

ABSTRACT

Title of Document: ASSESSING THE PRESENCE OF
ANTIBIOTIC-RESISTANT *ENTEROCOCCUS*
IN RECLAIMED WATER USED FOR SPRAY
IRRIGATION.

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Reclaiming wastewater is increasing in the US to combat dwindling freshwater supplies. This water potentially contains pathogenic bacteria; therefore, we evaluated the occurrence, concentration, and antimicrobial susceptibilities of *Enterococcus* spp.—an important opportunistic pathogen that remains a leading cause of nosocomial infections—in reclaimed water used for spray irrigation (SI). A total of 48 wastewater effluent and SI samples were collected in 2009 and 2010 from the Mid-Atlantic and Midwest regions of the US. Enterococci were isolated using membrane filtration, confirmed using biochemical tests and PCR, and tested for antimicrobial susceptibility using the Sensititre® dilution system. We detected total enterococci and vancomycin-resistant enterococci (VRE) in 68% (27/40) and 8% (3/40), respectively, of all SI samples. VRE and vancomycin-intermediate enterococci

(VIE) represented 2% (1/41) and 10% (4/41), respectively, of the total enterococci recovered from all SI sites. Our findings show that SI workers may be exposed to enterococci during spray irrigation activities.

ASSESSING THE PRESENCE OF ANTIBIOTIC-RESISTANT *ENTEROCOCCUS*
IN RECLAIMED WATER USED FOR SPRAY IRRIGATION.

By

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Dedication

For my supportive family.

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I would like to thank my parents and Michael for being supportive as I navigated toward my dream. Thank you, Uncle Richard, Uncle David, and Aunt Louise, for always pushing me to achieve my goals.

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Chapter 1: Introduction

Reclaimed Water Use

As the world population increases, the demand for freshwater also grows. With an expected increase of 80 million people a year, freshwater demand of about 60 billion cubic meters (almost 16 trillion gallons) a day is predicted (UN Water, 2013). In developed countries, freshwater demand is forecasted to increase by 18% by 2025, while a 50% increase is anticipated for developing countries (UN Water, 2013). It is estimated that 70%, 20%, and 10% of freshwater is currently used for irrigation, industry, and domestic use, respectively (UN Water, 2013).

Approximately, 128,000 million gallons of freshwater per day were used by the United States for irrigation alone in 2005, while industries used 18,200 million gallons of freshwater per day for cooling, diluting, or washing in 2005 (Barber, 2009; USGS, 2013). These numbers will continue to increase, as will the need for water (USGS, 2013). To combat this increase in freshwater demand, nations, including the United States, are reclaiming treated wastewater for potable and non-potable reuse (EPA, 2012). In the 2012 Guidelines for Water Reuse, the EPA defined reclaimed water as “[m]unicipal wastewater that has been treated to meet specific water quality criteria with the intent of being used for a range of purposes” (EPA, 2012).

Reclaimed water currently is used for urban (i.e. landscape irrigation), agricultural (i.e. watering crops), environmental (i.e. augmentation of wetlands), and industrial purposes (i.e. power production) in the United States (EPA, 2012).

Reclaiming wastewater has become a common practice in many parts of the world. Palestine uses reclaimed water for crop irrigation (Al-Sa'ed, 2007); Spain and Italy recharge aquifers using the reclaimed water (Levantesi, et al., 2010); and Japan utilizes reclaimed water for snowmaking, toilet-flushing, spray irrigation, and industrial activities (Tajima, et al., 2007). Israel is the leader of reclaiming wastewater, currently reclaiming 65% of their wastewater each year (Friedler, 2001). Israel plans to reclaim 90% of their wastewater, which is already used primarily for crop irrigation due to the country's arid climate (Friedler, 2001). As noted above, the United States has also adopted the practice of reusing wastewater due to the sustainable benefits of this practice. The United States produces 32 billion gallons of municipal effluent each day (Global Water Intelligence, 2010; Miller, 2006). Approximately 7-8% of this wastewater is reclaimed (Global Water Intelligence, 2010; Miller, 2006), and water reclamation in the United States is expected to increase over the next decade (EPA, 2012).

Current Guidelines/Regulations for Reclaimed Water

In 2012, the U.S. EPA published the 2012 Guidelines for Water Reuse (EPA, 2012). The guidelines defined reclaimed water usage; discussed planning, managing, and operating reclaimed water systems; explored water supply and environmental considerations; described main types of reuse applications; summarized state regulatory programs for water reuse; compared regional variations of water reuse; discussed treatment technologies; and presented global experiences of reclaimed water use (EPA, 2012). While the guidelines are extensive, the document is only a

guideline and not a federal regulation. Regulation is determined, currently, on a state by state basis.

The states vary on their use of reclaimed water since there is no federal regulation guiding them. The strictest guideline for reclaimed water use is the California Water Recycling Criteria (EPA, 2012). Reclaimed water must be filtered and disinfected according to the state's regulations before being used for unrestricted food crop irrigation and landscaping purposes (EPA, 2012). California established these guidelines since untreated wastewater is applied to crops in the developing world with accompanying adverse public health effects (EPA, 2012).

Microbial Contaminants in Reclaimed Water

Nonetheless, data regarding the presence of specific microbial contaminants in reclaimed water are lacking. Currently, the effectiveness of pathogen control in wastewater treatment is assessed through routine monitoring of the reclaimed water by using 100 mL samples to detect indicator bacteria, such as total or fecal coliforms (Costán-Logares, et al., 2008; Harwood, et al., 2005). Indicator bacteria are microorganisms that are used to estimate levels of fecal contamination in a water source (EPA, 2013). Indicator bacteria measurements are used in place of costly analytical tests which could detect specific organisms (Brookes, et al., 2005). The World Health Organization (WHO) currently recommends monitoring for fecal coliforms and intestinal nematodes in reclaimed water (Harwood, et al., 2005). Some states use total coliforms as the indicator organism, while the majority of states use fecal coliforms (Costán-Logares, et al., 2008; Harwood, et al., 2005). Despite the wide use of coliforms as pathogenic indicators, evidence shows that total and fecal

coliforms are not adequate representations of the presence or absence of pathogenic bacteria due to their high susceptibility to chemical disinfection and low correlation with protozoan parasites (Harwood, et al., 2005). Additionally, the correlation between indicator bacteria and pathogenic organisms is also seasonally dependent and site specific (Wilkes, et al., 2009). Studies suggest that perhaps other indicators, such as *Escherichia coli* or *Clostridium perfringens*, may show a stronger correlation with the presence of pathogens; however, similar to the coliform indicators, the suggested indicators do not correlate strongly with all pathogens (Brookes, et al., 2005; Costán-Logares, et al., 2008; Harwood, et al., 2005; Wilkes, et al., 2009). Therefore, current monitoring of reclaimed water may inaccurately assess the presence of pathogens.

Public Health Risks

With increasing reclaimed water use, concern about the potential public health impact due to microbial contamination of the reclaimed water becomes an important issue that needs to be addressed. Bacteria present at wastewater treatment plants, as well as the strains that remain in treated effluent, present opportunities for potential human exposure. For instance, reclaimed water can be sprayed on agricultural crops, which can be a major source of human exposure to pathogens.

One of the greatest concerns for human infection with regard to wastewater reuse is from exposure to antibiotic-resistant bacteria, which have been isolated from treated wastewater effluent (Ferreira da Silva, et al., 2006; Garcia, et al., 2007; Huang, et al., 2012; Martins de Costa, et al., 2006; Rosenberg Goldstein, et al., 2012). Antibiotic-resistant enterococci are one type of antibiotic-resistant bacteria that can cause life-threatening human infections. Enterococci are not completely eliminated

during wastewater treatment. Martins de Costa, et al. (2006) found that the presence of antimicrobials in urban and hospital effluent had created a large pool of resistance genes and that wastewater treatment processes failed to prevent the dissemination of antibiotic-resistant enterococci into the environment. Once in the environment, the bacteria may exchange resistance genes with other bacteria, creating a larger pool of antibiotic-resistant bacteria (Martins de Costa, et al., 2006).

Researchers have explored viruses, bacteria, and parasites present in wastewater (de Roda Husman, et al., 2009; Hunt, et al., 2010; Levantesi, et al., 2010; Ryu, et al., 2007). However, to our knowledge, there are no papers that address the presence of antibiotic-resistant enterococci in reclaimed water used at spray irrigation sites in the United States. This proposed research will address this knowledge gap, providing insights into the concentrations of enterococci in treated wastewater used in reuse applications.

Research Rationale

Objective: To evaluate the presence of total enterococci and, in particular, antibiotic-resistant enterococci in treated wastewater used in reclamation activities at spray irrigation sites.

Hypothesis: The reclaimed water samples will be positive for antibiotic-resistant enterococci and multidrug-resistant enterococci.

Gaps in Knowledge

To our knowledge, no research has been conducted to address antibiotic-resistant enterococci in reclaimed water used at spray irrigation sites in the United

States. Previous studies focused mainly on treated wastewater effluent before delivery to spray irrigation sites. Evaluating the presence of antibiotic-resistant enterococci and the changes in bacterial loads of this microorganism at spray irrigation sites is important since potential exposure to antibiotic-resistant enterococci in reclaimed water could occur after contact with this water source in spray irrigation settings.

Significance

Determining the presence or absence of antibiotic-resistant enterococci in reclaimed water would provide insights into one specific organism that humans may be potentially exposed to through reclamation activities. Evaluating the effectiveness of current wastewater treatment processes in the reduction/removal of enterococci, as well as the influence of storage practices on bacterial growth at spray irrigation sites, is also essential.

