ABSTRACT

Title of Thesis:	ANTIMICROBIAL RESISTANCE OF ENTEROCOCCI IN SURFACE AND RECYCLED WATER IN THE MID-ATLANTIC
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Increasing demands for agricultural water require identification of alternative water sources. *Enterococcus* species can exhibit antimicrobial resistance and transfer resistance traits to other bacterial taxa, including human pathogens. This study evaluated the distribution and antimicrobial resistance of *Enterococcus faecalis* and *E. faecium* in surface and recycled waters. In all, 365 isolates from 129 water samples collected over one year were identified by species, and 95 were selected for antimicrobial susceptibility testing. Season, water type, temperature and salinity were statistically significantly associated with species probability, and season with antimicrobial resistance of *E. faecalis*. 1.3% of *E. faecalis* and 5.0% *E. faecium* were pan-susceptible but 100% were susceptible to ampicillin, vancomycin, daptomycin and linezolid. Multidrug resistance was detected in 16% of *E. faecalis* and 70% of *E.*

faecium isolates. *Enterococcus* was ubiquitous in water and exhibited resistance to multiple antimicrobials, but resistance to antimicrobials of last resort for enterococcal infections was non-existent.

ANTIMICROBIAL RESISTANCE OF ENTEROCOCCI IN SURFACE AND RECYCLED WATER IN THE MID-ATLANTIC

by

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Dedication

For Joan and Helene.

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Chapter 1: Introduction

Water Use

The amount of water on earth is finite. Although water moves through the hydrologic cycle, changing forms, the sum stays the same. Approximately three quarters of the earth's surface is covered in water (Graham, Parkinson, & Chahine, 2010). While it might be presumed that there is enough water on earth for all of our needs, the majority of it (96.5%) lies in the salty oceans, seas and bays. Only 3.5% of the earth's 1.39 billion cubic kilometers of water is fresh. Furthermore, the majority of all freshwater (68.7%) is trapped in icecaps, glaciers, and permanent snow. This leaves 30.1% in groundwater, and less than 1.2% in surface waters (Graham et al., 2010).

Water is essential to life; approximately 60% of an adult's body weight is comprised of water, and the average sedentary adult should consume 1.5 liters of water per day to sustain normal functioning (Jéquier & Constant, 2010). As the human population continues to grow, an ever increasing strain is placed on our limited water supply. Population growth also leads to increased pressure on the agricultural industry to meet greater food production demands. In the U.S. alone, the population increased by 4% between 2010 and 2015, from 312.6 million to 325.0 million people (Dieter et al., 2018).

In the United States, an estimated 322 billion gallons per day (Bgal/d) of water are drawn to use in various industries. The three largest uses of withdrawn

water are thermoelectric power (132.9 Bgal/day, 41.3%), irrigation 118 Bgal/day, 36.6%) and public supply (39 Bgal/day, 12.1%). Total withdrawals decreased between 2010 and 2015, which can largely be attributed to a significant decrease (18%) in withdrawals for thermoelectric power (accounted for 89% of overall decrease). Withdrawals for irrigation slightly increased (2%) between 2010 and 2015 (from 116 Bgal/day to 118 Bgal/day) (Dieter et al., 2018). 87% (281 Bgal/d) of withdrawn water is considered "fresh"; defined as water containing less than 1000mg/L of dissolved solids. The remaining 13% (41 Bgal/d) is saline water (Dieter et al., 2018). Most of the saline water withdrawn is used for thermoelectric power; while small amounts are desalinated for other uses. Of the available freshwater, water withdrawn for irrigation exceeds that of thermoelectric power (118 Bgal/day, 42% vs 95 Bgal/day, 33.8%) (Dieter et al., 2018). Freshwater for irrigation is withdrawn from surface water and groundwater. Irrigation water includes urban and agricultural uses (Dieter et al., 2018).

Water for Agriculture

The agricultural sector uses approximately 70% of available freshwater worldwide, and demand is expected to increase 19% by 2050. Almost 40% of the global food supply is grown in areas void of sufficient natural precipitation (Water, n.d.). Lack of sufficient rainfall to sustain food production requires the use of irrigation, and places strain on surface and groundwater supplies. In 2017, almost 60 million tons of produce was grown in the US. More than one third of vegetables and two thirds of fruits and nuts are grown in California alone, but there are large

agricultural operations across the country (CDFA, n.d.; USDA, 2017; USDA, 2018a; USDA, 2018b). Drought conditions in these agricultural areas can have significant detrimental effects.

Drought events tend to align with crop growing seasons, and California has been affected by drought conditions for several years. However, the changing climate has also increased extreme weather events across the entire county. In the past 10 years, the US has suffered nine major multi-billion dollar drought events. The most recent occurred in North Dakota, South Dakota and Montana; reaching a price tag of 2.4 billion dollars in 2017. The most extensive drought since the 1930's occurred in 2012 and affected 22 states, for a cost of 32.7 billion dollars (NOAA, 2018). Even in the absence of drought, elevated temperatures increase production of water vapor through evaporation, transpiration and sublimation. This may increase the amount of water required for crop growth, and/or reduce available water supplies.

In 2015, US withdrawals of ground and surface water were fairly even (57.2 Bgal/d (48%) and 60.9 Bgal/d (52%) respectively), but in 2010, more water was withdrawn from groundwater (66.2 Bgal/d, 57%) than surface water (49.3 Bgal/d, 43%). While nationwide withdrawals of groundwater decreased between 2010 and 2015, they increased in California, due to a period of prolonged drought. Extended reliance on groundwater can lead to aquifer depletion, which may require many years to recharge (Dieter et al., 2018).

Conversely, the changing climate and extreme weather events have also increased heavy precipitation and flooding in parts of the US. Flooding may overwhelm sanitation facilities and contaminate water sources (UN Water, n.d.).

Although the quantity of water may be sufficient, the quality may be questionable. In light of these factors, and the increasing unpredictability of water availability in many areas (UN Water, n.d.), it is essential to identify "nontraditional", or alternative, water sources which can be used to augment and diversify existing agricultural water supply. These alternative water sources, which might including recycled, brackish, desalinated, fracking, agricultural runoff, aquaculture, livestock wastewater, and process waters (USDA, 2016), could potentially be employed to alleviate burden on water systems during times of water shortages.

Recycled, also known as reclaimed, water is municipal wastewater which has undergone treatment at a wastewater treatment plant and is ready for reuse (EPA, 2012). Reusing water for irrigation is a relatively new practice for many states. In 2015, at least ten states: Arizona, California, Colorado, Florida, Illinois, Kansas, Nevada, New Mexico, Texas, and Utah, used recycled water for irrigation (669 Mgal/day). Recycled water still accounted for less than 1% of total irrigation water, but represented a 42% increase from 2010 (472Mgal/day). It is likely that more than 10 states are using recycled water for irrigation, as 20 states have regulations, and an additional 8 set guidelines for non-food and processed food crop irrigation with recycled water. Fourteen states also have regulations, and an additional 5 set guidelines for food crop irrigation with recycled water (EPA, 2012). Water reuse could provide an alternative to aquifer reduction when surface water supplies are low.

Agricultural Water Quality

Regardless of water source, it is crucial to evaluate potential biological contaminants which may render the water unsuitable for the intended use. Generally, barring a major contamination event, groundwater is the safest water of the three mentioned water types, since water typically travels through the Earth's strata, which filters out microbial contaminants before allowing the water to pass into an underground aquifer. However, there are many factors which could influence microbial contamination of surface and recycled water. Pathogens, which have been reported as one of the top causes of impaired rivers, streams, bays and estuaries, may enter bodies of water due to fecal contamination from human or animal sources (EPA, 2017). It can be prohibitively expensive to test for specific pathogens; however, "indicator" bacteria can be used to assess fecal contamination of waters. The presence of these indicators, which are generally not pathogenic themselves, would suggest that the water may also contain pathogens.

Indicators

The four indicators used are total coliforms, fecal coliforms, *Escherichia coli*, and *Enterococcus*. These bacteria are common in the intestinal tract of warm-blooded animals, including humans, domesticated animals, and wildlife, which makes them useful as indicators of fecal contamination (EPA, 2006). Total coliforms are ubiquitous in nature. Although they are present in human and animal feces, and may indicate fecal contamination, coliforms can also be present in soil and plant material. Because of this they are no longer recommended as an indicator in recreational waters; however, they are still used to indicate potential contamination of drinking

water. A subset of total coliforms, fecal coliforms, are a better indicator of fecal contamination. Until 2012, fecal coliforms were the primary indicator of fecal contamination in recreational waters. For recreational freshwater, the Environmental Protection Agency (EPA) now recommends a specific fecal coliform, *E. coli*, as the appropriate indicator of fecal contamination (EPA 2012). Some states have changed their practices to accommodate this recommendation, but others have retained fecal coliforms as their primary indicator of human health risk from fecal contaminated recreational water. *Enterococcus*, which does not belong to the coliform group of bacteria, can also be used as an indicator for fecal contamination of fresh water. Unlike the coliforms, *Enterococcus* can survive in saltwater, similar to many pathogens, and is the EPA recommended indicator for brackish and marine recreational waters. While *E. coli* and *Enterococcus* are EPA recommended indicators for recreational waters, they have also been used as water quality indicators for other designated uses under the Clean Water Act.

