ABSTRACT

Title of Document: ANTIBIOTIC-RESISTANT BACTERIA IN WASTEWATER AND POTENTIAL HUMAN EXPOSURE THROUGH WASTEWATER REUSE.

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As community-acquired antibiotic-resistant bacterial infections occur with increasing frequency, it is important to identify possible environmental reservoirs for these organisms. My dissertation evaluated the presence of antibiotic-resistant bacteria in U.S. wastewater intended for reuse and the related public health implications. My objectives were to: 1) Evaluate wastewater from four U.S. wastewater treatment plants (WWTPs) for the presence of methicillin-resistant *Staphylococcus aureus* (MRSA); 2) Evaluate the occurrence of vancomycin-resistant enterococci (VRE) at four U.S. WWTPs from which treated wastewater is reused; and 3) Determine and compare MRSA, methicillin-susceptible *S. aureus* (MSSA), VRE, and vancomcyin-susceptible enterococci (VSE) colonization among American reclaimed water spray irrigators and controls.

Between 2009 and 2010, 44 wastewater samples were collected from four WWTPs, two in the Mid-Atlantic and two in the Midwest regions of the U.S. I analyzed samples for MRSA and VRE using standard membrane filtration. For the third objective, I collected 94 nasal and dermal swabs from 19 spray irrigators and 24 controls and analyzed them for MRSA, MSSA, VRE, and VSE. I confirmed all isolates and performed antimicrobial susceptibility testing by microbroth dilution. Statistical analyses included two-sample proportion tests and logistic regression.

MRSA and VRE were detected at all WWTPs. The percentage of MRSA-positive samples and concentration of VRE decreased as treatment progressed. Neither MRSA nor VRE were identified in tertiary-treated samples, but I identified both in an un-chlorinated effluent sample. No MRSA or VRE were detected in nasal or dermal samples from spray irrigators or controls. MSSA and VSE were detected in 26% and 11% of spray irrigators and 29% and 0% of controls, respectively. The odds of MSSA, MDR MSSA, and either MSSA or VSE colonization were not significantly different between the spray irrigators and controls.

My dissertation includes the first reports of MRSA at U.S. WWTPs and VRE at WWTPs whose effluent is intended for reuse. This is also the first U.S. evaluation of occupational exposure to antibiotic-resistant bacteria in reclaimed water. My findings provide additional scientific evidence that antibiotic-resistant bacteria can survive secondary-treated wastewater and may cause increased risks for infection among individuals exposed to reclaimed water.

# ANTIBIOTIC-RESISTANT BACTERIA IN WASTEWATER AND POTENTIAL HUMAN EXPOSURE THROUGH WASTEWATER REUSE

By

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Dissertation submitted to the Faculty of the Graduate School of the
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# Dedication

For my husband, Paul W. Goldstein, without whose love, support, and belief in my ability I would not have been able to accomplish this daunting task. I look forward to all the new exciting chapters in our life together.

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# Chapter 1: Introduction

A number of challenges currently threaten both the quality and quantity of freshwater supplies worldwide, including those of the United States. As of 2012, 783 million people, 11% of the world's population, lacked access to safe drinking water sources (WHO 2012). Unfortunately, access to safe water for drinking and other uses could become even more restricted in many areas as drought conditions occur with increasing frequency. In March 2013, nearly 45% of the U.S. was experiencing moderate droughts or worse (severe, extreme, or exceptional droughts) (NDMC-UNL 2013). As demand for freshwater increases, and water shortages become more common, reclaimed water (treated wastewater effluent) is increasingly being used for activities including irrigation, artificial snow production, and groundwater recharge (Levine and Asano 2004). As of 2011, 2.2 billion gallons per day of reclaimed water, or 5-6% of wastewater effluent, were being used across the U.S. for multiple types of activities (Miller 2011).

There are many potential benefits associated with reclaimed water use, including reduced energy consumption and costs from water treatment (EPA 2012). However, the possibility of exposing individuals who come into contact with reclaimed water to bacterial pathogens, as well as the public health implications of this exposure, need to be more fully evaluated. The primary goal of wastewater treatment is not to fully remove harmful contaminants, but to remove and degrade organic material (Maier 2009). Previous studies have analyzed specific microbial constituents in wastewater, and found that certain pathogens can survive wastewater treatment, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Börjesson et al. 2009; Caplin et al. 2008; da Silva et al. 2006; de Zutter and

van Hoof 1984; Harwood et al. 2001; Harwood et al. 2005; Iwane et al. 2001b; Mispagel and Gray 2005; Poole et al. 2005; Rose 2007; Schwartz et al. 2003; Volkmann et al. 2004). If MRSA, VRE, and other human pathogens do remain in reclaimed water, this may increase the risk of illnesses among humans that are occupationally or recreationally exposed to this alternative water source.

