BETA-LACTAM & CARBAPENEM RESISTANCE IN ENTEROBACTERIACEAE ISOLATED FROM FRESHWATER STREAMS IN A MIXED-USE WATERSHED

by

MARTINIQUE LEFEVRE EDWARDS

(Under the Direction of Erin K. Lipp)

ABSTRACT

Stream and river surface waters that span human-developed areas receive waste products, e.g., pharmaceuticals and fecal matter, from degraded sewer pipelines, water reclamation facility (WRF) effluent, improperly maintained septic systems, and impervious surface run-off. Recently, surface waters have received attention as potential reservoirs of antimicrobial resistance. In this study, we investigate the prevalence and diversity of Enterobacteriaceae resistant to beta-lactam and carbapenem antibiotics. On four dates spanning one year (2018), we collected surface water samples from 54 stream/river sites, and on three of the four dates, influent and effluent from 3 water reclamation facilities (WRFs). We detected extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in 22.5% (38/169) of surface water, 66.69% (6/9) influent, and 11.11% (1/9) effluent samples. Additionally, we detected carbapenem-resistant Enterobacteriaceae (CRE) in 13.0% (22/169) of surface water and 33.3% (3/9) effluent samples. This study yields preliminary evidence that warrants further investigation into the function of surface waters as reservoirs, or conduits, of antimicrobial resistance.

INDEX WORDS: *Escherichia coli*, antibiotic resistance, watershed, Enterobacteriaceae, beta-lactam, carbapenem, fecal contamination, surface water

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CHAPTER I

LITERATURE REVIEW

Antibiotics and antibiotic resistance (AR)

Antibiotics are chemicals produced by bacteria and fungi that are used to outcompete their bacterial neighbors for limited resources and to act as intercommunicative signaling molecules. Similarly, antibiotic resistance (AR) involves mechanisms used by bacteria to protect against these compounds as well as other environmental stressors, such as exposure to toxic chemicals. Recently, humankind has been able to purify and produce antibiotics en masse, resulting in their widespread dissemination for therapeutic use to treat multiple bacterial infections, including periodontitis, skin and soft tissue infections, and urinary tract infections (Feres et al. 2015; Pallin et al. 2015; Shepherd and Pottinger 2013). In response, bacteria worldwide are rapidly acquiring and expressing mechanisms to resist anthropogenic stressors, including antibiotics, heavy metals, and household and personal care products (Kennemann et al. 2011; Croucher et al. 2011; Graves et al. 2015; Tan et al. 2002; Dhillon et al. 2015). Over the past century, we have seen an alarming increase in the number of clinical infections resistant to antibiotics, with a growing number of those infections resistant to multiple antibiotics. This review will discuss the history and evolution of antibiotics as therapeutic treatment, the biological development of resistance mechanisms by bacteria, and ecology of AR in the environment. Interspersed throughout this review, a few key “superbugs” will take the spotlight as important and emerging threats to antibiotic treatment for infections.

Antibiotics

For much of human history, we have turned to plants, heavy metals, and bloodletting for medicinal prevention and treatment of disease (Cai et al. 2004; Benedek 2004; Parapia 2008). Not until recently, with the discovery of penicillin, have we looked to other biota for such remedies. Penicillin was extracted and purified from a *Penicillium* mold by Alexander Fleming in 1928 (Tan and Tatsumara 2015; Fleming 1929). Subsequently, researchers began to explore bacteria and fungi as wellsprings of antibiotic substances. In the early 1940s, Selman Waksman coined the term 'antibiotic' to describe a small molecule produced by microbes that disrupt the growth of other microbes (Clardy et al. 2010; Waksman 1943). He was fascinated in particular by the soil bacteria actinomycetes, which had the capability to produce several compounds that induced antibiosis of neighboring microbes (Ginsberg 2005; Waksman et al. 1953). In 1939, René Dubos, a former student of Waksman, isolated tyrothricin (an antibiotic mixture) from *Baccilus brevis* and was the first person to show that bacteria can secrete antibiotics in the presence of other bacteria (Ginsberg 2005; Dubos 1939). Years later, students H. Boyd Woodruff and Albert Schatz isolated streptothricin and streptomycin from actinobacteria (Waksman and Woodruff 1942; Schatz and Waksman 1944). Streptothricin was found to have delayed toxic effects on animal models and did not pass clinical trials; however, streptomycin passed effectively and is still used today to treat infectious diseases such as tuberculosis, mycobacterium avium complex, endocarditis, tularemia, and plague (Krause et al. 2016; Griffith et al. 2007; Elliott et al. 2004; Snowden and Stovall 2011; Galimand et al. 2006).

Since antibiotic-producing bacteria, such as actinomycetes, are commonly found in soil, it is thought that selective pressures in the environment, e.g., limited organic matter, water, space, or other resources, have driven bacteria and fungi to evolve antibiotic production to

compete with neighboring bacteria and fungi for nutrients and space (Waksman and Selman 1940). However, in addition to having inhibitory effects, antibiotics at low concentrations have been shown to serve dual roles as signaling molecules, for modulation of bacterial transcription, quorum-sensing, and quorum-quenching (Goh et al. 2002; Fajardo and Martinez 2008). For example, low concentrations of erythromycin and rifampicin were shown to stimulate or inhibit promoter-*lux* reporter constructs in a library of *Salmonella* Typhimurium (Goh et al. 2002). As another example, biosynthetic lantibiotic peptides, such as nisin produced by *Lactococcus lactis,* exhibit both antimicrobial and phermonone functions that are important in the regulation of quorum sensing of their biosynthesis (Kleerebezem 2004; Kleerebezem et al. 1997; Kuipers et al. 1995).

Currently, there are several ways that antibiotics can be classified, but the most common classification schemes are based on molecular structures, mechanisms of action, and spectra of activity on a bacterial cell (Etebu and Arikekpar 2016; Calderon and Sabundayo 2007). Common classifications of antibiotics based on chemical or molecular structure are as follows: beta-lactams, macrolides, tetracyclines, quinolones, aminoglycosides, sulphonamides, glycopeptides, and oxazolidinones (Etebu and Arikekpar 2016) (Table 1.1).

Dose-dependent effects of antibiotics. Antibiotics at different concentrations have varying effects on the receiving bacteria. At low concentrations, antibiotics can modulate transcription, at increased but sub-inhibitory concentrations, antibiotics can elicit a bacterial response to DNA damage (SOS response), and at high inhibitory concentrations, antibiotics can induce cell growth inhibition and death (Fajardo and Martinez 2008; Martinez 2008). However, the response an organism has to subinhibitory concentrations may depend on the bacterial species and type of antibiotic. For example, subinhibitory concentrations of tetracycline,

chloramphenicol, and aminoglycosides induce the SOS response in *Vibrio cholerae* but not in *E. coli*; whereas trimethoprim and fluoroquinolones induce the SOS response in both species (Baharoglu and Mazel 2011; Bernier and Surette 2013). Although many bacteria are intrinsically resistant antibiotics, the widespread dispersal of pharmaceutical antibiotics has placed unprecedented levels of selective pressures on bacteria associated with human, veterinary, and livestock hosts, causing acquired antibiotic resistance in bacteria to increase (Allen et al. 2010) (Figure 1.1).

Minimum Inhibitory Concentration and Breakpoints. The inhibitory concentration of any one antibiotic/drug varies from one strain of bacteria to another, dependent on the bacterium's reserve of enzymes, defensive proteins encoded in its DNA, location of infection, and concentration of pathogen (Rhodes et al. 2015; Munita and Arias 2016). Minimum inhibitory concentration (MIC) is defined as, quoted by JM Andrews (2001), "the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation". Laboratories employ MIC breakpoints when diagnosing clinical bacterial infections as susceptible or resistant to antibiotics as well as when developing new antimicrobial drugs (Andrews 2001). Susceptibility testing breakpoints, interpretive categories, and QC parameters guidelines are stored and updated on the Clinical and Laboratory Standards Institute (CLSI) website (https://clsi.org/) to assist clinicians in choosing appropriate antimicrobial therapy for patients.

Antibiotic Resistance

Intrinsic AR: Instrinsic resistance in bacteria is natural insensitivity in bacteria to antibiotics, a resistance that has been a part of the bacterial genome over evolutionary scales,

rather than from recent acquisition. ARG can therefore be seen across species, clades, and genera, rather than at the strain level. Intrinsic resistance mechanisms include reduction of permeability of cell wall to antibiotics (such as in *Pseudomonas aeruginosa*), alteration to the cell's active site so that an antibiotic cannot bind (such as in *Staphylococcus aureus*), or production of an enzyme that inhibits binding of antibiotic to the active site (such as in *Enterobacter* spp., *Citrobacter freundii*, and *Serratia marcescens*) (Hawkey 1998; Angus et al. 1982; Leski and Tomasz 2005; Ruppe et al. 2015). Many microbes produce antibiotics as secondary metabolites and use resistance mechanisms to protect themselves (Cundliffe and Demain 2010).

Acquired AR: Bacteria also quickly acquire resistance by undergoing gene mutation or by acquiring genes encoding enzymes that inhibit antibiotics' effects on the bacterial cell. Regarding gene mutation, there can be independent (single gene) and cooperative (multiple genes) mutations that lead to antibiotic resistance (Martinez and Baquero 2000). For example, in *E. coli,* an independent mutation to either the *gyr*A or *gyr*B gene, which encode for DNA gyrase, can lead to phenotypic resistance to quinolones such as nalidixic acid (Yoshida et al. 1990; Pourahmad Jaktaji and Mohiti 2010). Comparatively, multiple mutations spanning multiple genes may be required to acquire high levels of a resistance to a drug (Suzuki et al. 2014; Toprak et al. 2011). For example, in addition to mutations to *gyr*A or *gyr*B genes, an organism can have mutations to *par*C or *par*E, which encode a subunit of the quinolone target topoisomerase IV, making an organism resistant to both nalidixic acid and later-generation quinolones such as ciprofloxacin (Johnning et al. 2015; Saenz et al. 2003; Vila et al. 1995). Regarding acquisition of antibiotic resistance genes (ARG), this process can occur via gene mutation or transfer of genes during a bacterial cell's lifetime. ARG can be transferred to other bacteria through the process of

horizontal gene transfer (HGT) and can be located on plasmids, conjugative resistance transposons, and integron elements, all of which serve as platforms for the transfer of genes from one genomic location, or cell, to another (Bennett 2009). These mobile genetic elements make up two types: elements that move from one bacterial cell to another (including plasmids and conjugative resistance transposons) and elements that move from one genetic location to another within a single cell (including resistance transposons, gene cassettes, and ISCR [insertion sequence common region] elements) (Bennett 2009; McMillan et al. 2019; Toleman et al. 2006). In some regions of the world, the abundance of ARG has increased over the past several years, in part due to land use and antibiotic use in agriculture (Knapp et al. 2010). Similarly, ARG have widely disseminated between several regions of world, in part due to globalization/international travel (Bengtsson-Palme et al. 2015). Many mechanisms, including gene mutation and HGT, are responsible for such resistance. However, use of pharmaceutical antibiotics in agriculture and clinics or international travel may be assisting the bacterial acquisition of AR, as bacteria have been shown to acquire resistance even at low concentrations of antibiotics (Munita and Arias 2016; Gullberg et al. 2011; Gullberg et al. 2014).

Triggers of ARG transfer. There are several known chemical compounds that, when present at sub-inhibitory concentrations, promote the conjugative transfer of ARGs between bacterial cells. Such compounds include heavy metals, e.g., Copper(II), Silver(I), Chromium(VI), and Zinc(II), disinfectants, and by-products of disinfectants (Zhang et al. 2018; Zhang et al. 2016; Guo et al. 2015; Li et al. 2016). In a study by Zhang et al. (2018), *E. coli* cells were exposed to sub-inhibitory levels of heavy metals for two hours; these toxic chemicals induced the cells to undergo SOS stress in response to reactive oxygen species generated by the metals. This exposure led to significant upregulation of oxidative stress genes and SOS genes, increased cell

permeability, down-regulated cell regulation genes, and consequent activation of conjugation bridges to counteract free radical stress (Zhang et al. 2018). Additionally, biological and physical substrates, e.g., soil fungal networks or microplastics, can serve as environmental platforms on which bacteria colonize, form biofilms, and undergo conjugative transfer of ARGs, assisting the propagation of AR in a stressful environment (Nazir et al. 2017; Arias-Andres et al. 2018). This implies that ARG HGT can be enhanced by constituents of the environment, including those deemed as physical vectors or non-antibiotic anthropogenic waste present at sub-inhibitory concentrations.

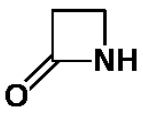
Top-of-the-list Superbugs

'Superbugs' describes bacterial organisms resistant to multiple antibiotic drugs. A CDC report published in 2013 described the top antibiotic resistant threats in the United States by partitioning the superbugs into three categories: Urgent, Serious, and Concerning (CDC AR Threats Report, 2013). Among eighteen superbugs, two chief bacteria that made the list are Carbapenem Resistant Enterobacteriaceae (CRE) (Urgent Threat) and Extended-spectrum Beta-Lactamase (ESBL)-producing Enterobacteriaceae (ESBL-E) (Serious Threat). Both groups of bacteria are commonly isolated in drug-resistant infections in healthcare settings (Lewis et al. 2007; Hilty et al. 2012; Schechner et al. 2011). Both groups also fall under the wider umbrella of organisms resistant to beta-lactam antibiotics.

Beta-lactams and beta-lactam resistance

Beta-lactams. Beta-lactam antibiotics comprise a large class of antibiotics that target infections associated with both Gram-positive and Gram-negative bacteria (CDC AR Threats Report, 2013; Williamson et al. 1986). Subset into five main classes, the beta-lactam antibiotics

include the 1) penicillin-derivatives, 2) cephalosporins, 3) carbapenems, 4) monobactams, and 5) carbacephems; each of these antibiotic classes are characterized by their similar chemical structure including a four-membered azetidinone ring (pictured below) (Bush and Bradford 2019).



2-Azetidinone, the most basic chemical structure of beta-lactam (photo courtesy of Wikipedia).

The cell wall of bacteria consists of peptidoglycan (PG), a polymer of cross-linked peptide and glycan chains, which contributes to cell shape and osmotic stability. It is especially thick in Gram-positive organisms. Beta-lactam antibiotics possess a mechanism to disrupt biosynthesis of the peptidoglycan cell wall by inactivating transpeptidase domains of penicillin binding proteins (PBPs), enzymes that mediate the terminal stages of cell wall synthesis (Cho et al. 2014). In all bacteria, PBPs work throughout a cell's lifetime to modulate localized, reparative mending of the PG layer when environmental stress is imposed on it and to assist in cell wall synthesis during cell division into two daughter cells (Spratt 1975). These processes are vulnerable stages in a bacterium's cell cycle and therefore are targeted by beta-lactam antibiotics (Ealand et al. 2018).

PBPs can be divided into high molecular weight (HMW) and low molecular weight (LMW) categories (Ealand et al. 2018). Beta-lactam antibiotics primarily target HMW transpeptidases (a type of PBP) to inhibit cell wall formation and repair. The function of HMW PBPs is to cross-link glycan chains via a transglycolysase domain and cross-link peptide bridges via a transpeptidase domain to form the PG layer (Ealand et al. 2018; Sauvage et al. 2008; Scheffers and Pinho 2005). If transpeptidase PBPs are disrupted, as by beta-lactam antibiotics, then the bacterial cell wall cannot repair itself, and the cell either cannot propagate or dies.

The beta-lactams, as stated above, can be divided into five classifications: the penicillin-derivatives, the cephalosporins, the monobactams, the carbapenems, and the carbacephems (Table 1.2). Penicillins are used to treat Gram-positive bacteria and some Gram-negative bacteria. The early-generation cephalosporins are generally most active against Gram-positive organisms and the later generations generally have broader spectrum activity against aerobic Gram-negative bacilli. Monobactams (Aztreonam is the only one commercially available) are administered for severe infections with aerobic Gram-negative bacilli. Carbapenems have the broadest spectra of activity of all beta-lactams against Gram-positive and Gram-negative bacteria (see section on Carbapenems on page 11) (Werth 2018, www.merckmanuals.com). Carbacephems are similar to cephalosporins but have greater chemical stability (Copper 1992).

Beta-lactamases. Beta-lactamase enzymes are capable of hydrolyzing beta-lactam antibiotics and are thought to be evolutionarily related to PBPs due to their shared similarities in chemical structure (Ozturk et al. 2015). Beta-lactamases have four molecular classes: A, B, C, and D, according to the Ambler classification method (Ambler 1980). Classes A, C, and D contain serine at the active sites of the enzyme (serine beta-lactamases). Class B beta-lactamases are comprised of at least one, but usually two, zinc ions (Zn^{2+}) that serve as metal cofactors at the histidine and cysteine active sites, hence the name metallo-β-lactamases (Massova and Mobashery1998; Palzkill 2013; Bush and Bradford 2019; Bebrone et al. 2009).

Believed to be 2 billion years old, serine beta-lactamases (Class A, C, D) are ancient enzymes; however, recent clinical advances in synthesis of antibiotics have placed greater pressures on bacterial selection for these enzymes, causing them to undergo a rapid evolution during the antibiotic era (in particular, Classes A and D) (Hall and Barlow 2004; Canton et al

2012; Bush and Jacoby 2010). Historically, Class A serine β-lactamases have been classified as those enzymes that favorably bind to penicillins as substrates, Class C favorably binds to cephalosporins, and Class D to oxacillin and other compounds structurally similar to penicillin (Ganta et al. 2009). Many Gram-negative bacteria intrinsically possess a chromosomally-mediated beta-lactamase, most likely due to stress caused by competition with beta-lactam-producing soil organisms in the environment (Bradford 2001; Ghuysen 1991). Class A TEM and SHV enzymes are believed to have appeared ~300-400 million years ago. These genes were encoded in the chromosomal DNA of Gram-negative bacteria (Hall and Barlow 2004). CTX-M enzyme, also Class A, was first isolated in 1986, but according to phylogenetic tests and speculation based on amino acid substitution rates, it is believed to have emerged 300-400 million years ago, with little divergence (7 clusters) since then (Hall and Barlow 2004; Bonnet 2004). Relatively recently (in historical terms), these beta-lactamase (*bla*) genes appeared on mobile genetic elements, like plasmids, that can be horizontally transferred from one bacterial cell to another (Hall and Barlow 2004; Bradford 2001). Over the past century, these *plasmid-mediated* beta-lactamases (TEM-1, the SHV-1, and the CTX-M) have grown rapidly in prevalence (Hall and Barlow 2004).

Extended-spectrum beta-lactamases. ESBLs are Class A plasmid-mediated beta-lactamases that hydrolyze most beta-lactam antibiotics, including penicillins and 1st, 2nd, and 3rd generation cephalosporins and aztreonam. Some of the most clinically problematic AR infections are those caused by extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E). ESBL-E are common pathogens responsible for intra-abdominal infections, urinary tract infections, and hospital-acquired pneumonia (Coque et al. 2008; Boontham and Soontornrak

2015). They do not digest cephamycins or carbapenems and are inhibited by clavulanic acid and other inhibitors (Pana and Zaoutis 2018; Paterson and Bonomo 2005; Dhillon and Clark 2012; Ghafourian et al. 2015). There are greater than 300 subtypes of ESBLs, with some of the most common encoded by alleles of *bla* genes CTX, TEM, OXA, and SHV (Haller et al. 2018). The CTX-M, TEM, and SHV serine beta-lactamases are a few of the most rapidly spreading beta-lactamases and are of utmost concern as we look toward treatment of drug resistant bacterial infections.

Beta-lactamase inhibitors. Growing concern over beta-lactamase-producing infections drove clinical researchers to search for beta-lactamase inhibitors (Brown 1986; Papp-Wallace et al. 2011). Decades later, beta-lactamase inhibitors are now coupled with beta-lactam antibiotics in order to assist the antibiotics in reaching the PBP targets of bacterial cells (Drawz and Bonomo 2010; Watkins et al. 2013). For example, clavulanate, sulbactam, and tazobactam are at times coupled with beta-lactam antibiotics amoxicillin, ampicillin, piperacillin, and ticarcillin to treat dire Enterobacteriaceae infections (Drawz and Bonomo 2010; Jeong et al. 2009).

Clavulanic acid was first isolated from the Gram-positive actinomycete bacterium *Streptomyces clavuligeres* in 1976 and resembles the 'nucleus' of penicillin (a 6-amino penicillanic acid that acts as the core of the molecule). However, clavulanic acid lacks an acyl-amino side chain, possesses oxygen instead of sulfur, and has a beta-hydroxy ethylidine substituent in the oxazolidine ring (Reading and Cole 1977; Papp-Wallace et al. 2011). Clavulanic acid serves as a suicide inhibitor of beta-lactamases.

Piperacillin/tazobactam is a beta-lactam/beta-lactamase inhibitor combination with a broad spectrum of activity against Gram-positive and Gram-negative aerobic and anaerobic bacteria, including those carrying beta-lactamases. Tazobactam can inhibit plasmid-mediated

beta-lactamases, staphylococcal penicillinase, and extended-spectrum Gram-positive beta-lactamases (Perry and Markham 1999). This drug combination, either alone or combined with amikacin (an aminoglycoside), could possibly be substituted for carbapenems in order to implement a "carbapenem-saving strategy" and reduce the rate of carbapenem resistance without increasing mortality (Ko et al. 2019; Pilmis et al. 2017).