Regulations

The Federal Water Pollution Control Act Amendments of 1972, also known as the Clean Water Act (CWA), governs surface water pollution in the US as it aims to "restore and maintain the chemical, physical, and biological integrity of the Nation's waters" (The Federal Water Pollution Control Act, 1972). The Act is administered by the EPA. Individual states set their own water quality standards, by first designating a use for each waterbody or segment of a waterbody. The state then sets an ambient water quality standard for each designated use type. The EPA provides guidelines for water quality standards (EPA, 2017b), and states may choose to follow the

recommendations or diverge from them based on appropriate scientific justification, but final standards must be approved by the EPA. States then identify and report to the EPA impaired waters – waters that do not meet the imposed standard (Carruth & Goldstein, 2013).

State and local authorities also set their own regulations on water reuse, as there are no federal regulations on this water type. EPA has provided guidelines on water reuse, the latest of which were published in 2012. The EPA guidelines for recycled water use for irrigation purposes include suggested secondary water treatment for surface irrigation of orchards and vineyards and nonfood crop irrigation, and tertiary/advanced treatment for landscape and golf irrigation and food crop irrigation. Many states have passed legislation regulating water reuse (EPA, 2012).

In 2016, the Produce Safety Rule of the Food Safety Modernization Act established standards for the growing, harvesting, packing, and holding of produce for human consumption. The rule includes standards for agricultural water based on the colony-forming unit (CFU) count of the fecal indicator *E. coli* in 100 mL of water. The rule does not include *Enterococcus*; however, it does allows farmers to use alternative water quality criteria, including the use of a different fecal indicator, if they can provide sufficient scientific data to support the change (Assar, 2017). Standards only apply to those produce crops in which the water may come into direct contact with the part of the plant which will be harvested. Implementation of the agricultural water standard for produce (excluding sprouts) was scheduled to begin in January 2018, but has been deferred while the FDA reexamines the science behind it. (FDA, 2018a).

The most recent National Rivers and Stream Assessment (2008/2009), reported enterococci exceeded threshold levels for protecting human health in 23% of river and stream miles (EPA, 2017). At least two states, Texas and Virginia, have added *Enterococcus* to their water quality testing policies (EPA 2012).

Research Rationale

Although species of the genus *Enterococcus* are commensal bacteria of the human gut, several are also opportunistic pathogens and can cause illness under certain conditions. *Enterococcus* spp. are one of the leading causes of nosocomial infections, and have been implicated in community acquired infections as well. Additionally, some enterococci have developed resistance to certain antibiotics, reducing the availability of effective treatments against these potentially life-threatening infections. Moreover, enterococci can transfer their resistance to other, more pathogenic bacteria (Kristich, Rice, & Arias, 2014). Thus, it is important to consider the potential for antimicrobial resistance when assessing the safety of irrigation water for food crops.

While several studies have examined enterococci in coastal, bay, river, pond, and recycled waters (Carey et al., 2016; Garcia et al., 2007; Moore, Guzman, & McGee, 2008; Rosenberg Goldstein et al., 2014; Sapkota, Curriero, Gibson, & Schwab, 2007; Tejedor Junco, González Martín, Pita Toledo, Lupiola Gómez, & Martín Barrasa, 2001), few have done so through the lens of agricultural irrigation water quality. We found one study which compared multiple water types, but only examined aminoglycoside resistance of *Enterococcus* (Rice, Messer, Johnson, &

Reasoner, 1995). To our knowledge no studies have compared enterococcal contamination and multi antimicrobial resistance of *Enterococcus* across multiple agricultural water types.

Objectives

The purpose of this study was to evaluate the distribution and antimicrobial resistance of two species of *Enterococcus* most relevant to human health, *E. faecalis* and *E. faecium*, in four water types: non-tidal fresh, tidal brackish, pond, and recycled, in the Mid-Atlantic region of the U.S. The specific objectives were to:

1) Identify *E. faecalis* and *E. faecium* isolates in *Enterococcus* isolates archived from water samples.
2) Evaluate single and multidrug resistance of *E. faecalis* and *E. faecium* isolates to a suite of antimicrobials.
3) Assess whether species type and antimicrobial resistance is associated with season, water type, temperature, pH, and/or salinity.

Hypothesis

Species and antimicrobial resistance of *Enterococcus* isolates varies by season and water type.

Chapter 2: Background

Antimicrobial Resistance

Bacteria and Discovery of Antibiotics

The human body is colonized by trillions of bacteria; containing at least as many bacterial cells as human cells (Sender, Fuchs, & Milo, 2016). A newborn's first bacterial cells are introduced at birth by passing through the birth canal. The first microbe received is *Lactobacillus*, without which the newborn could not break down the lactose in their mother's milk. Other bacteria make amino acids or vitamins essential to proper functioning, digest starch, break down fiber, and metabolize drugs. As children explore their environment, the amount of bacteria in their microbiome continues to increase until about the age of 3. After this age, the quantity of microbes in the body will remain near constant, however the composition of those microbes can vary greatly throughout life depending on different stressors. While many bacteria are innocuous, or even beneficial to human health, there are also pathogenic bacteria which can cause severe illnesses and/or infection. Before the advent of antibiotics, more soldiers died from bacterial illnesses than combat in World War I and the Civil War (Blaser, 2014).

By the time World War II broke out, the antibiotic sulfa was in use; however, its efficacy was severely limited. Attention was redirected to the first antibiotic discovered but not yet available, penicillin, in an effort to avoid the heavy infectious disease casualties of the previous wars. Sir Alexander Fleming discovered penicillin

by accident in 1928, but could never devise a method to produce more than small amounts of the antibiotic. In the hands of military scientists, the mass production of penicillin was underway by 1944 and used often to treat WWII soldiers (Ventola, 2015). Sulfa and penicillin were soon joined by streptomycin, tetracycline, erythromycin, chloramphenicol, and isoniazid, and the antibiotic era was born (Blaser, 2014).

There are 3 general ways that antibiotics work: by attacking the bacterial cell wall, disrupting the production of proteins, and disrupting the division and reproduction capabilities of the bacteria (Kapoor, Saigal, & Elongavan, 2017). Antibiotics were once considered miracle drugs, thought to be void of negative effects. So they were widely used and abused. Even today, nearly 50% of prescribed antibiotics are either unnecessary or dosed incorrectly (CDC, 2013).

There can be negative effects of antibiotic use though, such as adverse drug events including allergic reactions, diarrhea from over colonization of *C. difficile* since beneficial bacteria is also destroyed by antibiotics, interactions with other medications, and side effects such as nausea, diarrhea, and stomach pain. The overuse of antibiotics has also led to an increase in antimicrobial-resistant bacteria. The Centers for Disease Control and Prevention (CDC) has deemed antibiotic use "the single most important factor leading to antibiotic resistance around the world" (CDC, 2013).

Development of Antimicrobial Resistance

Antimicrobial resistance is the ability of a microorganism (such as bacteria) to reduce or eliminate the effectiveness of an antimicrobial (in this case antibiotic) against the microorganism. Antimicrobial resistance can be a natural development, as bacteria adapt to their surroundings and the antimicrobial activity of competing microbes in their environment. When a population faces stress, those that are best adapted survive (Blaser, 2014). Antimicrobial resistance has been discovered in pristine areas untouched by human activity, including Arctic wetlands, Brazilian caves, and the feces of Antarctic penguins (Pepper, Brooks, & Gerba, 2018). However the wide use of antibiotics has rapidly hastened the development of antimicrobial resistance. Antibiotics are fairly easy to obtain; while they are also overprescribed, some are available online or in countries which do not require a prescription (Ventola, 2015). Use of an antibiotic will eventually result in some bacteria developing resistance to that antibiotic (CDC, 2013; Ventola, 2015).

Antibiotics can be categorized as broad-spectrum or narrow-spectrum, effective against a wide or narrow range of bacteria respectively (CDC, 2013). While it is preferable to prescribe a narrow-spectrum antibiotic to target specific bacteria and avoid unnecessary side effects, broad-spectrum antibiotics are often prescribed instead. This is done because it may be deadly to wait until the problematic bacteria has been identified before beginning treatment. Unfortunately, broad-spectrum antibiotics kill more than just the intended bacteria, which allows surviving bacteria the ability to rapidly reproduce and take up residence in the newly freed space. This alters the bacterial composition in the digestive tract and potentially creates new

problems. A microbe that may have been manageable when its numbers were small may become unwieldly when allowed to multiply (Blaser, 2014). As the spectrum scope of the antibiotic increases, so does the selection for resistance. If resistance develops in the targeted bacteria, further treatment will be less effective (CDC, 2013).

