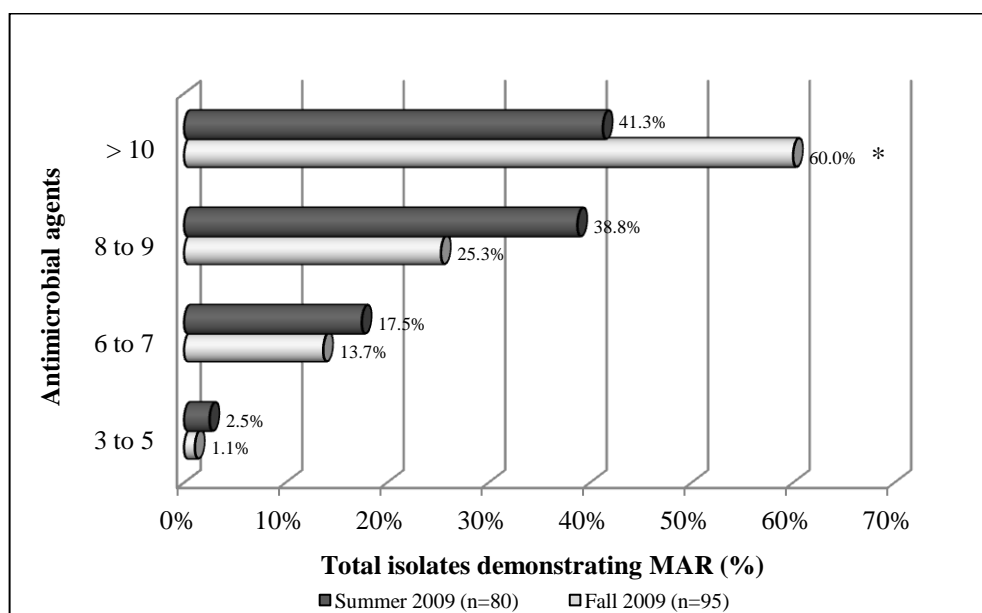


**Figure 3.7. Prevalence of antibiotic resistance (%) among *Enterococcus* spp. isolates collected during two sampling events (summer and fall) in a constructed lagoon system. An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n = number of isolates.**

Resistance was observed among all ten antimicrobial agents, with 100% (n=175) of total *Enterococcus* spp. isolates expressing resistance to three or more antimicrobial agents



(MAR) and was detected in all analysed sampling points (Table 3.5). Multiple antibiotic resistance profiles (%) among *Enterococcus* spp. isolates for each sampling event are shown in Figure 3.8. Although differences were observed between the summer and fall *Enterococcus* spp. isolates, the only significant difference ( $p < 0.05$ ) was between the number of isolates (%) demonstrating multiple antibiotic resistance to ten or more antimicrobials (Figure 3.8).

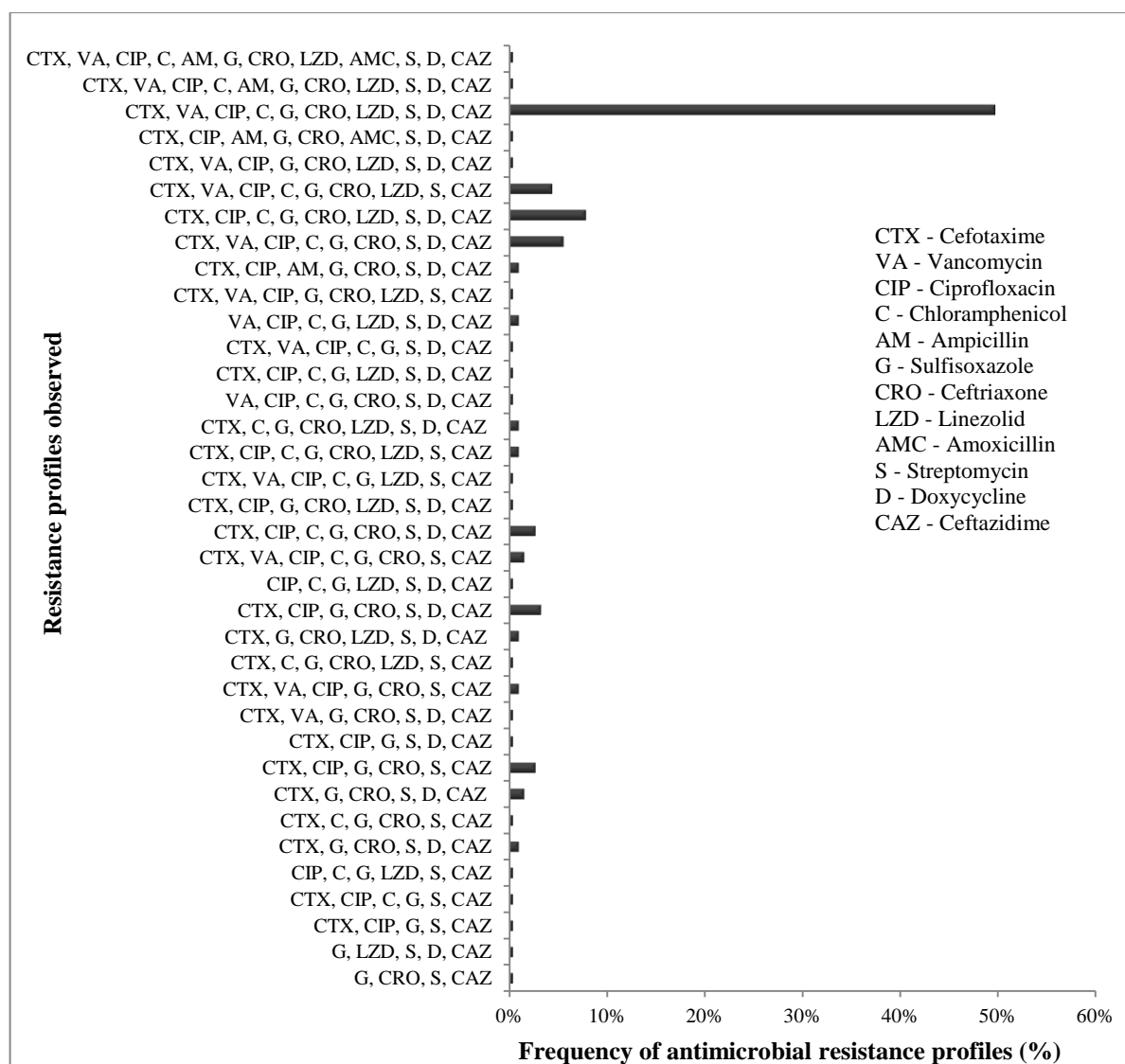


**Figure 3.8. Prevalence of multiple antibiotic resistance (MAR) profiles (%) among *Enterococcus* spp. isolates collected during two sampling events (summer and fall) in a constructed lagoon system. An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n = number of isolates.**

Approximately 41% of *Enterococcus* spp. isolates collected during the summer sampling event displayed resistance to ten or more antibiotics, while 60% of *Enterococcus* spp. isolates collected during the fall displayed simultaneous resistance to ten or more antimicrobials. The second most common resistance profile among both sampling events

(summer and fall) was between eight and nine antibiotics with 38.8% (n=31/80) and 25.3% (n=24/95), respectively, followed by 17.5% (n=14/80) and 13.7% (n=13/95) of summer and fall *Enterococcus* spp. isolates depicting simultaneous resistance to six or seven antibiotics, respectively. Overall, only 2.5% of summer and 1.1% of fall *Enterococcus* spp. isolates displayed simultaneous resistances to between three and five antimicrobial agents tested (Figure 3.8), with none of the isolates (0/175) expressing resistance to only one or two compounds.

All (175/175) resistant isolates demonstrated multiple-drug resistance (MDR) to three or more antimicrobial agents. Overall, 36 different resistance profiles were observed among *Enterococcus* spp. isolates collected from various locations within the constructed lagoon system. The most commonly observed resistance profile was to ten antimicrobials; cefotaxime, vancomycin, ciprofloxacin, chloramphenicol, sulfisoxazole, ceftriaxone, linezolid, streptomycin, doxycycline, and ceftazidime (CtxVaCipCGCroLzdSDCaz), representing nearly 50% (87/175) of the resistant isolates observed (Figure 3.9). The second most predominate profile was to cefotaxime, ciprofloxacin, chloramphenicol, sulfisoxazole, ceftriaxone, linezolid, streptomycin, doxycycline, and ceftazidime (CtxCipCGCroLzdSDCaz). These two different profiles accounted for more than half (57.7%) of the total drug resistant *Enterococcus* spp. isolates.



**Figure 3.9.** Frequency (%) of antimicrobial resistance profiles among *Enterococcus* spp. isolates (n=175) collected during two sampling events (summer and fall) in a constructed lagoon system; cefotaxime (CTX), vancomycin (VA), ciprofloxacin (CIP), chloramphenicol (C), ampicillin (AM), sulfisoxazole (G), ceftriaxone (CRO), linezolid (LZD), amoxicillin (AMC), streptomycin (S), doxycycline (D), ceftazidime (CAZ).

The results of this study illustrate that *Enterococcus* spp. from poultry processing wastewater in Canada are frequently resistant to multiple antimicrobials and that some of these patterns may very well reflect the use of approved antimicrobials in poultry. Antibiotics are approved in Canada through the Food and Drugs Act and Regulations

(Health Canada) as drugs, not feed additives; however, growth improvement doses of antibiotics are usually administered via feed or water. The following antibiotics are approved by Health Canada for growth improvement in livestock: chlortetracycline, virginiamycin, bacitracin, bambarmycins, lincomycin, salinomycin, penicillin, monensin, tylosin, and lasalocid (OMAFRA, 2005). However, antibiotics not approved in Canada (e.g. avoparcin) are used elsewhere in the world for growth promotion, and may subsequently be imported for “own use” by livestock producers. Typically, *Enterococcus* spp. are associated with both community- and hospital-acquired infections, thus presenting a therapeutic challenge because of their multi-drug resistance. One highly MAR *Enterococcus* spp. isolate was found to express resistance to all twelve antibiotics tested (CtxVaCipCAmGCroLzdAmcSDCaz), illustrating the potential amplification of antimicrobial resistance within environmental isolates. Resistance to tetracycline based antibiotics (i.e. doxycycline) among *Enterococcus* spp. is very common, especially among those of poultry origin in the United States (Molitoris *et al.*, 1986; Wiggins, 1996) and abroad (Butaye *et al.*, 2001; Yoshimura *et al.*, 2000). Tetracycline resistance has also been previously demonstrated to be linked closely to poultry production environments (van den Bogaard *et al.*, 2002), with these results conferring 84% resistance to tetracycline based antibiotics (doxycycline). Similar to the observations of enterococci of poultry origin from Denmark (Aarestrup *et al.*, 2000), resistance to aminoglycosides at high levels was observed among *Enterococcus* spp. populations of this study, with high-level streptomycin resistance predominating in each phenotype (100%). Rising levels of resistance to multiple antimicrobials dictate the urgent need for frequent and close monitoring of resistance in bacterial pathogens in both clinical and agricultural environments within Canada and abroad.

Without this measure of surveillance, the management of this problem on a gradual basis could result in a further decline of the effectiveness of antimicrobials and additionally lead to a reduction in the number of available antibiotics used to treat animal or human infections.

When comparing the difference between the prevalence of *E. coli* and *Enterococcus* spp. isolates displaying multiple antibiotic resistance to three or more antimicrobial agents, a significant difference ( $p < 0.05$ ) was observed. Among *Enterococcus* spp. isolates ( $n=175$ ), the most commonly observed phenotype was resistance to ten or more antibiotics, while *E. coli* isolates ( $n=140$ ) expressed the most common resistance pattern to between three and five antibiotics. Furthermore, only 5% (7/140) of total *E. coli* isolates demonstrated simultaneous resistance to ten or more antimicrobials, while 51% (89/175) of total *Enterococcus* spp. isolates showed multiple antibiotic resistances to ten or more of the antibiotics tested. These results are in agreement with Garcia *et al.* (2007) who studied the effect of wastewater treatment on antibiotic resistance in *E. coli* and *Enterococcus* spp. finding a much larger proportion of multiple antibiotic resistant *Enterococcus* spp. compared to that of *E. coli*. The differences between the levels of multiple antibiotic resistances as seen here could be attributed to the intrinsic hardiness of enterococci (Hayes *et al.*, 2003) that allow their long-term survival, even under adverse environmental conditions (Kühn *et al.*, 2000). Another possible explanation for the increased resistance to several antibiotics could be the acquisition of a multi-drug resistance plasmid. Multi-drug resistance is often achieved by the acquisition of a single mobile genetic cassette harboring genes coding for several different resistance mechanisms. In addition to the selective pressure exerted by antibiotic drugs themselves, other antibiotics and/or agents such as disinfectants and heavy

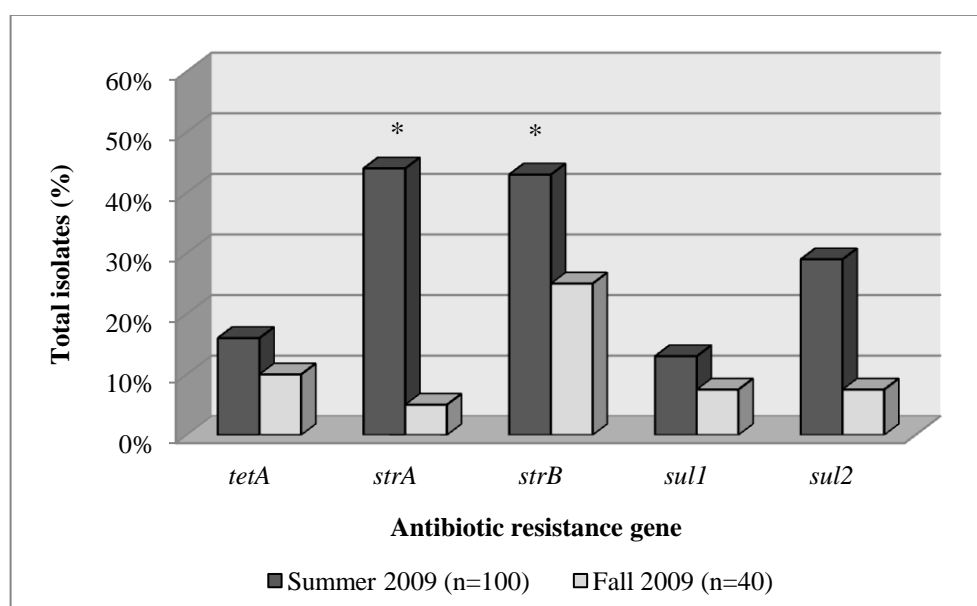
metals may also contribute to the maintenance of antibiotic resistance (Schluter *et al.*, 2007). Consequently, bacteria can retain resistance to drugs such as streptomycin and sulphonamides which are rarely used today, simply because their resistance genes are closely associated with contemporary antibiotic or heavy metal resistance mechanisms. Several multi-resistant plasmids harbouring transposons, each carrying up to 10 different ARGs, have been isolated from sewage treatment plants (Szczepanowski *et al.*, 2005). In addition, studies have shown an increased proportion of coliforms with resistance genes to different types of antibiotics during wastewater treatment in long-term retention lagoons (Bell *et al.*, 1983). The differences between *E. coli* and *Enterococcus* spp. as seen here, could also be attributed to the resistance gene transfer rates, which are affected by factors both internal and external to the bacterium. External influences include those which facilitate DNA transferability such as temperature, pH, detergents and organic solvents (Jury *et al.*, 2010); whereas, internal influences include the "SOS" response to DNA damage, which appears to increase the frequency of transfer of certain resistance traits. An SOS response regulates transcription in reply to external stresses such as UV radiation and certain antibiotics (ciprofloxacin, trimethoprim and  $\beta$ -lactams), thus causing metabolic changes and mutations facilitating survival and resistance (Cirz *et al.*, 2007).

### **3.4.3 Analysis of antimicrobial resistance genes (ARGs)**

Identification of the genes associated with antimicrobial resistance was accomplished using PCR analysis. In total, 140 *E. coli* isolates were screened for the presence of the following antimicrobial resistance genes; *tetA*, *strA*, *strB*, *sul1*, and *sul2*, representing three different

antibiotics (tetracycline, streptomycin, and sulfisoxazole), respectively. The selected ARGs were chosen because resistance to tetracyclines, sulfonamides, and streptomycin or spectinomycin in *E. coli* is generally the most prevalent among animal sources (Guerra *et al.*, 2003; Lanz *et al.*, 2003). The preferred genes were also selected based on previous antimicrobial resistance screening data, indicating high levels of resistance to tetracycline, streptomycin, and sulfisoxazole. Briefly, 20/140 *E. coli* isolates, carried the *tetA* gene; 46/140 carried the *strA* gene; 53/140 carried the *strB* gene; 16/140 carried the *sulI* gene; and 32/140 carried the *sul2* gene. Figure 3.10 illustrates the proportion of *E. coli* isolates that carried ARGs collected during two sampling events, summer and fall. When comparing the difference between the prevalence of screened isolates carrying ARGs in the summer versus the fall, a significant difference ( $p < 0.05$ ) was observed with the *strA* and *strB* genes. Approximately 44% of *E. coli* isolates collected during the summer sampling event ( $n=100$ ) possessed the *strA* gene, compared to only 5% of isolates collected during the fall ( $n=40$ ). Similarly, 43% of total *E. coli* isolates ( $n=100$ ) collected during the summer carried the *strB* gene, while 25% of fall isolates tested positive for the *strB* gene (Figure 3.10). No significant differences were observed among the other ARGs (*tetA*, *sulI*, *sul2*) when comparing the two sampling events. In the case of seasonal effects, the cool season (fall) may be reflective of reduced metabolic activity, in which microbial growth is reduced at lower temperatures, and therefore, the transfer of these genes may also be reduced. Typically warmer temperatures are associated with high microbial activity and reproduction which may also increase gene transfer. Recently, Graves *et al.* (2011) examined the distribution of ten ARGs in *E. coli* isolates from swine manure, lagoon effluent, and soil collected from a lagoon waste application field. These results are in accordance with those of Graves *et al.* (2011)

suggesting that bacteria with certain types of genes (i.e. *strA*, *strB*) vary in their response to environmental conditions, allowing potential differences in the recovery of *E. coli* carrying ARGs. Although, it is important to note that possession of a single or a few ARGs is not equivalent to its expression, as the dynamics of gene occurrence and ARG expression are complex and rely on a variety of intrinsic and extrinsic factors (Graves *et al.*, 2011).



**Figure 3.10. Proportion of total *E. coli* isolates carrying ARGs. *tetA*, tetracycline; *strA*, *strB*, streptomycin; *sul1*, *sul2*, sulfisoxazole. An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n = number of isolates.**

In the case of enterococci, a total of 175 isolates were screened for the presence of *tetA* (tetracycline) and *strA* (streptomycin). Among the 175 isolates tested, none of the isolates possessed either of the target genes. A possible explanation for the lack of detection of ARGs (*tetA*, *strA*, *strB*, *sul1*, *sul2*) among both *E. coli* and *Enterococcus* spp. isolates is that only 1 of the 38 recognized tetracycline resistance genes, 2 of the 3 recognized sulfonamide

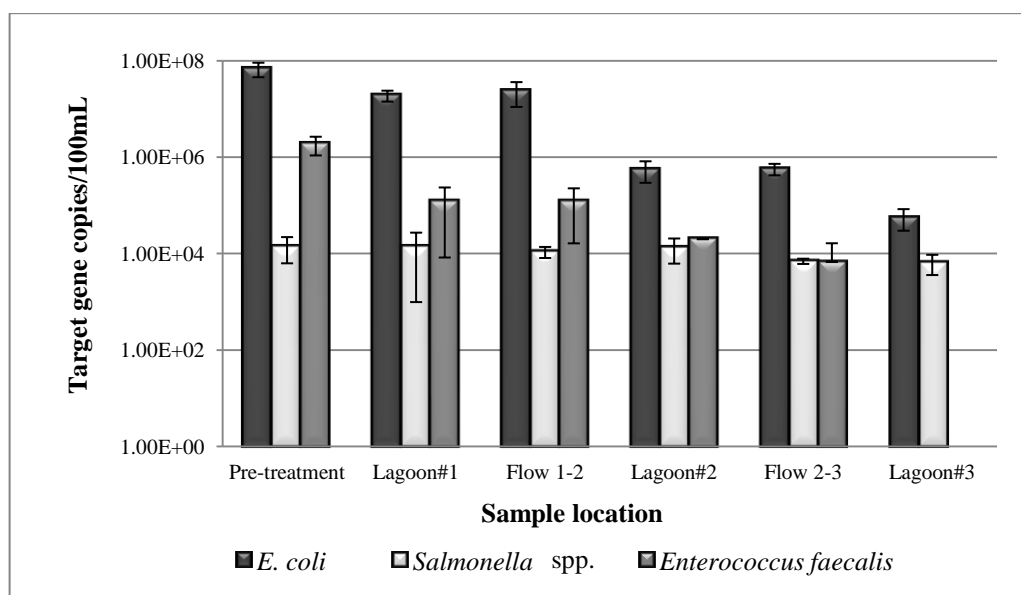


resistance genes, and 2 of the 5 recognized streptomycin resistance genes were screened. Thus, the absence of relevant resistance genes from isolates that were phenotypically resistant to other antimicrobials may reflect that the observed phenotypic resistance was caused by a resistance gene other than those investigated here or by nonspecific resistance mechanisms (e.g., an efflux pump) (Allen *et al.*, 2011; Aslam *et al.*, 2009; Samaha-Kfoury and Araj, 2003; Livermore *et al.*, 2001; Adrian *et al.*, 2000; Shaw *et al.*, 1993).

#### **3.4.4 Quantitative detection of bacterial pathogens and ARGs in a treatment lagoon system**

Real time quantitative PCR (qPCR) was used to determine the concentration of three bacterial pathogens; *E. coli*, *Enterococcus faecalis*, and *Salmonella* spp., and three ARGs; *tetA*, *ampC*, and *blaSHV*, within samples collected at various locations of the lagoon treatment system. Figure 3.11 illustrates the relative abundance (target gene copies/100 mL) of bacterial pathogens (*E. coli*, *Salmonella* spp., and *E. faecalis*) at different sampling points of the treatment lagoon system during the fall of 2010 (refer to Appendix A for additional qPCR data). The highest values were obtained for *E. coli* with approximately  $10^7$  target gene copies/100 mL detected in the initial stages of treatment (pre-treatment, lagoon#1, and flow through between lagoons #1 & 2). *E. coli* concentrations were reduced during the second stages of treatment (lagoon#2, flow through between lagoons #2 & 3), reaching a final concentration of approximately  $10^4$  target gene copies/100 mL detected in the final lagoon (lagoon#3). When comparing the relative concentrations of *E. coli* between different samples, a significant difference was observed ( $p < 0.05$ ) between the initial stages of

treatment (pre-treatment, lagoon#1, and flow through between lagoons #1 & 2) and the later stages of treatment (lagoon #2, flow through between lagoons #2 & 3, and lagoon #3). *Salmonella* spp. concentrations were fairly consistent throughout different stages of the treatment lagoon (no significant differences), while maintaining approximately  $10^4$  target gene copies/100 mL in the third lagoon. Concentrations of *E. faecalis* were also detected among all locations (with the exception of lagoon #3), with values of  $10^6$  and  $10^5$  target gene copies/100 mL detected during the initial stages of treatment, gradually falling to non-detectable limits in the third and final lagoon (Figure 3.11). *E. faecalis* gene concentrations were significantly higher ( $p < 0.05$ ) in the pre-treatment sample when compared to the levels observed among other sampling locations.

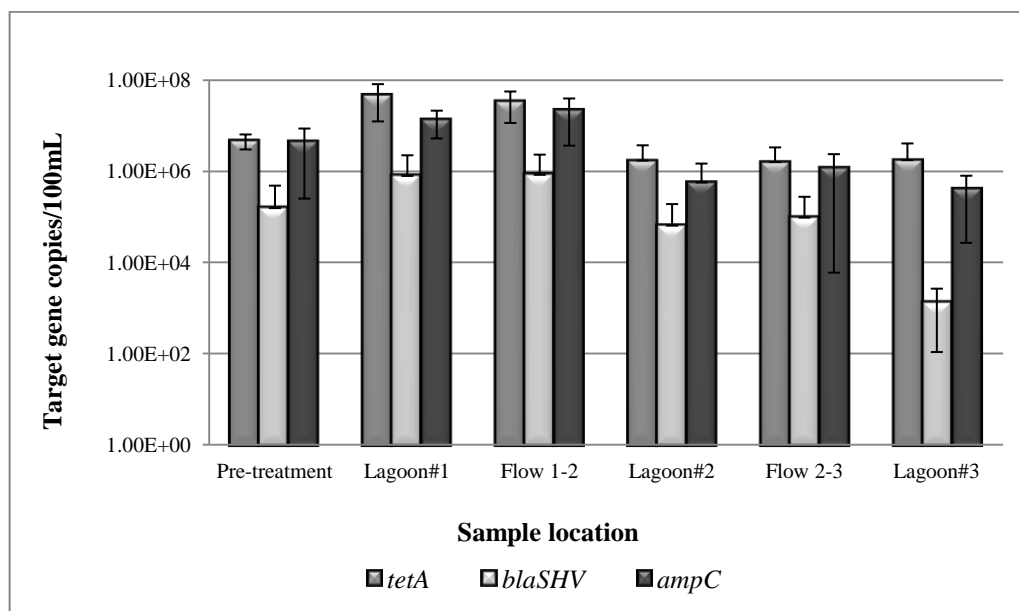


**Figure 3.11. Relative abundance (target gene copies/100 mL) of bacterial pathogens (*E. coli*, *Salmonella* spp., and *E. faecalis*) at different sampling points in the treatment lagoon system (Fall 2010). Error bars represent standard deviation around mean values from two independent qPCR runs in duplicate.**

An overall trend was observed that, although *E. coli* and *E. faecalis* tended to decrease in concentration as the waste effluents passed through different stages of treatment (multiple lagoons), *E. coli* and *Salmonella* spp. were still present in the third and final lagoon, with approximately  $10^4$  and  $10^3$  gene copies/100 mL, respectively. The following observed values are higher than those outlined in the Effluent Quality Guidelines as set by Environment Canada (400 faecal coliforms CFU/100 mL) (CSC, 2006). However, it may be inappropriate to directly compare the gene copy numbers with the cell numbers, as the former may overestimate the cell numbers as a result of the dead cell contribution to the wastewater DNA pool. Overall, the lagoon treatment system was found to reduce the level of bacterial pathogens, but does not guarantee the complete elimination of contamination with *E. coli* and *Salmonella*. Other studies also indicate that the sewage treatment process reduces the number of pathogens insufficiently (Strauch, 1998), with further treatment and disinfection required to render the water safe for release. From a public health point of view, the presence of these bacteria in the final lagoon effluent increases the likelihood of their release into surface waters, presenting a significant risk, and should therefore be considered in risk management regimes.

Figure 3.12 summarizes the concentrations of ARGs (*tetA*, *blaSHV* and *ampC*) obtained from different sampling points in the lagoon treatment system during the fall of 2010 (refer to Appendix A for additional qPCR data). Relative concentrations among all three ARGs were similar, with slightly higher *tetA* and *ampC* concentrations, reaching  $10^7$  target gene copies/100 mL in lagoon #1. All three ARGs screened were gradually reduced as the waste effluent passed through different stages of treatment (multiple lagoons); however,

concentrations remained relatively high with  $10^3$  -  $10^6$  target gene copies/100 mL in the final lagoon #3. No significant differences ( $p>0.05$ ) could be identified using t-test analysis between the different sampling locations among the three different ARGs. The *blaSHV* gene, conferring resistance to extended-spectrum  $\beta$ -lactamases, was detected among all sampling locations in lower concentrations when compared to the other ARGs, declining to  $10^3$  target gene copies/100 mL in the final lagoon.



**Figure 3.12. Relative abundance (target gene copies/100mL) of ARGs (*blaSHV*, *ampC*, and *tetA*) at different sampling points in the treatment lagoon system (Fall 2010). Error bars represent standard deviation around mean values from two independent qPCR runs in duplicate.**

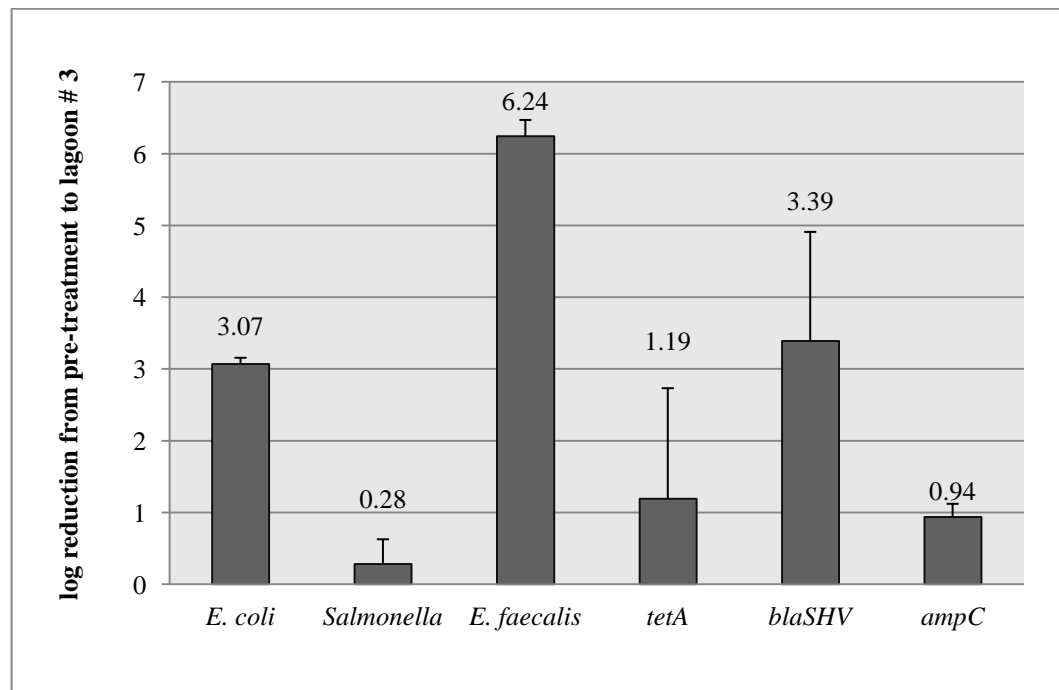
Although livestock operations are known to harbour elevated levels of antibiotic resistant bacteria, few studies have examined the potential of treatment lagoon systems to reduce ARGs. McKinney *et al.* (2010) studied *tet* and *sul* ARGs in livestock lagoons of various

operation type, configuration, and antibiotic occurrence. Their results indicate that *sul* ARGs were generally more recalcitrant than *tet* ARGs, with significant decreases in ARGs occurring between the first and last lagoon, and decreases between the intermediate lagoons not statistically significant (McKinney *et al.*, 2010). The results presented here agree with similar studies suggesting that alternative treatment systems, including constructed lagoons, are important sources of resistance genes to the environment (Auerbach *et al.*, 2007, Pruden *et al.*, 2006, McKinney *et al.*, 2010, Munir *et al.*, 2010). Wastewater has been said to stimulate horizontal gene transfer among microbial species (Aminov *et al.*, 2001; Lorenz and Wackemagel, 1994). Therefore, wastewater treatment plants and treatment lagoons could potentially increase the antibiotic resistance of surviving bacteria, and serve as important reservoirs for the spread of antibiotic resistance to opportunistic pathogens if the treatment processes are not effective.

#### **3.4.5 Antimicrobial removal efficiency of wastewater treatment in a constructed lagoon system**

Log removals were calculated based on the concentrations of pathogens and ARGs in the raw influent samples (pre-treatment) and the final lagoon effluent (lagoon #3) samples (Figure 3.13). *E. coli*, *E. faecalis*, and *Salmonella* spp. are commonly found in wastewater and sewage sludge and have been studied mainly using culture-based methods (Gantzer *et al.*, 2001; Godfree and Farrell, 2005; Sahlström *et al.*, 2004). Lee *et al.* (2006) used qPCR to quantify indicator and pathogenic bacteria during wastewater treatment and found that *E. coli* was more abundant in municipal wastewater than pathogens, including *Salmonella* spp.,

by 2-5 orders of magnitude. Similarly, Wéry *et al.* (2008) found that *E. coli* was more abundant in wastewater than *Clostridium perfringens*, *Salmonella* spp. and *Campylobacter jejuni* by, 3, 4, and 5 orders of magnitude, respectively. *E. coli* was found to be approximately 3-4 orders of magnitude higher in the pre-treatment sample compared to that of *Salmonella* spp.. The removal of indicator bacteria, including *E. coli* and *E. faecalis* by the lagoon treatment system was high, with an average log reduction of 3.07 and 6.24, respectively, between pre-treatment and lagoon #3 (final effluent stage) samples (Figure 3.13).



**Figure 3.13.** Average log reduction of target gene copies/100mL from pre-treatment to final lagoon (lagoon#3) samples among bacterial pathogens and ARGs. Error bars represent standard deviation around mean values from two independent qPCR runs in duplicate.

Several studies have examined the removal or inactivation of indicator bacteria and pathogens in wetlands, waste stabilization ponds, and facultative ponds (Perkins and Hunter 2000; Thurston *et al.*, 2001; Sinton *et al.*, 2002; Nelson *et al.*, 2004; Reinoso *et al.* 2008). However, the design of wetlands, waste stabilization ponds, and facultative ponds differ from aerated lagoons in terms of the presence of aquatic plants, aeration, retention time, etc. (US EPA 2002, 2008) and thus the data may not be comparable. Bacterial removal varies with the type of treatment system (and pond) but is generally in the range of 1-3 log units of indicator bacteria and pathogens (Bitton 2005). Such a reduction among indicator bacteria is most likely due to a variety of factors, including temperature, solar radiation, mixing and sedimentation, die-off, retention time, pH, and other local parameters that affect the removal of indicator microorganisms. Locas *et al.* (2010) studied the removal of indicator microorganisms from domestic wastewater by aerated lagoons and found similar removals among coliforms and enterococci, ranging from 3.7 to 5.5 log units. Pathogenic bacteria including *Salmonella* spp. showed very little reduction with an average log removal of 0.28 between the raw wastewater (pre-treatment) and final effluent stage (lagoon #3). Compared to other bacteria, *Salmonella* spp. in general have high survival rates in aquatic environments and are able to withstand a variety of stresses, including thermal and pH fluctuations, high osmolarity and low nutrient availability (Chao *et al.*, 1987; Winfield and Groisman, 2003). *Salmonella* spp. have also been described as being more resistant than *E. coli* to biotic factors (microbial predators or competing organisms) in sources of drinking water (Wright, 1989), perhaps due to a difference in adhesion to protective particles (Winfield and Groisman, 2003). A possible explanation for the detection of *Salmonella* spp. in the final lagoon could be because *Salmonella* spp. are able to withstand a wider variety of stresses

associated with environmental fluctuations, compared to that of indicator bacteria, and may ultimately persist in the water environment for some time (Percival *et al.*, 2004). Ghosh *et al.* (2009) examined the application of a biopolymeric flocculant for removal of *Salmonella* from poultry wastewater by the addition of the bioflocculant directly to the wastewater in different concentrations and compared to that of alum. Removal of *Salmonella* was found to be remarkably low with the standard dose of alum (Sobsey, 2002; Sobsey *et al.*, 2003). Similar observations on the low flocculation efficiency of alum in abattoir wastewaters have also been reported (Amuda and Alade, 2006). Therefore, another explanation for the limited reduction of *Salmonella* spp. through the lagoon treatment system could be due to the type and amount of flocculant used, however, this information was not accessible, hence, a direct conclusion cannot be made regarding *Salmonella* spp. removal and flocculation efficiency.

The removal efficiency of the following ARGs, *tetA*, *blaSHV* and *ampC* ranged from approximately 1-3 log removals from pre-treatment to final lagoon samples (Figure 3.13). The greatest decline in ARG concentration was observed for *blaSHV*, with an average log reduction of 3.39, whereas *ampC* and *tetA* were found to decrease by approximately one order of magnitude. Several recent studies have used culture-independent methods to study the occurrence of *tet* and *sul* genes in waste lagoons, groundwater, and other environmental compartments (Chee-Sanford *et al.*, 2001; Aminov *et al.*, 2002; Pruden *et al.*, 2006; McKinney *et al.*, 2010). For example, Aminov *et al.* (2002) found a limited reduction in *tet* gene abundance during lagoon storage, consistent with the detection of *tet* genes in groundwater downstream of a swine wastewater lagoon. Few studies have examined other types of resistance genes, including  $\beta$ -lactamase (*blaSHV*) and ampicillin (*ampC*) resistance



genes, in constructed lagoon systems. The results of this study illustrate the prevalence of ARGs (conferring resistance to three different classes of antibiotics) within the third lagoon, thereby, giving rise to the potential dissemination of ARGs into receiving water bodies. Currently, there are no provincial or national water quality regulations in Canada or the USA requiring testing on the release of antibiotic resistant organisms and ARGs into the environment. Previous suggestions (West *et al.*, 2011; Sayah *et al.*, 2005) propose the testing of ARGs in bacterial strains become part of the standard methods for examining and regulating water quality and wastewater discharge, especially in areas at high risk for pollution from human and animal waste. A link cannot be drawn between the detected ARGs to their host bacterial species nor can any conclusions be made about their ability to transfer into clinically relevant species. Unlike studies focusing on soil or sediments, there was an opportunity or advantage to filter and centrifuge samples to separate free DNA in solution from the bacterial cells. Therefore, it is likely that detected ARGs were all derived from intact or very recently ruptured cells. Conclusions cannot be made if these cells were viable or capable of horizontal transfer of resistance genes between organisms. Other factors are also clearly uncontrollable when studying lagoons, such as varying weather conditions, the scale of the feedlot operation, routine operating practices at each site, the mode of operation of any given lagoon, and ambient levels of solids in lagoons. Despite the recognized limitations, the work described herein provides a perspective on the occurrence and frequency of ARGs and antibiotic resistant bacteria in treatment lagoons receiving poultry abattoir wastewater. Further research is required to fully understand the implications for the proliferation of resistance in pathogens and commensals of concern to humans.

### 3.5 Conclusions

This chapter illustrates the value of traditional culture-based screening techniques in combination with molecular quantification through real-time PCR to help establish a preliminary environmental assessment of selected pathogens and ARGs for a lagoon treatment system receiving poultry slaughterhouse waste. Overall, the level of multiple antibiotic resistance (MAR) among culturable indicator bacteria was high, exhibited by 93% and 100% of *E. coli* and *Enterococcus* spp. isolates, respectively. The most common antibiotic resistance profile among *E. coli* was simultaneous resistance to between three and five antimicrobials, whereas the most common MAR profile among *Enterococcus* spp. isolates was resistance to ten or more antibiotics. Real time quantitative PCR was used to determine the concentration of three bacterial pathogens; *E. coli*, *Enterococcus faecalis*, and *Salmonella* spp., and three ARGs; *tetA*, *ampC*, and *blaSHV*, within samples collected at various points of the lagoon treatment system. Overall, the lagoon treatment system was found to reduce the level of bacterial pathogens, but does not guarantee the complete elimination of contamination with *E. coli* and *Salmonella*, with approximately  $10^4$  and  $10^3$  gene copies/100 mL detected in the third and final lagoon, respectively. All three ARGs were gradually reduced as the waste effluents passed through different stages of treatment (multiple lagoons); however, concentrations remained relatively high with  $10^3$  -  $10^6$  target gene copies/100 mL in the third lagoon. The focus of this study was limited to only a few select ARGs; however, numerous ARGs exist and may be present in the environment with their fates being unique to each specific environment. Thus, each ARG may have different behaviours with respect to fate and transport and response to physical, chemical, and/or biological treatment. Therefore, the results only reflect tetracycline and two classes of  $\beta$ -

lactam resistance within a constructed lagoon treatment system in southern Ontario, and should not be generally applied to all the environments in different geographic areas. Furthermore, due to the escalating problem of bacterial antibiotic resistance, there is a need to improve our understanding of the potential role of livestock operations both in the amplification and attenuation of antibiotic resistance levels. The results from this study do however contribute to an improved understanding regarding the occurrence, distribution, and fate of antibiotic resistance associated in livestock operations, while adding a new and important perspective to aid in the advancement of understanding this critical problem.