Carbapenems and carbapenemases

Also borne out of the search for beta-lactamase inhibitors was the first discovered carbapenem, thienamycin, that was extracted from *Streptomyces cattleya* on tomato paste-oatmeal agar in 1976 and, although unstable in aqueous solution, subsequently served as prototype to successive generations of carbapenems (Kahan et al. 1979; Papp-Wallace et al. 2011). Something unique to thienamycin that no other beta-lactam had prior to its discovery is its hydroxyethyl sidechain on the 4:5 fused ring. By contrast, the penicillins and cephalosporins have an acyl-amino substitute on the beta-lactam ring. As an additional difference, thienamycin possesses a *trans* configuration of this side chain as compared to the *cis* configuration of all other beta-lactams preceding it (Birnbaum et al. 1985).

Like all beta lactam antibiotics, carbapenems operate by interrupting the synthesis of the peptidoglycan cell wall via binding to PBPs. Four synthetic/semi-synthetic carbapenems are clinically approved for use in the United States: imipenem, meropenem, ertapenem, and doripenem (Doi and Chambers 2015). Carbapenems are effective in the presence of most Ambler class A, C, and D beta-lactamases and extended spectrum beta-lactamases produced by Gram-positive and Gram-negative aerobic and anaerobic bacteria, although they lack activity against *Enterococcus faecium*, methicillin-resistant *Staphyloccocus aureus* (MRSA), and

Stenotrophomonas maltophilia (Zhanel et al. 2007). As a result, they are regarded as the current treatment of choice for infections with ESBL-E (Papp-Wallace et al. 2011; Fritsche et al. 2005).

Imipenem is an amidine derivative of thienamycin and 5-10 times more stable (Rodloff and Torres 2006). It is distinguished from other beta-lactam antibiotics by broad-spectrum activity against aerobic and anaerobic Gram-positive bacteria and a selection of Gram-negative bacteria, including Enterobacteriaceae, *Pseudomonas aeruginosa*, and Bacteroides (Birnbaum et al. 1985). When prescribed, imipenem is combined with cilastatin, which protects imipenem from degradation by the kidney enzyme dehydropeptidase-1 (DHP-1) (Rodloff and Torres 2006). Carbapenems developed more recently (meropenem, ertapenem, doripenem) do not require the DHP-1 inhibitor cilastatin (Zhanel et al. 2007).

In 1996, meropenem entered the U.S. market as the second carbapenem for public use. Structurally similar to imipenem, meropenem differs in the addition of a methyl group at the Cl atom (Hellinger and Brewer 1999). Meropenem is slightly more active against Gram-negative bacilli, e.g., *Escherichia coli* and *Klebsiella pneumoniae*, compared to imipenem (Hellinger and Brewer 1999; Jones et al. 1989; Jorgenson et al. 1991).

Ertapenem, in contrast to imipenem and meropenem, has limited in-vitro activity against *Pseudomonas* and *Acinetobacter* species (Shah and Isaacs 2003; Wexler 2004). Like the two preceding carbapenems, it is active against both Gram-negative and Gram-positive bacterial pathogens. An advantage of ertapenem is its long; half-life, which results from the carbapenem's extensive protein binding (Zhanel et al. 2005; Hammond 2004). Ertapenem therefore requires a once-daily dosing, compared to the multiple-daily dosing of imipenem and meropenem (Zhanel et al. 2005; Hammond 2004).

Doripenem, due to a side chain at position 2, has increased activity against multi-drug resistant non-fermentative Gram-negative bacilli, oxacillin-susceptible *Staphyloccocus aureus*, and coagulase-negative staphylococci compared to the activities of imipenem and meropenem (Chen et al. 2005; Fritsche et al. 2005).

Each of these carbapenems, in appropriate doses, are fairly safe. At a low rate of incidence, patients have reported seizures when administered high dosages of imipenem-cilastin (Miller et al. 2011). However, meropenem appears to be less renal-toxic and neuro-toxic, mirroring the minimal side effects of other beta-lactam antibiotics. The reported rate of seizure activity due to administration of meropenem, doripenem, or ertapenem is less than 1% of cases (Norrby 2000; Miller et al. 2011). The carbapenems are poorly absorbed when ingested orally, therefore are administered intravenously or by injection (Codjoe and Donkor 2018).

Carbapenem-Resistant Enterobacteriaceae (CRE). Carbapenem antibiotics, despite exhibiting broad-spectrum bacteridical properties, have recently become ineffective against some cases of ESBL-producing Enterobacteriaceae. There exist several mechanisms by which Enterobacteriaceae develop resistance to carbapenems: bacterial alteration of PBP structures, bacterial recruitment of carbapenemases, e.g., Ambler Class B metallo-beta-lactamases that are capable of degrading carbapenems, changes to outer membrane porins that result in decreased cell permeability, and efflux pumps that pump carbapenems out of the periplasmic space of the cell (Zhanel et al. 2007; Meletis 2016). Carbapenemases are capable of hydrolyzing penicillins, cephalosporins, monobactams, and carbapenems and are quite diverse in target range (Queenan and Bush 2007). A few examples of carbapenemases acquired by CREs are KPC (Class A),

OXA-48-types (Class D), VIM (B), and NDM (B) (Hawkey and Livermore 2012; Navon-Venezia 2006).

In carbapenem-resistant Enterobacteriaceae, the main carbapenem-hydrolyzing-beta-lactamases are in Ambler Class A (e.g., *bla* KPC, IMI, SME) and Ambler Class B metallo-beta-lactamases (e.g., *bla* IMP, VIM, and NDM) (Nordmann et al. 2011; Queenan and Bush 2007; Hawkey and Livermore 2012; Poirel et al. 2012). There are also a growing number of cases of OXA-48-types (Class D) and AmpC (Class C) inducing partial-activity against carbapenems (Hawkey and Livermore 2012; Poirel et al. 2012; Nordmann et al. 2011).

The difference between extended-spectrum beta-lactamases (ESBL) and carbapenemases is that ESBLs hydrolyze the beta-lactams and 2nd and 3rd generation cephalosporins without inactivating carbapenems (Nordmann et al. 2014). For the most part, carbapenemases hydrolyze all of the beta-lactams, including carbapenems, cephalosporins, and monobactams, equipping them with a complete arsenal against beta-lactam antibiotics (Nordmann et al. 2014).

Spread of Antibiotic Resistance

Of potential importance is the role of the natural environment in drug-resistant infectious disease transmission (Hilleman 1996). Over the past several years, the environment has been recognized as reservoir for antibiotic resistance genes and AR-pathogens (Martinez 2008; Marti et al. 2014; Wright 2010). However, little is known about the conditions and circumstances under which the environmental antibiotic resistant elements pose as threats to public health (Berendonk et al. 2015; Bengtsson-Palme et al. 2018). On the contrary, better recorded are the mechanisms by which synthetic antibiotics and clinical AR-pathogens disseminate into the environment, such as through wastes (human sewage, sludge, and livestock manure) (Goulas et

al. 2018). Further research should be devoted to how environmental disturbances can release new AR-pathogens and parasites into new territories, making naïve host species never having been exposed to those pathogens susceptible to infection.

Role of surface water contamination in emergence and persistence of AR

Outside of clinical settings, the natural environment provides a reservoir for AR (Marti et al. 2014). Surface waters worldwide are impacted by anthropogenic waste, or by-products of human activities, in the forms of chemical, biological, and physical pollution (Fick et al. 2009). Examples of contaminants include fecal matter and its associated enteric pathogens, pharmaceuticals, and antibiotic residues (Fick et al. 2009; Pandey et al. 2014). The release of drugs and chemicals into the environment, via urine, leaking sewer pipelines, runoff from impervious roadways, or improper disposal of medicines, can lead to contamination of surface, ground, and drinking waters (Fick et al. 2009; Kuoppamaki et al. 2014; Kaye et al. 2006) and change the biochemistry of an aquatic ecosystem (Kümmerer 2009).

AR Enterobacteriaceae pathogens are associated with feces and urine (Parveen et al.1997; Edge and Hill 2005; Zhao et al. 2014) derived from warm-blooded animals, e.g., human, avian, wildlife, and farm animals. Aquatic ecosystems are contaminated with human and livestock waste in many regions of the world, including locations in the United States. Although human sewage in urbanized areas is usually piped underground for treatment at a wastewater treatment or water reclamation facility, at times these underground pipelines leak sewage or break (due to wear), contaminating nearby soils and underground water sources. Additionally, outflow of WRF effluent, overflow of combined sewer systems and sanitary sewer systems, and contamination from septic tanks can contribute human waste to local soils and rivers (McLellan 2015; Duda

1993). Lamba et al. (2018) conducted a study on antimicrobial resistance prevalence in a range of aquatic environments in India: 12 hospital effluent sites, 12 sewage treatment plants, 20 sewer drains, and 5 locations along the Yamuna River in New Delhi. Strikingly, they found significantly positive correlations between fecal coliform levels (a fecal indicator used to assess risk of gastrointestinal illness), CRE concentrations, and *bla* gene NDM-1 concentrations across all samples. CRE levels were generally highest at sites downstream from high population densities, indicating a need for wastewater management that is mindful of the adaptability of microorganisms to drugs disposed in wastewater in that area (Lamba et al. 2018).

Rodriguez-Mozaz et al. (2015) detected high concentrations of ARG copies in the influent of wastewater treatment plant and effluent of a hospital during 2011-2012 along the Ter River in Spain. Additionally, AR components were detected in the effluent of the treatment plant, indicating that the treatment plant did not remove antibiotics (i.e., fluoroquinolones ciprofloxacin and oflaxacin) and therefore the ARGs (e.g., *qnrS*, *bla*TEM, *ermB*) from inflowing sewage (Rodriguez-Mozaz et al. 2015). As a result, the receiving river contained higher concentrations of ARG pollutants downstream of the treatment plant outfall compared to upstream. There was a significant positive correlation between almost all ARGs analyzed (n=5) and the surface water concentration of antibiotics that could stimulate bacterial resistance (Rodriguez-Mozaz et al. 2015).

Wastewater reclamation facilities (WRFs) release treated wastewater into natural streams and rivers. However, the treatment processes within these plants do not remove 100% of all microbes or antimicrobial chemicals from the raw sewage that enters them. WRFs may actually serve as hotspots for HGT, making complete removal of AR-bacteria more difficult (Hultman et al. 2018; Rizzo et al. 2013). Over time, resistant bacteria can accumulate in the waterways from

persistent outfall of treated water, impacting wildlife and public health (Berglund et al. 2014). Additionally, biological amendmnets to soil, such as compost and manure, can increase the abundance of ARGs in the affected area (Tien et al. 2018; Heuer et al. 2018).

These mechanisms of consistent contamination have the potential to turn an aquatic environment into both a cocktail of low-level drugs and a reservoir for microbial horizontal gene transfer, which is a recipe for environmental dissemination of antibiotic resistance (Gullberg et al. 2011; Boxall 2004).

Management of Aging Infrastructure. One hurdle that will need to be overcome in coming years is that of aging water infrastructure in urban and semi-urban areas. Across the United States, wastewater enters treatment plants via public sewer lines, and in many municipalities, stormwater drains (ASCE 2017 Infrastructure Report Card). Wear of these pipelines, due to chronic water pressure, corrosion, heavy rainfall events, or tree root invasion, leads to leakage of sewage into the environment (Kessler 2011; Davies et al. 2001). The US EPA estimates that at least 23,000 to 75,000 sanitary sewer overflow events happen each year (ASCE 2017 Infrastructure Report Card; EPA 2004). Nearly 240 million Americans (76% of population) rely on the 14,748 treatment plants in the U.S., and by 2032, an expected 56 million more people will move from septic tank system to the centralized treatment plant system (ASCE 2017 Infrastructure Report Card). Management of old conveyance systems or construction of new water infrastructure is needed to meet future demands of flow throughput (ASCE 2017 Infrastructure Report Card). David Behar, the climate program director for the San Francisco Public Utilities Commission and spokesman for the Water Utility Climate Alliance in 2011, stated that most water utilities are still "living in an era of assessment rather than an era of adaptation" (Kessler 2011). To resolve disturbances to water infrastructure, we first need to

identify an impairment in infrastructure stability as it arises; then we can adapt to that change. In order to better monitor the integrity of underground sewer pipelines in urbanized areas, perhaps local utility offices can implement wireless sensor networks for leak detection (Sadeghioon et al. 2014). Civil and mechanical engineers at the University of Birmingham, UK, have developed an underground wireless sensor network called SmartPipes that uses force sensitive resistor technology to measure pressure change inside the pipe (Sadeghioon et al. 2014). Technologies like this that continually monitor the structural health of a pipe will hopefully be frequently implemented as we move forward.

An additional problem is impervious surfaces that down-regulate the absorption of fluids by soils, leading to flash floods of streams and rivers in urban areas. Urban planning strategies should keep waterways in mind, as they provide ecosystem services and could become potential reservoirs of disease transmission. If flash flood or channel modification disturbances outcompete self-regulation of streams and rivers, the biogeochemical properties of streams may reach a dysbiotic state to where the streams and rivers no longer provide the ecosystem services they should.

Conclusion

Microbes for billions of years have effectively adapted to their surroundings, whether living symbiotically or competitively. Those microbes (e.g., bacteria, fungi, and algae) in competitive environments have naturally developed and produced antibiotics to ward off competitors. Humans discovered one such (penicillin-type) antibiotic that later led to the launch of a worldwide antibiotic industry. Due to the widespread use and misuse of pharmaceutical antibiotics, there has been a rapid increase in the number of antibiotic-resistant infections over

the past century. AR-pathogens are isolated predominantly from clinical cases and hospital environments but also are increasingly found in the natural environment, with particular locations serving as hotspots for AR, to which humans can be exposed. Although the spread and evolution of AR may be daunting, there are communal measures that humankind can take to mitigate the harmful effects of resistance in the environment, such as rapidly responding to impairment in sewer and septic tank infrastructure. Two of the most crucial things that we can do as a species is to monitor and adapt, by removing point sources of biological and chemical pollutants. By taking action to mitigate pollution, ARG abundance in the environment could be reduced, and we too could remain nimble alongside the microbes.

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Table 1.1 Mechanisms for common classes of antibiotics.

Antibiotic Class	Mechanism	Reference
Beta-lactams	Bind to and inactivate peptidoglycan transpeptidase thereby inhibiting cell wall synthesis	Waxman and Strominger (1983)
Macrolides	Multiple, including inhibition of ERK1/2 and NF-κB immunomodulatory pathways	Kanoh and Rubin (2010), and references therein
Tetracyclines	Inhibit binding of aminoacyl-	Chopra and Roberts (2001);

	tRNA to the mRNA-ribosome complex thereby inhibiting protein synthesis	Chukwudi (2016), and references therein
Quinolones	Block ligation and increase concentration of enzyme-DNA cleavage complexes thereby inhibiting DNA synthesis	Aldred et al. (2014), and references therein
Aminoglycosides	Bind to bacterial 30S ribosomal subunit thereby inhibiting protein synthesis	Jana and Deb (2006)
Sulphonamides	Inhibit incorporation of para-aminobenzoic acid into folic acid thereby inhibiting folic acid synthesis	Smith and Powell (2000)
Glycopeptides	Multiple mechanisms to bind to peptidoglycan cell wall thereby inhibiting cell wall synthesis	Kang and Park (2015)
Oxazolidinones	Bind to 50S ribosomal subunit near to the 30S ribosomal subunit thereby inhibiting protein synthesis	Diekema and Jones (2001)

Table 1.2 A summary of beta-lactam classifications, their branded pharmaceutical names, and their spectra of bactericidal activity. Table adapted from writings of William A. Petri, Jr. in Chapter 53 of *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (12th Edition) (2011) and Copper (1992).

Class of Beta Lactam	**Drug Examples**	**Spectrum of Bactericidal Activity**
Penicillin and derivatives	Penicillin G, amoxicillin, oxacillin	Gram-positive, with later generations active against some Gram-negatives
Cephalosporins (1st generation)	cefazolin, cephalexin, Keflex	Gram-positive and some Gram-negative
Cephalosporins (2nd generation)	cefoxitin, cefprozil, cefuroxime	Gram-positive and Gram-negative. Some anti-anaerobe

		activity.
Cephalosporins (3rd generation)	Cefotaxime, ceftazidime, ceftriaxone	Gram-positive and Gram-negative Enterobacteriaceae, with some activity against *Pseudomonas aeruginosa*
Cephalosporins (4th generation)	cefepime	Same as 3rd generation but with increased stability to hydrolysis by chromosomal beta-lactamases
Monobactams	Aztreonam	Aerobic Gram-negative
Carbapenems	Imipenem, meropenem, doripenem	Broadest spectrum of any beta-lactam antibiotic
Carbacephems	Loracarbef (discontinued in United States)	Gram positive and slightly better Gram negative. (Usually grouped with 2nd generation cephalosporins)

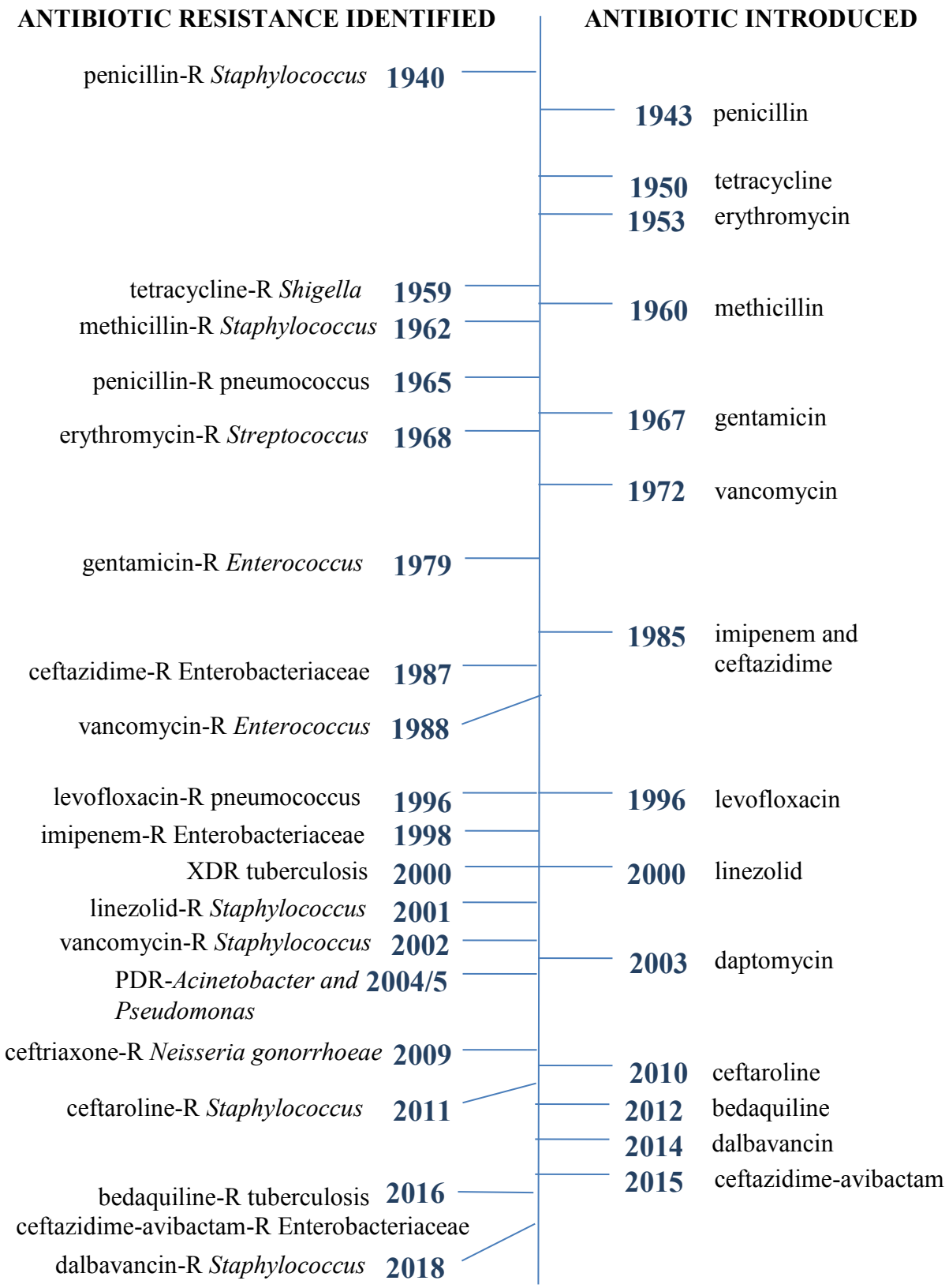

Fig 1.1. Timeline of antibiotic resistance compared to antibiotic approval for public use, adapted from CDC 2018.