Several studies concerning microbial constituents in recycled wastewater were conducted in the 1980s and 1990s as part of the Monterey Wastewater Reclamation Study for Agriculture (MWRSA) (Burau et al. 1987; Nelson et al. 2003; Sheikh et al. 1998). These studies all concluded that indicator organisms and pathogens are either not present or present at negligible levels in reclaimed water, suggesting that reclaimed water can be safely used for irrigation. However, the type of wastewater treatment required in California under Title 22—chlorination, dual-media filtration, coagulation, and flocculation—is not typical of wastewater treatment throughout the U.S., and more closely resembles drinking water treatment than wastewater treatment in some cases. To determine whether reclaimed water is a sustainable option for applications across the U.S., wastewater treated using more common treatment processes should be evaluated. To my knowledge, only four peer-reviewed studies examining the microbiology of wastewater intended for reuse have been published in the past decade, and all of these papers concluded that additional research and monitoring is needed before reclaimed water use continues to expand (Al-Bahry et al. 2009; Harwood et al. 2005; Jjemba et al. 2010; Rosario et al. 2009). Clearly, additional data are needed to better understand the presence of bacterial pathogens in recycled wastewater.

A number of organizations and agencies have endorsed the exploration and expansion of reclaimed water for non-potable uses (EPA 2012; NRC 2012; WateReuse 2012). However, other groups, including the Navajo Nation in Arizona, have expressed concerns about possible health consequences from exposure to reclaimed water (Macmillan 2012). At this time, no federal regulations exist for wastewater reuse, only guidelines, creating a lack of comprehensive standards for reclaimed water use in the U.S. (EPA 2012). In the U.S. Environmental Protection Agency (EPA)'s 2012 Guidelines for Water Reuse, EPA specifically emphasizes the need "to reduce the potential for AR [antibiotic resistance] proliferation, [therefore] future research should target identification of the major source(s) of AR (i.e., raw sewage, biosolids, or treated effluent), determine treatment conditions that promote AR development, and characterize the persistence of AR in the environment. Ultimately, this knowledge will assist in developing mitigation strategies and alleviating environmental and public health concerns" (EPA 2012).

The purpose of this dissertation was to address these calls for additional research concerning the presence of antibiotic-resistant bacteria in U.S. wastewater intended for reuse and related public health implications. There were three primary research objectives as part of this dissertation:

1. To evaluate municipal wastewater from four U.S. wastewater treatment plants (WWTPs) for the presence of MRSA.
2. To evaluate the occurrence and concentration of VRE at four U.S. WWTPs from which treated wastewater is reused at spray irrigation sites.
3. To determine and compare MRSA, methicillin-susceptible *S. aureus* (MSSA), VRE, and vancomycin-susceptible enterococci (VSE) colonization among reclaimed water

spray irrigation workers and office worker controls in the Mid-Atlantic region of the United States.

These research objectives are addressed in three different manuscripts included in this dissertation, and the overall dissertation is organized into six chapters described as follows. Chapter 2 provides background information on antibiotic-resistant bacterial infections, MRSA, VRE, wastewater treatment in the U.S., and reclaimed water use in the U.S., as well as current information about the presence of, and exposure to, pathogens and antibiotic-resistant bacteria in wastewater and reclaimed water. Chapter 3 is a manuscript titled "Methicillin-Resistant *Staphylococcus aureus* (MRSA) Detected at Four U.S. Wastewater Treatment Plants" published in *Environmental Health Perspectives* in November 2012 (Rosenberg Goldstein et al. 2012) that is the first published report of MRSA in U.S. wastewater. Of note, this manuscript was chosen by *Environmental Health Perspectives* as one of its Science Selections in the November 2012 issue ("Superbug Hideout: Finding MRSA in U.S. Wastewater Treatment Plants," http://ehp.niehs.nih.gov/120-a437a/). Chapter 4 is a manuscript titled "Occurrence of vancomycin-resistant enterococci at four U.S. wastewater treatment plants" that describes the first time VRE was detected in 1) wastewater at four U.S. WWTPs whose effluent is intended for reuse and 2) WWTPs located in the Mid-Atlantic region of the U.S. Chapter 5 is a manuscript titled "Occupational exposure to *Staphylococcus aureus* and *Enterococcus* spp. among spray irrigation workers using reclaimed water" that was submitted for publication in March 2013. This paper evaluated the risk for occupational exposure to antibiotic-resistant bacteria, specifically MRSA, VRE, MSSA, and VSE, from reclaimed water. Finally, Chapter 6 provides

the conclusions that can be drawn from this body of research, future directions for research concerning reclaimed water, and policy implications.