### **3.6 Recommendations & Future Research Needs**

- Further studies monitoring the prevalence and fate of different ARGs conferring resistance to other classes of antibiotics is needed, as each ARG may demonstrate different behaviours in response to wastewater treatment.
- Long term surveillance can help provide an indication of the mobility, persistence, and transfer of resistance genes and help address the issue of agriculture as a source of resistance genes for entry into the environment.
- Future studies could examine the prevalence, distribution, and fate of antibiotic resistant bacteria and ARGs in surface waters and/or groundwater downstream to the poultry waste lagoons, which allows for a greater understanding of the long-term impact and environmental migration of such contaminants.
- Additional analysis characterizing enterococcal and *E. coli* isolates from faecal samples of livestock species (poultry) and humans (poultry farmers/slaughterers)

may also help to determine the public health significance, including the carriage of virulence genes and antibiotic resistance. This may help establish if any epidemiological connection exists between these sources and water.

# 4

## **Prevalence of antimicrobial resistance among *Aeromonas*, *Yersinia*, and *Flavobacterium* populations and selected ARGs during winter operation of a constructed wetland receiving fish hatchery effluent<sup>3</sup>**

### **4.1 Introduction**

Bacterial communities in the environment can serve as important reservoirs of antibiotic resistance and this has implications with respect to the use of antibiotics in agriculture and

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<sup>3</sup>Helt, C.D., Wootton, B., Balch, G., Legge, R.L., Slawson, R.M. *Effect of oxytetracycline exposure on antibiotic resistance among fish pathogens and select antibiotic resistance genes in a constructed wetland receiving fish hatchery wastewater, in preparation.*

aquaculture industries. Resistance development has been reported in freshwater and marine environments, including rivers and streams (Schmidt *et al.*, 2000; Miranda and Zemelman, 2001; Ash *et al.*, 2002; Stachowiak *et al.*, 2009). Due to the limited number of treatment options for aquatic species in certain countries, including Canada, there is concern surrounding widespread resistance to even one class of antimicrobial compound. An effective and successful treatment option in the field of aquaculture is dependent on a number of factors, including knowledge of the species involved, history of disease occurrence at the farm, a prompt and accurate diagnosis, and the knowledge of antimicrobial susceptibility of the bacteria being treated (Uhland and Higgins, 2006). Stachowiak *et al.* (2009) examined the presence of antibiotic resistance among *E. coli* in a small stream receiving fish hatchery effluent that had no documented use of antibiotics in the hatchery for at least 6 months prior to their study. Their results indicate a significantly higher proportion of *E. coli* isolates resistant to tetracycline in the fish hatchery effluent and in both downstream water and sediment samples, as compared to either the upstream water or sediment samples (Stachowiak *et al.*, 2009). They concluded that the fish hatchery may possibly serve as a source of tetracycline-resistant microorganisms even in the absence of recent antibiotic use. In the field of aquaculture, both therapeutic and environmental problems have been addressed, as antimicrobial agents are released into the surrounding water during medical treatment of bacterial fish diseases (Aoki, 1992). Recent studies have confirmed the increased incidence of resistance among environmental isolates and fish pathogens associated with commercial fish production facilities (Petersen *et al.*, 2002; Alcaide *et al.*, 2005; Miranda *et al.*, 2003). The commercial production of fish for both human consumption and the improvement of recreational fishing through stream stocking

has increased worldwide by fourfold over the past several decades (Naylor and Burke, 2005; Cabello 2006; Goldberg and Naylor, 2005). Although the overall use of antibiotics in the fish production industry has increased in recent years, not all facilities routinely use antibiotics. Intermittent antibiotic use is common, employed only to treat active disease outbreaks, where time between uses can be long and in the order of months (Stachowiak *et al.*, 2009). However, even in the absence of continuous usage, antibiotic resistant microorganisms are able to persist in protected reservoirs such as sediments or the intestinal tracts of fish (Schmidt *et al.*, 2000; Petersen *et al.*, 2002).

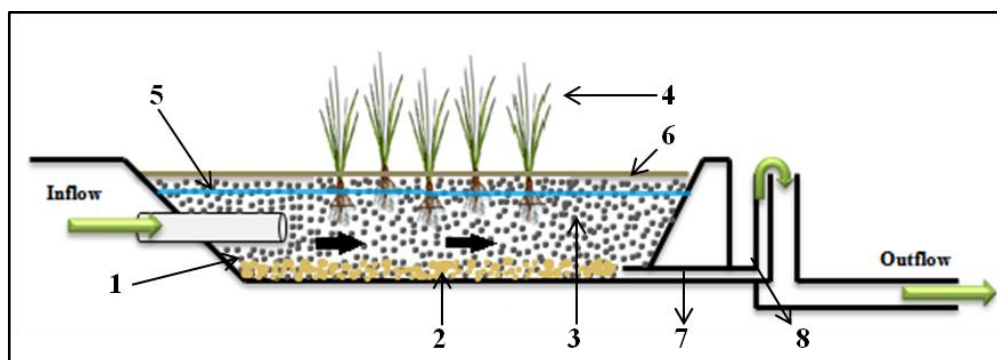
Major environmental pathogens involved in fish farm infections include the Gram negative species *Flavobacterium psychrophilum*, *Yersinia ruckeri*, and *Aeromonas salmonicida* and these are the etiological agents of cold water disease, enteric red mouth disease, and furunculosis, respectively (del Cerro *et al.*, 2002). Fish hatcheries and farms typically utilize antibiotics (e.g. oxytetracycline, ormetoprim, sulfadiazine) therapeutically to treat these common fish farm diseases (FDA, 2007; Heuer *et al.*, 2009), with administration through pelleted feed. Following treatment, antibiotics may be excreted in large amounts by fish without being metabolized with reports between 59% (Rigos *et al.*, 1999) to more than 90% (Cravedi *et al.*, 1987) of oxytetracycline (OTC) given to sea bass or rainbow trout, respectively. Antibiotics may also be dissolved in the water, settle to the bottom in uneaten feed, or sorb to particles. Freshwater fish farming has been shown to impact environmental *Aeromonas* spp., as indicated by an investigation of antimicrobial resistance at four Danish rainbow trout farms (Schmidt *et al.*, 2001). High levels of individual and multiple antimicrobial resistances were demonstrated in collected flavobacteria and aeromonads,

indicating a substantial impact of fish farming on several groups of bacteria associated with aquacultural environments (Schmidt *et al.*, 2001). Members of the genus *Aeromonas* readily develop single or multiple antimicrobial resistance phenotypes (Goni-Urriza *et al.*, 2000; Guardabassi *et al.*, 2000), and R plasmids are commonly found (Klein *et al.*, 1996; Adams *et al.*, 1998). Therefore, *Aeromonas* spp., represent an important environmental pathogen for monitoring the prevalence and distribution of antibiotic resistance. Seyfried *et al.* (2010) studied the occurrence of tetracycline resistance genes in aquaculture facilities with varying use of OTC and concluded the effluent from all farms, regardless of treatment regime, had higher tet<sup>R</sup> detection frequencies than the corresponding influent for all genes screened. Water from farms with recent OTC use had significantly higher tet<sup>R</sup> detection frequencies than did water from farms without OTC use (Seyfried *et al.*, 2010). It was suggested that both OTC treatment in aquaculture facilities and the farms themselves may be sources of tet<sup>R</sup> gene introduction to the environment (Seyfried *et al.*, 2010). Tetracyclines are among the therapeutic agents most commonly used in human and veterinary medicine, and are also frequently used in the aquaculture industry. In Canada and the United States, OTC can be added to animal and fish feed at subtherapeutic levels, acting to promote growth or to prevent/treat disease. Therefore, OTC was selected as the antibiotic of preference in this study due to the widespread use of tetracyclines in the aquaculture industry.

Recently, constructed wetlands (CWs) have been utilized to successfully treat agricultural, municipal, or industrial wastewaters. CWs are designed to take advantage of many processes that occur in natural wetlands, but in a more controlled setting; offering relatively low investment and operation costs, while producing high quality effluent with less dissipation of



energy (Song *et al.*, 2008). The basic classification of CWs is based on the type of macrophytic growth (emergent, submerged, free floating and rooted with floating leaves) and further classification can be based on the water flow (surface flow, sub-surface vertical or horizontal flow) (Vymazal, 2005). Biotic and abiotic purification mechanisms of CWs are based on the following processes (Gumbrecht, 1993; Hiley, 1995): mechanical screening and sedimentation, microbial degradation, biochemical nutrient removal of plant rhizomes, adsorption through ligand exchange, precipitation and chemical fixation of reactive soil ingredients. Recently, attention has focused on the ability of constructed wetlands to reduce pathogenic bacteria in wastewater (Karim *et al.*, 2004; Weber & Legge, 2008). The removal of microorganisms in constructed wetlands has shown varying degrees of effectiveness; however, several studies illustrate the improvement of microbial water quality using constructed wetlands (Green *et al.*, 1997; Song *et al.*, 2006). Horizontal sub-surface flow constructed wetlands (HF CWs) are based on the movement of wastewater in a more or less horizontal path, as it is fed in the inlet and slowly flows through porous medium under the surface of the bed until it reaches the outlet zone (Figure 4.1) (Vymazal, 2001).



**Figure 4.1.** Schematic representation of a horizontal sub-surface flow constructed wetland. 1, distribution zone filled with large stones; 2, impermeable liner; 3, bed media (e.g., gravel, crushed stones); 4, emergent macrophytes; 5, water level in the bed; 6, mulch layer; 7, collection drainage pipe; 8, outlet structure for maintaining water level in the bed. The arrows indicate only a general flow pattern (adapted from Vymazal, 2001).

The effluents from freshwater fish farms (e.g. trout) are typically 20–25 times more diluted than medium-strength municipal wastewaters, but have a relatively high flow rate (Vymazal, 2009). With respect to receiving water quality objectives, the most challenging element to remove from freshwater fish farm effluents is phosphorus (Comeau *et al.*, 2001). Few studies have been reported on the use of HF constructed wetlands to treat fish farm effluents in Canada (Comeau *et al.*, 2001; Naylor *et al.*, 2003; Chazarenc *et al.*, 2007). Naylor *et al.* (2003) studied the role of plants and substrates in the treatment of freshwater fish farm effluents using CWs, with results indicating effective pollutant removal despite the relatively low nutrient content. Organic matter (BOD, COD) and nitrogen removal was found to be superior under strong plant cover, whereas the removal of phosphorus was better achieved by the substrates containing the highest proportion of electric arc furnace (EAF) steel slag and limestone (Naylor *et al.*, 2003).

## **4.2 Research Needs & Objectives**

The concern with antibiotic resistance in freshwater environments in relation to fish farming activities is more recent and to date has been poorly documented (Guardabassi *et al.*, 2000; Schmidt *et al.*, 2000), especially in Canada and cold weather climates using constructed wetlands to treat fish hatchery waste. Treatment of aquaculture effluents from cold-water sub-surface flow CWs and monitoring the prevalence and distribution of antimicrobial resistance and ARGs among environmental pathogens has yet to be investigated. In order to improve our understanding of the dissemination of antimicrobial resistance (AMR) in a subsurface flow constructed wetland treating fish hatchery wastewater, the occurrence, distribution, and frequency of AMR among selected waterborne isolates are needed. In

addition, few studies have monitored the impact or effect of an antibiotic exposure on the level of antibiotic resistance among environmental bacteria within full-scale constructed wetlands. Helt *et al.* (2012) examined antibiotic resistance profiles of representative wetland bacteria and faecal indicators following exposure to ciprofloxacin in lab-scale constructed mesocosms, suggesting that the transient presence of an isolated exposure event of ciprofloxacin can impact the antibiotic resistance profiles among faecal indicators, and has the potential to impact constructed wetland treatment systems. To the best of my knowledge, this is the first study that examines and monitors antibiotic resistance profiles among selected fish pathogens (*Yersinia* spp., *Aeromonas* spp., *Flavobacterium* spp.) and the frequency of ARGs, both before and after an antibiotic exposure, within a sub-surface flow constructed wetland treating aquaculture wastewater. Therefore, the overall objective of the research reported in this chapter was to better understand the frequency of selected bacterial pathogens, antibiotic resistance, and ARGs, within multiple stages of a treatment wetland receiving fish hatchery waste during winter operation. This chapter will also contribute to a better understanding of the effect an influent antibiotic has on the level of antimicrobial resistance among selected environmental pathogens in a sub-surface flow constructed wetland system.

The specific objectives of this chapter are to:

1. Establish a phenotypic assessment and determine the prevalence of culturable *Yersinia* spp., *Aeromonas* spp., and *Flavobacterium* spp., from various locations in the sub-surface flow constructed wetland both before and after exposure to the antibiotic (oxytetracycline);

2. Confirm culturable *Yersinia* spp., and *Aeromonas* spp. isolates using molecular techniques (qualitative PCR), and further distinguish *Yersinia* spp. isolates between two common species; *Y. ruckeri* and *Y. enterocolitica*, using species-specific PCR;
3. Determine the frequency and distribution of antibiotic resistance among culturable *Yersinia* spp., *Aeromonas* spp., and *Flavobacterium* spp. isolates, within different locations of the constructed wetland. The prevalence of antibiotic resistance both before and after the OTC exposure will also be compared; and
4. Quantitatively detect selected ARGs (*ampC*, *tetA*, *blaSHV*) in various stages of the sub-surface flow constructed wetland using real-time quantitative PCR both before and after the OTC exposure.

## **4.3 Materials and Methods**

### **4.3.1 Sub-surface flow constructed wetland**

The sub-surface flow constructed wetland is located on the property of the Haliburton Highlands Outdoors Association (HHOA) Fish Hatchery in Haliburton, ON, and was constructed in 2008 as a subsurface flow cold climate treatment wetland receiving fish-farm waste. The wetland design for the HHOA Fish Hatchery includes two horizontal treatment cells (each cell had a dimension of approximately 2.0 m x 5.83 m), both planted with different species to compare treatment efficiencies of native Ontario plants. The CW is bisected and planted with common cattails (*Typha latifolia*; planted 2008) and wild rice (*Zizania palustris*; planted 2008) that died and were replaced with lake sedge (*Carex*

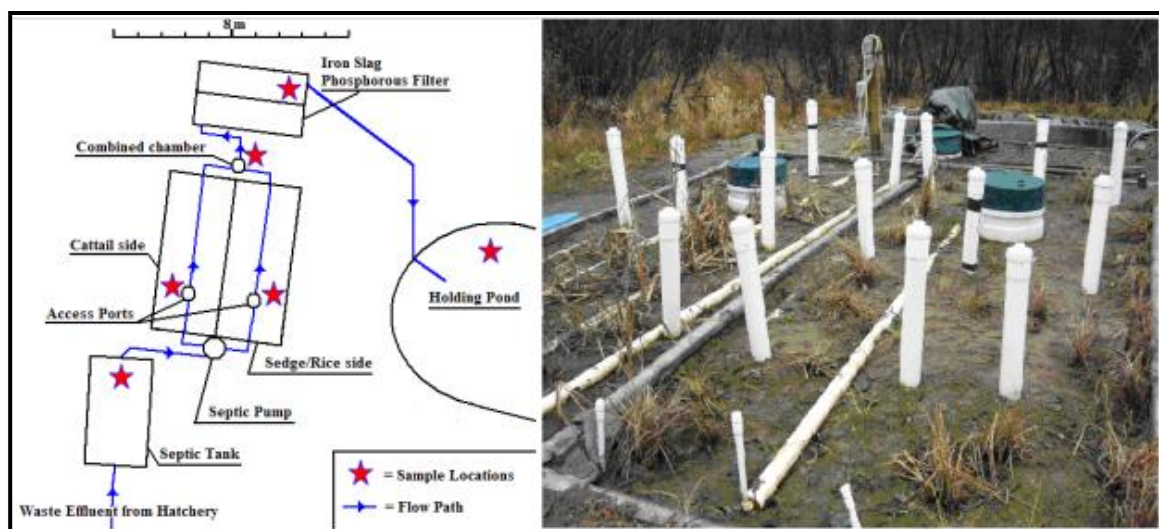
*lacustris*; planted 2009) to create two parallel flow paths through the CW. The first flush of lake trout raceway aquaculture wastewater is collected in a septic tank where the effluent is fed into the wetland cells (rate of approximately 2500 L/day) in batches by an on-demand pump. Once through the wetland, the treated water enters a combined chamber, after which it is subsequently pumped into a phosphorus filter comprised of iron slag. The phosphorus filter effluent is then pumped into a holding/settling pond (see Figure 4.2). Due to volume constraints, additional wastewater other than that with the most concentrated fish waste is routed to the existing settling ponds. From April 2009 to March 2010, the maximum carbonaceous biochemical oxygen demand (cBOD<sub>5</sub>) of the groundwater used by the hatchery as a source of water for the rearing of fish was 1.27 mg/L, while the average cBOD<sub>5</sub> concentration of the vacuumed wastewater entering the septic tank was 128 mg/L (Balch *et al.*, 2010). High cBOD<sub>5</sub> wastewater concentrations were expected because aquaculture effluents are mainly composed of organic solids from unconsumed food and fish feces. Lin *et al.* (2002) noted that aquaculture wastewater is a good candidate for treatment using constructed wetlands because of the high organic content of the solids present. From April 2009 to March 2010, the maximum total suspended solids (TSS) concentration of the groundwater used by the hatchery as a source of water for the rearing of fish was 2.8 mg/L and the average TSS concentration of the vacuumed wastewater entering the septic tank was 48.45 mg/L (Balch *et al.*, 2010). In sub-surface flow CWs, TSS concentrations are effectively reduced by the physical mechanisms of sedimentation, filtration and adsorption on biofilm attached to gravel and root systems. The overall hydraulic retention time for the waste effluent was approximately 38 hrs in the wetland and 13 hrs in the iron-slag phosphorus filter.

#### **4.3.2 Antibiotic addition**

A mass of 12.5 grams of oxytetracycline (OTC) was added to the exit of the septic tank as a means of spiking the wetland. The mass of OTC used was based on the average effluent loading to the wetland of approximately 2500 litres per day. The assumption made was that over a 24 hr duration, the initial spike of OTC would be diluted to a nominal concentration of approximately 5 mg/L (e.g., 5 ppm). A mass of 12.5 g OTC was added each morning for a total of 5 days; Feb 23<sup>rd</sup>-28<sup>th</sup>, 2010. The antibiotic exposure (spike) was done in accordance with a collaborative research plan under Dr. Brent Wootton (Principle Investigator, Fleming College; Balch *et al.*, 2010).

#### **4.3.3 Water sampling**

Samples were collected over six sampling dates between Feb-April 2010, and 6-7 locations were sampled throughout various stages of the wetland. A total of 35 samples were collected during this period. Six sampling events on Feb. 8<sup>th</sup>; 22<sup>nd</sup>; 24<sup>th</sup>; Mar. 1<sup>st</sup>; 15<sup>th</sup>, and Apr. 12<sup>th</sup> (2010), involved sampling locations identified in Figure 4.2. and are referred to as "Septic Out", taken from the effluent of the septic pump; "Cattail" and "Sedge", taken from the first access port on the cattail and sedge planted sides, respectively; "Final Pump", taken from the combined chamber; "Slag Filter", taken from the effluent of the iron slag phosphorus filter, and "Pond Out", taken from the holding/settling pond.



**Figure 4.2.** Schematic (left; Balch, G, pers. comm., Fleming College) and photograph (right; taken by Mitzel M.R, pers. comm., Wilfrid Laurier University) of the sub-surface flow CW. Wastewater flow is indicated by the arrows and sampling locations are designated by the stars.

Water sampling occurred at specified locations in the wetland and at the outlet of the wetland into the receiving pond 0 hr, 24 hr, 6, 20, and 48 days after the initial dosing on the first day of OTC addition (Balch *et al.*, 2010). Water samples were collected from seven different locations in the treatment system both before and after the antibiotic exposure event. Duplicate samples from each location were collected in sterile, 500-mL screw-capped polypropylene containers. Samples were shipped on ice (4°C) to the microbiology laboratory and analyzed within 24 hours of collection.

#### **4.3.4 Isolation and enumeration of selected environmental pathogens**

*Aeromonas* spp., *Yersinia* spp., and *Flavobacterium* spp. were isolated from each water sample according to standard methods for the examination of water and wastewater, by using the membrane filtration method for enumeration of *Aeromonas* spp., and the spread

plate method for enumeration of *Yersinia* spp., and *Flavobacterium* spp. (American Public Health Association; APHA, 1999). Diluted samples were filtered through 0.45- $\mu$ m, 47-mm mixed cellulose ester membrane filters (Difco, Fisher Scientific, Ottawa, Ontario, Canada), and then placed onto appropriate selective agar plates. Appropriate sample volumes, in duplicate, were filtered or plated and varied according to the group of organisms being enumerated and sample source (influent vs. treated effluent) to ensure countable plates with 20-200 colonies (Schraft and Watterworth, 2005). Plates were incubated for 24 hr at 35°C on ampicillin dextrin agar (ADA) for *Aeromonas* spp. (Havelaar *et al.*, 1987), 48 hr at 35°C on *Yersinia* selective agar containing *Yersinia* antimicrobial supplement CN (Cefsulodin-Novobiocin) for *Yersinia* spp. (Hench *et al.*, 2003), and 96-120 hr at 25°C on nutrient agar supplemented with 50  $\mu$ g/mL kanamycin for *Flavobacterium* spp. (Flint, 1985). All bacteriological media were obtained from Difco (Ottawa, ON). Positive colony characteristics of *Aeromonas* spp. were indicated by the presence of yellow colonies formed by the production of acid from dextrin fermentation on ADA, as indicated by the colour change from blue to yellow by the pH indicator, bromothymol blue. *Yersinia* Selective Agar (YSA) is a differential selective medium for the isolation of *Yersinia enterocolitica*. However, YSA may also be used for the isolation of *Yersinia* species other than *Y. enterocolitica*, including *Y. pseudotuberculosis*, *Y. frederiksenii*, and *Y. intermedia* (Bockemühl and Wong, 2003). Typical *Yersinia enterocolitica* colonies will have deep-red centers surrounded by a transparent, pale border giving the appearance of a "bull's-eye" on YSA. *Yersinia pseudotuberculosis* usually lacks the transparent zone around the colonies. Fermentation of mannitol in the presence of neutral red results in a characteristic bull's-eye colony of *Y. enterocolitica*, colorless with red centers on YSA (Bockemühl and Wong,



2003). Selective inhibition of Gram negative and Gram positive organisms is obtained by means of crystal violet, sodium deoxycholate and the antimicrobial agents, cefsulodin, irgasan, and novobiocin. A selective nutrient agar medium containing kanamycin at 50 µg/ml was developed for the isolation and enumeration of *Flavobacterium* spp. (Flint, 1985), producing typical yellow-pigmented colonies. Characteristic colonies for all organisms were recorded (and calculated in CFUs per 100 mL) and further streaked for purity onto Brain Heart Infusion (BHI) agar (Difco, Fisher Scientific) for *Aeromonas* spp. and *Yersinia* spp. (*Flavobacterium* spp. were not re-streaked onto BHI agar due to long incubation times and slow growth of the organism) and incubated at 35°C for 24 hr.

#### **4.3.5 Molecular confirmation of *Yersinia* spp. and *Aeromonas* spp.**

*Aeromonas* spp. isolates were identified and confirmed using genus-specific PCR primers of the *gyrB* gene, which has previously been designed and proves to be an excellent molecular marker for confirmation of the genus *Aeromonas* (Yáñez *et al.*, 2003). Similarly, *Yersinia* spp. isolates were characterized using species-specific PCR in order to distinguish between *Y. enterocolitica* and *Y. ruckeri*, as genus-specific PCR primers for *Yersinia* spp. have yet to be developed. Three sets of PCR primers (Table 4.1) were used as previously published (Yáñez *et al.*, 2003; Gibello *et al.*, 1999; Wannet *et al.*, 2001) and synthesized by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). PCR was performed using a BioRad™ I-cycler iQ PCR machine. PCR mixtures for *Aeromonas* spp., *Y. enterocolitica* and *Y. ruckeri* (25 µL) contained 5 X GoTaq Flexi Buffer (5 µL), 1.5 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleoside triphosphate mix, 1.0 U of GoTaq Flexi DNA Polymerase (Promega), various primer

concentrations (Table 4.1), and 2.5 µL DNA sample. The reaction mixture for *Aeromonas* spp. was subjected to 35 cycles of amplification as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and a final extension at 72°C for 1 min. PCR conditions for *Y. enterocolitica* started with a denaturation step at 94°C for 5 min, followed by 36 subsequent cycles consisting of heat denaturation at 94°C for 45 s, primer annealing at 62°C for 45 s, and extension at 72°C for 45 s. A final extension was performed at 72°C for 7 min to complete synthesis of all strands. PCR amplification for *Y. ruckeri* consisted of 25 cycles of denaturation for 1 min at 92°C, annealing at 60°C for 1 min, and extension for 1 min at 72°C, followed by a final extension step of 72°C for 5 min. Amplified products were analysed by electrophoresis on 1.5% agarose-SB (sodium borate) gels, stained with ethidium bromide and visualized on a UV transilluminator.

**Table 4.1. PCR primers, products, and reference strains for *Aeromonas* spp., and *Yersinia* spp. among sub-surface flow constructed wetland samples receiving fish hatchery effluent**

Strain	Primer	Sequence (5'–3')	Product size (bp)	Primer conc. for PCR mixture	Reference
<i>Aeromonas</i> spp.	gyrB3F gyrB14R	TCCGGCGGTCTGCACGGCGT TTGTCCGGGTGTACTCGTC	1100	0.8µM (2µL)	Yáñez <i>et al.</i> (2003)
<i>Yersinia enterocolitica</i>	Y1 Y2	AATACCGCATAACGTCTTCG CTTCTTCTGCGAGTAACGTC	330	0.5µM (1.25µL)	Wannet <i>et al.</i> (2001)
<i>Yersinia ruckeri</i>	YER8 YER10	GCGAGGAGGAAGGGTTAAGTG GAAGGCACCAAGGCATCTCT	575	1.0µM (2.5µL)	Gibello <i>et al.</i> (1999)

#### 4.3.6 Antimicrobial susceptibility testing

Antimicrobial resistance patterns were determined using the disc-diffusion method as set by the Clinical and Laboratory Standards Institute (CLSI, 2007). As *Flavobacterium* spp. require low temperature and extended incubation time the existing guidelines for

susceptibility testing (CLSI, 2007) had to be modified. *Aeromonas* spp., *Yersinia* spp., and *Flavobacterium* spp. isolates were inoculated into Brain Heart Infusion (BHI) broth (Difco, Fisher Scientific) and grown to a 0.5 McFarland turbidity standard (4-6 hrs; 18hrs for *Flavobacterium* spp.) and swabbed onto Mueller Hinton (Difco, Fisher Scientific) agar plates. The following antimicrobial agents were selected as important representatives of different antibiotic classes, with abbreviations and disc concentrations shown in brackets: streptomycin (S 10 µg), cefotaxime (CTX 30µg), vancomycin (VA 30 µg), ciprofloxacin (CIP 5 µg), chloramphenicol (C 30 µg), ceftriaxone (CRO 30 µg), amoxicillin (AMC 30 µg), ceftazidime (CAZ 30 µg), doxycycline (D 30 µg), ampicillin (AM 10 µg), linezolid (LZD 30 µg), sulfisoxazole (G 0.25 mg) and sulfamethoxazole-trimethoprim (SXT 23.75/1.25 µg). After incubation for 18-24 hours at 35°C, 32°C, and 30°C for *Aeromonas* spp., *Yersinia* spp., and *Flavobacterium* spp., respectively, the isolates were classified as susceptible, intermediate, or resistant by comparing the diameters of inhibition zones with the breakpoints according to CLSI recommendations (CLSI, 2007). Organisms considered intermediate by the Kirby–Bauer disc-diffusion method were recorded as resistant for the purpose of this study.

#### **4.3.7 DNA extraction**

DNA was extracted from 100 mL of water samples and filtered onto a sterile 47-mm 0.22 µm polycarbonate filter (Millipore), within 3 hours of sample arrival. Each filter was carefully placed into a Mo Bio PowerSoil (Mo Bio Laboratories Inc., Carlsbad, CA) bead tube using sterile forceps. The filter was then cut into small pieces using a new, sterile No. 11 blade (Feather, Fischer Scientific, Whitby, ON) on a sterile No. 3 handled scalpel for

approximately 5 minutes in the bead beating tube (as provided in the PowerSoil DNA kit). Genomic DNA was subsequently extracted from water samples and bacterial cultures (control strains) using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA), following the manufacturer's protocol. The quantity and purity of the DNA extract was determined spectrophotometrically using a Nanophotometer (Montreal Biotech Inc., Montreal, Canada) at an absorbance of 260 nm, and  $A_{260}/A_{280}$ , respectively. The extracted DNA was stored in the freezer at -20°C for long-term storage.

#### **4.3.8 Selection of PCR primers and TaqMan® probes**

All primers and probes (Table 4.2) were synthesized by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Three sets of real-time PCR primers and probes were previously designed (Ng *et al.*, 2001; Volkmann *et al.*, 2004; Böckelmann *et al.*, 2009) to detect selected ARGs in water samples. TaqMan probes and primer sets were designed to detect *ampC*, and *blaSHV* (Table 4.2), while *tetA* was quantified using the SYBR Green approach.

**Table 4.2. Primers and probes used for the detection of ARGs via qPCR within the sub-surface flow constructed wetland**

Primer or Probe	Target gene	Sequence (5'–3')	Reference	GenBank accession no.
ampCF <sup>a</sup>	<i>ampC</i>	GGGAATGCTGGATGCACAA	Volkmann <i>et al.</i> (2004)	AJ005633
ampCR <sup>b</sup>	<i>ampC</i>	CATGACCCAGTTCGCCATATC		
ampCP <sup>c</sup>	<i>ampC</i>	TEXASRED-CCTATGGCGTGAAAACCAACGTGCA–BHQ-1 <sup>d</sup>		
SHVF	<i>blaSHV-5</i>	AACAGCTGGAGCGAAAGATCCA	Böckelmann <i>et al.</i> (2009)	X55640
SHVR	<i>blaSHV-5</i>	TGTTTTTCGCTGACCGGCGAG		
SHVP	<i>blaSHV-5</i>	FAM–TCCACCAGATCCTGCTGGCGATAG–BHQ-1		
tetAF	<i>tetA</i>	GCTACATCCTGCTTGCCTTC	Ng <i>et al.</i> (2001)	X61367
tetAR	<i>tetA</i>	CATAGATCGCCGTGAAGAGG		
SYBR <sup>e</sup>				

<sup>a</sup> Forward primer, <sup>b</sup> Reverse primer, <sup>c</sup> TaqMan probe, <sup>d</sup> BHQ-1, black hole quencher, <sup>e</sup> SYBR Green

The abundances of three ARGs (*ampC*, *blaSHV*, *tetA*) were quantified against their respective standard using the appropriate real-time PCR primers and probes (Table 4.2) and assay conditions as optimized previously (Volkmann *et al.*, 2004; Böckelmann *et al.*, 2009; Ng *et al.*, 2001). Genomic DNA was extracted from the following strains and used as positive controls for qPCR; *Enterobacter cloacae* (ATCC#BAA-1143) containing *ampC*, *Klebsiella pneumoniae* (ATCC#700603) containing *blaSHV*, and *E. coli* containing *tetA* (plasmid DNA). The developed primer–probe systems were tested by duplicate amplification runs of the serially diluted reference strains. The C<sub>t</sub> value (cycle threshold) in the quantification graphs for each respective concentration was used to finally generate the standard curve.

#### 4.3.9 Quantitative Polymerase Chain Reaction

Real-time quantitative PCR was used for the quantification of *ampC*, *blaSHV-5*, and *tetA* genes in total DNA extracted from water samples, and was performed with an iCycler & iQ Real-Time PCR System (Bio-Rad Laboratories; Mississauga, ON, Canada). Each 25  $\mu$ L PCR reaction contained template genomic DNA, primers and TaqMan® probes added to DyNAmo™ Probe qPCR Mastermix (New England Biolabs, Pickering, ON, Canada) to a final concentration of 250 nM TaqMan® probes, and 1  $\mu$ M for each primer. A volume of up to 5  $\mu$ L of DNA was added to each reaction. The following three-step PCR program was used for the quantification of ARGs: (i) a uracil-DNA glycosylase step at 50°C for 2 min, (ii) an initial denaturation and *Taq* polymerase activation step at 95°C for 10 min, and (iii) 55 cycles of 95°C for 15 s and 60°C for 1 min (Böckelmann *et al.*, 2009). SYBR Green was used for the quantification of *tetA*, as TaqMan probe sequences have not yet been developed for the *tetA* gene. Previous studies have shown SYBR Green to be a reliable method for the quantification of *tet* genes detected in cattle farm waste (Yu *et al.* 2005), groundwater (Mackie *et al.* 2006), river sediments (Pei *et al.* 2006), and different water environments (Zhang *et al.*, 2009). Reactions for *tetA* were conducted with a final volume of 20  $\mu$ L, including 10  $\mu$ L Power SYBR Green PCR Mastermix, plus 2  $\mu$ L each primer (1 $\mu$ M) and 5  $\mu$ L template DNA. The following protocol was used for the quantification of *tetA*: 94°C for 3 min, followed by 45 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s (Zhang *et al.*, 2009). Tenfold dilution series of the standards for the respective genes were run along with the unknown samples. Each sample was tested in duplicate for each run and two independent runs were performed. Quantification was achieved using standard curves obtained from the amplification profiles of known concentrations of the respective standard. For each probe

and primer set tested against wastewater DNA using real-time PCR, a negative control was tested, which included primers, the TaqMan® probe, PCR mastermix, and sterile water in place of DNA template.

#### **4.3.10 Statistical analysis**

The chi-squared test and student t-test were used to conduct the statistical analysis of the results (i.e., for comparison of antibiotic resistance phenotypes before and after OTC exposure; between different stages of treatment; and for comparison of concentration means). A *p* value of <0.05 was considered statistically significant. All statistical computations were performed using Microsoft Excel version 2007 for Windows.

### **4.4 Results and Discussion**

#### **4.4.1 Enumeration of selected fish pathogens in a constructed wetland**

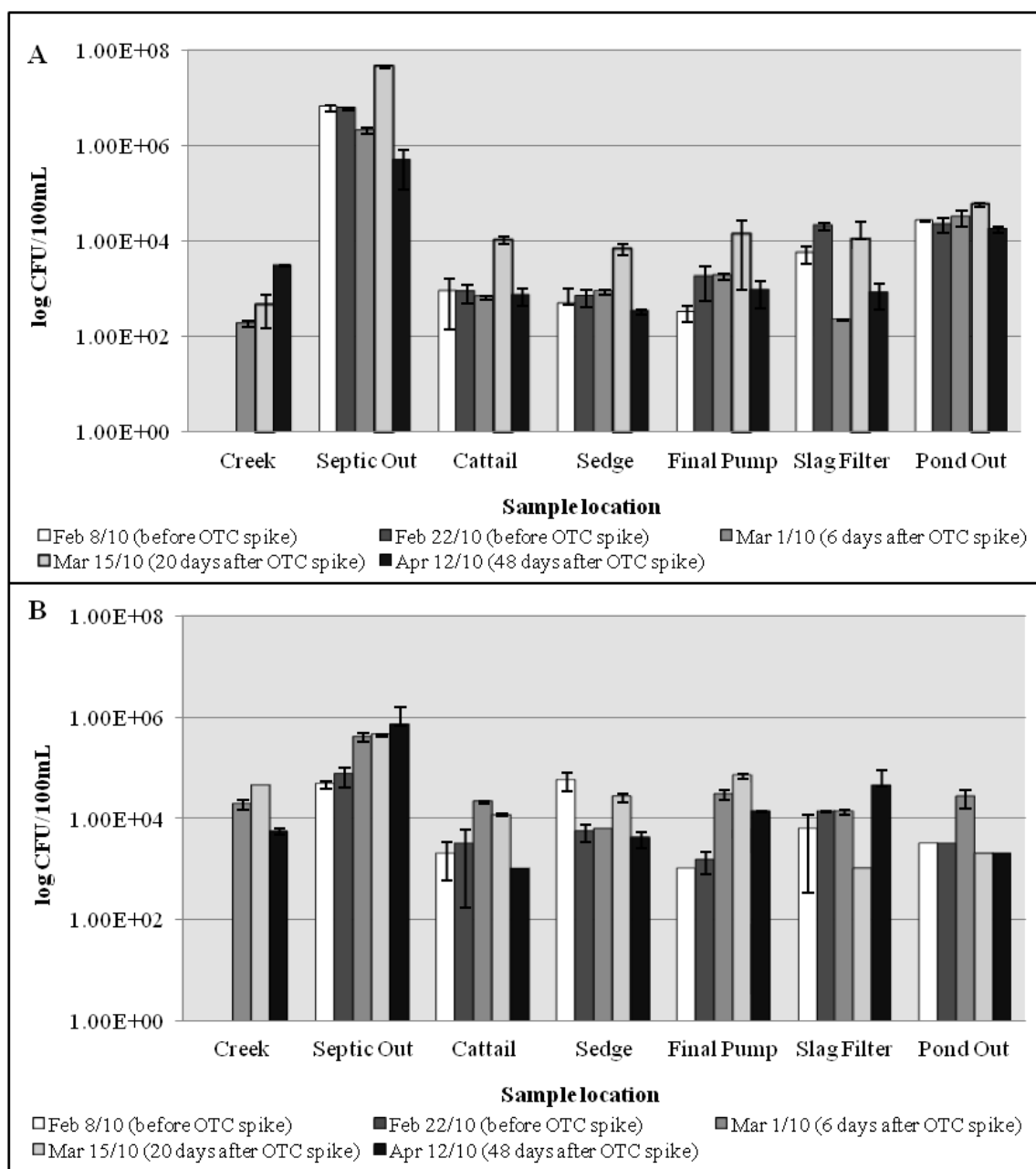
Culturable *Aeromonas* spp., *Yersinia* spp., and *Flavobacterium* spp., from various locations in the sub-surface flow CW were enumerated over the course of 10 weeks to evaluate the efficacy of a CW in the treatment of land-based fish farm discharge under cold climate environmental conditions, with a focus of evaluating removal of common fish pathogens. Total culturable *Aeromonas* spp., from seven different sampling locations are shown in Figure 4.3A, as well as bacterial counts on YSA for *Yersinia* spp., (Figure 4.3B) and enumeration of *Flavobacterium* spp. on nutrient agar supplemented with kanamycin (Figure 4.4).