Chapter 2: Background

Introduction

Increased application of reclaimed water—treated municipal wastewater—for agricultural and landscaping purposes is a rising practice in the US, as well as other nations around the world (Al-Sa'ed, 2007; EPA, 2012; Friedler, 2001; Levantesi, et al., 2010; Tajima, et al., 2007). Several pathogenic bacteria have been identified in wastewater treatment plant (WWTP) effluent, suggesting the presence of these bacteria in reclaimed water (Ferreira da Silva, et al., 2006; Garcia, et al., 2007; Huang, et al., 2012; Martins de Costa, et al., 2006; Rosenberg Goldstein, et al., 2012). *Enterococcus*, an important opportunistic pathogen that remains a leading cause of nosocomial infections, is one such bacterium whose presence in reclaimed water may potentially be harmful to human health (Fisher & Phillips, 2009).

Enterococcus

Genus Description

Enterococci are gram-positive, catalase negative, PYRase positive, facultative anaerobic organisms that are tolerant to an array of environmental conditions, such as extreme temperatures (5-50°C), variable pH (4.5-10), and high NaCl concentrations (Calfee, 2012; Fisher & Phillips, 2009; Moellering, 1992; Murray, 1990). These bacteria typically grow in chains and have the ability to grow in high levels of bile

(Fisher & Phillips, 2009; Moellering, 1992). Due to higher tolerance to chlorination, enterococci are used as fecal indicators (Castillo-Rojas, et al., 2013; Varela, et al., 2013). Fecal indicator bacteria, which are normally present in human feces, indicate levels of fecal contamination in a water source. Therefore, enterococci can be used as a potential predictor of the presence of other pathogenic bacteria (EPA, 2013).

Ecological Habitat and Distribution

Enterococci are present in the normal flora of the human intestinal tract and the female genital tract, and therefore, the bacteria are excreted in the feces (CDC, 2011; Moellering, 1992; Murray, 1990). Some species of enterococci may be exclusively isolated from environmental or veterinary sources (Murray, 1990). The following species of enterococci may be isolated from the environment, humans, or animals: *E. faecalis*, *E. faecium*, *E. durans*, *E. avium*, *E. raffinosus*, *E. gallinarum*, *E. casseliflavus*, *E. malodoratus*, *E. hirae*, *E. mundtii*, *E. solitaries*, and *E. pseudoavium* (Moellering, 1992). In production animals, *E. faecium* is the most commonly found (Fisher & Phillips, 2009; Murray, 1990). *E. mundtii* and *E. casseliflavus* are the most commonly isolated from plant sources, while *E. faecalis* and *E. faecium* are the most commonly isolated in the human gastrointestinal tract (Fisher & Phillips, 2009; Murray, 1990). *E. faecalis* constitutes approximately 85-90% of clinical isolates and ranges from 10^5 to 10^7 per gram in human feces, while *E. faecium* accounts for only 5-10% of clinical isolates and ranges from 10^4 to 10^5 per gram in human feces (Fisher & Phillips, 2009; Murray, 1990). *E. faecalis* is intrinsically resistant to quinupristin/dalfopristin (Synercid), macrolides, and lincosamides (Dina, et al., 2003;

Fisher & Phillips, 2009; Mazuski, 2008). *E. faecium* is intrinsically resistant to flavomycin (Sapkota, et al., 2012).

Pathogenicity

A few main factors contribute to the virulence of the *Enterococcus* species (Fisher & Phillips, 2009). The enterococci bacteria have the ability to colonize the human intestinal tract, adhere to multiple extracellular matrix proteins, and adhere to urinary tract epithelia, oral cavity epithelia, and human embryo kidney cells (Fisher & Phillips, 2009). Infections resulting from enterococci can occur both endogenously and exogenously (Castillo-Rojas, et al., 2013; Fisher & Phillips, 2009). Enterococci can translocate from the intestinal tract to the bloodstream, resulting in an endogenous infection initiating in the lymph nodes (Castillo-Rojas, et al., 2013; Fisher & Phillips, 2009). Also, exposure to contaminated objects, hands, food, or water may give rise to an exogenous enterococci infection (Castillo-Rojas, et al., 2013; Fisher & Phillips, 2009).

Nosocomial or community-acquired infections often are the result of an exposure to contaminated objects, such as healthcare workers' hands or food (Fisher & Phillips, 2009; Moellering, 1992; NYDOH, 2011). Enterococcal infections most often result in urinary tract infections, intra-abdominal and pelvis infections, and bacteremia (Moellering, 1992; Murray 1990; NYDOH, 2011). Enterococci can also cause endocarditis, CNS infections, neonatal meningitis, and surgical wound infections (Moellering, 1992, Murray 1990; NYDOH, 2011).

Enterococcus was the third most commonly reported pathogen causing healthcare-acquired infections (HAI) between 2006 and 2007, according to the CDC

National Healthcare Safety Network (NHSN) (Calfee, 2012; Hidron, et al., 2008; Hollenbeck & Rice, 2012). Twelve percent of 28,502 HAIs were associated with enterococci, and one-third of these reported infections were linked to vancomycin-resistant enterococci (VRE) (Calfee, 2012; Hidron, et al., 2008; Hollenbeck & Rice, 2012). Additionally, of the 81,139 pathogens causing the 69,475 HAIs reported to NHSN during 2009 and 2010, 14% were enterococci, and 3% of all reported pathogens were VRE (Sievert, et al., 2013). By 2010, enterococci became the second leading cause of healthcare acquired infections (Sievert, et al., 2013). Moreover, patients infected with enterococci have a high mortality rate of up to 61% (Fisher & Phillips, 2009). Most enterococcal infections are reported in hospitalized patients since the bacteria can easily be spread through contact with surfaces, such as equipment or hands contaminated with an infected person's feces (Fisher & Phillips, 2009; NYDOH, 2011).

Antibiotic Resistance Among Enterococci

Intrinsic resistance to a variety of antibiotics is common among enterococci species. For instance, *E. faecalis* is intrinsically resistant to macrolides, lincosamides, and streptogramin antibiotics (Dina, et al., 2003; Fisher & Phillips, 2009; Mazuski, 2008). Due to unique penicillin-binding proteins, enterococci can continue to synthesize its cell wall in the presence of β -lactam antibiotics, making some species of enterococci intrinsically resistant to penicillins, cephalosporins, and carbapenems (Moellering, 1992). In particular, *E. faecium*'s possession of low-affinity penicillin-binding proteins makes this enterococci species highly resistant to penicillin and ampicillin (Moellering, 1992). Single mutations can lead to high-level resistance to streptomycin and increased intrinsic resistance to the penicillins (Moellering, 1992).

In addition to intrinsic resistance to antimicrobials, enterococci can, and have, acquired resistance to certain antimicrobials. Acquired resistance can occur through horizontal gene transfer (HGT) or conjugation between bacteria, transformation, or transduction (Fisher & Phillips, 2009; Mazaheri, et al., 2011; Moellering, 1992). Specifically, genes can be exchanged through plasmids, transposons, or bacteriophages (Fisher & Phillips, 2009; Mazaheri, et al., 2011; Moellering, 1992). Evidence of gene exchange has been found between enterococci and staphylococci, streptococci, *Listeria*, *E. coli*, *Campylobacter coli*, and other gram positive bacteria (Fisher & Phillips, 2009; Moellering, 1992). The close contact in the gastrointestinal tract biofilm of enterococci with gram negative and other gram positive bacteria allows for exchange of genes by conjugation (Moellering, 1992). Rapid horizontal gene transfer occurs through a pheromone-induced conjugation system (Fisher & Phillips, 2009). Plasmid-free recipient cells secrete a specific sex pheromone peptide in order to initiate plasmid transference with the plasmid-sharing bacteria (Fisher & Phillips, 2009). Antibiotic resistance as well as virulence factors can be exchanged on transposons via plasmids through this process (Fisher & Phillips, 2009).

Vancomycin Resistance

Vancomycin is a glycopeptide used primarily to treat drug-resistant bacteria when other antibiotics fail (CDC, 2011; Varela, et al., 2013). Vancomycin was first clinically used as an antimicrobial to treat enterococci infections in 1972 (Fisher & Phillips, 2009). Only 15 years later, VRE was isolated in the United Kingdom and the United States (Fisher & Phillips, 2009; Mazuski, 2008). VRE infections increase the clinical treatment failure and mortality when compared to vancomycin-susceptible

enterococci infections (Fisher & Phillips, 2009). Mortality occurs in 75% of those with VRE bacteremia infections but in only 45% of those with vancomycin-susceptible enterococci infections (Fisher & Phillips, 2009).

Similar to methicillin-resistant *Staphylococcus aureus* (MRSA) infections, VRE infections are divided into two groups—hospital-acquired and community-acquired. The prevalence of community-acquired VRE may be on the rise due to the use of the growth promoter avoparcin, which was never approved in the United States, in animal feed outside of the United States (Fisher & Phillips, 2009; Mazuski, 2008). For hospital-acquired infections, CDC reported that between 1992 and 2004, there was a 20-fold increase in VRE-associated nosocomial infections (Fisher & Phillips, 2009).

Although seven known genes (*vanA-vanG*) confer vancomycin resistance, the three most prevalent genes are *vanA*, *vanB*, and *vanC* (Fisher & Phillips, 2009; Mazuski, 2008). These genes alter the binding target for vancomycin through the repression and activation of certain bacterial cell wall precursors (Mazuski, 2008). The *vanA* gene confers high-level resistance to vancomycin and teicoplanin; however, *vanB* confers moderate to high-level resistance to only vancomycin (Mazuski, 2008). Both *vanA* and *vanB* are associated with acquired resistance to vancomycin, while *vanC* is an intrinsic resistance gene that is most commonly found in *E. gallinarum*, *E. casseliflavus*, and *E. flavescens* (Fisher & Phillips, 2009; Mazuski, 2008). Since *vanC* is chromosomally located, this gene is non-transferable; however, *vanA* and *vanB* genes may be transferred to other gram-positive bacteria on plasmids during horizontal gene transfer (Fisher & Phillips, 2009).