# Chapter 2: Background

## *Antibiotic resistance*

Antibiotic resistance is a growing public health concern around the world and in the United States. Antibiotic-resistant bacteria are bacteria that have acquired resistance, or are inherently resistant, to antimicrobials that would otherwise limit their growth or kill them. Antibiotic-resistant bacteria present a threat to human health because they limit treatment options, often cause more serious infections than their antibiotic-susceptible counterparts, and are more likely to cause mortality (Drees et al. 2008; Siegel et al. 2006; Zhang et al. 2009). The number of hospitalizations with antibiotic-resistant infections in the United States increased approximately 3.6 times between 1997 and 2006 (Mainous et al. 2011). The increase in antibiotic-resistant bacteria and antibiotic-resistant bacterial infections could be the result of a number of factors including the overuse and misuse of antibiotics in humans, antibiotic use in animal and crop agriculture, antimicrobial substances in personal care products, and the incomplete removal of antimicrobials from wastewater treatment plants (WWTPs).

### Leading causes of antibiotic-resistant bacterial infections

Two of the leading causes of antibiotic-resistant bacterial infections are methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Mainous et al. 2011). These bacteria cause many infections acquired in hospital settings, and are also causing a growing number of infections among individuals who have not come into contact with a healthcare facility (community-acquired infections) (Gorak et al. 1999; Stevenson et al. 2005).

MRSA

*Staphylococcus aureus* is a bacterial pathogen associated with a wide range of human infections including skin and soft tissue infections, pneumonia, and septicemia (Bassetti et al. 2009). *S. aureus* is found throughout the environment in humans and animals, air, dust, water, milk, and food, although humans and animals are the primary reservoirs (FDA 2009). In addition to possessing characteristics that help it survive in the environment, *S. aureus* also has several characteristics that elicit immune responses in the body. Protein A, located on the surface of most strains of *S. aureus,* selectively binds to the receptors for immunoglobulin (P Murray et al. 2002). Once inside the body, *S. aureus* is able to survive partially due to its production of bound coagulase, a clumping factor that helps the organism bind to tissue surfaces. *S. aureus* is the only species of *Staphylococcus* to produce this enzyme (P Murray et al. 2002). Some strains of *S. aureus* also produce a highly heat-stable protein toxin, or enterotoxin (FDA 2009). It has been estimated that *S. aureus* is present in the nose, throat, hair, and skin of 50% or more of healthy individuals (FDA 2009). An even greater percentage of healthcare workers, hospital patients, and regular needle users are colonized with this bacterium (P Murray et al. 2002). According to samples collected in the 2003–2004 National Health and Nutrition Examination Survey (NHANES), between 27.2% and 30% of the United States population, or 75 to 82.9 million individuals, were nasally colonized with *S. aureus* (Gorwitz et al. 2008). Colonization with *S. aureus* can be either acute or chronic (Chambers 2001; Sanford et al. 1994). Colonization with *S. aureus* is more common among males, non-Hispanic whites and Mexican Americans, obese individuals, and individuals under 20 years old (Gorwitz et al. 2008). In neonates, *S. aureus* is often found in the oropharynx, umbilical stump, skin, and perineal area

(Adcock et al. 1998). In adults, *S. aureus* is often found in moist skin folds, in the nasopharynx, and gastrointestinal and urogenital tracts (P Murray et al. 2002). Although *S. aureus* colonization is widespread among humans, most individuals are asymptomatic rather than presenting for infection (Chambers 2001).

MRSA, a particular group of *S. aureus* that possesses genes conferring resistance to penicillinase-stable penicillins (methicillin and oxacillin), was first isolated in 1960 (Bassetti et al. 2009). MRSA infections are most often associated with skin and soft tissues infections (CDC 2009). A study by Gorwitz et al. (2008) that found the prevalence of MRSA colonization in the general U.S. population is approximately 1.5% based on the 2003-2004 NHANES data (Gorwitz et al. 2008). In 2005, MRSA infections were ultimately responsible for the death of approximately 18,650 individuals in the United States (Klevens et al. 2007).