Oxytetracycline (OTC) was added to the exit of the septic tank as a means of spiking the wetland, occurring over a five day period commencing February 23rd, 2010 and ending February 28th, 2010. With this dosing schedule, the microbial population of the wetland was sampled two times prior to the dosing of OTC and three times after the exposure of OTC. *Aeromonas* spp. and *Yersinia* spp. were enumerated both before and after the OTC exposure (total of five sampling dates); however, bacterial counts for *Flavobacterium* spp. were only determined following the OTC exposure (total of three sampling dates), as it was subsequently added as an additional pathogenic parameter later in the study.

The average concentration of total culturable *Aeromonas* spp., and *Yersinia* spp. in the constructed wetland were highest in the septic out sample throughout all sampling dates, reaching  $10^7$  and  $10^5$  CFU 100 mL<sup>-1</sup>, respectively (Figure 4.3). Concentrations of *Aeromonas* spp., and *Yersinia* spp. remained consistent throughout different stages of the treatment wetland, with very little difference between sample locations. However, increased levels of *Aeromonas* spp. were observed in the pond out sample, reaching final concentrations above  $10^4$  CFU 100 mL<sup>-1</sup>. Values in the pond indicate a slight increase, resulting from either an opportunity for growth or additional impacts. For example, occasionally the fish hatchery would experience volume constraints, prompting the release of additional wastewater (other than concentrated fish waste) into the existing settling ponds, which may ultimately lead to an increase among bacterial concentrations, including *Aeromonas* spp.. Overall, *Aeromonas* spp. was reduced throughout the wetland by approximately 2-3 orders of magnitude from the septic outflow to the final holding pond. It should also be noted that an increase of approximately 1-2 orders of magnitude was observed among *Aeromonas* spp. on Mar 15th



(20 days following the initial dosing of OTC), throughout all sampling locations (with the exception of pond out). This increase may be attributed to an alteration in cell surface properties, which could lead to either the detachment of the fixed microbial community or the impaired attachment ability within the constructed wetland after exposure to oxytetracycline. Similar studies have shown an increase in heterotrophic counts among interstitial bacterial communities in lab-scale constructed mesocosms following exposure to ciprofloxacin (Helt *et al.*, 2012) and acid mine drainage (Weber *et al.*, 2008). It is important to note that creek samples were only collected in March and April, as the creek was frozen over and covered with snow during the month of February. The receiving creek also provides pseudo-background environmental values as the samples were taken upstream of the pond outflow (Figure 4.3, 4.4).



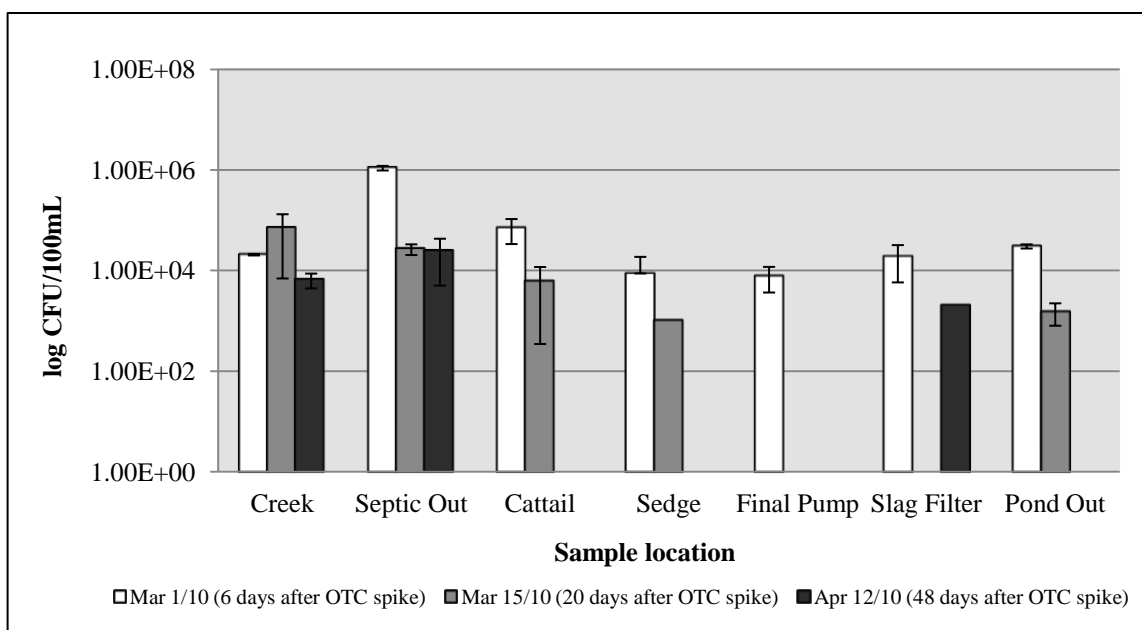
**Figure 4.3. Mean values of *Aeromonas* spp. (A) and *Yersinia* spp. (B) as colony-forming units (CFUs) per 100 mL from various locations in a sub-surface flow constructed wetland system collected over five sampling dates. Error bars indicate standard deviation.**

Overall, *Yersinia* spp. were reduced throughout the wetland by approximately 1-2 orders of magnitude from the septic outflow to the final holding pond, with approximately  $10^3$ - $10^4$

CFU 100 mL<sup>-1</sup> released into the receiving water body via the final holding pond. *Yersinia* spp. concentrations appeared to be unaffected by the OTC exposure, with concentrations remaining fairly constant before and after the antibiotic addition, as shown in Fig. 4.3B. In general, the constructed wetland showed increased efficiency at removing *Aeromonas* spp. (two to three log reduction), compared to that of *Yersinia* spp., and *Flavobacterium* spp., Fig. 4.4 (one to two log removal). However, this observation is somewhat expected, as the initial concentration of *Aeromonas* spp. present in the septic outflow was approximately two orders of magnitude higher than both *Yersinia* spp., and *Flavobacterium* spp. during each of the sampling events. The results are consistent with those of Bomo *et al.* (2003) who studied the removal of fish pathogenic bacteria in biological sand filters, and observed significantly higher ( $p < 0.05$ ) removal efficiencies for aeromonads (*A. hydrophila* and *A. salmonicida*) compared to *Y. ruckeri*. A main removal mechanism in constructed wetlands is attachment of bacteria to the biofilm covering the filter media surface (Kadlec and Wallace, 2009), where increased cell surface hydrophobicity will facilitate attachment to surfaces, in addition to cell surface structures and cell surface charges (Stenstøm, 1989; Zita and Hermansson, 1994; 1996). Bomo *et al.* (2003) measured the relative hydrophobicity of the different bacterial strains used in their study and showed that aeromonads had a higher cell surface hydrophobicity than *Yersinia ruckeri*. This is in agreement with the differences seen for bacterial removal efficiency, where a significantly higher removal rate was observed for the more hydrophobic aeromonads. Other studies have also documented that both *A. hydrophila* and *A. salmonicida* possess hydrophobic cell surface characteristics that facilitate adhesion to surfaces (Dalsgaard *et al.*, 1994; Enger and Thorsen, 1992; Rahman and Kawai, 1999). In contrast, *Y. ruckeri* was observed to have negative values for the measured hydrophobicity

(Bomo *et al.*, 2003). Other studies have also documented that the hydrophobic properties of the cell surface of *Y. ruckeri* are low and negative results are found for autoagglutination (Romalde *et al.*, 1990).

Bacterial counts for *Flavobacterium* spp. were only determined following the oxytetracycline exposure (total of three sampling dates), as it was subsequently added as an additional pathogenic parameter later in the study (determined after OTC exposure). The average total concentration of culturable *Flavobacterium* spp. in the constructed wetland is shown in Figure 4.4, reaching  $10^6$  CFU 100 mL<sup>-1</sup> in the septic out sample on Mar 1/10. Concentrations of *Flavobacterium* spp. collected six days following OTC exposure (Mar 1/10) remained fairly consistent throughout various stages of the treatment wetland, with very little difference between sample locations (resulting in a 1-2 log reduction from septic to final pond). Cattail and sedge cells were comparable with removal through the sedge cell being slightly better by about half a log. The number of *Flavobacterium* spp. decreased over the last two sampling events (20 and 48 days following OTC exposure), falling to non-detectable limits in most of the samples (with the exception of creek, septic, and slag filter) by Apr. 12/10 (Figure 4.4).



**Figure 4.4. Mean values of *Flavobacterium* spp. as colony-forming units (CFUs) per 100 mL from various locations in a sub-surface flow constructed wetland system collected over three sampling dates. Error bars indicate standard deviation.**

Although *Flavobacterium* spp. are associated with significant mortalities in fish culture, the ecology and biology of this bacterial species has been poorly examined. Limited information is available on the survival capacity of *Flavobacterium* spp. in the environment (Vatsos *et al.*, 2003; Madetoja *et al.*, 2003), and limited data exist on the enumeration or quantification of *Flavobacterium* spp. in fish farm wastewater. *Flavobacterium* spp. are considered to be very fastidious organisms, and problems with the culture of the bacterium have been reported (Daskalov *et al.*, 1999; Michel *et al.*, 1999). Furthermore, *Flavobacterium* spp. are considered to be an important group of bacteria involved in the cometabolic degradation of organo pollutants, which involves the transformation of a substance without nutritional advantage in the presence of a growth substrate (Fritsche and Hofrichter, 2008). Under adverse conditions, *Flavobacterium* spp. may enter a viable but nonculturable state, as is the case with many other bacterial species, such as *Salmonella* spp. (Dupray *et al.*, 1997) and

*Yersinia ruckeri* (Thorsen *et al.*, 1992). As a result, this characteristic prohibits the detection of this pathogen in environmental samples when culture-based methods are used for identification. Internal cellular influences also affect the ability of microbes to survive under different environmental conditions, such as the endogenous metabolism of the bacterium, the presence of material stored in the bacterial cell, the energy requirement of the cell, adenylate energy charge and the preservation of membrane potential (Vatsos *et al.*, 2003). The undetectable levels of *Flavobacterium* spp. during the final sampling event (Apr.12/10) in cattail, sedge, final pump, and pond samples could be attributed to the bacteria entering a viable but nonculturable state. Vatsos *et al.* (2003) studied the starvation of *F. psychrophilum* in broth, stream water and distilled water, concluding that the bacterium appeared to stop multiplying and became smaller and rounded when maintained in stream water. However, the viability of *F. psychrophilum* maintained in stream water (assessed using a live/dead kit) was found to be considerably higher than of bacteria maintained in broth (Vatsos *et al.*, 2003). It is possible that the cells maintained in the stream water had a lower metabolic activity, resulting in delayed cell death due to the limited nutrients in the water, while the bacteria maintained in broth could have increased cell death due to a possible accumulation of toxic by-products as a result of the bacteria metabolising the nutrients in the broth. The culturability of *Flavobacterium* spp. declined until it was no longer possible to obtain colonies on agar plates at the end of the trial at nineteen weeks (Vatsos *et al.*, 2003).

#### 4.4.2 Qualitative PCR to detect and characterize *Aeromonas* spp. and *Yersinia* spp.

Genotypic confirmation using qualitative PCR was performed on a total of 236 presumptive *Aeromonas* spp. isolates and 195 presumptive *Yersinia* spp. isolates, collected from various water samples throughout the sub-surface flow constructed wetland. Among these isolates, 71.6% were confirmed as *Aeromonas* spp. using genus-specific PCR (Table 4.3), reacting with the *gyrB* gene, which has previously been designed for confirmation of the genus *Aeromonas* (Yáñez *et al.*, 2003). The selective media ampicillin dextrin agar (ADA) was developed in 1987 and several reports have shown it to be one of the best media for recovery of *Aeromonas* spp. (Handfield *et al.*, 1996). ADA employs an antibiotic, ampicillin, and a detergent, deoxycholate, as selective agents (most *Aeromonas* spp. are intrinsically resistant to ampicillin and deoxycholate). Since all known aeromonads ferment dextrin, producing acid by-products, they appear as yellow colonies on ADA. Other non-dextrin fermenting bacteria appear green or colorless on ADA. It is possible that 28.4% of the colonies selected were not "true" yellow colonies on ADA, and therefore, only 71.6% (169/236) of total isolates amplified from the expected 1100 bp product.

**Table 4.3. Molecular confirmation of presumptive *Aeromonas* spp. isolates and distribution of *Y. ruckeri* and *Y. enterocolitica* among screened isolates in the constructed wetland**

Species	Total # isolates	Confirmed +ve by PCR	% Confirmed
<i>Aeromonas</i> spp.	236	169	71.6%
<i>Yersinia ruckeri</i>	195	0	0.0%
<i>Yersinia enterocolitica</i>	195	8	4.1%

Similarly, *Yersinia* spp. isolates were characterized using species-specific PCR in order to distinguish between *Y. enterocolitica* and *Y. ruckeri*, as genus-specific PCR primers for

*Yersinia* spp. have not been established (Table 4.3). Among the total number of screened presumptive *Yersinia* spp. isolates (n=195), only 4.1% (8/195) were characterized as *Y. enterocolitica*, and zero were considered to be *Y. ruckeri*. *Y. enterocolitica* is considered to be an important human pathogen with a global distribution (Bottone, 1999; Ostroff, 1995). Surveillance data worldwide illustrate an extensive increase in the number of non-outbreak-related isolates and cases of yersiniosis reported in the last two decades. This notice has led to the referral of *Y. enterocolitica* as a potential emerging enteric human pathogen (Ostroff, 1995; Tauxe, 1997). Besides *Y. enterocolitica*, two other *Yersinia* species, *Y. pseudotuberculosis* and *Y. pestis*, have long been known to cause human disease (Carniel and Mollaret 1990; Bottone 1999). The remaining eight species (*Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. aldovae*, *Y. rohdei*, *Y. bercovieri*, *Y. mollaretii* and *Y. ruckeri*) have not been studied extensively and because of the absence of classical *Yersinia* virulence markers, they have been generally considered to be environmental and nonpathogenic species. The majority of these species are found in the environment, isolated from freshwater sources such as rivers, lakes (Massa *et al.* 1988), drinking water (Kuznetsov and Timchenko 1998), and sewage (Ruhle *et al.* 1990; Ziegert and Diesterweg 1990). From the results of this study, it is important to note that presumptive *Yersinia* spp. isolates require more discriminate analysis, as only two (out of 11) known *Yersinia* species were screened. Yersinia Selective Agar (YSA) was used as a differential selective medium for the isolation of *Yersinia enterocolitica*. However, YSA may also be used for the isolation of *Yersinia* species other than *Y. enterocolitica*, including *Y. pseudotuberculosis*, *Y. frederiksenii*, and *Y. intermedia* (Bockemühl and Wong, 2003). Therefore, it is possible that species other than *Y. enterocolitica* were selected for on YSA; however, due to the inability to screen isolates



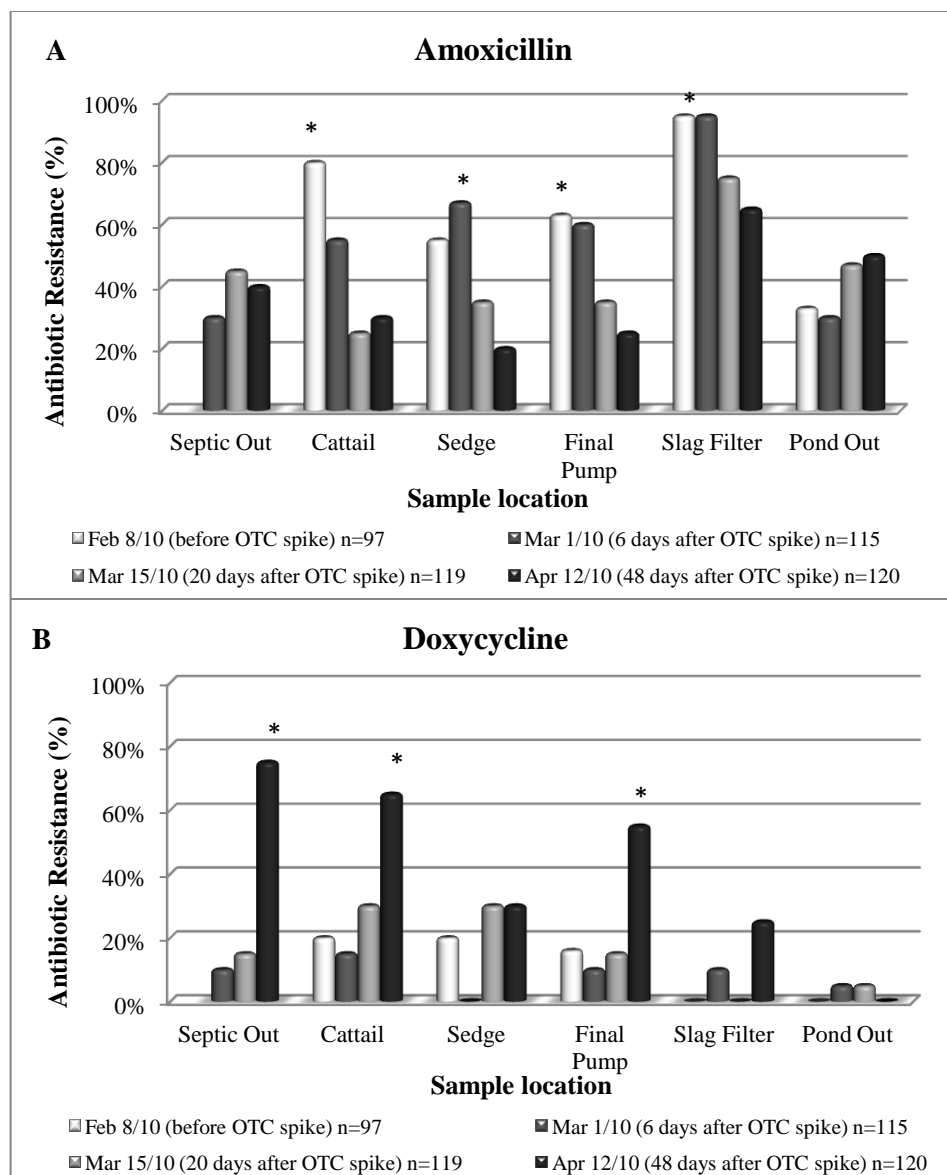
using genus-specific primers it was not possible to confirm all presumptive *Yersinia* spp. isolates using the selected PCR primers. Falcao *et al.* (2004) examined water and sewage for the presence of Yersinia other than *Y. pseudotuberculosis* and *Y. pestis* and found that of the 11 known species of *Yersinia*, only four (*Y. enterocolitica*, *Y. intermedia*, *Y. frederiksenii*, and *Y. kristensenii*) were isolated from the tested water sources and sewage. The distribution of the 144 *Yersinia* strains was classified as *Y. enterocolitica* (67 strains), *Y. intermedia* (64 strains), *Y. frederiksenii* (9 strains), and *Y. kristensenii* (3 strains) (Falcao *et al.*, 2004). Therefore, it is possible that a large proportion of the "unclassified" *Yersinia* spp. isolates are among the other common environmental strains, including *Y. intermedia*, *Y. frederiksenii*, and *Y. kristensenii*. The low prevalence of *Y. enterocolitica* (4.1%) among culturable isolates from water samples was comparable to results of other researchers. For example, Sulakvelidze *et al.* (1996) analysed environmental *Yersinia* isolates and found only 14 (1%) of 1,295 samples from environmental sources, including soil and water were *Y. enterocolitica* or *Y. enterocolitica*-like species. Similarly, Brennhovd *et al.* (1992) isolated *Yersinia* spp. from only a small proportion (4.2%) of polluted surface water samples. The detection of *Y. ruckeri* among 195 culturable *Yersinia* spp. isolates in this study was zero. Previous studies have shown that the incidence of *Y. ruckeri* and occurrence of enteric redmouth outbreaks, in freshwater fish farms has been correlated with season and water temperature (Rodgers 1991, 1992; Romalde *et al.*, 1994). Therefore, it is possible that the low detection among culturable *Yersinia* spp. isolates found here is related to the season and water temperature, with the majority of ERM outbreaks occurring at water temperatures ranging from 11 to 18°C (Rodgers 1992). Romalde *et al.* (1994) monitored the incidence of *Y. ruckeri* for two fish farms during a one-year period and found peaks of isolation in the

autumn samples, with CFU/mL increasing with temperature, and peaks observed between 14 and 18°C. However, temperatures higher than 19-20°C were found to inhibit the appearance of *Y. ruckeri* (Romalde *et al.*, 1994). The influent and effluent water temperatures observed for Feb-April ranged between 5-10°C depending on the sampling date, suggesting that the water temperature may have been too cold to allow for the persistence of *Y. ruckeri*. A comparable study monitoring bacterial fish pathogens over a 2-year period in a freshwater trout farm found only one outbreak of disease caused by *Y. ruckeri* serotype O1 and the infection coincided with a rise in water temperature from 5 to 12 °C (Dalsgaard & Madsen, 2000).

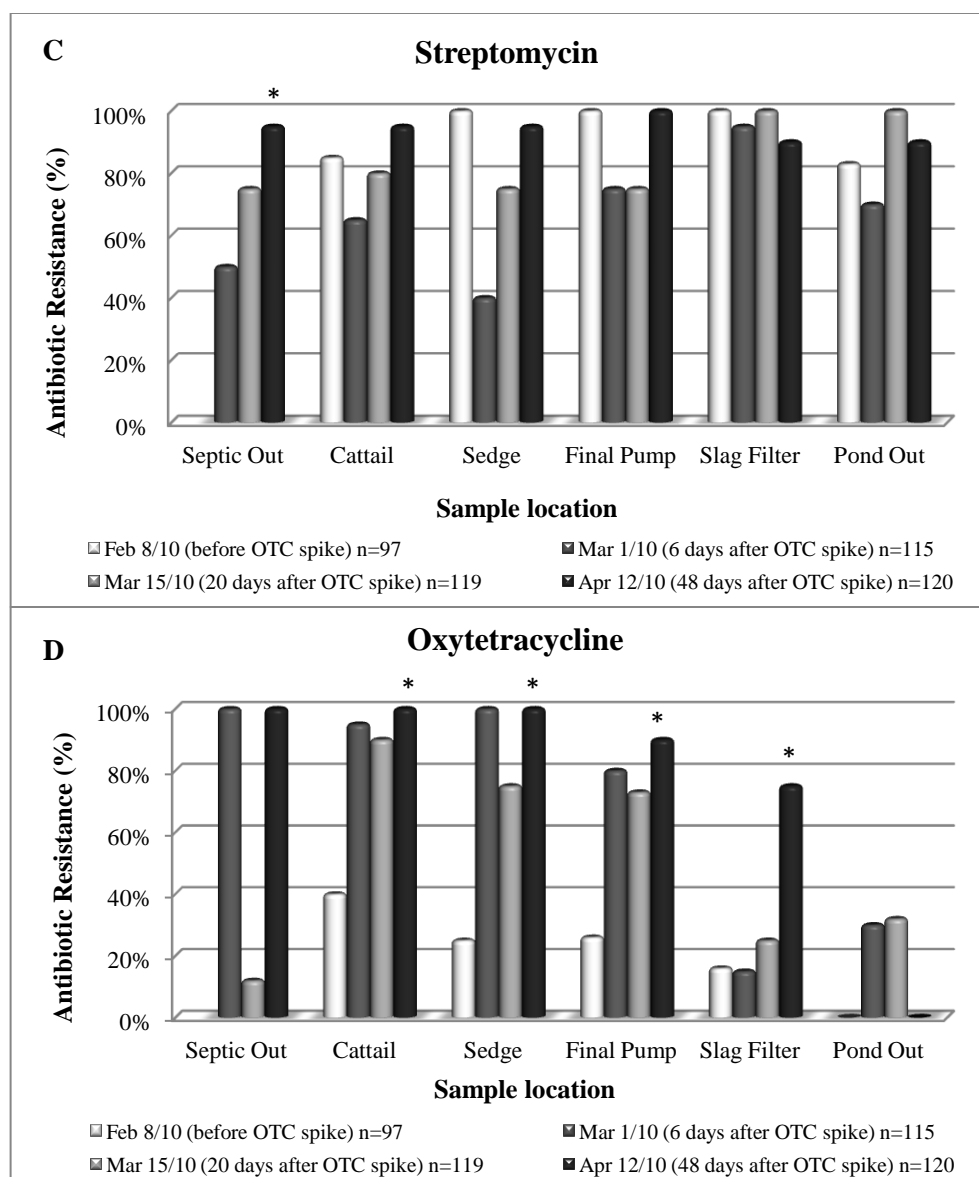
#### **4.4.3 Frequency and distribution of antibiotic resistance among *Aeromonas* spp., *Yersinia* spp., and *Flavobacterium* spp.**

The antibiotic susceptibility results were obtained for *Aeromonas* spp., *Yersinia* spp., and *Flavobacterium* spp. isolates from different sampling locations in the sub-surface flow constructed wetland both before and after the OTC exposure (with the exception of *Flavobacterium* spp.). Among *Aeromonas* spp., the most commonly observed resistance profiles for all sampling events were to amoxicillin, doxycycline, streptomycin, and oxytetracycline, as shown in Figure 4.5. The resistance level among doxycycline, streptomycin, and oxytetracycline in various stages of the constructed wetland demonstrated a significant increase ( $p < 0.05$ ) following OTC exposure (6, 20, and/or 48 days post inoculation; Figure 4.5). The initial resistance levels to OTC among *Aeromonas* spp. isolates from the cattail, sedge, final pump, and slag filter samples before the antibiotic exposure (Feb 8/10) were 45%, 25%, 26%, and 16%, respectively, increasing to 100%, 95%, 100%,

and 80%, 7 days post oxytetracycline exposure (Figure 4.5D). The level of OTC resistance among *Aeromonas* spp. remained high, reaching 100% in most samples by April 12/10 (48 days following antibiotic spike). Similarly, the level of doxycycline resistance increased significantly ( $p < 0.05$ ) following OTC exposure (48 days), reaching 75%, 65%, 55%, among the septic out, cattail, and final pump samples, respectively (Figure 4.5B). Amoxicillin was the only antibiotic that revealed a significant ( $p < 0.05$ ) decrease in resistance among *Aeromonas* spp. isolates following the OTC exposure (48 days). Initial amoxicillin resistance levels among *Aeromonas* spp. isolated from cattail, sedge, final pump, and slag filter samples were 80%, 55%, 63%, and 95%, respectively, decreasing to approximately 30%, 20%, 25%, and 65%, by April 12/10 (Figure 4.5A). Streptomycin resistance remained relatively consistent throughout the constructed wetland samples (slight differences between sampling events), maintaining nearly 100% resistance among all samples 48 days following the OTC exposure (Figure 4.5C). The overall comparison between different sampling locations in the constructed wetland revealed apparent differences in levels of antibiotic resistance among *Aeromonas* spp. isolates, both before and after OTC exposure. The differences in antibiotic resistance levels observed between samples are most likely due to the ecological complexity or the temporal and spatial variations in wetland processes occurring in the various stages of the sub-surface flow constructed wetland system. However, little differences were noted between cattail and sedge samples, with antibiotic resistance levels among *Aeromonas* spp. isolates remaining comparable between the two planted wetland “cells” (Figure 4.5).



**Figure 4.5. Prevalence of antibiotic resistance (%) among *Aeromonas* spp. to amoxicillin (A), doxycycline (B), streptomycin (C), and OTC (D) in various sampling locations in the constructed wetland before and after (6, 20, 48 days) the OTC exposure. An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n= number of isolates.**



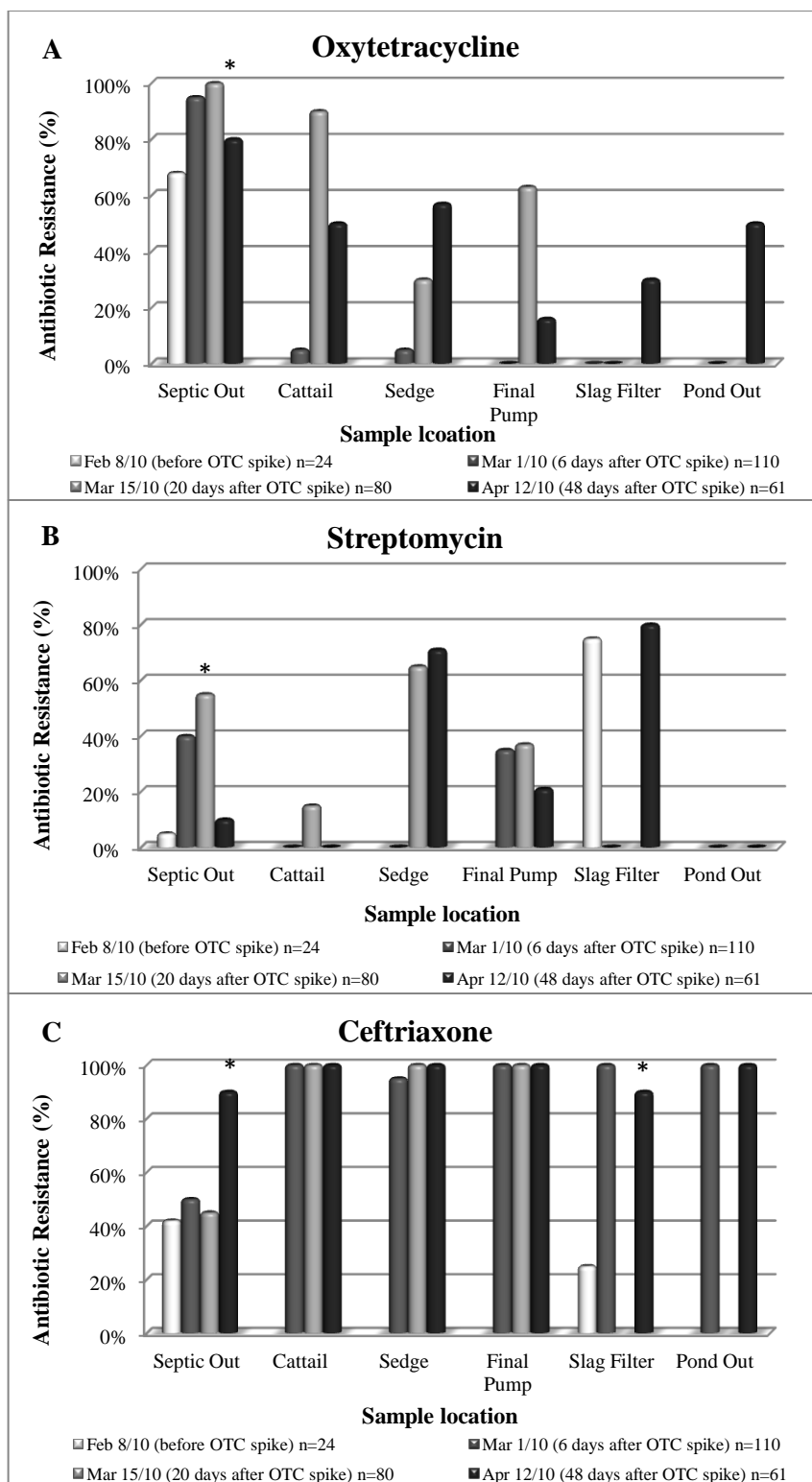
**Figure 4.5. Prevalence of antibiotic resistance (%) among *Aeromonas* spp. to amoxicillin (A), doxycycline (B), streptomycin (C), and OTC (D) in various sampling locations in the constructed wetland before and after (6, 20, 48 days) the OTC exposure. An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n= number of isolates.**

The results for *Aeromonas* spp. illustrate the potential for a single antibiotic to create a significant increase in antibiotic resistance to tetracycline based antibiotics. The tetracyclines, including both OTC (first generation) and doxycycline (second generation), are a family of antibiotics that inhibit protein synthesis by preventing the attachment of

aminoacyl-tRNA to the ribosomal acceptor (A) site (Chopra and Roberts, 2001). Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of Gram positive and Gram negative bacteria, and protozoan parasites. The favourable antimicrobial properties of these agents and the absence of major adverse side effects have led to their extensive use in the therapy of human and animal infections. Furthermore, in some countries, including Canada and the United States, tetracyclines are added at subtherapeutic levels to animal feeds to act as growth promoters (OMAFRA, 2005). Resistance to tetracyclines has emerged in many commensal and pathogenic bacteria due to the acquisition of *tet* genes. Twenty-nine different tetracycline resistance (*tet*) genes and three OTC resistance (*otr*) genes have been characterized (Chopra and Roberts, 2001). Eighteen of the *tet* genes and one of the *otr* genes code for efflux pumps, and seven of the *tet* genes and one of the *otr* genes code for ribosomal protection proteins. All the *tet* efflux genes code for membrane-associated proteins which export tetracycline from the cell, thereby reducing the intracellular drug concentration and protecting the ribosomes in the cell (Chopra and Roberts, 2001). The increase in resistance to OTC and doxycycline among *Aeromonas* spp. following the 5-day OTC exposure may be attributed to the acquisition or upregulation of certain efflux pump-encoded genes. In addition, the majority of *tet* genes in bacteria have been associated with mobile plasmids, conjugative transposons, and integrons (Recchia and Hall, 1995; Roberts 1996, 1997), enabling the potential movement of *tet* genes from species to species via conjugation. Resistance to several antibiotics as seen here could also be due to a single physiological stress response/adaption. Exposure of bacteria to sub-therapeutic levels of antimicrobial concentrations is thought to increase the occurrence of antimicrobial resistance. Langlois *et al.* (1986) found that in a pig herd where the animals were

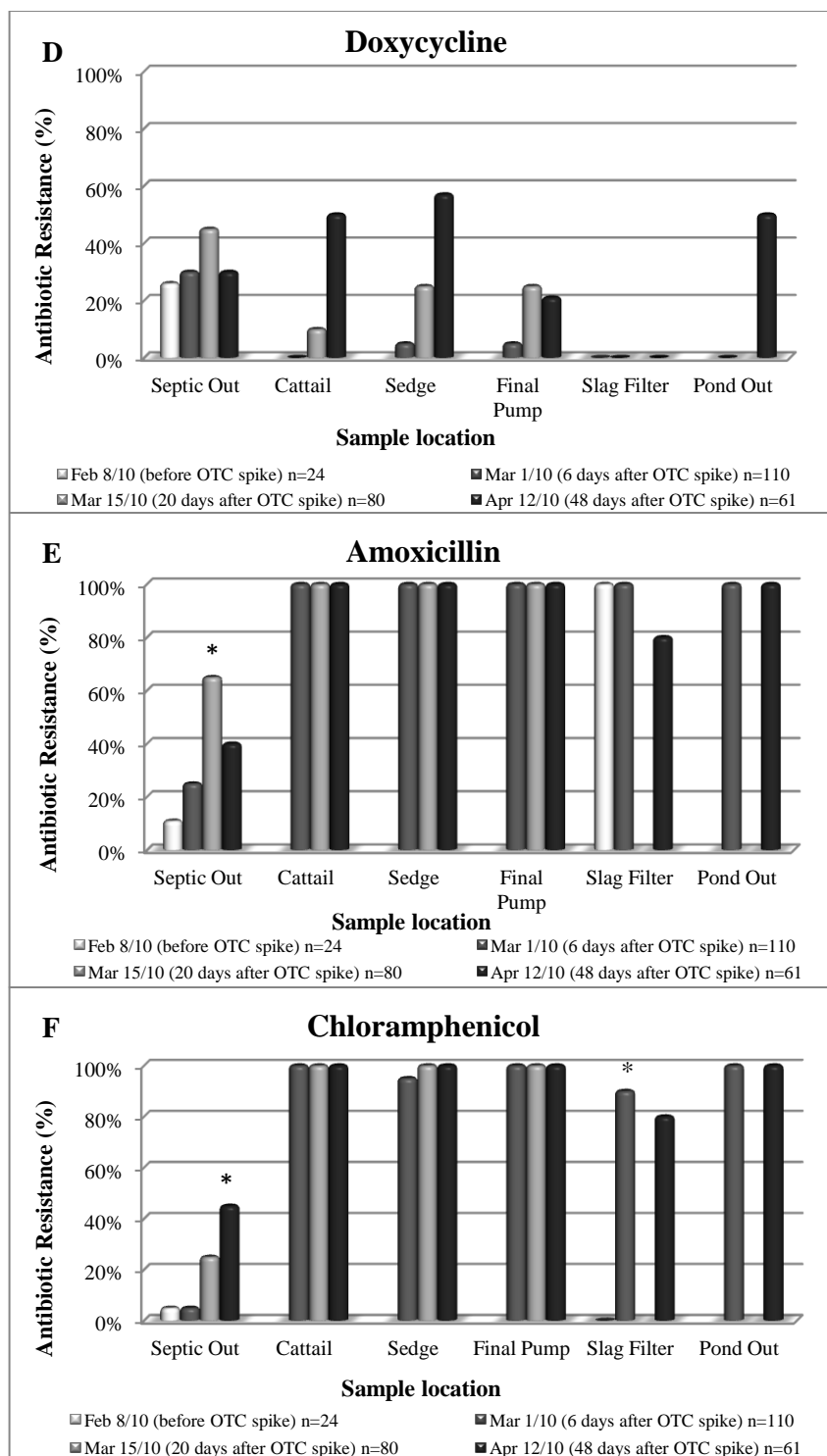
continuously exposed to antibiotics, both as feed additives and therapeutic agents, almost 100% of the faecal coliform bacteria were tetracycline resistant. Similarly, Helt *et al.* (2012) studied the antibiotic resistance profiles among faecal indicators following ciprofloxacin exposure in constructed mesocosms and concluded that the transient presence of an isolated exposure event can lead to an increase in resistance, not only to ciprofloxacin, but to other classes of antibiotics as well. The results obtained for *Aeromonas* spp. isolates are in agreement with those of others, suggesting that antibiotic exposure (or the use of OTC in fish farming) can enhance the frequency of OTC-resistant microorganisms (DePaola *et al.*, 1995; Samuelsen *et al.*, 1992; Hansen *et al.*, 1993; Kerry *et al.*, 1996) and consequently of OTC-resistant fish pathogens (Björklund *et al.*, 1991).

The prevalence of antibiotic resistance among *Yersinia* spp. isolates to six common antibiotics before and after the addition of OTC is shown in Figure 4.6. It is important to note that the number of isolates (n) screened for antibiotic resistance at each sampling date was limited to the number of culturable isolates enumerated at that time (due to difficult growth requirements/conditions). Therefore, the initial background levels of antimicrobial resistance observed before the OTC exposure could only be determined from *Yersinia* spp. enumerated from the septic out and slag filter samples (as *Yersinia* spp. isolates from all other samples produced no growth during the antimicrobial susceptibility test method).