The composition of the VRE's cell wall is altered in order to resist vancomycin (Fisher & Phillips, 2009). The peptidoglycan precursor D-Ala-D-Ala, which is vancomycin-susceptible, is changed to D-Ala-D-Lactate (D-Lac), which has 1,000 times less affinity for vancomycin (Fisher & Phillips, 2009). Another precursor, D-Ala-D-Ser (D-Ser), has a 7-fold decrease in affinity for vancomycin (Fisher & Phillips, 2009). These two peptidoglycan precursors essentially remove the susceptible target of vancomycin (Fisher & Phillips, 2009). Two genes, *vanS/vanR*, are involved in the repression of the binding site of vancomycin (Fisher & Phillips, 2009). With the presence of vancomycin, the *vanS* sensor kinase is activated, initiating the production of either the D-Lac or D-Ser peptidoglycan precursor and the repression of D-Ala-D-Ala (Fisher & Phillips, 2009).

Bacteria in Wastewater

Human medical waste has been identified as a possible source of environmental contamination for antibiotic-resistant bacteria (Varela, et al., 2013). VRE and ciprofloxacin-resistant enterococci were isolated in hospital effluent at densities of 10^2 to 10^3 CFU/mL (Varela, et al., 2013). The VRE isolates were identified as *E. faecalis* and *E. faecium*, and the isolates expressed multidrug resistance to ciprofloxacin, tetracycline, erythromycin, and gentamicin (Varela, et al., 2013). This pattern was identified in both the hospital effluent as well as WWTP effluent (Varela, et al., 2013).

At municipal WWTPs, antibiotic-resistant bacteria have already been identified (Araujo, et al., 2010; Börjesson, et al., 2009; Börjesson, et al., 2010; Ferreira da Silva, et al., 2006; Huang, et al., 2012; Martins de Costa, et al., 2006;

Rahimi, et al., 2007; Rosenberg Goldstein, et al., 2012). MRSA was identified at various stages of treatment at plants in the United States and Sweden (Börjesson, et al., 2009; Börjesson, et al., 2010; Rosenberg Goldstein, et al., 2012,). Antibiotic-resistant *Enterococcus* spp. were recovered at WWTPs in Utah, Iran, and Portugal (Araujo, et al., 2010; Ferreira da Silva, et al., 2006; Garcia, et al., 2007; Martins de Costa, et al., 2006; Rahimi, et al., 2007).

Insufficient eradication of antibiotic-resistant bacteria at WWTPs may also play a crucial role in contamination of the environment at spray irrigation sites using reclaimed water. Some studies have already identified pathogenic bacteria in reclaimed water and effluent samples. Martins de Costa, et al. (2006) found that the use of antimicrobials had created a large pool of resistance genes and that sewage treatment processes failed to prevent the dissemination of antibiotic-resistant enterococci into the environment. Rosenberg Goldstein, et al. (2012) identified MRSA and methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates in WWTP effluent while chlorination was not taking place at one sampling site, suggesting the possibility of the presence of these bacteria in reclaimed water as well. MRSA was found in one sample, and MSSA was found in two samples (Rosenberg Goldstein, et al., 2012). Additionally, antibiotic-resistant enterococci were recovered in treated wastewater effluent in Utah, China, and Portugal (Garcia, et al., 2007, Huang, et al., 2012, Ferreira da Silva, et al., 2006, Martins de Costa, et al., 2006). In particular, VRE was isolated in treated wastewater effluent in Texas and the United Kingdom (Caplin, et al., 2008, Beier, et al., 2008). Furthermore, at spray irrigation sites, soil contamination with *Enterococcus* has been reported in central Mexico

(Dalkmann, et al., 2012). The absolute numbers of antibiotic resistance genes and of *Enterococcus* isolates in the soil increased after prolonged years of spray irrigation, leading the authors to believe that the treated wastewater was the source of contamination (Dalkmann, et al., 2012).

Other studies examining bacteria in wastewater have focused mainly on bacterial indicator organisms. In Spain, *E.coli* was cultured from raw wastewater effluent flowing from a secondary treatment facility (Bichai, et al., 2012). Fecal coliforms were identified in secondary treatment effluent at levels above 3.5 log units during irrigation season in Tunisia (Bahri, et al., 2001). This spray irrigation water was not in compliance with WHO Guidelines (Bahri, et al., 2001). After tertiary treatment with chlorination disinfection, only 87%, 85%, 53%, and 98% of *E.coli*, total coliforms, *Pseudomonas aeruginosa*, and *E. faecalis*, respectively, were removed from the wastewater effluent in Mexico (Coronel-Olivares, et al., 2011). *Salmonella* species were identified in secondary treatment effluent from two WWTPs in Spain and Italy after a culture-based and DNA extraction method was completed (Levantesi, et al., 2010). Water used for spray irrigation contained 1.2×10^2 to 2.1×10^3 *Salmonella* gene copies/100 mL (Levantesi, et al., 2010). Additionally, *E.coli* and enterococci were present at concentrations of about 1 CFU/100 mL in the spray irrigation water (Levantesi, et al., 2010).

Objectives of This Thesis Project

In this study, we evaluated the occurrence, concentration, and antimicrobial susceptibilities of *Enterococcus* spp. at three spray irrigation sites that receive treated

wastewater from three different WWTPs in the Mid-Atlantic and Mid-West regions of the United States.

Chapter 3: Assessing the Presence of Antibiotic-Resistant *Enterococcus* in Reclaimed Water Used for Spray Irrigation

Abstract

Reclaiming municipal wastewater for agricultural, environmental, and industrial purposes is increasing in the United States to combat dwindling freshwater supplies. Assessing the presence of pathogenic bacteria in this reclaimed water is necessary. To our knowledge, data regarding the presence of *Enterococcus*, an opportunistic pathogen responsible for both hospital-acquired and community-acquired infections, at spray irrigation sites in the United States is lacking. Therefore, the occurrence, concentration, and antimicrobial susceptibility of *Enterococcus* in reclaimed water used for spray irrigation were evaluated in this study. A total of 8 wastewater effluent samples and 40 reclaimed water samples used for spray irrigation were collected in 2009 and 2010 from one wastewater treatment plant (WWTP) and its associated spray irrigation site in the Mid-Atlantic region of the United States and two WWTPs and their associated spray irrigation sites in the Midwest region. Enterococci were isolated using standard membrane filtration. Isolates were confirmed using biochemical tests and PCR. Antimicrobial susceptibility testing was conducted using the Sensititre® microbroth dilution system. Data were analyzed by two-way tables with measures of association and analysis of variance. We detected total enterococci and vancomycin-resistant enterococci (VRE) in 68% (27/43) and 8% (3/40), respectively, of all spray irrigation samples. VRE and vancomycin-intermediate enterococci (VIE) represented 2% (1/41) and 10% (4/41), respectively,

of the total enterococci recovered from all spray irrigation sites. At the Mid-Atlantic spray irrigation site, UV radiation decreased the total enterococci to undetectable levels. However, storage in open-air ponds at all three sites resulted in increased concentrations of enterococci compared to that of wastewater effluent inflow to the sites. Thirty-two percent of the total enterococci were identified as multi-drug resistant (MDR) (resistant to ≥ 2 antibiotic classes). More MDR isolates were identified as *E. faecium* (n=6) than *E. faecalis* (n=1). Our findings show that spray irrigation workers may be exposed to enterococci, particularly antibiotic-resistant enterococci, during spray irrigation activities.

Introduction

As the world population increases and water use escalates, freshwater resources continue to dwindle. To combat increases in freshwater demand, nations, including the United States, are reclaiming treated wastewater for potable and nonpotable reuse (EPA, 2012). In the 2012 Guidelines for Water Reuse, the EPA defined reclaimed water as “[m]unicipal wastewater that has been treated to meet specific water quality criteria with the intent of being used for a range of purposes” (EPA, 2012). Reclaimed water currently is used for urban (i.e. landscape irrigation), agricultural (i.e. watering crops), environmental (i.e. augmentation of wetlands), and industrial purposes (i.e. power production) (EPA, 2012). With increasing reclaimed water use, concern about the potential public health impacts due to microbial contamination of reclaimed water is an important issue that needs to be addressed.

Previous studies have shown that a number of bacterial pathogens can survive wastewater treatment including methicillin-resistant *Staphylococcus aureus* (MRSA),

Escherichia coli, *Salmonella*, and enterococci (Levantesti, et al., 2010; Nagulapally, et al., 2009; Rosenberg Goldstein, et al., 2012; Rosenberg Goldstein, et al., 2013).

VRE, in particular, have recently been isolated from wastewater effluent (Garcia, et al., 2007; Nagulapally, et al., 2009; Rosenberg Goldstein, et al., 2013) and could persist in distribution systems that relay reclaimed water to spray irrigation sites.

Enterococci are gram-positive, facultative anaerobic organisms that are present in the normal flora of warm-blooded animals and are tolerant to an array of environmental conditions, including extreme temperatures (5-50°C), variable pH levels (4.5-10), and high NaCl concentrations (Calfee, 2012; Fisher & Phillips, 2009; Moellering, 1992; Murray, 1990). Due to the higher tolerance of enterococci to chlorination, these microorganisms could withstand wastewater treatment processes, including tertiary treatments involving chlorination (Castillo-Rojas, et al., 2013; Varela, et al., 2013).

