CHARACTERIZATION OF ANTIBIOTIC RESISTANCE PROFILES OF SURFACE WATER BACTERIA IN AN URBANIZING WATERSHED

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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August 2016

Major Subject: Water Management and Hydrological Science

ABSTRACT

Wastewater treatment plants (WWTP) are typically incapable of addressing the influx of antibiotics (AB), and may act as a harbor for the selection and proliferation of antibiotic resistant bacteria (ARB). In order to examine the influence of WWTP discharge on the AB resistance profiles of surface water bacteria in an urban stream setting, *E. coli* isolates and total heterotrophic bacteria populations were cultivated from 6 sampling sites up and downstream of WWTPs, and evaluated for resistance to selected ABs. Samples were collected over a 9-month period in the Carter's Creek watershed of College Station, TX. *E. coli* isolates were tested for resistance to ampicillin, tetracycline, sulfamethoxazole, ciprofloxacin, cephalothin, cefoperazone, gentamycin, and imipenem using the Kirby-Bauer disc diffusion method. HPCs were cultivated on R2A amended with ampicillin, ciprofloxacin, tetracycline, and sulfamethoxazole. Significant associations ($p < 0.05$) were observed between the locations of sampling sites relative to WWTP discharge points and the rate of *E. coli* isolate resistance to tetracycline, ampicillin, cefoperazone, ciprofloxacin, and sulfamethoxazole; and an increased rate of isolate multi-drug resistance. The abundance of AB-resistant HPCs was significantly greater ($p < 0.05$) downstream of WWTPs for all treatments; however, there was no spatially significant difference when normalized to total HPCs cultivated with no AB. Results suggest that the effects of human development, specifically the discharge of treated WWTP effluent into surface waters, are potentially significant contributors to the spread and persistence of AB resistance in the surrounding watershed.

DEDICATION

To my parents, Allan and Debra, who have given their unwavering support in every great, decent, and terrible decision I've ever made.

ACKNOWLEDGEMENTS

I would like to thank my advisor and committee chair, Dr. Terry Gentry, for his outstanding mentorship and inexhaustible patience, for striking the balance between providing direction and staying hands-off enough to encourage independent work.

Thanks to my committee members: Dr. John Brooks, whose extensive knowledge of antibiotic resistance played a pivotal role in developing this project, Dr. Karthikeyan, and Dr. Franco Marcantonio, for their guidance and support throughout the course of this research. Thanks also to Dr. Aitkenhead-Peterson for lending her enthusiasm and expertise for the thesis defense.

I owe a great deal to Heidi Mjelde for catching most of my mistakes before I made them, and for putting up with me while I stumbled around hogging refrigerator space and spilling water all over her lab for two years. Thanks to Maitreyee Mukherjee for her help setting up the PCR work. Thanks to Jason Paul, Soofia Farooqui, Drew Pendleton, and Omar Elhassan for helping out in the lab and in the field; I'm sorry for all the yelling, snakes, and wet socks.

Finally, thank you to all my friends and colleagues at Texas A&M for making my time here inspiring, provoking, exhausting, and unforgettable.

NOMENCLATURE

AMR	Antimicrobial Resistance
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistance Genes
B/CS	Bryan/College Station
CFU	Colony Forming Unit
HPC	Heterotrophic Plate Count
WWTP	Wastewater Treatment Plant

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION

1.1 Antibiotic Resistance

The development of antibiotics led to historically groundbreaking advancements in public health. Through the first 8 decades of the 20th century, the infectious disease mortality rate was reduced by 95% (Armstrong *et al.*, 1999). Surgical infection rates were reduced from 40% to 2% (Zaffiri *et al.*, 2012). As the prevalence of antibiotic use has increased in modern society due to their effectiveness and impact in mitigating bacterial health risks, so has the occurrence of bacteria developing resistance to widely used antibiotics. The rates of antibiotic resistance in pathogenic bacteria have been increasing rapidly over the last several decades (Jones *et al.*, 2008), and the occurrence of antibiotic resistance has been identified as a critical issue by the U.S. Center for Disease Control (CDC), World Health Organization (WHO), and numerous other global authorities in public health (Pruden, 2014).

Resistance traits in bacteria can propagate by gene transfer of antibiotic resistance genes (ARGs) between organisms and by spontaneous mutational changes that alter the interactions between the target and antibiotic agent within the cell (Pepper *et al.*, 2015). Horizontal gene transfer between organisms can occur through conjugation, the direct cell-to-cell transfer of plasmid DNA through the extension of a pilus; transduction, the transfer of genetic information through bacteriophages; and transformation, the uptake of free DNA from the cell's environment (Pepper & Gentry, 2015). Conjugation is thought to be the most common method of transfer of ARGs in

environments with high cell counts (Courvalin, 1994, Pepper & Gentry, 2015), and allows for the transfer of genetic material between unrelated species (Davison, 1999), meaning that ARGs in nonpathogenic organisms can be transferred to pathogens. In the presence of antibiotics, selective pressure is placed on microbial communities, encouraging the proliferation of organisms that possess resistance traits (Baquero *et al.*, 2008).

Newly utilized antibiotics have generally seen a significant decrease in their effectivity within ten years of their development (Palumbi, 2001), and the rate of discovery of new antibiotics is decreasing while the emergence of resistance traits in bacteria continues to grow in tandem with increasing populations and antibiotic use (Pruden, 2014). There is also indication that bacterial resistance to specific antibiotics will stabilize and persist in the environment even after discontinued use of that antibiotic (Andersson, 2003), as the mechanisms that enable resistance promote additional environmental resilience and minimize survival costs of the organism (Levy & Marshall, 2004).

1.2 Multidrug Resistance

The proliferation of multi-drug resistant microbes and their corresponding impact on morbidity and mortality rates related to infections has been identified as a major threat to US public health and national security by the National Academy of Science's Institute of Medicine, the federal Interagency Task Force on Antimicrobial Resistance, and the Infectious Diseases Society of America (IDSA) (Spellberg *et al.*, 2008). Multi-drug resistant Gram-negative bacterial infections have been reported to increase the length of hospital stays (Blot *et al.*, 2010, Lye *et al.*, 2012), limit the efficacy of entire regiments of antibiotic classes (Rice, 2006), and generally increase the need for novel mechanisms and antibacterial agents for treatment (Chopra *et al.*, 2008, Nikaido & Pagès, 2012, Naqvi *et al.*, 2013, Worthington & Melander, 2013).

Multi-drug resistance is still a loosely defined term, with little consensus on the specific definitions of 'multi-','extreme-','extensive-', or 'pan-' drug resistance; definitions are formed based on relevance to the environment they are describing, making reliable reference between surveillance studies difficult (Magiorakos *et al.*, 2012). It generally develops as a combination of different resistance mechanisms, utilizing limited outer-membrane permeability and efflux pumps to resist and remove antimicrobial agents from the cell before they have a chance to achieve an actionable concentration (Tenover, 2006). Mitigation of the development multi-drug resistance generally focuses on preventing the generation of environments that expose diverse microbial populations to sub-lethal concentrations of broad-spectrum antimicrobial agents (Dzidic & Bedeković, 2003).

1.3 Environmental Conveyance

Major contributors to the spread of antibiotic resistance include excessive use in humans and animals, overcrowding and increased rates of transmission between people in communities and hospitals, and the failure of implementing and executing proper hygiene and disinfection practices (Gopal Rao, 2012). While the mechanisms by which ARBs and ARGs are transported and spread through the environment are still being researched, significant connections have been established between human activity and the conveyance of resistance traits through agricultural operations, aquatic environments, and sediments (Pei *et al.*, 2006, Baquero *et al.*, 2008).

1.3.1 Agricultural Operations

Non-point source introduction of resistant bacteria has largely been attributed to the application of antibiotics to feedlot operations, for use in the treatment of infections, disease prevention, and as a prophylactic to increase biological rates of production (Brooks *et al.*, 2015). The subsequent land application of the manure/litter from the feedlots is then washed downstream into the watershed during rainfall events (Gunther *et al.*, 1984). The occurrence of a number of AB resistant pathogens have been observed in connection to the extensive use of ciprofloxacin in poultry operations (Humphrey *et al.*, 2005), sparking concerns of foodborne conveyance and infection of the resistant bacteria (Pepper *et al.*, 2015). Bacterial resistance to penicillin, cephalosporin, and tetracycline has been found in swine lagoon effluent, with increased multi-drug resistance found in nurseries with younger piglets (Brooks & McLaughlin, 2009). Chee-Sanford *et al.*

(2001) demonstrated that swine lagoon effluent could directly contribute to ARGs in soil and groundwater.

1.3.2 Wastewater Treatment Plants

Pharmaceutical compounds and resistant bacteria may also be introduced to wastewater treatment systems through hospital, industrial, and residential wastewater discharge, and then introduced to the environment (Zuccato *et al.*, 2010, Amador *et al.*, 2015, Verlicchi *et al.*, 2015). In terms of point-source inputs, wastewater treatment plants (WWTP) represent perhaps the most significant impact of human activity and urbanization on the surrounding watershed. Contemporary municipal WWTPs are typically incapable of specifically addressing the influx of antibiotics (Adams *et al.*, 2002), and may produce an environment where bacteria in the wastewater interact with relatively high concentrations of antibiotics. WWTP sludge has been suspected to foster an ideal environment for the exchange and development of resistant genes, providing additional advantages not available to microbes in the natural environment (Nicholls, 2003). Treatment plants and the associated urbanization may act as a harbor for the selection process of resistant bacteria and resistance genes to eventually be introduced back into the aquatic environment (Makowska *et al.*, 2016).

Investigation into the contribution of wastewater treatment plants to resistance in the environment has expanded greatly in the past decade. Wastewater treatment has been found to be generally ineffective against certain strains of resistant enterococci, specifically with resistance to ciprofloxacin, erythromycin, and tetracycline (da Silva *et al.*, 2006), with the prevalence of ciprofloxacin resistance actually increasing through the

treatment process. The presence of sulfonamide resistance genes in a river environment was found to increase significantly downstream of a swine feedlot WWTP (Hsu *et al.*, 2014). Iwane *et al.* (2001) found that *Escherichia coli* isolates obtained along the Tama River in Tokyo, Japan expressed increasing resistance to antibiotic agents as sampling moved downstream, and was attributed to treatment plant discharge. Studies tend to vary with respect to the efficiency in which resistant organisms are removed during the treatment process, the microbial species expressing resistance in the effluent, and the antimicrobial agents to which the organisms express resistance (Sayah *et al.*, 2005, Janezic *et al.*, 2013).

The confluence of antibiotics and resistant bacteria in WWTPs has also given rise to the concern of microbes developing resistance to multiple drugs. WWTPs may foster an environment that enables rapid exchange of genetic material through the microbial community, facilitating the sharing of ARGs and increasing rates of multi-drug resistance in bacteria. Czekalski *et al.* (2012) found that while WWTPs reduced total bacterial loads in the effluent, there was an observed increase in multidrug resistant bacteria and ARGs which were then found to accumulate in the sediment of the plant outlet. *Aeromonas* and *Pseudomonas aeruginosa* isolates obtained from some water reservoirs were found to express 50 and 100% multi-drug resistance, respectively (Blasco *et al.*, 2008). One potential culprit in the exchange of ARGs in WWTPs is the activated sludge comprising an integral part of most treatment processes (Wellington *et al.*, 2013). One study suggested that sewage sludge may be a main reservoir for

fluoroquinolone residues, found to persist in the environment if transferred in the absence of adequate sludge management practices (Golet *et al.*, 2003).

Furthering the understanding of the impact urbanization and wastewater effluent has on the presence of antibiotic resistance in the environment will aid in future efforts to address antibiotic resistance through treatment plant process design. Current knowledge of how to directly treat wastewater for resistant bacteria and the effect of current treatment processes on resistant bacteria is very limited. Studies have been conducted to improve our understanding of current processes and to investigate new possible solutions, including ozonation, charcoal/sand filtration, and nanotechnology (Lüddeke *et al.*, 2015, Sharma *et al.*, 2015).