**Figure 4.6. Prevalence of antibiotic resistance (%) among *Yersinia* spp. to OTC (A), streptomycin (B), ceftriaxone (C), doxycycline (D), amoxicillin (E), and chloramphenicol (F) in various sampling locations in the constructed wetland before and after (6, 20, 48 days) the OTC exposure. An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n= number of isolates.**





**Figure 4.6. Prevalence of antibiotic resistance (%) among *Yersinia* spp. to OTC (A), streptomycin (B), ceftriaxone (C), doxycycline (D), amoxicillin (E), and chloramphenicol (F) in various sampling locations in the constructed wetland before and after (6, 20, 48 days) the OTC exposure. An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n= number of isolates.**

The resistance level among *Yersinia* spp. to oxytetracycline, streptomycin, ceftriaxone, amoxicillin, and chloramphenicol collected from the septic out sample was found to significantly increase ( $p < 0.05$ ) following OTC exposure (after 6, 20, and/or 48 days post inoculation; Figure 4.6). The initial proportion of tested isolates displaying resistance to OTC among *Yersinia* spp. from the septic out and slag filter samples before the antibiotic exposure (Feb 8/10) were 68% and 0%, increasing to 100% (20 days post spike) and 30% (48 days post spike), respectively (Figure 4.6A). Resistance to amoxicillin (Figure 4.6E), ceftriaxone (Figure 4.6C), and chloramphenicol (Figure 4.6F) among *Yersinia* spp. in almost all sampling locations was considerably more common than to doxycycline (Figure 4.6D), streptomycin (Figure 4.6B), and OTC, reaching nearly 100% in all samples (with the exception of select septic and pond out samples) following OTC exposure (6, 20, and 48 days post spike). In *Y. enterocolitica*, resistance to penicillins and cephalosporins has been attributed to the production of  $\beta$ -lactamases (Hornstein *et al.*, 1985; Tzelepi *et al.*, 1999). Cornelis and Abraham (1975) studied the  $\beta$ -lactamases produced by a number of *Y. enterocolitica* strains of human and animal origin extensively and characterised two chromosomally mediated enzymes; including a constitutive penicillinase termed enzyme A and an inducible cephalosporinase labelled enzyme B. A possible explanation for the high levels of resistance to amoxicillin and ceftriaxone (third generation cephalosporin) among *Yersinia* isolates tested may be attributed to the production of  $\beta$ -lactamases, including both penicillinases and cephalosporinases. Sharifi Yazdi *et al.* (2011) studied antibiotic susceptibilities of *Y. enterocolitica* and other *Yersinia* species recovered from meat and chicken, and reported high levels of resistance to cephalotin (98%) and ampicillin (58%) among *Yersinia* isolates, followed by much lower resistance levels to tetracycline (25%) and

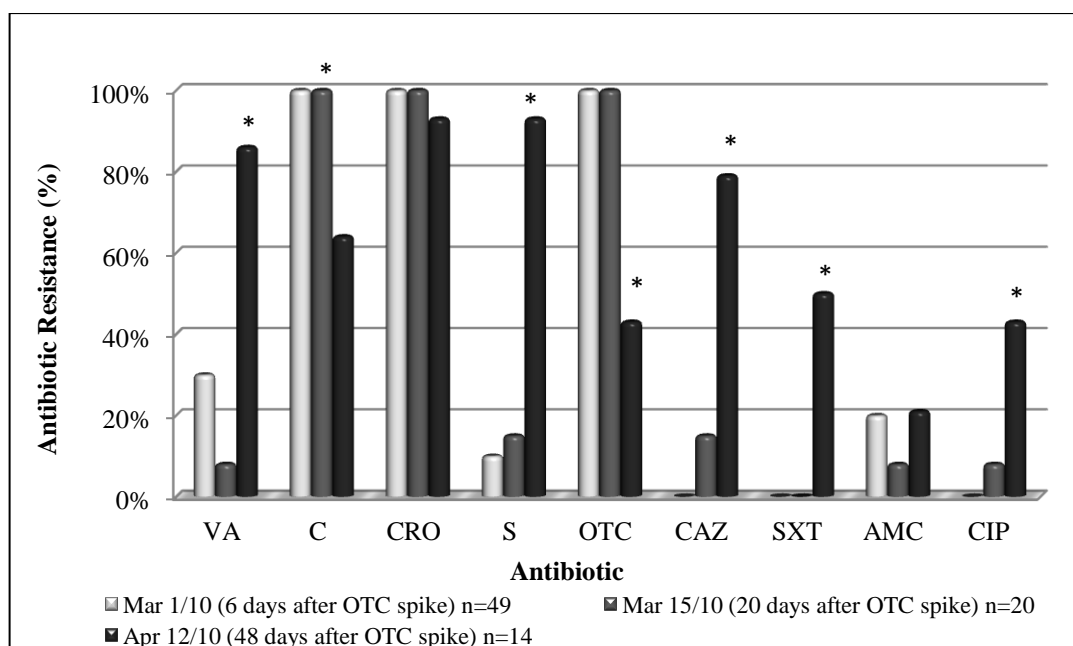
streptomycin (42%), respectively. Results here are in agreement with those of Sharifi Yazdi *et al.* (2011), indicating a much higher level of resistance to cephalosporins, and penicillins (amoxicillin), compared to tetracycline-based antibiotics or aminoglycosides (streptomycin).

These results illustrate the significant impact that antibiotic use in trout farming and fish hatcheries may have on environmental bacteria and fish pathogens, as antibiotic resistance levels remained high in the pond or outlet samples (Figures 4.5, 4.6 & 4.7). Similarly, Schmidt *et al.* (2000) observed a significant increase in antibiotic resistance among culturable aeromonads in outlet or pond samples when compared to inlet samples. The results for *Yersinia* spp. illustrate the potential for a single antibiotic to create a significant increase in antibiotic resistance, not only to the antibiotic itself, but to additional antibiotics as well. A possible explanation for the increased resistance among several antibiotics could be the acquisition of a multi-drug resistance plasmid. Plasmid-mediated transmission is the most common mechanism of horizontal gene transfer (HGT) (Norman *et al.*, 2009) with subinhibitory concentrations of antibiotics facilitating the process of antibiotic resistance development (Davies *et al.*, 2006). For example, antimicrobials have been shown to enhance gene transfer and recombination (Couce and Blazquez, 2009), partially through the activation of the SOS system (Guerin *et al.*, 2010). In addition, antimicrobials have been shown to induce phage production from lysogens. Such factors may play an important role in the proliferation of gene exchange in aquatic environments. Similarly, Westen (1996) concluded that the use of one antimicrobial agent can lead to increased levels of resistance not only to a specific drug but to many others, including those which use different modes of antibacterial action (cross resistance). The results obtained for *Yersinia* spp. enumerated

from the 'septic out' sample supports this concept, in which the introduction of one antibiotic can lead to an increase in resistance, not only to OTC (tetracycline class), but to other classes of antimicrobials as well (cephalosporins, penicillins, aminoglycosides and chloramphenicol). The results reported in this work increase concern regarding the creation of reservoirs of transferable antimicrobial resistance in the fish farming industry and the importance of evaluating the role of these environments in the dissemination and evolution of resistance genes and their vectors. The presence of high numbers of antibiotic resistant bacteria in the outflow (holding pond) of a sub-surface flow constructed wetland used to treat fish hatchery wastewater has ecological and public health implications and emphasizes the need for further studies in relation to the genes encoding resistance in different bacterial species. Further epidemiological and molecular investigations are needed in order to evaluate the presence of genetically mobile ARGs in the human and animal food chain (Levy, 1989; Young, 1993; Sørum, 1998). Also, these results prompt the necessity of establishing policies (Gould, 1999) to reduce current levels of antibiotic use in the fish farming industry.

The prevalence of *Flavobacterium* spp. isolates resistant to nine common antibiotics in the septic out sample of the constructed wetland following the addition of OTC is shown in Figure 4.7. It is important to note the differences in the number of isolates screened for antibiotic resistance during each sampling date was limited to the number of culturable isolates enumerated at that time (due to difficult growth requirements/conditions). Therefore, due to the fastidious nature of *Flavobacterium* spp. antibiotic resistance profiles were limited to only one sampling location (septic out) and three sampling events (as growth

was limited or absent among other sampling locations when performing the antimicrobial susceptibility test method).



**Figure 4.7. Prevalence of antibiotic resistance (%) among *Flavobacterium* spp. isolated after the OTC exposure in the 'Septic Out' sample. The following antimicrobial agents were selected as important representatives of different antibiotic classes (abbreviations are shown in brackets); vancomycin (VA), chloramphenicol (C), ceftriaxone (CRO), streptomycin (S), oxytetracycline (OTC), ceftazidime (CAZ), sulfamethoxazole-trimethoprim (SXT), amoxicillin (AMC), and ciprofloxacin (CIP). An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n = number of isolates.**

The resistance level among the following antibiotics demonstrated a significant ( $p < 0.05$ ) increase from Mar 1/10 (6 days post OTC spike) to April 12/10 (48 days post spike) in the septic out sample: vancomycin (VA), streptomycin (S), ceftazidime (CAZ), sulfamethoxazole-trimethoprim (SXT), and ciprofloxacin (CIP), as shown in Figure 4.7. The levels of antimicrobial resistances before the OTC exposure were not determined, therefore, it is difficult to make any conclusions regarding the effect of an antibiotic exposure on the

prevalence of resistance among *Flavobacterium* spp.. However, a significant increase in resistance among isolates was observed between sampling events, with VA, S, CAZ, SXT, and CIP displaying 30%, 10%, 0%, 0%, and 0% resistance on Mar 1/10, increasing to 86%, 93%, 79%, 50%, and 43%, respectively, by the final sampling event. The increase in resistance between sampling events may be attributed to the OTC exposure, as also hypothesized with *Aeromonas* spp., and *Yersinia* spp., thereby creating a physiological stress response/adaption. Exposure of bacteria to sub-therapeutic levels of antimicrobial concentrations is thought to increase the occurrence of antimicrobial resistance (Davies *et al.*, 2006). Antimicrobial resistance in bacteria can be the result of a number of genetic mechanisms, including, chromosomal mutations, expression of a latent resistance gene, or the acquisition of new genetic material via conjugation, transduction, or transformation. Some bacterial species are intrinsically resistant to a range of antibiotics (Guardabassi and Courvalin, 2006). The most common plasmid in *Flavobacterium psychrophilum* strains, designated pCP1, has been sequenced (Alvarez *et al.*, 2004), and contains four open reading frames, one of which encodes a plasmid replication gene, and the remaining three genes having unknown functions (Alvarez *et al.*, 2004). R plasmids have not yet been reported in *F. psychrophilum*, therefore, chromosomally determined mechanisms of resistance are presumed to be more important in this bacterium (Schmidt *et al.*, 2000). The high level of antibiotic resistance observed among *Flavobacterium* spp. isolates here is most likely a result of either chromosomal mutations (after the OTC exposure) or the acquisition of genetic elements encoding resistance that became incorporated into the chromosome. Often a single organism can possess several multiple drug resistance (MDR) efflux pumps, for example the Mex systems of *Pseudomonas aeruginosa* (Poole and Srikumar, 2001) or the

Acr systems of the *Enterobacteriaceae* (Yu *et al.*, 2003). Therefore, it is also possible that the activation of an efflux pump could have resulted in multi-drug resistance that conferred a selective advantage among *Flavobacterium* spp. isolates, allowing for increased drug resistance to multiple antibiotics observed on the last sampling date (48 days following OTC spike). Resistance to amoxicillin (AMC) and ceftriaxone (CRO) remained fairly consistent over the three sampling events, maintaining 21% and 93% resistance to AMC and CRO, respectively, by the final sampling event. Previous studies have shown inconsistent results with regard to amoxicillin resistance among *Flavobacterium* spp.. For example, Didinen *et al.* (2005) and Kum *et al.* (2008) reported the resistance of 13 and 20 *F. psychrophilum* isolates to AMC, 15.4% and 15%, respectively; whereas, both Ispir *et al.* (2004) and Diler *et al.* (2003) reported sensitivity to AMC for *F. psychrophilum* isolates. These results are similar to those of others with *Flavobacterium* spp. exhibiting a relatively low level of resistance to AMC, with an approximate average of 16% between all three sampling events.

Resistance to chloramphenicol (C) and oxytetracycline (OTC) among *Flavobacterium* spp. isolates was exceptionally high during the first two sampling events, displaying 100% resistance to both antimicrobials. However, resistance to both antibiotics was shown to decrease significantly ( $p < 0.05$ ) following the second sampling event, maintaining resistance levels of 64% and 43% on the final sampling event for chloramphenicol and OTC, respectively. The results for these two antimicrobials suggest that after a period of time with no antibiotic exposure a decrease in antibiotic resistance may be observed among *Flavobacterium* spp. isolates. Similar studies have shown that it is possible to limit the occurrence of resistance to some degree by removing the selective pressure or antimicrobial

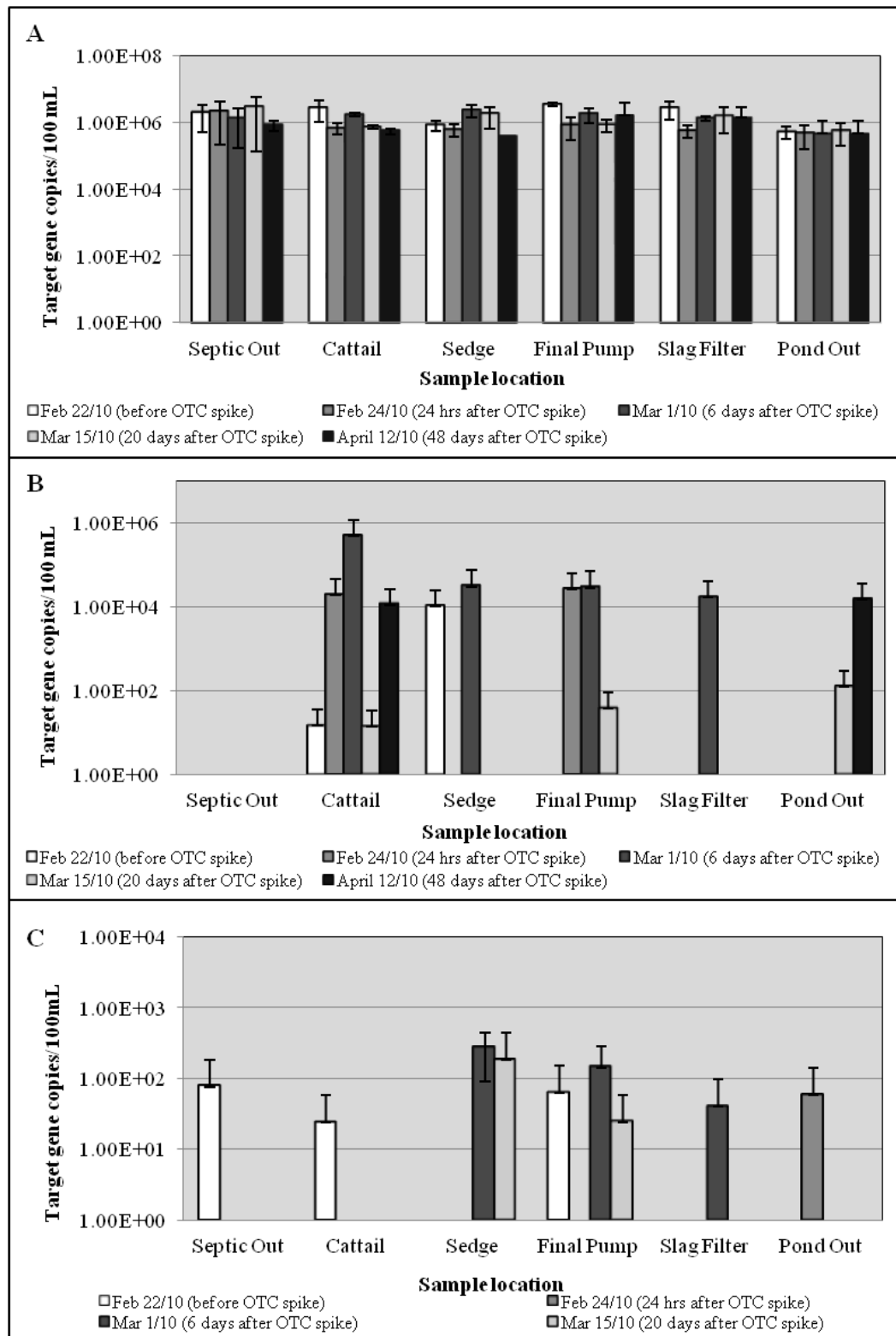
agent (Klare *et al.*, 1999; Pantosti *et al.*, 1999; Aarestrup *et al.*, 2001; Helt *et al.*, 2012). Helt *et al.* (2012) observed a significant decline ( $p < 0.05$ ) in doxycycline resistance among *Enterococcus* spp. isolates, decreasing approximately 40% following exposure to ciprofloxacin (56 days post inoculation). These results support this observation in that a 57% decrease in OTC resistance was reported from the first to the last sampling event (48 days post OTC exposure).

#### **4.4.4 Quantitative detection of ARGs in a sub-surface flow constructed wetland**

Real time quantitative PCR (qPCR) was used to determine the concentration of three ARGs, including *tetA*, *ampC*, and *blaSHV*, in samples from various locations throughout the constructed wetland system. Figure 4.8 summarizes the relative abundance (target gene copies/100 mL) of ARGs obtained from different locations in the constructed wetland. The frequency of *tetA* among all locations was significantly higher ( $p < 0.05$ ) than other ARGs (*blaSHV*, *ampC*), with concentrations reaching approximately  $10^6$  target gene copies/100 mL (Figure 4.8A). Relative concentrations of *tetA* were similar among sampling events and sampling locations, with little reduction observed from the septic sample to the final holding pond sample (during each sampling event). Previous studies have investigated the occurrence of specific culturable antibiotic-resistant bacteria in hatcheries and fish farms (Akinbowale *et al.*, 2006; Chelossi *et al.*, 2003; Miranda and Zemelman., 2002; Nonaka *et al.*, 2007); however, few have considered a genotypic and cultivation-independent examination of ARGs associated with antibiotic exposure (Seyfried *et al.*, 2010; Miranda *et al.*, 2003). Seyfried *et al.* (2010) studied the occurrence of tetracycline resistance genes in



aquaculture facilities with varying use of OTC and concluded that water from farms with recent OTC use had significantly higher tet<sup>R</sup> detection frequencies than did water from farms without recent OTC use. It was suggested that both OTC treatment in aquaculture facilities and the farms themselves may be sources of tet<sup>R</sup> gene introduction to the environment (Seyfried *et al.*, 2010). Few studies have examined other classes of antimicrobials, including  $\beta$ -lactamase (*blaSHV*) and ampicillin (*ampC*) resistance genes, in aquaculture facilities and constructed wetlands. This is the first study to examine the frequency of different classes of ARGs (*tetA*, *blaSHV*, *ampC*) using real-time quantitative PCR in a constructed wetland treating fish hatchery wastewater. The results illustrate the prevalence of ARGs (conferring resistance to different classes of antibiotics) in different stages of the sub-surface flow constructed wetland, thereby, giving rise to the potential dissemination of ARGs into receiving water bodies.



**Figure 4.8. Relative abundance (target gene copies/100mL) of *tetA* (A), *ampC* (B), and *blaSHV* (C) at different sampling points in the sub-surface flow constructed wetland. Error bars represent standard deviation around mean values from two independent qPCR runs in duplicate.**

Overall, the OTC exposure didn't have an effect on the concentration of *tetA* resistance genes in the constructed wetland, with frequencies remaining high ( $10^6$  target gene copies/100 mL) both before and after the antibiotic exposure. Seyfried *et al.* (2010) also found the frequency of *tet*<sup>R</sup> gene detection in sedimentation pond water to be approximately the same, regardless of antibiotic treatment. Previous investigators have shown that even in the absence of OTC, elevated levels of culturable resistant bacteria could be detected in sediments where fish feed had accumulated and decomposed (McPhearson *et al.*, 1991; Vaughan *et al.*, 1996). This could help explain the high occurrence of *tetA* in various sample locations both before and after OTC exposure. Noteworthy is the high frequency of *tetA* in the 'pond out' sample or settling pond, maintaining average concentrations of  $10^5$  -  $10^6$  target gene copies/100 mL, being subsequently released into receiving aquatic environments (i.e. creek). Stachowiak *et al.* (2009) examined the impact of fish hatchery effluent on the presence of antibiotic resistant microorganisms in a small stream, and their results support the need for long-term monitoring of commercial fish-farming operations to assess their impact on antimicrobial resistance in the environment. In their study, the proportion of *E. coli* isolates resistant to tetracycline in the hatchery effluent and in both the downstream water and sediment samples was significantly higher than in either the upstream water or sediment, supporting the possibility of the hatchery as a source of tetracycline-resistant microorganisms even in the absence of recent antibiotic use (Stachowiak *et al.*, 2009).

Concentrations of *ampC* (Figure 4.8B) and *blaSHV* (Figure 4.8C), conferring resistance to extended-spectrum  $\beta$ -lactamases, were sporadic and detected in most sampling locations at least once over the duration of the study. Non-detectable levels of both ARGs were observed

among three to four of the six sampling locations before OTC exposure. The average frequency of *ampC* was found to reach  $4.66 \times 10^5$ ,  $3.00 \times 10^4$ ,  $2.81 \times 10^4$  and  $1.63 \times 10^4$  target gene copies/100 mL in the cattail, sedge, final pump, and slag filter samples, respectively, on Mar 1/10. Whereas, concentrations of *blaSHV* also increased following OTC exposure, reaching  $2.66 \times 10^2$ ,  $1.42 \times 10^2$ , and  $4.02 \times 10^1$  target gene copies/100 mL in sedge, final pump, and slag filter samples, respectively. Similarly, concentrations of both *ampC* and *blaSHV* were observed on Mar 15/10, decreasing to approximately  $10^1$ - $10^2$  target gene copies/100 mL in cattail, final pump, and pond samples for *ampC*, and  $10^1$ - $10^2$  target gene copies/100 mL in sedge and final pump samples for *blaSHV*. However, both *ampC* and *blaSHV* could not be detected during the final sampling event, with the exception of *ampC* in the cattail and pond out samples (Figure 4.8B). A possible explanation for the increase in *ampC* and *blaSHV* concentrations shortly following OTC exposure could be due to resistance mechanisms occurring by horizontal gene transfer. If a susceptible population acquires a resistance gene, through the acquisition of plasmids, or transposable elements, the recipient cells transmit the resistance gene to their descendants and, furthermore, may act as donors to susceptible cells. In the absence of a fitness cost, both processes rapidly allow for the development of a resistant population, occurring even in the absence of antimicrobials (Garcia-Migura *et al.*, 2007; Pallecchi *et al.*, 2008; Prelog *et al.*, 2009). Several resistance determinants associated with mobile genetic elements have been described previously with regard to aquaculture (Kim and Aoki, 1998; Rosser and Young, 1999; Kruse and Sorum, 1994), with R plasmids detected in *Y. ruckeri* (Klein *et al.*, 1996) and the genus *Aeromonas* (Sandaa *et al.*, 1992; Starliper and Cooper, 1998). Low concentrations of certain antibiotics, including fluoroquinolones and  $\beta$ -lactams, have also been reported to fuel mutagenesis and

to increase the risk for emergence of resistance (Couce & Blázquez, 2009). Therefore, it is possible that the OTC exposure promoted mutagenesis in bacteria, allowing for the development and amplification of specific ARGs in the population. The specific causes for the disappearance of *ampC* and *blaSHV* gene concentrations among most of the sampling locations 49 days following OTC exposure remains unknown. However, a possible theory accounting for the undetectable levels of selected ARGs in the final sampling event is the removal of a selective pressure, in which case resistance traits can be lost over time from the population, occurring as a gradual process as it is typically not a huge metabolic burden to most cells (Sorensen *et al.*, 2005). Another possible explanation contributing to the disappearance of *ampC* and *blaSHV* could be attributed to a reduction in ARG-bearing organisms, however, based on these data it cannot be determined whether it is the genes or the gene-containing organisms that are being lost. Either way, this study provides estimates as to how long resistance traits (different classes of resistance genes) might be retained or removed in wetland systems and holding ponds during the treatment of fish hatchery wastewater. Specifically, it contributes to our understanding surrounding the environmental fate of antibiotic resistance and ARGs in a sub-surface flow constructed wetland treating fish hatchery wastewater, which may result in improved management strategies and environmental policies directed at minimizing antibiotic gene release into the environment.

## **4.5 Conclusions**

This chapter presents results from traditional culture-based screening techniques in combination with molecular quantification using real-time PCR to help establish a preliminary environmental assessment of selected fish pathogens and ARGs in a sub-surface

flow constructed wetland treating fish hatchery wastewater. The results obtained for the level of antibiotic resistance among *Aeromonas* spp., *Yersinia* spp., and *Flavobacterium* spp. supports the hypothesis that an isolated antibiotic exposure event to an aquatic system can lead to an increase in resistance, not only to the chosen antibiotic but to other classes of antibiotics as well. The data support the notion that an intermittent antibiotic exposure event could have an impact on the development of antibiotic resistance among fish pathogens and frequency of ARGs in various stages of a sub-surface flow constructed wetland. Real time quantitative PCR was used to determine the concentration of three ARGs; *tetA*, *ampC*, and *blaSHV*, in samples collected from multiple locations in the constructed wetland both before and after the OTC exposure. Overall, the constructed wetland system was found to moderately reduce the level of fish pathogens, but does not guarantee the complete elimination of *Aeromonas* spp., *Yersinia* spp., and *Flavobacterium* spp. during winter month operation, with approximately  $10^3$ - $10^4$  CFU 100 mL<sup>-1</sup> released into the receiving water body via the final holding pond. All three ARGs were detected using real-time quantitative PCR, with *tetA* concentrations far exceeding *ampC* and *blaSHV* concentrations, maintaining  $10^6$  target gene copies/100 mL in all sampling locations (during each sampling event). The focus of this study was limited to only a few select ARGs; however, numerous ARGs exist and may be present in the environment, with their fates being unique to specific environments. Thus, each ARG may have different behaviours with respect to fate and transport and response to physical, chemical, and/or biological treatment. Therefore, the results only reflect tetracycline and two classes of  $\beta$ -lactam resistance among a sub-surface flow constructed wetland and should not be generally applied to all environments in different geographic areas. Furthermore, due to the escalating problem of antibiotic resistance, there is

a need to improve our understanding of the potential role of aquaculture operations and fish hatchery facilities both in the amplification and attenuation of antibiotic resistance levels in the environment.

#### **4.6 Recommendations & Future Research Needs**

- Further studies monitoring the prevalence and fate of different ARGs conferring resistance to other classes of antibiotics is needed, as each ARG may demonstrate different behaviours in response to treatment using a sub-surface flow constructed wetland.
- Long term surveillance (including seasonal comparisons between different months of the year) monitoring the impact of multiple stressors on CWs could help provide an indication of the mobility, persistence, and transfer of resistance genes, thereby assisting with the issue of aquaculture as a source of resistance genes for entry into the environment.
- Future studies could examine the prevalence, distribution, and fate of antibiotic resistant bacteria and ARGs in surface waters and/or groundwater upstream and downstream to the fish hatchery facility, which allows for a greater understanding of the long-term impact and environmental migration of such contaminants.
- Additional understanding of the effects antimicrobials have on wetland systems could be established through direct observation and characterization of biofilms. This knowledge would help both microbiologists and water treatment engineers gain a greater understanding for the effects antibiotics have on wetland systems and overall antibiotic resistance levels.

# 5

## **Distribution, quantification, and dissemination of selected pathogens and antibiotic resistance genes (ARGs) in a pilot-scale BNR system receiving wastewater from a full-scale WWTP<sup>4</sup>**

### **5.1 Introduction**

Wastewater treatment plants (WWTP) represent important potential reservoirs of human and animal commensal bacteria in which antibiotic resistant organisms and ARGs may persist in the final effluent and be subsequently released into the environment (Ferreira da Silva, *et al.*,

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<sup>4</sup>Helt, C.D., Parker, W.J., Legge, R.L., Slawson, R.M. *Distribution and molecular quantification of selected pathogens and antibiotic resistance genes within a pilot-scale biological nutrient removal (BNR) system receiving municipal wastewater, in preparation.*



2007; Reinthaler *et al.*, 2003). Resistance is common where antibiotics are heavily used, with antibiotic resistant bacteria shown to be present in wastewater, surface water, ground water, sediments and soils, and increasingly in aquatic environments (Baquero *et al.*, 2008; Martinez *et al.*, 2008; Zhang *et al.*, 2009). Due to the high microbial biomass and the abundance of nutrients, WWTPs serve as potential "hot spots" for horizontal gene transfer, frequently involving the passage of plasmids and transposons encoding antibiotic resistance (Guardabassi *et al.*, 2002). Bacteria in wastewater treatment processes may enter the environment through typical end products, that is effluent or biosolids, or through atypical routes such as seepage through cracks in tanks, flooding, or animal contact (Garcia *et al.*, 2007). The combination of municipal sewage, hospital wastewater, and surface water, may facilitate the spread of antibiotics, ARGs, and antibiotic resistant bacteria in wastewater treatment plants (Schluter *et al.*, 2007; Zhang *et al.*, 2009). Large amounts of antibiotics are released into municipal wastewater due to incomplete metabolism in humans or due to disposal of unused antibiotics (Nagulapally *et al.*, 2009), which eventually find their way into different natural environmental compartments. The role of wastewater treatment plants in reducing the load of antibiotic resistant bacteria and ARGs present in raw sewage is not well known (Rijal *et al.*, 2009). However, it has been suggested that certain conditions in wastewater treatment plants might increase the number of antibiotic resistant bacteria during the treatment process (Silva *et al.*, 2006). For example, Zhang *et al.* (2009) suggested that wastewater treatment contributes to the selective increase of antibiotic resistant bacteria (e.g. *Acinetobacter* spp.) and the occurrence of multi-drug resistant bacteria in aquatic environments. A key factor for the acquisition and proliferation of antibiotic resistance amongst bacteria appears to be the co-occurrence of high concentrations of faecal bacteria

and sub-lethal antibiotic levels (Jury *et al.*, 2010). The presence of low concentrations of antibiotics in sewage treatment plant (STP) wastewaters may serve as a selective pressure for the proliferation of resistant organisms. The assumption that antibiotic sensitive bacteria can acquire resistance in STPs as a consequence of lateral resistance gene transfer is probable, given that several transmissible plasmid harbouring resistance genes have been isolated from sewage plants (Szczepanowski *et al.*, 2005; Schluter *et al.*, 2003). However, demonstrating transfer of such genes between bacteria in STPs remains challenging, as most studies have been performed under laboratory conditions. For example, Soda *et al.* (2008) recently reported the *in vitro* transfer of the multi-resistant plasmid RP4 (IncP-1) from an *E. coli* donor to activated sludge bacterial recipients. Such experiments give an indication of realistic events but do not truly mirror conditions found in STPs.

The conventional activated sludge (CAS) process and its variations (including the sequencing batch reactor process) are the most widely used biological or secondary treatment processes in Ontario (OMOE, 2008). A number of biological processes including suspended growth, fixed film, and hybrid (combined suspended and fixed-film) systems are well known and commonly used in North America (OMOE, 2008). Biological nutrient removal (BNR) removes total nitrogen (TN) and total phosphorus (TP) from wastewater through the use of microorganisms under different environmental conditions in the treatment process (Metcalf and Eddy, 2003). There are a number of BNR process configurations available. Some BNR systems are designed to remove only TN or TP, while others remove both. The configuration most appropriate for any particular system depends on the target effluent quality, operator experience, influent quality, and existing treatment processes

(Jeyanayagam, 2005). Although the exact configurations of each system differ, BNR systems designed to remove TN must have an aerobic zone for nitrification and an anoxic zone for denitrification, and BNR systems designed to remove TP must have an anaerobic zone free of dissolved oxygen and nitrate (USEPA, 2007). Some of the advantages associated with the BNR process include: no (or reduced) chemicals needed, reduced sludge production, reduced metal concentrations in effluent and sludge, improved sludge settleability and dewatering characteristics, reduced oxygen requirements, reduced process alkalinity requirements, increased oxygen transfer efficiency in the aeration basin and reduced effluent nitrogen concentration (OMOE, 2008).

Hospital sewage typically carries increased numbers of antibiotic resistant bacteria compared to other municipal sewage (Grabow and Prozesky, 1973; Kümmerer *et al.*, 1999; Prado *et al.*, 2008). Because hospital sewage also contains elevated levels of antibiotics (Gros *et al.*, 2006), the increased resistance may reflect a selective pressure imparted by the elevated levels of these compounds. Controlled pilot scale biological reactors can be used to help facilitate our understanding of the impact of antibiotics and the movement of resistance determinants (ARGs) in the bacterial population of a sewage treatment plant. The objective of this study was to quantify select ARGs and potential pathogens including *tetA*, *ampC*, *blaSHV*, *E. coli*, *Enterococcus faecalis* and *Salmonella* spp. using real-time quantitative PCR, and to observe antibiotic resistance profiles among common faecal indicators, in the raw influent, activated sludge, and final effluent of a pilot scale biological nutrient removal (BNR) treatment system. This is the first study to monitor the distribution of and quantify selected ARGs, pathogens and antibiotic resistance profiles among faecal indicators in a

pilot-scale BNR system receiving wastewater from the Skyway WWTP in Burlington, ON. Recently, Munir *et al.* (2010) studied the release of antibiotic resistant bacteria (ARB) and genes (*tetW*, *tetO*, *SulI*) in the effluent and biosolids of five wastewater utilities in Michigan and found that the concentrations of ARGs in the MBR (Membrane Biological Reactor) effluent were 1-3 logs less than that of conventional treatment utilities. Disinfection (chlorination and UV) processes did not contribute to a significant reduction of ARGs and ARB with very little change in concentrations of ARGs and ARB between pre- and post-disinfected effluents (Munir *et al.*, 2010).

Human exposure to ARGs and ARB, which may be pathogenic in nature, could occur in a number of different ways. However, the water matrix is considered to play an important part in providing a medium for the transfer of resistance genes and resistant bacteria to the environment (Baquero *et al.*, 2008; Iwane *et al.*, 2001). Wastewater treatment plants hold an important place in the elimination or dissemination of antibiotic resistant microbes as the treatment systems and their operational conditions may impact the fate of resistant bacteria or resistant genes (Iwane *et al.*, 2001). Quantitative polymerase chain reaction (qPCR) makes possible the enumeration of cells in environmental samples, and not only those which are culturable. This technique has been used previously to study pathogenic bacteria in manure (Lebuhn *et al.*, 2003) or soil (Marsh *et al.*, 1998) and has recently been applied to detect bacterial pathogens and ARGs during municipal wastewater treatment (Lee *et al.*, 2006; Wéry *et al.*, 2008; Munir *et al.*, 2010; Shannon *et al.*, 2007; Volkmann *et al.*, 2004). Conventional detection of antibiotic resistances through phenotypic characterization of *E. coli* and *Enterococcus* spp. isolates was performed in combination with genotypic

identification of ARGs and select bacterial pathogens using molecular based techniques (qPCR). Moreover, qPCR allows for the detection of resistance determinants in viable but non culturable microorganisms. In this study, samples of raw wastewater, treated effluent, and activated sludge were monitored for antibiotic resistant *E. coli* and *Enterococcus* spp., ARGs (*tetA*, *ampC*, and *blaSHV*) and frequency of *Salmonella* spp., *E. faecalis*, and *E. coli* using qPCR assays. Tetracycline and beta-lactam resistance genes (*tetA*, *ampC* and *blaSHV*) were chosen in this study because tetracyclines and beta-lactam antibiotics are the most commonly used antimicrobials in human and veterinary medicine (Boxall *et al.*, 2003; Chopra and Roberts, 2001). Selected pathogens included one enteric pathogen, *Salmonella* spp., which is mainly responsible for foodborne outbreaks and is frequently isolated from wastewater and sludge, as well as the bacteria commonly used as indicators of faecal contamination: *E. coli* and *Enterococcus faecalis*. In addition, these bacterial groups are present at different concentrations in wastewater and are known to survive differently in the environment, therefore, it is important to quantify and compare their fates and removal throughout the treatment process.

## **5.2 Research Needs & Objectives**

The enumeration of antibiotic-resistant bacteria and ARGs in various stages of a pilot-scale biological nutrient removal (BNR) treatment system will prove helpful in understanding both the baseline and potential movement of selected pathogens, antibiotic resistance among faecal indicators, and frequency and fate of ARGs throughout wastewater treatment systems. Controlled pilot scale biological reactors can be used to help facilitate our understanding of

the impact of antibiotics and the movement of resistance determinants (ARGs) in the bacterial population of a sewage treatment plant. Few studies have examined both the occurrence and fate of pathogens and different classes of ARGs using a combined approach of traditional culture-based screening and molecular quantification using real-time PCR. Previous studies have focused on the quantification of other tetracycline resistance genes (*tetW* and *tetO*) and sulfonamide genes (*SulI*) in wastewater treatment systems; however, other classes of antibiotics including penicillins, and beta-lactams are more commonly used in human and veterinary medicine, and must be examined. Furthermore, the results of this study should help establish a preliminary assessment quantifying ARGs and selected pathogens in a biological nutrient removal system receiving full-scale wastewater in Ontario, Canada. Therefore, the overall objective of the research reported in this chapter was to better understand the distribution, frequency and potential dissemination of selected bacterial pathogens and ARGs, conferring resistance to three classes of antimicrobials: tetracyclines, penicillins, and  $\beta$ -lactam resistance (conferring broad resistance to penicillins and cephalosporins) in samples of raw wastewater, activated sludge, and treated effluent from a pilot-scale BNR system receiving authentic wastewater.

The specific objectives are to:

1. Determine the prevalence of antibiotic resistance among culturable *E. coli* using the disc-diffusion method, while comparing any differences in the level of antibiotic resistance between different stages of treatment (raw wastewater, activated sludge, treated effluent);

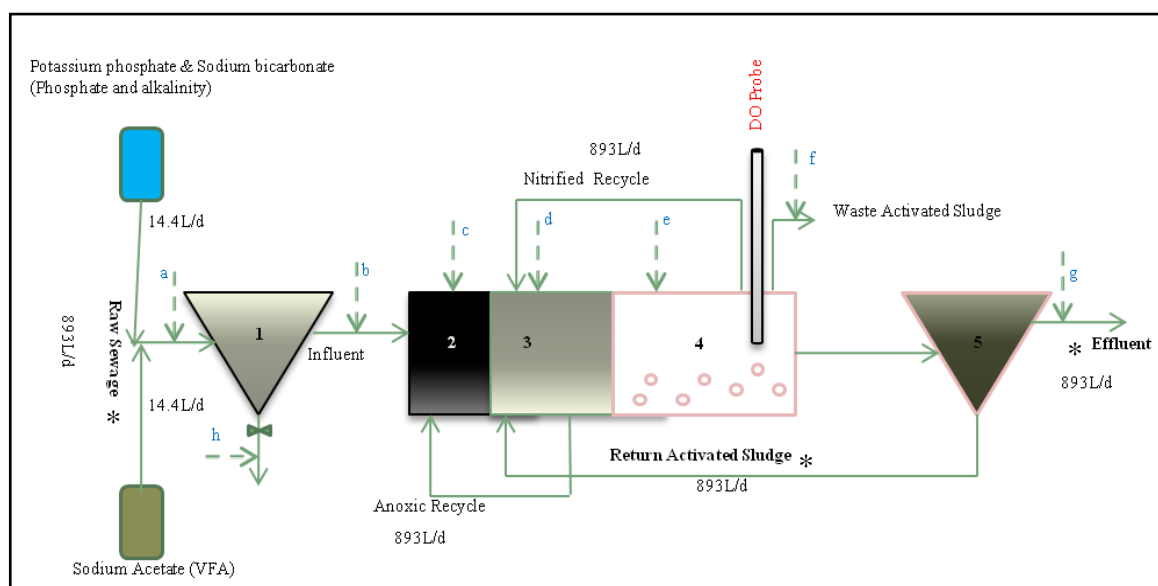
2. Establish the frequency of multiple antimicrobial resistance (MAR) profiles and antimicrobial resistance profiles among culturable faecal indicators (*E. coli* and *Enterococcus* spp.);
3. Determine the occurrence of antimicrobial resistance among culturable *Enterococcus* spp. isolates using the disc-diffusion method and compare any differences between the frequency of antibiotic resistance among various stages of treatment (raw wastewater, activated sludge, treated effluent);
4. Quantify selected bacterial pathogens (*E. coli*, *Salmonella*, *E. faecalis*) and ARGs (*ampC*, *tetA*, *blaSHV*) in various stages of the pilot-scale BNR system using real-time qPCR; and
5. Determine the efficiency of wastewater treatment using a pilot-scale BNR system receiving wastewater effluent from a full-scale WWTP through the comparison of pathogen and ARG log removals.