Enterococcus was the third most commonly reported pathogen causing healthcare-acquired infections (HAI) between 2006 and 2007, according to the CDC National Healthcare Safety Network (NHSN) (Calfee, 2012; Hidron, et al., 2008; Hollenbeck & Rice, 2012). Twelve percent of 28,502 HAIs were associated with enterococci, and one-third of these reported infections were linked to vancomycin-resistant enterococci (VRE) (Calfee, 2012; Hidron, et al., 2008; Hollenbeck & Rice, 2012). Additionally, of the 81,139 pathogens causing the 69,475 HAIs reported to NHSN during 2009 and 2010, 14% were enterococci, and 3% of all reported pathogens were VRE (Sievert, et al., 2013). By 2010, enterococci became the second leading cause of healthcare acquired infections (Sievert, et al., 2013). Moreover,

patients infected with enterococci have a high mortality rate of up to 61% (Fisher & Phillips, 2009). Most enterococcal infections are reported in hospitalized patients since the bacteria can easily be spread through contact with surfaces, such as equipment or hands contaminated with an infected person's feces (Fisher & Phillips, 2009; NYDOH, 2011).

Human medical waste has been identified as a possible source of environmental contamination of antibiotic-resistant bacteria (Varela, et al., 2013). Vancomycin-resistant and ciprofloxacin-resistant enterococci were isolated in hospital effluent at densities of 10^2 to 10^3 CFU/mL (Varela, et al., 2013). In addition to human medicine, antibiotic use in veterinary, agriculture, and fish farm applications is a factor that may increase the antibiotic-resistant bacteria in water, thereby being a possible source of environmental contamination at spray irrigation sites (Furtula, et al., 2013; Varela, et al., 2013). Additionally, antibiotic-resistant enterococci were recovered in treated wastewater effluent in Utah, China, and Portugal (Ferreira da Silva, et al., 2006; Garcia, et al., 2007; Huang, et al., 2012; Martíns de Costa, et al., 2006). In particular, VRE was isolated in treated wastewater effluent in Texas and the United Kingdom (Beier, et al., 2008; Caplin, et al., 2008). Furthermore, at spray irrigation sites, soil contamination has been located in central Mexico (Dalkmann, et al., 2012). The absolute numbers of antibiotic resistance genes and of *Enterococcus* isolates in the soil increased after prolonged years of spray irrigation, leading the authors to believe that the treated wastewater was the source of contamination (Dalkmann, et al., 2012).

To our knowledge, there are no published studies analyzing reclaimed water used at spray irrigation sites in the U.S. for the presence of total enterococci and VRE. In this study, we evaluated the occurrence, concentration, and antimicrobial susceptibilities of enterococci recovered from three spray irrigation sites that receive treated wastewater from three different WWTPs in the Mid-Atlantic and Mid-West regions of the U.S..

Materials and Methods

Sampling sites

Spray irrigation samples collected at reclamation sites were primarily studied in order to identify the presence or absence of *Enterococcus* at the point of use. Samples from three spray irrigation sites were analyzed. All sites were chosen based on the willingness of the site operator to participate. Mid-Atlantic spray irrigation site 1 (SI1) receives wastewater effluent from a Mid-Atlantic WWTP, which is a tertiary WWTP in an urban area. Domestic and hospital wastewater comprise the influent at the Mid-Atlantic plant, and the effluent is used for spray irrigation at landscaping sites. Once the WWTP treated effluent reaches SI1, it passes through a double-walled aluminum screen and is then treated with ultraviolet (UV) radiation. After UV treatment, the water is pumped into an open-air storage pond at a rate of 230,000 gallons per day with a peak capacity of 4 million gallons. Water is then pumped from the storage pond to a pump that distributes the water to spray heads. Water samples were retrieved at multiple steps during the treatment process at SI1. The treatment steps at SI1 are illustrated in Figure 1a.

Midwest spray irrigation site 1 (NE1) receives effluent from Midwest WWTP1, which is a tertiary WWTP in a rural area whose influent includes domestic wastewater and agriculturally influenced stormwater. Chlorination occurs at this site during the summer. This chlorinated effluent is used also for spray irrigation at landscaping sites, particularly golf courses. Midwest spray irrigation site 2 (NE2) receives effluent from Midwest WWTP2, a secondary WWTP. The influent is comprised of domestic wastewater, wastewater from a food production facility, and agriculturally influenced stormwater. The unchlorinated effluent is used for landscaping and crop irrigation. At both spray irrigation sites in the Midwest, there is no further treatment of the wastewater effluent once the water is piped directly from the WWTPs. The wastewater is stored in open ponds at both Midwest spray irrigation sites. The treatment steps at NE1 and NE2 are illustrated in Figure 1b.

Sampling

A total of forty spray irrigation samples and eight effluent samples were included in this study. Thirty-two samples were collected from Mid-Atlantic SII, three samples were collected from Midwest NE1, and five samples were collected from Midwest NE2. One sample was collected from Mid-Atlantic WWTP1, three samples were collected from Midwest WWTP1, and four samples from Midwest WWTP2. The samples were collected between October 2009 and October 2010 (Rosenberg Goldstein, et al., 2012). Exact timing of sample collection was determined by the site operators (Rosenberg Goldstein, et al., 2012). All samples were collected in 1-L sterile polyethylene Nalgene® Wide Mouth Environmental

Sample Bottles and transported to the laboratory at 4°C (Rosenberg Goldstein, et al., 2012).

Isolation

Membrane filtration was used to isolate total enterococci and VRE from the water samples (EPA, 2002). One liter of each spray irrigation sample was filtered through 0.45 µm, 47 mm mixed cellulose ester filters (Millipore, Billerica, MA). Filters were then plated in duplicate on membrane-Enterococcus Indoxyl-β-D-Glucoside (mEI) agar (EMD Millipore, Billerica, MA) to isolate total enterococci and mEI agar modified with 16 µg/mL of vancomycin to isolate VRE. Plates were incubated at 41°C for 24 hr. Colonies with blue halos were considered presumptive total enterococci and VRE. These colonies were purified on Brain Heart Infusion (BHI) agar (Becton, Dickinson and Company, Franklin Lakes, NJ) and archived in Brucella broth (Becton, Dickinson and Company) with 15% glycerol at -80°C. *E. faecalis* ATCC 29212 was used as a positive control and phosphate buffered saline was used as a negative control throughout the isolation process.

Identification

Total enterococci and VRE were confirmed using the Gram stain, the catalase test, and by detection of pyrrolidonyl peptidase (pyr) activity (Remel, Lenexa, KS). For confirmation, a multiplex PCR assay developed by Micallef et al. (2013) was used. Genomic DNA was extracted by heat lysis as described previously (Micallef, et al., 2013). The PCR reaction targeted the D-alanine:D-alanine ligase (*ddl*) genes of *E. faecalis* and *E. faecium*, the vancomycin resistance-encoding *vanC1* and *vanC2/3* genes of *E. gallinarum* and *E. casseliflavus*, respectively, and an internal control

targeting a 350 base pair portion of the 16S rRNA gene. PCR amplification consisted of an initial denaturing step of 95°C for 3 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. Positive controls used for PCR amplification were *E. faecalis* ATCC 51299, *E. faecium* ATCC 51559, *E. casseliflavus* ATCC 25788, and *E. gallinarum* ATCC 49573. Molecular grade water was used as a negative control for PCR amplification.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed on all PCR-confirmed *Enterococcus* isolates ($n = 41$) using the Sensititre® microbroth dilution system (Trek Diagnostic Systems Inc., Cleveland, OH) following the manufacturer's recommendations. Cultures incubated overnight were transferred to sterile, demineralized water (Trek Diagnostic Systems) to achieve a 0.5 McFarland standard. Then, 50 µL of each suspension was transferred to sterile cation-adjusted Mueller Hinton broth (Trek Diagnostic Systems), and 50 µL of the broth solution was then dispensed into GPN3F minimal inhibitory concentration (MIC) plates (Trek Diagnostic Systems) that included the following antibiotics (range of concentrations in µg/ml): erythromycin (ERY; 0.25–4 µg/mL), clindamycin (CLI; 0.12–2 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), gentamicin (GEN; 2–16, 500 µg/mL), levofloxacin (LEVO; 0.25–8 µg/mL), linezolid (LZD; 0.5–8 µg/mL), ceftriaxone (AXO; 8–64 µg/mL), streptomycin (STR; 1,000 µg/mL), penicillin (PEN; 0.06–8 µg/mL), rifampin (RIF;

0.5–4 µg/mL), gatifloxacin (GAT; 1–8 µg/mL), ciprofloxacin (CIP; 0.5–2 µg/mL), trimethoprim/sulfamethoxazole (SXT; 1/19–4/76 µg/mL), and oxacillin+2%NaCl (OXA+; 0.25–8 µg/mL). *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 strains were used for quality control. Then, 50 µL of each suspension was transferred to sterile cation-adjusted Mueller Hinton broth (Trek Diagnostic Systems), and 50 µL of the broth solution was then dispensed into CMV5ACDC minimal inhibitory concentration (MIC) plates (Trek Diagnostic Systems) that included the following antibiotics (range of concentrations in µg/ml): erythromycin (ERY; 0.50–8 µg/mL), quinupristin/dalfopristin (SYN; 1–32 µg/mL), vancomycin (VAN; 0.5–32 µg/mL), tetracycline (TET; 4–32 µg/mL), gentamicin (GEN; 128–1024 µg/mL), linezolid (LZD; 0.5–8 µg/mL), streptomycin (STR; 512–2048 µg/mL), penicillin (PEN; 0.50–16 µg/mL), and ciprofloxacin (CIP; 0.12–4 µg/mL). *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were used as quality control strains. All plates were read manually. MICs were recorded as the lowest concentration of an antimicrobial that completely inhibited bacterial growth [Clinical and Laboratory Standards Institute (CLSI), 2010]. Resistance break points published by the CLSI were used (CLSI, 2010). Multidrug resistance (MDR) was defined as resistance to two or more classes of antibiotics.