1.3.3 Natural Environments

Antibiotic resistance also occurs naturally in the environment, and is not solely a product of modern antibiotic use. Genes coding for resistance to tetracycline and vancomycin have been found frozen in 30,000 year old permafrost samples (D'Costa *et al.*, 2011), and antibiotics have been active agents in the microbial community dating back to the emergence of vertebrate fish (Allen *et al.*, 2010). *Streptomyces* isolates obtained from diverse urban, agricultural, and forest soils were found to be, without exception, resistant to at least 6 different antibiotics (D'Costa *et al.*, 2006), indicating that soils likely act as a natural source for antibiotic resistance. While anthropogenic effects may be contributing to the selective propagation of antibiotic resistance in the environment, the resistance profiles of any natural setting will always be determined in

part by the characteristics of the indigenous microbial community (Wellington *et al.*, 2013).

1.4 Study Goals

This study aims to investigate the relationship between urban development and the occurrence and persistence of antibiotic resistance in the surrounding aquatic environment using bacterial resistance data produced by cultivation-based methods. In order to characterize the antimicrobial resistance profile of surface water bacteria in an urban stream setting, *E. coli* isolates and total heterotrophic bacteria populations were cultivated from six sampling sites within the Carter's Creek watershed of Bryan/College Station (B/CS), TX, and evaluated for resistance to selected antibiotics. Sites were selected based on their relative location to local WWTP effluents in the watershed, and the position up and downstream of developed urban areas. Rates of antibiotic resistance for *E. coli* isolates and heterotrophic communities were compared by sampling site and their relative position with respect to WWTPs to determine if WWTP discharge may affect the antibiotic resistance profiles of surface water bacteria in the surrounding environment.

2. MATERIALS AND METHODS

2.1 Study Area

Six sampling sites were established within the boundaries of the Carter's Creek watershed (Figure 1) in B/CS on the main stems of Carter's Creek and Burton Creek. Sites were selected to represent areas both up and downstream of the more heavily developed areas of the Carter's Creek watershed, and up and downstream of two WWTPs. Sites 1, 3, 5, and 6 were located on the main stem of Carter's Creek, and sites 2 and 4 located on the main stem of Burton Creek. Most sites (all but site 3) were located at the intersection of the respective creek and an overpassing bridge. Sites 2, 4, 5, and 6 were sampled upstream of the bridge crossing, and site 1 was sampled directly underneath the overpass. Site 3 was sampled on the stream stem of Carter's Creek running adjacent to the highway, upstream of its confluence with Burton Creek. Site 2 was located at the outlet of a channelized stretch of Burton Creek, characterized by shallow flow with substantial algal growth on the concrete surface. All of the sampling sites selected in this study have been regular water quality monitoring sites for the Texas Commission on Environmental Quality (TCEQ) since the commencement of an ongoing Carter's Creek watershed Total Maximum Daily Load (TMDL) project in August, 2007. Specific information concerning the sampling sites is included in Table 1.

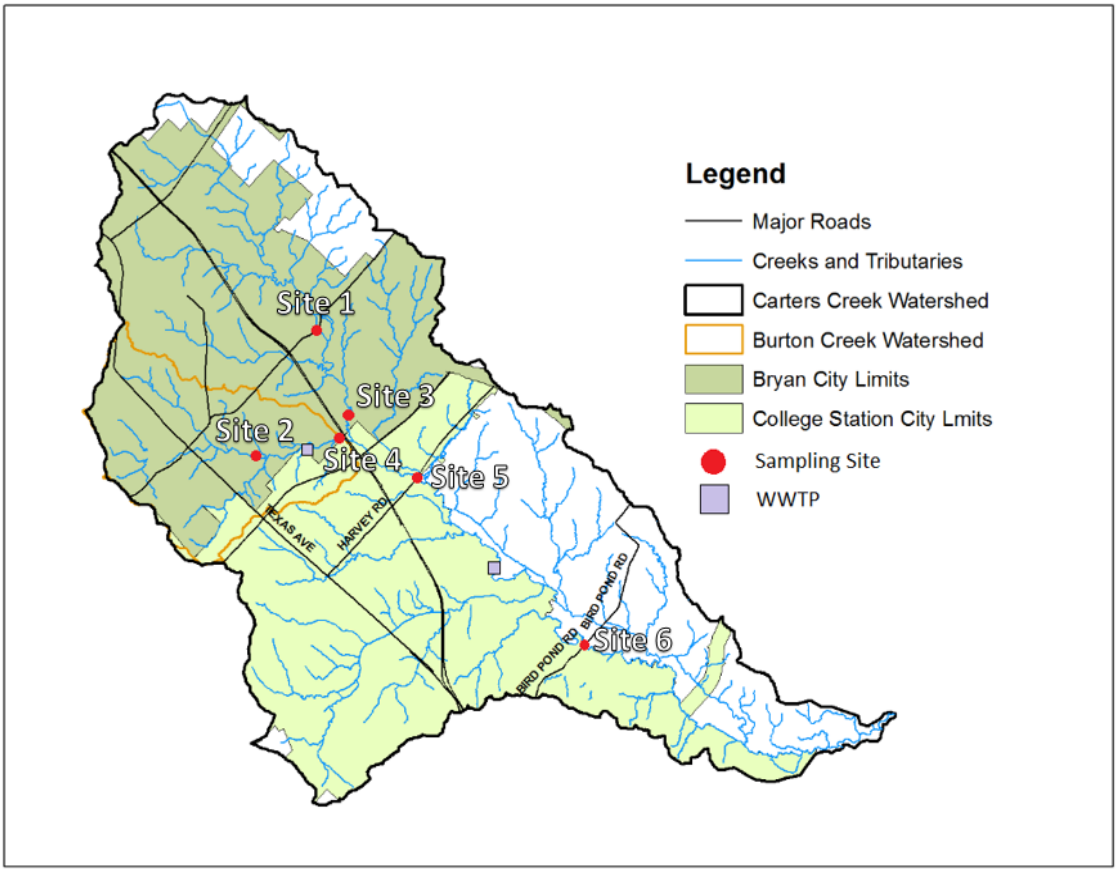

Figure 1: Map of the Carter's Creek watershed and locations of the six sampling sites and the two WWTPs

Site selection was considerably driven by relative location to WWTP effluents. There are two major WWTPs operating and discharging effluent to the Carter's Creek watershed: the Burton Creek Wastewater Treatment Facility (BCWWTF) and the Carter's Creek Wastewater Treatment Plant (CCWWTP). The larger of the two plants, the CCWWTP, began operation in 1956 and now operates at a capacity of approximately 35 million liters (9.5 million gallons) per day. The BCWWTF, commissioned in 1987, is authorized for a maximum discharge of 30 million liters (8 million gallons) per day, and

discharges into an unnamed tributary approximately 1,000 meters upstream of Burton Creek's outlet into Carter's Creek (TCEQ, 2006). Sites 1, 2, and 3 were located upstream of any WWTP discharge points, while sites 4, 5, and 6 were located downstream of at least one. Sites 4 and 5 were located downstream of one WWTP, the Burton Creek Wastewater Treatment Facility, and Site 6 was located downstream of both the Burton Creek and Carter's Creek treatment facilities. There is a third WWTP servicing the southern area of College Station, discharging its effluent into Lick Creek. However, flows from Lick Creek do not interact with waters from the Carter's Creek watershed until both creeks have converged with the Navasota River on their western borders.

Table 1: Site locations and coordinates

Site Number	Description	Coordinates	Position in relation to WWTP
1	Carter's Creek at Briarcrest Drive	30°40'04.3"N 96°19'13.2"W	Upstream
2	Burton Creek at Tanglewood Drive	30°38'26.8"N 96°20'06.6"W	
3	Carter's Creek upstream of Burton Creek	30°38'40.3"N 96°18'43.2"W	
4	Burton Creek at Route 6, downstream of WWTP	30°38'39.2"N 96°18'50.4"W	Downstream
5	Carter's Creek at Harvey Road	30°38'09.5"N 96°17'45.2"W	
6	Carter's Creek at Bird Pond Road	30°36'10.6"N 96°15'00.1"W	

2.2 Sample Collection

A total of six separate sampling events were conducted over a 9-month period between July, 2015 and April, 2016. Surface water samples were collected using ~500 mL Whirl-Pak® sterile bags (eNasco, Fort Atkinson, WI) and a sampling pole. Water samples were collected from the mid-point of the stream flow approximately 3 cm below the surface. Samples were put on ice for transfer back to the laboratory and processed within 6 hours of collection. *E. coli* isolates were collected for antibiotic susceptibility testing from 5 of the 6 sampling events. Heterotrophic plate counts were obtained for all 6 sampling events.

2.3 *E. coli* Isolation and Antibiotic Susceptibility Testing by Kirby-Bauer Disc Diffusion Method

Four concentrations of each sample were prepared (1.0, 0.1, 0.01, and 0.001) by ten-fold serial dilutions in phosphate-buffered saline solution (PBS). Ten mL of each dilution was then filtered through a 0.45 µm filter membrane (Millipore, Billerica, MA) by vacuum filtration. Filter membranes were placed on 47 mm Difco® Modified mTEC agar plates (Becton, Dickinson and Company, Sparks, MD) and incubated at 35 °C for 2 hours and then 44.5 °C for 24 hours in accordance with EPA Method 1603 (USEPA, 2002). Following incubation, ten presumed *E.coli* (magenta) colonies for each of the six sampling site sets were randomly selected, transferred to Difco® Tryptic Soy agar (Becton, Dickinson and Company, Sparks, MD) using a sterile loop, and incubated at 35 °C for 24 hours. *E. coli* cell suspensions were prepared by transferring two colonies of each isolate into tubes with 5 mL of BBL® Tryptic Soy Broth (Becton, Dickinson and

Company, Sparks, MD) and incubating at 35 °C for 3 hours while shaking at 150 rpm. Tubes were checked for turbidity against a pre-prepared 0.5 McFarland standard corresponding to a $10^7 – 10^8$ CFU/mL bacterial cell count in the broth.

After incubation, sterile swabs were used to inoculate 100 mm Mueller Hinton Agar (MHA) plates (Neogen Corporation, Lansing, Michigan). Antibiotic resistance of the *E. coli* isolates was determined by the Kirby-Bauer method for antibiotic susceptibility (Bauer *et al.*, 1966) . Eight antibiotic susceptibility discs (Becton, Dickinson and Company, Franklin Lakes, NJ) of tetracycline (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg), sulfamethoxazole/trimethoprim (23.75/1.25 µg), imipenem (10 µg), gentamicin (120 µg), cefoperazone (75 µg), and cephalothin (30 µg) were stamped onto each MHA plate using a BBL® Sensi-Disc® 8-place Dispenser (Becton, Dickinson and Company, Franklin Lakes, NJ). The MHA plates were then incubated at 35 °C for 16 – 24 hours and the diameters of the inhibition zones measured to determine resistance, intermediate resistance, or susceptibility of each isolate to the antibiotics according the Clinical and Laboratory Standards Institute (CLSI) standards. Reference organisms, *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27852 (American Type Culture Collection, Manassas, VA), were used as controls to ensure consistency during the antibiotic disc diffusion process.

It should be noted that for an initial screening sampling event, erythromycin (15 µg) was used in place of cefoperazone. The replacement of erythromycin with cefoperazone as the eighth antimicrobial agent for this study occurred due to

inconsistencies in the inhibition zones and the absence of established reference data relating to the control organisms for that antibiotic.