## **5.3 Materials and Methods**

### **5.3.1 Pilot-scale BNR system and sampling**

The pilot-scale biological nutrient removal (BNR) treatment system is located in Burlington, ON and receives raw wastewater (passed through screens) from the Skyway wastewater treatment plant (WWTP) in Burlington, ON. The Skyway WWTP is a secondary treatment plant consisting of screening, raw sewage pumping, de-gritting, primary clarification, activated sludge, final clarification and two-stage anaerobic digestion. The final effluent is

seasonally disinfected using ultra-violet irradiation prior to discharge into Hamilton Harbour. The pilot-scale BNR system utilizes anaerobic, anoxic, and oxic (A/A/O) processes as illustrated in Figure 5.1 (Ogunlaja, 2011). The conventional activated sludge (CAS)-BNR (Plug Flow reactor) system operates under the following conditions: a solids retention time (SRT) of 40 days, hydraulic retention time (HRT) of 10 hours, temperature between 12-13°C, and the influent flow rate is approximately 0.6 L/min. Three sites were selected for sampling (indicated by the asterisks in Fig. 5.1): raw sewage, activated sludge, and treated effluent.



**Figure 5.1. Schematic depicting the treatment processes in the pilot scale BNR system. 1- primary settler, 2- anaerobic zone, 3- anoxic zone, 4- aerobic zone, 5- secondary settler (courtesy of Ogunlaja, 2011, reproduced with permission). Sampling sites are designated by asterisks.**

Sampling was carried out between May 2011 and September 2011 with a total of three sampling events during this period. Two-litre wastewater samples (raw wastewater and treated effluent) and one-litre activated sludge (AS) samples were collected in sterile



sampling bottles, allowing for representative samples. Samples were stored on ice and transported to the microbiology laboratory for immediate processing. The bacterial biomass in the raw influent sample (1500 mL) and activated sludge sample (500 mL) was concentrated by centrifugation at  $16,000 \times g$  for 20 min at 4 °C, followed by storage of the pellets (water solids) at -20°C until DNA extraction was performed for molecular analysis. Volumes of 250-500 mL of treated effluent were filtered using 0.45- $\mu$ m, sterile mixed cellulose ester membrane filters (Difco, Fisher Scientific, Ottawa, Ontario, Canada) using a sterile filtration apparatus.

### **5.3.2 DNA extraction**

For extracting DNA from treated effluent samples, filters were carefully placed into a Mo Bio PowerSoil (Mo Bio Laboratories Inc., Carlsbad, CA) bead tube using sterile forceps. The filter was then cut into small pieces using a new, sterile No. 11 blade (Feather, Fischer Scientific, Whitby, ON) on a sterilized No. 3 handled scalpel for approximately 5 minutes in the bead tube. Genomic DNA was subsequently extracted from raw wastewater and activated sludge samples (pellets) and bacterial cultures (control strains) using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA), following the manufacturer's protocol. The quantity and purity of the DNA extract was determined spectrophotometrically using a Nanophotometer (Montreal Biotech Inc., Montreal, Canada) at an absorbance of 260 nm, and  $A_{260}/A_{280}$ , respectively. The extracted DNA was stored in the freezer at -20°C for long-term storage.

### 5.3.3 Isolation and enumeration of faecal bacteria

*Enterococcus* spp. and *E. coli* were isolated from raw wastewater and treated effluent samples using standard membrane filtration methods (American Public Health Association; APHA, 1999). Dilutions were filtered through 0.45- $\mu$ m, 47-mm mixed cellulose ester membrane filters (Difco, Fisher Scientific, Ottawa, Ontario, Canada), and then placed onto appropriate selective agar plates. mEnterococcus agar (Difco, Fisher Scientific) was used for the detection and enumeration of *Enterococcus* spp., followed by confirmation with bile esculin agar (Difco, Fisher Scientific). mFC-BCIG agar was used for the detection and isolation of *E. coli* (Kon *et al.*, 2007), made with mFC basal agar (Difco, Fisher Scientific) and 100  $\mu$ g 5-bromo-4-chloro-3-indolyl  $\beta$ -d-glucuronide cyclohexyl ammonium salt (Medox Diagnostics, Ottawa, Ontario, Canada) per liter. Incubation conditions for the agar plates were as follows: mEnterococcus plates,  $35 \pm 2^\circ\text{C}$  for 48 hr and mFC-BCIG plates,  $44.5 \pm 0.5^\circ\text{C}$  for 24 hrs (hot waterbath). After 48 hr, membrane filters from mEnterococcus agar were placed onto pre-warmed bile esculin agar plates and incubated at  $44 \pm 0.5^\circ\text{C}$  for 2 hrs. Positive colony characteristics of *Enterococcus* spp. ranged from pink to dark red on mEnterococcus agar and produced a brown to black precipitate on bile esculin agar. Blue colonies arising on mFC-BCIG agar plates were considered positive for *E. coli* (indicative of a positive reaction for  $\beta$ -glucuronidase). Samples were prepared in duplicate, and enumeration counts between 20 and 200 (Schraft and Watterworth, 2005) colonies per plate were considered for analysis. Characteristic colonies (confirmed using multiplex and/or colony PCR) were recorded (and calculated in CFUs per 100 mL filtered sample) and further streaked for purity onto Brain Heart Infusion (BHI) agar (Difco, Fisher Scientific) for

*Enterococcus* spp. or Luria-Bertani (LB) agar (Bioshop Canada Inc., Burlington, Ontario) for *E. coli* and incubated at  $35 \pm 2^{\circ}\text{C}$  for 24hrs.

#### **5.3.4 Antimicrobial susceptibility testing**

Antimicrobial resistance patterns were determined using the disc-diffusion method as set by the Clinical and Laboratory Standards Institute (CLSI, 2007). *Enterococcus* spp. and *E. coli* isolates were inoculated into Brain Heart Infusion (BHI) broth (Difco, Fisher Scientific) and grown to a 0.5 McFarland turbidity standard (4-6 hrs) and swabbed onto Mueller Hinton (Difco, Fisher Scientific) agar plates. The following antimicrobial agents were selected as important representatives of different antibiotic classes, with abbreviations and disc concentrations shown in brackets: streptomycin (S 10  $\mu\text{g}$ ), vancomycin (VA 30  $\mu\text{g}$ ), ciprofloxacin (CIP 5  $\mu\text{g}$ ), chloramphenicol (C 30  $\mu\text{g}$ ), ceftriaxone (CRO 30  $\mu\text{g}$ ), cefotaxime (CTX 30  $\mu\text{g}$ ), amoxicillin (AMC 30  $\mu\text{g}$ ), minocycline (MI 30  $\mu\text{g}$ ), doxycycline (D 30  $\mu\text{g}$ ), ampicillin (AM 10  $\mu\text{g}$ ), sulfadiazine (SD 0.25 mg), sulfamethoxazole-trimethoprim (SXT 23.75/1.25  $\mu\text{g}$ ) and tetracycline (TE 30  $\mu\text{g}$ ). After incubation for 16-18 hours at  $35 \pm 2^{\circ}\text{C}$ , the isolates were classified as susceptible, intermediate or resistant by comparing the diameters of inhibition zones with the breakpoints according to CLSI recommendations (CLSI, 2007). Organisms considered intermediate by the Kirby–Bauer disc-diffusion method were recorded as resistant for the purpose of this study.

### 5.3.5 Selection of PCR primers and TaqMan® probes

All primers and probes (Table 5.1) were synthesized by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Six sets of real-time PCR primers and probes were previously designed (Ng *et al.*, 2001; Volkmann *et al.*, 2004; Lee *et al.*, 2006; Böckelmann *et al.*, 2009) to detect pathogens and/or ARGs in wastewater samples. TaqMan probes and primer sets were designed to detect the following organisms and ARGs: *E. coli*, *Enterococcus faecalis*, *Salmonella* spp., *ampC*, and *blaSHV* (Table 5.1), while *tetA* was quantified using the SYBR Green approach.

**Table 5.1. Primers and probes for real-time qPCR to detect pathogens and ARGs**

Primer or Probe	Target gene	Sequence (5'-3')	Reference	GenBank accession no.
ampCF <sup>a</sup> ampCR <sup>b</sup> ampCP <sup>c</sup>	<i>ampC</i> <i>ampC</i> <i>ampC</i>	GGAATGCTGGATGCACAA CATGACCCAGTTCGCCATATC TEXASRED- CCTATGGCGTGAAAACCAACGTGCA-BHQ-1 <sup>d</sup>	Volkmann <i>et al.</i> (2004)	AJ005633
SHVF SHVR SHVP	<i>blaSHV-5</i> <i>blaSHV-5</i> <i>blaSHV-5</i>	AACAGCTGGAGCGAAAGATCCA TGTTTTTCGCTGACCGGCGAG FAM-TCCACCAGATCCTGCTGGCGATAG-BHQ-1	Böckelmann <i>et al.</i> (2009)	X55640
tetAF tetAR SYBR <sup>e</sup>	<i>tetA</i> <i>tetA</i>	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	Ng <i>et al.</i> (2001)	X61367
Efaecal-F ( <i>Enterococcus faecalis</i> ) Efaecal-R Efaecal-PR	groES (Heat-shock protein) groES groES	TGTGGCAACAGGGATCAAGA TTCAGCGATTGACGGATTG FAM-TCGTTCGTGCATTAGA- BHQ-1	Lee <i>et al.</i> (2006)	AF335185
Eco-F ( <i>Escherichia coli</i> ) Eco-R Eco-PR	uidA (Glucuronidase) uidA uidA	GTCCAAAGCGGCGATTG CAGGCCAGAAGTCTTTTTCCA HEX-ACGGCAGAGAAGGTA- BHQ-1	Lee <i>et al.</i> (2006)	S69414
Sal-F <i>Salmonella</i> spp. Sal-R Sal-probe	invA (Invasion protein) invA invA	CGTTTCCTGCGGTACTGTTAATT AGACGGCTGGTACTGATCGATAA HEX-CCACGCTCTTCGTCT- BHQ-1	Lee <i>et al.</i> (2006)	U43272

<sup>a</sup> Forward primer, <sup>b</sup> Reverse primer, <sup>c</sup> TaqMan probe, <sup>d</sup> BHQ-1, black hole quencher, <sup>e</sup> SYBR Green

### 5.3.6 Amplification of standards for real-time qPCR assays

The abundance of six genes, three ARGs (*ampC*, *blaSHV*, *tetA*) and three bacterial pathogens (*E. coli*, *E. faecalis*, *Salmonella* sp.) were quantified against their respective standard using the appropriate real-time PCR primers and probes (Table 5.1) and assay conditions optimized previously (Volkman *et al.*, 2004; Lee *et al.*, 2006; Böckelmann *et al.*, 2009). Genomic DNA was extracted from the following strains and used as positive controls for qPCR; *E. coli* (ATCC#25922), *E. faecalis* (ATCC#49532), *Salmonella* sp. (ATCC#13311), *Enterobacter cloacae* (ATCC#BAA-1143) containing *ampC*, *Klebsiella pneumoniae* (ATCC#700603) containing *blaSHV*, and *E. coli* containing *tetA* (plasmid DNA). The signal intensity produced by the amplification process was plotted on a linear scale. A threshold value above the background was chosen for the significant increase of the fluorescence signal, as indicated in the plot. Thus, the intercepts of the fluorescence signals with the threshold line determined the threshold cycle values  $C_t$  as a quantifiable measure of the abundance of targeted DNA in the sample. The serially diluted suspensions of the reference strains corresponded with the continuously increasing  $C_t$  values, which is essential for a quantification of targets. The developed primer–probe systems were tested by duplicate amplification runs of the serially diluted reference strains. Calibration curves were prepared for each gene with serial dilutions of positive controls over 7 orders of magnitude.

### 5.3.7 Quantitative Polymerase Chain Reaction

Real-time qPCR was used for the quantification of *ampC*, *blaSHV*-5, and *tetA* genes and *Enterococcus faecalis*, *E. coli*, and *Salmonella* sp. organisms in total DNA extracted from

water samples, performed with an iCycler & iQ Real-Time PCR System (Bio-Rad Laboratories; Mississauga, ON, Canada). Each 25  $\mu$ L PCR amplification contained template genomic DNA, primers and TaqMan® probes added to DyNAmo™ Probe qPCR Mastermix (New England Biolabs, Pickering, ON, Canada) to a final concentration of 250 nM TaqMan® probes, and 1  $\mu$ M for each primer. A volume of up to 5  $\mu$ L of DNA was added to each reaction. The following three-step PCR program was used for the quantification of pathogens and ARGs: (i) a uracil-DNA glycosylase step at 50°C for 2 min, (ii) an initial denaturation and *Taq* polymerase activation step at 95°C for 10 min and (iii) 55 cycles of 95°C for 15 s and 60°C for 1 min (Shannon *et al.*, 2007; Böckelmann *et al.*, 2009). SYBR Green was used for the quantification of *tetA*, as TaqMan probe sequences have not yet been developed for the *tetA* gene. Previous studies have shown SYBR Green to be a reliable method for the quantification of *tet* genes detected in cattle farm waste (Yu *et al.*, 2005), groundwater (Mackie *et al.*, 2006), river sediments (Pei *et al.*, 2006), and different water environments (Zhang *et al.*, 2009). Reactions for *tetA* were conducted with a final volume of 20  $\mu$ L, including 10  $\mu$ L Power SYBR Green PCR Mastermix, plus 2  $\mu$ L each primer (1 $\mu$ M), and 5-8  $\mu$ L template DNA. The following protocol was used for the quantification of *tetA*: 94°C for 3 min, followed by 45 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s (Zhang *et al.*, 2009). Tenfold dilution series of the standards for the respective genes were run along with the unknown samples. Each sample was tested in duplicate for each run and two independent runs were performed. Quantification was achieved using standard curves obtained from the amplification profiles of known concentrations of the respective standard. For each probe and primer set tested against wastewater DNA using real-time PCR, a

negative control was tested, which included primers, the TaqMan® probe, PCR mastermix, and sterile water in place of DNA template.

### **5.3.8 Statistical analysis**

The chi-squared test and student t-test were used to conduct the statistical analysis of the results (i.e., for comparison of antibiotic resistance during different stages of treatment and for comparison of concentration means). The null hypothesis that the concentration of ARGs (or antibiotic resistance) was not different between different samples was rejected at a *p*-value less than or equal to 0.05. All statistical computations were performed using Microsoft Excel version 2007 for Windows.

## **5.4 Results and Discussion**

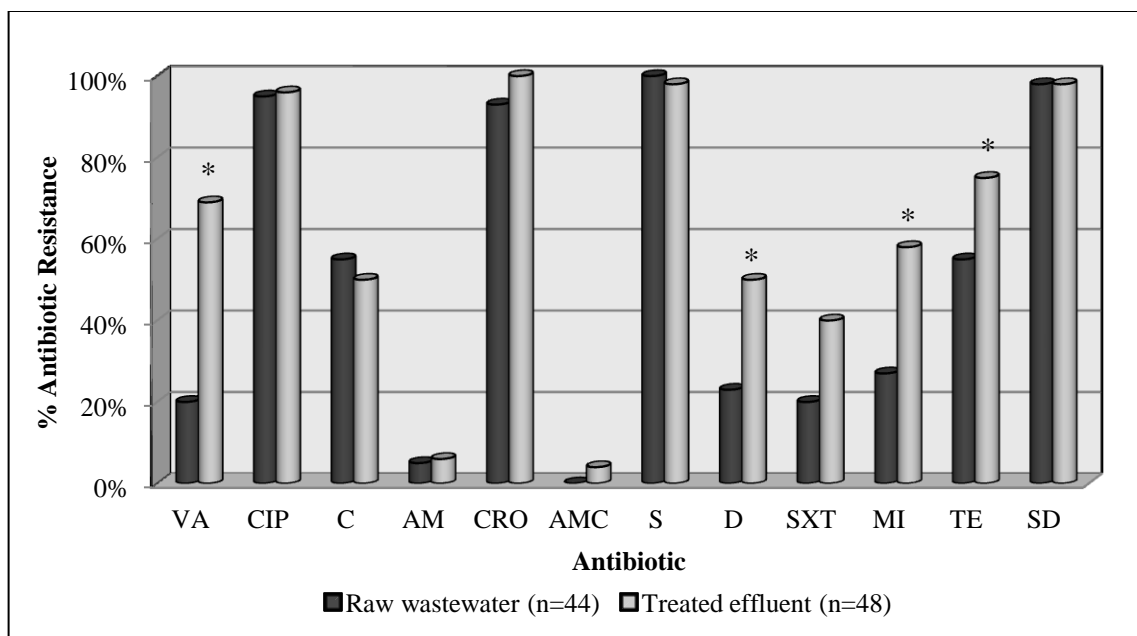
### **5.4.1 Prevalence of antibiotic resistance among faecal indicator organisms**

The susceptibility results were obtained for 92 *Enterococcus* spp. isolates and 99 *E. coli* isolates from raw wastewater and treated effluent samples collected from the pilot-scale treatment system. Resistance profiles to twelve common antimicrobials for each sample are shown in Figures 5.2 and 5.3 for *Enterococcus* spp. and *E. coli* isolates, respectively. In the case of enterococci, the most commonly observed resistances for raw and treated wastewater were to ciprofloxacin, ceftriaxone, streptomycin, and sulfadiazine (Figure 5.2), with resistances between 93-100% in both samples. Although enterococci are commensal organisms in the guts of most animals, their intrinsic resistance to many antimicrobial agents

and their ability to efficiently acquire antibiotic resistance determinants makes them a major concern, as their role in nosocomial infections has increased due to their ability to acquire high-level resistance to antibiotics, making them difficult to treat (Linden and Miller, 1999). When comparing the levels of antibiotic resistance among raw wastewater and treated effluent samples, an increase in resistance to seven antibiotics was observed in the treated effluent, with a significant increase ( $p < 0.05$ ) to vancomycin (VA), doxycycline (D), minocycline (MI), and oxytetracycline (TE) (Figure 5.2). Resistance to vancomycin was 20% in the raw wastewater and significantly increased to nearly 70% in the treated effluent. Similarly, *Enterococcus* spp. isolates demonstrated relatively low levels of resistance of 23%, 27%, and 55% to doxycycline, minocycline, and oxytetracycline in raw wastewater, respectively. However, resistance significantly increased ( $p < 0.05$ ) to 50%, 58%, and 75% to D, MI, and TE, respectively in treated effluent samples. This suggests that elements of wastewater treatment could possibly provide opportunities for a significant increase in antibiotic resistance levels among culturable *Enterococcus* spp.. It has been previously reported that the wastewater treatment process can have an influence on antibiotic resistance through selective pressures (i.e. exposure to antibiotics, variations in temperature or flow rate) and can lead to an overall increase in the concentration of antibiotic resistant bacteria (Zhang *et al.*, 2009; Silva *et al.*, 2006; Reinthaler *et al.*, 2003). A previous study has suggested that antibiotic and antibiotic resistance gene concentrations are lower at increased temperatures during the biological treatment in a dairy lagoon (Pei *et al.*, 2007) and a second study reported decreased antibiotic concentrations in river sediment during high-flow compared to low-flow sampling (Pei *et al.*, 2006). Similarly, Zhang *et al.* (2009) observed increased susceptibility to antibiotics among *Acinetobacter* spp. strains isolated from a high-



temperature (31°C), high-flow event compared to those strains isolated from a low-temperature (8°C), low-flow event. High temperature may be more effective for the biodegradation of antibiotics, and high-flow can allow for the dilution of antibiotics and biomass, thereby decreasing the selective pressure for antibiotic resistance. Therefore, wastewater treatment processes could allow for an increase in the antibiotic resistance of surviving bacteria and serve as important vehicles for the spread of antibiotic resistance to opportunistic pathogens if the treatment is insufficient. Results are in accordance with this observation, suggesting that it is possible for wastewater treatment using a BNR utility to allow for an increase in antimicrobial resistance among *Enterococcus* spp. isolates to specific antibiotics. Results suggest that drug resistance of enterococci might be influenced by hospital sewage, which is fed to municipal treatment plants and mixed with household sewage. In this study, the raw wastewater entering the pilot-scale BNR system comes from the Skyway WWTP, which receives hospital sewage from the Joseph Brant Memorial Hospital in Burlington, ON. Because hospital sewage also contains elevated levels of antibiotics (Gros *et al.*, 2006) the increased resistance may reflect a selective pressure imparted by the elevated levels of these compounds.

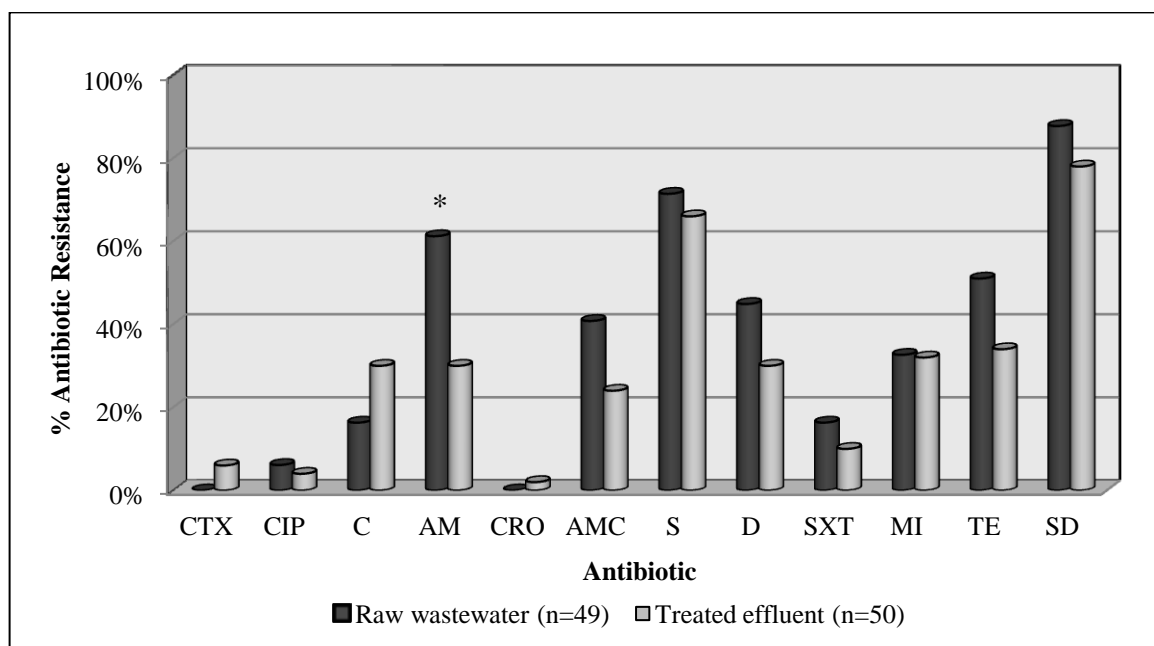


**Figure 5.2.** Prevalence of antibiotic resistance (%) among *Enterococcus* spp. isolates from raw wastewater and treated effluent samples. The following antimicrobial agents were selected as important representatives of different antibiotic classes (abbreviations are shown in brackets); vancomycin (VA), ciprofloxacin (CIP), chloramphenicol (C), ampicillin (AM), ceftriaxone (CRO), amoxicillin (AMC), streptomycin (S), doxycycline (D), sulfamethoxazole-trimethoprim (SXT), minocycline (MI), oxytetracycline (TE), and sulfadiazine (SD). An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n = number of isolates.

Conversely, ampicillin (AM) and amoxicillin (AMC) seemed to be highly active against *Enterococcus* spp., with tested isolates displaying fairly low levels of antibiotic resistance (Figure 5.2). Similar studies assessing the level of antimicrobial resistance among wastewater enterococcal strains have also shown low levels of resistance to ampicillin and amoxicillin (Martins da Costa *et al.*, 2006; Ferreira da Silva *et al.*, 2006, Łuczkiwicz *et al.*, 2010), confirming high susceptibility of these bacteria to penicillin-based antibiotics.

Among *E. coli* the most commonly observed resistances for bacteria from raw wastewater were to sulfadiazine (SD), streptomycin (S), ampicillin (AM), oxytetracycline (TE), doxycycline (D), amoxicillin (AMC) and minocycline (MI) which displayed resistance

levels of approximately 88%, 71%, 61%, 51%, 45%, 41%, and 33%, respectively (Figure 5.3). The finding that resistances to penicillins, aminoglycosides, tetracyclines and sulfonamides are more prevalent in *E. coli* than resistances to quinolones (CIP) and third generation cephalosporins (CTX and CRO) is consistent with previous reports (Reinthal *et al.*, 2003; Watkinson *et al.*, 2007b; Hu *et al.*, 2008; Servais and Passerat, 2009). This is also in good agreement with the extent to which these antimicrobial classes have been prescribed to date. Penicillins, tetracyclines, aminoglycosides and sulfonamides have been extensively used for decades, while the introduction of quinolones (and more particularly fluoroquinolones) and third generation cephalosporins into clinical use is more recent (Davies, 2007).

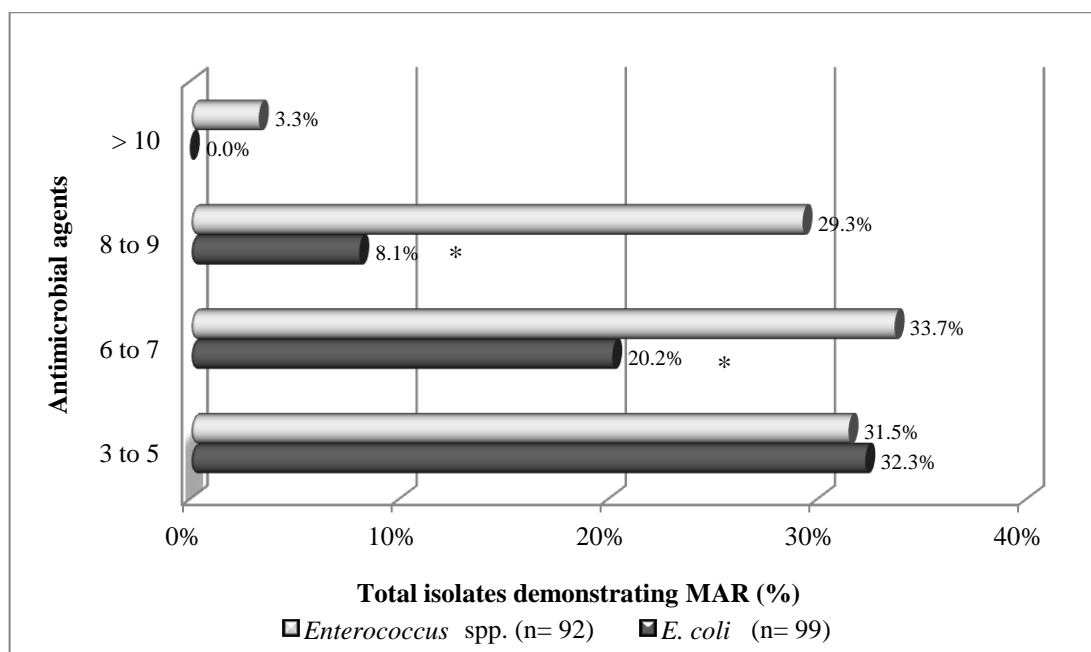


**Figure 5.3. Prevalence of antibiotic resistance (%) among *E. coli* isolates from raw wastewater and treated effluent samples. The following antimicrobial agents were selected as important representatives of different antibiotic classes (abbreviations are shown in brackets); cefotaxime (CTX), ciprofloxacin (CIP), chloramphenicol (C), ampicillin (AM), ceftriaxone (CRO), amoxicillin (AMC), streptomycin (S), doxycycline (D), sulfamethoxazole-trimethoprim (SXT), minocycline (MI), oxytetracycline (TE), and sulfadiazine (SD). An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n = number of isolates.**

When comparing the level of antibiotic resistance between raw wastewater and treated effluent samples, chi-squared tests revealed no significant differences among almost all antimicrobials, with the exception of ampicillin. A significant decrease ( $p < 0.05$ ) was found among culturable *E. coli* isolates resistant to ampicillin in the raw wastewater compared to the treated effluent, decreasing from approximately 61% to 30%, respectively (Figure 5.3). Therefore, the results of *E. coli* are in contrast to those obtained for *Enterococcus* spp., which suggest that wastewater treatment using BNR can lead to an increase in antibiotic resistance.

A possible explanation accounting for the high level of resistance observed among both faecal indicators (*E. coli* and *Enterococcus* spp.) in this study could be simply due to the type of sample being analyzed. Sewage constitutes a favourable environment, consisting of variable mixtures of bacteria, nutrients, and antimicrobial agents (Hirsch *et al.*, 1999) that might promote growth of resistant bacteria or gene transfer between bacteria in the sewage or in the biofilm that may line pipelines (Iversen *et al.*, 2002). Resistance was observed among all twelve antimicrobial agents with 60.6% and 97.8% of total *E. coli* ( $n=99$ ) and *Enterococcus* spp. ( $n=92$ ) isolates expressing the phenotype of resistance to three or more antimicrobial agents (MAR) and were detected in both raw and treated wastewater samples. Multiple antibiotic resistance profiles among *Enterococcus* spp. and *E. coli* isolates are shown in Figure 5.4. Differences were observed between *E. coli* and *Enterococcus* spp. isolates and multiple antibiotic resistance profiles with significant differences ( $p < 0.05$ ) noted among the frequency of isolates displaying simultaneous resistance to six and seven antibiotics, and eight to nine antibiotics (Figure 5.4). The most common resistance profile

among total *Enterococcus* spp. and *E. coli* isolates were 33.7% and 32.3%, displaying simultaneous resistance to between six and seven, and three to five antibiotics, respectively (Figure 5.4).



**Figure 5.4. Prevalence of multiple antimicrobial resistance (MAR) profiles (%) among *E. coli* and *Enterococcus* spp. isolates collected from raw wastewater and treated effluent samples in a pilot-scale treatment system. An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n = number of isolates.**

In total, 29.3% of *Enterococcus* spp. isolates demonstrated MAR to eight and nine antimicrobials, with 3.3% exhibiting simultaneous resistance to ten or more antibiotics. When comparing the level of MAR with total *E. coli* isolates, only 8.1% displayed resistance to between eight and nine antimicrobials, and none of the observed *E. coli* isolates were found to display resistance to ten or more of the tested antibiotics. Similarly, Łuczkiwicz *et al.* (2010) studied antimicrobial resistance of faecal indicators in a municipal wastewater treatment plant and found as much as 9% of *E. coli* isolates expressed the phenotype of

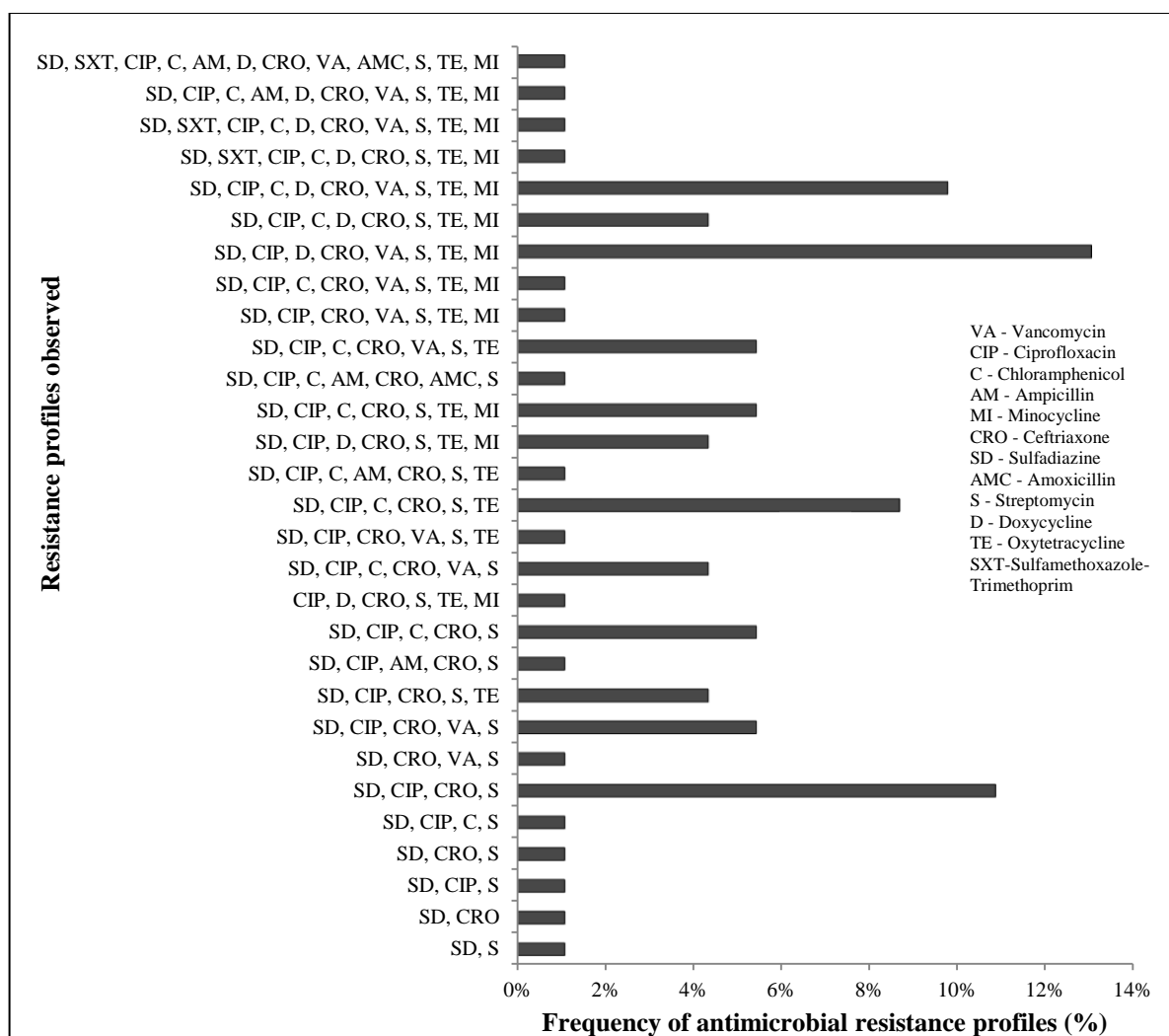
resistance to three or more classes of antimicrobial agents. In the case of enterococci, MAR resistance patterns were detected among 29% of isolates (Łuczkiwicz *et al.*, 2010). Different environmental factors can influence the positive selection of wastewater isolates expressing multiple antibiotic resistance patterns. Specifically, hospital wastewater is often contaminated by antimicrobial agents, which even in subinhibitory concentrations may promote resistance and the survival of resistant strains (Kim *et al.*, 2007; Al-Ahmad *et al.*, 1999). Also, horizontal gene transfer is considered to be the main mechanism of resistance dissemination (Soda *et al.*, 2008), since the presence of antimicrobial resistance genes on mobile genetic elements (plasmids, transposable elements or integron-specific gene cassettes) has been reported among wastewater isolates (Schluter *et al.*, 2007; Tennstedt *et al.*, 2003). In addition, wastewater processes based on activated sludge carry a high cell density and have prolonged biomass retention times, which may enhance the opportunity for resistance dissemination (Soda *et al.*, 2008; Marcinek *et al.*, 1998; Łuczkiwicz *et al.*, 2010; Novo and Manaia, 2010). The MAR results (expressing phenotypic resistance to three or more antibiotics) are considerably higher than those reported by Łuczkiwicz *et al.* (2010) reaching nearly 61% and 98% among *E. coli* and *Enterococcus* spp. isolates, respectively. A possible explanation for this could be the type of wastewater treatment system employed (BNR with activated sludge), in addition to the mixture or composition of wastewater being treated, both of which can greatly influence the prevalence of MAR. Therefore, it is probable that the high levels of MAR observed here could be due to the high microbial cell density within the BNR activated sludge treatment system, favouring the transfer of antibiotic resistance determinants. Another promising explanation for the increased multiple antibiotic resistance among *E. coli* and *Enterococcus* spp. could be the longer hydraulic retention time

(10 hr in this study), as typically employed with biological treatment based on activated sludge processes, compared to biological treatment in fixed film reactors based on trickling filter (TF) or submerged aerated filter (SAF) processes, which rely on much shorter retention times (Novo and Manaia, 2010). Longer HRTs and SRTs may contribute to the development of a resistant bacterial population, as horizontal gene transfer may be favored (Summers, 2006; Kelly *et al.*, 2009), ultimately creating an increase in the prevalence of multiple antimicrobial resistance, as was observed here.

Overall, 29 different resistance profiles were observed among *Enterococcus* spp. isolates collected from raw and treated wastewater in the pilot scale BNR treatment system. In total, 97.8% (90/92) of resistant isolates demonstrated multiple-drug resistance (MDR) to three or more antimicrobial agents. The most commonly observed resistance profile was to eight antimicrobials; sulfadiazine, ciprofloxacin, doxycycline, ceftriaxone, vancomycin, streptomycin, oxytetracycline, and minocycline (SdCipDCroVaSTeMi), representing 13% (12/92) of the resistant isolates observed (Figure 5.5). The second and third most predominate profiles were to sulfadiazine, ciprofloxacin, ceftriaxone, streptomycin (SdCipCroS), and to sulfadiazine, ciprofloxacin, chloramphenicol, doxycycline, ceftriaxone, vancomycin, streptomycin, oxytetracycline, and minocycline (SdCipCDCroVaSTeMi) accounting for 10.9% (10/92), and 9.8% (9/92) of total resistant *Enterococcus* spp. isolates, respectively. The fourth most common MDR profile accounting for 8.7% of total *Enterococcus* spp. isolates was to sulfadiazine, ciprofloxacin, chloramphenicol, ceftriaxone, streptomycin, and oxytetracycline (SdCipCCroSTe). Overall, these four different profiles accounted for almost half, 42.4% of the total drug resistant *Enterococcus* spp. isolates.

Resistance to only one or two antimicrobial agents was observed in 2.2% (2/92) of tested isolates, in response to streptomycin, sulfadiazine and/or ceftriaxone. One highly MAR *Enterococcus* sp. isolate was found to express resistance to all twelve antibiotics tested (SdSxtCipCAmDCroVaAmcSTeMi), illustrating the potential amplification of antimicrobial resistance in environmental isolates. *Enterococcus* species rank among the leading causes of nosocomial infections, costing nearly \$0.5 billion each year in the United States for the treatment of approximately 800,000 enterococcal infections (Richards *et al.*, 2000). The large majority of infection-derived clinical isolates belong to the species *E. faecalis*, although *E. faecium* remains the species demonstrating a disproportionately greater level of resistance to multiple antibiotics (Harbarth *et al.*, 2002; Mundy *et al.*, 2000). Widespread use of vancomycin and extended-spectrum cephalosporins in U.S. hospitals has likely contributed to the emergence and dramatic increase of vancomycin-resistant enterococci (VRE) over the past 20 years (Edmond *et al.*, 1995; Kirst *et al.*, 1998). In total, 45.7% (42/92) of *Enterococcus* spp. isolates from this study displayed resistance to vancomycin (Figure 5.5). Similarly, Sahlström *et al.* (2009) studied vancomycin resistant enterococci in Swedish sewage sludge and found that *E. faecium*, compared to other species of enterococci isolated from sewage sludge, had higher minimum inhibitory concentrations (MICs) to vancomycin.