CHAPTER II

BETA-LACTAM AND CARBAPENEM RESISTANCE IN A MIXED-USE WATERSHED

[1] Edwards ME, Hiott LM, Westrich JR, Frye JF, Cho S, Ottesen EA, Davis J, Seim R, Francis L, Mcdonald JM, Ramadan HH, Jackson CR, O'Malley M, Robertson M, Lipp EK. To be submitted to Applied and Environmental Microbiology

Abstract

We conducted a watershed-wide surveillance study to determine the prevalence and distribution of beta-lactam resistance in Enterobacteriaceae from urban and sub-urban surface waters in the Piedmont Region of northeast Georgia (USA). Over the course of one year (four quarterly samples), 54 sites were sampled (169 surface water samples). Enterobacteriaceae isolates that were PCR positive for at least one of eleven beta-lactamase (*bla*) genes tested were detected in 102/169 (60.36%) of surface water samples were PCR positive for at least one of eleven beta-lactamase (*bla*) genes tested. Fourty-seven isolates (from 38 samples) were phenotypically confirmed as ESBL-producing Enterobacteriaceae (ESBL-E), and 25 isolates (from 22 samples) were phenotypically confirmed as carbapenem-resistant Enterobacteriaceae (CRE). Among the surface water isolates phenotypically confirmed as ESBL-E or CRE, 17 (of 71) had multiple resistance genes (up to 4). Additionally, wastewater influent and effluent from three municipal treatment plants that serve residential neighborhoods, industrial facilities, and hospital facilities were sampled. Enterobacteriaceae carrying *bla* gene(s) were detected in all treatment plants, in both influent and effluent, at least once during the three sampling dates. Additionally, phenotypically confirmed ESBL-E were present in influent of all three plants and effluent of one plant. Phenotypically confirmed CRE were not present in influent but were detected in effluent of two plants. Among the influent and effluent isolates confirmed as ESBL-E or CRE, 4 (of 14) carried multiple resistance genes (up to 3). ESBL-E and CRE species isolated over the study were primarily *Escherichia coli*, *Enterobacter* spp., and *Serratia fonticola* in surface water and *E. coli, Enterobacter* spp. and *Klebsiella* spp. in influent and effluent. Fourteen surface water and 4 influent isolates that were confirmed as phenotypic ESBL-E or CRE had multiple resistance genes (up to 4 genes per isolate). Over the four study dates, frequency of

isolation of ESBL-E in surface water was highest in January and November and lowest in July and April, while frequency of isolation of CRE in surface water was highest in July and November and lowest in January and April. Results of this study suggest that surface water ecosystems impacted by urban and suburban development may be an important reservoir of significant AR genes and emerging AR pathogens.

Introduction

Antimicrobial resistance (AMR) is a growing issue in management of infectious disease bacteria and is increasingly recognized in environmental settings across the world, with implications for environmentally transmitted pathogens (e.g., food and waterborne). Over the past few decades, there has been an increase in hard-to-treat infections due to antibiotic-resistant bacteria (ARB) (Sakoulas and Moellering 2008; Arias and Murray 2009) as well as an increase in ARB in the natural environment (Wellington et al. 2013). Although bacteria may be intrinsically resistant to some antibiotics (Brown and Reynolds 1980; Sanchez et al. 2009), bacteria can also acquire resistance through gene mutation or through uptake of antibiotic resistance genes (ARG) via horizontal gene transfer from neighboring bacterial cells. Environmental pollution with waste products that may contain antibiotics or enteric bacteria, and nutrients can change the composition, diversity, and behavior of environmental microbes, sometimes resulting in the acquisition and upregulation of ARG in response to environmental stress. Freshwater streams and rivers worldwide carry high loads of pollutants derived from pharmaceuticals, lawn fertilizers, stormwater runoff, and excrement that run off from urban or highly modified areas (Fick et al. 2009; Lehman et al. 2011; Pandey et al. 2014). Consequently, surface waters may be important as possible ‘hot spots’ for emergence of ARB and ARGs and

for their roles in water transmission of pathogens (Hamner et al. 2006; Hunter 2003; Rodriguez-Mozaz et al. 2015; Skariyachan et al. 2015).

AR within the clinical setting is well-studied, but despite the potential importance of environmental reservoirs in transmission and as potential hot-spots for recombination and emergence of novel AR profiles, there is much less known about the extent of AR among bacterial pathogens in the environment. Of particular interest are water bodies contaminated with antibiotics, ARG, and ARB, from recreational sources, agricultural sources, waste water treatment plant effluent, or discharge of undertreated sewage (Biyela et al. 2004; Rizzo et al. 2013; Lamba et al. 2018). Although environmental hotspots for AR have recently been foci of study, less is known about the prevalence and diversity of resistant species across an entire watershed.

In this study, we describe the prevalence and genetic and phenotypic diversity of ESBL-producing Enterobacteriaceae (ESBL-E) and carbapenem-resistant Enterobacteriaceae (CRE) present in the surface waters of streams and influent and effluent of water reclamation facilities (WRF) of a mixed-use watershed. ESBL-E and CRE were listed by the CDC in the 2013 Antibiotic Resistance (AR) Threats Report as two of the most urgent and serious antibiotic resistant bacterial agents (CDC AR Threats Report, 2013) and have the potential for transmission through environmental routes, especially water (Laurens et al. 2018). We aimed to characterize these AR-pathogens, their number and type of *bla* genes, and their phenotypic resistance to beta-lactam antibiotics as well as aimed to identify associations between these pathogens and environmental parameters, i.e., fecal indicator bacterial levels, impervious surface cover, and sampling date.

Materials and Methods

Study area. Water samples were collected from streams and rivers comprising the Upper Oconee Watershed (United States Geological Survey Hydrological Unit Code 03070101) in Northeast Georgia (Figure 2.1). Encompassing 538.7 km^2, the watershed lies completely within the Piedmont province, which consists of rolling hills and various topographical and hydrological features (Metropolitan N. Georgia Water Planning District 2017). The watershed's two major rivers, the Middle Oconee and North Oconee (referred to as MIDO and NORO, respectively), converge into the Oconee (referred to as BICO) near the southernmost part of the sampling region. Sample stations span headwaters to sixth-order river segments and include diverse land covers and uses, including urban, residential, rural, and agricultural land uses. In conjunction with the Upper Oconee Watershed Network (UOWN) (http://uown.org/UOWN-Wordpress/), we selected 54 stream and river sites spanning 14 subwatersheds differing in average surrounding development (0.2 - 26.9%) and impervious surface cover (0.7 – 42%), using data from National Land Cover Database (NLCD) 2011 (NLCD, at https://www.mrlc.gov) and ArcMap (http://desktop.arcgis.com/en/arcmap/) to locate the stream sites. UOWN is a volunteer-based non-profit that leads quarter-annual water collections to monitor for biological, chemical, and physical water quality. Due to the UOWN resource and history of fecal contamination (Appendix A), the UOW was chosen as a model watershed for this study.

Water sample collection. Surface water samples (n=169) were collected from 40-44 stream stations in the Upper Oconee Watershed, GA, USA during four collection periods over a one year period (54 total). (Table 2.1; Table 2.2). All samples were collected synoptically by volunteers of the Upper Oconee Watershed Network (UOWN) within a 2-h window in January

(winter, n=42), April (spring, n=40), July (summer, n=43), and November (fall, n=44) of 2018. Twenty-four stream stations were sampled on all four dates. Grab samples (single samples) (100-1000 mL of water) were collected from just below the surface in the main flow of the stream using either sterile Whirl-Pak® bags or autoclaved 1-L polyprolyene bottles. Influent (n=9) and effluent (n=9) from the three water reclamation facilities (WRF) in Athens, GA were also sampled over three collection periods (January, April, July). WRF samples were collected by WRF personnel into sterile 2-L polypropylene bottles as 24 h composite samples (ending during the 2 h stream sample collection window). All samples were stored on ice and processed within 4 h of collection.

Fecal indicator bacteria. *E. coli* levels were determined EPA Method 1603 (EPA 2014), with a slight modification in incubation conditions. Briefly, water samples were vacuum-filtered in duplicate for at least two volumes onto 47 mm 0.45µm diameter pore size mixed cellulose ester membranes (Millipore Sigma, Cat. No. HAWG047S6). Membranes were placed on modified membrane-Thermotolerant *Escherichia coli* (mTEC) agar (Difco™ Modified mTEC Agar, BD). Plates were incubated at 35 °C for 2h +/- 30 min before being placed into a sealed closed cell foam box and transferred to a 44.5 °C incubator for 18 – 24 h (Feng et al. 2002; Laitsch et al. 2012). A recording thermometer was used to ensure that temperature varied by less than 0.2 °C (Laitsch et al. 2012). All red/magenta colonies were counted as *E. coli* and data were reported as CFU 100 ml^{-1}.

CRE and ESBL-producers. To screen for presumptive ESBL-producers, water samples were membrane-filtered (10 and 100 ml) in duplicate, as described above, and membranes placed onto CHROMagar™ ESBL. Plates were incubated at 37 °C for 18 – 24 h. Additonally, 100 ml of each

sample was filtered, membranes placed in sterile 15 mL polypropylene tubes with 9 mL of modified Buffered Peptone Water (Hardy Diagnostics CRITERION™), and enriched overnight at 37 °C. A loopful of overnight growth was then streaked in duplicate onto CHROMagar™ ESBL and CHROMagar™ mSuperCARBA agar plates and incubated at 37 °C for 18 – 24 h for the detection of ESBL-E and CRE, respectively. Up to four well-isolated blue (presumptive *Klebsiella*, *Enterobacter*, or *Citrobacter*) and pink (presumptive *E. coli*) colonies per plate were picked (for both directly plated ESBL and enrichments for ESBL and CRE) and re-streaked to isolation onto CHROMagar™ ESBL or mSuperCARBA, respectively. Subsequently, 1 colony (or 2 if mixed growth) per plate was picked and streak-purified through a second round.

All presumptive ESBL-producing and CRE isolates were streaked twice onto blood agar (BA) (BBL™ TSA 5% Sheep Blood) for biochemical identification testing, whole cell template preparation, and cryopreservation. Whole cell templates were made by resuspending one colony in 200 µl of PCR-grade water in 0.5 mL sterile microcentrifuge tubes and stored at 4 °C for PCR. For cryopreservation, 1-5 isolated colonies from the same BA2 plate were resuspended in 1.5 mL Lysogeny Broth, Miller (LB; BD Difco™) with 30% glycerol in 2 mL cryovial tubes and stored at -80 °C.

Bacterial identification. Isolates on BA2 were identified to species via the bioMérieux VITEK® 2 system using Gram-negative identification (VITEK® 2 GN ID) cards (bioMérieux, Durham, NC, USA). Any isolates that were reported to the species level with < 90% certainty were re-streaked and retested through the VITEK® system**.**

Polymerase chain reaction for *bla* gene detection. We used targeted PCR to detect 11 beta-lactamase (*bla*) genes in all presumptive ESBL-E and CRE isolates that were screened through VITEK®: CMY-2 (Zhao et al. 2001), CTX-M (Bonnet et al. 2003), KPC (Fernando et al. 2016; Mulvey et al. 2011), NDM-1 (Fernando et al. 2016; Mulvey et al. 2011), OXA-1 (Féria et al. 2002), SHV (Colom et al. 2003), TEM (Brinas et al. 2002), IMI-NmcA (Voets et al. 2011), IMP (Yousefi et al. 2010), VEB (Dallenne et al. 2010), and VIM (Fernando et al. 2016; Mulvey et al. 2011)**.** We amplified the *bla* gene targets in Applied Biosystems 2720 thermal cyclers using 24 µl reactions (22 µl master mix and 2.2 µl DNA). Primer sequences and amplicon sizes can be found in Table S1. Reaction conditions and master mix concentrations can be found in Table S2.

Antibiotic susceptibility panel testing. Antibiotic susceptibility tests were performed on VITEK-confirmed Enterobacteriaceae isolates carrying at least one *bla* gene via the Sensititre™ semi-automated antimicrobial susceptibility system (TREK Diagnostic Systems Inc., Cleveland, OH, USA). We used the ESB1F (ESBL-specific) panels to analyze select Enterobacteriaceae isolates' susceptibility to 16 antibiotics using the Minimum Inhibitory Concentration (MIC) breakpoints set by Clinical and Laboratory Standards Institute (CLSI). The control strains used for MIC determination were *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 29213. The 16 antimicrobials and MIC breakpoints (resistance) (µg ml^{-1}) contained in the ESB1F panels were: ampicillin (≥32), cefazolin (≥8/≥32), cefepime (≥16), cefotaxime (≥4), cefotaxime/clavulanic acid (no interpretation), cefoxitin (≥32), cefpodoxime (≥8), ceftazidime (≥16), ceftazidime/clavulanic acid (no interpretation), ceftriaxone

(≥4), cephalothin (≥32), ciprofloxacin (≥1), gentamicin (>16), imipenem (≥4), meropenem (≥4), piperacillin/tazobactam (≥128/4) (CLSI M100 and M31, 2018).

Enterobacteriaceae isolates were considered to be ESBL-producing if they were resistant to cefotaxime and/or ceftazidime and experienced a ≥ 3-twofold decrease in MIC of cefotaxime + clavulanic acid versus cefotaxime alone, and/or a ≥ 3-twofold decrease in MIC of ceftazidime + clavulanic acid versus ceftazidime alone (CLSI 2018). The Sensititre SWIN software system (TREK Diagnostics) was used to read and report antimicrobial susceptibility results.

Statistical analysis and data visualization. *E. coli* concentrations were not normally distributed (Shapiro Wilks test, W = 0.41, *p-value* = < 2.2e-16) (Figure S1), therefore, differences in median levels between sampling dates were calculated via Kruskal-Wallis one-way analysis of variance followed by Dunn's Multiple Comparison Test, a post-hoc non-parametric test. All sites (169 samples) were used for this analysis; however, eight samples (2 January and 6 November samples) were removed due to lack of *E. coli* data. One surface water sample was processed for *E. coli* but not ESBL-E or CRE and was not included in statistical tests. Across all 161 samples, three models (Poisson regression, negative binomial regression, and observation-level random effects) were used to test the relationship between *E. coli* concentrations and percent impervious surfaces.

Univariate logistic regression was used to test associations between *E. coli* concentrations or percent impervious surface with the presence/absence of isolates that carried single or multiple *bla* genes. Ordinal logistic regression (using the "polr()" function in the MASS package within RStudio) was used to evaluate association between *E. coli* concentrations and the maximum number of *bla* genes detected in an isolate (max*bla* factor levels = 0, 1, 2, 3, 4).

We employed Pearson's $\chi 2$ test to examine the association between sample date and a variety of response variables, including the frequency of samples that exceeded the EPA 410 CFU *E. coli* 100 ml^{-1} threshold, the presence/absence of at least one *bla* genes, presence/absence of two or more *bla* genes, presence/absence of phenotypic ESBL-producing Enterobacteriaceae, and presence/absence of phenotypic carbapenem-resistant Enterobacteriaceae. Only isolates identified as Enterobacteriaceae were considered for presence of *bla* genes and ESBL-producing and carbapenem-resistant characteristics (other bacteria, even if a *bla* gene was detected, were excluded from further testing, i.e., the sample was considered negative if either no *bla* containing isolates were detected or if only non-Enterobacteriaceae *bla* containing isolates were detected). Analyses used each sample (instead of each isolate) as a statistical unit.

Figures were made and statistical analyses were conducted using RStudio (RStudio Team 2016) and the following packages: ggplot2 (figure production), PMCMR (Kruskal-Wallis and Dunn test), MASS (ordinal logistic regression), sandwich (Poisson regression), msm (Poisson regression), and AER (Poisson regression, negative binomial, observation-level random effects).

Results

Fecal indicator bacteria. Over the course of the study, *E. coli* levels in surface water ranged from 10 to > 10,000 CFU 100 ml^{-1}. Effluent samples ranged from undetectable to 6,200 CFU 100 ml^{-1}. *E. coli* levels in influent were all ≥ 48,500 CFU 100 ml^{-1}. Of 162 surface water samples analyzed for *E. coli*, 32.7% (53/162) exceeded the 410 CFU 100 ml^{-1} statistical threshold value (STV) (RWQC 2012) and two of the nine WRF effluent samples exceeded that threshold. *E. coli* levels by sample date ranged from a median of 162 CFU 100 ml^{-1} in January to 385 CFU 100 ml^{-1} in July. Concentrations in July were significantly higher than those in January or November (*p*-

value < 0.001; Fig. 2.2). *E. coli* levels exceeded the STV in 22.5% of January samples (n = 40), 41.5% of April samples (n = 41), 46.5% of July samples (n = 43), and 18.4% of November samples (n = 38) ($\chi 2 = 9.031$, df = 3, p-value = 0.02888), with frequency of samples exceeding the threshold significantly higher in July.

Among the 24 sites that were sampled on all four dates, MIDO616 had the highest median concentration (2,493 CFU 100 ml^{-1}) and NORO527 had the lowest (163 CFU 100 ml^{-1}) (Figure 2.2); however, there was no statistically significant difference in concentration among the sites. Grouped by subwatershed, both MIDO (n = 18) and NORO (n = 6) sites had similar levels of *E. coli* with median values of 308 and 315 CFU 100 ml^{-1}, respectively. None of the BICO sites were sampled on all 4 dates and therefore were not included in the comparison. Among the 24 sites sampled across all dates, there was no site at which *E. coli* levels exceeded the STV at all four dates, but the threshold was exceeded for 3 of the 4 dates at five sites (MIDO609, MIDO617, MIDO709, NORO114, and NORO501) and 2 of the 4 dates at four sites (MIDO611, MIDO613, MIDO616, and MIDO826). Only 6 sites had *E. coli* levels that never exceeded the threshold (MIDO606, MIDO608, MIDO610, MIDO612, MIDO712, and NORO514).

ESBL-E and CRE across samples. Presumptive ESBL-producing and carbapenem-resistant colonies were isolated from 88.17% (149/169) of surface water samples, 100% (9/9) of influent samples, and 100% (9/9) of effluent samples. Isolates were subsequently confirmed as Enterobacteriacaeae from 66.27% (112/169) of surface water samples, 100% (9/9) influent, and 77.78% (7/9) effluent. Finally, isolates confirmed to carry at least one targeted *bla* gene were detected from 60.35% of surface water samples (102/169), 100% (9/9) influent, and 66.67%

(6/9) effluent. Enterobacteriaceae with the ESBL-producing phenotype were isolated from 32.49% (38/169) of surface water samples, 66.67% (6/9) influent, and 11.11% (1/9) effluent (Table 2.1; Table 2.2). Enterobacteriaceae with the carbapenem-resistant phenotype were isolated from 13.02% (22/169) surface water samples, 0.00% (0/9) influent, and 33.33% (3/9) effluent (Table 2.1; Table 2.2). Presumptive ESBL and CRE isolates carried up to four *bla* genes (one July *K. pneumoniae* isolate carried 4 *bla* genes; one January, one July and two November *E. coli* isolates carried 3 *bla* genes; one April *K. pneumoniae* isolate carried 3 *bla* genes; one November *R. planticola* isolate carried 3 *bla* genes) with a median carriage rate of 1 *bla* gene (over all sample types).

Presumptive ESBL-E and CRE isolates. In all, 368 stream, 30 influent, and 18 effluent isolates were recovered from CHROMagar ESBL and CHROMagar mSuperCARBA plates. Of those, 194 stream, 18 influent, and 12 effluent isolates were confirmed as Enterobacteriaceae**.** Finally**,** 47 stream, 9 influent, and 2 effluent isolates were confirmed as ESBL-E, and 25 stream and 3 effluent isolates were phenotypically confirmed as CRE; no influent isolates were confirmed as CRE (Table 2.3; Table S3). Isolates identified as Enterobactericeae (n=30) from WRF influent and effluent samples (from January, April, and July) were similar to those identified in surface water samples (Table 2.3; Table S3).

Isolate Analysis. *Serratia fonticola* was the most commonly isolated organism from streams on CHROMagar ESBL and made up 32.9% (121) of all 368 stream isolates and 62.37% (121) of the 194 Enterobacteriaceae stream isolates (Table 2.3). Of the 121 *S. fonticola* isolates, 111 carried *bla*CTX-M, and 21 of these isolates were confirmed as ESBL-producers (Table 2.3), predominantly in the winter sampling date (Table S4). No *Serratia fonticola* were isolated from

influent or effluent samples or from any sample type plated on CHROMagar mSuperCARBA; however six *S. fonticola* isolates from CHROMagar ESBL were also carbapenem-resistant.

Following *S. fonticola, Enterobacter cloacae* complex (ECC) were the next most commonly identified Enterobactericeae from ESBL (n=7) and CRE (n = 19) plates, especially for isolates confirmed as CRE. ECC confirmed as CRE carried the *bla*IMI-NmcA gene(s) (18 isolates from streams, 2 isolates from effluent) (Table 2.3; Table S3). All ECC CRE surface water isolates (n=18) were collected in July and November (from 13 stations) and were confirmed to have resistance to imipenem (Table 2.4). All were resistant to meropenem, with the exception of one November and three July isolates. All 18 ECC CRE surface water isolates were susceptible to piperacillin-tazobactam (Table 2.4), and none were confirmed as ESBL-producers. ECC CRE isolates were recovered more than once at only two of thirteen stations (BICO101 and NORO527, during both July and November). There were two confirmed ECC CRE effluent isolates, which carried *bla*IMI-NmcA and were both resistant to imipenem and one resistant to meropenem.