### Mechanisms of action and mechanisms of resistance

Methicillin, or the more commonly prescribed oxacillin, binds to penicillin binding protein (PBP) in the *S. aureus* cell wall, disrupting synthesis of the peptidoglycan layer, and leading to the death of *S. aureus* (Deurenberg and Stobberingh 2008). The *mecA* gene encodes for penicillin binding protein 2a (PBP2a) which does not allow methicillin or any β-lactam antibiotics to bind (Deurenberg and Stobberingh 2008).

In addition to being resistant to the penicillin class of antibiotics, MRSA isolates are also generally resistant to a greater percentage of other types of antibiotics compared to methicillin-susceptible *S. aureus* (MSSA) (Tenover et al. 2008). For example, 90.5% of MRSA isolates

compared to 36.9% of MSSA isolates recovered from 43 U.S. medical centers included in a large-scale study by Richter et al. (2011) were resistant to erythromycin (Richter et al. 2011).

### MRSA characterization

MRSA is often characterized using a number of different techniques including SCC*mec* typing, *pvl* gene detection, and pulsed field gel electrophoresis (PFGE). *Staphylococcal* cassette chromosome *mec* (SCC*mec*) is a mobile genomic island on which the *mecA* gene is located (Deurenberg and Stobberingh 2008). SCC*mec* also contains ccr genes (cassette chromosome recombinases) that control integration and excision of SCC*mec* from the genome (Deurenberg and Stobberingh 2008). There are currently eight SCC*mec* types that have been identified (Center 2013). Panton Valentine leukocidin (PVL) targets and destroys white blood cells (neutrophils), the first line of defense in the human immune system against bacterial infection (Center 2013). PVL kills leukocytes by damaging the cell membrane (Deurenberg and Stobberingh 2008). Based on a large-scale study in the U.S. through NHANES, most MSSA isolates do not carry the *pvl* gene, and *pvl* gene possession among MRSA isolates depends on the MRSA strain type (Tenover et al. 2008). PFGE is used to define MRSA strain types based on banding patterns. PFGE groups USA100 and USA300 are the most common MRSA strain types in the United States (Tenover et al. 2008). USA100 are typically associated with hospital-acquired MRSA, whereas USA300 are more commonly associated with community-acquired MRSA (Tenover et al. 2008).

### Hospital-acquired MRSA

For the past four decades, MRSA infections have been largely associated with hospital environments and referred to as hospital-acquired MRSA (HA-MRSA) (Bassetti et al. 2009; Gorwitz et al. 2008). By the end of 1998, the prevalence of MRSA isolates from intensive care units was almost 50% and this percentage is expected to increase (Chambers 2001; Diekema et al. 2001). Most HA-MRSA strains are resistant to several classes of antimicrobials including aminoglycosides, erythromycin, clindamycin, and tetracycline (Flynn and Cohen 2008).

### Community-acquired MRSA

In the late 1990s, community-acquired MRSA (CA-MRSA) infections began to appear in otherwise healthy people who had no known risk factors for these infections (Bassetti et al. 2009; Gorak et al. 1999). CA-MRSA is associated with skin and soft tissue infections as well as severe systemic infections including sepsis and necrotizing pneumonia (Malik et al. 2006). CA-MRSA can be defined by absence of risk factors (exposure to healthcare settings) or by genetic markers. CA-MRSA usually possess *pvl* genes, are SCC*mec* types IV, V, or VII, are related to strain type USA300, and are typically more virulent than HA-MRSA (Deurenberg and Stobberingh 2008). The incidence of CA-MRSA has continued to increase in the United States (Klein 2009). The percentage of USA300 MRSA among total MRSA isolates increased over 9% between 2001-2002 and 2003-2004 (Tenover et al. 2008). While outbreaks of CA-MRSA have occurred among individuals sharing close contact with others in schools, prisons, nursing homes, and locker rooms, other possible environmental reservoirs of MRSA have yet to be comprehensively explored (Diekema et al. 2001).