Statistical Analysis

Descriptive statistics include the percentages of wastewater samples positive for enterococci and VRE by spray irrigation site. Two-sample mean comparison tests and analysis of variance were used to compare concentrations at each spray irrigation site and between spray irrigation sites, respectively. Two-way tables with measures

of association were performed between *Enterococcus* spp. with respect to the percent of resistance and intermediate-resistance of each group of isolates. In all cases, p -values ≤ 0.05 were defined as statistically significant. All statistical analyses were performed using Stata/IC 10 (StataCorp LP, College Station, TX).

Results

Presence and Concentration of *Enterococcus*

Enterococcus was detected at all spray irrigation sites in this study (Table 1). Total enterococci were identified in the majority of samples, except in those taken immediately after UV treatment and in one pond sample at Mid-Atlantic SI1 during the June sampling. From all sampling sites, 68% (27/40) of spray irrigation samples were positive for enterococci: 59% (19/32) of samples from Mid-Atlantic SI1; 100% (3/3) of samples from Midwest NE1 spray irrigation site; and 100% (5/5) of samples from Midwest NE2 spray irrigation site. The percentage difference in positive samples between the three locations (SI1, NE1, and NE2) was not statistically significant.

At the Mid-Atlantic SI1 and Midwest NE1 sites, the concentration of enterococci increased between the WWTP effluent samples and upon reception to the spray irrigation site. At the Midwest NE2 sites, total enterococci decreased between the WWTP effluent and upon reception to the spray irrigation site. None of these changes were statistically significant.

The concentration of total enterococci decreased to undetectable levels after UV treatment at Mid-Atlantic SI1 but increased after delivery to and storage in the open-air pond (Table 1). After UV treatment, the average concentration of total

enterococci at Mid-Atlantic SII increased during pond storage and upon delivery to the pumphouse (Figure 2). The order of magnitude increase in total enterococci between the open-air storage pond and the inlet to the pumphouse at Mid-Atlantic SII was statistically significant ($p=.048$).

In total, 41 enterococci isolates were recovered from the three spray irrigation sites: 36 isolates at Mid-Atlantic SII, 4 isolates at Midwest NE1, and 1 isolate at Midwest NE2. PCR was used to identify the species of forty-one isolates (Table 2). Overall, 44% (18/41) of *Enterococcus* spp. were identified as *E. faecalis*, and 27% (11/41) were identified as *E. faecium*. Additionally, 12% (5/41), 5% (2/41), and 12% (5/41) were identified as *E. casseliflavus*, *E. gallinarum*, and an unidentified *Enterococcus* species, respectively.

Presence of VRE and VIE

VRE and vancomycin-intermediate enterococci (VIE) represented 2% (1/41) and 10% (4/41), respectively, of the total enterococci recovered from all spray irrigation sites. VRE and VIE were detected only at Mid-Atlantic SII in 3% (1/32) and 9% (3/32) of the samples, respectively. No VRE or VIE were detected at the Midwest spray irrigation sites. The VRE isolate was recovered before UV radiation and was identified as an *Enterococcus* species other than *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus*. The VIE isolates were isolated in samples from the inlet to the pumphouse and were identified as *E. casseliflavus* (75%) and an *Enterococcus* species not targeted in the PCR amplification (25%). While the total number of enterococci increased between the open-air storage pond and the inlet to

the pumphouse at Mid-Atlantic SII, the number of VIE isolates also increased; however, this increase was not statistically significant (Table 1).

Antibiotic Resistance Patterns

The MIC₅₀ (MIC for 50% of the bacteria are less than or equal to this MIC) and MIC₉₀ (MIC for 90% of the bacteria are less than or equal to this MIC) of *E. faecalis* and *E. faecium* isolates for each antibiotic were almost identical. Besides the difference in the antibiotic quinupristin/dalfopristin, only the MIC₅₀ for penicillin was larger for *E. faecalis*, and only the MIC₉₀ for ciprofloxacin was larger for *E. faecium* (Table 3).

At Mid-Atlantic SII, 94% (17/18) of the *E. faecalis* isolates from Mid-Atlantic SII were only resistant to quinupristin/dalfopristin, representing intrinsic antibiotic resistance. Forty-five percent (5/11) of the *E. faecium* isolates from Mid-Atlantic SII were resistant to multiple antibiotics used to treat enterococci infections including erythromycin, quinupristin/dalfopristin, penicillin, and ciprofloxacin (Figure 3). The percentage difference in resistance of quinupristin/dalfopristin and ciprofloxacin between the *E. faecalis* and *E. faecium* isolates was statistically significant ($p=.045$). Also, some *E. faecalis* and *E. faecium* isolates were intermediately resistant to erythromycin, quinupristin/dalfopristin, linezolid, and ciprofloxacin (Figure 4).

At the Midwest NE1 and NE2 sites, all isolates were identified as *E. faecalis* with 100% intrinsic resistance to quinupristin-dalfopristin. Additionally, three isolates were intermediately resistant to erythromycin, and one isolate was intermediately resistant to ciprofloxacin. While the number of enterococci isolates

increased as the reclaimed water was processed at Mid-Atlantic S11, the percentage of antibiotic-resistant enterococci decreased (Figure 5).

One VRE isolate and four VIE isolates were isolated from Mid-Atlantic S11. The isolates were resistant or intermediately resistant to a number of other clinically relevant antibiotics including erythromycin, tetracycline, ciprofloxacin, clindamycin, linezolid, and quinupristin-dalfopristin (Figure 6).

Multi-drug Resistance

Thirty-two percent (13/41) of the enterococci isolates from all three spray irrigation sites were identified as MDR. Overall, 26% (5/19) were identified as *E. faecium*, 16% (3/19) were identified as a species not targeted in the PCR reaction, 10.5% (2/19) were identified as *E. casseliflavus*, 10.5% (2/19) were identified as *E. gallinarum*, and 5% (1/19) were identified as *E. faecalis*.

Discussion

Occurrence of *Enterococcus*

Previous studies have detected *Enterococcus* spp. in treated wastewater used at spray irrigation sites in Israel; however, to our knowledge, our study is the first to identify enterococci in reclaimed water at spray irrigation sites in the Mid-Atlantic and Midwest regions of the United States (Benami, et al., 2013). Similar to other studies' findings of VRE and *Enterococcus* in treated effluent, we detected enterococci in the samples delivered from the WWTP to the spray irrigation sites (Beier, et al., 2008; Caplin, et al., 2008; Ferreira da Silva, et al., 2006; Garcia, et al., 2007; Huang, et al., 2012; Martins de Costa, et al., 2006; Rosenberg Goldstein, et al., 2013).

However, our study identified that there was potentially an environmental exposure,

perhaps biofilms—communities of microorganisms in which cells stick to each other on a hydrated surface—in the delivery pipe system from the WWTP to the spray irrigation sites, that increased the concentration of total enterococci as well as VRE at the spray irrigation sites.

At the Mid-Atlantic spray irrigation site where the treated wastewater was disinfected through UV radiation treatment, total enterococci were significantly reduced to an undetectable level. Consistent with findings from previous studies, our study identified UV radiation as a successful disinfectant for enterococci (Connor-Kerr, et al., 1998; Luczkiewicz, et al., 2011; Nagulapally, et al., 2009). Of particular note, Nagulapally, et al. (2009) determined that VRE was eliminated to undetectable levels in WWTP effluent after UV disinfection. These results were also consistent with our study's findings. However, as seen by the increase in concentration of total enterococci after storage in the open-air pond, the benefits of the UV-disinfection are eliminated probably due to prolonged environmental exposure. California state law requires UV-disinfection of reclaimed water used for crop irrigation, suggesting that a more stringent processing system at spray irrigation sites may be necessary (EPA, 2012).

Species Diversity

Of the species-identified enterococci, 71% of the total enterococci isolates were identified as *E. faecium* and *E. faecalis*. These two species of *Enterococcus* are the predominant species of enterococci located in the human gastrointestinal tract, therefore it is not surprising to find them in significant numbers in wastewater (Fisher & Phillips, 2009; Murray, 1990). While *E. faecalis* was the most predominant species isolated in this study, *E. faecium* isolates represented the majority of the

multi-drug resistant bacteria. *E. faecalis* is also the most commonly isolated from human clinical samples of enterococci (Aaerestrup, et al., 2000, Fisher & Phillips, 2009; Murray, 1990).

The identification of VRE and VIE as *E. casseliflavus* and another untargeted species instead of as *E. faecium* and *E. faecalis* suggests an environmental source since *E. casseliflavus* and *E. mundtii* are the most commonly isolated from plants sources (Fisher & Phillips, 2009; Murray, 1990). The open-air storage pond at all three spray irrigation sites allows for prolonged exposure to environmental contamination. As previously determined, antibiotic-resistant enterococcal contamination can occur through interaction with the open-air pond and urban runoff, animal excrement, animal farm runoff, and plants (Furtula, et al., 2013; Moore, et al., 2008; Vignaroli, et al., 2011). However, the resistance associated with *E. casseliflavus* could also be attributed to the *vanC* gene, which provides intrinsic resistance to this species (Fisher & Phillips, 2009; Mazuski, 2008).

Antibiotic Resistance Patterns

Thirty-two percent of the total enterococci were MDR. In addition, all of these MDR isolates were intermediately resistant to at least one antibiotic. Intermediately-resistant bacteria are a potential public health threat due to their ability to evolve into resistant bacteria. There are currently no established recommendations for treatment of VRE; however, the antibiotics daptomycin and linezolid have been found to be effective against this pathogenic strain (Casal, et al., 2012; Eliopoulos, 2009; Gallagher, et al., 2009). According to our study, 29% of enterococci isolates are already intermediately-resistant to linezolid, suggesting that future use of this antibiotic may be in jeopardy.