In 1996, the National Antimicrobial Resistance Monitoring System (NARMS) was established to track antimicrobial-resistant threats in bacteria common in food animals which could cause illness in humans. NARMS is a collaboration between the CDC, United States Food and Drug Administration (FDA), United States Department of Agriculture (USDA) and state and local health departments. NARMS currently evaluates antimicrobial resistance of *Enterococcus* in food animals and retail meats (Karp et al., 2017).

Sixty percent of human infectious diseases are zoonotic (van Doorn, 2014). Many of the antibiotics used to treat human disease are also used for animals, both ones kept as pets and others reared for food. The Center for Veterinary Medicine of the Food and Drug Administration (FDA) approves antibiotic use in animals (FDA, 2018b). Sixty percent of the antimicrobials used for US food producing animals are also essential for medicinal use in humans; the vast majority of which are tetracyclines (FDA, 2017). Antibiotics may be used to prevent, control, or treat disease in animals, but were also once used to promote growth of poultry and livestock by increasing muscle mass or enhancing feed efficiency. Their use as growth promoters has caused controversy, especially since the use of antimicrobials in animals has contributed to the antimicrobial resistance problem (CDC, 2013; Ventola, 2015).

The first country to ban the use of antibiotics for growth promotion was Sweden in 1986. The European Union followed suit in 1999, and they are now banned for growth promotion purposes across the entire continent (Blaser, 2014). While antimicrobial use for growth promotion has not been banned in the US, the FDA did request that the poultry and livestock industries halt the practice in 2013. A number of antimicrobials which were once available over the counter now require a prescription or veterinarian supervision (FDA, 2013). Antibiotics and antibiotic-resistant bacteria may be passed along to humans directly, through consumption of meat or dairy products, or indirectly, through fertilizer which may transfer to crops or runoff into nearby water bodies which may then be used as irrigation water (Ventola, 2015).

Severity of Antimicrobial Resistance Threat

Bacteria may be intrinsically resistant to antimicrobials, or they may acquire resistance through adaptation upon exposure to antimicrobial drugs or transfer of genetic elements from other bacteria. Intrinsic resistance is obtained through vertical transmission of resistance, in which a parent cell divides and reproduces, passing on the resistant genes to its offspring. Horizontal transmission of resistance occurs when two bacterial cells give, gain, or swap genes which bestow resistance (Blaser, 2014; Ventola, 2015) Every time a new antibiotic is discovered and distributed, resistance to that antibiotic has eventually developed. It is essential that the search for new antimicrobials continues; however, incentives for pharmaceutical companies to conduct the required research have severely decreased since the early

1980's. Since resistance quickly develops, and physicians now attempt to keep low resistance antimicrobials reserved for treatment of last resort, the profit margin remains low (Ventola, 2015).

The CDC conservatively estimates that more than two million people in the US develop serious antibiotic-resistant infections per year, resulting in at least 23,000 deaths. More die from complications related to an antibiotic-resistant infection (CDC, 2013). Antimicrobial-resistant infections may result in high health and financial costs. Alternatives to a normally prescribed antibiotic may be more toxic to the patient and more expensive to administer, and recovery time is extended. It is estimated that the annual cost of antibiotic-resistant infections is $20 billion in healthcare and $35 billion in lost productivity (Ventola, 2015). The CDC has prioritized the most worrisome antibiotic-resistant bacteria by three threat levels: urgent, serious, and concerning. Vancomycin Resistant Enterococcus (VRE) has been listed as a "serious" threat, meaning it is a "significant antibiotic-resistant threat ... [which] may become urgent without ongoing public health monitoring and prevention activities" (CDC, 2013).

Enterococcus

Genus

There are more than 50 different species within the *Enterococcus* genus (Parte, 2014). Enterococci are gram positive cocci which can occur singly, in pairs, or in short chains. They are catalase negative, and the majority of them are pyrrolidonyl peptidase (pyr) positive . *Enterococcus* was once considered to be a member of

Streptococcus Group D; the first three species identified were *Streptococcus faecalis*, *S. faecium*, and *S. durans*. The "enterococcal group" of streptococci grew in temperatures ranging from 10 to 45°C, at pH 9.6, in 6.5% NaCl, and could survive 60°C for 30 min. In 1984, *S. faecalis* and *S. faecium* were reclassified to the new genus *Enterococcus* as *Enterococcus faecalis* and *E. faecium* (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012; Lebreton, Willems, & Gilmore, 2014).

Enterococcus has been found in water, soil, plants, the gut of humans, animals, birds, reptiles and insects, and in food as starter cultures or probiotics (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006; Byappanahalli et al., 2012; Micallef et al., 2013; Lebreton et al., 2014). Several of the most well researched species have been found in all (*E. faecalis*, *E. faecium*, *E. durans*, *E. gallinarum*) or all but food (*E. casseliflavus*, *E. mundtii*) groups. Their ability to grow in saline conditions marked their selection as the EPA recommended indicator for fecal contamination in brackish and marine waters (Lebrerton et al., 2014).

Enterococcus in Water Environments

Enterococci are a regular component of water microbiota and are also used as a fecal contamination indicator; however, there is debate about the extent to which enterococci presence can be attributed to fecal sources. While many species found in water have been sourced to fecal origin, including recent additions *E. hamoperoxidus* and *E. moraviensis*, first isolated from surface water (Svec et al., 2001; Taučer-Kapteijn, Hoogenboezem, Hoogenboezem, Haas, & Medema, 2017), several, including *E. aquimarinus* (seawater), *E. silesiacus* (drinking water), *E. rivorum*

(pristine brook), *E. silesiacus* and *E. quebecensis* (surface water) have not yet been attributed to a fecal source (Byappanahalli et al., 2012; Lebreton et al., 2014).

Enterococci which did not originate in the water source can come from many different sources, all of which can be categorized as point source or non-point source. Point source discharges are regulated through the National Pollutant Discharge Elimination System (NPDES) permitting program authorized by the Clean Water Act (CWA). The CWA prohibits point source discharge without a permit, or discharge of amounts exceeding that which has been approved in the permit (Carruth & Goldstein, 2013). Municipal wastewater, industrial wastewater, and concentrated animal feeding operations (CAFOs) are considered point sources and are regulated under the NPDES permitting program (The Federal Water Pollution Control Act, 1972). Several other activities which are not regulated by the CWA may contribute to water contamination. Enterococcus may be shed during recreational use by boaters and bathers (Boehm & Sassoubre, 2014), or enter water through urban runoff from activities such as lawn irrigation or car washing. There may also be direct or indirect fecal deposition by wildlife or livestock, or other agricultural activities which could result in runoff entering nearby waterbodies (Byappanahalli et al., 2012). Agricultural runoff, from activities such as crop production, grazing, and animal feeding operations, are among the most common sources of water contamination for rivers, streams, lakes, ponds, and reservoirs (EPA, 2017). Rainfall or snowmelt may cause a surge of bacterial water contamination, wherein storms may increase runoff and/or overwhelm sewage facilities contributing to fecal contamination of waters (Boehm & Sassoubre, 2014).

Enterococcus can survive in fresh and marine (saline) waters, however crucial nutrients are required in order for them to multiply (Lebreton et al., 2014). Research has shown that enterococci can survive longer and multiply in mesocosms containing submerged aquatic vegetation when compared to those which do not contain the vegetation. It is presumed that cladophora, a macrophytic green alga found in fresh and marine waters, contains the required nutrients to promote growth of *Enterococcus* to high concentrations, as it has been shown to harbor a significant quantity of enterococci and enteric pathogens. Sediments can also be a significant bacterial reservoir; *Enterococcus* has been isolated from marine and freshwater sediments. Enterococci are able to survive longer in sediment than water, partially due to resistance to solar inactivation, starvation, and predation (Byappanahalli et al., 2012).

Enterococcus in Mammals

Only a small fraction of *Enterococcus* spp. are of concern to human health. The species which are most often found in the intestinal tract and feces of mammals are *E. faecalis, E. faecium, E. hirae*, and *E. durans*. *E. faecalis* and *E. faecium* are most abundant in the human intestinal tract and account for most enterococcal disease. *E. hirae* and *E. durans*, as well as *E. avium*, *E. gallinarum*, *E. casseliflavus, E. mundtii*, *E. raffinosus, E. cecorum, E. sanguinicola, E. pallens, E. canintestini, E. dispar,* and *E. gilvus* have also been shown to cause human infection (Lebreton et al., 2014; Byappanahalli et al., 2012).