2.4 PCR Isolate Confirmation

PCR amplification of the *E. coli* specific *uidA* sequence was used to confirm all isolates collected as *E. coli* (Bower *et al.*, 2005). Cell suspensions of each presumed *E. coli* isolate were prepared by suspending bacterial lawn growth from the MHA agar in 100 µL of sterile, distilled water. PCR mixtures (50 µL) were prepared consisting of 25 µL of GoTaq® G2 Green Master Mix (Promega, Madison, WI), 1.75 µL (350 nM) each of the forward (uidA1318F) and reverse (uidA1698R) primers (Integrated DNA Technologies, Coralville, IA), 5 µL of cell suspension as the template DNA, and 16.5 µL of sterile nuclease-free water. *E. coli* 25922 isolates were used for the positive control. Primer sequences, target, and reference are shown in Table 2.

Table 2: PCR primers for amplification of the *E. coli* specific *uidA* gene

Primer	Sequence	Target	Reference
uidA1318F	5’CCGATCACCTGTGTCAATGT 3’	*E.coli* β - glucuronidase	(Bower *et al.*, 2005)
uidA1698R	5’GTTACCGCCAACGCGCAATA 3’		

PCR conditions included 1 initial heating cycle at 94 °C for 4 minutes; followed by 35 cycles at 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds; a final cycle at 72 °C for 6 minutes, and then held at 4 °C. DNA electrophoresis was performed in a 2% agarose gel (Amresco, Solon, OH) stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO) and a 1X Tris-Borate-EDTA (TBE) (Fisher BioReagents, Fair Lawn, NJ) buffer solution. A 100 bp ExACTGene™ DNA ladder (Fisher BioReagents, Fair Lawn, NJ) was used as the marker.

2.5 Heterotrophic Plate Counts

Four concentrations of each of the six water samples were prepared (1.0, 0.1, 0.01, and 0.001) by ten-fold serial dilutions in PBS. Thirty microliters of each dilution were spread-plated onto five sets of 47 mm plate Bacto® Reasoner's 2A (R2A) agar (Difco Laboratories, Detroit, MI) amended with the following antibiotics: 32 µg/mL ampicillin (Ward's Science, Rochester, NY), 16 µg/mL tetracycline (Alfa Aesar, Ward Hill, MA), 4 µg/mL ciprofloxacin (TCI America, Portland, OR), 50.4 µg/mL sulfamethoxazole (Chem-Impex International Inc., Wood Dale, IL), and un-amended R2A with no antibiotic. Antibiotic concentrations in the agar were determined based on prior studies and are generally around half the strength of either the IV or oral dosage concentrations (Pei *et al.*, 2006, Gao *et al.*, 2012, Garcia-Armisen *et al.*, 2013). Plates also contained 200 µg/mL of cycloheximide (Amresco, Solon, OH) as a fungicide. All plates were incubated at 28 °C for five days before obtaining bacterial CFU plate counts.

2.6 Statistics

E. coli isolate responses to antibiotic susceptibility disc diffusion were categorized as either susceptible or resistant (including intermediate resistance) and assigned a binary value for each response: 1 for resistant and 0 for susceptible. Isolates and isolate responses could then be grouped into a number of various categories and tested for significant associations by chi-square analysis. Groupings were generally done by pairing binary data from two individual sampling sites or two groups of sampling sites, generating two-by-two grids with one degree of freedom. Significant differences were determined by Chi square sums of 3.84 or greater, or $p < 0.05$ for one degree of freedom. Statistical analysis of the heterotrophic bacteria and box plot production was done using SAS® University Edition (SAS Institute, Cary, NC). Significant differences in the abundance and normalized resistance rates of heterotrophic ARB were evaluated using one-way ANOVA by least-significant-difference (LSD) comparison. Significant differences were checked for homogeneity of variance by Levene's test, and in cases where significant differences in homogeneity were found in the data set significant difference was determined by Welch's ANOVA. Relationships were considered to be significant at $p < 0.05$.

3. RESULTS

3.1 PCR Isolate Confirmation

Isolates were confirmed as *E. coli* through PCR amplification of *uidA* with *E. coli*-specific primers and an expected amplicon size of approximately 400 bp (Bower *et al.*, 2005). Figure 2 shows the results of PCR amplicon gel electrophoresis of all 300 isolates. Out of the total 300 isolates collected for this study, 280 (93%) were confirmed as *E.coli*. Any isolate that returned negative results had a second cell suspension prepared at a higher concentration, and both the original suspension and concentrated suspension were run again to confirm the negative result. Positive and negative controls produced the expected results for each assay. Several isolates initially produced negative results, but when the additional cell suspension was made and a second reaction was performed to confirm the negative results, the isolates returned positive. The 20 isolates that were not confirmed were excluded from the results and statistical analysis of the study.

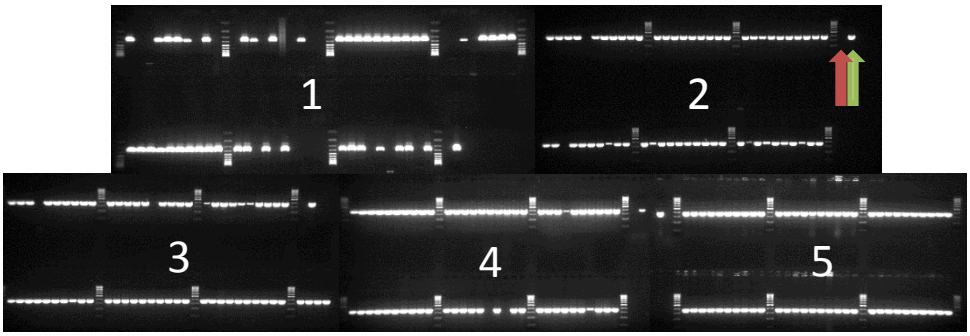

Figure 2: Results of PCR amplicon gel electrophoresis of *uidA* (~400 bp) for all 300 *E. coli* isolates obtained from sampling events 1 – 5. Green arrow = positive control, red arrow = negative control.

3.2 *E. coli* Isolate Antibiotic Susceptibility

3.2.1 Individual Antibiotics

A total of 280 confirmed isolates across all sampling sites and events were tested for susceptibility to 8 antibiotics. Inhibition zone diameters were measured and recorded in millimeters, and compared to CSLI standards to determine if each isolate was susceptible or resistant to each antibiotic. Isolates displaying intermediate resistance were categorized as resistant. The number of isolates expressing resistance to individual antimicrobial agents by sampling site are displayed in Table 3.

Table 3: Number of *E. coli* isolates (%) expressing resistance to antibiotics, by sampling site

Antibiotic	Disc ID	Sampling Site						Total (n=280)
		Upstream of any WWTP			Downstream of ≥ 1 WWTP			
		1 (n=49)	2 (n=44)	3 (n=46)	4 (n=47)	5 (n=44)	6 (n=50)	
Tetracycline	TE-30	2 (4)	4 (9)	0 (0)	8 (17)	10 (23)	14 (28)	38 (14)
Ampicillin	AM-10	3 (6)	1 (2)	6 (13)	8 (17)	9 (20)	14 (28)	41 (15)
Cefoperazone	CFP-75	1 (2)	0 (0)	0 (0)	1 (2)	3 (7)	3 (5)	8 (3)
Ciprofloxacin	CiP-5	0 (0)	0 (0)	0 (0)	2 (4)	3 (7)	7 (14)	12 (4)
Sulfamethoxazole/ Trimethoprim	SXT	1 (2)	0 (0)	0 (0)	2 (4)	3 (7)	7 (14)	13 (5)
Gentamycin	GM-10	2 (4)	0 (0)	0 (0)	1 (2)	2 (5)	3 (6)	8 (3)
Cephalothin	CF-30	44 (90)	38 (86)	37 (80)	36 (77)	38 (86)	43 (86)	236 (84)
Imipenem	IPM-10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Only 12% of all isolates were susceptible to all 8 antibiotics. The relatively low number of susceptible isolates can mostly be attributed to the high rate of cephalothin resistance found across all sampling sites. A large proportion (84%) of all isolates expressed resistance or intermediate resistance to cephalothin, with rates of resistance at each individual sampling site falling consistently between 77 - 90% of the isolates collected. The next highest rates of resistance after cephalothin occurred with respect to ampicillin and tetracycline at 15% and 14% of all isolates, respectively. Resistance to ampicillin was expressed in 41 isolates, with resistance rates falling between 2 – 13% for isolates obtained upstream of WWTP discharges and 17 – 28% for isolates obtained downstream of WWTP discharges; and resistance to tetracycline was expressed in 38 isolates, with resistance rates falling between 0 – 9% for isolates obtained upstream of WWTP discharges and 17 – 28% for isolates obtained downstream of WWTP discharges. Resistance to cefoperazone, gentamycin, ciprofloxacin, and sulfamethoxazole/trimethoprim was found in a fewer number of isolates, at rates of 3%, 3%, 4%, and 5%, respectively. All four resistances were found more frequently in the isolates obtained from downstream sampling sites. Gentamycin resistance was the only instance in which isolate resistance was found to occur more frequently in one of the upstream sites than in one of the downstream sites (site 1 vs. site 4). All 280 isolates were susceptible to imipenem.

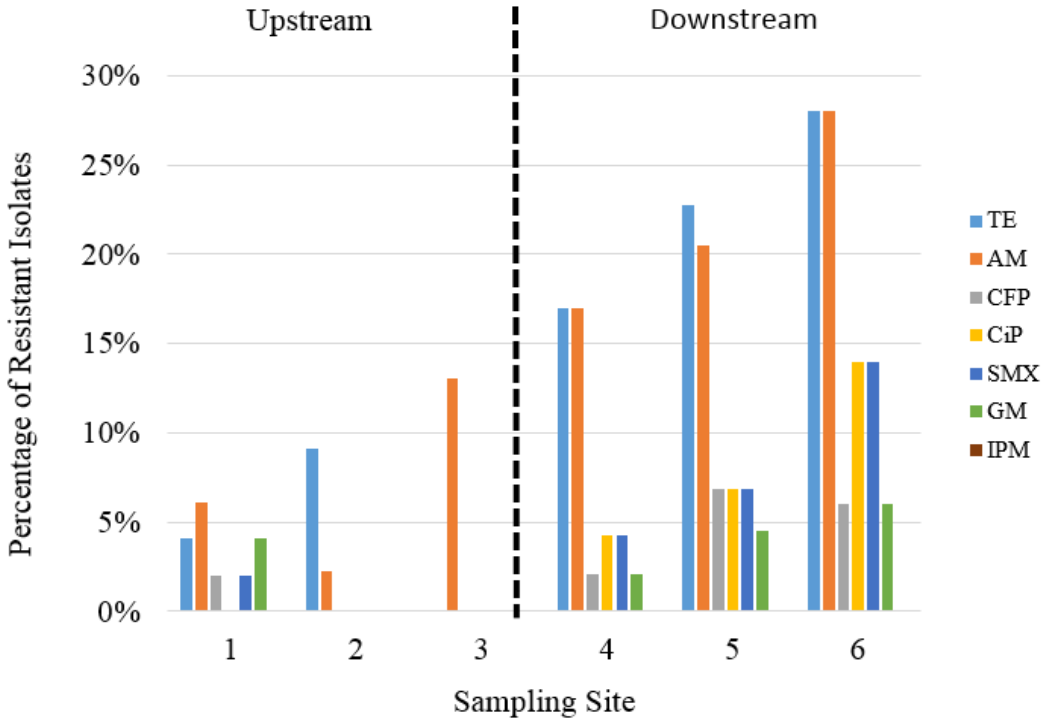

Figure 3: Percentage of resistant isolate responses to seven of eight antibiotics by sampling site. Cephalothin is excluded for visibility of less frequently occurring AB resistances. IPM, imipenem, GM, gentamycin, SMX, sulfamethoxazole, CiP, ciprofloxacin, CFP, cefoperazone, AM, ampicillin, TE, tetracycline.