Escherichia coli was the third major Enterobacteriaceae isolated from surface waters, comprising 23 isolates (22 isolated on CHROMagar ESBL and 1 on CHROMagar mSuperCARBA). All 23 *E. coli* isolates were confirmed to have at least 1 of 11 targeted *bla* genes (Table 2.3). Additionally, 14 of these isolates had $\geq$2 *bla* genes. 21 isolates were confirmed as phenotypic ESBL-producers and 21 of these isolates carried the *bla*CTX-M gene. Seven presumptive ESBL *E. coli* were isolated from influent and one *E. coli* was isolated from effluent. Six of the 7 influent isolates carried *bla*CTX-M and were confirmed phenotypic ESBL-producers. The single *E. coli* effluent isolate did not carry any targeted *bla* genes and was not tested for phenotypic resistance. Other *bla* genes present in all presumptive ESBL *E. coli*

isolates were *bla*CMY-2, *bla*OXA-1, *bla*SHV, and *bla*TEM. Approximately 56.67% (17/30) of presumptive ESBL *E. coli* isolates, over all sample types, carried multiple (2 or 3) *bla* genes, and all but one *E. coli* isolate grown on CHROMagar ESBL were confirmed as phenotypic ESBL-producers. Only those *E. coli* isolates that carried *bla*CTX-M were phenotypic ESBL-producers. Two presumptive CRE *E. coli* isolates were isolated on CHROMagar mSuperCARBA, one from surface water (MIDO 826) and one from effluent in April. The surface water isolate carried the *bla* genes KPC and TEM (Table 2.4); however, it was not confirmed as carbapenem-resistant, according to the CLSI MIC breakpoints (Table S4). The effluent isolate did not carry any targeted *bla* genes and was not tested for antibiotic susceptibility.

Citrobacter braakii, freundii, and *sedlakii* were cultured from surface waters on CHROMagar ESBL and from WRF influent and effluent on CHROMagar mSuperCARBA. Only *C. braakii* and *C. freundii* carried at least one *bla* gene. Five presumptive ESBL stream isolates were identified as *Citrobacter freundii* and all carried *bla*CMY-2. Four of the five isolates were detected in November and were isolated from 4 separate surface water sites (MIDO 605, MIDO 609, MIDO 802, and NORO 501). *C. freundii* was isolated from CHROMagar mSuperCARBA from one January influent sample. The isolate carried *bla*KPC and *bla*SHV and was susceptible to both imipenem and meropenem. A presumptive CRE isolate was also detected in effluent in April and carried *bla*KPC and SHV but was susceptible to both imipenem and meropenem (Table S4).

All of the *Klebsiella pneumoniae* isolates (12 total; 5 stream, 3 influent, and 4 effluent) isolated from CHROMagar ESBL and CHROMagar mSuperCARBA carried *bla* genes KPC and/or SHV. Two surface water, one influent, and two effluent *K. pneumoniae* isolates were confirmed as phenotypic ESBL-producers. One surface water isolate was confirmed as

carbapenem-resistant. One isolate, cultured from MIDO 802, a stream site located in a forested park and downstream from a residential neighborhood, carried 4 *bla* genes: CTX-M, OXA-1, SHV, and TEM (Table S4). One *Klebsiella oxytoca* isolate was collected from MIDO 826, downstream from a hospital, in April and carried *bla*KPC and *bla*TEM, however, this isolate was not confirmed to phenotypically express ESBL.

Although not Enterobacteriaceae, *Aeromonas* spp. were commonly isolated from ESBL and CRE plates and represented 91 (24.73%) of all stream isolates, 10 (55.56%) influent isolates, and 3 (23.08%) effluent isolates. Eight stream isolates of *Aeromonas hydrophila/caviae* complex were positive for *bla*TEM gene and five positive for *bla*CTX-M. All *Aeromonas* isolates positive for *bla*CTX-M were also positive for *bla*TEM (carried both genes) (Table S4). Four influent *Aeromonas* isolates carried the *bla* genes CTX-M, KPC, and/or SHV. No *Aeromonas* isolates were tested for phenotypic antibiotic susceptibility.

Environmental Correlates and Predictors of ESBL-E and CRE.

Fecal indicator bacteria. *E. coli* concentrations were not significantly correlated with the presence/absence of Enterobacteriaceae isolates carrying *bla* genes across stream sites, nor were they significantly correlated with the presence/absence of Enterobacteriaceae carrying multiple (2+) *bla* genes. Moreover, *E. coli* concentrations were not significantly correlated with presence/absence of Enterobacteriaceae ESBL-producer phenotype or carbapenem resistance phenotype among Enterobacteriaceae. There was no relationship between *E. coli* concentration and the maximum number of detected *bla* genes per sample. Based on an ordinal logistic regression univariate model, there was no relationship between *E. coli* concentration and the maximum number of detected *bla* genes per sample.

Samples with *E. coli* concentrations exceeding the EPA STV were not significantly associated with presence of detected *bla* genes or ESBL-E or CRE phenotype in samples from both ESBL and CRE media. However, there was a significantly higher frequency of exceedance of the EPA STV in the summer and fall sampling dates (see *Sampling date* subsection below).

***Impervious surfaces*.** Percent impervious surfaces were not significantly correlated with the presence/absence of Enterobacteriaceae isolates carrying *bla* genes across stream sites, nor were they significantly correlated with the presence/absence of Enterobactericeaece carrying multiple (2+) *bla* genes. Moreover, percent impervious surfaces were not significantly correlated with presence/absence of Enterobacteriaceae ESBL producer phenotype or carbapenem resistance phenotype among Enterobacteriaceae.

***Sampling date*.** The frequency of Enterobacteriaceae carrying 1+ *bla* gene was significantly higher than expected in the April and July ($\chi 2$ =14.236, df = 3, p-value = 0.003, Table 2.5). Similarly, *E. coli* concentrations were more likely to exceed 410 CFU 100 mL^{-1} in April and July (χ^2 = 9.031, df = 3, p-value = 0.02888, Table 2.6). However, phenotypic ESBL-E had a higher frequency of detection in January and November ($\chi 2$ = 20.5, df = 3, p-value < 0.001, Table 2.7). There was no significant association between sampling date and frequency of detection of phenotypic CRE or between sampling date and frequency of samples containing isolates carrying ≥2 *bla* genes for either CRE or ESBL-E.

Most (80%) of the *bla*-gene-carrying Enterobacteriaceae isolates in influent and effluent were detected in the April and July sampling dates (no WRF samples were collected in November 2018). Regarding possible transfer of AR pathogens from influent to effluent, we did not isolate the same species carrying the same *bla*-genes in both the influent and effluent of a single WRF on a single sampling date.

Site. Enterobacteriaceae isolates carrying ≥2 *bla* genes were derived from 16 samples across 12 surface water sites, and found at all dates. Those 12 sites were: BICO101, MIDO103, MIDO609, MIDO712, MIDO719, MIDO802, MIDO804, MIDO826, NORO114, NORO527, NORO609, and NORO615, spanning 8 watersheds. From seven sites, we isolated both ESBL-E and CRE from the same sample/sampling date. From ten sites, we isolated ESBL-E and/or CRE on two or more sampling dates, and from three sites (BICO101, MIDO719, NOROINF), we isolated ESBL-E on three sampling dates.

Discussion

Streams and rivers are vital resources for drinking water and recreation. Today, we face worldwide dissemination of antibiotic resistant microbial pathogens, antimicrobial drugs, and ARG pollution in waterbodies (Li et al. 2014; Schwab et al. 2005; Zhang et al. 2009). Research is needed to monitor environmental waters as potential reservoirs for rapidly emerging AR threats, such as ESBL-producing and carbapenem-resistant Enterobacteriaceae. The overall objective of this study was to elucidate the prevalence, distribution, and diversity of extended-spectrum beta-lactam and carbapenem resistance within a mixed-use watershed. This is a highly impacted watershed interlinked with urban, agricultural, and industrial land usage (Fisher et al. 2000; Fisher et al. 2001; Cho et al. 2018). Although not a predictor of ESBL or CRE isolates (or carriage of *bla* genes), *E. coli* levels were high across this watershed and followed similar seasonal patterns as noted for the AR isolates. Out of 162 water samples, 53 (32.72%) (from 31 of 54 stream sites) exceeded the EPA recommended threshold level. AR was also high, with isolates carrying one or more *bla* genes detected in 102 water samples (60.36%). Confirmed ESBL-E isolates were detected in 38/169 samples, and CRE in 22/169 water samples. In surface

waters, fecal indicator concentrations were greatest in July and April, frequency of ESBL-E isolates was greatest in January and November, and frequency of CRE isolates was greatest (although not significantly) in November and July sampling dates.

ESBL-producing Enterobacteriaceae. The most frequently isolated Enterobacteriaceae species from the Upper Oconee Watershed was *Serratia fonticola* with almost all isolates carrying the CTX-M ESBL gene. *Serratia fonticola* has been shown to intrinsically carry beta-lactamase enzymes similar to *bla*CTX-M (62-75% identity) (Bonnet 2004; Peduzzi et al. 1997), which explains the high rate of CTX-M detected in our isolates. We detected *Serratia fonticola* in predominantly three seasons: January through July, but not in November of 2018, and the ESBL-producing phenotype was seen predominantly in the winter sampling date. *Serratia fonticola*, first described by Gavini et al. (1979), are naturally found in freshwater. *Serratia* species are opportunistic and capable of secreting several virulence factors, making most of them clinically significant pathogens if present in infection (Kurz et al. 2003; Mahlen 2011). *S. fonticola* are underrepresented in the number of clinical reports of infection with *Serratia* spp. (Aljorayid et al. 2016); however, it is possible they serve as environmental donors of ARG to human pathogens, such as during quorum sensing and biofilm formation (Houdt et al. 2007). The capability of *S. fonticola* isolates detected in this study to transfer ARG is uncertain, as the location (chromosomal or plasmid) of the *bla*CTX-M gene in these isolates was not determined.

After *Serratia fonticola*, the most abundant Enterobacteriaceae taxa confirmed as ESBL-E were *E. coli*, *Citrobacter* spp., and *Klebsiella* spp. Only those *E. coli* isolates that carried *bla*CTX-M were phenotypic ESBL-producers. Castanheira et al. (2008) surveyed US medical centers for clinical prevalence of β-lactamase in Enterobacteriaceae and reported that the

majority of recovered *bla*CTX-M genes were in *E. coli* (25/28 isolates), collected from 12 of 15 (80%) medical centers spanning the contiguous United States. In China, Wu et al. (2018) isolated ESBL-producing *E. coli* from chickens, in which the most commonly isolated ESBL-encoding gene was *bla*CTX-M-type (92.7%). In Chinese river sediments, Lu et al. (2010) uncovered predominantly *bla*CTX-M as ESBL gene in Enterobacteriaceae. This finding is in concordance with our study, as *bla*CTX-M was detected in 78.49% (135/172) of our Enterobacteriaceae surface water isolates.

Although three *Citrobacter* species (*C. braakii, freundii,* and *sedlakii*) were isolated on ESBL medium, none of the *C. sedlakii* isolates carried *bla* genes tested in this study. It is likely that isolates of this species and others that grew on ESBL or CRE media without detected *bla* genes did indeed carry *bla* genes that were not targeted in this study. For example, the *C. sedlakii* isolates may have carried the *bla*SED-1 gene, which encodes a Class A beta-lactamase and has partial homology with CTX-M enzymes (Naas et al. 2008; Petrella et al. 2001; Edelstein et al. 2003).

Non-Enterobacteriaceae isolates carrying *bla* genes were also frequently recovered in this watershed (e.g., *Aeromonas hydrophila/caviae, Chryseobacterium gleum*, and *Pseudomonas oryzihabitans*). These species are commonly found in environmental freshwaters and can serve as donors of AR genes, via horizontal gene transfer, to Enterobacteriaceae known to be human pathogens; however the environmental conditions required for this process to occur are unclear (Read and Woods 2014). Environmental studies suggest that the environment has long been a reservoir for ARG; however, not until recently has there been evidence of ARG mobilization into human pathogens (Marti et al. 2014). Heightened ARG have frequently been detected in aquatic environments impacted by anthropogenic impacts (Chen et al. 2013; Pruden et al. 2012).

Furthermore, even low concentrations of antibiotic and other pharmaceutical pollutants in waters can lead to upregulation and horizontal transfer of ARG (Baharoglu and Mazel 2011; Bernier and Surette 2013). The release of anthropogenic chemicals should not be ignored as a trigger for dissemination of ARG. The environment, combined with accidental introduction of waste containing human pathogens and chemicals, has great potential as conduit for AR.

Carbapenem-Resistant Enterobacteriaceae. The *Enterobacter cloacae* complex (ECC) stream isolates (n=18) (sites=13) from derived from CHROMagar mSuperCarba carried *bla*IMI-NmcA, which encodes class A serine carbapanemases. Carbapenem- and multi-drug-resistant ECC have globally increased in incidence, but rarely has ECC carrying *bla* genes IMI-NmcA been isolated from the USA, let alone the aquatic environment (Fernandez et al. 2011; Davin-Regli and Pagès 2015; Annavajhala et al. 2019). In 2003, the first report of isolation of NmcA carbapenem-hydrolyzing enzyme from a clinical isolate of ECC in North America was published (Pottumarthy et al. 2003). We are witnessing a global emergence of carbapenem-resistant ECC infections, largely due to a plethora of clonal lineages, overexpression of AmpC/inhibition of membrane permeability, and uptake of carbapenemase genes in plasmids (Annavajhala et al. 2019). However, little research (with the exception of Aubron et al. 2005) has been published on *Enterobacter cloacae* complex species carrying IMI-NmcA carbapenemase genes in river systems; this study, to our knowledge, is one of the first to capture its prevalence in surface waters across a watershed.

We recovered one (non-carbapenem-hydrolyzing) *E. coli* stream isolate from CRE-selective agar that carried the KPC and TEM *bla* genes however was not confirmed as resistant to imipenem/meropenem. Globally, carbapenemase-producing *E. coli* have been isolated from

hospital patients, food animals, and wastewater (Navon-Venezia et al. 2006; Hoelle et al. 2019). Navon-Venezia et al. (2006) isolated KPC-2 imipenem-hydrolyzing enzyme from four medical patients in Israel in *E. coli* clones that originated from USA. Hoelle et al. (2019) isolated 322 AR *E. coli* from US wastewater effluent, of which 65 were imipenem-resistant. Of those 65 isolates, 62% were positive for one or more and 32% were positive for two or more carbapenemase and ESBL genes, of which *bla*VIM and *bla*KPC were most common (Hoelle et al. 2019). In 2012, a *bla*KPC-2 gene was detected in *E. coli* in a Portugal river system (Poirel et al. 2012). Our finding of a carbapenemase-gene-carrying *E. coli* isolate in a stream, although not common, is not unprecedented. Non-*Klebsiella* Enterobacteriacae carrying *bla*KPC are becoming more frequently documented, in both environmental and clinical samples (Poirel et al. 2012; Xu et al. 2015; Arnold et al. 2011).

Like the presumptive ESBL-producers, *K. pneumoniae* cultured on CHROMagar mSuperCarba (n=2 isolates) carried *bla*KPC and/or *bla*SHV regardless of sample type (stream, influent, effluent), but neither were phenotypically confirmed as carbapenem resistant. *Klebsiella pneumoniae* carbapenemase (KPC) was first identified in the USA in 1996 (Yigit et al. 2001). Since then, the *bla*KPC has spread among Gram-negative organisms and over international borders (Munoz-Price et al. 2013). Bacteria carrying these enzymes can hydrolyze penicillins, all cephalosporins, monobactams, carbapenems, and β-lactamase inhibitors (Munoz-Price et al. 2013; Papp-Wallace et al. 2010), making treatment difficult. In 2011-2012, Galler et al. (2013) isolated a *K. pneumoniae* isolate carrying *bla*KPC-2 from Austrian wastewater, and in 2011, Oliveira et al. (2013) isolated 3 *bla*KPC-2-producing *K. pneumoniae* from two urban rivers in Brazil. Although *bla*KPC-producing *K. pneumoniae* isolates have been collected in river systems in other regions of the world (Oliveira et al. 2013; Jelic et al. 2013), reports of its presence in

rivers are not as common in the United States. However, in 2016, Mathys et al. (2019) recovered four *bla*KPC-producing *K. pneumoniae* isolates in surface waters in the vicinity of wastewater treatment plants in the Nevada (1 upstream of plant), Mississippi (1 downstream), and Michigan (1 up- and 1 downstream) states. Our study confirms the presence of *bla*KPC in Enterobacteriaceae in environmental surface waters.

Wastewater. We did not culture the same organism carrying the same *bla* gene(s) twice (both influend and effluent) in the same plant and sampling date. This indicates either that we isolated too few isolates (2-3 colonies per sample) to effectively detect the same organism before and after WRF treatment, or that an organism originally present in the influent was indeed killed by the WRFs' disinfection systems, equipped with an aeration basin, biosolid accumulation clarifier, and ultra-violet light disinfectant system (ACC Public Utilities Department 2019). We did, however, culture 26 isolates, 8 of which were from effluent, that carried at least 1 *bla* gene. Three of these effluent organisms were confirmed as ESBL-producers (Two *K. pneumoniae* carrying *bla*SHV and/or *bla*KPC and one *Citrobacter* sp. carrying *bla*SHV), three as carbapenem-resistant (ECC and *E. asburiae* carrying *bla*KPC and ECC carrying *bla*IMI-NmcA). ESBL-E and CRE have been isolated from treated wastewaters worldwide, including in Algeria, Croatia, and Spain (Alouache et al. 2014; Hrenovic et al. 2017; Ojer-Usoz et al. 2014). In the United States, Hoelle et al. (2019) collected 322 *E. coli* isolates from secondary effluent of seven geographically dispersed WRFs and found that 20% (65/322) were resistant to imipenem and 73% (235/322) were multi-drug resistant. The release of AR microbes into a river system can have downstream effects on the gene possession of freshwater microbial communities, by introducing selective AR genes to environmental bacteria (Amos et al. 2014), in particular in

streambed microbial biofilms (Proia et al. 2016; Lehmann et al. 2016). Furthermore, besides microbes, pharmaceuticals may enter a freshwater body through WRF effluent and accumulate in river biofilms, triggering horizontal gene transfer (Aubertheau et al. 2017; Kristiansson et al. 2011; Huerta et al. 2016).

Temporal and impervious surface effects on AR patterns. Although limited to a single year, in this study system, summer (July) was the most impacted sampling date. *E. coli* concentrations, percent of sites exceeding the EPA threshold for *E. coli*, and presence of *bla* genes in Enterobacteriaceae were all significantly elevated (and presence of CRE in samples non-significantly elevated) in the summer collection. Winter was underrepresented in these contamination metrics. However, detection of ESBL-E in surface water samples was significantly highest in the winter sampling date. Our study area included a large university and a population that shifts in predictable patterns, most notably a decrease in population in the summer months. Therefore, this putative seasonal trend is not likely associated with increased inputs to WRFs or septic systems, but rather to other environmental factors, such as seasonal changes in surface water temperature.

One hypothesis to explain this seasonal phenomenon (summer elevation of *E. coli* and ARG) is that temperature may enhance the environmental growth and transfer of ARG in pathogens (Gautam et al. 2011). Another hypothesis is the differing flow rates (and transport of sediments) between seasons. In a Cuban river system, Knapp et al. (2018) found an increased ARG transport rate downstream in the wet season (high flow rate) compared to the dry season (low flow rate). Furthermore, spatial ARG distribution in the water column was more even in the wet season compared to dry season (Knapp et al. 2018). Although our study was too short in

duration to draw seasonal conclusons, the findings in literature that temperature/seasonal variation have an effect on ARG prevalence suggest a need for accounting seasonality into antibiotic resistance dissemination models. Generally, MacFadden et al. (2018) found that a 10 ^{o} C increase in ambient temperature from one city to another in the United States was associated with 4.2% and 2.2% increases in the percent of reported clinical cases of *E. coli* and *Klebsiella pneumoniae* not susceptible to a particular antibiotic, and these associations were consistent across most classes of antibiotics. The authors provided possible explanations for this phenomenon, including that temperature facilitates bacterial HGT and environmental growth and that temperature is associated with complex human social behaviors. Although not completely understood, change in environmental factors are linked to change in dissemination of AR and clinical cases of infection.

Conclusion

This study investigated the prevalence and diversity of ESBL-producing Enterobacteriaceae (ESBL-E) and carbapenem-resistant Enterobacteriaceae (CRE) throughout a United States watershed and in the influent and effluent of three wastewater reclamation facilities serving a municipality within that watershed. Most frequently detected ESBL-E in freshwater streams were *Serratia fonticola* (*bla*CTX-M), *E. coli* (*bla*CTX-M), and *Citrobacter* (*bla*CMY-2, but not phenotypically confirmed as ESBL-producing). Wastewater effluent did not have an abundance of phenotypically confirmed ESBL-E, with the exception of two *K. pneumoniae* isolates carrying *bla*SHV and one *Enterobacter asburiae* isolate carrying *bla*KPC that was also confirmed to be resistant to imipenem. The most frequently detected CRE in freshwater streams were *Enterobacter cloacae* complex (ECC) (*bla*IMI-NmcA), and in

wastewater effluent the phenotypically confirmed CRE isolates were ECC (*bla*IMI-NmcA and *bla*KPC) and *E. asburiae* (*bla*KPC). It is rare to detect *bla*IMI-NmcA in ECC, especially in a river system. Summer was the season most impacted by high (≥410 CFU 100 ml^{-1}) *E. coli* concentrations as well as high abundance of Enterobacteriaceae isolates carrying at least 1 targeted *bla* gene in both presumptive ESBL and CRE isolates. Winter was underrepresented in these contamination metrics and spring and fall seasons varied by selective (ESBL or CRE) agar medium. Winter was the season with significantly highest frequencies of ESBL-E detection; while, CRE were most frequently detected in fall and summer. Fecal and ARG contamination within this watershed is high and varies by sampling date. In coming years when studying the ecology of antibiotic resistance, we should acknowledge the environment as a large reservoir of ARG, dynamically changing with environmental factors.

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Table 2.1. Number and percent of stream samples (n=169) with isolated bacteria suspected to have phenotypic and genotypic resistance to beta lactams, including carbapenems. Bacteria were isolated on CHROMagar ESBL and CHROMagar mSuperCARBA. Carbapenemase genes (carbapenem-hydrolyzing beta-lactamases) tested were KPC, NDM-1, IMI-NmcA, IMP, and VIM, of which we detected KPC and IMI-NmcA.