Enterococcus is a commensal bacteria, primary located in the small and large intestine. Enterococci are also common in the oral cavity, but are rare in the acidic

environment of the stomach. Enterococci are usually a small (≤1%) proportion of the intestinal microbiome, but the use of antibiotics can reduce competition and allow antimicrobial-resistant enterococci to over colonize the gastrointestinal tract (Lebreton et al., 2014). This overgrowth can lead to their becoming an opportunistic pathogen, and excretion of enterococci in feces may allow transfer to other areas through fecal contamination. *E. faecalis* and *E. faecium* can cause many illnesses, including endocarditis, bacteremia, and central nervous system, urinary tract, abdominal, and pelvic infections (Byappanahalli et al., 2012).

Antimicrobial Resistance

Enterococcus spp. have intrinsic resistance to certain antibiotics, including some aminoglycosides, cephalosporins, clindamycin and semisynthetic penicillinase-stable penicillins, and acquired resistance to others, such as vancomycin (Byappanahalli et al., 2012; Aguedelo Higuita & Huycke, 2014). *E. faecium* is much more resistant to vancomycin than *E. faecalis*. Studies have shown that more than 50% of *E. faecium* pathogenic strains exhibit resistance to vancomycin, as well as ampicillin and high doses of aminoglycosides (Aguedelo Higuita & Huycke, 2014).

The β-lactam ampicillin is the antibiotic of choice to treat susceptible enterococcal infections in the absence of allergy. The glycopeptide vancomycin may be used in case of allergy to β-lactams. For severe infections, such as endocarditis, an aminoglycoside may be added for its synergistic activity with β-lactams and glycopeptides (Kristich et al., 2014). In cases of β-lactam and glycopeptide resistance (or β-lactam allergy and glycopeptide resistance) a limited number of other

antibiotics, such as, the duo quinupristin/dalfopristin, daptomycin and linezolid can be employed; however there have been cases of resistance to these as well (Maraki, Samonis, Dimopoulou, & Mantadakis, 2014; Kelesidis, Humphries, Uslan, & Pegues, 2011; Kainer et al., 2007). *E. faecalis* are also intrinsically resistant to quinupristin/dalfopristin, so this therapy could only be perused for treatment of *E. faecium* infections (Aguedelo Higuita & Huycke, 2014).

Enterococcus is a leading cause of nosocomial infections. Among gram-positive nosocomial infections, it is second only to *Staphylococcus* (Miller, Munita, & Arias, 2014). *E. faecalis* and *E. faecium* are often found in healthcare environments. *E. faecalis* is most often involved with nosocomial infections, while *E. faecium* is most resistant to antimicrobials (Byappanahalli et al., 2012). Enterococci are among the leading causes of hospital-acquired infections (HAIs) (Lebrerton et al., 2014). Between 2011 and 2014, *E. faecalis* (7.4%), *E. faecium* (3.7%) and other *Enterococcus* spp. (3.6%) contributed 14.7% of all HAIs. Considered together, enterococci were the most common pathogen associated with central line-associated bloodstream infections (CLABSIs) and the second most common pathogen among the 4 HAI types: CLABSIs, catheter-associated urinary tract infections (CAUTIs), surgical site infections (SSIs), and ventilator-associated pneumonias (VAPs). 82.2%-83.8% of *E. faecium*, yet only 9.3%-10.1% of *E. faecalis* associated with CLABSIs tested resistant to vancomycin (Weiner et al., 2016). Annually, approximately 20,000 (30%) of nosocomial enterococcal infections are resistant to vancomycin, resulting in about 1300 deaths (CDC, 2013). Enterococcal infections can also be acquired in the community. Some bloodstream infections and urinary tract infections have been

attributed to community-acquired enterococcal infections (Billington et al., 2014; Kim, Lim, Kim, Park, & Kim, 2013).

Antimicrobial resistance of enterococcus isolated from the environment has been well documented in the literature. Among *E. faecalis* and *E. faecium* of environmental origin, Dicuonzo et al. (2001) found that 75% of *E. faecalis* isolates were resistant to ciprofloxacin, and a large percentage of *E. faecium* isolates were resistant to ciprofloxacin (100%), chloramphenicol (71%), tetracycline (57%), and ampicillin (43%). Micallef et al. (2013) found low resistance of *E. faecalis* isolates (7.1%) to ciprofloxacin, but high resistance (71.4%) to rifampicin, and intrinsic resistance (100%) to quinupristin/dalfopristin. Among *E. faecium* isolates, Micallef et al. (2013) found high resistance to penicillin (100%), ampicillin (66.7%) and rifampicin (66.7%). McGowan et al. (2006) found high resistance of *E. faecalis* isolates in meat to bacitracin (51.3%), lincomycin (66.3%), and quinupristin/dalfopristin (67.5%). They also found high resistance of *E. faecium* isolates in meat to bacitracin (80%), flavomycin (80%), kanamycin (80%), lincomycin (60%), and quinupristin/dalfopristin (60%). Bacitracin, lincomycin, and flavomycin were used as growth promotors in animals.

Vancomycin-resistant *Enterococcus* (VRE) has been listed by the CDC as one of the top 18 drug resistant threats to the US (CDC, 2013). Vancomycin-resistant *Enterococcus faecium* has also been assigned a "high priority" status on the World Health Organization's list of antibiotic-resistant bacteria for which new antibiotics are needed (WHO, 2017). *E. faecium* also causes the majority of multidrug resistant enterococcal infections (Kristich et al., 2014).

Enterococci can transfer antimicrobial resistance to other, more pathogenic, bacteria through the horizontal transfer of mobile genetic elements. Vancomycin resistance has been transferred to *Staphylococcus aureus*, including methicillin resistant *S. aureus* (Kristich et al., 2014; Miller et al., 2014). *S. aureus* is among the top 5 pathogens which cause foodborne illness in the United States (CDC, 2017). Vancomycin-resistant *S. aureus* is also among the top 18 drug resistant threats to the US (CDC, 2013). The opportunistic nature of *Enterococcus* and its ability to transfer resistance to pathogens necessitates its inclusion in safety assessment of agricultural water.

Chapter 3: Antimicrobial Resistance of Enterococci in Surface and Recycled Water in the Mid-Atlantic

Methods

This project is part of a larger study which intends to "facilitate the adoption of transformative on-farm water treatment solutions that enable the safe use of nontraditional irrigation water on food crops" (CONSERVE, n.d.).

Study Sample

One L grab samples were collected in wide mouth propylene Nalgene bottles (Thermo Scientific) from 11 sites in Maryland (3 recycled wastewater, 4 non-tidal fresh, 2 tidal brackish, and 2 pond) on up to 16 sampling dates between October 24, 2016 and October 16, 2017. Samples (n=129) were transported on ice for processing within 24 hours post collection. *Enterococcus* spp. were isolated from samples following EPA method 1600. This method used membrane filtration and membrane Enterococcus Indoxyl-B-D-Glucoside (mEI) agar to detect enterococci (EPA, 2002b). Up to three *Enterococcus* isolates per water sample (Table 1) were confirmed by catalase and pyr tests (Remel, Lenexa, KS), suspended in 1 ml Brucella Broth with 15% glycerol in labeled cryogenic tubes, and frozen at -80°C for long term storage.

Species Identification

365 isolates were regrown on Brain Heart Infusion Agar (Hardy Diagnostics, Santa Maria, CA) and incubated at 35-37°C for 24 hours. A 1µL loopful of bacteria

was removed from the plate and suspended in 100 µL 7.5% Chelex solution in a 1.5 mL microcentrifuge tube. Tubes were heated for 10 minutes at 101°C and centrifuged at 8,000 rpm for 1 minute.