A column chart of isolate resistance responses by sampling site and antibiotic shows an increase in the rate of resistant responses in the downstream sampling sites (Figure 3). Isolates collected from the downstream sampling sites expressed resistance more frequently and to a higher variety of antimicrobial agents than the upstream sites. Sampling site 1 displays the most diversity in resistance to different agents in the upstream group, due to one isolate sampled during event 2 expressing resistance to six agents. The number of total resistant responses also appears to increase as the sampling sites progress further downstream in the downstream group. Cephalothin resistance is

presented separately in Figure 4 as to not visually overwhelm the less frequently occurring antibiotic resistances. Cephalothin resistance occurred at a greater frequency and more consistently across all sampling sites than the other antibiotic resistances.

Chi-square analysis was used to determine significant differences in the rates of isolate resistance to individual antibiotic agents by location. Sampling sites (independent

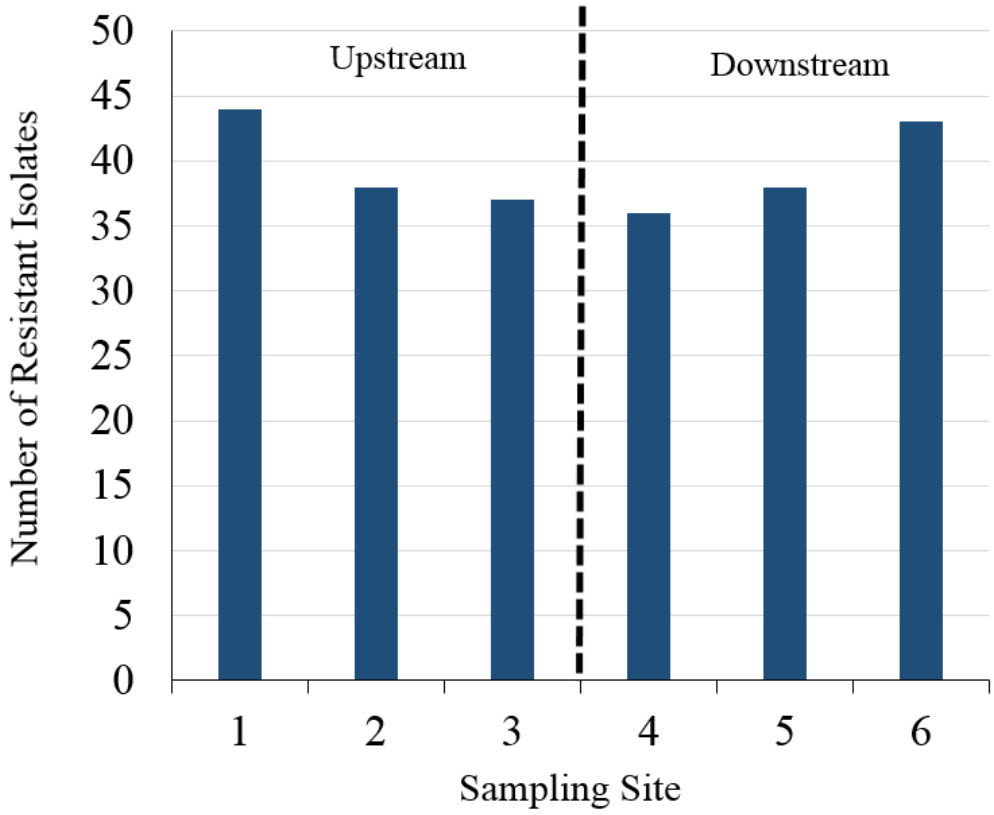

Figure 4: Column chart of cephalothin resistant *E. coli* isolate responses. Resistance occurred at a greater frequency than other antibiotic resistances, more consistently across upstream and downstream sampling sites.

variable) were tested against each other individually, and also as two major groupings of sites: (1) upstream of any WWTP discharge (sites 1 -3) and (2) downstream of at least one WWTP discharge (sites 4 -6). Isolate responses to each antibiotic (dependent variable) were assigned a binary value, 0 for susceptible and 1 for resistant, and summed for each category. A total of eight chi-square tests, one for each of the eight antibiotics for which isolate resistance was tested, were performed for each sampling site set.

Chi-square tests for isolate resistance by individual sampling site (Figure 5) showed significant differences ($p < 0.05$) between at least one pair of sites for ampicillin, sulfamethoxazole, tetracycline, and ciprofloxacin. The majority of these occurred between site pairings in which one site was upstream of a WWTP and the other site was downstream of a WWTP. Only one test reported a significant difference between two sites with the same relative location to a WWTP. This result was reported for the rate of isolate resistance to tetracycline between sites 2 and 3, corresponding to the Burton Creek site upstream of the WWTP and the Carter's Creek site upstream of its confluence with Burton Creek, respectively.

When sampling sites were categorized into either an upstream (sites 1 – 3) or downstream (sites 4 – 6) group, a significant difference ($p < 0.05$) was found to exist in isolate rates of resistance to ampicillin, tetracycline, cefoperazone, ciprofloxacin, and sulfamethoxazole. While cefoperazone resistance did not increase significantly between any individual sampling sites, there was a significant increase when compared between the upstream and downstream groups.

Ampicillin	Sampling Site					
	1	2	3	4	5	6
1		0.83	1.33	**2.81**	**4.24**	**8.33**
2			3.64	**5.55**	**7.22**	**11.55**
3				**0.29**	**0.89**	**3.25**
4					0.18	1.67
5						0.72
6						

Sulfamethoxazole	Sampling Site					
	1	2	3	4	5	6
1		0.91	0.95	**0.39**	**1.29**	**4.77**
2			-	**1.91**	**3.11**	**6.66**
3				**2.00**	**3.24**	**6.95**
4					0.29	2.73
5						1.27
6						

Tetracycline	Sampling Site					
	1	2	3	4	5	6
1		0.96	1.92	**4.30**	**7.17**	**10.45**
2			4.38	**1.25**	**3.06**	**5.41**
3				**8.57**	**11.76**	**15.08**
4					0.47	1.67
5						0.34
6						

Gentamycin	Sampling Site					
	1	2	3	4	5	6
1		2.78	2.91	**0.96**	**0.11**	**0.00**
2			-	**0.95**	**2.05**	**2.73**
3				**0.99**	**2.14**	**2.85**
4					0.42	0.92
5						0.10
6						

Cefoperazone	Sampling Site					
	1	2	3	4	5	6
1		0.91	0.95	**0.00**	**1.29**	**1.00**
2			-	**0.95**	**3.11**	**2.73**
3				**0.99**	**3.24**	**2.85**
4					1.19	0.92
5						0.03
6						

Cephalothin	Sampling Site					
	1	2	3	4	5	6
1		0.26	1.65	**3.01**	**0.26**	**0.33**
2			0.57	**1.43**	**0.00**	**0.00**
3				**0.20**	**0.57**	**0.53**
4					1.43	1.42
5						0.00
6						

Ciprofloxacin	Sampling Site					
	1	2	3	4	5	6
1		-	-	**2.13**	**3.45**	**7.38**
2			-	**1.91**	**3.11**	**6.66**
3				**2.00**	**3.24**	**6.95**
4					0.29	2.73
5						1.27
6						

Imipenem	Sampling Site					
	1	2	3	4	5	6
1		-	-	**-**	**-**	**-**
2			-	**-**	**-**	**-**
3				**-**	**-**	**-**
4					-	-
5						-
6						

Figure 5: Chi-square test values for rates of isolate resistance between all sampling sites by antibiotic. A significant difference ($p < 0.05$) between sites existed for test values > 3.84 (critical value for 1 degree of freedom). Values for which one site was upstream and the other was downstream are bolded. Shaded cells are tests that reported a significant difference in isolate resistance rates for that antibiotic. Cells with no value (-) indicate that no isolate resistance existed at one of the sites.

3.2.2 Multi-drug Resistance

Binomial resistance values determined by the number of resistant responses of each isolate were tallied and sorted into five groups, isolates resistant to 0, 1, 2, 3, or ≥ 4 agents, and organized by sampling site (Table 4). Out of the 280 isolates, the majority (88%) showed resistance to at least 1 antibiotic agent. A total of 28 isolates (10% of total) showed resistance to 2 agents, 9 (3% of total) showed resistance to 3 agents, and 17 (6% of total) showed resistance to 4 or more agents. Of the 54 multi-drug resistant isolates collected (resistant to at least 2 agents), 41 (76%) were obtained from downstream sites (sites 4 -6). All isolates resistant to 3 agents and all but one of the isolates that were resistant to 4 agents were collected from one of the downstream sites.

Table 4: Number (%) of multi-drug resistant *E. coli* isolates by sampling site

Site Number	**Number (% by site) of Isolates with Resistance to *n* agents:**					**Total**
	n = 0	n = 1	n = 2	n = 3	n ≥ 4	
1	5 (10)	39 (80)	4 (8)	0 (0)	1 (2)	49
2	5 (11)	35 (80)	4 (9)	0 (0)	0 (0)	44
3	7 (15)	35 (76)	4 (9)	0 (0)	0 (0)	46
4	8 (17)	29 (62)	5 (11)	2 (4)	3 (6)	47
5	6 (14)	24 (55)	6 (14)	3 (7)	5 (11)	44
6	2 (4)	31 (62)	5 (10)	4 (8)	8 (16)	50
All Sites	33 (12)	193 (69)	28 (10)	9 (3)	17 (6)	280

Resistance responses were also sorted by type of antibiotic and number of agents that each isolate was resistant to (Table 5). Cephalothin resistance was again the most frequently occurring (95%) antibiotic resistance in the sample set of multi-drug resistant isolates (resistant to 2 or more agents). Out of all isolates that were resistant to at least one antibiotic, 74% were only resistant to cephalothin, and cephalothin resistance accounted for 95% of all single-drug resistant isolates. Isolates only resistant to tetracycline, ampicillin, or cefoperazone were found sparingly, each representing less than 2% of the single-drug resistant isolates. No isolates were only resistant to ciprofloxacin, sulfamethoxazole, gentamycin, or imipenem.

Isolates resistant to 2 or more agents were generally resistant to cephalothin and either tetracycline (41%), or ampicillin (48%). Resistance to 3 agents occurred less frequently than resistance to 4 or more agents, at only 4% of resistant isolates. All isolates showing resistance to 4 or more antibiotics were resistant to cephalothin, and over 80% of these isolates were also resistant to tetracycline, and 90% to ampicillin. Sulfamethoxazole resistance was only found in isolates resistant to 3 or more agents. Resistance to cefoperazone, ciprofloxacin, sulfamethoxazole, and gentamycin was generally accompanied by several other resistances.

Table 5: Number of *E. coli* isolates expressing resistance to each antibiotic, grouped by the number of agents the isolate was resistant to

Antibiotic	Number (%) of Resistant Isolates when Isolate is Resistant to *n* agents:				Total
	n = 1	n = 2	n = 3	n ≥ 4	
Tetracycline	4 (2)	12 (41)	8 (89)	14 (82)	38 (15)
Ampicillin	3 (1.5)	14 (48)	8 (89)	16 (94)	41 (17)
Cefoperazone	1 (0.5)	0 (0)	0 (0)	7 (41)	8 (3)
Ciprofloxacin	0 (0)	2 (7)	0 (0)	10 (59)	12 (5)
Sulfamethoxazole/Trimethoprim	0 (0)	0 (0)	3 (33)	13 (76)	13 (5)
Gentamycin	0 (0)	1 (3)	1 (11)	6 (35)	8 (3)
Cephalothin	184 (95)	28 (97)	7 (78)	17 (100)	236 (95)
Imipenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Chi-square analysis was performed for *E. coli* isolate multidrug resistance to examine any significant associations ($p < 0.05$) between all individual sampling sites, between the two groups of sampling sites representing creek stem regions upstream and downstream of the WWTPs, and for each individual sampling site when compared against all other sampling sites. Three separate definitions of multidrug resistance were examined for each grouping: isolates resistant to 2 or more agents, 3 or more agents, and 4 or more agents.