Season	# of samples	% samples with Enterobacteriaceae isolate(s)	% samples with Enterobacteriaceae isolate(s) carrying at least 1 *bla* gene	% samples with Enterobacteriaceae isolate(s) carrying at least 1 *bla* gene and confirmed to have ESBL-producing phenotype	% samples with Enterobacteriacaeae isolate(s) carrying at least 1 *bla* gene and confirmed to have carbapenem-resistant phenotype
W18	42	11.83	11.24	11.24	1.18
SP18	40	18.34	17.16	2.37	1.78
SM18	43	19.53	19.53	2.37	4.73
F18	44	16.57	12.43	6.51	5.33
All Seasons Combined (# or %)	169	66.27	60.36	22.49	13.02

Table 2.2. Presumptive ESBL-producers and suspect carbapenem resistant Enterobacterieaceae in wastewater influent and effluent. Influent and effluent isolates that **1)** *were identified as Enterobacteriaceae* & **2)** *carried at least 1 of the 11 targeted bla genes* were tested for phenotypic antibiotic susceptibility using MIC breakpoints set by CLSI and NARMS (n = 12 from wastewater influent and n = 8 from wastewater effluent; no wastewater samples were collected in Fall 2018).
Enterobacteriaceae isolates were confirmed as ESBL-producing if **1)** *resistant to cefotaxime and/or ceftazidime* & **2)** *impeded by cefotaxime + clavulanic acid and/or ceftazidime + clavulanic acid.*

Water Reclamation Facility	# of Enterobacteriaceae isolates	# of Enterobacteriaceae isolates carrying at least 1 *bla* gene	# of Enterobacteriaceae isolates carrying at least 1 *bla* gene and confirmed as ESBL-producing	# of Enterobacteriaceae isolates carrying at least 1 *bla* gene and confirmed as carbapenem-resistant
A				
Influent	7	7	4	0
Effluent	5	2	0	1
B				
Influent	6	6	1	0
Effluent	7	5	2	2
C				
Influent	5	5	4	0
Effluent	1	1	0	0
Grand Total				
Influent	18	18	9	0
Effluent	13	8	2	3

Table 2.3. Taxonomic diversity, presence of beta-lactamase (*bla*) genes, and phenotypic AR of bacterial isolates (n=368) selected from CHROMagar ESBL and CHROMagar mSuperCARBA. All isolates (n=368) were screened for beta-lactamase (*bla*) genes (CMY-2, CTX-M, KPC*, OXA-1, SHV, TEM, NDM-1*, IMI-NmcA*, IMP*, VEB, VIM*). Carbapenemase genes (carbapenem-hydrolyzing beta-lactamases) are denoted by *. No NDM-1, VEB, VIM, or IMI-NmcA beta-lactamase genes were detected. Only Enterobacteriaceae isolates carrying 1+ *bla* genes were tested for ESBL-producing phenotype.

	# of isolates	# of isolates confirmed with *bla* gene(s)	# of isolates with >1 *bla* gene	# of isolates with >2 *bla* genes	*bla* genes							# of isolates confirmed to have ESBL-E phenotype	# of isolates confirmed to have CRE phenotype
					CMY-2	CTX-M	KPC*	OXA-1	SHV	TEM	IMI-NmcA*		
Enterobacteriaceae organisms													
Low discrimination/*Citrobacter braakii* or *freundii*	1	1	0	0	0	**1**	0	0	0	0	0	0	0
Citrobacter braakii	3	2	0	0	0	**2**	0	0	0	0	0	0	0
Citrobacter freundii	5	5	0	0	**5**	0	0	0	0	0	0	0	0
Citrobacter sedlakii	4	0	0	0	0	0	0	0	0	0	0	-	-
Enterobacter asburiae	1	0	0	0	0	0	0	0	0	0	0	-	-
Enterobacter cancerogenus	2	0	0	0	0	0	0	0	0	0	0	-	-
Enterobacter cloacae complex	26	19	5	0	0	0	0	0	**1**	**1**	**18**	1	18
Escherichia coli	23	23	14	4	**7**	**21**	**1**	**1**	**1**	**11**	0	21	0
Klebsiella oxytoca	1	1	1	0	0	0	**1**	0	0	**1**	0	0	0
Klebsiella pneumoniae	5	5	3	2	0	**1**	**3**	**1**	**4**	**2**	**0**	2	1
Raoultella planticola	2	2	1	1	0	**1**	0	**1**	**1**	**1**	**0**	2	0
Serratia fonticola	121	110	0	0	0	**111**	0	0	0	0	0	21	6
SUBTOTAL:	**194**	**168**	**24**	**7**	**12**	**137**	**5**	**3**	**7**	**16**	**18**	**47**	**25**
non-Enterobacteriaceae organisms													
Acinetobacter baumanii complex	5	0	0	0	0	0	0	0	0	0	0	-	-
Aeromonas hydrophila/caviae	75	7	5	0	0	5	0	0	0	8	0	-	-
Aeromonas sobria	15	0	0	0	0	0	0	0	0	0	0	-	-
Aeromonas sobria/veronii	1	0	0	0	0	0	0	0	0	0	0	-	-
Burkholderia cepacia group	5	0	0	0	0	0	0	0	0	0	0	-	-
Burkholderia gladioli	1	0	0	0	0	0	0	0	0	0	0	-	-
Chromobacterium violaceum	7	1	0	0	0	1	0	0	0	0	0	-	-
Chryseobacterium gleum	15	2	0	0	0	1	0	0	1	0	0	-	-
Low discrimination/ *Pseudomonas aeruginosa or fluorescens or putida*	4	0	0	0	0	0	0	0	0	0	0	-	-
Pseudomonas aeruginosa	16	0	0	0	0	0	0	0	0	0	0	-	-
Pseudomonas fluorescens	2	0	0	0	0	0	0	0	0	0	0	-	-
Pseudomonas oryzihabitans	9	1	0	0	0	1	0	0	0	0	0	-	-
Pseudomonas putida	1	0	0	0	0	0	0	0	0	0	0	-	-
Sphingobacterium spiritivorum	1	0	0	0	0	0	0	0	0	0	0	-	-
Sphingomonas paucimobilis	1	0	0	0	0	0	0	0	0	0	0	-	-
Stenotrophomonas maltophilia	15	0	0	0	0	0	0	0	0	0	0	-	-
Unidentified	1	0	0	0	0	0	0	0	0	0	0	-	-
SUBTOTAL:	**174**	**11**	**5**	**0**	**0**	**8**	**0**	**0**	**1**	**8**	**0**	**-**	**-**
GRAND TOTAL:	**368**	**179**	**29**	**7**	**12**	**145**	**5**	**3**	**8**	**24**	**18**	**47**	**25**

Table 2.4. Phenotypic Antibiotic Susceptibility Results for Carbapenem-Resistant *Enterobacter cloacae* complex (ECC) stream isolates. Imipenem and Meropenem are members of the carbapenem class of antibiotics. Piperacillin-tazobactam is occasionally used as an alternative to carbapenems for infections with ESBL-producing bacteria (Pilmis et al. 2017). IMIPEN = Imipenem; MEROPE = Meropenem; PIPTAZ = Piperacillin-tazobactam

Season	Stream Site	Organism ID	*bla* Gene	IMIPEN MIC (µg/mL)	Organism Susceptibility to IMIPEN	MEROPE MIC (µg/mL)	Organism Suscpetibility to MEROPE	PIPTAZ MIC (µg/mL)	Organism Susceptibility to PIPTAZ
SM18	BICO 101	ECC	ImI-NmcA	> 16	RESIST	= 2	INTER	<= 4	SUSC
SM18	MIDO 605	ECC	ImI-NmcA	= 8	RESIST	= 2	INTER	<= 4	SUSC
SM18	MIDO 605	ECC	ImI-NmcA	> 16	RESIST	= 8	RESIST	<= 4	SUSC
SM18	MIDO 608	ECC	ImI-NmcA	> 16	RESIST	> 8	RESIST	<= 4	SUSC
SM18	MIDO 610	ECC	ImI-NmcA	= 16	RESIST	<= 1	SUSC	<= 4	SUSC
SM18	NORO 520	ECC	ImI-NmcA	> 16	RESIST	= 8	RESIST	<= 4	SUSC
SM18	NORO 527	ECC	ImI-NmcA	= 16	RESIST	= 8	RESIST	<= 4	SUSC
F18	BICO 101	ECC	ImI-NmcA	= 16	RESIST	= 8	RESIST	<= 4	SUSC
F18	MIDO 609	ECC	ImI-NmcA	> 16	RESIST	> 8	RESIST	<= 4	SUSC
F18	MIDO 609	ECC	ImI-NmcA	> 16	RESIST	= 8	RESIST	<= 4	SUSC
F18	MIDO 719	ECC	ImI-NmcA	> 16	RESIST	> 8	RESIST	<= 4	SUSC
F18	MIDO 802	ECC	ImI-NmcA	> 16	RESIST	= 4	RESIST	<= 4	SUSC
F18	MIDO 826	ECC	ImI-NmcA	> 16	RESIST	= 4	RESIST	<= 4	SUSC
F18	NORO 514	ECC	ImI-NmcA	> 16	RESIST	= 8	RESIST	<= 4	SUSC
F18	NORO 514	ECC	ImI-NmcA	> 16	RESIST	= 4	RESIST	<= 4	SUSC
F18	NORO 527	ECC	ImI-NmcA	= 16	RESIST	= 2	INTER	<= 4	SUSC
F18	NORO 615	ECC	ImI-NmcA	> 16	RESIST	= 8	RESIST	<= 4	SUSC
F18	Lake Herrick	ECC	ImI-NmcA	> 16	RESIST	= 8	RESIST	<= 4	SUSC

Table 2.5. Contingency table for chi-squared comparison between sampling date and presence of *bla* genes across samples (169 samples [χ^2 = 14.236, df = 3, p-value = 0.003]). All values displayed are outcomes of presence.

Season	Observed	Expected
January	19	25.349
April	29	24.142
July	33	25.953
November	21	26.556

Table 2.6. Contingency table for chi-squared comparison between sampling date and presence of *E. coli* exceedance of 410 CFU threshold across samples (161 samples [$\chi^2 = 9.031$, df = 3, p-value = 0.029]. All values displayed are outcome of presence.

Season	Observed	Expected
January	9	12.671
April	16	12.671
July	19	13.621
November	7	12.037

Table 2.7. Contingency table for chi-squared comparison between sampling date and presence of phenotypic ESBL-producing Enterobacteriaceae across samples (169 samples [$\chi^2 = 20.5$, df = 3, p-value < 0.01]). All values displayed are outcomes of presence.

Season	Observed	Expected
January	19	9.444
April	4	8.994
July	4	9.669
November	11	9.893

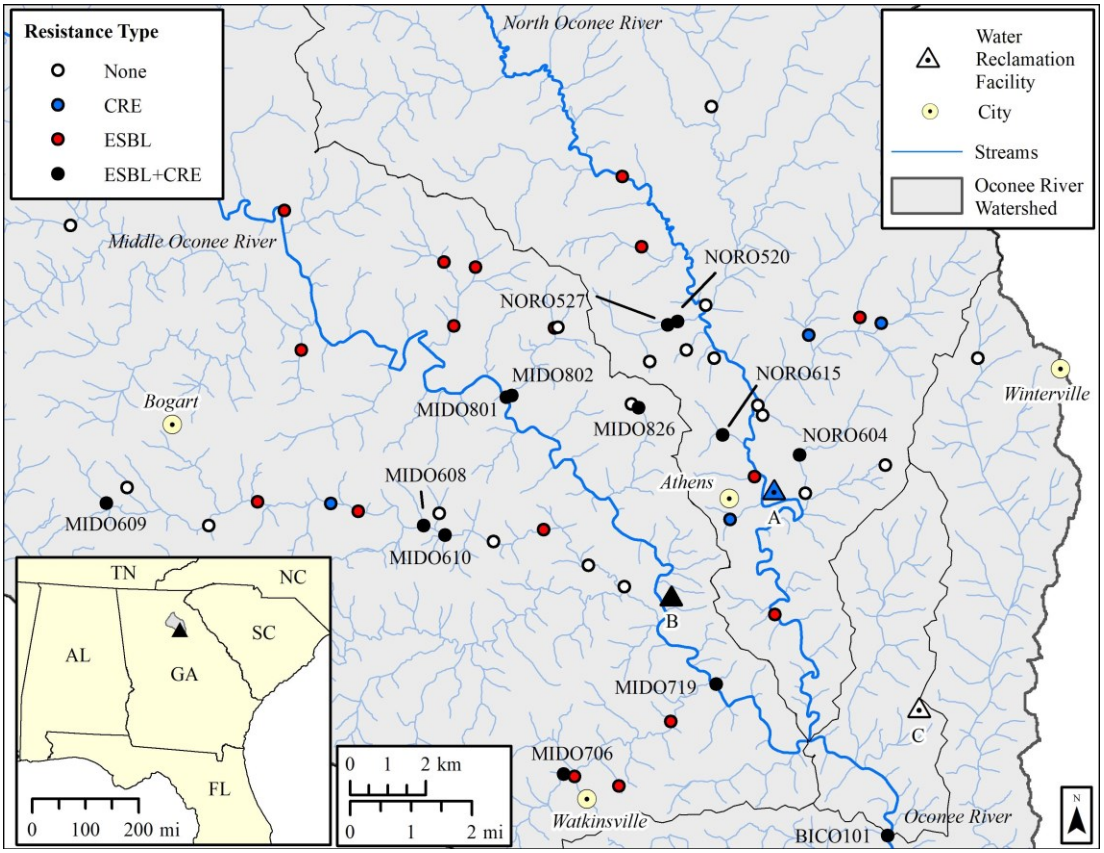

Figure 2.1. Map of stream and WRF sites sampled in this study. White = No ESBL-producing Enterobacteriaceae (ESBL-E) or carbapenem-resistant Enterobacteriaceae (CRE) detected in effluent (n=9). Blue = CRE detected in effluent. Red = ESBL-E detected in effluent. Black = Both ESBL-E and CRE detected in effluent.

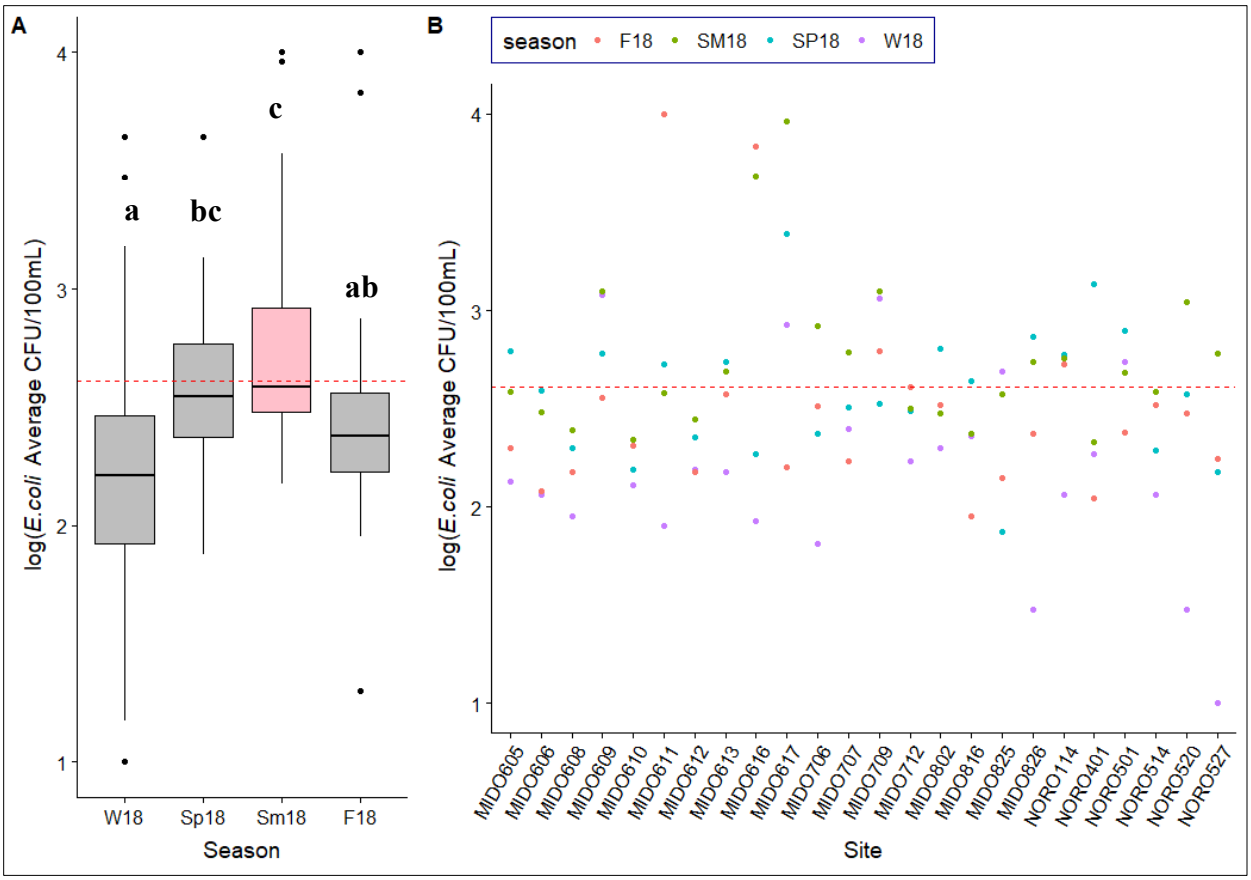

Figure 2.2. **A-** Box and Whisker plot of log(average *E. coli* CFU) for each of 54 surface water sites sampled in winter (W18), spring (Sp18), summer (Sm18), and fall (F18). Summer sites on average exceeded the US EPA's recommended Standard Threshold Value of 410 *E. coli* CFU/100 mL STV (pink), suggesting seasonal shifts in contamination throughout this watershed. For both figures, a red dashed line denotes this threshold.
B- Scatter plot visualization of *E. coli* Avg. CFU/100 mL for the 24 sites sampled 4 sampling dates (colors key to salmon = winter, green = spring, cyan = summer, lavender = fall).

Table S1. *bla* gene primer sequences, amplicon sizes, literature reference, and primer concentration.

Primer	Sequence (5' - 3')	Amplicon size (base pairs)	Reference	Primer concentration
CMY-2-F	GACAGCCTCTTTCTCCAC	1000 bp	Zhao et al. (2001)	10 µm
CMY-2-R	TGGAACGAAGGCTACG			
CTX-M-F	CGCTTTGCGATGTGCAG	550 bp	Bonnet et al. (2003)	5 µm
CTX-M-R	ACCGCGATATCGTTGGT			
KPC-F	ATGTCACTGTATCGCCGTC	863 bp	Fernando et al. (2016);	1 µm
KPC-R	AATCCCTCGAGCGCGAGT		Mulvey et al. (2011)	
NDM-F	GGTGCATGCCCGGTGAAATC	660 bp	Fernando et al. (2016);	1 µm
NDM-R	ATGCTGGCCTTGGGGAACG		Mulvey et al. (2011)	
OXA-1-F	TATCTACAGCAGCGCCAGTG	199 bp	Féria et al. (2002)	1 µm
OXA-1-R	CGCATCAAATGCCATAAGTG			
SHV-F	AGGATTGACTGCCTTTTTG	393 bp	Colom et al. 2003	10 µm
SHV-R	ATTTGCTGATTTCGCTCG			
TEM-F	TTCTTGAAGACGAAAGGGC	1150 bp	Brinas et al. (2002)	10 µm
TEM-R	ACGCTCAGTGGAACGAAAAC			
IMI-NmcA-F	GGTGTCTACGCTTTAGACACTGGCTC	536 bp	Voets et al. (2011)	10 µm
IMI-NmcA-R	GCACGAATACGCGCTGCACCGG			
IMP-F	CATGGTTTGGTGGTTCTTGT	528 bp	Yousefi et al. (2010)	10 µm
IMP-R	GTAMGTTTCAAGAGTGATGC			
VEB-F	CATTTCCCGATGCAAAGCGT	648 bp	Dallenne et al. (2010)	1 µm
VEB-R	CGAAGTTTCTTTGGACTCTG			
VIM-F	GTTTGGTCGCATATCGCAAC	645 bp	Fernando et al. (2016);	10 µm
VIM-R	AATGCGCAGCACCAGGATAGAA		Mulvey et al. (2011)	

Table S2. Thermal cycler reaction conditions and Master Mix concentrations per four samples. **Row 1:** corresponding to *bla* genes CMY-2, CTX-M, KPC, NDM-1, OXA-1, SHV, and VEB. Boxed sections of reaction conditions in Row 1 were programmed as 30 cycles. **Row 2:** corresponding to *bla* genes TEM, IMP, VIM, and IMI-NmcA. Boxed sections of reaction conditions in Row 2 were programmed as 25 cycles.