Public Health Implications

As previously stated, *Enterococcus* species have the ability to cause life-threatening human infections, and enterococci increase the mortality of an infected person by up to 61% (Fisher & Phillips, 2009). The greatest concern for human infection from spray irrigation sites is from exposure to antibiotic-resistant bacteria, which have been previously isolated from treated wastewater effluent (Ferreira da Silva, et al., 2006; Garcia, et al., 2007; Huang, et al., 2012; Martins de Costa, et al., 2006). This study shows that enterococci, in particular VRE and VIE, are present in reclaimed water at spray irrigation sites. Enterococcal presence at spray irrigation sites presents immediate occupational concerns to the spray irrigation workers through inhalation, dermal, or accidental ingestion exposure. Previous studies have identified an increase in gastrointestinal illness in workers at WWTPs; however, the association of the illness to a specific pathogenic bacterium is inconclusive (Seuri, et al., 2005; McCunney, 1986). In addition, some reclaimed water is used for crop irrigation or as a source for drinking water, increasing the potential exposure concerns to the general public (EPA, 2012).

One of the predominant clinical concerns associated with enterococcal infection is that enterococci are difficult to clinically treat due to their intrinsic and acquired resistance (Mazuski, 2008). Most enterococci have intrinsic resistance to the penicillins and the cephalosporins, and some have acquired resistance to tetracyclines, macrolides, lincosamines, fluoroquinolones, and aminoglycosides (Mazuski, 2008). *E. faecalis* is intrinsically resistant to quinupristin/dalfopristin (Synercid), macrolides, and lincosamides (Dina, et al., 2003; Fisher & Phillips, 2009;

Mazuski, 2008). *E. faecium* is intrinsically resistant to flavomycin (Sapkota, et al., 2012).

Another significant public health concern is intra- and inter-species transfer of antimicrobial resistance genes. Martins de Costa, et al. (2006) found that the use of antimicrobials had created a large pool of resistance genes and that sewage treatment processes failed to prevent the dissemination of antibiotic-resistant enterococci into the environment. Once in the environment, the bacteria may exchange resistance genes with other bacteria, creating a larger pool of antibiotic-resistant bacteria.

Currently, 30% of enterococci isolated from ICUs are VRE, and approximately 10-19% of patients colonized with VRE are also colonized with MRSA (Mazuski, 2008).

The use of vancomycin to treat MRSA may be a factor in the increase of VRE in healthcare settings (Mazuski, 2008). Potential transmission of vancomycin resistance to staphylococci is a rising public health concern, especially since it has been found that enterococci and *Staphylococcus aureus* can exchange genetic material (Fisher & Phillips, 2009; Mazuski, 2008). The first case of vancomycin-resistant *Staphylococcus aureus* (VRSA) was reported in 2003, and the continual rise of VRSA and vancomycin-intermediate *Staphylococcus aureus* (VISA) will have a significant impact on management of these infections (Mazuski, 2008).

Limitations

We identified a few limitations to our study, which is common among field studies. We were limited with sampling opportunities due to the site operators' discretion, limiting the number of samples to analyze for enterococci isolates. Due to the limited sample size, analyzing statistical significance of percentage differences may not accurately represent the true relationship. Additionally, direct comparison

between the three spray irrigation sites was limited due to differences in sampling location and treatment and storage processes at each site. Also, enrichments of the enterococci were not completed due to the authors' desire to analyze concentration data. Therefore, some bacteria may not have been accounted for in addition to the bacteria that may have been injured between sampling and laboratory plating. This study's data can also not be generalized to the whole country since only three spray irrigation sites in two regions of the country were examined.

Conclusions

To our knowledge, our study is the first to demonstrate the occurrence, concentration, and antimicrobial susceptibility of enterococci present in reclaimed water at spray irrigation sites in the Mid-Atlantic and Midwest regions of the United States. We found an increase in concentration of total enterococci after storage in an open-air pond, suggesting an environmental source for the increase in total enterococci and VIE. VRE, VIE, and MDR enterococci were identified at the spray irrigation sites which raises public health concerns with regard to potential human exposures to the reclaimed water.

Table 1. Average concentration of total enterococci and vancomycin-resistant enterococci (VRE) by spray irrigation site and treatment or storage step across all sample collection dates

	Total Enterococci (CFU/100 mls)	VRE (CFU/100 mls)
Mid-Atlantic SI1		
WWTP Effluent	2.1×10^{-2}	0
Before UV	3.18×10^1	0
After UV	0	0
Pond	2.70×10^{-1}	0
Inlet to Pumphouse	1.84×10^2	2.00×10^{-1}
Mid-West NE1		
WWTP Effluent	1.26×10^1	2.98
Pond	7.44×10^1	3.56×10^{-1}
Mid-West NE2		
WWTP Effluent	7.32×10^1	0
Hose	4.83	0

Table 2. Number and percentage of total enterococci isolated by species and spray irrigation site

<i>Enterococcus</i> <i>species</i>	Number of Isolates (%) ^a			
	Mid-Atlantic SI1	Midwest NE1	Midwest NE2	Total
<i>E. faecalis</i>	13 (36.1)	4 (100)	1 (100)	18 (43.9)
<i>E. faecium</i>	11 (30.6)	0 (0)	0 (0)	11 (26.8)
<i>E. casseliflavus</i>	5 (13.9)	0 (0)	0 (0)	5 (12.2)
<i>E. gallinarum</i>	2 (5.5)	0 (0)	0 (0)	2 (4.9)
Other	5 (13.9)	0 (0)	0 (0)	5 (12.2)

Table 3. Minimum inhibitory concentration (MIC) ranges, MIC_{50s}, and MIC_{90s} (µg/mL) for nine antibiotics.

Antimicrobial	All <i>Enterococcus</i> Isolates (n=41)			<i>E. faecalis</i> (n=18)			<i>E. faecium</i> (n=11)		
	MIC range	MIC ₅₀	MIC ₉₀	MIC range	MIC ₅₀	MIC ₉₀	MIC range	MIC ₅₀	MIC ₉₀
ERY	≤.25 to 4	2	2	≤.25 to 4	1	2	≤.25 to 4	2	4
GEN	≤2 to ≤128	≤128	≤128	8 to ≤128	≤128	≤128	2 to ≤500	≤128	≤128
STR	≤512 to 1000	≤512	1000	≤512 to 1000	≤512	1000	≤512 to 1000	≤512	1000
SYN	0.5 to 16	8	16	2 to 16	16	16	0.5 to 16	4	16
VAN	≤0.5 to ≥64	1	8	≤1 to 4	1	1	≤0.5 to 2	1	1
TET	≤2 to ≥64	≤4	≤4	≤2 to ≤4	≤4	≤4	≤2 to ≥32	≤4	≤4
LZD	1 to 4	2	4	1 to 4	2	4	2 to 4	2	4
PEN	.06 to ≥32	2	4	2 to 8	4	4	.06 to ≥32	2	4
CIP	0.5 to ≥4	1	2	1 to 2	1	2	0.5 to ≥4	1	≥4

Figure 1. Spray irrigation site treatment processes at the Mid-Atlantic SII (1a) and the Midwest NE1 and NE2 (1b) sites.

Figure 1. Spray irrigation site treatment processes at the Mid-Atlantic SII (1a) and the Midwest NE1 and NE2 (1b) sites.

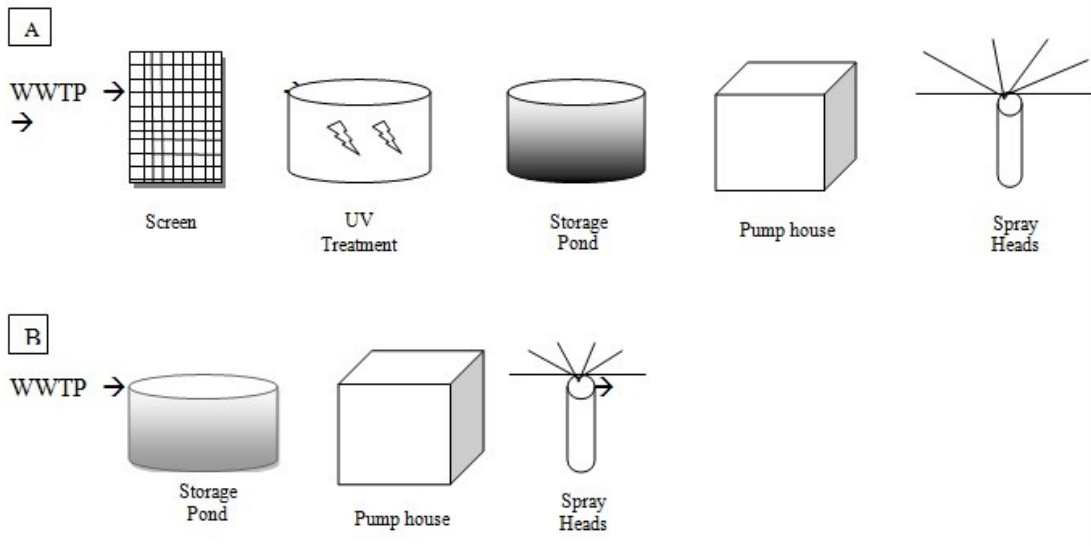


Figure 2. Concentration (CFU/100mL) of total enterococci at different sampling locations at Mid-Atlantic SII.