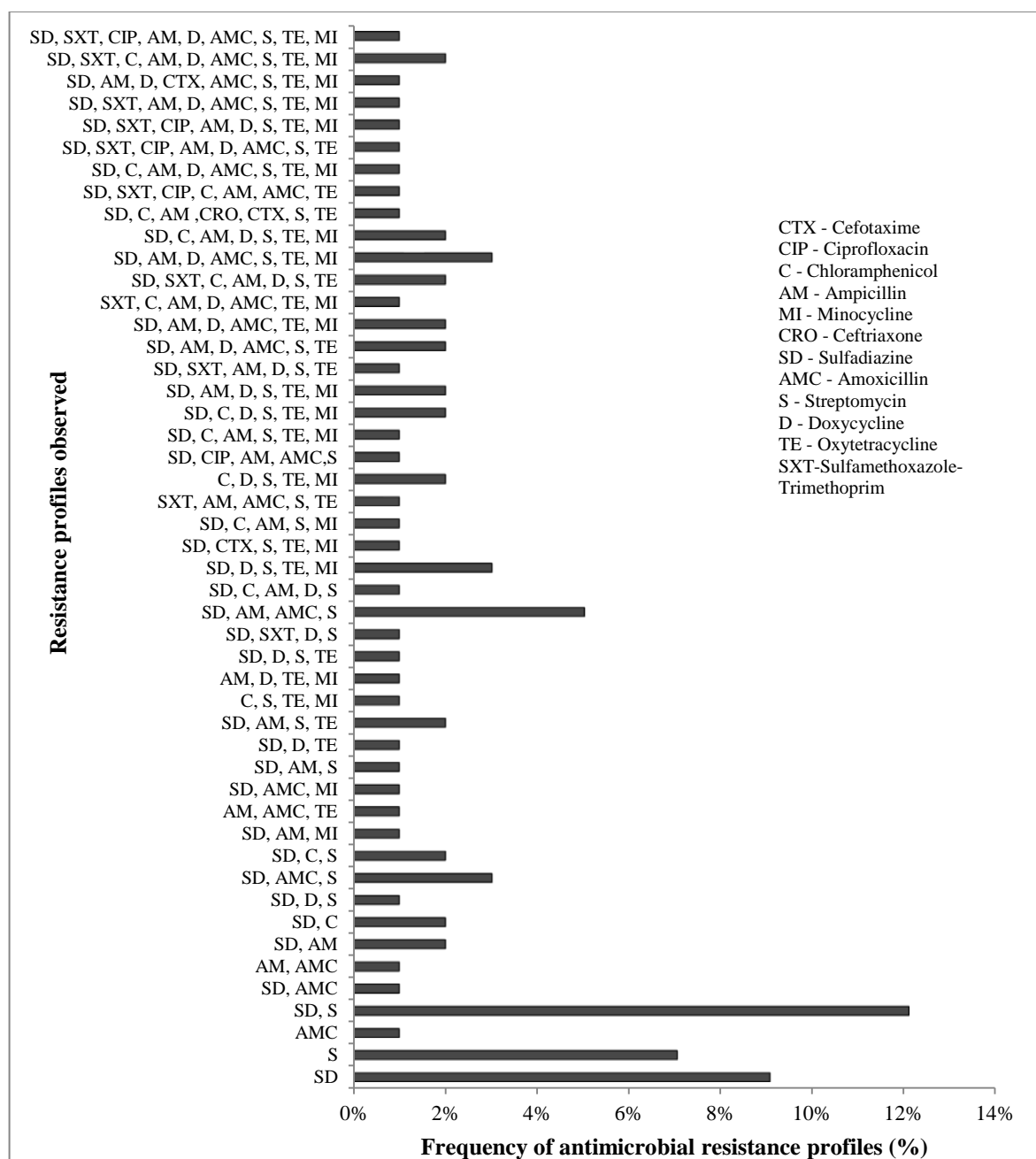




**Figure 5.5. Resistance profiles among *Enterococcus* spp. isolated from wastewater samples in the pilot-scale BNR system (n=92); vancomycin (VA), ciprofloxacin (CIP), chloramphenicol (C), ampicillin (AM), minocycline (MI), ceftriaxone (CRO), sulfadiazine (SD), amoxicillin (AMC), streptomycin (S), doxycycline (D), oxytetracycline (TE), sulfamethoxazole-trimethoprim (SXT).**

In the case of *E. coli* isolates, a total of 48 different resistance profiles were observed from raw and treated wastewater samples in the pilot scale BNR treatment system (Figure 5.6). Overall, 60.6% (60/99) of resistant isolates demonstrated multiple-drug resistance (MDR) to three or more antimicrobial agents. The most commonly observed resistance profile was to sulfadiazine and streptomycin (SdS) representing 12.1% of total isolates, followed by single

drug resistance to sulfadiazine (Sd) and streptomycin (S), representing 9.1% and 7.1% of total *E. coli* isolates, respectively. The fourth most abundant resistance profile was to sulfadiazine, ampicillin, amoxicillin, and streptomycin (SdAmAmcS) accounting for approximately 5.1% of total *E. coli* isolates (Figure 5.6). These four different profiles accounted for approximately 33.4% of the total drug resistant *E. coli* isolates. Resistance to only one antimicrobial was observed in 19.2% (19/99) of total *E. coli* isolates, with 4% (4/99) of *E. coli* isolates exhibiting sensitivity to all twelve antimicrobials. Single drug resistance (SDR) was observed in response to sulfadiazine, streptomycin, and amoxicillin. In total, only three isolates (3%) displayed resistance to one or more of the third-generation cephalosporins (CTX and/or CRO), as shown in Figure 5.6. In addition, MDR isolates displayed high levels of resistance to sulfonamide (sulfadiazine and/or sulfamethoxazole-trimethoprim), aminoglycoside (streptomycin), penicillin (ampicillin and/or amoxicillin) and tetracycline (doxycycline, oxytetracycline, and/or minocycline) based antibiotics, representing approximately 82%, 71%, 51%, and 49% of total *E. coli* isolates, respectively. The results are in agreement with Pignato *et al.* (2009), who examined *E. coli* isolates from raw and treated municipal wastewaters and found the highest resistance levels were to ampicillin, tetracycline, sulfamethoxazole, and streptomycin.



**Figure 5.6. Resistance profiles among *E. coli* isolated from wastewater samples in the pilot-scale BNR system (n=99); cefotaxime (CTX), ciprofloxacin (CIP), chloramphenicol (C), ampicillin (AM), minocycline (MI), ceftriaxone (CRO), sulfadiazine (SD), amoxicillin (AMC), streptomycin (S), doxycycline (D), oxytetracycline (TE), sulfamethoxazole-trimethoprim (SXT).**

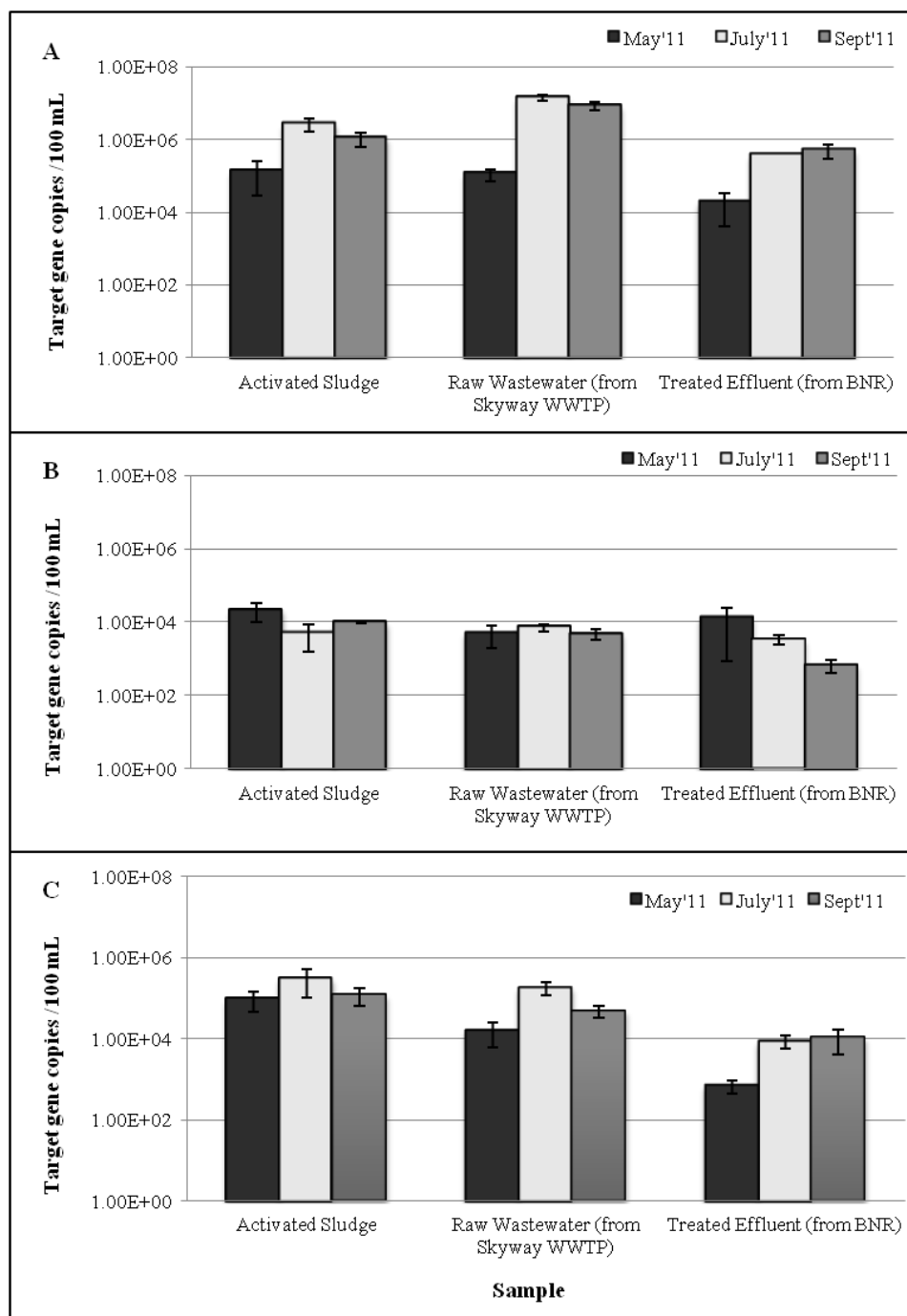
Antibiotic resistance, particularly MDR, is a major public health threat and the presence of resistant organisms in treated wastewater is an emerging concern worldwide. The potential

for this resistance to be transferred to native populations or other pathogenic species is largely unknown and warrants further investigation.

#### **5.4.2 Quantitative detection of bacterial pathogens and ARGs in a pilot-scale BNR treatment system**

Real time quantitative PCR (qPCR) was used to determine the concentration of three bacterial pathogens; *E. coli*, *Enterococcus faecalis*, and *Salmonella* spp., and three ARGs; *tetA*, *ampC*, and *blaSHV* in raw and treated wastewater samples, as well as activated sludge samples collected over three sampling events. Figure 5.7 illustrates the relative abundance (target gene copies/100 mL) of bacterial pathogens (*E. coli*, *Salmonella* spp., and *E. faecalis*) over three sampling events in activated sludge, raw wastewater, and treated effluent samples. The highest values were obtained for *E. coli* with approximately  $10^7$  target gene copies/100 mL detected during the second sampling event (July'11) in raw wastewater samples. Similar concentrations of *E. coli* were obtained in the activated sludge samples, reaching approximately  $10^6$  target gene copies/100 mL during the second and third sampling events. *E. coli* concentrations were reduced slightly during the treatment process, reaching a final concentration of approximately  $10^4$  -  $10^5$  target gene copies/100 mL detected in the treated effluent. Overall, treatment reduced *E. coli* concentrations by only one order of magnitude in most cases, with the exception of the second sampling event (July'11), in which a two log reduction was observed from raw wastewater to treated effluent (Figure 5.7A). *Salmonella* spp. concentrations were fairly consistent with no significant differences observed between

the three sampling events and different stages of treatment, maintaining approximately  $10^3$ - $10^4$  target gene copies/100 mL in the raw wastewater and treated effluent (Figure 5.7B).



**Figure 5.7. Relative abundance (target gene copies/100mL) of *E. coli* (A), *Salmonella* spp. (B), and *E. faecalis* (C) in wastewater samples from the BNR pilot-scale system. Error bars represent standard deviation around mean values from two independent qPCR runs in duplicate.**

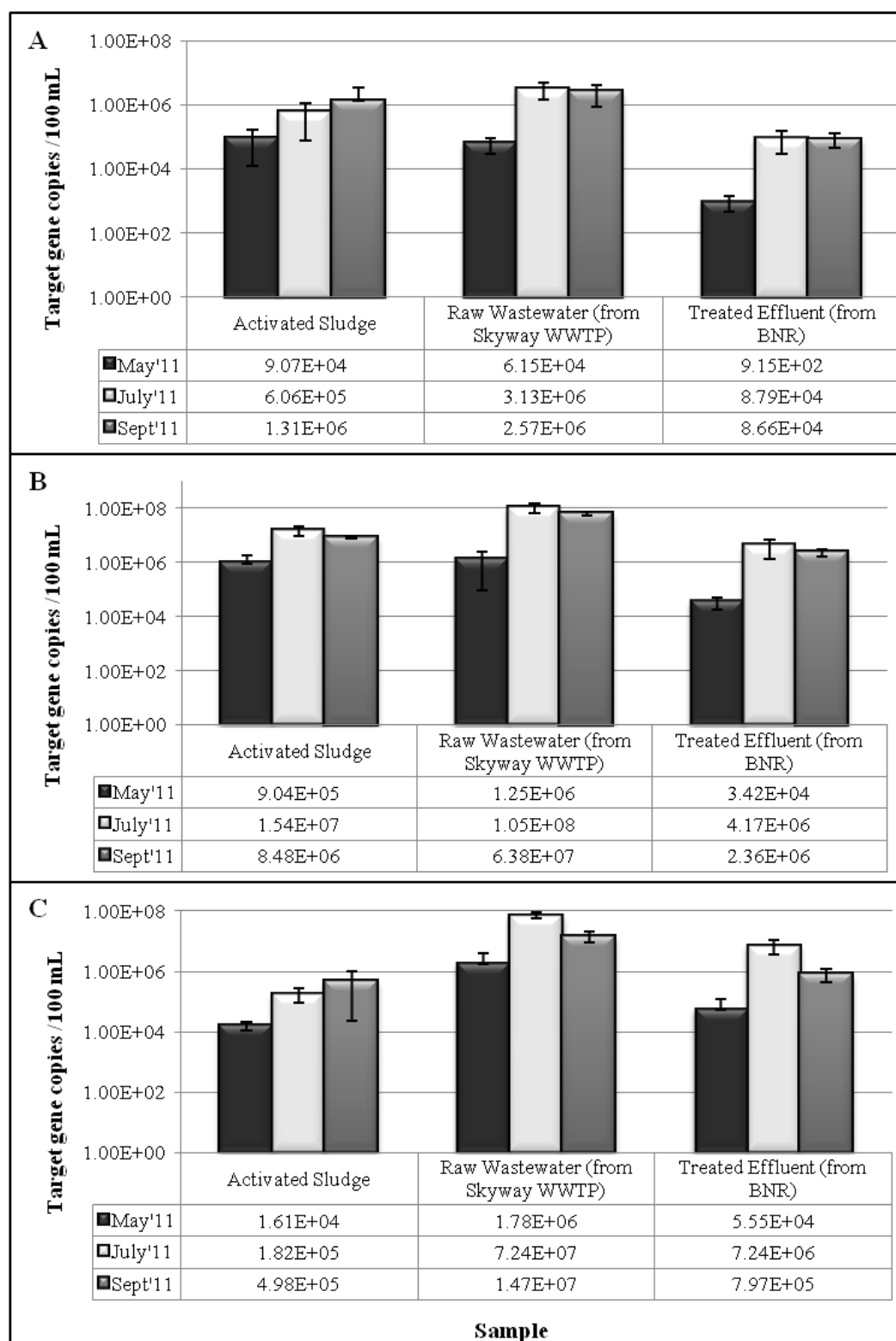
The BNR pilot-scale system showed very little removal, if any, for *Salmonella* spp.. During the first sampling event (May'11), average *Salmonella* spp. concentrations were  $5.03 \pm 3.01 \times 10^3$  target gene/100 mL in raw wastewater, however, a slight increase was observed in the final treated effluent, reaching an average concentration of  $1.29 \pm 1.21 \times 10^4$  target gene/100 mL (Figure 5.7B). It is important to note the limitation associated with quantitative PCR, and the inability to distinguish between DNA in viable cells and freely available DNA in the environment or contained in dead cells (Rudi *et al.*, 2005). Therefore, the observed increase in *Salmonella* spp. in the treated effluent may not be an indication of increased viable or culturable *Salmonella* spp., but rather an increase in *Salmonella* spp. DNA from either live or dead bacterial cells. Compared to other bacteria, *Salmonella* spp. have high survival rates in aquatic environments and are able to withstand a variety of stresses, including thermal and pH fluctuation, high osmolarity and low nutrient availability (Chao *et al.*, 1987; Winfield and Groisman, 2003). Similarly, Lee *et al.* (2006) used qPCR to quantify indicator and pathogenic bacteria during wastewater treatment and found that *E. coli* was more abundant in municipal wastewater than representative pathogens, including *Salmonella* spp., by 2-5 orders of magnitude. The results are in agreement with previously published studies (Lee *et al.*, 2006; Wéry *et al.*, 2008) in that *E. coli* was present in much higher concentrations among all samples compared to that of *E. faecalis* or *Salmonella* spp., by approximately two and three orders of magnitude, respectively. Concentrations of *E. faecalis* were detected among all samples, with values of  $10^4$  -  $10^5$  target gene copies/100 mL detected in the activated sludge and raw wastewater samples, but were reduced in the treated effluent falling to  $10^2$  -  $10^4$  in these samples (Figure 5.7C).

The observed treated effluent values are higher than those specified in the Effluent Quality Guidelines as set by Environment Canada (400 faecal coliforms CFU/100 mL) (CSC, 2003). However, it may be inappropriate to directly compare gene copy numbers with cell numbers, as the former may overestimate cell numbers as a result of dead cell contribution to the wastewater DNA pool (Lee *et al.*, 2006). Overall, the pilot-scale BNR system receiving raw wastewater from the Skyway WWTP was found to reduce the level of bacterial pathogens, specifically *E. coli* and *E. faecalis*, but does not guarantee the complete removal of contamination by faecal indicators and pathogenic species, including *Salmonella* spp.. Other studies have also shown that the sewage treatment process may reduce the number of pathogens insufficiently (Strauch, 1998), with further treatment and disinfection required to render the water safe for discharge.

Figure 5.8 summarizes the concentrations of ARGs (*blaSHV*, *ampC*, *tetA*) obtained from activated sludge, raw wastewater, and treated effluent samples, over three separate sampling events, in the pilot-scale BNR treatment system. Relative concentrations among all three ARGs in raw wastewater samples were similar, with target gene copies/100 mL between  $10^6$  -  $10^8$ , and slightly higher *tetA* and *ampC* concentrations, reaching  $10^7$  and  $10^8$  target gene copies/100 mL, respectively (Figure 5.8). All three ARGs were reduced by approximately 1-2 orders of magnitude, as the raw wastewater passed through the treatment system, maintaining *blaSHV* concentrations of  $10^2$  -  $10^4$  target gene copies/100 mL in the treated effluent (Figure 5.8A). Concentrations of *ampC* (Figure 5.8B) and *tetA* (Figure 5.8C) were reduced to approximately  $10^4$  -  $10^6$  target gene copies/100 mL in the final treated effluent. Average concentrations of all three ARGs increased following the first sampling event

(May'11), with *blaSHV*, *ampC*, and *tetA* raw wastewater concentrations in the order of  $10^4$ ,  $10^6$ , and  $10^6$  target gene copies/100 mL, respectively, escalating to  $10^6$ ,  $10^8$ , and  $10^7$  target gene copies/100 mL during subsequent sampling events (July, Sept'11), respectively. A plausible explanation for the increase in ARGs could perhaps be attributed to the increase in temperature within the treatment system during the summer months, as the BNR system maintained an approximate temperature of 12-13°C during the first sampling event (May), while rising to 18°C during the second (July) and third (Sept.) sampling events. In the case of seasonal effects, the cooler months may be reflective of reduced metabolic activity, in which microbial growth may be reduced at lower temperatures, and therefore, the transfer of these genes may also be reduced. Typically warmer temperatures are associated with high microbial activity and reproduction which may also increase gene transfer, thereby allowing for an overall increase in antibiotic resistance gene concentrations as observed in this study. In particular, the results for the detection of *E. coli* within raw wastewater samples (Figure 5.7A) are in accordance with this statement, as an increase by approximately two orders of magnitude ( $10^5$  to  $10^7$  target gene copies/100 mL) was observed during the warmer months (July and September).





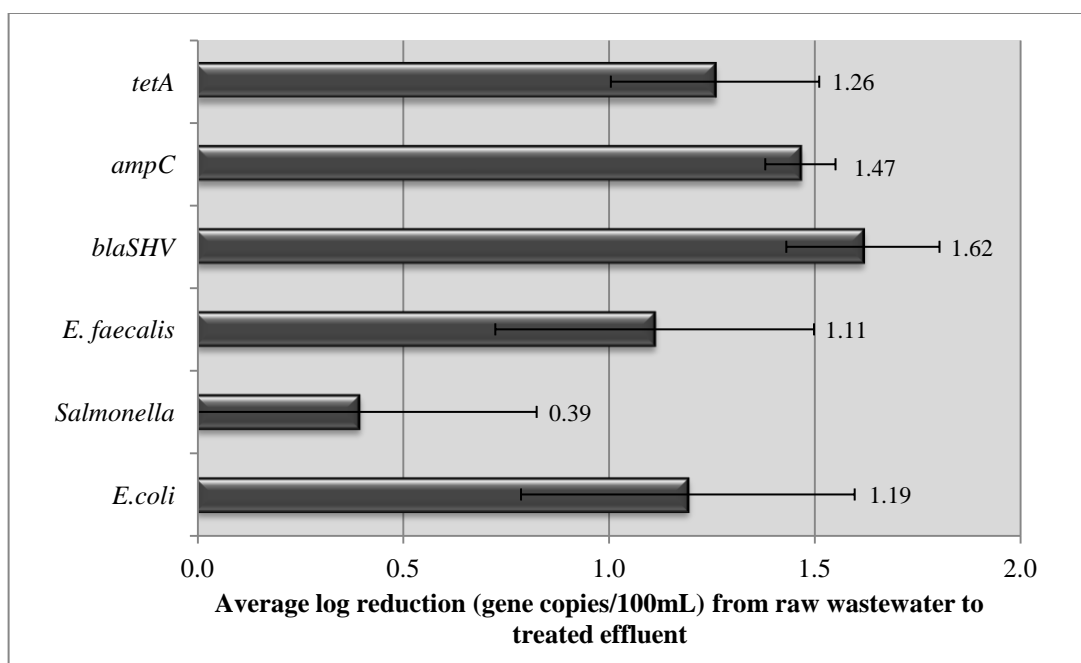
**Figure 5.8. Relative abundance (target gene copies/100mL) of *blaSHV* (A), *ampC* (B), and *tetA* (C) genes in wastewater samples from the BNR pilot-scale system. Error bars represent standard deviation around mean values from two independent qPCR runs in duplicate.**

Tetracycline resistance is commonly encountered in natural and human-affected environments (Esiobu *et al.*, 2002; Auerbach *et al.*, 2007; Patterson *et al.*, 2007). Both *tetA* and *tetB* are located on conjugative plasmids or transposons (Roberts 1996), which have been shown to disseminate between different bacterial species in water environments (Rhodes *et al.* 2000). The conjugative plasmids and transposons may also carry other types of antibiotic and heavy metal resistance genes (Roberts, 1996). Therefore, high concentrations of tetracycline resistance genes (i.e. *tetA*) may indicate a potential risk for simultaneous spread of other genes encoding resistance. The results are in accordance with similar studies, suggesting WWTPs and alternative treatment systems are a significant source of resistance genes to the environment (Auerbach *et al.*, 2007, Pruden *et al.*, 2006, McKinney *et al.*, 2010, Munir *et al.*, 2010). Wastewater has been said to stimulate horizontal gene transfer among microbial species (Aminov *et al.*, 2001; Lorenz and Wackemagel, 1994). As a result, wastewater treatment plants and alternative systems could potentially serve as important reservoirs for the spread of antibiotic resistance among faecal indicators and pathogens if the treatment process proves unsuccessful in removing ARGs. The water environment itself is considered to play an important role in providing a medium for the transfer of resistance genes and resistant bacteria to the broader environment (Baquero *et al.*, 2008; Iwane *et al.*, 2001). High concentrations of ARGs were found in the treated effluent (reaching  $10^6$  target gene copies/100 mL), which may ultimately serve as a potential route for entry of ARGs and antibiotic resistant bacteria into the natural environment. Wastewater treatment plants hold important jurisdiction over the elimination or spread of antibiotic resistant microbes as the treatment systems and their operational conditions might influence the fate of resistant bacteria or resistance genes (Iwane *et al.*, 2001). However, the extent of

human exposure to ARGs and antibiotic resistant bacteria is still not well understood, and future studies on human exposure to these resistant contaminants are necessary.

#### **5.4.3 Removal efficiency of bacteria and ARGs in a pilot-scale BNR treatment system**

Average log removals for all three sampling events were calculated based on the concentrations of pathogens and ARGs in raw wastewater samples and final treated effluent samples, as represented in Figure 5.9. *E. coli* was approximately 3-4 orders of magnitude higher in the pre-treatment sample (raw wastewater) compared to that of *Salmonella* spp., and approximately 2-3 orders of magnitude higher than *E. faecalis*. The removal of indicator bacteria, including *E. coli* and *E. faecalis* by the pilot-scale BNR system was relatively low, with an average log reduction of 1.19 and 1.11, respectively, between raw wastewater and treated effluent samples (Figure 5.9). Bacterial removal varies with the type of treatment system, but is generally expected to be in the range of 1-3 log units of indicator bacteria and pathogens (Bitton 2005). However, in this study, pathogenic bacteria including *Salmonella* spp. showed very little reduction, with an average log removal of 0.39 between the raw wastewater and treated effluent. Compared to other bacteria, *Salmonella* spp. have high survival rates in aquatic environments and are able to withstand a variety of stresses (Winfield and Groisman, 2003). *Salmonella* spp. have also been described as being more resistant than *E. coli* to biotic factors (microbial predators or competing organisms) in sources of drinking water (Wright, 1989), perhaps due to a difference in adhesion to protective particles (Winfield and Groisman, 2003).



**Figure 5.9. Average log reduction of target gene copies/100mL for all three sampling events from raw wastewater to treated effluent samples among bacterial pathogens and ARG's. Error bars represent standard deviation.**

The removal efficiency of the ARGs *tetA*, *ampC*, and *blaSHV* were very similar with log removals between one and two orders of magnitude from raw wastewater to treated effluent (Figure 5.9). The greatest decline in ARG concentration was observed among *blaSHV*, with an average log reduction of 1.62, whereas *ampC* and *tetA* were found to decrease by approximately 1.47 and 1.26 orders of magnitude, respectively. Munir *et al.* (2010) studied the release of antibiotic resistant bacteria and genes in effluent and biosolids from five wastewater utilities and found much higher log removals of ARGs (*tetW*, *tetO*, *sulI*) in the membrane biological reactor (MBR) facility (range of removal: 2.57-7.06 logs) compared to that in conventional treatment plants (range of removal: 2.37- 4.56 logs). These results differ from those of Munir *et al.* (2010) in that the average log removal of bacterial pathogens and ARGs was considerably less, decreasing by less than two orders of magnitude from raw

wastewater to treated effluent. Differences in log removals could be attributed to a number of factors including the type of wastewater (i.e. if raw wastewater contains hospital effluent it is more likely to have increased bacterial loads, and therefore, could be more difficult to remove), the particular treatment system being studied, the specific ARG of interest, or multiple selective pressures in the environment. Wastewater utilities appear to be a potential source of emerging ARGs and bacteria in the environment. The results illustrate high concentrations of tetracycline, and beta-lactamase genes (*ampC*, *blaSHV*) in raw wastewater, activated sludge, and treated effluent samples analysed in this study, signifying the potential dissemination of ARGs into receiving water bodies.

## 5.5 Conclusions

This chapter describes the use of traditional culture-based antibiotic screening techniques in combination with molecular quantification through real-time PCR to help establish a preliminary assessment of selected pathogens and ARGs in a pilot-scale BNR treatment system receiving full-scale municipal and hospital wastewater. Overall, the level of multiple antibiotic resistance (MAR) among culturable indicator bacteria was high, encompassing approximately 61% and 98% of total *E. coli* and *Enterococcus* spp. isolates, respectively. The most common antibiotic resistance profile among *E. coli* was simultaneous resistances to sulfadiazine and streptomycin (SdS) representing 12.1% of total isolates, whereas the most common MAR profile for *Enterococcus* spp. was resistance to eight antimicrobials; sulfadiazine, ciprofloxacin, doxycycline, ceftriaxone, vancomycin, streptomycin, oxytetracycline, and minocycline (SdCipDCroVaSTeMi), representing 13% (12/92) of total

isolates. Real time quantitative PCR was used to determine the concentration of three bacterial pathogens; *E. coli*, *Enterococcus faecalis*, and *Salmonella* spp., and three ARGs; *tetA*, *ampC*, and *blaSHV*, in activated sludge, raw wastewater, and treated effluent samples collected over three sampling events (May, July, and Sept'11). Overall, the pilot-scale BNR treatment system was found to minimally reduce the level of bacterial pathogens and ARGs, with average log removals ranging between 0.39 and 1.62 (ideal removals typically range between two and five orders of magnitude). Concentrations of all three ARGs remained relatively high with  $10^4$  -  $10^6$  target gene copies/100 mL present in the final treated effluent. Although, the focus of this study was limited to only a few select ARGs; numerous ARGs may be present in wastewater and sewage sludge, with their fates being unique to each specific environment. Thus, it is important to consider each ARG may have different behaviours with respect to fate and transport and response to physical, chemical, and/or biological treatment. The results generated from this study do however, present new data on the prevalence and fate of faecal indicators, selected pathogens, and ARGs, on a real scale, during a wastewater treatment process using a pilot scale BNR system.

## **5.6 Recommendations & Future Research Needs**

- Future studies could examine the prevalence, distribution, and fate of antibiotic resistant bacteria and ARGs in different types of wastewater treatment utilities (i.e. membrane biological reactor, oxidative ditch, sludge treatment processes), while comparing the different operational processes and conditions which may ultimately affect the occurrence and frequency of ARGs.

- Additional studies are needed in order to understand the effects of disinfection on the concentration of ARGs and antibiotic resistant bacteria in wastewater treatment plants.
- Further studies monitoring the prevalence and fate of different ARGs conferring resistance to other classes of antibiotics are needed, as each ARG may demonstrate different behaviours in response to wastewater treatment.
- Supplementary studies could monitor the prevalence and fate of bacterial pathogens and ARGs at the point of discharge from the WWTP (i.e. rivers, lakes), as well as examining the extent of human exposure to ARGs and antibiotic resistant bacteria (which may include the biological fitness of resistance genes, the opportunities to reach new hosts, and the ability of bacterial species to transfer resistance genes).

# 6

## **Prevalence and transferability of tetracycline and streptomycin determinants in *E. coli* and *Salmonella* spp. isolated from wastewater effluents<sup>5</sup>**

### **6.1 Introduction**

The transfer of antibiotic resistance genes (ARGs) among microorganisms has long been recognized as a serious threat, contributing to the evolution and emergence of antibiotic resistant bacteria, thereby reducing the therapeutic potential against pathogens (Levy and Marshall, 2004; Davies, 2007; Hawkey and Jones, 2009; Merlin *et al.*, 2011). Many

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<sup>5</sup>Helt, C.D., Legge, R.L., Slawson, R.M. *Prevalence and transferability of tetracycline and streptomycin determinants in *E. coli* and *Salmonella* spp. isolated from wastewater effluents*, in preparation.



ecosystems can support the dissemination of resistance genes (Witte, 2000; Hawkey and Jones, 2009), although some environments seem to promote increased transfer rates and as such have been defined as “hot spots” (van Elsas and Bailey, 2002; Salyers *et al.*, 2004). Wastewater treatment plants (WWTPs), including activated sludge processes, are known to combine high bacterial cell density, antibiotics, and resistant bacteria, promoting the development of such hot spots (Dröge *et al.*, 2000; Schlüter *et al.*, 2007), although the environmental parameters driving the transfer of genes still need to be fully understood. Conventional wastewater treatment reduces the numbers of enteric microbes, but reductions in treatment processes can vary extensively and wastewater effluents may still contain high numbers of faecal microorganisms. The removal efficiency of pathogenic and indicator microorganisms in wastewater treatment plants vary according to the treatment process type, retention time, other biological flora present in activated sludge, O<sub>2</sub> concentration, pH, temperature and the efficiency in removing suspended solids (Teitge *et al.*, 1986; Yaziz and Lloyd, 1979). Pathogenic microorganisms have been demonstrated even to multiply in wastewaters or wastewater treatment plants (Emparanza-Knorr and Torrella, 1995). Plasmids are considered to play a key role in the transfer of ARGs (Hawkey and Jones, 2009) and have been isolated from anthropogenic environments including activated sludge (Dröge *et al.*, 2000; Schlüter *et al.*, 2007). There are four general mechanisms of resistance, all of which are controlled by the action of specific genes: enzymatic inactivation or modification of antimicrobial agents, impermeability of the bacteria cell wall or membrane, active expulsion of the drug by the cell efflux pump, and alteration in target receptors (Prescott *et al.*, 2000). Bacteria acquire ARGs through mobile elements, such as plasmids, transposons, and integrons (Prescott *et al.*, 2000; Rubens *et al.*, 1979) which result in

mutations in genes responsible for antibiotic uptake or binding sites (Spratt, 1994) or activation of portions of bacterial chromosomes (Aleksun and Levy, 1999; Hachler *et al.*, 1999). Gram negative bacteria and related organisms often harbour different plasmids which confer to them multiple antibiotic resistance to many unrelated antibiotics, contributing to survival in microbially hostile environments. Horizontal transfer of ARGs through conjugative plasmids often occurs among coliforms and other Gram negative bacteria which are dominant in sewage and aquatic environments, making them important bacteria in the development of multiple antibiotic resistances to several antibiotics (Shakibaie *et al.*, 2009). *E. coli* is a natural intestinal inhabitant of humans and other warm-blooded animals and a significant pathogen associated with gastrointestinal infection, urinary tract infections and a variety of other extraintestinal infections (Donnenberg, 2005). *E. coli* shed into the environment can survive for significant time periods (Flint, 1987; McQuaig *et al.*, 2006; Rhodes and Kator, 1988) and the detection of its presence in food or water is widely used as a microbiological indication of faecal contamination and the possible presence of enteric pathogens (Krumperman, 1983). Data on the significance of environmental contamination with antibiotic-resistant *E. coli* for human health are limited. Previous reports have shown that antimicrobial-resistant strains of bacteria are present in a variety of effluents such as hospital effluent discharge (Jakobsen *et al.*, 2007; Prado *et al.*, 2008), inflow to a WWTP (Mesa *et al.*, 2006) and treated effluent from a WWTP (Martins da Costa *et al.*, 2006; Chitnis *et al.*, 2004; Moura *et al.*, 2007; Vilanova *et al.*, 2004). WWTPs treating hospital effluent may be associated with relatively high discharge levels of antimicrobial-resistant *E. coli* compared to those of a plant treating municipal effluent that does not include hospital wastewater (Reinthalder *et al.*, 2003). Another important enteric pathogen commonly found in

wastewater is *Salmonella*, however, little information exists on the frequency of antimicrobial resistance and transferability of ARGs among *Salmonella* spp. isolated from WWTPs in Canada. The survival of *Salmonella*, despite treatment, implies the possibility of selection of resistant strains, or the acquisition of resistance through the transference of genetic material. Similar to other bacteria, *Salmonella* can acquire resistance through mutations in their genetic material or through the uptake of resistance genes on mobile genetic elements via horizontal gene transfer (Foley and Lynne, 2008; Thomas, 2011).

National and international surveillance programs have been developed to monitor the frequency and progression of drug resistance in many zoonotic pathogens. In North America, these national programs consist of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) and the National Antimicrobial Resistance Monitoring System (NARMS). These programs focus on and report the phenotypic expression of drug resistance among enteric pathogens and commensal bacteria, including *Salmonella* and *E. coli*, however; genotypic characterization is rarely reported (Boerlin *et al.*, 2005; Li *et al.*, 2007; Andrysiak *et al.*, 2008). Knowledge of the genes responsible for resistance and potential transferability of such genes is imperative for understanding the development and spread of antimicrobial resistance.

Antimicrobial agents are commonly used therapeutically in animals and humans for control of bacterial infections and may be incorporated into commercial livestock and poultry feed at subtherapeutic doses for growth promotion. This practice is believed to enhance selection

of resistant bacteria more than the therapeutic use of antimicrobial agents in response to clinical disease (Van den Bogaard *et al.*, 2001) and may potentially lead to antibiotic resistance in humans acquired through the human food chain (Barton, 1998; Witte, 1998). A possible strategy thought to curtail this problem is to eliminate the use of antimicrobial agents needed for human treatment as feed additives (Richmond, 1972; Swann Committee, 1969; WHO, 1997), however; an ongoing debate concerning whether and to what extent feed additives contribute to the development of resistance in human bacterial pathogens still remains (Casewell *et al.*, 2003; Prescott *et al.*, 2000). In addition to the consequences for human health, concerns have been raised surrounding the contamination of aquatic environments, including surface water with resistant bacteria from livestock operations and human septage. Resistant bacteria have been isolated from a variety of sources, including domestic sewage, drinking water, surface water, rivers, and lakes (McKeon *et al.*, 1995; Mulamattathil *et al.*, 2000; Sayah *et al.*, 2005). The levels of antibiotic resistance that have been reported range from 72% (Mulamattathil *et al.*, 2000) up to 100 and 87% for faecal and nonfaecal coliforms, respectively (McKeon *et al.*, 1995).