	CMY-2	**CTX-M**	**KPC**	**NDM-1**	**OXA-1**	**SHV**	**VEB**
	95° for 15 min	95° for 15 min	95° for 15 min	95° for 15 min	95° for 15 min	95° for 15 min	94° for 10 min
	94° for 1 min	95° for 1 min	94° for 30 sec	94° for 30 sec	94° for 1 min	94° for 1 min	94° for 40 sec
1	61° for 1 min	54° for 1 min	60° for 1 min 30 sec	60° for 1 min 30 sec	62° for 1 min	62° for 1 min	60° for 40 sec
	72° for 1 min	72° for 1 min	72° for 1 min	72° for 1 min	72° for 1 min	72° for 1 min	72° for 1 min
	72° for 10 min	72° for 10 min	72° for 7 min	72° for 7 min	72° for 10 min	72° for 10 min	72° for 7 min
	4° forever	4° forever	4° forever	4° forever	4° forever	4° forever	4° forever
dH2O	72.34µl	72.34 µl	76.4µl	76.4µl	72.34µl	72.34 µl	76 µl
30mM MgCl	6.66µl	6.66µl	6.66µl	6.66µl	6.66µl	6.66µl	5µl
F & R primer	4µl	4µl	2µl	2µl	4µl	4µl	3µl
dNTPs	2µl	2µl	2µl	2µl	2µl	2µl	2µl
Taq	1µl	1µl	1µl	1µl	1µl	1µl	1µl

	TEM	**IMP**	**VIM**	**IMI-NmcA**
	95° for 15 min	95° for 4 min	95° for 4 min	94° for 1 min
	95° for 1 min	94° for 1 min	94° for 1 min	94° for 30 sec
2	55° for 1 min	56° for 1 min	56° for 1 min	60° for 40 sec
	72° for 2 min	72° for 45 sec	72° for 45 sec	72° for 1 min
	72° for 5 min	72° for 7 min	72° for 7 min	72° for 1 min
	4° forever	4° forever	4° forever	4° forever
dH2O	72.34µl	76µl	77.1µl	dH2O: 73.1
30mM MgCl	6.66µl	5µl	8.3µl	DMSO: 5µl
F & R primer	4µl	3µl	0.8µl	30mM MgCl: 5µl
dNTPs	2µl	2µl	2µl	F primer: 2.0µl
Taq	1µl	1µl	1µl	R primer: 2.4µl
				dNTPs: 2µl
				Taq: 1µl

Table S3. Taxonomic diversity, presence of *bla* genes, and presence ESBL-producing Enterobacteriaceae (ESBL-E) and CRE phenotype in isolates from wastewater reclamation facility effluent.

	# of isolates	# of isolates confirmed with *bla* gene(s)	# of isolates with >1 *bla* gene	# of isolates with >2 *bla* genes	*bla* genes							# of isolates confirmed to have ESBL-E phenotype	# of isolates confirmed to have CRE phenotype
					CMY-2	CTX-M	KPC*	OXA-1	SHV	TEM	IMI-NmcA*		
Enterobacteriaceae organisms													
Citrobacter braakii/freundii	1	1	1	0	0	**1**	0	0	**1**	0	0	1	0
Enterobacter asburiae	1	1	0	0	0	0	**1**	0	0	0	0	0	1
Enterobacter cloacae complex	4	2	0	0	0	0	**1**	0	0	0	**1**	0	2
Escherichia coli	2	0	0	0	0	0	0	0	0	0	0	0	0
Klebsiella pneumoniae	4	4	0	0	0	0	**1**	0	**3**	0	0	2	0
SUBTOTAL:	**12**	**8**	**1**	**0**	**0**	**1**	**3**	**0**	**4**	**0**	**1**	**3**	**3**
non-Enterobacteriaceae organisms													
Acinetobacter baumanii complex	1	0	0	0	0	0	0	0	0	0	0	0	0
Aeromonas hydrophila/caviae	2	0	0	0	0	0	0	0	0	0	0	0	0
Aeromonas sobria	1	0	0	0	0	0	0	0	0	0	0	0	0
Burkholderia cepacia group	1	0	0	0	0	0	0	0	0	0	0	0	0
Stenotrophomonas maltophilia	1	0	0	0	0	0	0	0	0	0	0	0	0
SUBTOTAL:	**6**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**-**	**-**
GRAND TOTAL:	**18**	**8**	**1**	**0**	**0**	**1**	**3**	**0**	**4**	**0**	**1**	**3**	**3**

Table S4. See page 95 for "Supplementary file of isolates recovered from Upper Oconee Watershed."
1 indicates an attribute was detected, and 0 indicates an attribute was not detected for attributes Enterobacteriaceae, ESBL-E (ESBL-producing Enterobacteriaceae), and CRE (carbapenm-resistant Enterobacteriaceae). AR pattern legend: ampicillin (AMP), cefazolin (FAZ), cefepime (FEP), cefotaxime (FOT), cefoxitin (FOX), Cefpodoxime (POD), ceftazidime (TAZ), ceftriaxone (AXO), cephalexin (PHA), ciprofloxacin (CIP), gentamicin (GEN), imipenem (IMI), meropenem (MER), piperacillin/tazobactam (P/T4).

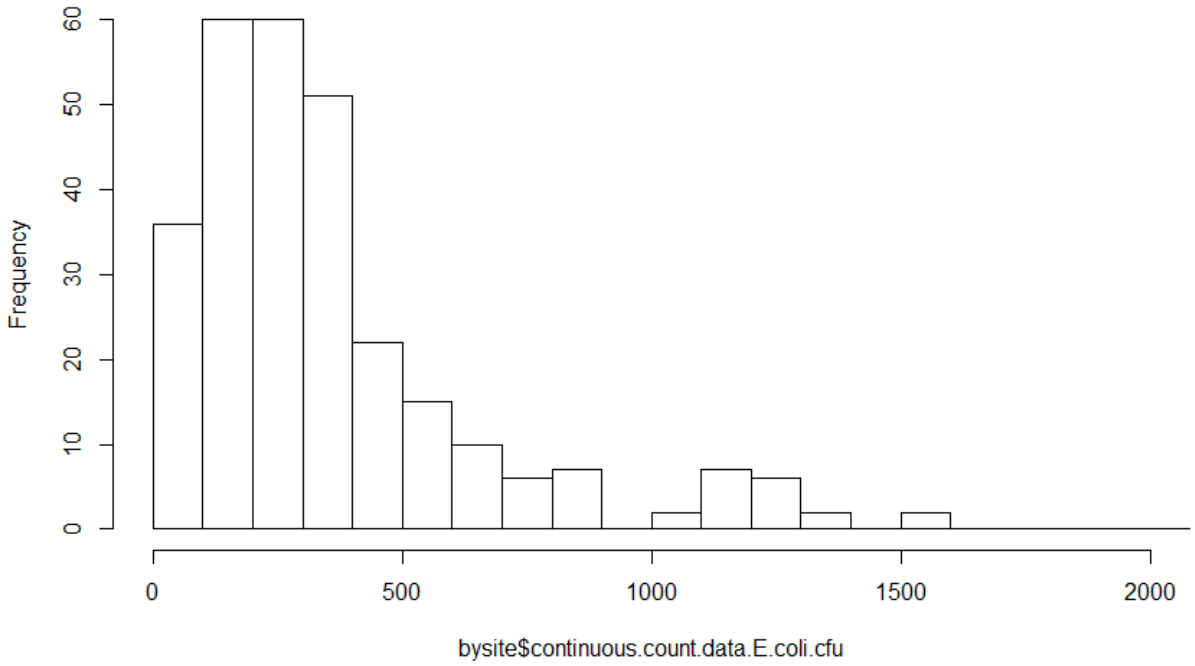

Figure S1. Histogram depicting the frequency of *E. coli* counts over 307 stream and river samples in the Upper Oconee Watershed, GA, USA, spanning 4 sampling dates in 2018.

CHAPTER III

CONCLUSIONS

This study is one of the first few to observe and characterize ESBL-producing and carbapenem-resistant bacteria across an entire watershed. In a partnership with the Upper Oconee Watershed Network, a non-profit devoted to water-quality monitoring in the Upper Oconee Watershed, we were capable of sampling 54 stream sites within a 4 hr window, which, on multiple occasions, allowed for a geographically-broad sampling event within a short time frame. On four sampling dates in 2018, water samples were collected from 40-44 stream sites (54 total), resulting in 169 surface water samples for analysis. In addition, on three of the four sampling dates, influent and effluent samples were collected from three water reclamation facilities (WRF)s, resulting in 18 total WRF samples.

Fecal contamination was approximated by the concentration of *E. coli* fecal indicator bacterium, which ranged from 10 to over 10,000 CFU 100 ml^{-1} in surface waters and undetectable to 6,200 CFU 100 ml^{-1} in effluent samples. Concentrations in July were significantly higher than in January or November. EPA's recommended STV, above which suggests an unacceptable level of risk for gastrointestinal illness, is 410 CFU 100 ml^{-1}, and was exceeded in 32.92% (53/162) of surface water samples and 22.22% (2/9) of WRF effluent samples.

Bacterial isolates obtained from two media types carrying at least one *bla* gene were collected from 102 different surface water samples, and those carrying two or more *bla* genes were isolated from 15 samples, spanning 12 stream sites. Additionally, isolates carrying at least

one *bla* gene were isolated from each of five WRF effluent samples, but only one effluent isolate carried multiple *bla* genes. Surface water Enterobacteriaceae isolates carrying *bla* genes of any quantity were identified as *Serratia fonticola* (*bla*CTX-M), *Enterobacter cloacae* complex (*bla*IMI-NmcA), *E. coli* (*bla*CMY-2, *bla*CTX-M, *bla*OXA-1, *bla*SHV, *bla*TEM), *K. pneumoniae* (*bla*CTX-M, *bla*KPC, *bla*OXA-1, *bla*SHV, *bla*TEM), *Klebsiella oxytoca* (*bla*KPC and *bla*TEM), *R. planticola* (*bla*CTX-M, *bla*OXA-1, *bla*SHV, *bla*TEM), and *Citrobacter* species (*bla*CMY-2 and *bla*CTX-M). Whether these *bla* genes were intrinsic to the environmental microbes or acquired by other microbes that may have originated from fecal matter is uncertain; however, these data establish a baseline for future AR studies in this watershed.

Phenotypically-confirmed ESBL-E were isolated from 22.49% (38/169) of surface water samples and phenotypically confirmed CRE isolated from 13.02% (22/169) of surface water samples. We isolated ESBL-E in two or three sampling dates from three sites. If this study were to extend past this one year, focus should be placed on these three sites in particular, as they are known to repeatedly carry Enterobacteriaceae resistant to a broad spectrum of beta-lactams. Regarding effluent, one WRF sample carried ESBL-producing *K. pneumoniae* and three effluent samples carried carbapenem-resistant *Enterobacter* spp.

Sampling date had an effect on the frequency of *E. coli* and presence of *bla* genes in streams (with frequency highest in summer month). In contrast, the highest frequency of detection of ESBL-E was in the winter sampling date. While the study period of only one year makes it difficult to draw firm conclusions about the role of season, we suspect that water temperature may play a part in the replication of enteric pathogens, and ARG abundance, in the aquatic environment.

Future work

In future studies, effort should be extended into the sediments of streams and rivers, as here is where there may be concentrated amounts of microbes, living in biofilms rather than planktonic lifestyle. During disturbance events, e.g., heavy rainfall and/or flash floods, the sediment particulates and associated microbes can be suspended into the water column, changing the perceived prevalence of ARG and ARG-carrying pathogens in that water body. This study encapsulates the surface layer of waters; however, recreators who swim do come into contact with the benthic layer and whatever resides there. To form a holistic picture of AR ecology, we must begin to understand the interactions between antibiotics, ARG, and ARG-carrying pathogens and the bottom-dwelling, sometimes anaerobic, environment of the benthos.

Moderate levels of pharmaceuticals, even sub-inhibitory levels of antibiotics, can induce a stress response in microbes and speed up the transfer of plasmid-encoding AR genes, especially if bacteria accumulate into a biofilm (Salcedo et al. 2015; Balcazar et al. 2015). One characteristic of a biofilm is the secretion of an extra-cellular polysaccharide matrix; this could help protect microbes from external stressors (Balcazar et al. 2015). Additionally, the microbes residing in the center of a microbial clump could better shield from external stressors, e.g., antibiotics, compared to living out a planktonic, freely suspended lifestyle. The close proximity of cells within a biofilm also contributes to the transfer of ARG, a process that can be prompted by pharmaceutical contaminants in the water. Most likely, since antibiotics tend to exist at sub-clinical concentrations in waters, the antibiotics serve as signaling molecules more so than actual killing molecules. These signaling molecules trigger several intra- and inter-cellular processes, including inter-cell communications (quorum-sensing), the formation of biofilms, gene transcription and expression, and synthesis of microbial virulence factors, among other cascade

effects (Balcazar et al. 2015, An et al. 2019). In order to better understand the cascade effects of WRF effluent discharge into waters, we should look not only at the surface waters but the sediments of these waters, whose contents are re-suspended into the water column when disturbed.

A tool separate from the culture method used in this study that explores gene expression of microbes is metatranscriptomics. The contribution of the microbiome to human physiology remains elusive. Lesser known is the contribution of such microbiomes to aquatic ecosystem dynamics. In future studies, the expression, not solely the presence, of *bla* genes should be studied to be paired with phenotypic susceptibility tests (Rowe et al. 2017). This would more holistically capture the linkage between bacterial response to stressors and genes' service as templates for tolerance or resistance.

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APPENDIX A

HISTORICAL FECAL CONTAMINATION IN UPPER OCONEE WATERSHED

In October 2017-December 2018, the dynamics of fecal contamination was studied intensively. *E. coli* was cultured from 9 stream sites (8 urbanized and 1 reference) in the UOW, at a high frequency of 2-3 times per month. The results indicated dynamic but consistently high *E. coli* levels throughout the 15-month period, with average monthly *E. coli* exceeding the EPA's recommended 410 CFU 100 mL^{-1} in 11 of the 15 months (Figure A1). When *E. coli* was averaged per site over the 15-month period, each of the 9 sites exceeded the 410 CFU threshold (Figure A2). This established that there is fecal contamination in a subset of urbanized streams in the UOW and prompted further investigation into the spread of human-pathogenic microbes and AMR in this river network.

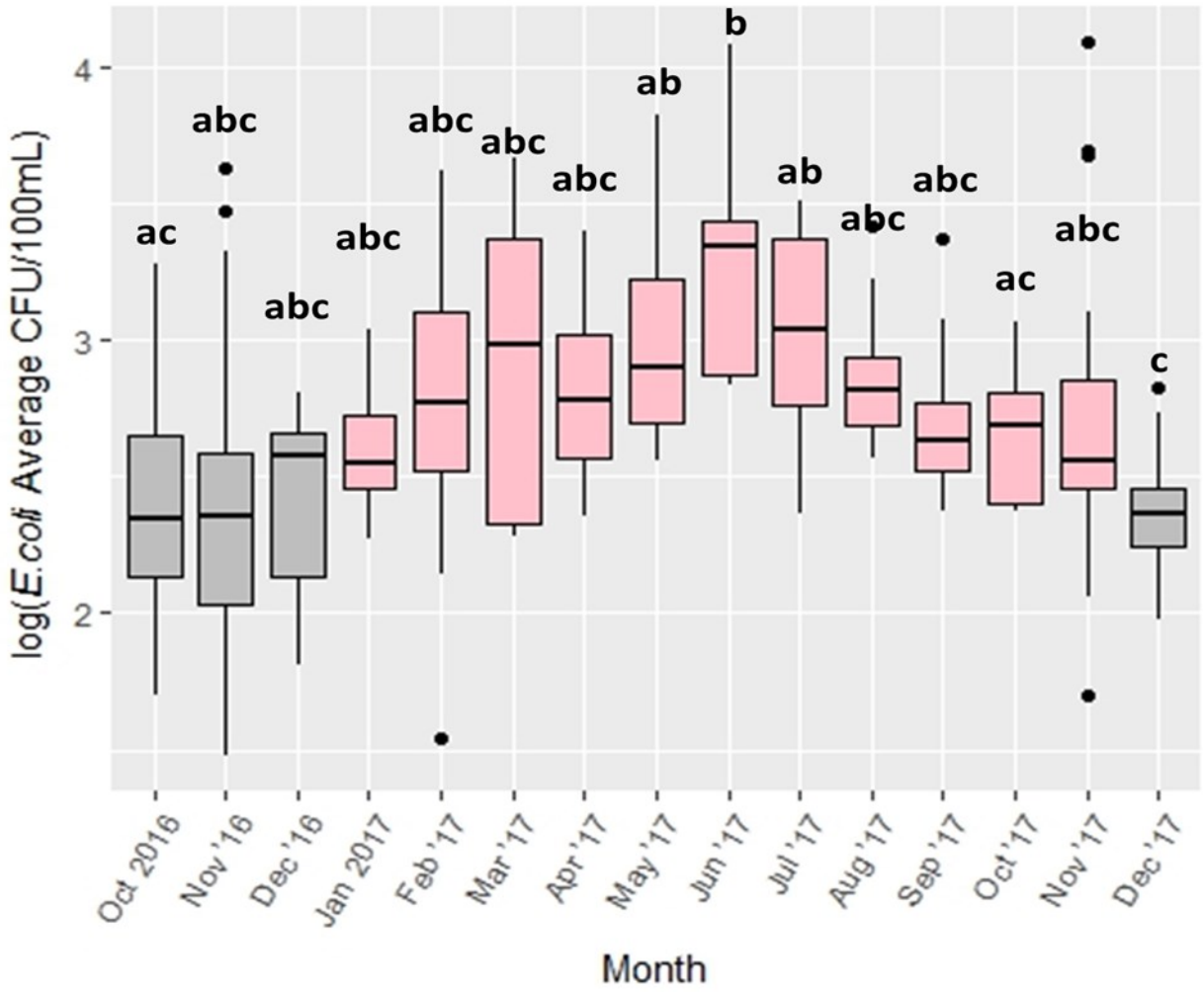

Figure A1. Box and whisker plot of average monthly log(E. coli Average CFU/100mL water) in stream surface waters. Samples were collected biweekly (Oct 2016 – Dec 2017) at 9 sites over an urban gradient. Grey indicates *E. coli* levels below the EPA recommended standard threshold value of 410 CFU $100mL^{-1}$ for recreational exposure. Pink indicates *E. coli* levels that exceeded this threshold (EPA 2012 Recreational Water Quality Criteria). Summer months had significantly higher levels of *E. coli*.

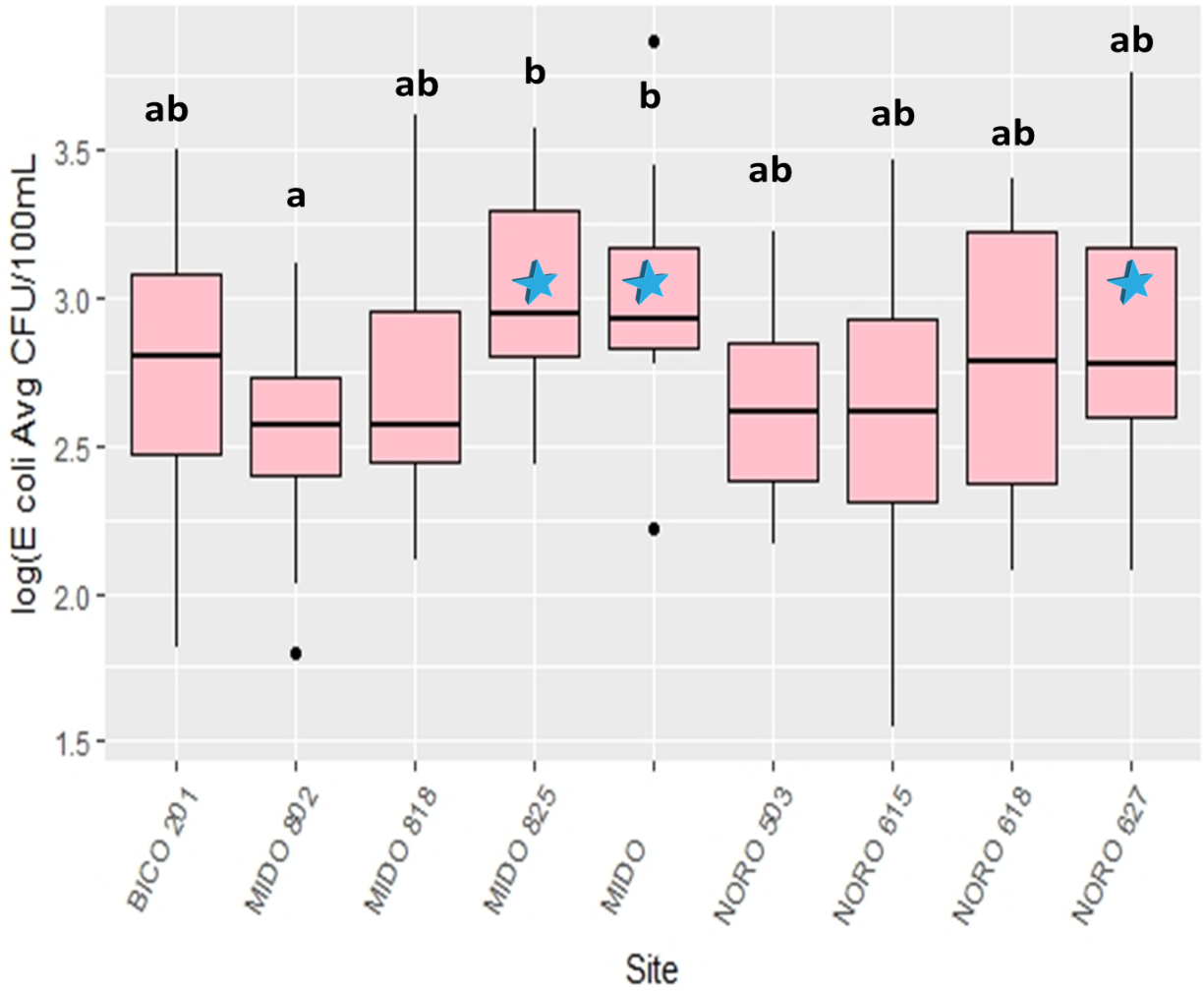

Figure A2. Box and whisker plot depicting log(E. coli Average CFU/100mL water) for each of 9 stream sites over a 15-month period. MIDO 825, MIDO (Hospital creek), and NORO 627 had 15-month average *E. coli* levels that exceeded 1000 CFU/100mL and are indicated by [blue star].