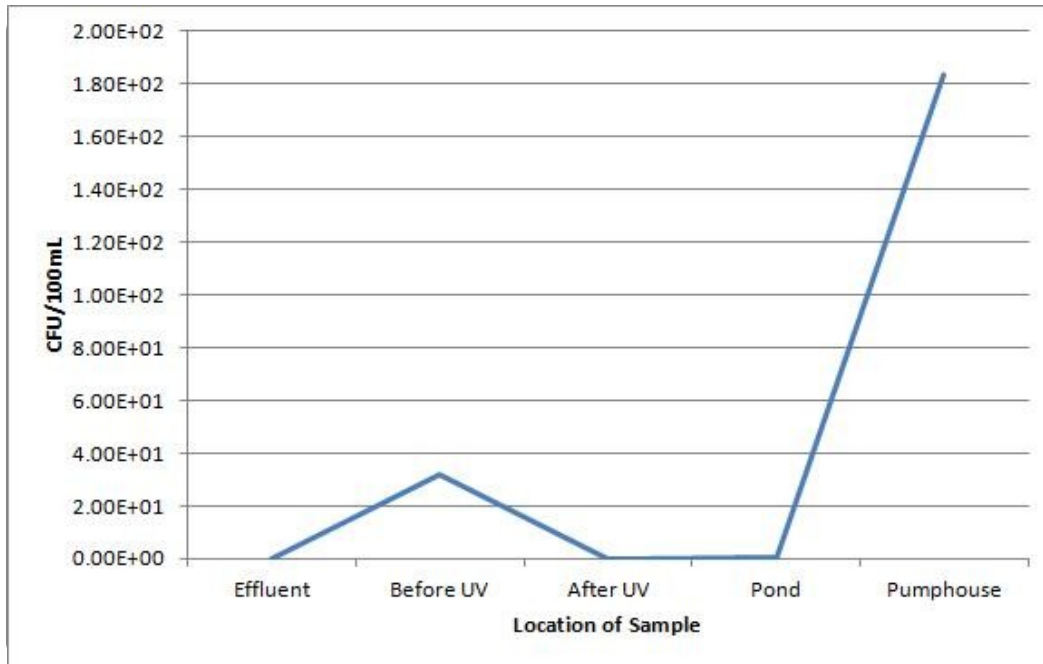
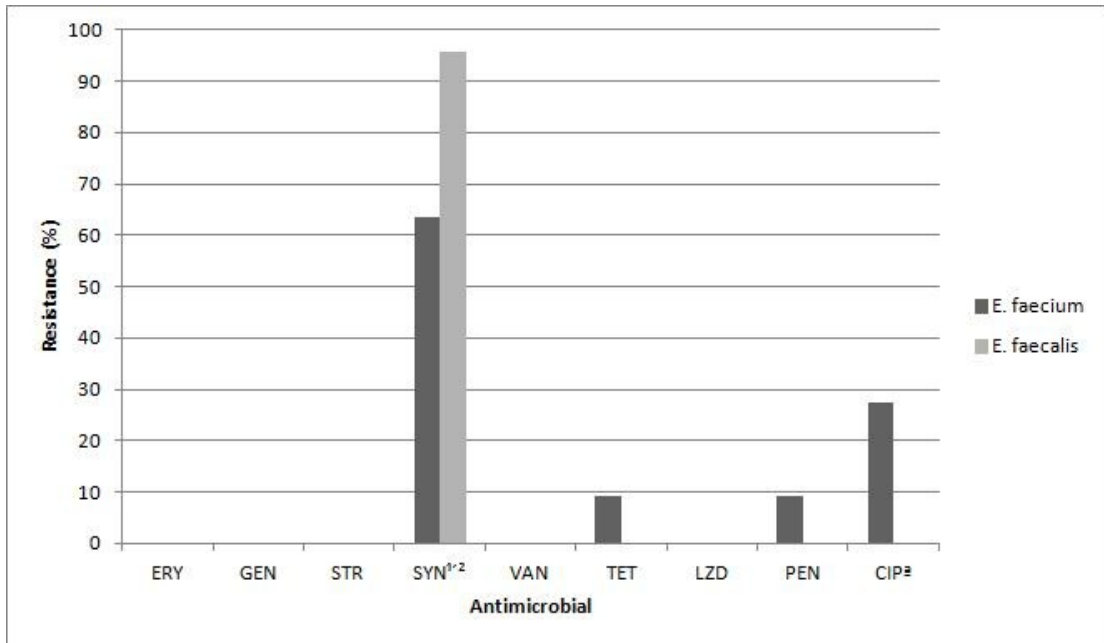


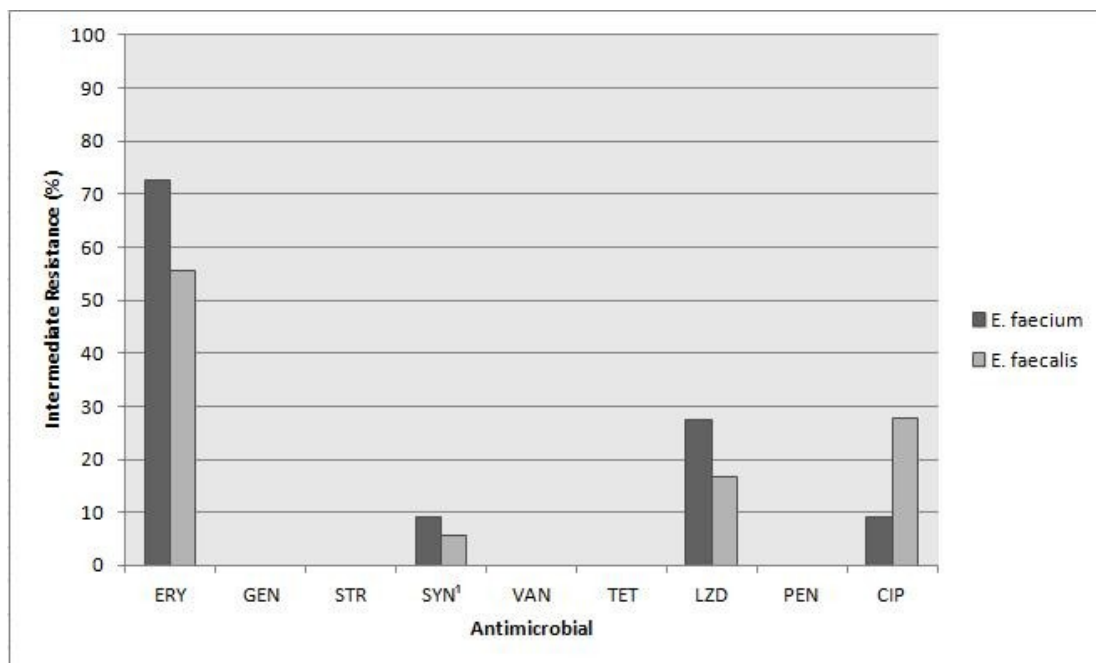
Figure 3. Antimicrobial resistance patterns among *E. faecalis* and *E. faecium* isolated from all three spray irrigation sites.



¹Statistically significant

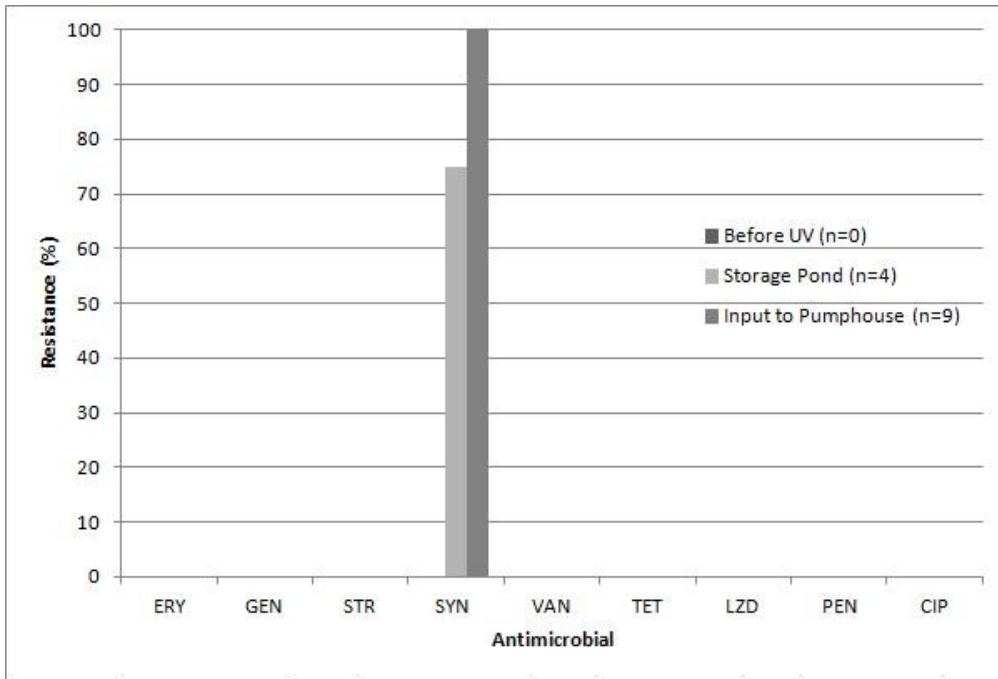
²*E. faecalis* is intrinsically resistant to SYN.

Figure 4. Antimicrobial intermediate-resistance patterns among *E. faecalis* and *E. faecium* isolated from all three spray irrigation sites.



¹*E. faecalis* is intrinsically resistant to SYN.

Figure 5a. Antimicrobial resistance patterns among *E. faecalis* recovered from Mid-Atlantic SII.



E. faecalis is intrinsically resistant to SYN.

Figure 5b. Antimicrobial resistance patterns among *E. faecium* recovered from Mid-Atlantic SII.