Tetracycline and streptomycin are two antimicrobials that belong to two families (tetracyclines and aminoglycosides) of broad-spectrum antibiotics and their efficacy, low cost and lack of side effects make them a popular choice among human and veterinary medicine (Boxall *et al.*, 2003; Chopra and Roberts, 2001). In the case of enteric pathogens including *E. coli* and *Salmonella* spp. resistance to tetracyclines and streptomycin or spectinomycin is generally the most prevalent (Guerra *et al.*, 2003; Lanz *et al.*, 2003). Therefore, the following ARGs were selected for study, *tetA*, *strA*, and *strB*. Several genes

including *strA* and *strB* are known to be associated with streptomycin resistance (Chiu *et al.*, 2006; Hur *et al.*, 2011) and are commonly distributed in *Salmonella* spp. (Pezella *et al.*, 2004; Weill *et al.*, 2006) and *E. coli* (Boerlin *et al.*, 2005; Graves *et al.*, 2011). Most environmental *tet* genes code for transport proteins, which pump the antibiotics out of the bacterial cell, thereby maintaining a low intracellular concentration allowing the ribosomes to function normally (Roberts, 2002). Among the forty tetracycline resistance genes discovered thus far (Thaker *et al.*, 2010), *tetA* and *tetC* have frequently been measured from environmental sites including activated sludge (Guillaume *et al.*, 2000), fish farming ponds (Dang *et al.*, 2007), surface water (Poppe *et al.*, 2006) and swine lagoons (Macauley *et al.*, 2007). The prevalence of tetracycline and streptomycin resistance represents a useful marker to monitor resistance genes (Ozgumus *et al.*, 2007) and it can provide a good model for ecological studies of antimicrobial resistance (Karami *et al.*, 2006).

While the reservoir for ARGs in populations of clinical isolates has been, and continues to be documented in view of the importance for the treatment of infections, no such database exists for bacterial populations in soil and/or water. The incidence of bacteria which are resistant to one or more antibiotics has been traditionally studied by plating on antibiotic-containing nutrient media, or by screening bacterial isolates for their antibiotic resistance patterns. More recently, molecular tools have enabled scientists to make progress in studying the epidemiology of ARGs and mobile genetic elements at the genetic level. Therefore, the objective of this study was to examine waterborne *Salmonella* spp. and *E. coli* isolates collected from raw and/or treated wastewater effluents and determine the prevalence of antimicrobial resistance using both phenotypic and genotypic methods. The ability of

*Salmonella* spp. and *E. coli* isolates to transfer ARGs (*tetA*, *strA*, *strB*) to a recipient strain was also assessed.

## **6.2 Research Needs & Objectives**

Genotypic evaluation of drug resistance in environmentally obtained strains of *Salmonella* spp. and *E. coli* is limited. In order to have a better understanding of the extent to which wastewater effluents are associated with the spread and dissemination of antimicrobial resistance (AMR), the prevalence, diversity, distribution, and conjugal transfer of resistance genes in waterborne isolates is needed. Establishing how the resistance is mediated in *E. coli* and *Salmonella* spp. isolates, whether plasmid mediated or chromosomally-encoded, is essential to understanding the complexity of resistance among environmental isolates. Few studies have examined the transferability via conjugation of ARGs in *E. coli* and *Salmonella* spp. isolated from wastewater (West *et al.*, 2011), with previous reports focusing on *E. coli* isolates from food-producing animals (Gow *et al.*, 2008), humans (Schwaiger *et al.*, 2010; Tuckman *et al.*, 2003), companion animals (Costa *et al.*, 2008), meat products (Koo and Woo, 2011) and raw seafood (Nawaz *et al.*, 2009). Understanding the ability of enteric pathogens to transfer their resistance determinants to other bacteria (Gram negative and/or Gram positive) will prove useful in determining the impact of sewage and WWTPs on the dissemination of ARGs into the broader environment.

The specific objectives of this chapter are to:

1. Determine the phenotypic characteristics and antibiotic resistance profiles among culturable *E. coli* and *Salmonella* spp. isolates using the disc-diffusion method;
2. Investigate the distribution and diversity of the following ARGs (*tetA*, *strA*, *strB*) conferring resistance to tetracycline and/or streptomycin among waterborne *E. coli* and *Salmonella* spp. donor isolates;
3. Examine the *in vitro* transfer via conjugation assays to ascertain the transferability of tetracycline (*tetA*) and/or streptomycin (*strA* and *strB*) resistance determinants and antibiotic resistance patterns using multi-drug resistant *E. coli* and *Salmonella* spp. isolates as donors and *E. coli* K12 as the recipient; and
4. Utilize molecular techniques to confirm plasmid-mediated conjugal transfer of ARGs through PCR detection among transconjugant isolates.

## **6.3 Materials and Methods**

### **6.3.1 Bacterial strains**

Previously isolated waterborne *Salmonella* spp. (n=16) and *E. coli* (n=14) strains collected from various environmental compartments including poultry lagoons, raw wastewater from a municipal WWTP, and treated effluent from a pilot-scale BNR system, were selected to study the distribution of tetracycline and streptomycin resistance determinants, antibiotic resistance profiles, and the conjugal transfer of selected ARGs to an antibiotic-susceptible

strain of *E. coli* K12 JM109, rendered chromosomally resistant to nalidixic acid, referred to as *E. coli* nal<sup>R</sup>. All strains were confirmed by polymerase chain reaction (PCR) as previously described (Lee *et al.*, 2006). Primers used in this study to confirm *Salmonella* spp. and *E. coli* are shown in Table 6.1.

### 6.3.2 Detection of tetracycline and streptomycin resistance genes

All isolates of tetracycline and streptomycin-resistant strains were tested by single PCR analysis for the presence of genetic determinants (*tetA*, *strA*, and *strB*) causing this resistance. The oligonucleotide primers and predicted sizes of the amplified products used in this study are shown in Table 6.1 as previously described (Faldynova *et al.*, 2003; Levings *et al.*, 2005). The amplifications were carried out using an iCycler thermal cycler (Bio-Rad Laboratories; Mississauga, ON, Canada). These three antimicrobial resistance genes confer resistance to two different classes of antibiotics, including tetracyclines and aminoglycosides (streptomycin).

**Table 6.1. PCR primers used for the detection of *E. coli*, *Salmonella* spp., and ARGs**

Primer	Nucleotide Sequence (5'–3')	Product (bp)	Accession no.	Reference
Eco-F Eco-R	GTCCAAAGCGGCGATTTG CAGGCCAGAAGTTCTTTTCCA	<100	S69414	Lee <i>et al.</i> (2006)
Sal-F Sal-R	CGTTTCCTGCGGTACTGTAAATT AGACGGCTGGTACTGATCGATAA	82	U43272	Lee <i>et al.</i> (2006)
strA-F strA-R	CTTGGTGATAACGGCAATTC CCAATCGCAGATAGAAGGC	548	M95402	Levings <i>et al.</i> (2005)
strB-F strB-R	ATCGTCAAGGGATTGAAACC GGATCGTAGAACATATTGGC	509	M95402	Levings <i>et al.</i> (2005)
tetA-F tetA-R	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	210	X61367	Faldynova <i>et al.</i> (2003)



The reaction mixtures (20 µL) contained 5 X GoTaq Flexi Buffer (4.0 µL), 2.5 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleoside triphosphate mix, 1.0 U of GoTaq Flexi DNA Polymerase (Promega), 2.5 µL of each primer (10 µM), and colony or template (2.5 µL) DNA. The PCR conditions were 95°C for 5 min for an initial denaturation, followed by 35 cycles at 94°C for 60 s, annealing for 60 s at 55°C and a final extension at 72°C for 1.5 min. The size of each PCR product was determined by comparing to a Quick-Load 100 bp DNA ladder (New England Biolabs Ltd., Pickering, ON). PCR products were examined on 1.6% 1X sodium borate agarose gels and confirmed under UV light after ethidium bromide (EB) staining.

### **6.3.3 Antimicrobial susceptibility testing**

Antimicrobial resistance patterns were determined using the disc-diffusion method as set by the Clinical and Laboratory Standards Institute (CLSI, 2007). *Salmonella* spp. and *E. coli* isolates were inoculated into Brain Heart Infusion (BHI) broth (Difco, Fisher Scientific) and grown to a 0.5 McFarland turbidity standard (4-6 hrs) and swabbed onto Mueller Hinton (Difco, Fisher Scientific) agar plates. The following antimicrobial agents were selected as important representatives of different antibiotic classes, with abbreviations and disc concentrations shown in brackets: streptomycin (S 10 µg), cefotaxime (CTX 30µg), sulfadiazine (SD 0.25 mg), ciprofloxacin (CIP 5 µg), chloramphenicol (C 30 µg), sulfamethoxazole-trimethoprim (SXT 23.75/1.25 µg), amoxicillin (AMC 30 µg), ceftazidime (CAZ 30 µg), doxycycline (D 30 µg), ampicillin (AM 10 µg), minocycline (MI 30 µg), and tetracycline (TE 30 µg). After incubation for 16-18 hours at 35±2°C the isolates were classified as susceptible, intermediate or resistant by comparing the diameters of

inhibition zones with the breakpoints according to CLSI recommendations (CLSI, 2007). Organisms considered intermediate by the Kirby–Bauer disc-diffusion method were recorded as resistant for the purpose of this study.

#### **6.3.4 Plasmid extraction**

All donor strains of *Salmonella* and *E. coli* and transconjugant strains were subjected to a plasmid extraction assay, conducted with a plasmid extraction kit (Qiagen, Mississauga, ON) according to the manufacturer's recommendations for extraction of plasmids. The extracted plasmid DNA was stored at -20°C until used for further analysis.

#### **6.3.5 Conjugation experiments**

Conjugation was performed as described previously (Provence and Curtiss, 1994; Poppe *et al.*, 2006; Thomas, 2011). Conjugation experiments were carried out on isolates susceptible to nalidixic acid and resistant to one or more of the following antibiotics: streptomycin, tetracycline, minocycline, and/or doxycycline (n=9), using the nalidixic acid resistant *E. coli* K12 as a recipient strain. Selected *Salmonella* and *E. coli* isolates were taken from frozen (-80°C) stock cultures or recently isolated and refrigerated (4°C) cultures and grown in LB broth overnight at 37°C in an orbital shaking waterbath. Nalidixic acid (Nal) resistant recipient strain, *E. coli* K12 JM109 was also grown overnight (24 h at 37°C) under the same conditions. Following overnight growth, each recipient and donor were streaked onto LB agar plates containing nalidixic acid (50 µg/mL) to ensure the recipient maintained Nal

resistance and that the donors were sensitive. A total of 9 mL of the overnight recipient culture was placed into pre-warmed 50 mL flasks at 45°C for 15 minutes. One mL of each donor was placed into one flask containing the recipient (9 mL) and swirled to mix cells. The flasks were subsequently incubated in a waterbath at 37°C (without shaking) for 24 h and then 100 µL was plated in duplicate directly onto LB containing nalidixic acid (50 µg/mL) plus streptomycin (100 µg/mL), nalidixic acid (50 µg/mL) plus tetracycline (30 µg/mL), and nalidixic acid (50 µg/mL) plus streptomycin (100 µg/mL) and tetracycline (30 µg/mL). Following 24 h and 48 h incubation at 37°C, transconjugant cells that grew on LB + Nal + antimicrobial compound were examined and counted. PCR was used to confirm transconjugants carried the same resistance determinants (*tetA*, *strA*, and/or *strB*) as their donors.

#### **6.3.6 Statistical analysis**

The chi-squared test and student t-test were used to conduct the statistical analysis of the results (i.e., for comparison of antibiotic resistance). The null hypothesis, that the prevalence of antimicrobial resistance (or ARGs) was not different between different species, was rejected at a *p*-value less than or equal to 0.05. All statistical computations were performed using Microsoft Excel version 2007 for Windows.

## 6.4 Results and Discussion

### 6.4.1 Prevalence of antibiotic resistance among *Salmonella* spp. and *E. coli*

In the current study, previously characterized multi-drug resistant *E. coli* and *Salmonella* spp. isolates were selected as important potential donors for bacterial conjugation experiments. All tested waterborne isolates of *E. coli* (n=14) and *Salmonella* spp. (n=14) collected from various environmental compartments including poultry lagoons, raw wastewater from a municipal WWTP, and treated effluent from a pilot-scale BNR system, displayed resistance to streptomycin (100%) and sulfadiazine (100%). The frequencies of resistance to twelve individual antimicrobials for *E. coli* and *Salmonella* spp. isolates are shown in Table 6.2. Of the 14 *E. coli* isolates, 100% showed resistance to tetracycline, streptomycin and sulfadiazine, followed by 93% (n=13), 85.7% (n=12), and 78.6% (n=11) displaying resistance to minocycline, chloramphenicol and doxycycline, respectively. One *E. coli* isolate was found to carry resistance to 3<sup>rd</sup> generation cephalosporins (CAZ and CTX) in combination with the quinolone ciprofloxacin and all tetracyclines (TE, D, and MI), sulfadiazine, and streptomycin (resistant to eight antimicrobials). Health Canada has categorized antimicrobial drugs based on their importance to human medicine (Health Canada, 2012) with Category I drugs including 3<sup>rd</sup> generation cephalosporins and quinolones, considered to be of significant importance in human medicine and often used as the last resort for treatment of invasive diseases (Shea *et al.*, 2004; Government of Canada, 2007). These drugs are also classified as critically important antimicrobial compounds by the World Health Organization (WHO, 2009). Among the  $\beta$ -lactam antibiotics, resistance was greatest to ampicillin at 57.1% (8/14), followed by resistance to amoxicillin at 28.6% (4/14),

whereas resistance to sulfamethoxazole-trimethoprim was observed in 6/14 (42.9%) of *E. coli* isolates (Table 6.2).

**Table 6.2. Phenotype distribution of resistance among waterborne *Salmonella* spp. and *E. coli***

No. (%) of isolates resistant to compound <sup>a</sup>												
	SD	SXT	CIP	C	AM	D	CAZ	CTX	AMC	S	TE	MI
<i>E. coli</i> (n=14)	14 (100)	6 (42.9)	2 (14.3)	12 (85.7)	8 (57.1)	11 (78.6)	1 (7.1)	1 (7.1)	4 (28.6)	14 (100)	14 (100)	13 (92.9)
<i>Salmonella</i> spp. (n=14)	14 (100%)	0	0	7 (50)	0	5 (35.7)	0	0	0	14 (100)	8 (57.1)	9 (64.3)

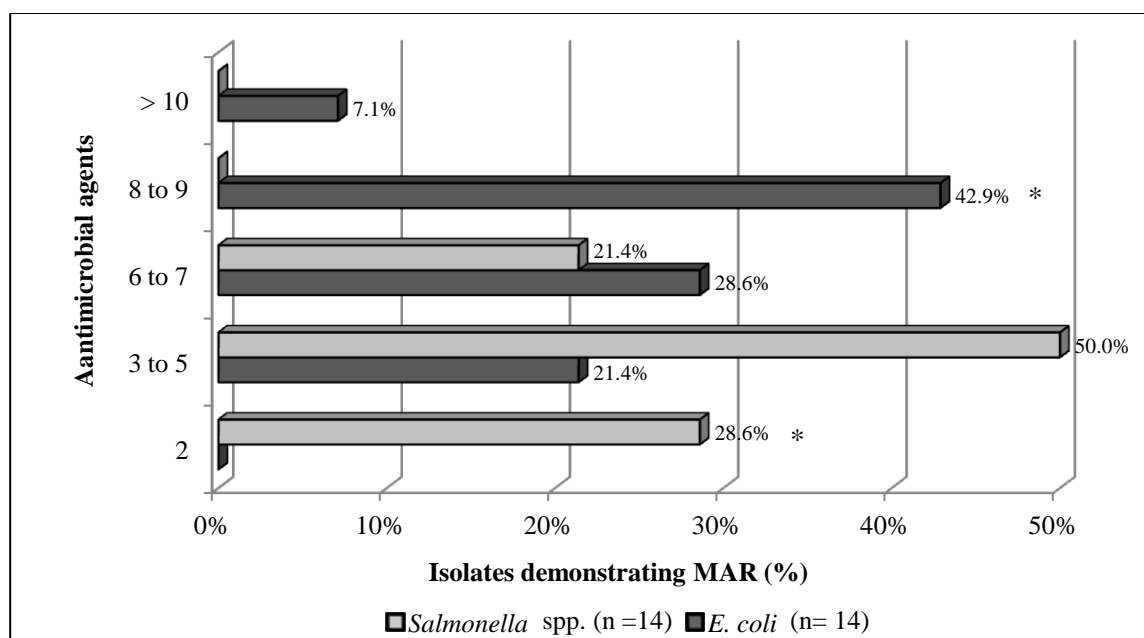
<sup>a</sup> SD, sulfadiazine; SXT, sulfamethoxazole-trimethoprim; CIP, ciprofloxacin; C, chloramphenicol; AM, ampicillin; D, doxycycline; CAZ, ceftazidime; CTX, cefotaxime; AMC, amoxicillin; S, streptomycin; TE, tetracycline; MI, minocycline; n= number of isolates

Overall, the highest levels of resistance observed among *Salmonella* spp. isolates (n=14) were 100% to streptomycin and sulfadiazine, followed by resistance to minocycline at 64.3%, tetracycline at 57.1%, chloramphenicol resistance at 50% and doxycycline resistance at 35.7% (Table 6.2). None of the *Salmonella* spp. strains tested were found to be resistant to 3<sup>rd</sup> generation cephalosporins (CAZ and CTX), ciprofloxacin,  $\beta$ -lactams (AMC and AM), or sulfamethoxazole-trimethoprim. Likewise, Varga *et al.* (2008) examined the prevalence of AMR in *Salmonella* spp. isolated from swine faecal samples and found no resistance to amoxicillin, ciprofloxacin, and 3<sup>rd</sup> generation cephalosporins among *Salmonella* spp. isolates and very low resistance levels of 0.2% and 6.1% to sulfamethoxazole-trimethoprim and ampicillin, respectively. When comparing the prevalence of antibiotic resistance among *E. coli* and *Salmonella* spp., significant differences (p<0.05) were observed among the following antimicrobials: sulfamethoxazole-trimethoprim, chloramphenicol, ampicillin, doxycycline, amoxicillin, and tetracycline. *E. coli* isolates displayed higher levels of resistance to almost all antibiotics tested when compared to *Salmonella* spp., with the

exception of sulfadiazine, and streptomycin (both displayed 100% resistance). *E. coli* exhibited significantly higher ( $p<0.05$ ) frequency of resistance to SXT, C, AM, D, AMC, and TE. The results are in accordance with previous studies (Varga *et al.*, 2008; Van *et al.*, 2007), where significantly higher resistance frequencies were observed in *E. coli* compared to *Salmonella* spp. isolates.

Similarly, the results are in agreement with Koo and Woo (2011) who demonstrated that 100% of *E. coli* isolates ( $n=121$ ) from tested meat products were resistant to tetracycline. Tetracycline resistance sometimes appeared in combination with resistance to streptomycin (68%), ampicillin (66%), ciprofloxacin (44.6%), sulfamethoxazole-trimethoprim (41.3%), chloramphenicol (26.4%), and gentamicin (19%). Their results also reveal resistance to multiple antibiotics in combination with tetracycline, streptomycin, and sulfadiazine resistance. Overall, the results from this study revealed 100% of *E. coli* isolates displayed multiple antibiotic resistance (MAR) to at least three or more antimicrobial agents (Figure 6.2). The MAR results are slightly higher than those observed by Koo and Woo (2011), with 76.9% (93/121) of *E. coli* isolates shown to be multi-drug resistant. Varga *et al.* (2008) showed that among tested *E. coli* and *Salmonella* spp. isolates, 70.2% and 28% were resistant to two or more antimicrobials, respectively. In this case resistance levels were higher than those reported by Varga *et al.* (2008), with 100% and 74.1% of tested *E. coli* and *Salmonella* spp. isolates displaying simultaneous resistance to three or more antimicrobial agents. The increased levels of multiple antibiotic resistance here compared to those of previous studies (Koo and Woo, 2011; Varga *et al.*, 2008) may be attributed to a variety of factors, including; differences between host source and sample type (i.e. wastewater, meat

products, swine feces), environmental factors, or previous exposure to antimicrobial agents. It is reasonable to believe that isolated *E. coli* and *Salmonella* spp. (raw municipal wastewater, poultry lagoon wastewater) had increased levels of antibiotic resistance compared to *E. coli* isolated from meat or meat products (Koo and Woo, 2011), since wastewater creates an ideal environment for the development of antibiotic resistant bacteria, due to the combination of high bacterial cell density, abundance of nutrients, and antibiotics, which frequently involves the passage of plasmids encoding antibiotic resistance (Guardabassi *et al.*, 2002). Figure 6.1 depicts the prevalence and distribution of MAR among *E. coli* and *Salmonella* spp. isolates, with differences noted between all categories of antimicrobial agents (i.e. 3 to 5) and species; however, significant differences were only apparent between multiple resistances to eight or nine antibiotics and two antibiotics. When comparing *E. coli* and *Salmonella* spp. isolates, the most common profiles were 42.9%, and 50%, displaying simultaneous resistances to between eight and nine antibiotics and three to five antibiotics, respectively (Figure 6.1). Overall, 28.6% of *E. coli* isolates were resistant to six or seven antimicrobials, followed by 21.4% resistant to between three and five antibiotics, and 7.1% of total *E. coli* isolates displaying resistance to ten or more of the tested antimicrobials. The second most common resistance profile among *Salmonella* spp. isolates was simultaneous resistance to two antimicrobials with 28.6%, followed by 21.4% depicting simultaneous resistance to six or seven antimicrobials. None of the observed *Salmonella* spp. isolates were found to display resistance to eight or more of the tested antibiotics (Figure 6.1).



**Figure 6.1. Prevalence of multiple antimicrobial resistance (MAR) profiles (%) among waterborne *Salmonella* spp. and *E. coli* isolates. An asterisk indicates a significant difference ( $p < 0.05$ , 2-tailed unequal variance t-test); n = number of isolates.**

Overall, *E. coli* displayed significantly higher ( $p < 0.05$ ) levels of MAR to between eight and nine antibiotics with 42.9% compared to 0% among *Salmonella* spp. isolates; however, *Salmonella* spp. displayed a significantly higher proportion of isolates resistant to two antimicrobials with 28.6%, compared to 0% among *E. coli*. Chandran *et al.* (2008) studied the prevalence of multiple drug resistant *E. coli* serotypes revealing more than 95% of *E. coli* isolates were MAR, very similar to those found in this study (100%). Much lower frequency of resistance, ranging from 31 to 75%, has been previously reported for *E. coli* isolates from various aquatic environments (Gomathinayagam *et al.*, 1994; Park *et al.*, 2003). Recent studies have also shown that antibiotics can accumulate in the environment and even persist for up to a year (Zuccato *et al.*, 2000; Hamscher *et al.*, 2002; Jacobsen *et al.*, 2004; Schlüsener and Bester, 2006). This may suggest a possible explanation for the increase of MAR bacteria. It has also been suggested that MAR microorganisms are more fit



or robust than their nonresistant counterparts and are therefore able to withstand or survive under harsh conditions (McKeon *et al.*, 1995; McGee, 2003; Walsh *et al.*, 2005). Rising levels of resistance to multiple antimicrobials dictate the urgent need for frequent and close monitoring of resistance in bacterial pathogens in both aquatic and agricultural environments in Canada and abroad. Without this measure of surveillance, a further decline in the effectiveness of antimicrobials is probable, leading to a reduction in the number of available antibiotics used to treat bacterially-mediated human infection and disease.

#### **6.4.2 Distribution of tetracycline and streptomycin resistance genes**

Identification of the genes associated with streptomycin and tetracycline resistance was accomplished using PCR analysis. In total, a select number of *E. coli* (n=5) and *Salmonella* spp. (n=7) isolates were chosen (based on phenotypic characterization using culture-based AMR screening) as multi-drug resistant donors and screened for the presence of *tetA*, *strA*, and *strB*, conferring resistance to tetracycline and streptomycin. The selected ARGs were chosen since resistance to tetracyclines, and streptomycin or spectinomycin in *E. coli* and *Salmonella* spp. are generally the most prevalent (Guerra *et al.*, 2003; Lanz *et al.*, 2003; Graves *et al.*, 2011). Briefly, 80% of *E. coli* isolates (4/5) carried the *tetA* and *strA* genes, and 100% (5/5) carried the *strB* gene. Of the 7 *Salmonella* spp. isolates, 100% carried all three genes examined. Table 6.3 summarizes the phenotypic and genotypic antimicrobial resistance profiles of the selected *E. coli* and *Salmonella* spp. isolates, including the recipient strain, *E. coli* K12. It is also important to note that all donor strains remained sensitive to nalidixic acid (50 µg/mL) upon phenotypic analysis.

**Table 6.3. Phenotypic and genotypic resistance profiles of *E. coli* and *Salmonella* spp. donor and recipient strains**

Donor strain	Isolate #	Origin	AMR Profile	Gene (s)
<i>E. coli</i>	L12-15	Poultry lagoon <sup>a</sup>	SdCipDCazCtxSTeMi	<i>strA, strB</i>
	T17	Treated effluent <sup>b</sup>	SdCAmDSTeMi	<i>tetA, strA, strB</i>
	T25	Treated effluent	SdCDSTeMi	<i>tetA, strA, strB</i>
	T28	Treated effluent	SdSxtCipCAmDAmcSTeMi	<i>tetA, strA, strB</i>
	R15	Raw wastewater <sup>c</sup>	SdSxtCAmDAmcSTeMi	<i>tetA, strB</i>
<i>E. coli</i> K12	Recipient Strain		Nal	ND
<i>Salmonella</i> spp.	S6	Poultry lagoon	SdCDSTeMi	<i>tetA, strA, strB</i>
	S8	Poultry lagoon	SdCDSTeMi	<i>tetA, strA, strB</i>
	S9	Poultry lagoon	SdSTe	<i>tetA, strA, strB</i>
	S10	Poultry lagoon	SdDSTeMi	<i>tetA, strA, strB</i>
	S1	Poultry lagoon	SdCDSTeMi	<i>tetA, strA, strB</i>
	S4	Poultry lagoon	SdS	<i>tetA, strA, strB</i>
	S5	Poultry lagoon	SdDSTeMi	<i>tetA, strA, strB</i>

<sup>a</sup> Poultry lagoon site is described in Chpt. 3; <sup>b</sup> Treated effluent is from the pilot-scale BNR system as described in Chpt. 5; <sup>c</sup> Raw wastewater entering the pilot-scale BNR system as described in Chpt. 5. SD, sulfadiazine; SXT, sulfamethoxazole-trimethoprim; CIP, ciprofloxacin; C, chloramphenicol; AM, ampicillin; D, doxycycline; CAZ, ceftazidime; CTX, cefotaxime; AMC, amoxicillin; S, streptomycin; TE, tetracycline; MI, minocycline; Nal, nalidixic acid; ND- not detectable, no determinant identified.

In previous studies, it was found that most *Salmonella* spp. strains resistant to tetracycline carried the *tetA* gene (Chen *et al.*, 2004; Pezzella *et al.*, 2004; Hur *et al.*, 2011). Here it was found that all donor isolates resistant to tetracycline (with the exception of S4, which carried the *tetA* gene but did not exhibit phenotypic tetracycline resistance) harboured the *tetA* gene. Similarly, all *Salmonella* spp. and *E. coli* donor isolates demonstrated phenotypic resistance to streptomycin, with 100% carrying either one or both streptomycin resistance determinants (*strA* and *strB*) (Table 6.3). Several genes, including *strA*, *strB*, and *aad* are known to be associated with streptomycin resistance (Chiu *et al.*, 2006), and all three genes are known to be widely distributed among *Salmonella* spp. and *E. coli* (Weill *et al.*, 2006; Hur *et al.*, 2011; Graves *et al.*, 2011). Streptomycin, tetracycline, and sulfa drugs are more commonly used in

animal production than other antibiotics (Chee-Sanford *et al.*, 2009) which may be linked to the frequent occurrence of these ARGs among *Salmonella* spp. isolates. The high occurrence of antibiotic resistance and ARGs among selected *E. coli* and *Salmonella* spp. isolates in this study could be due to the source or origin of the selected isolates, collected from poultry lagoon wastewater, raw municipal wastewater and treated effluent. Different environmental factors can influence the positive selection of wastewater isolates with multiple antibiotic resistance patterns. For example, as stated previously, wastewater processes based on activated sludge, may allow for enhanced resistance dissemination due to high cell density (Soda *et al.*, 2008; Marcinek *et al.*, 1998) as well as by the prolonged retention time of biomass in the system (Łuczkiewicz *et al.*, 2010; Novo and Manaia, 2010). This theory is consistent with the *E. coli* isolates in this study, collected from raw wastewater and treated effluent (using activated sludge BNR system), with 100% of isolates displaying multi-drug resistance phenotypes, and almost half (43%) with simultaneous resistance to eight or nine antimicrobial compounds.

#### **6.4.3 Transfer of tetracycline and/or streptomycin resistance genes by conjugation**

The potential transfer of tetracycline and streptomycin resistance genes was demonstrated through broth conjugation experiments using multi-drug resistant *Salmonella* spp. (n=7) and *E. coli* (n=5) isolates as donors, and *E. coli* K12 as the recipient. Of the twelve strains examined, conjugation was successfully observed in nine (75%) (Table 6.4), occurring with both *Salmonella* spp. and *E. coli* donors. The antibiotic susceptibility test of transconjugants revealed that donors could transfer all or part of their resistance phenotypes to the recipients. Table 6.4 illustrates the antimicrobial resistance patterns of all donor and transconjugant

strains, including the genes transferred (*tetA*, *strA*, *strB*) via plasmid mediated resistance (plasmid DNA was screened for the presence of all three ARGs using PCR analysis). Six strains (50%) were capable of transferring all three resistance genes (*tetA*, *strA*, and *strB*) to the recipient strain, resulting in 58.5% (38/65) of total transconjugant strains acquiring all three resistance determinants (Table 6.4). Overall, 63% (41/65) of total transconjugant isolates acquired at least one of the three selected resistance genes; however, 100% of transconjugants displayed a resistance phenotype similar to that of the respective donor strain (complete or partial transfer of resistance pattern). For example, the donor isolate *E. coli* T17 displayed a resistance phenotype of SdCAmDSTeMi, with the transconjugant acquiring a similar resistance pattern of SdCSTeMi; however, upon genotypic analysis none of the selected ARGs (*tetA*, *strA*, *strB*) could be detected. A possible explanation for the lack of genotypic confirmation among selected ARGs (*tetA*, *strA*, *strB*) for both *E. coli* and *Salmonella* spp. transconjugant isolates could be due to the small number of ARGs screened. Only 1 of the 38 recognized tetracycline resistance genes and 2 of the 5 recognized streptomycin resistance genes were analysed. Thus, the inability to detect relevant resistance genes in transconjugants that were phenotypically resistant may reflect the fact that the observed resistance was due to a resistance gene other than those assayed here or by nonspecific resistance mechanisms (e.g., an efflux pump) (Allen *et al.*, 2011). Donor strain *E. coli* T28 with a resistance profile of SdSxtCipCAmDAmcSTeMi, displaying resistance to ten antimicrobials, was able to completely transfer its resistance phenotype to 10 (90.1%) transconjugants, and partially transfer (SxtCipCAmSdMiDSTe) to one transconjugant (9.1%), as shown in Table 6.4. In addition, all transconjugants (from donor T28) were found to carry the three resistance genes, *tetA*, *strA*, and *strB*. Therefore, the results of the

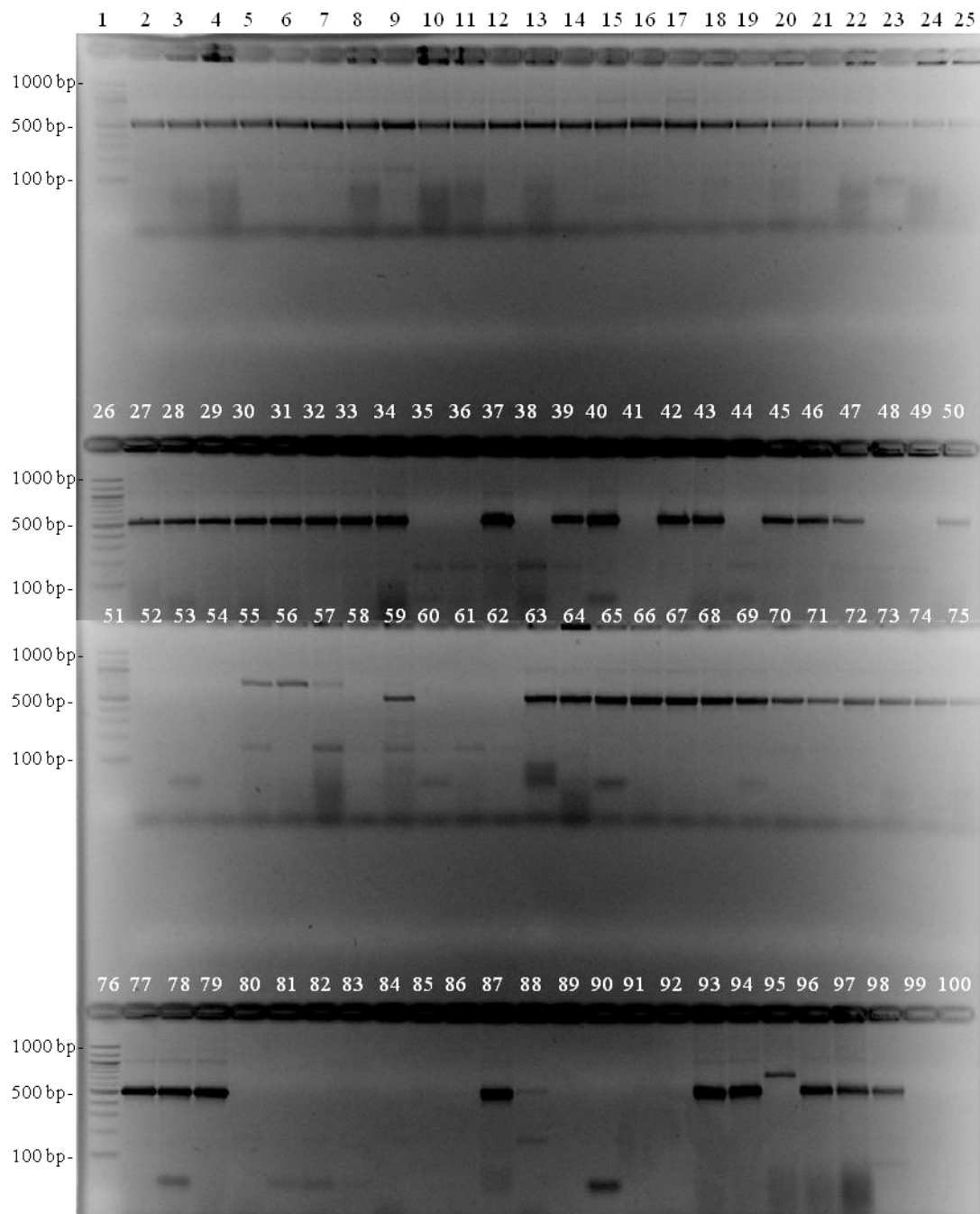
conjugation experiments reveal that many of the multi-drug resistant donors were able to completely or partially transfer phenotypic and/or genotypic patterns including ARGs.

**Table 6.4. Antimicrobial resistance patterns of selected MDR *E. coli* and *Salmonella* spp. donor isolates and transconjugants**

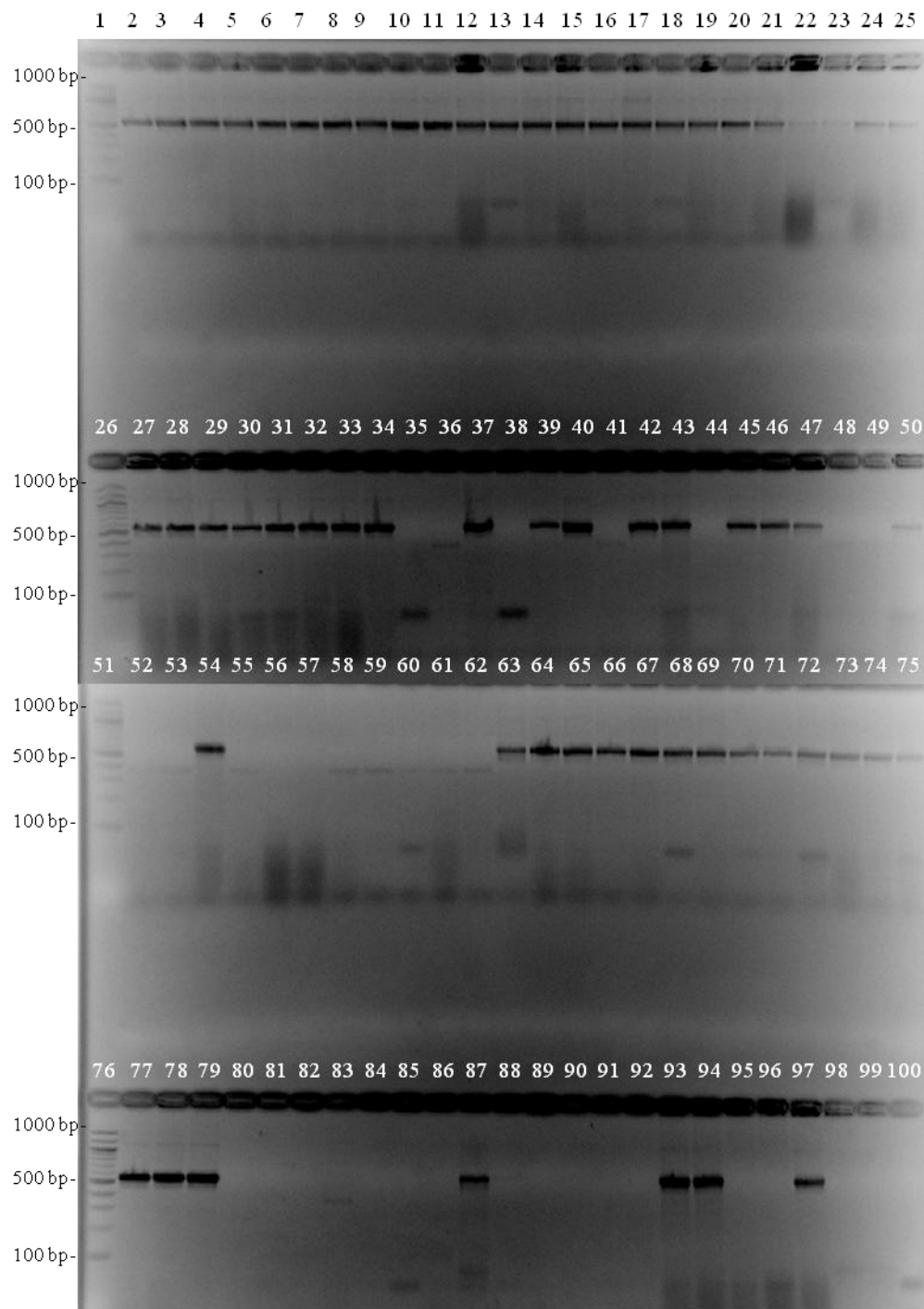
Donor Strain	Donor resistance phenotype	Transconjugant	Transconjugant resistance phenotype	No. (%) of transconjugants with profile	Gene(s) transferred (plasmid-mediated resistance)
<i>E. coli</i> T25	SdCDSTeMi	T25 + <i>E. coli</i> K12 (n=20)	CSdMiDSTe CMiDSTe	16 (80) 4 (20)	<i>tetA</i> , <i>strA</i> , <i>strB</i> (n=20, 100%)
<i>E. coli</i> T28	SdSxtCipCAmDAmcSTeMi	T28 + <i>E. coli</i> K12 (n=11)	SxtCipCAmSdMiDAmcSTe SxtCipCAmSdMiDSTe	10 (90.1) 1 (9.1)	<i>tetA</i> , <i>strA</i> , <i>strB</i> (n=11, 100%)
<i>E. coli</i> T17	SdCAmDSTeMi	T17 + <i>E. coli</i> K12 (n=1)	SdCSTeMi	1 (100)	ND
<i>E. coli</i> R15	SdSxtCAmDAmcSTeMi	R15 + <i>E. coli</i> K12 (n=1)	SdSxtCAmDAmcSTeMi	1 (100)	<i>tetA</i> , <i>strA</i> , <i>strB</i> (n=1, 100%)
<i>E. coli</i> L2-15	SdCipDCazCtxSTeMi	L2-15 + <i>E. coli</i> K12 (n=15)	CtxSxtCipCSdMiDTeSCaz CtxCipCSdMiDTeSCaz CtxSxtCipSdMiDTeSCaz	9 (60) 3 (20) 3 (20)	<i>tetA</i> , <i>strA</i> , <i>strB</i> (n=3, 20%)
<i>Salmonella</i> spp. #6	SdCDSTeMi	S6 + <i>E. coli</i> K12 (n=1)	SdDSTeMi	1 (100)	<i>tetA</i> , <i>strA</i> , <i>strB</i> (n=1, 100%)
<i>Salmonella</i> spp. #8	SdCDSTeMi	S8 + <i>E. coli</i> K12 (n=8)	CipCSdMiDSTe SdCDSTeMi CipCSdMiDTe CipSdMiDSTe	3 (37.5) 1 (12.5) 3 (37.5) 1 (12.5)	<i>tetA</i> , <i>strA</i> , <i>strB</i> (n=2, 25%) <i>strA</i> , <i>strB</i> (n=1, 12.5%) <i>tetA</i> , <i>strA</i> (n=1, 12.5%)
<i>Salmonella</i> spp. #9	SdSTe	S9 + <i>E. coli</i> K12 (n=4)	CSdMiDSTe SdMiDSTe CSdMiSTe	2 (50) 1 (25) 1 (25)	ND
<i>Salmonella</i> spp. #10	SdDSTeMi	S10 + <i>E. coli</i> K12 (n=4)	CSdMiDSTe CipCSdMiDTe	2 (50) 2 (50)	<i>strB</i> (n=1, 25%)

SD, sulfadiazine; SXT, sulfamethoxazole-trimethoprim; CIP, ciprofloxacin; C, chloramphenicol; AM, ampicillin; D, doxycycline; CAZ, ceftazidime; CTX, cefotaxime; AMC, amoxicillin; S, streptomycin; TE, tetracycline; MI, minocycline; n= number of isolates; ND- not detectable, no determinant identified.