Table 1: Number of archived isolates per water sample

Site	Water Type	Fall 2016		Winter		Spring			Summer					Fall 2017	
		24Oct	14Nov	23Jan	13Mar	17Apr	08May	12Jun	26Jun	17Jul	08Aug	21Aug	11Sep	25Sep	16Oct
MA01	RW					1	3		3	3		3	3	3	3
MA02	RW		3				3			3		2	3	1	3
MA03	NF	2	3	3	3	2	3		3	3	3	3	3	3	3
MA04	TB	3	3	3	3	3	3	3	3	3	3		3	3	3
MA05	NF	3	3	3	3	3	3	3	3	3	3	3	3	3	3
MA06	RW		3		1	3	3				3		3	2	1
MA07	NF	3	1	3	3	3	3	3	3	3	3	3	3	3	3
MA08	TB	3	3	3		3		3	3	3	3	3	3	3	3
MA09	NF	3	3	3	1	3	3	3	3	1	3	3	3	3	3
MA10	PW	3	3	3	3	3	3	3	2	3	3	3	3	3	3
MA11	PW	3	3			3	3	3	3	3	3	2	1	3	3

E. faecalis and *E. faecium* were identified by PCR amplification targeting the *ddl* and *sodA* genes (Table 2). The *ddl* gene encodes D-Alanine:D-alanine ligase and the *sodA* gene encodes superoxide dismutase (Evers, Reynolds, & Courvalin, 1994; Poyart, Quesnes & Trieu-Cuot, 2000). Initial PCR included targeting of the *ddl* genes and an internal control targeting a 350 base pair section of the 16S rRNA gene. A number of isolates presented as both species despite serial bacterial isolation. For these isolates, PCR amplification was repeated targeting the *sodA* gene. PCR of 16S rRNA was omitted in the second PCR amplification. Two µl aliquots were used as DNA template in 20 µl PCR reactions containing 1× PCR buffer, 2 mM $MgCl_2$, 0.15 mM dNTPs, 0.1 µM each of forward and reverse primers for 16S rDNA, 0.2 µM each for all *ddl* or *sod*A genes and 0.6 Units of Taq DNA Polymerase (New England Biolabs, Ipswich, MA; Integrated DNA Technologies, Coralville, IA). PCR amplification consisted of a denaturing step at 95°C for 3 minutes, followed by 35

cycles of denaturing at 94°C (*ddl*) or 95°C (*sodA*) for 30 seconds, annealing at 54°C (*ddl*) or 49°C (*sodA*) for 30 seconds and extension at 72°C for 30 seconds. The amplification ended with a final extension for 5 minutes. *E. faecalis* ATCC 29212 and *E. faecium* ATCC 51559 were used as positive controls, and molecular grade water was used as a negative control.

Table 2: PCR primers used in this study

Primer	Size (bp)	Primer Pair Sequences	Reference
ddlE. faecalis	941	5'-ATCAAGTACAGTTAGTCTTTATTAG-3' 5'-ACGATTCAAAGCTAACTGAATCAGT-3'	Kariyama et al., 2000
ddlE. faecium	658	5'-TTGAGGCAGACCAGATTGACG-3' 5'-TATGACAGCGACTCCGATTCC-3'	Kariyama et al., 2000
sodAE. faecalis	360	5'-ACTTATGTGACTAACTTAACC-3' 5'-TAATGGTGAATCTTGGTTTGG-3'	Jackson et al., 2004
sodAE. faecium	215	5'-GAAAAAACAATAGAAGAATTAT-3' 5'-TGCTTTTTTGAATTCTTCTTTA-3'	Jackson et al., 2004
16S rRNA	350	5'-AGTTTGATCCTGGCTCAG-3' 5′-CTGCTGCCTCCCGTA-3'	Sacchi et al., 2002

PCR products were resolved on a 1.5% (ddl) or 2% (sodA) agarose gel (Lonza, Rockland, ME) in a 1X Tris-borate-EDTA buffer. A 100bp DNA ladder (New England Biolabs) was used as a molecular size marker. Products were visualized under UV light in the BIO-RAD Molecular Imager® Gel Doc™ XR+ System.

Antimicrobial Susceptibility Testing

Where available, one isolate per species of interest (*E. faecalis*, *E. faecium*) was selected from each water sample for antimicrobial susceptibility testing to avoid the possibility of clonal isolates. Antimicrobial susceptibility testing was performed with the Sensititre®AIM™ automated inoculation system using a 96-well microtiter GPN3F plate containing the following antimicrobials (abbreviation, range of

concentrations): erythromycin (ERY, 0.25-4 µg/mL), clindamycin (CLI, 0.12-2 µg/mL), gentamicin (GEN, 2-16 and 500 µg/mL), streptomycin (STR, 1000 µg/mL), quinupristin/dalfopristin (SYN, 0.12-4 µg/mL), daptomycin (DAP, 0.25-8 µg/mL), vancomycin (VAN, 1-128 µg/mL), tetracycline (TET, 2-16 µg/mL), ampicillin (AMP, 0.12-16 µg/mL) , rifampicin (RIF, 0.5-4 µg/mL), levofloxacin (LEVO, 0.25-8 µg/mL), linezolid (0.5-8 µg/mL), penicillin (PEN, 0.06-8 µg/mL), ciprofloxacin (CIP, 0.5-2 µg/mL), trimethoprim/sulfamethoxazole (SXT, 1/19-4/76 µg/mL), ceftriaxone (AXO, 8-64 µg/mL), gatifloxacin (1-8 µg/mL), oxacillin + 2% NaCl (OXA+, 0.5-8 µg/mL) (Thermo Fisher Scientific, Waltham, MA.). Quality control organisms were *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 (CLSI, 2018).

Approximately 3-5 colonies from an overnight culture were transferred to sterile demineralized water to achieve a homogenized suspension equivalent to a 0.5 McFarland standard using the Sensititre™ Nephelometer (Thermo Scientific). Ten mL of this suspension were added to 11mL of sterile cation-adjusted Mueller Hinton broth (Thermo Scientific). Fifty µl of the broth inoculum was delivered by the Sensititre®AIM™ to each well of the GPN3F plate through a disposable dosing head. Plates were covered, incubated at 35°C and manually read after 16-20 hour incubation for all antibiotics excepting vancomycin and high-concentration gentamycin and streptomycin. Plates were manually read after 24 hour incubation for vancomycin and high-concentration gentamycin. If the isolate remained susceptible to high-concentration streptomycin after 24 hours, the plate was read after an additional 24 hour incubation for a total incubation of 48 hours (CLSI, 2012; CLSI, 2018). In accordance with the Clinical and Laboratory Institute (CLSI) approved interpretive

criteria, minimal inhibitory concentrations (MICs) (Table 3) were recorded as the lowest concentration (µg/mL) of an antimicrobial that completely inhibited bacterial growth for all but linezolid. The linezolid MIC was recorded as the lowest concentration (µg/mL) that inhibited 80 – 90% of bacterial growth when compared to the plate positive control (CLSI, 2018). Resistance to two or more classes of antimicrobials was termed multidrug resistance.

Table 3: MIC interpretive criteria (adapted from CLSI, 2018)

	MIC Interpretive Criteria (µg/mL)		
Antimicrobial	Susceptible	Intermediate	Resistant
ampicillin	≤ 8	-	≥16
ciprofloxacin	≤ 1	2	≥ 4
daptomycin	≤ 4	-	-
erythromycin	≤ 0.5	1 - 4	≥ 8
gatifloxacin	≤ 2	4	≥ 8
gentamicin	-	-	500
levofloxacin	≤ 2	4	≥ 8
linezolid	≤ 2	4	≥ 8
penicillin	≤ 8	-	≥ 16
quinupristin/dalfopristin	≤ 1	2	≥ 4
rifampicin	≤ 1	2	≥ 4
streptomycin	-	-	1000
tetracycline	≤ 4	8	≥ 16
vancomycin	≤ 4	8 - 16	≥ 32

Statistical Analysis

Analysis was conducted with SAS 9.4 (SAS Institute, Cary, NC). Descriptive statistics included number and proportions of species and antimicrobial resistance. Differences in proportions were assessed using the chi-squared and Fisher's exact tests. Multinomial logistic regression was used to measure association of season, water type, temperature, pH and salinity with species type. Binomial logistic regression was used to assess unadjusted and adjusted odds for *E. faecalis* or *E. faecium* probability, and to measure association of season, water type, temperature,

pH and salinity with single and multi-drug resistance of *E. faecalis* and *E. faecium*. For all analyses, p values ≤ 0.05 were deemed statistically significant.

Results

Species Identification

From a total of 365 isolates, 148 isolates were identified as *E. faecalis* (40.5%), 28 as *E. faecium* (7.7%) and 189 as other (51.8%) (Table 4). By season,

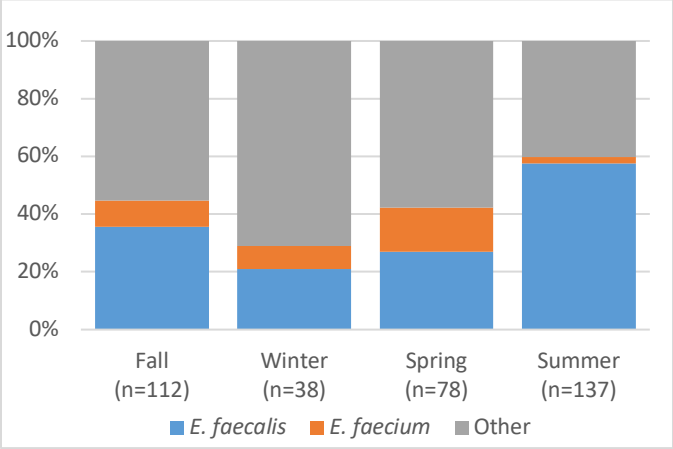
Table 4: Distribution of Enterococcus spp. by season, water type, and site

	n	*E. faecalis* 148	*E. faecium* 28	Other 189
Season				
Fall	112	40	10	62
Winter	38	8	3	27
Spring	78	21	12	45
Summer	137	79	3	55
Water Type				
Nontidal Fresh	157	74	11	72
Tidal Brackish	75	45	2	28
Pond	74	18	4	52
Recycled	59	11	11	37
Site				
MA01	22	4	2	16
MA02	18	6	1	11
MA03	37	15	1	21
MA04	39	13	2	24
MA05	42	23	3	16
MA06	19	1	8	10
MA07	40	16	1	23
MA08	36	32	0	4
MA09	38	20	6	12
MA10	41	13	2	26
MA11	33	5	2	26

the highest number of isolates were collected in summer (n=137), followed by fall (n=112), spring (n=78) and winter (n=38) (Figure 1). Season is a factor in species (p <0.01). Unadjusted, *E. faecalis* is more likely to be found in summer than fall, winter or spring. When adjusted for the other variables, it is more likely to be found in summer than fall or spring. Unadjusted, *E. faecium* is more likely to be found in

spring and fall than in summer. When adjusted for the other variables, it is more likely to be found in spring than winter or summer (Table 5).