## VRE

VRE are a public health concern because they cause a large percentage of hospital-acquired infections, increase mortality rates, and can transfer antibiotic resistance genes within the genus as well as to other bacteria. Enterococci are commensal bacteria found in the gastrointestinal (GI) tracts of most humans and animals (Fisher and Phillips 2009). The most common species found in human feces are *E. faecium* and *E. faecalis* (Fisher and Phillips 2009). Twelve percent of hospital-acquired infections in the U.S. are caused by *Enterococcus* spp. (Fisher and Phillips 2009). Enterococci, including VRE, can cause urinary tract, bloodstream (bacteremia), and wound infections, as well as meningitis, and endocarditis (CDC 2008; P Murray et al. 2002; Spacek and Vinetz 2009). VRE was first reported in England in 1988 (Uttley et al. 1988). In the United States, the incidence of VRE infections in hospitals doubled between 2000 and 2006 (Ramsey and Zilberberg 2009). VRE infections are associated with increased mortality rates (Fisher and Phillips 2009; Ramsey and Zilberberg 2009). Genes conferring resistance to vancomycin are intrinsic in certain species of enterococci (*vanC* in *E. casseliflavus*, *E. flavescens*, and *E. gallinarum*), however the *vanA* and *vanB* genes occur on moveable plasmids (Fisher and Phillips 2009).

### Mechanisms of action and mechanisms of resistance

Vancomycin, a glycopeptide antibiotic, alters cell wall synthesis by binding to the D-Ala-D-Ala C-terminus of the pentapeptide which disrupts further the production of more

crosslinkages (Courvalin 2006). *van* genes alter the target site for vancomycin by altering cell wall composition from Ala-D-Ala-D to Ala-D-Ala-Lactate which reduces affinity for the antibiotic (Fisher and Phillips 2009).

### Transfer of resistance genes

In addition to the transfer of vancomycin resistance genes between strains of enterococci, vancomycin resistance can also be transferred from enterococci to other types of bacteria, including *S. aureus* (NIAID 2009; Sievert et al. 2008). *Enterococcus* spp. can exchange antimicrobial resistance genes with *Staphylococcus* spp. on plasmids or transposons (Han et al. 2009). The Michigan Department of Community Health reported the first clinical isolate of vancomycin-resistant *S. aureus* (VRSA) in 2002 (Sievert et al. 2008). VRSA is of particular concern because of the limited treatment options for this type of infection. As of 2012, only 13 clinical cases of VRSA had been confirmed in the United States, but the incidence of VRSA infections could continue to rise (CDC 2012). Hanrahan et al. (2000) found that vancomycin and ampicillin resistance genes make up part of the same large, transferable chromosomal element, causing resistance to these two antibiotics to often occur in the same microorganisms (Hanrahan et al. 2000). Resistance genes can also be transferred between environmental and clinical settings, such as from clinical strains released into wastewater to community strains contained in the wastewater (Guardabassi and Dalsgaard 2004). Because of enterococci's ability to survive for long periods of time in water (18-30 days at room temperature), it could be cycled between clinical and environmental settings, and cause re-exposure among human communities (Lleò et al. 2005; NIAID 2009).

Community-acquired VRE

Community-associated VRE (CA-VRE) (no previous hospital stay reported) has rarely been reported in the U.S. (McDonald 1997; Stevenson 2005), however, the introduction of VRE into hospital settings from outside environments has been documented in both the U.S. and internationally (Anzar et al. 2004; Raja et al. 2005; Stevenson 2005). A study in Germany in 1999 found a 0.9% prevalence of VRE among a "healthy" student population, not admitted to a hospital for colonization or infection with VRE (Wendt et al. 1999). The first CA-VRE urinary tract infection was reported in Spain in 2004 and the first skin and soft tissue CA-VRE infection was reported in Malaysia in 2005 (Anzar et al. 2004; Raja et al. 2005). In a study by Stevenson et al. (2005) of rural U.S. hospitals, 22% of patients with positive VRE cultures had either been in the hospital for less than 48 hours or were outpatients, prompting classification as a CA-VRE infection (Stevenson 2005).