The conjugation results showed that more than half, or 63% (41/65) of selected transconjugant isolates acquired tetracycline and/or streptomycin resistance genes through conjugative plasmids (Table 6.4), as confirmed using plasmid DNA extraction (Figures 6.3, 6.4 & 6.5). Similarly, Koo and Woo (2011) studied the transfer of *tet* genes by conjugation in meatborne *E. coli* and found that almost all isolates (98.3%) containing at least one *tet* gene (among *tetA* to *tetD*) were able to transfer tetracycline resistance to the recipient strain, *E. coli* J53. PCR was used to confirm the presence of tetracycline and streptomycin resistance determinants (*tetA*, *strA*, and *strB*) among extracted plasmid DNA from all donor and transconjugant isolates. Figures 6.3, 6.4, and 6.5 depict representative gels of transconjugant isolates (n=85), donor isolates, and the recipient strain (*E. coli* K12) carrying the *strB* gene (509 bp product), *strA* gene (548 bp product), and *tetA* gene (210 bp product), conferring resistance to streptomycin and tetracycline, respectively.



**Figure 6.3.** Visualization of PCR products amplified from transconjugant plasmid DNA isolates (n=85) carrying the *strB* gene (509 bp) conferring resistance to streptomycin. Lanes 1, 26, 51, & 76 contained the DNA standard; lanes 23, 63, 87, 97, 98, 93, 94, 95, 96 contained *E. coli* T17, T28, T25, L2-15, R15, *Salmonella* S6, S8, S9, & S10 donor isolates, respectively; lane 99 contained *E. coli* K12 recipient strain, lane 100 contained negative control; lanes 2-92 (excluding 23, 63, 87) contained transformed strains grown on N+S, N+T, or N+T+S.



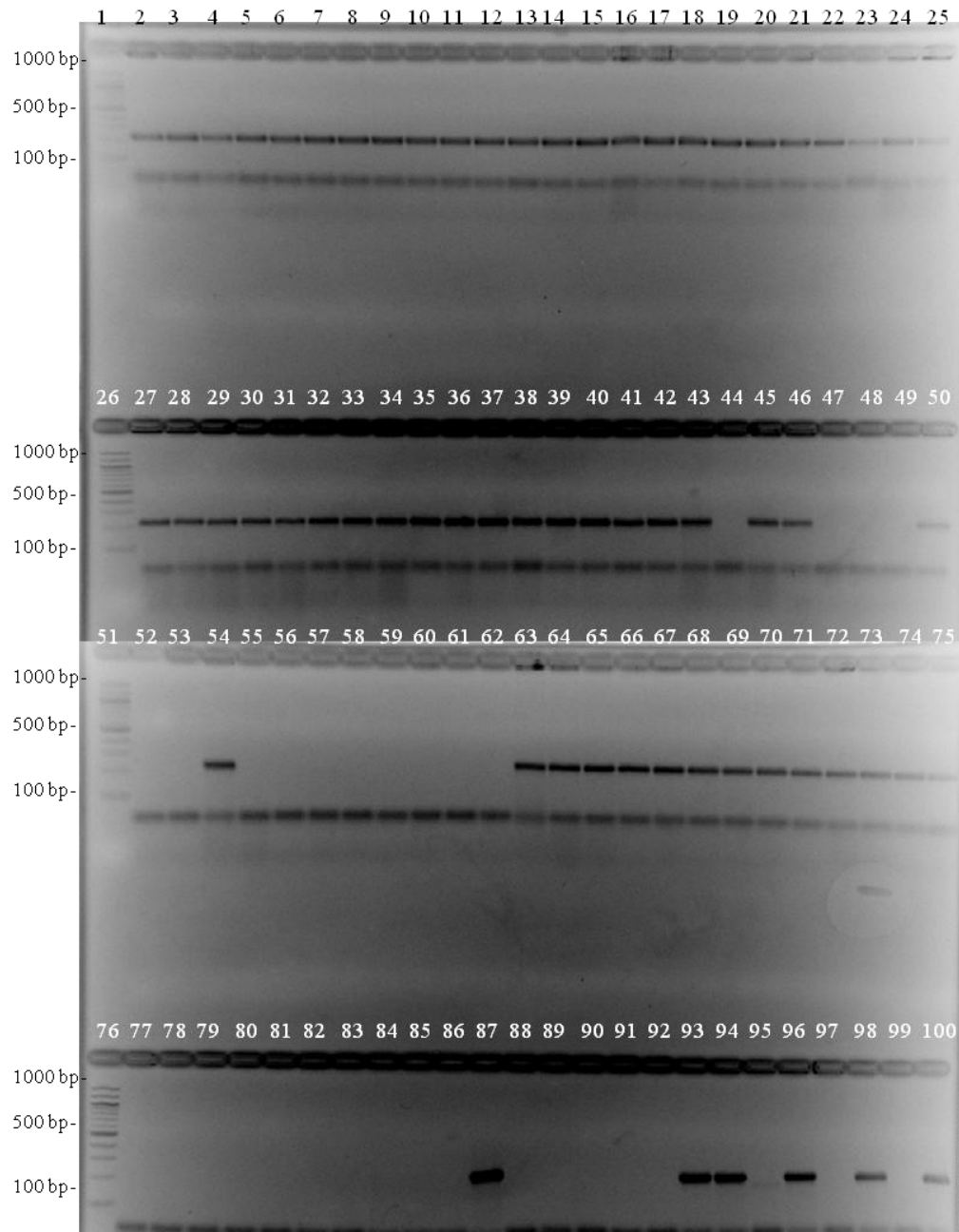
**Figure 6.4.** Visualization of PCR products amplified from transconjugant plasmid DNA isolates (n=85) carrying the *strA* gene (548 bp) conferring resistance to streptomycin. Lanes 1, 26, 51, & 76 contained the DNA standard; lanes 23, 63, 87, 97, 98, 93, 94, 95, 96 contained *E. coli* T17, T28, T25, L2-15, R15, *Salmonella* S6, S8, S9, & S10 donor isolates, respectively; lane 99 contained *E. coli* K12 recipient strain, lane 100 contained negative control; lanes 2-92 (excluding 23, 63, 87) contained transformed strains grown on N+S, N+T, or N+T+S.



Recently, Karczmarczyk *et al.* (2011) studied the horizontal transfer of antimicrobial resistance among *E. coli* isolates from cattle farms, and observed 10 of the 16 (62.5%) *strA-strB*-positive isolates transferred these markers, suggesting the association of these genes with conjugative or mobilizable plasmids. The results found here are in agreement with Karczmarczyk *et al.* (2011), in that of the 12 *strA* and/or *strB* positive donor isolates, 7 (58%) were capable of transferring one or both of these resistance markers. The *strA-strB* gene pair can be found in the transposon Tn5393, carried by large self-transmissible plasmids (L'Abée-Lund and Sørum, 2000). However, further investigation is required to clarify whether the *strA-strB* genes predominating in this study are linked with a similar transposon.

Previous studies have shown that *tet* genes are largely associated with the class 1 integrons by conjugative plasmids, which are able to transfer horizontally (Agerso and Sand vang, 2005). Similarly, *tet* genes that confer resistance to tetracyclines in the *Enterobacteriaceae* family have also been analyzed by other authors, including Pasquali *et al.* (2004). Martin *et al.* (2008) characterized ARGs linked to class 1 and 2 integrons among *Salmonella* spp. strains isolated from swine, and specifically examined the ability of integron-associated resistance determinants to transfer through conjugation. The results of their study demonstrated that *tetA* genes and class 1 integrons harbouring the *aadA1* cassette were transferred from 2 of 16 donor *Salmonella* strains to *E. coli* J53 (resistant to sodium azide), and a high molecular mass plasmid was identified in transconjugant strains (Martin *et al.*, 2008). It is known that integrons are not able to self-mobilize; however, if they are associated with mobile elements, they can potentially transfer themselves horizontally,

which could represent an important risk in the dissemination of multi-drug resistance (Rowe-Magnus and Mazel, 2001).



**Figure 6.5. Visualization of PCR products amplified from transconjugant plasmid DNA isolates (n=85) carrying the *tetA* gene (210 bp) conferring resistance to tetracycline. Lanes 1, 26, 51, & 76 contained the DNA standard; lanes 23, 63, 87, 97, 98, 93, 94, 95, 96 contained *E. coli* T17, T28, T25, L2-15, R15, *Salmonella* S6, S8, S9, & S10 donor isolates, respectively; lane 99 contained *E. coli* K12 recipient strain, lane 100 contained positive *tetA* control; lanes 2-92 (excluding 23, 63, 87) contained transformed strains grown on N+S, N+T, or N+T+S.**

In summary, these results confirm the role of environmental bacteria (isolated from wastewater treatment facilities) as a reservoir of antibiotic resistance bacteria and ARGs, containing mobile genetic elements, which are capable of disseminating and transferring ARGs, signifying a potential problem for human and animal health. In order to facilitate and further understand the emergence of AMR, additional studies with comprehensive collections of environmental samples are needed to help increase our understanding of molecular genetic mechanisms involved in the dissemination and transfer of ARGs from waterborne pathogens to humans.

## 6.5 Conclusions

This chapter highlights the importance of studying phenotypic antimicrobial resistance among waterborne *E. coli* and *Salmonella* spp. isolates, in combination with genotypic AMR analysis, including the ability to transfer resistance determinants, to further understand the epidemiology of drug resistance among commensal and pathogenic bacteria, especially when multi-drug resistance is expressed to drugs of high human health importance. In addition, molecular techniques confirmed plasmid-mediated conjugal transfer of streptomycin and tetracycline resistance determinants among transconjugants.

The results reveal resistance to multiple antibiotics in combination with tetracycline, streptomycin, and sulfadiazine resistance. Overall, 100% and 71.4% of *E. coli* and *Salmonella* spp. isolates, respectively, exhibited multiple antibiotic resistance (MAR) to at least three or more antimicrobial agents, with 100% of *E. coli* and *Salmonella* spp. isolates

demonstrating resistance to streptomycin and sulfadiazine. Identification of three resistance determinants (*tetA*, *strA*, *strB*) conferring resistance to tetracycline and streptomycin was performed on selected multi-drug resistant *Salmonella* spp. and *E. coli* isolates (to be used as potential donors for conjugation). Briefly, 80% of *E. coli* isolates carried the *tetA* and *strA* genes, and 100% carried the *strB* gene. Similarly, 100% of *Salmonella* spp. isolates harboured all three ARGs, including *tetA*, *strA*, and *strB*.

The potential transfer of tetracycline and streptomycin resistance genes was demonstrated through broth conjugation experiments using multi-drug resistant *Salmonella* spp. (n=7) and *E. coli* (n=5) isolates as donors, and *E. coli* K12 as the recipient. Of the twelve strains examined, conjugation was successfully observed in 75%, occurring in both *Salmonella* spp. and *E. coli* donor isolates. Six strains (50%) were capable of transferring their *tetA*, *strA*, and *strB* resistance genes to the recipient strain, resulting in 58.5% of total transconjugant strains acquiring all three resistance determinants. Results of the conjugation experiments reveal that many of the multi-drug resistant donors were able to completely or partially transfer phenotypic and/or genotypic patterns including ARGs via mobile elements, such as conjugative plasmids. Plasmid DNA was extracted from all donor and transconjugant strains, revealing the presence of a 509 bp PCR product from those isolates harbouring the *strB* gene, a 548 bp product revealing the presence of the *strA* gene, and a 210 bp product from those isolates carrying the *tetA* gene. In summary, the results confirm the role of wastewater-associated bacteria (isolated from wastewater treatment utilities) as a reservoir of antibiotic resistance bacteria and ARGs, containing mobile genetic elements, which are capable of disseminating and transferring ARGs. As concerns about water quality and

environmental contamination by human and agricultural effluents have increased, it has become progressively more important to consider the prevalence and transferability of ARGs to human pathogens.

## **6.6 Recommendations & Future Research Needs**

- Additional studies could examine the transferability of antibiotic resistance and ARGs among environmental isolates from different sources, including activated sludge, animal production waste (i.e. manure), soil, lakes, and/or surface water, which may increase our understanding of molecular genetic mechanisms involved in the dissemination and transfer of ARGs.
- Further studies could examine different mechanisms of ARG transfer among environmental bacteria other than conjugation, for example transference by transposons or phages, which may also contribute to the dissemination of multi-drug resistance.
- Further analysis could be carried out to determine the diversity and genetic relatedness of plasmids (through restriction fragment length polymorphism, RFLP) carrying the *tetA*, *strA*, and *strB* collected in this study to further understand the endemic nature of these plasmids in waterborne *E. coli* and *Salmonella*. Plasmid sequencing may also provide a more comprehensive understanding of the plasmids associated with tetracycline and streptomycin resistance.
- Additional studies could elaborate on PCR detection of resistance genes (i.e. those associated with  $\beta$ -lactam, chloramphenicol, and sulfonamide resistance, etc.)

integrons (genes associated with class 1 and 2 integrons), transposons (i.e. Tn21, Tn3), and virulence genes (associated with pathogenicity islands).

# 7

## **Thesis Conclusions & Recommendations**

### **7.1 Thesis Summary & Principle Contributions**

There is growing concern that the ubiquitous nature of antibiotic resistance and ARGs among environmental pathogens from a variety of wastewater effluents, suggests that the aquatic environment, and specifically alternative wastewater treatment systems, may act as reservoirs for drug resistant bacteria and ARGs, thereby contributing to the wide spread dissemination of antibiotic resistance. The overall objective of this work was to study the prevalence, fate, and potential transfer of resistance and ARGs among selected potential pathogens in bacterial communities of alternative wastewater treatment systems, while considering the impact of treatment strategies on the expression of antibiotic resistance in pathogenic bacteria. A detailed analysis was initially conducted with respect to the

characterization and quantification of microbial populations (including antibiotic resistant bacteria) in a variety of treatment systems and waste effluent sources. In order to aid in the investigation of the overall objective, the following short term objectives were proposed:

- A) To confirm and monitor the prevalence of selected faecal-indicators (*E. coli*, *Enterococcus* spp.) and selected environmental pathogens (*Yersinia* spp., *Aeromonas* spp., *Flavobacterium* spp. & *Salmonella* spp.) through screening and culture-based phenotypic characterization in various types of alternative wastewater treatment systems (bench-scale mesocosms, sub-surface flow constructed wetland, constructed lagoon, pilot-scale biological nutrient removal (BNR) system). Quantification of selected environmental pathogens using real-time quantitative PCR (qPCR) was also performed;
- B) To determine antibiotic resistance profiles for 10-12 common antibiotics using representative isolates of *E. coli*, *Enterococcus* spp., *Salmonella* spp., *Yersinia* spp., *Aeromonas* spp., and *Flavobacterium* spp., using culture-based techniques;
- C) To quantify, track, and compare the presence of the following ARGs using real-time qPCR; *ampC*, *tetA*, and *blaSHV-5* (conferring resistance to various antimicrobials) in different alternative wastewater treatment systems, including constructed wetlands, constructed lagoons, and pilot-scale WWTPs;
- D) To assess the ability to completely or partially transfer antibiotic resistance patterns and ARGs via conjugation experiments using multi-drug resistant isolates (*E. coli* and *Salmonella* spp.) as donors, and *E. coli* K12 as the recipient; and
- E) To better understand or assess the impact of changing conditions or environmental stress (i.e. antibiotic exposure) on the level of antibiotic resistance among



representative faecal indicators (*Escherichia coli* and *Enterococcus* spp.) and selected environmental pathogens (*Aeromonas* spp., *Yersinia* spp., & *Flavobacterium* spp.) in constructed wetland systems.

#### **7.1.1 Antibiotic resistance profiles of wetland bacteria and faecal indicators following ciprofloxacin exposure in lab-scale constructed mesocosms (Objectives A-B & E)**

One of the major objectives of this thesis (Objective E) was to better understand the impact of an environmental stressor (i.e. antibiotic exposure) on the presentation and potential development of antibiotic resistance among representative faecal indicators (*E. coli* and *Enterococcus* spp.) in lab-scale constructed wetlands (mesocosms). Little information is available on the prevalence of antibiotic resistance among indicator and pathogenic microorganisms in wetlands designed for wastewater treatment or in natural wetland systems. Therefore, this study was developed to examine and monitor antibiotic resistance profiles among both faecal indicators (*E. coli* and *Enterococcus* spp.) and total heterotrophs before and after the exposure to an antibiotic, in a lab-scale system. The information gained from this study proves useful when examining antibiotic resistance levels and profiles among other alternative wastewater treatment systems, including full-scale constructed wetlands and lagoons. The results obtained for the level of antibiotic resistance among *E. coli* isolates supports the idea that the introduction of an antibiotic to an aquatic system can lead to an increase in resistance, not only to the same class of antibiotics, but to other classes of antibiotics as well. A significant increase in antibiotic resistance was observed among *E. coli* isolates following ciprofloxacin exposure, not only to ciprofloxacin (quinolone class), but to other classes of antimicrobials, including cephalosporins, penicillins, tetracyclines,

and sulfonamides. The increase in antibiotic resistance among *E. coli* isolates could be attributed to the upregulation of certain efflux pump-encoded genes following the 5-day ciprofloxacin exposure. Another possible explanation for the increased resistance among several antibiotics could be the acquisition of a multi-drug resistance plasmid. Plasmid-mediated transmission is the most common mechanism of horizontal gene transfer (Norman *et al.*, 2009), with subinhibitory concentrations of antibiotics facilitating the process of antibiotic resistance development (Davies *et al.*, 2006).

#### **7.1.2 Prevalence of antimicrobial resistance among *Aeromonas*, *Yersinia*, and *Flavobacterium* populations and selected ARGs during winter operation of a constructed wetland receiving fish hatchery effluent (Objectives A-C & E)**

To better understand and further elaborate on the occurrence and presentation of antibiotic resistance following an antibiotic exposure (Objective E), an additional study was performed with an operational sub-surface constructed wetland treating aquaculture wastewater. There is increasing concern surrounding the use of antibiotics in agriculture and aquaculture industries, and due to the limited number of treatment options for aquatic species in certain countries, including Canada, widespread resistance to even one antibiotic class is of increasing concern. Major environmental pathogens involved in fish farm infections include the Gram negative species *Flavobacterium psychrophilum*, *Yersinia ruckeri*, and *Aeromonas salmonicida*; the etiological agents of cold water disease, enteric red mouth disease, and furunculosis, respectively (del Cerro *et al.*, 2002). In Canada and the United States, oxytetracycline is permitted as an additive to animal and fish feed at subtherapeutic levels, acting to promote growth or to prevent/treat disease. Oxytetracycline was selected as the

antibiotic of preference in this study, due to the widespread use of tetracyclines in the aquaculture industry. The concern with antibacterial resistance in freshwater environments in relation to fish farming activities is more recent and thus poorly documented, especially in Canada and cold weather climates using constructed wetlands to treat fish hatchery waste. Treatment of aquaculture effluents from cold-water sub-surface flow CWs and monitoring the prevalence and distribution of antimicrobial resistance and ARGs among environmental pathogens has yet to be investigated. As such, an appropriate study was conducted which examined and monitored antibiotic resistance profiles among selected fish pathogens (*Yersinia* spp., *Aeromonas* spp., *Flavobacterium* spp.) (Objective A & B) and the frequency of ARGs (Objective C), both before and after OTC exposure, for a sub-surface flow constructed wetland treating aquaculture wastewater. Results obtained for the level of antibiotic resistance among *Aeromonas* spp., *Yersinia* spp., and *Flavobacterium* spp. supports the idea that an isolated antibiotic exposure event (oxytetracycline) to an aquatic system can lead to an increase in resistance, not only to the chosen antibiotic (tetracycline), but to other classes of antibiotics (cephalosporins, aminoglycosides, glycopeptides, penicillins, and sulfonamides) as well. The data is in accordance with previous research using lab-scale constructed mesocosms (Helt *et al.*, 2012), supporting the notion that an intermittent antibiotic exposure event could have an impact on the development of antibiotic resistance, specifically among fish pathogens and the frequency of ARGs in various stages of a sub-surface flow constructed wetland.

### **7.1.3 Monitoring the occurrence, distribution, and frequency of selected bacterial pathogens and antibiotic resistance genes in treatment lagoons receiving poultry waste effluent (Objectives A-C)**

The widespread use of antibiotics in animal husbandry and agriculture for treating or preventing infection and promoting growth, can play an important role in the development and spread of antibiotic resistance. Little information is available regarding the fate of antibiotics in the environment and their link to the emergence of the resident resistant genotypes. The annual production of livestock and poultry waste coupled with antibiotic usage in North America is significant, serving potentially as a large source of both antibiotics and antibiotic-resistant bacteria into the environment. The treatment of domestic sewage in natural systems such as constructed wetlands and lagoons has become a popular approach for small communities in rural regions. Constructed lagoon systems represent a common and effective method to purify communal sewage in rural areas, with relatively low costs and high purification rates (Steinmann *et al.*, 2003). Few studies have examined both the occurrence and fate of antibiotic resistance among environmental bacterial pathogens (Objectives A & B) and different classes of ARGs (Objective C) in poultry abattoir wastewater, using a combined approach of traditional culture-based screening and molecular quantification through real-time PCR. Therefore, a study was developed to examine the presence and behaviour of the following ARGs; *ampC* (ampicillin resistance), *blaSHV* (extended  $\beta$ -lactam resistance conferring broad resistance to penicillins and cephalosporins), *tetA* (tetracycline resistance) and selected bacteria or pathogens (*E. coli*, *Enterococcus faecalis*, *Salmonella* spp.) in a constructed treatment lagoon system receiving poultry slaughterhouse waste. To monitor the efficiency of waste treatment lagoons and ensure

adequate removal concentrations of indicator organisms and bacterial pathogens/ARGs from the final effluent to receiving water bodies, the number of pathogenic bacteria and ARGs was measured using quantitative PCR during various stages of treatment. The results of this study help establish a preliminary environmental assessment of lagoon treatment systems receiving poultry slaughterhouse waste. The level of multiple antibiotic resistance (MAR) among culturable indicator bacteria was high, 93% and 100% for *E. coli* and *Enterococcus* spp. isolates, respectively. The most common profile among *E. coli* was simultaneous resistances to between three and five antimicrobials, whereas among *Enterococcus* spp. isolates resistance to ten or more antibiotics was the most common. The constructed lagoon treatment system reduced the level of bacterial pathogens, but does not guarantee the complete elimination of contamination with *E. coli* and *Salmonella*. Approximately  $10^4$  and  $10^3$  gene copies/100 mL were detected in the third and final lagoon, respectively. All three ARGs were gradually reduced as the waste effluents passed through different stages of treatment (multiple lagoons); however, concentrations remained relatively high with  $10^3$  -  $10^6$  target gene copies/100 mL in the third lagoon. Due to the escalating problem of antibiotic resistance, there is a need to improve our understanding of the potential role of livestock and slaughterhouse operations both in the amplification and attenuation of antibiotic resistance levels. However, the results from this study contribute to our understanding regarding the occurrence, distribution, and fate of antibiotic resistance in livestock operations, while adding a new and important perspective to aid in the advancement of this critical problem.

#### **7.1.4 Distribution, quantification, and potential dissemination of selected pathogens and antibiotic resistance genes in a pilot-scale BNR system receiving wastewater from a full-scale WWTP (Objectives A-C)**

Wastewater treatment plants (WWTP) represent important potential reservoirs of human and animal commensal bacteria in which antibiotic resistant organisms and ARGs may persist in the final effluent and subsequently be released into the environment. The role of wastewater treatment plants in reducing the load of antibiotic resistant bacteria and ARGs present in raw sewage is not well known. However, it has been suggested that certain conditions in wastewater treatment plants might actually lead to an increase in the number of antibiotic resistant bacteria during the treatment process (Silva *et al.*, 2006; Zhang *et al.*, 2009). The presence of low concentrations of antibiotics in sewage treatment plant wastewaters may serve as a selective pressure for the proliferation of resistant organisms (Jury *et al.*, 2010). The conventional activated sludge (CAS) process and its variations (including sequencing batch reactor processes) is the most widely used biological or secondary treatment process in Ontario (OMOE, 2008). Controlled pilot scale biological reactors can contribute to our understanding of the impact of antibiotics and the movement or potential transfer of resistance determinants in the bacterial population of a sewage treatment plant. This study quantified ARGs and pathogens, including, *tetA*, *ampC*, *blaSHV*, *E. coli*, *Enterococcus faecalis*, and *Salmonella* spp., using real-time quantitative PCR, and characterized the antibiotic resistance profiles among common faecal indicators, in the raw influent, activated sludge, and final effluent of a pilot scale biological nutrient removal (BNR) treatment system (Objectives A-C). Furthermore, the results provide a preliminary assessment of selected pathogens and ARGs in a pilot-scale BNR treatment system receiving full-scale municipal

and hospital wastewater. The level of multiple antibiotic resistance (MAR) among culturable indicator bacteria was high, with approximately 61% and 98% of total *E. coli* and *Enterococcus* spp. isolates, respectively. The most common antibiotic resistance profile among *E. coli* was simultaneous resistances to sulfadiazine and streptomycin (SdS), whereas the most common MAR profile for *Enterococcus* spp. was resistance to eight antimicrobials; sulfadiazine, ciprofloxacin, doxycycline, ceftriaxone, vancomycin, streptomycin, oxytetracycline, and minocycline (SdCipDCroVaSTeMi). The pilot-scale BNR treatment system minimally reduced the level of bacterial pathogens and ARGs, with average log removals ranging between 0.39 and 1.62. Concentrations of all three ARGs remained relatively high with  $10^4$  -  $10^6$  target gene copies/100 mL present in the final treated effluent. Wastewater utilities could be a potential source of emerging ARGs and bacteria in the environment, with results illustrating high concentrations of tetracycline (*tetA*), and beta-lactamase genes (*ampC*, *blaSHV*) in raw wastewater, activated sludge, and treated effluent samples, exemplifying the potential dissemination of ARGs into receiving water bodies.

#### **7.1.5 Prevalence and transferability of tetracycline and streptomycin determinants in *Escherichia coli* and *Salmonella* spp. isolated from wastewater effluents (Objectives C & D)**

The transfer of ARGs among microorganisms has long been recognized as a serious threat, contributing to the evolution and emergence of antibiotic resistant bacteria, thereby reducing the therapeutic potential against pathogens (Hawkey and Jones, 2009; Merlin *et al.*, 2011). Gram negative bacteria and related organisms often harbour different plasmids, which confer to them multiple antibiotic resistance to many unrelated antibiotics, contributing to survival

in microbially hostile environments, such as sewage. Horizontal transfer of ARGs through conjugative plasmids often occurs among coliforms and Gram negative bacteria which are dominant in sewage and aquatic environments, making them important bacteria in the development of multiple antibiotic resistances to several antibiotics (Shakibaie *et al.*, 2009). Genotypic evaluation of drug resistance in environmentally obtained strains of *Salmonella* spp. and *E. coli* is limited. In order to have a better understanding of the extent to which wastewater effluents are associated with the spread and dissemination of antimicrobial resistance (AMR), the prevalence, diversity, distribution, and conjugal transfer of resistance genes in waterborne isolates is needed (Objectives C & D). Therefore, waterborne *Salmonella* spp. and *E. coli* isolates collected from raw and/or treated wastewater effluents were examined for antimicrobial resistance using both phenotypic and genotypic methods, including the ability of *Salmonella* spp. and *E. coli* isolates to transfer ARGs (*tetA*, *strA*, *strB*) via conjugation to a recipient *E. coli* strain. Overall, 100% and 71.4% of *E. coli* and *Salmonella* spp. isolates, respectively, exhibited multiple antibiotic resistance (MAR) to at least three or more antimicrobial agents, with 100% of *E. coli* and *Salmonella* spp. isolates demonstrating resistance to streptomycin and sulfadiazine. Identification of three resistance determinants (*tetA*, *strA*, *strB*) conferring resistance to tetracycline and streptomycin was performed on selected multi-drug resistant *Salmonella* spp. and *E. coli* isolates, with 80% of *E. coli* isolates carrying the *tetA* and *strA* genes, and 100% carrying the *strB* gene. Similarly, 100% of *Salmonella* spp. isolates harboured all three ARGs, including *tetA*, *strA*, and *strB*. The potential transfer of tetracycline and streptomycin resistance genes was demonstrated through broth conjugation experiments using multi-drug resistant *Salmonella* spp. and *E. coli* isolates as donors, and *E. coli* K12 as the recipient. Of the twelve strains examined,



conjugation was successfully observed in 75%, occurring in both *Salmonella* spp. and *E. coli* donor isolates. Six strains were capable of transferring all three resistance genes (*tetA*, *strA*, and *strB*) to the recipient strain, resulting in 58.5% (38/65) of total transconjugant strains acquiring all three resistance determinants. The results of the conjugation experiments reveal that many of the multi-drug resistant donors were able to completely or partially transfer phenotypic and/or genotypic patterns including ARGs via mobile elements, such as conjugative plasmids, or integrons. The results confirm the role of environmental bacteria (isolated from wastewater treatment utilities) as a reservoir of antibiotic resistance bacteria and ARGs, containing mobile genetic elements, which are capable of disseminating and transferring ARGs.

When comparing different alternative wastewater treatment systems, the results gathered here indicate that relatively high concentrations of environmental bacteria and pathogens (*E. coli*, *Enterococcus* spp., and *Salmonella* spp.), in addition to ARGs (*tetA*, *ampC*, and *blaSHV*) are released into the receiving water environment, regardless of treatment type and/or sewage composition. Taken together, this study provides evidence that antibiotic resistant bacteria and ARGs are not fully eliminated in some wastewater treatment processes, and can therefore potentially be disseminated downstream of the treatment system via the final effluent. Pollution by ARB and ARGs can increase the opportunities for human pathogens to acquire resistance. The release of residues containing microbiota of human origin, into environments containing bacteria enriched in resistance elements may increase the possibility of acquiring novel resistance genes by human-associated bacteria. For this reason, it has been suggested that the release of residues from hospitals containing both

human commensal and pathogenic bacteria (resistant and susceptible) as well as antibiotics, should be reduced to a minimum in order to avoid exchange of genetic material (Martinez *et al.*, 2009). Contact with human microbiota and other types of microbiota in different ecosystems (from soil sediments of ground water to animal microbiota) will increase the potential for genetic variation and the possible emergence of novel resistance mechanisms that are consequently re-introduced into the human environment (Baquero *et al.*, 2008).

An important part of the dispersal and emergence of antimicrobial resistance in bacteria depends on a complex interaction of factors involved in the evolution and spread of resistance mechanisms. Much evidence suggests that environmental habitats, especially water bodies and wastewater effluents, are ideal vectors for antibiotic resistance dissemination due to the integration of environmental, human, and/or animal related bacteria. As observed in this research, the ubiquitous nature of multi-drug resistant bacteria (including *Salmonella* spp., *E. coli* & *Enterococcus* spp.) in water and wastewater effluents, the presence of diverse ARGs of human and veterinary health significance, as well as the transfer of resistance determinants through conjugative plasmids to recipient bacteria, suggests that environmental exposure through contact with or consumption of contaminated water is probable. However, a lack of critical information still exists regarding the movement of resistance genes in and between microbial populations in the environment. The efficiency of each treatment system and removal of pathogens and/or ARGs must be viewed with discretion, as factors including sewage source, species composition, type of wastewater treatment system, operational conditions, and environmental selective pressures, can significantly influence the outcomes. In addition, the extent of human exposure to ARGs and

antibiotic resistant bacteria is still not well understood, and future studies on human exposure to these resistant contaminants are necessary.

## **7.2 Thesis Recommendations**

Recommendations and future work are detailed and specifically outlined in each chapter of this dissertation, however, some general recommendations can be proposed. Further epidemiological and molecular studies examining waterborne isolates and pathogens isolated from food, humans, and animals, are necessary in order to provide additional insight into establishing whether or not an epidemiological connection exists. Advancements in molecular microbiology such as, non-culture based techniques and high throughput DNA sequencing technology have evolved the field of environmental microbiology, and will only continue to aid in the ability to characterize the antibiotic resistome (pool of ARGs in the environment) in wastewater treatment facilities and the broader aquatic environment. In addition, molecular techniques may be used to help address the questions surrounding the fate and distribution of antibiotic resistance plasmids originating from wastewater treatment facilities and entering the environment (i.e. How stable and transferable are resistance plasmids once they enter the environment?). More studies are needed to provide novel insights into the evolution and dissemination of resistance plasmids and ARGs (via horizontal gene transfer) during the wastewater treatment process and after discharge from the treatment system. One strategy to help minimize or reduce the prevalence of antibiotic resistance in the environment is to curtail the nontherapeutic uses of antimicrobial agents

(i.e. as feed additives); however, there is ongoing debate as to whether or not and to what extent feed additives contribute to the development of resistance in human bacterial pathogens. In addition, improved government surveillance programs in Canada to include environmental sources (i.e. water monitoring, animals, food sources, etc.) are critical to understanding the associations between water and public health. To modify the progression of antimicrobial resistance in Canada and limit the potential negative effects associated with resistance, a national antimicrobial resistance policy program is critical, while integrating an effective governance structure and appropriate sustained funding.

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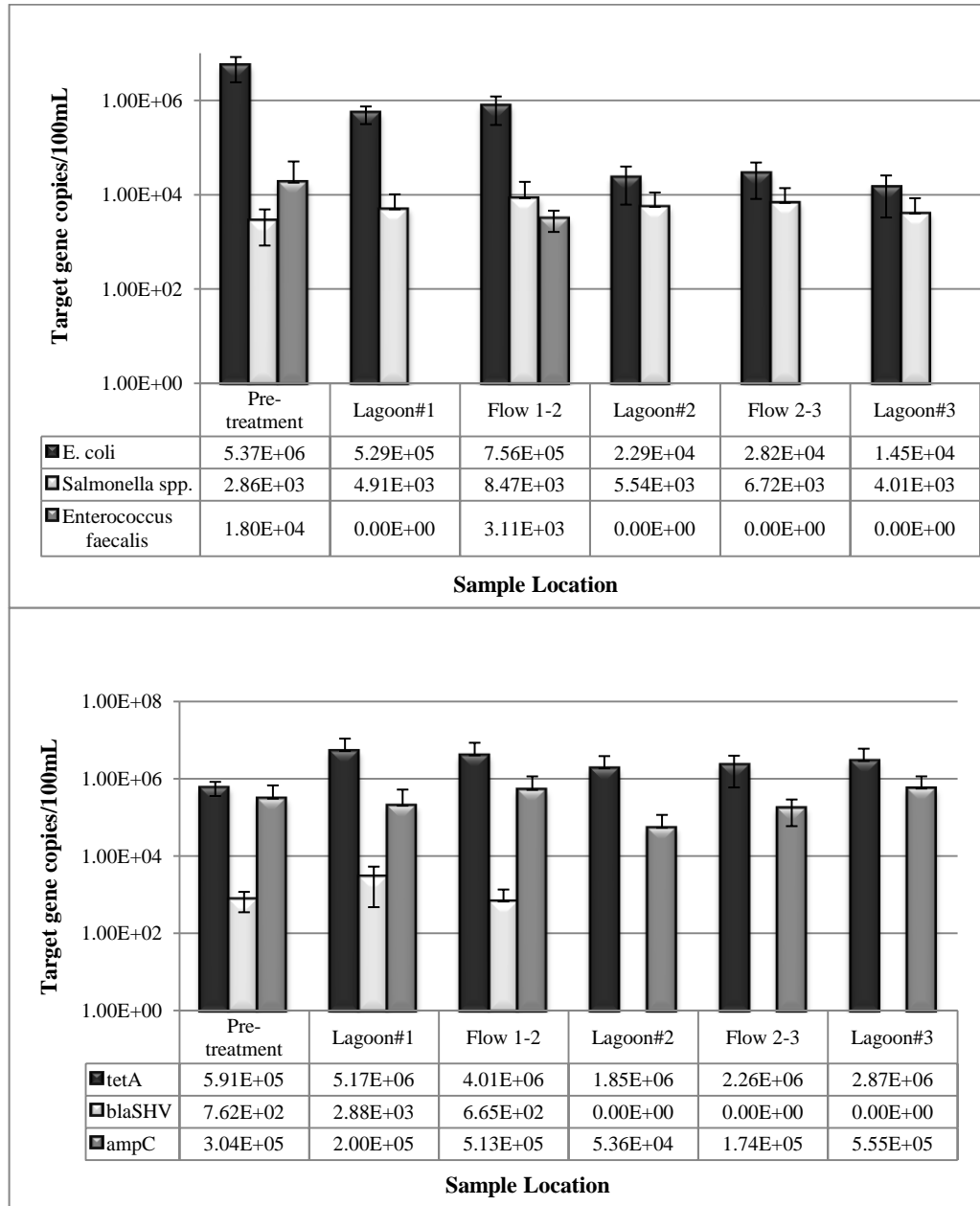
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## Appendix A – Quantitative PCR results for selected pathogens and ARGs collected during the summer (July) 2010 from a constructed lagoon system



**Figure A1. Relative abundance (target gene copies/100mL) of pathogens (*E. coli*, *Salmonella* spp., *E. faecalis*) and ARGs (*blaSHV*, *ampC*, and *tetA*) at different sampling points in the treatment lagoon system (July 2010). Error bars represent standard deviation around mean values from two independent qPCR runs in duplicate.**