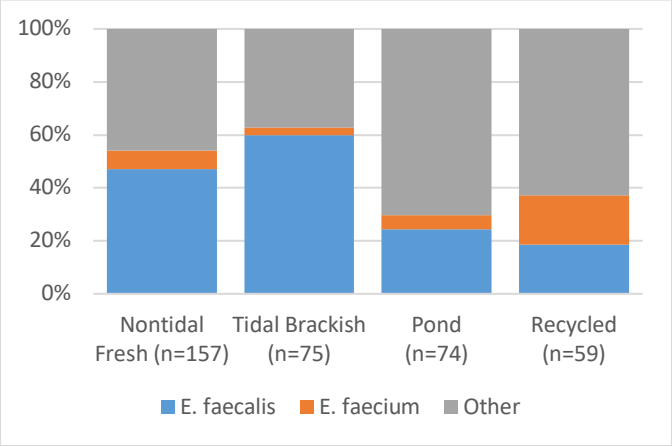
Figure 1: Distribution of Enterococcus spp. by season.



By water type, the highest number of isolates were collected in nontidal fresh (n=157), followed by tidal brackish (n=75), pond (n=74) and recycled (n=59) (Figure 2). Water type is a factor in species (p <0.01). Unadjusted, *E. faecalis* is more likely to be found in non-tidal fresh and tidal brackish than in pond or recycled water. When adjusted for the other variables, it is more likely to be found in non-tidal fresh than pond or recycled water. *E. faecium* is more likely to be found in recycled than non-tidal fresh, tidal brackish, or pond water (unadjusted and adjusted for other variables) (Table 5).

When unadjusted, pH, temperature, and salinity were also associated with *E. faecalis* probability, and temperature was associated with *E. faecium* probability. When adjusted for all other variables in table 5, salinity was associated with *E.*

Figure 2: Distribution of Enterococcus spp. by water type.



faecalis probability and temperature remained associated with *E. faecium* probability. *E. faecalis* probability increased as salinity increased, and *E. faecium* probability decreased as temperature increased (Table 5).

Antimicrobial Susceptibility Testing

Sixty six percent of water samples contained one or both species of interest, with representatives selected for antimicrobial susceptibility testing. Testing was conducted on 95 isolates (75 *E. faecalis*, 20 *E. faecium*) from 85 water samples (Table 6). Cephalosporins (ceftriaxone), clindamycin, trimethoprim-sulfamethoxazole, and low-concentration aminoglycosides (gentamicin) are not clinically effective against *Enterococcus*, and thus were excluded from the results. High concentration gentamycin and streptomycin were included, as resistance to those represent resistance to the often prescribed synergistic combination of penicillin/aminoglycoside (CLSI, 2018).

Table 5: Unadjusted and adjusted odds ratios of an Enterococcus isolate being E. faecalis or E. faecium

	E. faecalis		*E. faecium*	
	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Season				
Summer vs Fall	**2.452 (1.466-4.099)**	**2.017 (1.006-4.043)**	**0.228 (0.061-0.851)**	0.462 (0.100-2.139)
Summer vs Winter	**5.108 (2.182-11.954)**	2.139 (0.561-8.161)	0.261 (0.051-1.351)	1.314 (0.123-14.058)
Summer vs Spring	**3.697 (2.02-6.765)**	**3.504 (1.683-7.295)**	**0.123 (0.034-0.451)**	**0.194 (0.046-0.810)**
Fall vs Winter	2.083 (0.872-4.975)	1.061 (0.351-3.207)	1.144 (0.298-4.395)	2.843 (0.524-15.421)
Fall vs Spring	1.508 (0.801-2.838)	1.737 (0.832-3.628)	0.539 (0.22-1.319)	0.419 (0.157-1.122)
Spring vs Winter	1.382 (0.547-3.49)	0.611 (0.176-2.112)	2.121 (0.561-8.019)	**6.781 (1.086-42.346)**
Water Type				
Recycled vs NonTidal Fresh	**0.257 (0.124-0.531)**	**0.191 (0.081-0.446)**	**3.042 (1.240-7.460)**	**6.034 (2.056-17.711)**
Recycled vs Tidal Brackish	**0.153 (0.069-0.341)**	0.365 (0.129-1.033)	**8.365 (1.775-39.411)**	**7.148 (1.349-37.885)**
Recycled vs Pond	0.713 (0.307-1.657)	0.580 (0.238-1.410)	**4.010 (1.206-13.340)**	**4.120 (1.093-15.534)**
NonTidal Fresh vs Tidal Brackish	0.594 (0.340-1.039)	1.914 (0.854-4.287)	2.750 (0.594-12.733)	1.185 (0.230-6.095)
NonTidal Fresh vs Pond	**2.774 (1.497-5.139)**	**3.043 (1.345-6.886)**	1.318 (0.405-4.288)	0.683 (0.185-2.515)
Tidal Brackish vs Pond	**4.667 (2.308-9.434)**	1.590 (0.578-4.375)	0.479 (0.085-2.701)	0.576 (0.088-3.765)
pH	**0.414 (0.297-0.578)**	0.738 (0.483-1.129)	0.906 (0.527-1.558)	0.534 (0.244-1.167)
Temperature	**1.067 (1.029-1.107)**	1.066 (0.996-1.141)	**0.920 (0.867-0.976)**	**0.876 (0.783-0.980)**
Salinity	**1.471 (1.229-1.761)**	**1.487 (1.198-1.847)**	0.736 (0.042-1.226)	0.612 (0.201-1.867)

Adjusted for all other variables reported in table. Bold values are statistically significant (p <0.05). OR = odds ratio, CI = confidence interva

Table 6: Water samples containing species of interest

Site	Water Type	Water Samples	*E. faecalis*	*E. faecium*	*E. faecalis* **and/or** *E. faecium*
MA01	RW	8	3 (37.5%)	2 (25.0%)	4 (50.0%)
MA02	RW	7	4 (57.14%)	1 (14.29%)	4 (57.14%)
MA03	NF	13	7 (53.85%)	1 (7.69%)	7 (53.85%)
MA04	TB	13	8 (61.54%)	2 (15.38%)	9 (69.23%)
MA05	NF	14	12 (85.71%)	2 (14.29%)	12 (85.71%)
MA06	RW	8	1 (12.5%)	4 (50.0%)	5 (62.50%)
MA07	NF	14	7 (50.0%)	1 (7.14%)	7 (50.0%)
MA08	TB	12	12 (100%)	0 (0%)	12 (100.0%)
MA09	NF	14	12 (85.71%)	4 (28.57%)	13 (92.86%)
MA10	PW	14	6 (42.86%)	1 (7.14%)	7 (50.0%)
MA11	PW	12	3 (25.0%)	2 (16.67%)	5 (41.67%)
Totals		129	75 (58.14%)	20 (15.50%)	85 (65.89%)

One *E. faecalis* isolate (1.3%) was susceptible to all excepting quinupristin/dalfopristin for which it is intrinsically resistant, and one *E. faecium* isolate (5.0%) was susceptible to all antimicrobials. All isolates (n=95) were susceptible to ampicillin, vancomycin, daptomycin, and linezolid. *E. faecalis* isolates (n=75) were also susceptible to penicillin, and most (97.3%) were susceptible, while two were intermediate, to gatifloxacin. *E. faecium* isolates were susceptible to high concentration gentamicin and gatifloxacin.