Significant associations were found between several sampling site pairings for all classifications of multi-drug resistance (Figure 6). All significant associations between

individual sampling sites and rates of multi-drug resistance occurred between upstream and downstream sites; none were found between sites within the same upstream or downstream group.

≥ 2 Agents	Sampling Site 1	2	3	4	5	6
Sampling Site 1		0.03	0.06	**2.23**	**6.66**	**8.11**
2			0.00	**2.59**	**6.98**	**8.37**
3				**2.88**	**7.51**	**8.98**
4					1.30	1.95
5						0.05
6						

≥ 3 Agents	Sampling Site 1	2	3	4	5	6
Sampling Site 1		0.91	0.95	**3.03**	**6.91**	**10.46**
2			-	**4.95**	**8.80**	**12.11**
3				**5.17**	**9.18**	**12.62**
4					1.06	2.99
5						0.47
6						

≥ 4 Agents	Sampling Site 1	2	3	4	5	6
Sampling Site 1		0.91	0.95	**1.13**	**3.34**	**5.83**
2			-	**2.90**	**5.30**	**7.69**
3				**3.03**	**5.53**	**8.03**
4					0.70	2.23
5						0.42
6						

Figure 6: Chi-square test values for rates of isolate resistance between all sampling sites by extent of multi-drug resistance. A significant difference ($p < 0.05$) between sites existed for test values > 3.84 (critical value for 1 degree of freedom). Values for which one site was upstream and the other was downstream are bolded. Shaded cells are tests that reported a significant difference in multi-drug resistance rates for that site pairing. Cells with no value (-) indicate that no multi-drug resistance occurred at one of the sites.

A significant association ($p < 0.001$) was found to exist between the number of isolates expressing resistance to ≥ 2, ≥ 3, and ≥ 4 antibiotic agents and whether the isolate was collected upstream of any WWTP (sites 1, 2, and 3) vs. downstream of at least 1 WWTP (sites 4, 5, and 6). There was not a significant association ($p > 0.05$) between the upstream and downstream sample site groups and the occurrence of isolate resistance to 1 or more agents (data not shown).

Six chi-square tests were performed to compare rates of multi-drug resistance isolates between each individual sampling site and all other five sites for isolates resistant to 2 or more, 3 or more, or 4 or more antibiotic agents (Table 6); and between each individual sampling event and all other five events for isolates resistant to 2 or more, 3 or more, or 4 or more antibiotic agents (Table 7). These tests are intended to provide information concerning which sites or sampling events are contributing the most significance to variations in the antibiotic resistance profiles of surface water *E. coli* in the watershed, independent of whether the site is contributing significantly less or significantly more resistant isolates compared to the other sampling sites. Ideally, all of these tests would not have a significant association with the data set, indicating that the overall trend in multi-drug resistance is not significantly affected by only one single sampling event.

Table 6 shows sites that were identified as having a significant association with the number of multi-resistant isolates produced when compared against all other sampling sites and the p-values of each association. Downstream sampling sites 5 and 6 contributed significantly to the variation between sites in the amount of isolates resistant

to ≥ 2 and ≥ 3 antibiotic agents, with site 6 contributing significantly to variations between sites with isolates resistant to ≥ 4 agents as well. Upstream of the WWTPs, sites 2 and 3 showed a significant difference in the numbers of isolates resistant to ≥ 3 agents. Of all the sampling events and for all definitions of multi-drug resistant isolates, only sampling event 2 had a significant ($p < 0.01$) difference for the number of isolates resistant to 2 or more antibiotics (Table 7). Ideally, all of these tests would not have a significant association with the data set, indicating that the overall trend in multi-drug resistance is not significantly affected by only one single sampling event.

Table 6: Chi-square significant associations for individual sampling sites for 3 groupings of multidrug *E. coli* isolate resistance. Shaded cells are site/multidrug combinations producing a significant association, df =1

Site Number	p values for sites with a significant association with respect to isolates resistant to *n* agents:		
	n ≥ 2	n ≥ 3	n ≥ 4
1	p > 0.05	p > 0.05	p > 0.15
2	p > 0.05	p < 0.025	p > 0.05
3	p < 0.05	p < 0.02	p > 0.05
4	p > 0.25	p > 0.25	p > 0.25
5	p < 0.025	p < 0.05	p > 0.10
6	p < 0.005	p < 0.0005	p < 0.0025

Table 7: Chi-square significant associations for sampling events for 3 groupings of multidrug *E. coli* isolate resistance. Shaded cells are site/multidrug combinations producing a significant association, df =1

Event Number	p values for events with a significant association with respect to isolates resistant to *n* agents:		
	$n \geq 2$	$n \geq 3$	$n \geq 4$
1	$p < 0.01$	$p > 0.25$	$p > 0.25$
2	$p > 0.25$	$p > 0.25$	$p > 0.25$
3	$p > 0.25$	$p > 0.25$	$p > 0.25$
4	$p > 0.25$	$p > 0.25$	$p > 0.25$
5	$p > 0.15$	$p > 0.25$	$p > 0.25$

3.3 Heterotrophic Plate Counts

Heterotrophic bacterial plate counts were obtained during six sampling events in order to examine the antibiotic resistance profiles of the cultivable, heterotrophic bacterial community in the watershed. The log-transformed bacterial concentrations of each treatment category for all sampling events and sampling sites are displayed in Table 8. The limit of detection was one CFU in 30 µL of undiluted sample, or 1.52 $\log_{10}$ CFU/mL. There were five instances in which no bacteria were culturable within the sample volume and concentration limit; four of the ciprofloxacin-amended plates, and one of the tetracycline-amended plates. These results were reported as below the limit of detection, and were represented as ½ the limit of detection (16.67 CFU/mL) for statistical analysis.

Table 8: Log_{10}-transformed concentrations (log_{10} CFU/mL) of heterotrophic bacteria and antibiotic resistant heterotrophic bacteria for each antibiotic by sampling event and sampling site. AM, ampicillin, CiP, ciprofloxacin, TE, tetracycline, SMX, sulfamethoxazole

Sampling Event	Date	AB	Concentration (log_{10} CFU/mL) by Sampling Site					
			1	2	3	4	5	6
#1	7/13/2015	None	3.05	6.08	3.52	5.28	4.78	4.21
		AM	2.67	3.22	3.03	4.20	3.85	2.70
		CiP	< 1.52	2.12	1.52	2.52	2.52	2.12
		TE	1.82	< 1.52	2.52	3.82	3.10	2.12
		SMX	2.88	3.88	2.82	4.29	3.70	3.15
#2	9/7/2015	None	5.10	5.29	4.59	5.41	5.23	5.43
		AM	3.52	3.70	3.29	4.04	3.90	3.65
		CiP	3.82	3.70	3.75	4.11	4.18	4.26
		TE	2.52	2.90	2.22	3.47	2.90	2.87
		SMX	3.88	4.18	3.53	4.70	4.32	4.52
#3	11/5/2015	None	4.32	4.46	4.29	5.17	4.59	5.17
		AM	2.82	3.17	2.85	3.56	3.85	3.56
		CiP	3.56	3.48	3.59	3.94	4.08	3.94
		TE	2.52	2.52	2.37	2.87	3.22	2.87
		SMX	4.12	3.87	3.64	4.66	4.19	4.66
#4	1/20/2016	None	5.17	5.50	5.05	5.21	5.17	5.20
		AM	2.70	4.37	2.94	3.99	4.00	3.64
		CiP	3.32	3.78	3.69	3.46	3.72	3.50
		TE	1.52	2.85	1.82	3.18	3.11	2.67
		SMX	3.34	2.85	3.43	4.56	4.73	4.48
#5	2/16/2016	None	5.04	5.16	4.58	5.30	5.27	5.41
		AM	2.37	4.04	2.12	4.22	4.17	3.77
		CiP	< 1.52	1.82	< 1.52	3.79	2.88	2.97
		TE	2.43	2.92	2.56	3.64	3.65	3.48
		SMX	3.99	4.52	3.22	5.20	4.85	4.43
#6	4/6/2016	None	4.70	5.09	4.17	5.65	5.39	5.39
		AM	3.22	3.43	3.11	4.41	4.41	4.03
		CiP	1.52	2.22	< 1.52	3.90	3.87	3.21
		TE	3.56	3.15	3.17	3.95	3.92	3.95
		SMX	2.90	3.52	2.43	4.95	4.64	4.01

3.3.1 Abundance of Antibiotic Resistant Bacteria in Heterotrophic Bacteria Populations

For the total concentrations of each subset of heterotrophic bacteria cultivations, the R2A agar with no antibiotic produced the highest overall concentration with a median value of 1.47×10^5 CFU/mL and a mean value of 1.68×10^5 CFU/mL (Figure 7). Sulfamethoxazole resistant bacteria were the next highest with a median concentration of 1.18×10^4 CFU/mL, followed by ampicillin and ciprofloxacin with median concentrations of 4.00×10^3 CFU/mL and 3.10×10^3 CFU/mL, respectively. Tetracycline resistant heterotrophic bacteria had the lowest overall concentration in the study area with a median concentration of 7.67×10^2 CFU/mL. Variance in the total population for each treatment was considerably large, with standard deviations larger than the mean values.

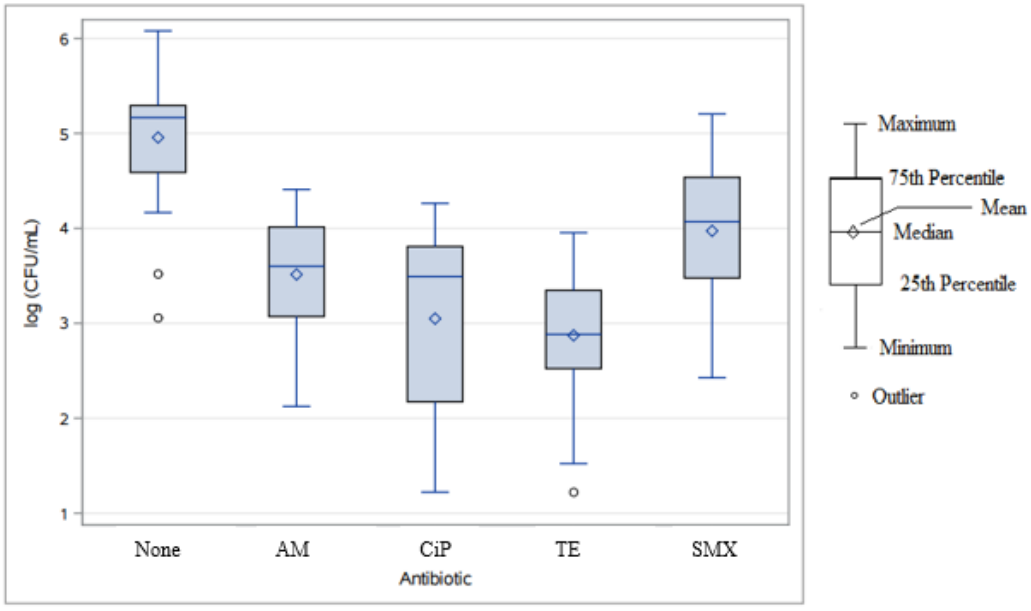

Figure 7: Box plot of log-transformed concentration distributions (log_{10} CFU/mL) of heterotrophic bacteria by antibiotic agent across all sampling events and sampling sites.

Concentrations of bacteria in the R2A treatment with no antibiotic were relatively consistent with the exception of a few outliers (Figure 8), in the case of sampling site 1 falling two orders of magnitude below the median value. Concentrations in the sample set varied significantly across sampling sites ($p < 0.025$) and between upstream (sites 1 -3) and downstream (sites 4 – 6) site groups relative to WWTP discharge ($p < 0.02$), but not by sampling event ($p > 0.15$). Median concentrations were near or above 1×10^5 CFUs/mL, except for site 3.