APPENDIX B

FECAL INDICATOR ALTERNATIVES TO *E. COLI*

E. coli is commonly used as a fecal indicator bacterium for freshwater bodies (USEPA RWQC 2012). AR in water bodies may be associated with human fecal pollution (Karkman et al. 2019). In this study, *E. coli* did not serve as a good predictor of AR. If we want to employ a human-specific predictor of fecal contamination, then we should instead look to microbial source tracking (MST). Human fecal contamination markers, e.g., bacteriophages crAssphage and ϕB124-14 that infect *Bacteroides*, could be studied as predictors of both human-sourced fecal contamination and human-sourced AR pathogens (Karkman et al. 2019). The class 1 integron (CL1) integrase gene has also been used as a proxy for anthropogenic pollution and ARG abundance (Gillings et al. 2015), but this gene may be co-selected with ARG (Karkman et al. 2019), suggesting lack of independence between the two.

Compared to the use of bacteria as human fecal indicators, advantages of using phage are prolonged environmental persistence, high abundance, and ability to replicate within the bacterial host cell (Ogilvie et al. 2018; Gomez-Donate et al. 2011). Unlike the bacterial microbiome, the human virome largely consists of unknown genomic sequences, inviting ample opportunities for discovery of novel viruses and earning the human virome the colloquial name "biological dark matter" (Dutilh et al. 2014; Mokili et al. 2012; Ogilvie et al. 2012). A couple of viral bacteriophages that have received attention are crAssphage and ϕB124-14. CrAssphage infects bacteria *Bacteroides intestinales* and is the most abundant virus in the human gut (Guerin et al. 2018). ϕB124-14 infects a subset of human gut-associated *Bacteroides fragilis* strains

(Ogilvie et al. 2012). Both have no other homologues in our historical record of viral genomes and seem to be highly specific to their host cells. Although ϕB124-14 was originally associated with a generalized mammalian gut microbiome (human, porcine, bovine), target regions have since been refined to those found in only human (Ogilvie et al. 2018). Together, crAssphage and particular regions of ϕB124-14 can be used to target human-specific and livestock-specific sources of fecal contamination (Karkman et al. 2019).

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APPENDIX C

FINANCIAL COSTS OF ANTIBIOTIC RESISTANCE AND ALTERNATIVES TO ANTIBIOTICS

Potential Economic Consequences of Clinical Antibiotic Resistant Infections

Today there are, due to rapid evolution of antibiotic resistance, people whose infections cannot be treated with any of our antibiotics. The etiological agents driving these infections have evolved defense mechanisms to antibiotic chemical attacks. Compared to other pharmaceuticals on the market that are taken for a lifetime, e.g., those that control blood pressure or relieve depression, antibiotics are taken only for a matter of days or weeks, a duration that is not as effective at offsetting the costs of development (Schaes 2010). According to the UN's April 2019 report on the state of affairs of drug resistance, in some countries, 35% of common human infections are resistant to antibiotic therapies. In some middle and low-income countries, 80-90 percent of bacterial infection cases are resistant to some antibiotic therapies (WHO IACG 2019). The magnitude of antibiotic resistance effects on humanity is not well-known, but it is estimated that resistant bacterial infections cause at least 700,000 deaths each year, including 230,000 deaths from MDR-tuberculosis alone (WHO IACG 2019; The Review on Antimicrobial Resistance 2014; WHO 2018). These pathogenic-bacteria-induced death rates are projected to jump to 10 million by the year 2050, based on scenarios modeled by RAND Europe and KPMG (The Review on Antimicrobial Resistance 2014). Discovering new antibiotics is becoming

increasingly challenging and the economics of developing new antibiotics is becoming less and less profitable (Shrestha et al. 2018).

Nosocomial treatment of antibiotic resistant bacterial infections can rack up costs of healthcare. Bartsch et al. (2017) developed a CRE clinical and economic outcomes model, based on infection type, mode of therapy, probability of mortality, and probability of stay in either ICU or general ward, in order to better assess the economic burden of CRE infections in the US (Bartsch et al. 2017). They estimated that, assuming a CRE infection incidence of 2.93 per 100,000 persons and meropenem as the antibiotic of choice, these infections would cost hospitals $275 million, third-party payers $147 million, and society $553 million annually (Bartsch et al. 2017). The cost to society and quality-adjusted life years lost would also depend on mortality rate due to infection (Bartsch et al. 2017).

Communal action to curb resistant infections. Despite these portentous projections, there are several incentives that we as a human populace can deliver to mediate the drug resistance situation. If every household were to donate $2 to the research of antibiotic resistance and development of new drugs, then we could perhaps curb the rapid return to a pre-antibiotic era (OECD 2018). Educating about antibiotic resistance and providing testimonials and case studies in the form of stories can be tools to invoke empathy and possibly provoke action to support prevention and treatment of bacterial infections.

Mitigation of Costly Antibiotic Resistance Effects

Alternatives to antibiotics. Some alternatives to chemical antibiotics are bacteriophage therapy (where a virus infects bacterial cell) and probiotic therapy to establish mutualistic bacteria that dilute and compete with the pathogen bacteria (Imperial and Ibana 2016). However, not much

research has yet been done for either treatment plan. Few phages have been isolated and characterized thus far; however, they are some of the most abundant genetic agents on Earth (Hendrix et al. 1999; Chan et al. 2013), with host ranges from narrow to quite broad (Hyman and Abedon 2010). To treat AR-infections such as diabetes mellitus foot infections and cystic fibrosis, researchers have effectively developed cocktails that mix multiple phages together (Mendes et al. 2014; Morello et al. 2011). Probiotics, mainly of the genera *Lactobacillus* and *Bifidobacterium*, have been used to treat various infections, particularly infections of mucosal surfaces such as the gut and vagina (Nami et al. 2015). However, despite their potentially beneficial health effects, probiotics may also serve as conduits of antibiotic resistance spread, through exchange of mobile antibiotic resistance genes (Imperial and Ibana 2016). With more research and greater dispersion of education campaigns, perhaps the modern human mindset regarding infectious disease can shift from a chemical warfare to more of a park management, where we cultivate the microbes on and within us as if we as hosts are the managers of a park (Costello et al. 2012).

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Enterobacteriace

Sampling date	Media	Site	Organism	ae	bla genes	AR pattern
Winter 2018	ESBL	BICO 101	Escherichia coli	1	CMYCTXTEM	AMPFAZFOTFOXPODTAZAXOCEPCIPGEN
Winter 2018	CRE	BICO 101	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	CRE	BICO 201	Burkholderia cepacia group	0	None	Not tested
Winter 2018	CRE	CC EFF	Burkholderia cepacia group	0	None	Not tested
Winter 2018	CRE	CC EFF	Stenotrophonomonas maltophilia	0	None	Not tested
Winter 2018	ESBL	CC INF	Escherichia coli	1	CMYOXA	AMPFAZFOTFOXPODTAZAXOCEPCIPGEN
Winter 2018	ESBL	CC INF	Aeromonas caviae or hydrophila	0	None	Not tested
Winter 2018	ESBL	MIDO 301	Serratia fonticola	1	CTX	AMPFAZFOTPODAXOCEP
Winter 2018	ESBL	MIDO 601	Serratia fonticola	1	CTX	AMPFAZFOTPODAXOCEP
Winter 2018	ESBL	MIDO 601	Serratia fonticola	1	CTX	AMPFAZFOTPODAXOCEP
Winter 2018	ESBL	MIDO 606	Serratia fonticola	1	CTX	AMPFAZFOTFOXPODXOCEP
Winter 2018	CRE	MIDO 608	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	CRE	MIDO 608	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	ESBL	MIDO 609	Serratia fonticola	1	CTX	AMPFAZFOTPODAXOCEP
Winter 2018	CRE	MIDO 609	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	ESBL	MIDO 609	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	CRE	MIDO 609	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	ESBL	MIDO 610	Serratia fonticola	1	CTX	AMPFAZFEPFOTFOXPODAXOCEP
Winter 2018	ESBL	MIDO 611	Serratia fonticola	1	CTX	AMPFAZFOTPODAXOCEP
Winter 2018	ESBL	MIDO 611	Pseudomonas fluorescens	0	None	Not tested
Winter 2018	ESBL	MIDO 612	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	CRE	MIDO 612	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	ESBL	MIDO 612	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	MIDO 613	Pseudomonas aeruginosa or fluorescens	0	None	Not tested
Winter 2018	ESBL	MIDO 616	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	MIDO 617	Pseudomonas aeruginosa/fluorescens	0	None	Not tested
Winter 2018	CRE	MIDO 706	Burkholderia cepacia group	0	None	Not tested
Winter 2018	ESBL	MIDO 706	Serratia fonticola	1	CTX	AMPFAZFOTPODXOCEP
Winter 2018	ESBL	MIDO 707	Serratia fonticola	1	CTX	AMPFAZFOTFOXPODAXOCEP

Winter 2018	ESBL	MIDO 707	Serratia fonticola	1	CTX	AMPFAZFOTPODAXOCEP
Winter 2018	ESBL	MIDO 709	Serratia fonticola	1	CTX	AMPFAZFOTFOXPODAXOCEP
Winter 2018	ESBL	MIDO 709	Pseudomonas oryzihabitans	0	None	Not tested
Winter 2018	ESBL	MIDO 712	Pseudomonas fluorescens	0	None	Not tested
Winter 2018	ESBL	MIDO 719	Serratia fonticola	1	CTX	AMPFAZFOTPODAXOCEP
Winter 2018	ESBL	MIDO 719	Pseudomonas oryzihabitans	0	CTX	Not tested
Winter 2018	ESBL	MIDO 719	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	MIDO 801	Serratia fonticola	1	CTX	AMPFAZFOTPODAXOCEPIMI
Winter 2018	ESBL	MIDO 801	Pseudomonas oryzihabitans	0	None	Not tested
Winter 2018	ESBL	MIDO 802	Serratia fonticola	1	CTX	AMPFAZFOTFOXPODAXOCEP
Winter 2018	ESBL	MIDO 816	Serratia fonticola	1	CTX	AMPFAZFOTFOXPODAXOCEP
Winter 2018	ESBL	MIDO 817	Serratia fonticola	1	CTX	AMPFAZFOTFOXPODAXOCEP
Winter 2018	CRE	MIDO 817	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	ESBL	MIDO 817	Serratia fonticola	1	CTX	AMPFAZFEPFOTFOXPODTAZAXOCEP
Winter 2018	ESBL	MIDO 820	Serratia fonticola	1	CTX	AMPFAZFOTFOXPODAXOCEP
Winter 2018	ESBL	MIDO 820	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	MIDO 820	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	MIDO 825	Pseudomonas aeruginosa or putida	0	None	Not tested
Winter 2018	ESBL	MIDO EFF	Enterobacter asburiae	1	KPC	AMPFAZFOTFOXPODAXOCEP
Winter 2018	ESBL	MIDO EFF	Aeromonas caviae or hydrophila	0	None	Not tested
Winter 2018	ESBL	MIDO EFF	Enterobacter cloacae complex	1	None	Not tested
Winter 2018	ESBL	MIDO EFF	Enterobacter cloacae complex	1	None	Not tested
Winter 2018	CRE	MIDO INF	Citrobacter freundii	1	KPCSHV	AMPFAZFOTPODAXOCEPCIP
Winter 2018	ESBL	MIDO INF	Aeromonas caviae or hydrophila	0	None	Not tested
Winter 2018	ESBL	MIDO INF	Aeromonas caviae or hydrophila	0	KPC	Not tested
Winter 2018	ESBL	NORO 114	Pseudomonas oryzihabitans	0	None	Not tested
Winter 2018	CRE	NORO 114	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	ESBL	NORO 114	Serratia fonticola	1	CTX	AMPFAZFOTFOXPODAXOCEP
Winter 2018	ESBL	NORO 401	Pseudomonas oryzihabitans	0	None	Not tested
Winter 2018	CRE	NORO 501	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	ESBL	NORO 502	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	NORO 502	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	NORO 511	Serratia fonticola	1	CTX	AMPFAZFOTFOXPODAXOCEP
Winter 2018	ESBL	NORO 517	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	NORO 518	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	NORO 520	Enterobacter asburiae	1	None	Not tested

Winter 2018	ESBL	NORO 527	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	NORO 527	Pseudomonas oryzihabitans	0	None	Not tested
Winter 2018	CRE	NORO 603	Burkholderia cepacia group	0	None	Not tested
Winter 2018	ESBL	NORO 603	Pseudomonas aeruginosa/fluorescens/putida	0	None	Not tested
Winter 2018	ESBL	NORO 603	Pseudomonas oryzihabitans	0	None	Not tested
Winter 2018	ESBL	NORO 604	Serratia fonticola	1	CTX	AMPFAZFOTPODAXOCEPIMI
Winter 2018	CRE	NORO 604	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	ESBL	NORO 604	Pseudomonas oryzihabitans	0	None	Not tested
Winter 2018	CRE	NORO 604	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	ESBL	NORO 611	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	NORO 611	Pseudomonas aeruginosa	0	None	Not tested
Winter 2018	ESBL	NORO EFF	Escherichia coli	1	None	Not tested
Winter 2018	ESBL	NORO EFF	Acinetobacter baumannii complex	0	None	Not tested
Winter 2018	ESBL	NORO INF	Escherichia coli	1	CTXTEM	AMPFAZFOTFOXPODAXOCEP
Winter 2018	ESBL	NORO INF	Stenotrophomonas maltophilia	0	None	Not tested
Winter 2018	CRE	NORO INF	Stenotrophonomonas maltophilia	0	None	Not tested
Summer 2018	CRE	BICO 101	Aeromonas hydrophila/caviae	0	TEM	Not tested
Summer 2018	ESBL	BICO 101	Chryseobacterium gleum	0	None	Not tested
Summer 2018	CRE	BICO 101	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMI
Summer 2018	ESBL	BICO 101	Escherichia coli	1	CTXSHVTEM	AMPFAZFOTFOXPODTAZAXOCEPCIP
Summer 2018	CRE	CC EFF	Aeromonas sobria	0	None	Not tested
Summer 2018	ESBL	CC EFF	negative		NA	Not tested
Summer 2018	ESBL	CC INF	Escherichia coli	1	CTXOXA	AMPFAZFOTPODAXOCEPCIP
Summer 2018	ESBL	CC INF	Klebsiella pneumoniae	1	SHV	AMPFAZFOTPODTAZAXOCEPGEN
Summer 2018	CRE	CC INF	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	CRE	MIDO 103	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	CRE	MIDO 103	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	MIDO 103	Serratia fonticola	1	CTX	FAZ
Summer 2018	ESBL	MIDO 103	Escherichia coli	1	CMYCTX	AMPFAZFOTPODAXOCEP
Summer 2018	CRE	MIDO 305	negative	0	NA	Not tested
Summer 2018	ESBL	MIDO 305	Chryseobacterium gleum	0	None	Not tested
Summer 2018	ESBL	MIDO 305	Chryseobacterium gleum	0	None	Not tested

Summer 2018	CRE	MIDO 604	negative	0	NA	Not tested
Summer 2018	ESBL	MIDO 604	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 604	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	ESBL	MIDO 605	Chryseobacterium gleum	0	CTX	Not tested
Summer 2018	CRE	MIDO 605	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMI
Summer 2018	CRE	MIDO 605	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER
Summer 2018	ESBL	MIDO 605	Serratia fonticola	1	None	Not tested
Summer 2018	ESBL	MIDO 606	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 606	Burkholderia cepacia group	0	None	Not tested
Summer 2018	CRE	MIDO 608	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER
Summer 2018	ESBL	MIDO 608	Burkholderia cepacia group	0	None	Not tested
Summer 2018	ESBL	MIDO 608	Serratia fonticola	1	CTX	FAZ
Summer 2018	ESBL	MIDO 609	Serratia fonticola	1	CTX	AMPFAZPODCEP
Summer 2018	ESBL	MIDO 609	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 609	Sphingobacterium spiritivorum	0	None	Not tested
Summer 2018	CRE	MIDO 610	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMI
Summer 2018	ESBL	MIDO 610	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 610	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	ESBL	MIDO 611	Chryseobacterium gleum	0	None	Not tested
Summer 2018	ESBL	MIDO 611	Unidentified	0	None	Not tested
Summer 2018	ESBL	MIDO 612	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 612	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	CRE	MIDO 613	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	MIDO 613	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 613	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 616	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 616	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 617	Serratia fonticola	1	CTX	AMPFAZCEPIMI
Summer 2018	ESBL	MIDO 617	negative	0	NA	Not tested

Summer 2018	CRE	MIDO 706	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	MIDO 706	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 706	Serratia fonticola	1	CTX	AMPFAZPODAXOCEPIMI
Summer 2018	CRE	MIDO 707	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	MIDO 707	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	ESBL	MIDO 707	Stenotrophomonas maltophilia	0	None	Not tested
Summer 2018	ESBL	MIDO 707	Chryseobacterium gleum	0	None	Not tested
Summer 2018	ESBL	MIDO 709	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 709	Sphingomonas paucimobilis	0	None	Not tested
Summer 2018	CRE	MIDO 712	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	MIDO 712	Chryseobacterium gleum	0	None	Not tested
Summer 2018	ESBL	MIDO 712	negative	0	NA	Not tested
Summer 2018	CRE	MIDO 719	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	MIDO 719	Escherichia coli	1	CMYCTX	AMPFAZFOTPODAXOCEP
Summer 2018	ESBL	MIDO 719	negative	0	NA	Not tested
Summer 2018	ESBL	MIDO 801	Chryseobacterium gleum	0	None	Not tested
Summer 2018	ESBL	MIDO 801	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	CRE	MIDO 802	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	MIDO 802	Klebsiella pneumoniae	1	CTXOXASHVTEM	AMPFAZFOTPODAXOCEPGEN
Summer 2018	ESBL	MIDO 802	Pseudomonas aeruginosa	0	None	Not tested
Summer 2018	ESBL	MIDO 802	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	CRE	MIDO 804	Acinetobacter baumannii complex	0	None	Not tested
Summer 2018	ESBL	MIDO 804	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	ESBL	MIDO 804	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	CRE	MIDO 816	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	MIDO 816	negative	0	NA	Not tested
Summer 2018	ESBL	MIDO 816	negative	0	NA	Not tested
Summer 2018	ESBL	MIDO 817	Serratia fonticola	1	None	Not tested

Summer 2018	ESBL	MIDO 817	Serratia fonticola	1	CTX	AMPFAZAXOCEP
Summer 2018	ESBL	MIDO 820	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 820	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	MIDO 825	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	ESBL	MIDO 825	negative	0	NA	Not tested
Summer 2018	ESBL	MIDO 826	negative	0	NA	Not tested
Summer 2018	ESBL	MIDO 826	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	CRE	MIDO EFF	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	MIDO EFF	Klebsiella pneumoniae	1	KPC	AMPFAZPODCEPCIP
Summer 2018	ESBL	MIDO EFF	Slashline Enterobacter cloacae	1	KPC	AMPFAZFOTFOXPODAXOCEPCIPIMIP/T4
Summer 2018	ESBL	MIDO INF	Enterobacter asburiae	1	KPC	AMPFAZFOXPODCEP
Summer 2018	ESBL	MIDO INF	Escherichia coli	1	CMYCTXOXA	AMPFAZFOTPODTAZAXOCEPCIP
Summer 2018	CRE	MIDO INF	Klebsiella pneumoniae	1	KPC	AMPFAZPODAXOCEP
Summer 2018	CRE	NORO 108	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	NORO 108	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	ESBL	NORO 108	Escherichia coli	1	CTX	AMPFAZFOTPODAXOCEPCIP
Summer 2018	ESBL	NORO 114	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	NORO 114	negative	0	NA	Not tested
Summer 2018	CRE	NORO 401	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	NORO 401	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	ESBL	NORO 401	Serratia fonticola	1	CTX	AMPFAZPODCEP
Summer 2018	CRE	Lake Chapman	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	Lake Chapman	Stenotrophomonas maltophilia	0	None	Not tested
Summer 2018	ESBL	Lake Chapman	Stenotrophomonas maltophilia	0	None	Not tested
Summer 2018	ESBL	NORO 501	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	ESBL	NORO 501	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	NORO 502	negative	0	NA	Not tested
Summer 2018	ESBL	NORO 502	negative	0	NA	Not tested
Summer 2018	ESBL	NORO 503	Aeromonas hydrophila/caviae	0	None	Not tested