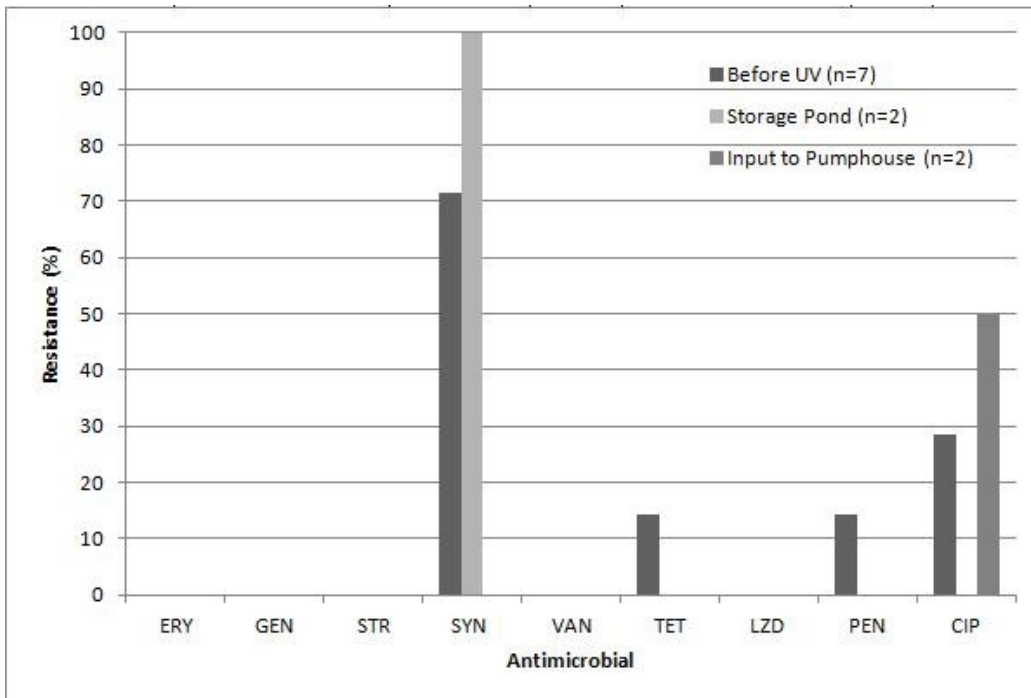
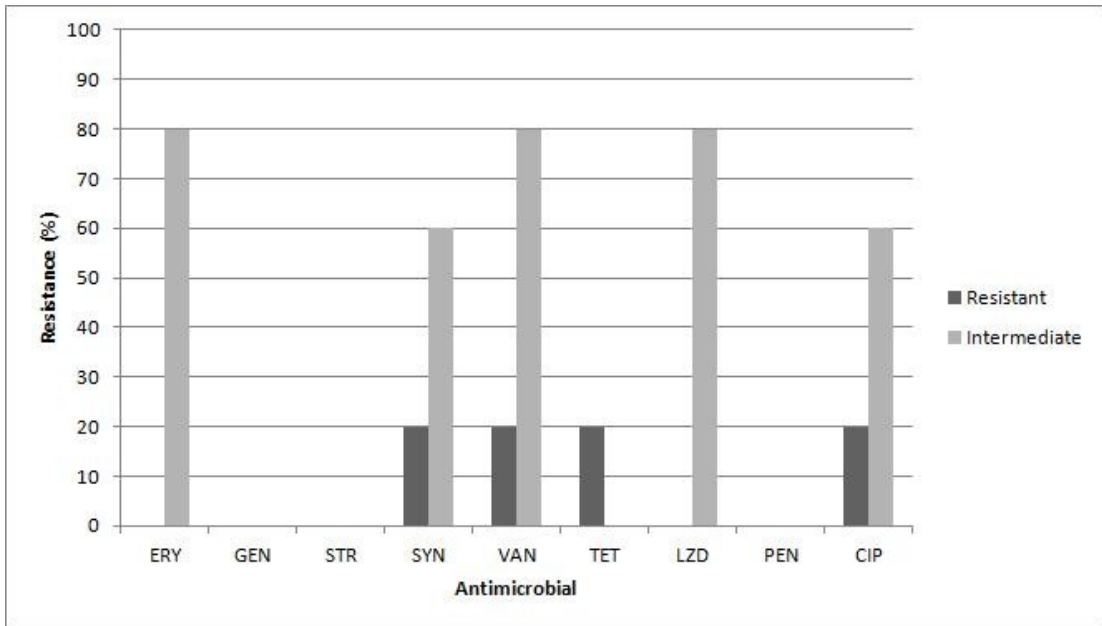


Figure 6 Antimicrobial resistance patterns among vancomycin-resistant enterococci (VRE) and vancomycin-intermediate enterococci (VIE).



Chapter 4: Public Health Implications and Conclusions

Public Health Implications

As previously stated, *Enterococcus* spp. have the ability to cause life-threatening human infections, and antibiotic-resistant enterococci increase the mortality of an infected person by up to 61% (Fisher & Phillips, 2009). One of the greatest concerns for human infection from spray irrigation sites is from exposure to antibiotic-resistant bacteria, which have been previously isolated from treated wastewater effluent (Garcia, et al., 2007, Huang, et al., 2012, Ferreira da Silva, et al., 2006, Martins de Costa 2006). This study shows that enterococci, in particular VRE and VIE, are present in low numbers in reclaimed water at spray irrigation sites. Enterococcal presence at spray irrigation sites presents immediate occupational concerns to the spray irrigation workers through inhalation, dermal, or accidental ingestion exposures. Previous studies have identified an increase in gastrointestinal illness in workers at WWTP; however, the association of the illness to a specific pathogenic bacterium has been inconclusive (Seuri, et al, 2005; McCunney, 1986).

In addition, some reclaimed water is used for crop irrigation or as source water for drinking water treatment plants, increasing the exposure concern to the general public as well (EPA, 2012). One public health concern for the general public involves eating fruits and vegetables washed with reclaimed water since *Enterococcus* has already been identified on tomato plants after irrigation with primary treated wastewater and secondary treated wastewater (Manios, et al., 2006).

One of the predominant clinical threats of enterococcal infection is that enterococci are difficult to clinically treat due to their intrinsic and acquired resistance (Mazuski, 2008). Most enterococci have intrinsic resistance to the penicillins and the cephalosporins, and some have acquired resistance to tetracyclines, macrolides, lincosamines, fluoroquinolones, and aminoglycosides (Mazuski, 2008). *E. faecalis* is intrinsically resistant to quinupristin/dalfopristin (Synercid), macrolides, and lincosamides (Dina, et al., 2003; Fisher & Phillips, 2009; Mazuski, 2008). *E. faecium* is intrinsically resistant to flavomycin (Sapkota, et al., 2012). Treating infections to these pathogenic bacteria has become increasingly challenging due to the bacteria's intrinsic and acquired antimicrobial resistance.

Thirty-two percent of the total enterococci in this study were MDR bacteria. In addition, all of these MDR bacteria were intermediately resistant to at least one antibiotic. Intermediately-resistant bacteria are a potential public health threat due to their ability to evolve into resistant bacteria. Currently, vancomycin is used primarily to treat drug-resistant bacteria when other antibiotics fail (CDC, 2011; Varela, et al., 2013). Vancomycin was first clinically used as an antimicrobial to treat enterococci infections in 1972; however, VRE surfaced only 15 years later (Fisher & Phillips, 2009). VRE infections increase the clinical treatment failure and mortality when compared to vancomycin-susceptible enterococci infections (Fisher & Phillips, 2009). Mortality occurs in 75% of those with VRE bacteremia infections but in only 45% of those with susceptible strain infections (Fisher & Phillips, 2009). There are currently no established recommendations for treatment of VRE; however, the antibiotics daptomycin and linezolid have been found to be effective against this pathogenic

strain (Casal, et al., 2012; Eliopoulos, 2009; Gallagher, et al., 2009). According to our study, 29% of enterococci isolates are already intermediately-resistant to linezolid, suggesting that future use of this antibiotic may be in jeopardy.

Another significant public health concern is intra- and inter-species transfer of antimicrobial resistance genes. Martins de Costa, et al. (2006) found that the use of antimicrobials had created a large pool of resistance genes and that sewage treatment processes failed to prevent the dissemination of antibiotic-resistant enterococci into the environment. Once in the environment, the bacteria may exchange resistance genes with other bacteria, creating a larger pool of antibiotic-resistant bacteria. Currently, 30% of enterococci isolated from ICUs are VRE, and approximately 10-19% of patients colonized with VRE are also colonized with MRSA (Mazuski, 2008). The use of vancomycin to treat MRSA may be a factor in the increase of VRE in healthcare settings (Mazuski, 2008). Potential transmission of vancomycin resistance to staphylococci is a rising public health concern, especially since it has been found that enterococci and *Staphylococcus aureus* can exchange genetic material (Fisher & Phillips, 2009; Mazuski, 2008). The first case of vancomycin-resistant *Staphylococcus aureus* (VRSA) was reported in 2003, and the continual rise of VRSA and vancomycin-intermediate *Staphylococcus aureus* (VISA) will have a significant impact on management of these infections (Mazuski, 2008).

Concluding Thoughts

More studies are needed to assess the inhalation, ingestion, and dermal exposure risk to spray irrigation workers and the general public with regard to the use of reclaimed water. This may include exploring the total bacterial diversity of the

reclaimed water as well as the presence of additional antibiotic-resistant bacteria. Epidemiology studies following the illnesses of spray irrigation site workers is also important. Future studies on this topic are relevant due to the United States' increased use of reclaimed water.

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