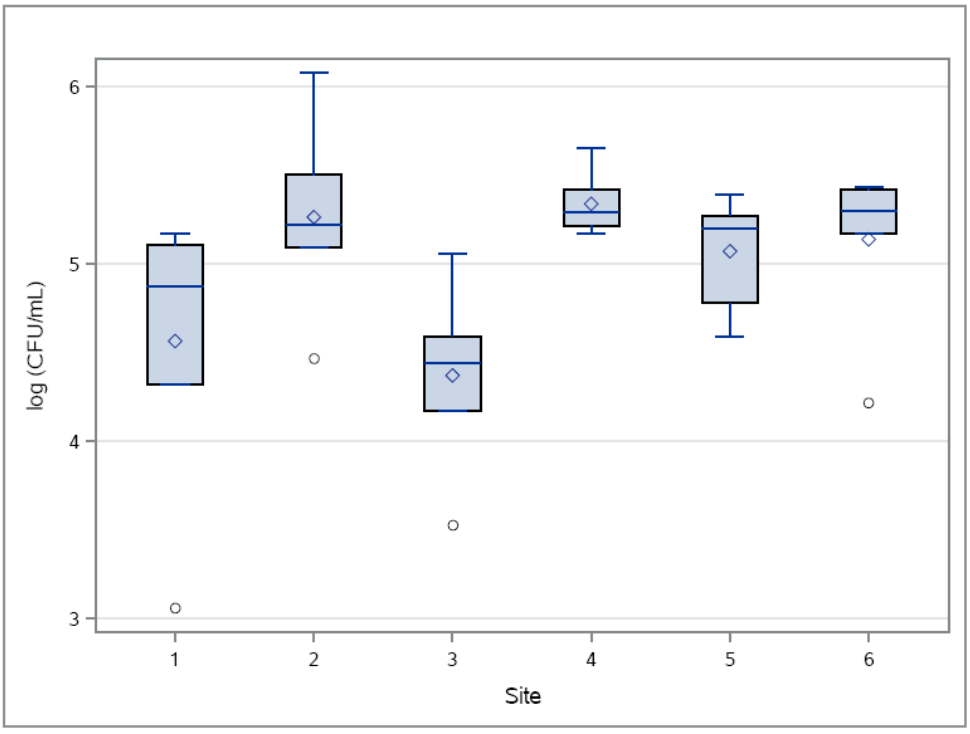

Figure 8: Box plot for total heterotrophic bacteria concentrations (log_{10} CFU/mL) in the control group (un-amended R2A media) across sampling sites for all sampling events

Abundance of ampicillin-resistant bacteria varied significantly between sampling sites ($p < 0.0001$), primarily due to the variance occurring between sites 1, 2, 3, and 6 and sites 4 and 5 (Figure 9). There was no temporal effect on the occurrence of ampicillin-resistant bacteria, and CFUs observed on R2A plates did not vary significantly by sampling event ($p > 0.65$). Ampicillin resistant bacteria were found in significantly greater ($p < 0.0001$) concentrations in the downstream group relative to WWTP discharge, with mean concentrations of 1.3×10^4 and 1.2×10^4 CFU/mL from sites 4 and 5, respectively.

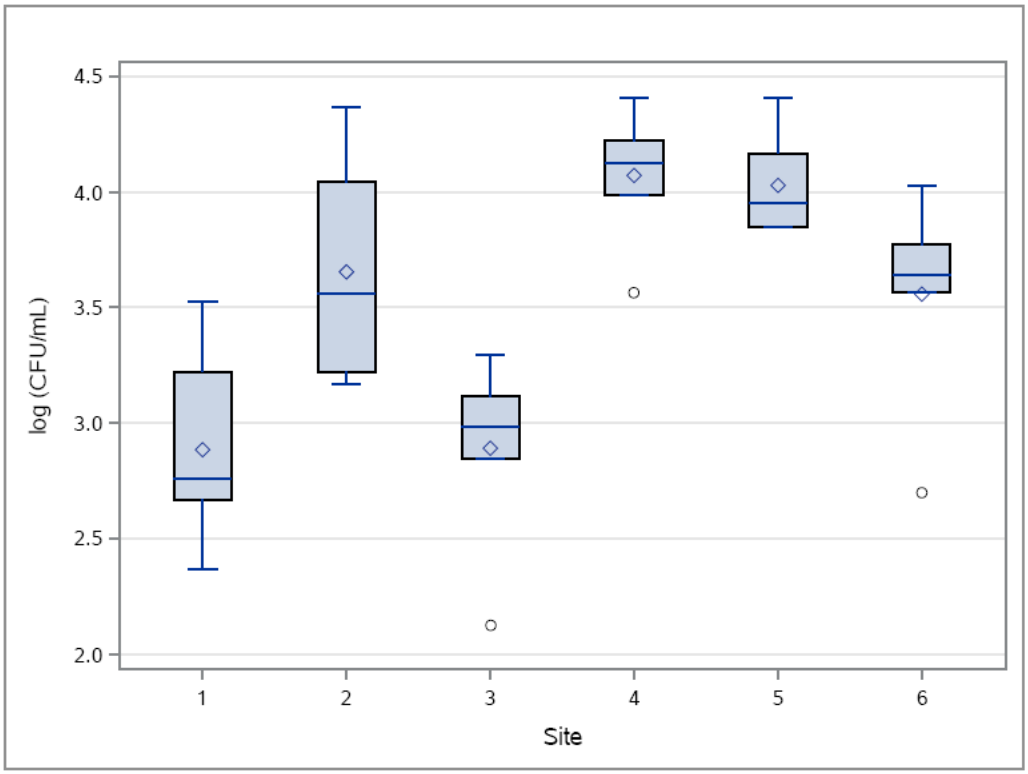

Figure 9: Concentrations (log_{10} CFU/mL) of ampicillin-resistant heterotrophic bacteria across sampling sites for all sampling events

Concentrations of ciprofloxacin resistant bacteria were more broadly distributed, with several sites having a range of over two orders of magnitude across all sampling events with mean concentrations ranging from 2.07×10^3 to 6.79×10^3 CFU/mL (Figure 10). Ciprofloxacin resistant bacteria were also considerably less abundant. Minimum concentration values for the data set fell below 10^2 CFU/mL for half of the sampling events, and 4 of the 5 results that were below the limit of detection came from ciprofloxacin-amended R2A agar. There was a significant variation ($p < 0.0001$) in the abundance of ciprofloxacin resistant bacteria between sampling events, but no significance differences in concentrations between the individual sampling sites ($p > 0.10$). However, despite generally low abundance and spatial variation between sites, a significant difference was also determined to exist between sites when sites were grouped by their position relative to WWTP discharge for ciprofloxacin resistant bacteria ($p < 0.01$).

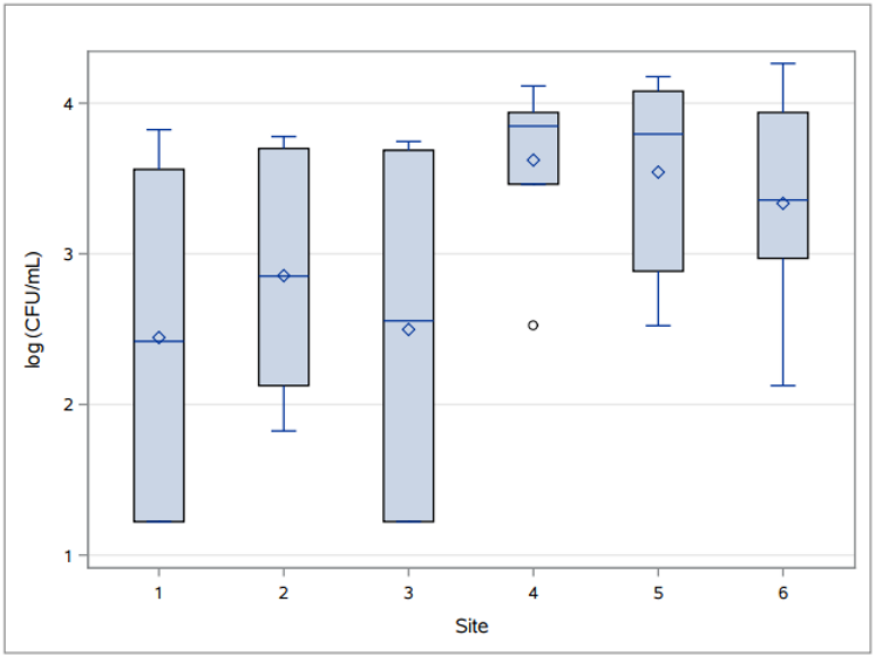

Figure 10: Concentrations (log_{10} CFU/mL) of ciprofloxacin-resistant heterotrophic bacteria across sampling sites for all sampling events

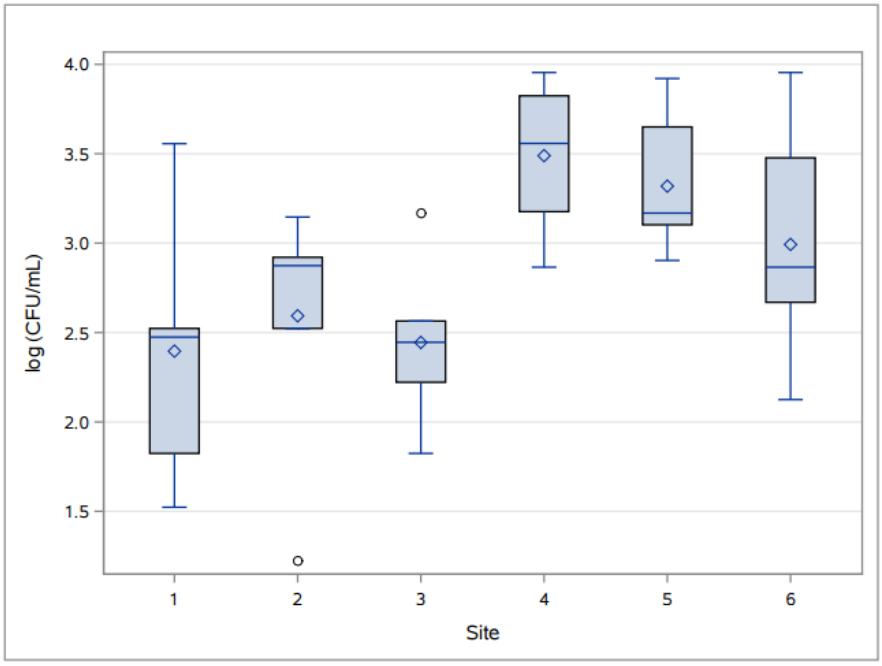

Figure 11: Concentrations (log_{10} CFU/mL) of tetracycline-resistant heterotrophic bacteria across sampling sites for all sampling events