Resistance was detected in *E. faecalis* isolates (n=75) to erythromycin (5.3%), high-concentration gentamicin (4.0%), high-concentration streptomycin (2.7%), quinupristin/dalfopristin (90.7%), tetracycline (10.7%), rifampicin (3.3%), ciprofloxacin (10.7%), and levofloxacin (1.3%). Resistance was detected in *E. faecium* isolates (n=20) to erythromycin (5.0%), high-concentration streptomycin (10.0%), quinupristin/dalfopristin (45.0%), tetracycline (20.0%), penicillin (5.0%), rifampicin (75.0%), and ciprofloxacin (20.0%) (Table 7, Figure 3). The only significant difference in resistance of *E. faecalis* and *E. faecium* isolates was to

quinupristin/dalfopristin (p<0.0001), but that can be expected as *E. faecalis* is intrinsically resistant to the antimicrobial (Figure 4).

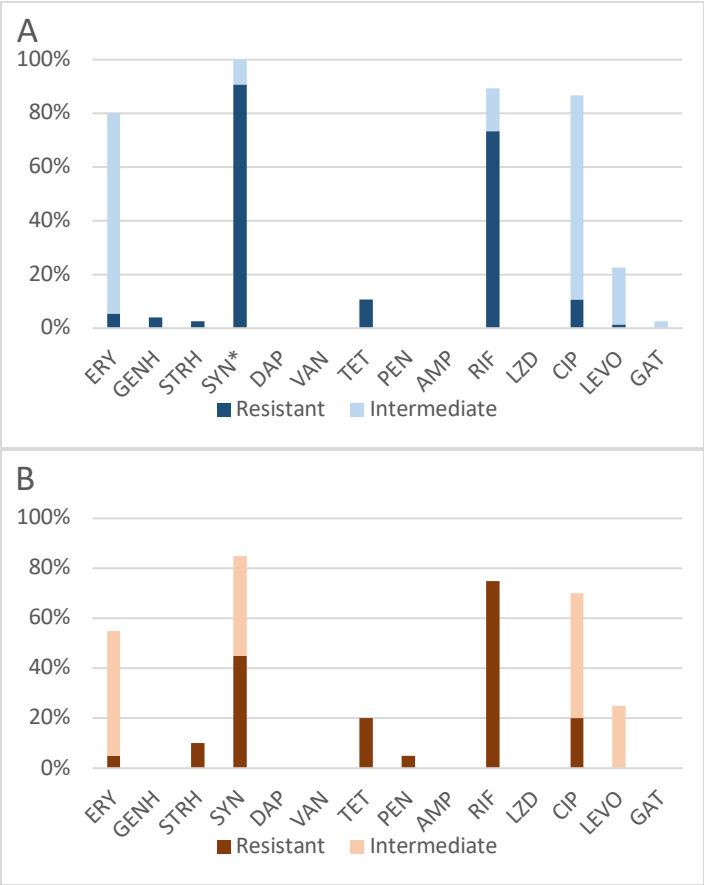
Table 7: Resistance of E. faecalis and E. faecium isolates to 14 antimicrobials

	E. faecalis (n=75)		*E. faecium* (n=20)	
Antimicrobial	Resistant	Intermediate	Resistant	Intermediate
ERY	4	56	1	10
GENH	3	NA	0	NA
STRH	2	NA	2	NA
SYN*	68	7	9	8
DAP	NA	NA	NA	NA
VAN	0	0	0	0
TET	8	0	4	0
PEN	0	NA	1	NA
AMP	0	NA	0	NA
RIF	55	12	15	0
LZD	0	0	0	0
CIP	8	57	4	10
LEVO	1	16	0	5
GAT	0	2	0	0

**E. faecalis* is intrinsically resistant to Q/D (SYN); NA: no corresponding MIC

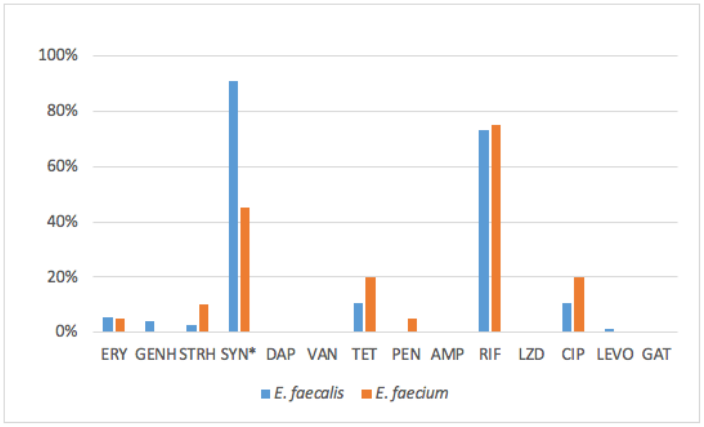
Season is statistically significantly associated with *E. faecalis* resistance to erythromycin (p <0.01), high-concentration streptomycin (p =0.0432) and tetracycline (p <0.01). Unadjusted, *E. faecalis* resistance to erythromycin is higher in winter than summer (OR 39.447, 95% CI: 1.474-<999.999) and resistance to tetracycline is higher in winter than fall (OR 15.663, 95% CI: 1.534-159.951), winter than summer (OR 22.995, 95% CI: 2.296-230.337) and spring than summer (OR 10.733, 95% CI: 1.281-89.949), but the statistically significant associations disappear once adjusted for the other variables. Neither season for *E. faecalis* nor water type for either species are statistically significantly associated with any of the antimicrobials tested.

Figure 3: Proportion of E. faecalis and E. faecium isolates resistant and intermediate to selected antimicrobials A: E. faecalis (n=75); B: E. faecium (n=20)



**E. faecalis* intrinsically resistant to SYN

Figure 4: Proportion of E. faecalis and E. faecium isolates resistant to selected antimicrobials.



**E. faecalis* intrinsically resistant to SYN

Multidrug Resistance

Twelve (16.0%) *E. faecalis* (excluding syn as *E. faecalis* is intrinsically resistant) and 14 (70.0%) *E. faecium* isolates were resistant to two or more antimicrobial classes (MDR) (p <0.0001). Of the *E. faecalis* MDR isolates, seven (9.3%) were resistant to two classes, four (5.3%) were resistant to three classes, and one (1.3%) was resistant to four classes. *Of the E. faecium* isolates, 11 (55%) were resistant to two classes, two (10.0%) were resistant to three classes, and one (5.0%) was resistant to four classes (Table 8). Season is statistically significantly associated with *E. faecalis* MDR (p <0.01) (Figure 5). Unadjusted, *E. faecalis* MDR is higher in winter than fall (OR 11.002, 95% CI: 1.272-95.195), winter than summer (OR 10.669, 95% CI: 1.458-78.076), spring than fall (OR 7.333, 95% CI: 1.072-50.141) and spring than summer (OR 7.111, 95% CI: 1.258-40.205) but the statistically significant associations disappear once adjusted for the other variables. By water type, MDR of *E. faecalis* and *E. faecium* was highest in tidal brackish, followed by non-tidal fresh, recycled and pond; however, the differences were not statistically significant.

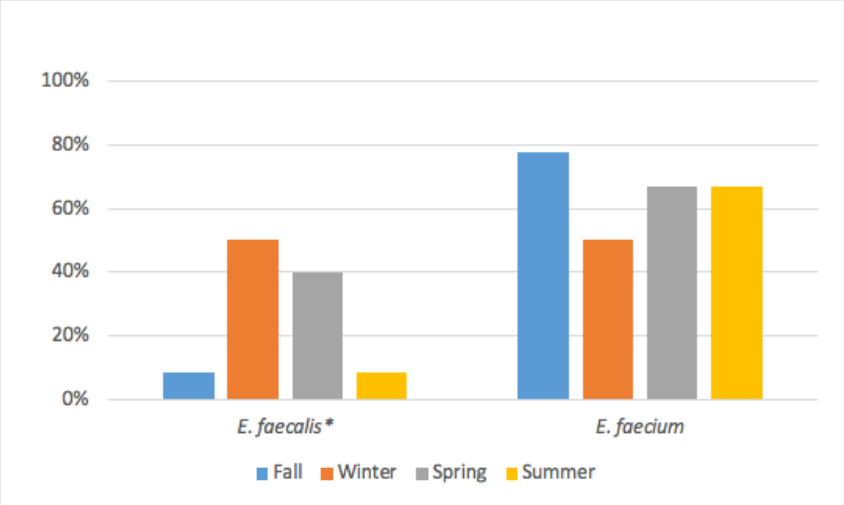
Discussion

Although 70% of *E. faecium* isolates were multidrug resistant, the resistance profiles did not include antimicrobials used for serious enterococcal infection. There is still the potential that the water sources contain pathogenic bacteria since they do contain enterococcal species. From an antimicrobial resistance perspective, it seems

Table 8: Multidrug Resistance profiles of E. faecalis and E. faecium isolates

Species	# of classes	Antimicrobials	# of isolates
E. faecalis	2	RIF+CIP	5
		GENH+TET	1
		TET+CIP	1
	3	ERY+GENH+TET	1
		ERY+GENH/STRH+TET	1
		ERY+TET+RIF	1
		TET+RIF+CIP/LEVO	1
	4	ERY+STRH+TET+RIF	1
E. faecium	2	SYN+RIF	7
		RIF+CIP	1
		ERY+RIF	1
		TET+CIP	1
		TET+RIF	1
	3	TET+RIF+CIP	1
		STRH+TET+PEN	1
	4	STRH+SYN+RIF CIP	1

Figure 5: Proportion of MDR E. faecalis and E. faecium isolates by season



*SYN excluded from MDR for *E. faecalis*

the risk of transferring serious antimicrobial-resistant *Enterococcus* from these waters to produce would be low. There were no significant differences in resistance to any of the antimicrobials tested between water types, but it may be more important to know which types of effluent are being deposited in a water source. The risk of vancomycin resistant *Enterococcus* may be higher for waterbodies which intake

treated wastewater from hospital waste (Garcia et al., 2007). While season is a factor in resistance of *E. faecalis* isolates to erythromycin, high-concentration streptomycin, and tetracycline, several options remain for treatment of enterococcal infection.