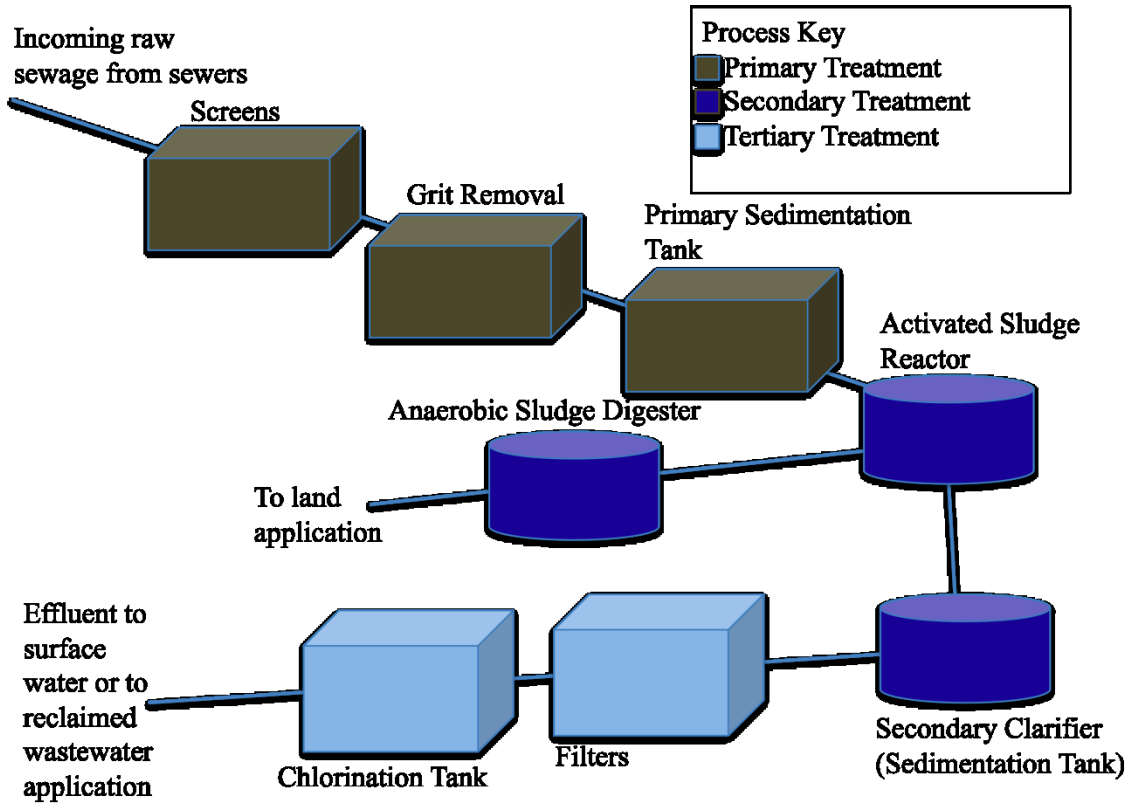
*Possible environmental sources of antibiotic-resistant bacteria*

As community-acquired antibiotic-resistant bacterial infections continue to occur with more frequency, it is increasingly important to identify possible environmental reservoirs for these organisms. Wastewater and reclaimed water have both been identified as possible exposure pathways for CA-MRSA and CA-VRE (Börjesson et al. 2009; McDonald 1997; Poole et al. 2005; Rosenberg Goldstein et al. 2012). To better understand the potential for distribution of antibiotic-resistant bacteria from wastewater and reclaimed water, it is important to first understand how wastewater is treated, current knowledge about pathogens in wastewater, how reclaimed water is distributed and used, and known information about pathogens in reclaimed water.

*Wastewater Treatment in the United States*

Because wastewater is treated mainly to remove organic solids, microorganisms that pose a threat to human health, including antibiotic-resistant bacteria, are not necessarily completely removed during wastewater treatment in the United States. Because antimicrobials and bacteria are both present in wastewater, it has been hypothesized that antimicrobial compounds present in wastewater could exert selective pressure for resistant bacteria or that resistance genes could be transferred in wastewater (Oh et al. 2009; Rosenberg Goldstein et al. 2012). More than 16,000 WWTPs in the United States treat approximately 121 billion liters of wastewater per day (EPA 2013).

**Figure 1. Typical wastewater treatment plant process steps**



Typical wastewater treatment in the United States can be categorized into three main steps, primary, secondary, and tertiary treatment (Figure 1) (Maier 2009). Primary treatment consists of the physical removal of large objects from raw sewage. Primary treatment usually includes screening of large objects, grit removal, and sedimentation (EPA 2004a; Maier 2009). Secondary treatment is the use of biological processes to remove suspended solids and microorganisms from water that has already undergone primary treatment (Maier 2009). Secondary treatment is the minimum level of treatment required by the United States government through the Clean Water Act (EPA 2004a). The most common type of secondary treatment in the U.S. is the activated sludge process, which uses nitrifying microorganisms and increased levels of oxygen to break down suspended organic materials (Maier 2009). After the activated sludge process, the wastewater is slowed in secondary clarifiers to induce die-off in the microbial population. The secondary clarifiers also allow for physical removal of remaining organic material with mechanical arms. The secondary treatment processes can remove as much as 90% of organic material from wastewater (EPA 2004a). Tertiary treatment is any treatment beyond secondary treatment, including disinfection and filtration (Maier 2009). Free chlorine is the most common disinfectant used in the tertiary treatment process, but ultraviolet light and ozone are also used (EPA 2004a).