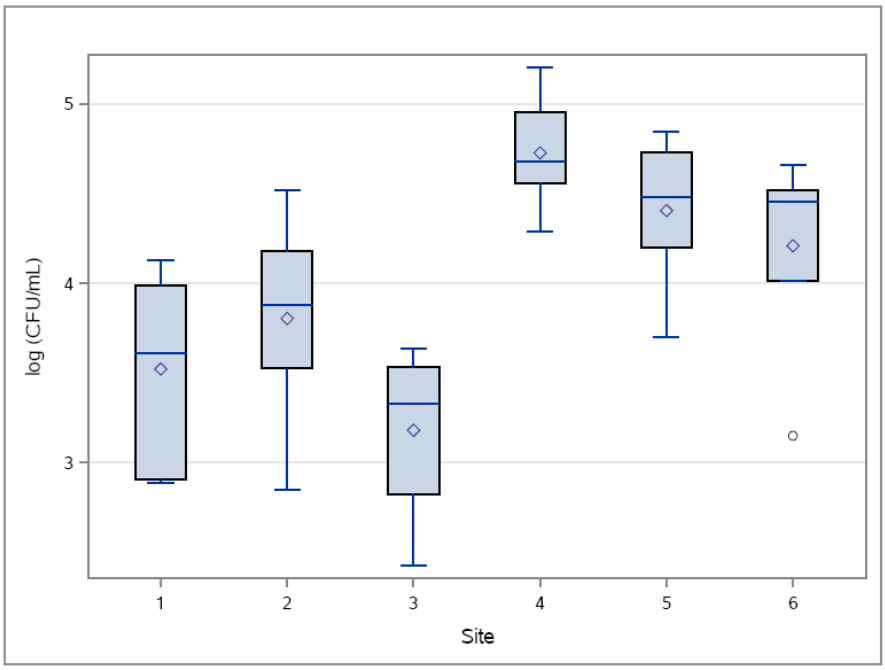

Figure 12: Concentrations (log_{10} CFU/mL) of sulfamethoxazole-resistant heterotrophic bacteria across sampling sites for all sampling events

Tetracycline resistance in heterotrophic bacteria produced a few outliers due to an atypically compact distribution of concentrations at site 2 (Figure 11), in contrast to an otherwise expansive distribution and large standard deviations as seen in the other treatments. Standard deviations of resistant bacteria concentrations in the tetracycline treatment at sites 2 and 3 were one order of magnitude or more lower than what was typically seen in other treatments. The abundance of tetracycline resistant bacteria varied significantly by sampling site ($p < 0.007$) and sampling event ($p < 0.02$), mainly due to considerably higher concentrations sampled during event 6.

Sulfamethoxazole resistant bacteria were the most prominent across all sampling sites in this study with the highest mean concentration of resistant bacteria at any sampling site of 6.67×10^4 CFU/mL (Figure 12). Sampling site had a significant

influence ($p < 0.0001$) on the concentration of resistant bacteria, mainly due to consistently higher concentrations found at sites downstream from WWTPs. Temporal variations by sampling event did not significantly affect results ($p > 0.20$).

The mean concentrations of resistant bacteria obtained upstream of a WWTP in the tetracycline and sulfamethoxazole treatments were an order of magnitude below the mean concentrations in their respective downstream sites. Significant differences in the abundance of ARB were found to exist between upstream and downstream sites for both the tetracycline ($p < 0.0001$) and sulfamethoxazole treatments ($p < 0.0001$).

3.3.2 Antibiotic Resistant Bacteria Normalized to Total Heterotrophic Population

Heterotrophic bacteria populations are diverse and possess a considerable amount of intrinsic variance in the way they occur and interact in the environment. By normalizing the abundance of antibiotic resistant heterotrophic bacteria in the study area to the total heterotrophic bacteria population, a better understanding can be formed concerning the extent of antibiotic resistance relative to total numbers. Figure 13 shows log-transformed ratios of resistant bacteria CFUs normalized to total HPCs with no antibiotic. One-way ANOVA and LSD tests were conducted to determine significance between normalized resistance rates and sampling site, sampling event, and relative position up or downstream of a WWTP.

In contrast to the results of the total abundance of heterotrophic ARBs, there were no significant ($p > 0.05$) spatial associations for the ratios of resistant bacteria normalized to total HPCs. Sampling site and relative location to a WWTP had no significant effect on the proportion of resistant bacteria in each treatment population.

While sampling event did have a significant effect on the normalized ratios of tetracycline and ciprofloxacin resistant bacteria within the total heterotrophic community ($p < 0.05$), no other significance existed for the other two antibiotics, or for any influence between sampling site or relative location to WWTP discharge.

While there were no significant differences within each treatment group, there were differences between the proportions of the different ARB within the total HPC population. Though no significant association existed between normalized sulfamethoxazole ratios by site, event, or relative location to a WWTP, sulfamethoxazole ratios in the total heterotrophic community were significantly higher than all of the resistant bacteria ratios for tetracycline, ampicillin, and ciprofloxacin ($p < 0.001$).

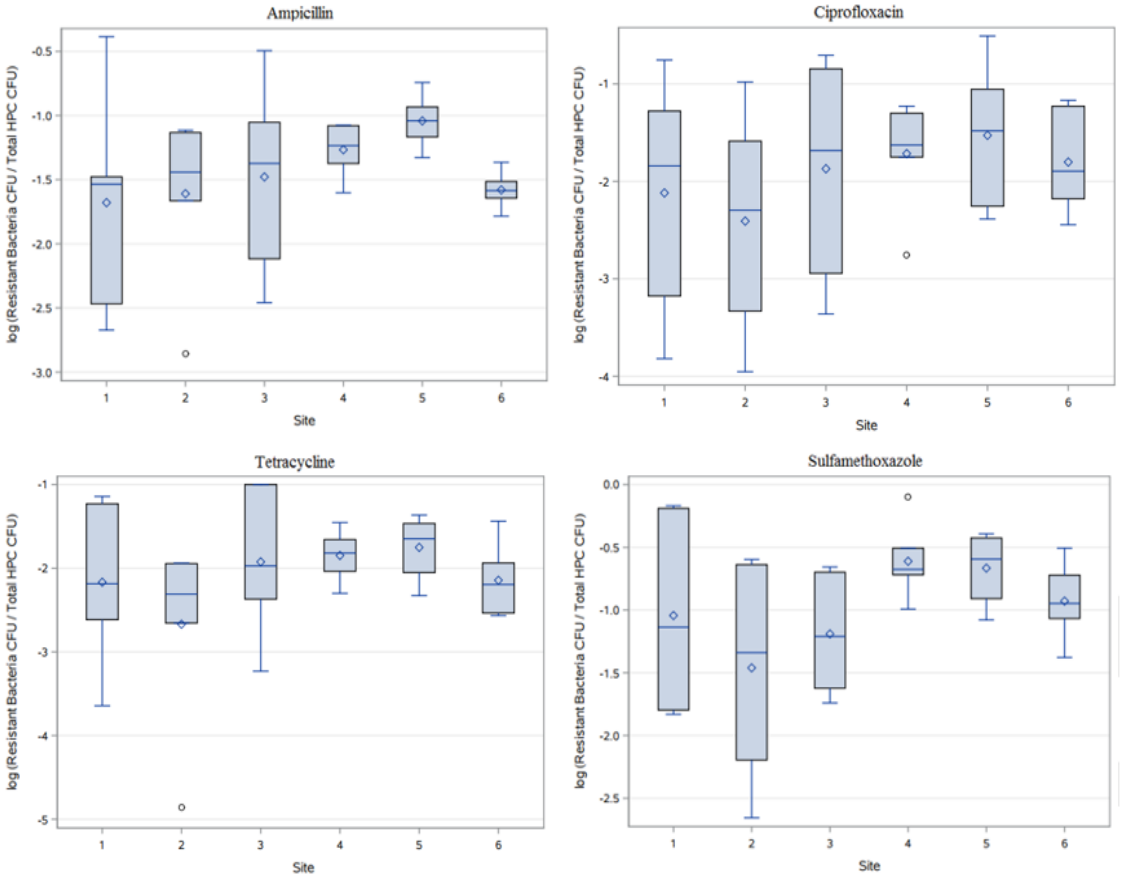

Figure 13: Normalized ratios of the concentrations of antibiotic resistance bacteria to the total heterotrophic population for four antibiotics across six sampling sites.

4. DISCUSSION

4.1 Antibiotic Resistance in *E. coli* Isolates

Ampicillin, sulfamethoxazole, and ciprofloxacin, or closely related drugs (amoxicillin), are among the top 5 antibiotics prescribed for use for adults in the U.S. (Shapiro *et al.*, 2013, Van Boeckel *et al.*, 2014, Hicks *et al.*, 2015), and all have been found to occur in WWTPs in varying concentrations and design conditions (Batt *et al.*, 2007). In the current study, a significant association ($p < 0.05$) was found to exist between the location of sampling sites relative to WWTPs (upstream group vs. downstream group) and isolates expressing resistance to ampicillin, ciprofloxacin, cefoperazone, sulfamethoxazole, and tetracycline. This lends support to the hypothesis that WWTP effluent may be contributing to the conveyance of antibiotic resistance bacteria downstream from discharge points. The absence of significant associations between rates of isolate resistance among upstream sites indicates that these differences are not solely dependent on variations between all sampling sites, but also their relative position to WWTP discharge points. Only one significant difference was found within the upstream group, between sites 2 and 3, on two separate streams.

The occurrence of antibiotic resistance may not always imply an outside effect, and can be an intrinsic property of the natural environment. In this study, cephalothin represented the highest rate (84%) of resistance found in all isolates. The high rate of resistance to cephalothin and the uniformity with which it is expressed across all sampling sites suggests that this resistance trait is naturally occurring in the watershed.

This is remarkably consistent with results attained in other studies performed with *E. coli* isolates obtained from surface waters in Michigan and Illinois, finding rates of isolate resistance to cephalothin at 80.6% and 80% (Sayah *et al.*, 2005, Janezic *et al.*, 2013). Cephalothin resistance also represented 95% of the 193 isolates resistant to only 1 antibiotic, dramatically inflating the abundance of isolates classified as resistant to at least 1 antibiotic. If cephalothin was excluded from the antibiogram for this study, an additional 184 isolates (66% of total) would be classified as susceptible to all agents.

The rate of isolate resistance to tetracycline (14% of all isolates) was found to be lower than expected when compared to similar research. Previous studies have found the occurrence of tetracycline resistance to be prevalent in watersheds associated with agricultural and animal feed lot operations (Jindal *et al.*, 2006, Rajić *et al.*, 2006), with resistance rates of over 90% found in *E. coli* isolates obtained from swine lagoon effluent (Brooks & McLaughlin, 2009). One study, also conducted in the Carter's Creek watershed in College Station, TX, found a substantial occurrence of tetracycline resistant bacteria and tetracycline resistant genes in sediment and surface water samples collected from the watershed; however, the majority were found in greater abundance bound in stream sediment samples than in surface water samples (Sullivan & Karthikeyan, 2012). In contradiction with the results of this study, Sullivan & Karthikeyan (2012) also found that while the occurrence of tetracycline resistance genes increased downstream of WWTPs, concentrations of tetracycline resistant bacteria were not significantly affected. However, this study used a different cultivation media and a different method for determining resistance rates, and investigated a more diverse microbial population. It is

possible that the effect of WWTP discharge on tetracycline resistance rates in *E. coli* isolates does not occur in the same manner for a more diverse population. Additionally, cultivation viability and antibiotic resistance fractions have been shown to be significantly influenced by media selection and conditions (Oh *et al.*, 2009).

Imipenem is a group 2 carbapenem generally reserved as a last line of defense against particularly resilient Gram-negative pathogens (Nicolau *et al.*, 2012). As a result the agent is prescribed sparingly and resistance to the agent would not be expected to develop in the microbial population sampled in this study. Accordingly, out of all the isolates collected from all sampling sites, all 280 were susceptible to imipenem. Imipenem resistance does not appear to be occurring in the surface water bacteria of the Carter's Creek watershed.

Overall, the significant increase in the rates of AB resistance to agents associated with frequent human use suggests that there is a human influence on the resistance rates of surface water bacteria moving downstream through B/CS. While more precise investigation into possible sources and sinks for ARBs and ARGs in the watershed is needed to confirm major causes, it seems likely that WWTP discharge may be contributing to some degree.