All isolates but one were susceptible to penicillin, and all to ampicillin, which are typically used as the first treatment option for enterococcal infections. One *E. faecium* isolate was resistant to penicillin, but not ampicillin; thus ampicillin could be used to treat an infection of this type. Sometimes an aminoglycoside is used with a penicillin to increase treatment efficacy, unless the enterococcal infection is resistant to high-concentration aminoglycosides. This synergistic treatment would be ineffective against one *E. faecalis* isolate, which was resistant to high-concentration gentamicin and high-concentration streptomycin. Three other *E. faecalis* and two *E. faecium* isolates were resistant to one, but not both, aminoglycosides tested. However, as all isolates were susceptible (none detected as intermediate) to ampicillin, it is unlikely an aminoglycoside would be employed.

In cases of penicillin allergy, vancomycin may be the initial treatment prescribed. All isolates tested were susceptible to vancomycin, and were also susceptible to two of the next lines of treatment for enterococcal infections which are reserved for cases of penicillin and vancomycin resistance (or penicillin allergy and vancomycin resistance): daptomycin and linezolid. Although resistance to vancomycin was not observed here, it has been well documented in the literature (Willems et al., 2005), and although it remains fairly low, resistance to daptomycin and linezolid has been observed as well (Maraki et al., 2014; Kelesidis et al., 2011; Kainer et al., 2007). Quinupristin/dalfopristin can also be used for vancomycin

resistant *E. faecium* infections only, as *E. faecalis* isolates are intrinsically resistant to it. However, resistance to quinupristin/dalfopristin was also observed in 45% of the *E. faecium* isolates in this study.

Aminoglycosides (gentamicin, streptomycin), macrolides (erythromycin), penicillins (penicillin, ampicillin), and quinolones (ciprofloxacin) are critically important antimicrobials for both human and animal health. Other critically important antimicrobials for human health are glycopeptides (vancomycin), lipopeptides (daptomycin), and oxazolidinones (linezolid). Rifampicin is a critically important antimicrobial agent for human health and a highly important antimicrobial agent for animal health, and tetracyclines are highly important for human health, but critically important for animal health (OIE, 2015).

In agriculture, water is only one potential pathway for exposure of crops to *Enterococcus*. Often, soil amendments are used to provide nutrients for plant growth, which may contain animal or human waste as fertilizer. Cattle waste may contain near the same level of risk as human waste, while chicken and pig waste may be considerably lower (Soller, Schoen, Bartrand, Ravenscroft, & Ashbolt, 2010). Persistence of enterococci in freshwater may also be higher for those of bovine fecal origin than human origin (Korajkic et al., 2013). Crop contamination can also occur by direct fecal deposit from area wildlife, or improper personal hygiene of agricultural workers.

Enterococcus is only one potential contaminant in water – the Produce Safety Rule requires examination of *E. coli* concentrations (FDA, 2018a). Chemical and physical parameters should also be reviewed to provide a holistic picture of the

quality of surface and recycled waters. Another lab working on the same larger project is evaluating antibiotic residues in these water samples. Several of the antibiotics used to evaluate antimicrobial resistance in this study are undergoing evaluation in the other lab: penicillin, ampicillin, erythromycin, ciprofloxacin, linezolid, tetracycline, and vancomycin. The results from this study can be combined with the results of that study to evaluate a potential association between antibiotic residues and resistance of enterococci in these waters.

The direct use of recycled water for agricultural purposes, bypassing deposition into a water source, could reduce disruption to a waterbody's existing ecosystem. Nutrients such as nitrogen and phosphorus often remain in the recycled water after wastewater treatment plant processing, and direct use of that water could reduce the amount of fertilizer needed for crops, and reduce the development of algal blooms from nutrient overload in lakes (Muhid, Davis, Bunn, & Burford, 2013; Toze, 2006).

Limitations

Antimicrobial resistance analysis could not be conducted by site because the number of *E. faecium* isolates was too low. The second year of water sampling has recently finished, perhaps the addition of those isolates will allow an analysis by site. We were able to conduct analysis by water type.

We used the standard gram positive (GPN3F) plate for testing. There is another gram positive NARMS plate (CMV3AGPF) available that includes antibiotics to treat VRE infections, including tigecycline which is an antimicrobial of last resort for enterococcal infections. The isolates we tested were low in resistance to

most of the potential treatment options for enterococcal infections, including vancomycin. The GPN3F plate includes antimicrobials to which resistance is commonly found in environmental isolates, such as ampicillin and rifampin, however, the use of the CMV3AGPF plate may be a better choice for future studies evaluating antimicrobial susceptibility of enterococci to clinically important antibiotics, such as vancomycin.

The microbroth dilution method can be used to assess antimicrobial resistance to penicillin and ampicillin for enterococci which are resistant due to production of low-affinity PBP's, but not those which are resistant due to production of β-lactamase. β-lactamase producing resistant isolates can be identified by a nitrocefin-based β-lactamase test, however the test was not performed in this study due to the rarity of β-lactamase producing enterococci (CLSI, 2012).

Chapter 4: Public Health Implications and Conclusions

Although it is good news that the *E. faecalis* and *E. faecium* isolates in this study did not display a high proportion of resistance to the antimicrobials used for serious enterococcal infection, their presence as fecal indicators suggest there may be other, more pathogenic bacteria in the water. The CDC has estimated that of the foodborne illnesses for which a cause could be identified, almost 50% are attributed to produce (Painter et al., 2013). While water is only one potential source of produce contamination in the farm to fork continuum, foodborne illness outbreaks can have far reaching consequences as some of the food produced in the U.S. is exported to other countries though the global food trade (Johnson, 2016). Conversely, there is growing interest in the "locavore" movement, or eating foods grown within a smaller, local area. Often this results in purchasing from smaller producers, and although an outbreak may be much more contained, there could also be increased risk because of less regulation on small farm production (Micallef & Buchanan, 2017).

Additionally, enterococci may shift to a viable but non-culturable (VBNC) state in the absence of a nutrient rich environment (Painter et al., 2013). Due to this factor and the random selection of isolates for archival in this study, it is probable that more than 65.89% of the water samples contained *E. faecalis* and/or *E. faecium*.

Many individuals could be exposed to antimicrobial-resistant bacteria on the farm to fork continuum. Agricultural workers may be exposed to the bacteria during irrigation of crops with a dose differential based on irrigation type (spray, drip, or surface), or during cultivation of crops. They may then transfer bacteria to their family members through take home exposures. Cultivated crops may also transfer

bacteria to distributors, processors and consumers. In cases of agricultural runoff, antimicrobial-resistant bacteria may be reintroduced to the same or another water source where it can affect swimmers, boaters, or fishermen (Dickin, Schuster-Wallace, Qadir, & Pizzacalla, 2016).

Consumption of produce contaminated with antimicrobial-resistant *Enterococcus* could introduce the bacteria to the gut microbiome, potentially creating a new reservoir of bacteria. While it may not cause any problems in a healthy individual, it could contribute to nosocomial or community acquired infections if spread through improper sanitation. The opportunistic pathogenic nature of *Enterococcus* and its ability to transfer microbial resistance to other, more pathogenic bacteria, necessitate close attention to use of enterococcal contaminated water on food crops. With the passage of the Food Safety Modernization Act (FSMA), the focus on foodborne illness has shifted from one of reaction to prevention. Although FSMA's Final Rule on Produce Safety only addresses *E. coli*, *Enterococcus* in irrigation water should not be ignored (Xu, Buchanan, & Micallef, 2016). While food can become contaminated at any location on the farm to fork continuum, care at the earliest stages of production can greatly reduce the risk of foodborne illness to consumers.

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