WWTPs in the United States range from less than secondary to tertiary treatment facilities. In 2008, approximately 96.5 million individuals living in the United States were serviced by 7,332 secondary, or less than secondary, WWTPs, which account for slightly less than half (49.6%) of all municipal WWTPs in the United States (EPA 2010). Approximately 34% of municipal WWTPs go beyond secondary treatment and 15% of WWTPs now produce no discharge (EPA 2010).

*Pathogens in wastewater*

Bacterial pathogens occur in large quantities in wastewater because they are excreted in the feces of colonized individuals. A study at a Polish WWTP identified 34 species of bacteria and 14 species of fungi in incoming wastewater (Prazmo et al. 2003). Of the bacteria and fungi that were identified, 16 of the bacterial species and four of the fungal species had possible allergenic and/or immunogenic properties, which could affect individuals exposed to the wastewater (Prazmo et al. 2003). Reduction of enteric microorganisms can vary greatly among regions, WWTPs, and treatment steps, and wastewater effluent often still contains detectable levels of these microorganisms (Koivunen et al. 2003; Rose 2007). The number of pathogenic microorganisms and percentage of antibiotic-resistant microorganisms can increase at certain stages during the wastewater treatment process, most notably in the activated sludge process (secondary treatment process) (de Zutter and van Hoof 1984; Emparanza-Knorr and Torrella 1995; Kayser et al. 1987; Koivunen et al. 2003; LeChevallier et al. 1988). Prazmo et al. (2003) found that the concentration of total microorganisms in wastewater was two to three times greater in the primary and secondary treatment steps compared to the final treatment step (Prazmo et al. 2003).

*Occupational exposure to microbial pathogens among wastewater treatment plant workers*

Based on data from numerous studies, WWTP workers often report higher levels of GI and respiratory disease symptoms than other populations, although there is conflicting evidence about increased risks of infection due to specific pathogens identified in wastewater (Glas et al.

2001; Khuder et al. 1998; Seuri et al. 2005; Thorn and Kerekes 2001). A review of the literature through 2001 by Thorn and Kerekes (2001) found that several studies identified WWTP workers as more likely to report GI and respiratory symptoms and more likely to have antibodies against hepatitis A (Thorn and Kerekes 2001). Few of the studies reviewed by Thorn and Kerekes (2001) evaluated the risk of bacterial infections among WWTP workers (2001). Khuder et al. (1998) found that a greater proportion of WWTP workers reported gastroenteritis than controls, but no specific microbial agents were identified as causing these symptoms (Khuder et al. 1998). A Finnish study interested in determining whether the increased risk of diarrheal disease among WWTP workers was attributable to *Salmonella* found that, although more GI symptoms were reported by WWTP workers than controls, there was no significant difference in *Salmonella* antibody levels between the two groups (Seuri et al. 2005).

*Reclaimed water use in the U.S.*

Human exposure to wastewater is expanding beyond occupational exposure of WWTP workers with the increased use of treated wastewater. Historically, treated wastewater has been discharged into surface water bodies, but as freshwater sources are increasingly stressed, the planned reuse of treated wastewater is expanding around the world and across the U.S., creating many more opportunities for exposure to wastewater occupationally, as well as residentially and recreationally (EPA 2012; Miller 2011). Water supplied through publically-owned distribution systems in the U.S. is all treated to drinking water level quality, although this level of treatment is not necessary for all uses, such as industrial and landscaping applications. Using treated wastewater, often referred to as reclaimed water, for non-potable uses reduces discharge to

surface waters and reduces costs associated with treating water to meet drinking water standards (EPA 2012). The federal government and individual states encourage land application of wastewater as an alternative to the traditional discharge of wastewater into surface waters (EPA 2012). Amendments to the Federal Water Pollution Control Act require that during the planning phase for new WWTPs, land application of wastewater be considered as an end use (MDE 2009).