4.2 Multi-drug Resistance in *E. coli* Isolates

A substantial fraction (19%) of all 280 *E. coli* isolates expressed resistance to 2 or more antibiotics. Multi-drug resistance was found to increase significantly ($p < 0.05$) in the sites downstream of a WWTP for isolates resistant to ≥ 2, ≥ 3, and ≥ 4 agents. Other studies have observed high rates in the development of multi-drug resistance in *E.*

coli isolates in WWTP processes (Korzeniewska *et al.*, 2013, Amador *et al.*, 2015), found to be primarily driven by the transfer of conjugative plasmids (Silva *et al.*, 2006). A number of WWTP disinfection practices have been shown to have negligible effects on reducing rates of multi-drug resistant bacteria, and in a number of cases increasing it (Bouki *et al.*, 2013). Even if the effluent of the WWTPs in this study area does have considerably low levels of multi-drug resistant bacteria, these low concentrations have been shown in other studies to persist and propagate through the environment once discharged. ARGs not necessarily bound to culturable organisms are also likely escaping treatment processes and contributing to the development of multidrug resistance (Kümmerer, 2009). Resistance to cefoperazone, sulfamethoxazole, ciprofloxacin, and gentamycin was more frequently found in multi-drug resistant isolates, and rarely as the only type of resistance. This suggests that resistance to these agents is either driven by similar modes of defense coded by resistance genes to other agents, or that the acquisition of resistance to these agents usually occurs in tandem with other antibiotic resistances.

The high rates of resistance to cephalothin across all 6 sampling sites inflated multi-drug resistance rates, present in 95% of the 248 isolates resistant to at least 1 antibiotic. This increases the importance of the multi-drug resistance classifications of isolates resistant to 3 or more and 4 or more agents, due to cephalothin resistance effectively acting as a resistance baseline for this sample set. While the strictest definition for multi-drug resistance is resistant to one or more agents, ‘resistance to three or more classes’ has become increasingly standard for defining multi-drug resistance in

Gram-positive and Gram-negative bacteria (Magiorakos *et al.*, 2012). Still, a substantial number, 9% of all 280 isolates, expressed resistance to at least 3 antibiotics. This rate is more in line with other reports of the prevalence of multi-drug resistant *E. coli* in surface waters (Blaak *et al.*, 2015), though these rates likely differ considerably as a function of antibiotics tested and sampling site. A large majority (86%) of these isolates were collected downstream of a WWTP, and all significant increases in rates of isolate resistance to 3 or more agents occurred when comparing an upstream site to a downstream site. While some degree of multidrug resistance appears to exist naturally, the results suggest that WWTPs in the study area are contributing significantly to multi-drug resistant bacteria in the surface water.

4.3 Antibiotic Resistance in Total Heterotrophic Bacteria Populations

A significant increase in the concentrations of antibiotic resistant bacteria downstream of WWTP discharge was found for all four agents tested in the total heterotrophic bacterial community. Heterotrophic bacteria populations are diverse and possess a considerable amount of intrinsic variance in the way they occur and interact in the environment (Garcia-Armisen *et al.*, 2013). By normalizing the abundance of antibiotic resistant heterotrophic bacteria in the study area to the total heterotrophic bacteria population, a better understanding can be made concerning the extent of antibiotic resistance relative to total numbers. Unfortunately, this diversity also makes it difficult to establish a reliable standard for which to compare resistance rates against. Concentrations of ampicillin, ciprofloxacin, and tetracycline resistant bacteria were generally confined to a range of 1% to 10% of the total heterotrophic community when

compared to the control, though in some instances spiking to between 20% and 40% of the total population. However, these large spikes in the ratios of resistant bacteria to the control CFU were generally due to significantly lower counts in the control during a sampling event or at a sampling site and not because the relative CFU of resistant bacteria increased. In contrast, sulfamethoxazole resistant bacteria were frequently found to represent from 20% to 80% of the total heterotrophic population, ratios significantly ($p < 0.001$) higher than all other resistant bacteria. This same trend in sulfamethoxazole resistant bacteria was found to exist throughout numerous processes sampled in a municipal wastewater treatment plant (Gao *et al.*, 2012), also finding that while the total abundance of resistant bacteria were reduced in the effluent, that reduction was consistent with the reduction in total HPC populations. The similarities in heterotrophic sulfamethoxazole resistance observed in the downstream sites in this study may indicate contribution of resistance traits originating from WWTP effluent.

The occurrence of a significant increase in the concentrations of tetracycline resistant bacteria downstream of WWTPs in the total heterotrophic populations appears to contradict the findings of Sullivan & Karthikeyan (2012), research also conducted in the Carter's Creek watershed. Sullivan & Karthikeyan (2012) found no effect of WWTP location on the prevalence of tetracycline resistant bacteria in surface water, but did see an increase in the abundance of tetracycline ARGs. While molar concentrations of tetracycline used in both studies were similar, the discrepancy might be explained by differences in the cultivation media: Sullivan & Karthikeyan (2012) used nutrient-rich agar and this study used nutrient-limited R2A agar. Differences in cultivation media can

significantly affect the counts of culturable ARB even from identical samples (Garcia-Armisen *et al.*, 2013). Sullivan & Karthikeyan (2012) also found no seasonal variability in the occurrence of tetracycline resistant genes or bacteria. While there were significant variations in the abundance and normalized rates of tetracycline and ciprofloxacin resistant heterotrophic bacteria found in this study, the variations did not appear to be related to seasonal changes, and are likely due to natural occurrence of variance in the environment.

While there was a significant increase in the abundance of AB resistant bacteria in the downstream sites, there was no significant increase when the concentrations were normalized to total HPCs with no antibiotic in the cultivation media. This indicates that while the total amount of resistant bacteria is increasing downstream through the watershed, it is increasing proportionately with the total population. This could be explained in a number of ways. WWTP effluent may be introducing viable bacteria back into the watershed that has experienced no significant increase in its proportion of resistant bacteria, increasing total abundance without increasing normalized rates of resistance. Constituents of the effluent, residual suspended solids and dissolved nutrients, may also be facilitating increased growth rates of the pre-existing heterotrophic bacteria in the downstream surface water. With more favorable growth conditions, the total heterotrophic population, and the abundance of resistant bacteria, increases proportionately.

The characteristic diversity of heterotrophic bacteria and the natural variance in environmental resistance profiles perhaps contributes to overwhelming any

anthropogenic trends that might exist. Total heterotrophic population CFUs on control plates during antimicrobial studies can vary dramatically (3 orders of magnitude) (Pei *et al.*, 2006), making it difficult to normalize results of antibiotic bacteria within the population.

4.4 Temporal Considerations

While not a central focus of this study, variations in rates of individual antimicrobial agent resistance and multi-drug resistance also occurred due to temporal variations between sampling events. Several significant associations ($p < 0.05$) existed between the abundance and normalized rates of HPC resistance. Chi-square tests on individual sampling events indicated one sampling event (event 2) that had a significant correlation ($p < 0.0005$) with the rates of multi-drug resistant *E. coli* isolates. Ideally, all of these tests would not have a significant association with the data set, indicating that the overall trend in multi-drug resistance is not significantly affected by only one single sampling event.

These variations can be mitigated through more stringent control of environmental conditions or, more realistically, by increasing the isolate sample size so that the impact of one outlying data set is reduced. Increased rates of antibiotic resistance might be triggered by sewage line leaks or other independent events that introduce an additional source of bacteria into the water, though no significant irregularities in bacterial plate counts were observed in this study. It is also possible that a relationship exists between the prevalence of isolate antibiotic resistance and temporal variance related to changes in season, stream flow, local population, or WWTP operations. More

research tied to these variables would need to be done to determine the impact of temporal and seasonal variations on the resistance profiles of surface water bacteria.

4.5 Significance of Cultivation-Based Approach

The scope of this project was limited to only cultivable bacteria, and therefore does not completely represent the entire microbial community as viable-but-not-culturable (VBNC) bacteria have not been considered. Cultivable populations may only represent a small portion of the entire microbial community (Smit *et al.*, 2001), and it has been shown that variability in the microbial community in response to the presence of various antibiotics can be exaggerated when evaluated by cultivation-based methods (Garcia-Armisen *et al.*, 2013). Further, cultivation viability and antibiotic resistance fractions have been shown to be influenced by media selection and conditions, and a fully standardized method may still need to be developed for consistent results between studies (Oh *et al.*, 2009). While many studies are now being conducted to evaluate ARGs by molecular methods, developing cultivation-based methods for the analysis of antimicrobial resistance in the environment is critically important. Cultivation-based methods demonstrate the phenotypic expression of the ARGs present, and provide the benefit of showing actual outcome over potential outcomes. They may also be preferred in cases where the resources available to an agency justifies the use of cultivation-based methods.

4.6 Mitigation and Prevention

While an understanding of the role that WWTPs play in the conveyance of antibiotic resistance through the environment is important for developing effective

management practices, domestic mitigation of antibiotic resistance is unlikely to be the key limiting factor in the proliferation of ARB. The overuse and application of antibiotics in industrializing nations with relaxed or non-existent regulation may ultimately prove to have a much more significant impact concerning the development of antibiotic resistance on a global scale (Istúriz & Carbon, 2000). Most serious conversations about controlling the spread of AB resistance center around limiting use and proper clinical procedures to prevent multi-drug resistant bacteria from ever developing in the first place (Stephan & Matthew, 2005). International efforts are being made to identify critical priorities for stable control of AB resistance, and to promote urgent mitigation actions including public education, improvements in sanitation and public health, limitations on use, old and new antibiotics, and alternative non-antibiotic disease prevention measures (Bush *et al.*, 2011).

5. CONCLUSIONS

The goal of this study was to investigate the relationship between human activity and urbanization on the occurrence of antibiotic resistance in the surrounding watershed using cultivation-based methods. The results suggest that the effects of human activity and development, specifically by the introduction of treated WWTP effluent into local surface waters, are potentially significant contributors to the spread and persistence of antibiotic resistance in nearby surface water environments.

The relative location of sampling points up or downstream of a WWTP discharge point had a significant effect on the concentration of antibiotic resistant *E. coli* isolates that were observed from that site. Downstream isolates showed an increased resistance to ampicillin, ciprofloxacin, cefoperazone, sulfamethoxazole, and tetracycline, and were more often resistant to a higher quantity of different antibiotics. These effects were mirrored in the total heterotrophic bacteria community, with a significant increase in the abundance of ampicillin, ciprofloxacin, sulfamethoxazole, and tetracycline resistant bacteria in the surface water downstream of WWTP discharge points. However, when normalized to total HPCs, this significance did not persist; the inherent diversity of heterotrophic communities may encourage antibiotic resistance profiles that are more resilient to variations in the environment and overshadow some anthropogenic influences. Particulate and nutrient constituents of the WWTP effluent may also be facilitating enhanced growth conditions for heterotrophic populations preexisting in the

environment, producing proportional increases in abundance of both resistant and total bacteria.

More research needs to be conducted concerning the transport and storage of microbial antibiotic resistance in the environment. Resistance profiles may vary considerably between surface water and sediment-bound bacteria. Further understanding of the interrelationships between ARB concentrations, ARG concentrations, antibiotic agents, microbial species, and environmental media will help to eventually enable the modeling of antibiotic resistance transfer through the environment. Standardization of techniques to evaluate antibiotic resistance in the environment may be beneficial in elucidating these mechanisms, as the extent of resistance has been shown to vary dramatically in relation to cultivation media and sampling site selection even within the same watershed.

The results do not conclusively identify WWTP discharge as the cause of the increased rates of resistance; there are many factors that could be contributing to the trends seen in the data. A more specific investigation and more constrained system focusing on the inflows, outflows, and process components at the WWTPs would be beneficial in further determining the extent of its contribution to resistance in the environment. The larger scale watershed approach is still important, as measurements at the outflow may be artificially lowered by downstream reactivation of bacteria initially neutralized during UV treatment. A broader picture can be drawn of occurrence of resistance in the environment by looking at resistance trends across the watershed, informing further investigation into more precise contributors.

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