Summer 2018	ESBL	NORO 503	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	NORO 510	negative	0	NA	Not tested
Summer 2018	ESBL	NORO 510	negative	0	NA	Not tested
Summer 2018	CRE	NORO 514	Aeromonas sobria	0	None	Not tested
Summer 2018	ESBL	NORO 514	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	NORO 514	Serratia fonticola	1	CTX	AMPFAZPODCEP
Summer 2018	ESBL	NORO 517	negative	0	NA	Not tested
Summer 2018	ESBL	NORO 517	Stenotrophomonas maltophilia	0	None	Not tested
Summer 2018	ESBL	NORO 518	Serratia fonticola	1	CTX	AMPFAZPODCEP
Summer 2018	ESBL	NORO 518	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	CRE	NORO 520	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER
Summer 2018	ESBL	NORO 520	Chryseobacterium gleum	0	None	Not tested
Summer 2018	ESBL	NORO 520	Serratia fonticola	1	CTX	AMPFAZCEP
Summer 2018	ESBL	NORO 527	Chryseobacterium gleum	0	None	Not tested
Summer 2018	CRE	NORO 527	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER
Summer 2018	ESBL	NORO 527	Chryseobacterium gleum	0	None	Not tested
Summer 2018	CRE	NORO 605	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	NORO 605	Chryseobacterium gleum/indologenes	0	None	Not tested
Summer 2018	ESBL	NORO 605	Serratia fonticola	1	CTX	AMPFAZPODCEP
Summer 2018	CRE	NORO 609	Aeromonas hydrophila/caviae	0	None	Not tested
Summer 2018	ESBL	NORO 609	Serratia fonticola	1	CTX	FAZCEP
Summer 2018	ESBL	NORO 609	Chryseobacterium gleum	0	None	Not tested
Summer 2018	ESBL	NORO 615	Chryseobacterium gleum	0	SHV	Not tested
Summer 2018	ESBL	NORO 615	Chyreseobacterium gleum	0	None	Not tested
Summer 2018	CRE	NORO EFF	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER

2018 Summer						
2018 Summer	ESBL	NORO EFF	negative		NA	Not tested
2018 Summer	ESBL	NORO INF	Escherichia coli	1	CMYCTX	AMPFAZFEPFOTPODTAZAXOCEPC P
2018 Summer	CRE	NORO INF	Klebsiella pneumoniae	1	KPC	AMPFAZFOTPODTAZAXOCEPP/T4
2018 Summer	ESBL	NORO INF	Kluyvera cryocrescens	1	CTX	FAZ
2018 Summer	CRE	NORO INF	Slashline Enterobacter cloacae complex	1	KPC	AMPFAZFOTFOXPODTAZAXOCEP
Spring 2018	CRE	BICO 101	Klebsiella pneumoniae	1	KPC	AMPFAZFOTFOXPODTAZAXOCEP
Spring 2018	ESBL	BICO 101	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	ESBL	BICO 101	Acinetobacter baumanii complex	0	None	Not tested
Spring 2018	CRE	BICO 101	Aeromonas sobria	0	None	Not tested
Spring 2018	CRE	CC EFF	Klebsiella pneumoniae	1	SHV	AMP

Spring 2018	ESBL	CC INF	Kluyvera cryocrescens	1	SHV	AMPFAZPODTAZCEP
Spring 2018	ESBL	CC INF	Kluyvera cryocrescens	1	SHV	AMPFAZPODTAZAXOCEP
Spring 2018	CRE	CC INF	Aeromonas caviae or hydrophila	0	None	Not tested
Spring 2018	CRE	CC INF	Aeromonas caviae or hydrophila	0	SHV	Not tested
Spring 2018	CRE	MIDO 103	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	CRE	MIDO 103	Aeromonas sobria	0	None	Not tested
Spring 2018	ESBL	MIDO 103	Pseudomonas aeruginosa	0	None	Not tested
Spring 2018	CRE	MIDO 301	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 301	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 301	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 301	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	MIDO 305	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 305	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 305	Aeromonas sobria	0	None	Not tested
Spring 2018	ESBL	MIDO 305	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	MIDO 601	Acinetobacter baumanii complex	0	None	Not tested

Spring 2018	ESBL	MIDO 601	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 601	Aeromonas sobria	0	None	Not tested
Spring 2018	ESBL	MIDO 601	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 605	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 605	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 605	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 605	Serratia fonticola	1	CTX	AMPFAZPODCEP
Spring 2018	CRE	MIDO 606	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 606	Pseudomonas oryzihabitans	0	None	Not tested
Spring 2018	CRE	MIDO 608	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 608	Serratia fonticola	1	CTX	FAZFOTCEP
Spring 2018	CRE	MIDO 608	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 608	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	MIDO 609	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 609	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	MIDO 609	Aeromonas sobria	0	None	Not tested

Spring 2018	ESBL	MIDO 609	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	ESBL	MIDO 610	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	ESBL	MIDO 610	Serratia fonticola	1	CTX	AMPFAZPODCEP
Spring 2018	CRE	MIDO 611	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 611	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	MIDO 611	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 611	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 612	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 612	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 612	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 612	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	MIDO 613	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	CRE	MIDO 613	Aeromonas sobria	0	None	Not tested
Spring 2018	CRE	MIDO 614	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 614	Chromobacterium violaceum	0	None	Not tested
Spring 2018	CRE	MIDO 614	Aeromonas sobria	0	None	Not tested

Spring 2018	ESBL	MIDO 614	Chromobacterium violaceum	0	None	Not tested
Spring 2018	ESBL	MIDO 616	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 616	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 616	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 617	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 617	Chromobacterium violaceum	0	None	Not tested
Spring 2018	ESBL	MIDO 617	Chromobacterium violaceum	0	None	Not tested
Spring 2018	CRE	MIDO 706	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 706	Serratia fonticola	1	CTX	AMPFAZPODCEP
Spring 2018	CRE	MIDO 706	Aeromonas sobria	0	None	Not tested
Spring 2018	ESBL	MIDO 706	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 707	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 707	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 707	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 707	Serratia fonticola	1	None	Not tested
Spring 2018	CRE	MIDO 709	Aeromonas hydrophila/caviae	0	None	Not tested

Spring 2018	ESBL	MIDO 709	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	ESBL	MIDO 709	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	MIDO 712	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	CRE	MIDO 712	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	CRE	MIDO 802	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 802	Serratia fonticola	1	CTX	AMPFAZPODCEP
Spring 2018	ESBL	MIDO 802	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	MIDO 816	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 816	Serratia fonticola	1	CTX	FAZPODCEP
Spring 2018	CRE	MIDO 816	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 816	Serratia fonticola	1	CTX	FAZCEP
Spring 2018	CRE	MIDO 817	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 817	Serratia fonticola	1	None	Not tested
Spring 2018	CRE	MIDO 817	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 817	Serratia fonticola	1	None	Not tested
Spring 2018	CRE	MIDO 820	Aeromonas hydrophila/caviae	0	None	Not tested

Spring 2018	ESBL	MIDO 820	Serratia fonticola	1	CTX	AMPFAZPODCEF
Spring 2018	CRE	MIDO 820	Aeromonas sobria/veronii	0	None	Not tested
Spring 2018	ESBL	MIDO 820	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	MIDO 825	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 825	Serratia fonticola	1	CTX	AMPFAZPODCEP
Spring 2018	CRE	MIDO 825	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	MIDO 825	Serratia fonticola	1	CTX	AMPFAZPODCEP
Spring 2018	ESBL	MIDO 826	Klebsiella oxytoca	1	KPCTEM	AMPFAZFOTPODTAZAXOCEPCIPPT4
Spring 2018	CRE	MIDO 826	Klebsiella pneumoniae	1	KPCSHVTEM	AMPFAZPODTAZAXOCEPCIP
Spring 2018	CRE	MIDO 826	Escherichia coli	1	KPCTEM	AMPFAZFOTFOXPODTAZAXOCEPCEN
Spring 2018	ESBL	MIDO 826	Klebsiella pneumoniae	1	KPCSHVTEM	AMPFAZCEPIMI
Spring 2018	ESBL	MIDO EFF	Klebsiella pneumoniae	1	SHV	AMPFAZPODTAZAXOCEP
Spring 2018	ESBL	MIDO EFF	Klebsiella pneumoniae	1	SHV	AMPFAZCEPIMI
Spring 2018	ESBL	MIDO INF	Kluyvera ascorbata	1	CTX	AMPFAZFOXPODCEP

Spring 2018	ESBL	MIDO INF	Kluyvera ascorbata	1	None	Not tested
Spring 2018	CRE	MIDO INF	Aeromonas caviae or hydrophila	0	KPC	Not tested
Spring 2018	CRE	MIDO INF	Aeromonas caviae or hydrophila	0	CTXKPC	Not tested
Spring 2018	ESBL	NORO 108	Serratia fonticola	1	None	Not tested
Spring 2018	ESBL	NORO 114	Pseudomonas putida	0	None	Not tested
Spring 2018	CRE	NORO 114	Aeromonas sobria	0	None	Not tested
Spring 2018	ESBL	NORO 114	Serratia fonticola	1	None	Not tested
Spring 2018	CRE	NORO 401	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 401	Chromobacterium violaceum	0	CTX	Not tested
Spring 2018	CRE	NORO 401	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 401	Chromobacterium violaceum	0	None	Not tested
Spring 2018	CRE	Lake Chapman	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	CRE	Lake Chapman	Aeromonas sobria	0	None	Not tested
Spring 2018	ESBL	Lake Chapman	Burkholderia gladioli	0	None	Not tested
Spring 2018	CRE	NORO 501	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 501	Serratia fonticola	1	CTX	AMPFAZPODCEP

Spring 2018	CRE	NORO 501	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 501	Serratia fonticola	1	CTX	Not tested
Spring 2018	CRE	NORO 503	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 503	Serratia fonticola	1	None	Not tested
Spring 2018	CRE	NORO 503	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 503	Serratia fonticola	1	CTX	AMPFAZPODCEPIMI
Spring 2018	CRE	NORO 510	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 510	Serratia fonticola	1	CTX	AMPFAZPODCEP
Spring 2018	CRE	NORO 510	Aeromonas sobria	0	None	Not tested
Spring 2018	ESBL	NORO 510	Serratia fonticola	1	CTX	AMPFAZPODCEP
Spring 2018	CRE	NORO 511	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 511	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	NORO 511	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 511	Serratia fonticola	1	CTX	AMPFAZPODCEP
Spring 2018	ESBL	NORO 514	Serratia fonticola	1	CTX	AMPFAZCEPIMI
Spring 2018	CRE	NORO 514	Aeromonas hydrophila/caviae	0	None	Not tested

Spring 2018	CRE	NORO 514	Aeromonas sobria	0	None	Not tested
Spring 2018	ESBL	NORO 514	Serratia fonticola	1	CTX	AMPFAZPODCEP
Spring 2018	CRE	NORO 520	Enterobacter cloacae complex	1	None	Not tested
Spring 2018	ESBL	NORO 520	Escherichia coli	1	CTX	AMPFAZFOTPODAXOCEP
Spring 2018	CRE	NORO 520	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 520	Escherichia coli	1	CTX	AMPFAZFOTPODCEP
Spring 2018	CRE	NORO 527	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 527	Escherichia coli	1	CTXTEM	AMPFAZFOTPODAXOCEPCIPGEN
Spring 2018	CRE	NORO 527	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 527	Escherichia coli	1	CTXTEM	AMPFAZFOTPODAXOCEPCIPGEN
Spring 2018	CRE	NORO 605	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	CRE	NORO 605	Aeromonas sobria	0	None	Not tested
Spring 2018	ESBL	NORO 605	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	NORO 609	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 609	Serratia fonticola	1	CTX	AMPFAZFOXPODAXOCEP
Spring 2018	CRE	NORO 609	Aeromonas sobria	0	None	Not tested

Spring 2018	ESBL	NORO 609	Serratia fonticola	1	CTX	AMPFAZFOXCEP
Spring 2018	CRE	NORO 615	Acinetobacter baumanii complex	0	None	Not tested
Spring 2018	ESBL	NORO 615	Serratia fonticola	1	CTX	AMPFAZCEP
Spring 2018	CRE	NORO 615	Aeromonas hydrophila/caviae	0	None	Not tested
Spring 2018	ESBL	NORO 615	Serratia fonticola	1	None	Not tested
Spring 2018	CRE	NORO EFF	Citrobacter braakii/freundii	1	CTXSHV	FAZFOXCEP
Spring 2018	CRE	NORO EFF	Escherichia coli	1	None	Not tested
Spring 2018	CRE	NORO INF	Aeromonas sobria	0	None	Not tested
Spring 2018	ESBL	NORO INF	Escherichia coli	1	CTX	AMPFAZFOTPODAXOCEP
Spring 2018	ESBL	NORO INF	Escherichia coli	1	CTX	AMPFAZFOTPODAXOCEP
Spring 2018	CRE	NORO INF	Aeromonas caviae/hydrophila	0	None	Not tested
Fall 2018	ESBL	BICO 101	Escherichia coli	1	CTX	AMPFAZFOTPODAXOCEP
Fall 2018	ESBL	BICO 101	Klebsiella pneumoniae	1	SHV	AMPFAZPODCEP
Fall 2018	CRE	BICO 101	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXPHAIMIMER
Fall 2018	CRE	Lake Herrick	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER
Fall 2018	ESBL	Lake Herrick	negative		NA	Not tested

Fall 2018	CRE	MIDO 301	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 301	Serratia fonticola	1	None	Not tested
Fall 2018	ESBL	MIDO 601	negative	0	None	Not tested
Fall 2018	CRE	MIDO 601	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 604	Aeromonas hydrophila/caviae	0	CTXTEM	Not tested
Fall 2018	ESBL	MIDO 604	Aeromonas hydrophila/caviae	0	CTXTEM	Not tested
Fall 2018	ESBL	MIDO 604	Aeromonas hydrophila/caviae	0	CTXTEM	Not tested
Fall 2018	CRE	MIDO 604	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 605	Citrobacter freundii	1	CMY	AMPFAZFOTFOXPODTAZAXOCEP
Fall 2018	CRE	MIDO 605	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 606	negative	0	NA	Not tested
Fall 2018	CRE	MIDO 606	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 608	negative	0	NA	Not tested
Fall 2018	CRE	MIDO 608	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 609	Citrobacter freundii	1	CMY	AMPFAZFOTFOXPODTAZAXOCEP
Fall 2018	CRE	MIDO 609	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER

Fall 2018	CRE	MIDO 609	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER
Fall 2018	ESBL	MIDO 610	Aeromonas hydrophila/caviae	0	TEM	Not tested
Fall 2018	ESBL	MIDO 610	Aeromonas hydrophila/caviae	0	CTXTEM	Not tested
Fall 2018	CRE	MIDO 610	negative	0	NA	Not tested
Fall 2018	CRE	MIDO 611	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 612	Aeromonas hydrophila/caviae	0	TEM	Not tested
Fall 2018	ESBL	MIDO 612	Aeromonas hydrophila/caviae	0	None	Not tested
Fall 2018	ESBL	MIDO 612	Aeromonas hydrophila/caviae	0	None	Not tested
Fall 2018	CRE	MIDO 612	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 613	Citrobacter braakii/freundii	1	CTX	AMPFAZCEP
Fall 2018	CRE	MIDO 613	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 616	Aeromonas hydrophila/caviae	0	CTXTEM	Not tested
Fall 2018	CRE	MIDO 616	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 617	Citrobacter braakii	1	CTX	AMPFAZCEP
Fall 2018	CRE	MIDO 617	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 706	Enterobacter cloacae complex	0	None	Not tested

Fall 2018	CRE	MIDO 706	negative	0	NA	Not tested
Fall 2018	CRE	MIDO 707	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 709	Citrobacter braakii	1	CTX	AMPFAZPODAXOCEP
Fall 2018	CRE	MIDO 709	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 709	Serratia fonticola	1	CTX	AMPFAZCEP
Fall 2018	ESBL	MIDO 712	Escherichia coli	1	CTXTEM	AMPFAZFOTPODAXOCEP
Fall 2018	ESBL	MIDO 712	Escherichia coli	1	CTXTEM	AMPFAZFOTPODAXOCEPGEN
Fall 2018	CRE	MIDO 712	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 719	Citrobacter sedlakii	1	None	Not tested
Fall 2018	ESBL	MIDO 719	Citrobacter sedlakii	1	None	Not tested
Fall 2018	CRE	MIDO 719	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER
Fall 2018	ESBL	MIDO 719	Escherichia coli	1	CTXSHV	AMPFAZFOTPODTAZAXOCEPCIPOEN
Fall 2018	ESBL	MIDO 801	Citrobacter freundii	1	CMY	AMPFAZFOTFOXPODAXOCEP
Fall 2018	ESBL	MIDO 801	Enterobacter cloacae complex	1	Noe	Not tested
Fall 2018	CRE	MIDO 801	negative	0	NA	Not tested

Fall 2018	ESBL	MIDO 802	Citrobacter freundii	1	CMY	AMPFAZFOTFOXPODTAZAXOCEP
Fall 2018	CRE	MIDO 802	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXPODCEPIMIMER
Fall 2018	ESBL	MIDO 804	Citrobacter braakii	1	None	Not tested
Fall 2018	ESBL	MIDO 804	Escherichia coli	1	CMYCTXTEM	AMPFAZFOTPODAXOCEPCIP
Fall 2018	CRE	MIDO 804	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 805	Enterobacter cloacae complex	1	None	Not tested
Fall 2018	ESBL	MIDO 805	Enterobacter cloacae complex	1	None	Not tested
Fall 2018	CRE	MIDO 805	negative	0	NA	Not tested
Fall 2018	CRE	MIDO 816	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 820	Enterobacter cancerogenus	1	None	Not tested
Fall 2018	ESBL	MIDO 820	Enterobacter cancerogenus	1	None	Not tested
Fall 2018	CRE	MIDO 820	negative	0	NA	Not tested
Fall 2018	CRE	MIDO 825	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 825	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 826	Enterobacter cloacae complex	1	None	Not tested
Fall 2018	CRE	MIDO 826	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER

Fall 2018	ESBL	MIDO 826	Escherichia coli	1	CMYCTXTEM	AMPFAZFOTPODAXOCEP
Fall 2018	ESBL	MIDO 611	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 707	negative	0	NA	Not tested
Fall 2018	ESBL	MIDO 816	negative	0	NA	Not tested
Fall 2018	CRE	NORO 108	negative	0	NA	Not tested
Fall 2018	ESBL	NORO 114	Escherichia coli	1	CMYCTX	AMPFAZFOTPODAXOCEP
Fall 2018	ESBL	NORO 114	Escherichia coli	1	CTXTEM	AMPFAZFOTPODAXOCEPGEN
Fall 2018	CRE	NORO 114	negative	0	NA	Not tested
Fall 2018	ESBL	NORO 401	negative	0	NA	Not tested
Fall 2018	CRE	NORO 401	negative	0	NA	Not tested
Fall 2018	CRE	Lake Chapman	negative	0	NA	Not tested
Fall 2018	ESBL	NORO 501	Citrobacter freundii	1	CMY	AMPFAZFOTFOXPODTAZAXOCEP
Fall 2018	CRE	NORO 501	negative	0	NA	Not tested
Fall 2018	ESBL	NORO 503	Acinetobacter baumanii complex	0	None	Not tested
Fall 2018	CRE	NORO 503	negative	0	NA	Not tested
Fall 2018	ESBL	NORO 510	Citrobacter sedlakii	1	None	Not tested

Fall 2018	CRE	NORO 510	negative	0	NA	Not tested
Fall 2018	ESBL	NORO 511	Chromobacterium violaceum	0	None	Not tested
Fall 2018	CRE	NORO 511	negative	0	NA	Not tested
Fall 2018	ESBL	NORO 514	Aeromonas hydrophila/caviae	0	None	Not tested
Fall 2018	CRE	NORO 514	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER
Fall 2018	CRE	NORO 514	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER
Fall 2018	ESBL	NORO 520	Enterobacter cloacae complex	1	SHVTEM	AMPFAZFOTFOXPODTAZAXOCEP
Fall 2018	ESBL	NORO 520	Escherichia coli	1	CTX	AMPFAZFOTPODAXOCEPCIP
Fall 2018	CRE	NORO 520	negative	0	NA	Not tested
Fall 2018	CRE	NORO 527	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMI
Fall 2018	ESBL	NORO 527	Escherichia coli	1	CTX	AMPFAZFOTPODAXOCEPCIP
Fall 2018	ESBL	NORO 527	Raoultella planticola	1	SHV	AMPFAZPODTAZAXOCEP
Fall 2018	ESBL	NORO 605	Enterobacter cloacae complex	1	None	Not tested
Fall 2018	CRE	NORO 605	negative	0	NA	Not tested
Fall 2018	ESBL	NORO 609	Escherichia coli	1	CTXTEM	AMPFAZFOTPODAXOCEP
Fall 2018	CRE	NORO 609	negative	0	NA	Not tested

Fall 2018	ESBL	NORO 611	Citrobacter sedlakii	1	None	Not tested
Fall 2018	CRE	NORO 611	negative	0	NA	Not tested
Fall 2018	CRE	NORO 615	Enterobacter cloacae complex	1	IMI-NmcA	AMPFAZFOXCEPIMIMER
Fall 2018	ESBL	NORO 615	Escherichia coli	1	CTX	AMPFAZFOTPODAXOCEPCIP
Fall 2018	ESBL	NORO 615	Raoultella planticola	1	CTXOXATEM	AMPFAZFOTPODTAZAXOCEPCIPOEN
Fall 2018	ESBL	NORO 627	Escherichia coli	1	CTX	AMPFAZFOTPODAXOCEPGEN
Fall 2018	CRE	NORO 627	negative	0	NA	Not tested
Fall 2018	ESBL	NORO108	negative	0	NA	Not tested
Fall 2018	ESBL	Lake Chapman	negative	0		Not tested