Reclaimed water is already being used across the United States for a number of different activities. In 2011, 2.2 billion gallons of reclaimed water were used per day in the United States (Miller 2011). Reclaimed water is used for a number of reasons including, but not limited to: replenishing groundwater supplies, water conservation, additional treatment of wastewater, and reduced cost (Levine and Asano 2004). Reclaimed water is used in both non-potable (land application), and to a lesser extent, potable (sources for drinking water treatment plants) projects (EPA 2012). Land applications of reclaimed water include low-rate irrigation, overland flow, and high-rate infiltration (groundwater recharge) (Maier 2009; Tien 2007). The choice of reuse method usually depends on site-specific conditions, method of wastewater treatment, and intended use of reclaimed water (Maier 2009). There are more than 200 U.S. water reclamation facilities that supply water to over 1,600 parks, playgrounds, and schoolyards (Crook 2005).

At this time, no federal regulations exist for wastewater reuse, only guidelines. Regulations are legally enforceable, whereas guidelines are suggestions for use, but cannot be enforced (EPA 2012). EPA's 2012 Guidelines for Water Reuse provide technical approaches for planning and operating reuse facilities, case studies of current reuse applications, and public health considerations related to reuse (EPA 2012). Each state determines whether to develop regulations or guidelines to oversee the use of reclaimed water within their state. As of

September 2012, 30 states had passed regulations concerning the use of reclaimed water, 15 states had developed guidelines, and five states had neither regulations nor guidelines (EPA 2012).

In the past, secondary (biological) treatment and disinfection were considered standard for reusing treated wastewater (EPA 2012). Currently, many additional advanced treatments exist that can be applied to wastewater effluent before reuse such as reverse osmosis or membrane filtration (EPA 2012; NRC 2012). The majority of U.S. water reclamation facilities disinfect water before distribution, but not all treated wastewater intended for reuse is distributed through formal reclamation facilities (Crook 2005; Rosenberg Goldstein et al. 2012). Some reuse sites are provided with water directly from WWTPs, which supply varying levels of treated wastewater (Rosenberg Goldstein et al. 2012). Some states still allow the use of primary (physical removal of solids has taken place) and secondary (biological) treated wastewater, but with additional requirements for buffer zones around application sites, as is the case in the State of Maryland (Crook 2005; MDE 2009). However, buffer zones would not reduce occupational exposure for individuals applying reclaimed water by hand. Most states that have reuse guidelines or regulations rely on indicator organism monitoring, most commonly total and fecal coliforms, to provide information about the microbial quality of reclaimed water (Crook 2005; Levine et al. 2008). However, it has been noted in a number of studies that there is no significant correlation between indicator bacteria and most pathogens in reclaimed water (Harwood et al. 2005; Jjemba et al. 2010; Levine et al. 2008).

*Pathogens in reclaimed water*

Pathogens, including viruses, bacteria, and protozoa, have been detected in reclaimed water in previous studies (Brissaud et al. 2008; Harwood et al. 2005; Jjemba et al. 2010; Rose et al. 1996). The studies that have been completed and published provide evidence that human pathogens are often present, sometimes at concentrations below recommended levels set by national or international health and environmental agencies, although this is not always the case (Brissaud et al. 2008; Crook 2005). A study of pathogen concentrations in reclaimed water intended for spray irrigation of landscape areas at a resort in France never detected helminth eggs, *Salmonella*, or enteroviruses in effluent or reclaimed water (Brissaud et al. 2008). The researchers did identify *E. coli* and enterococci in reclaimed water samples, but at concentrations below European Union recommended levels for bathing waters (Brissaud et al. 2008). Of particular importance for public health, in the Brissaud et al. (2008) study *Legionella* spp. were detected in wastewater effluent and storage ponds supplying spray heads at concentrations between $2 \times 10^5 - 3 \times 10^7$, however *L. pneumophila* was either not detected or detected at levels below quantification (Brissaud et al. 2008). In a study by Harwood et al. (2005), enteric viruses, *Giardia*, and infectious *Cryoptospordium* oocysts, were detected in 31%, 80%, and 70% of reclaimed water samples, respectively (Harwood et al. 2005).

In addition to the ability of some pathogens to survive wastewater treatment, there may be regrowth of injured or unculturable bacteria in the reclaimed water distribution system, as noted by Jjemba et al. (2010). Jjemba et al. (2010) found regrowth of indicator and pathogenic bacteria in four reclaimed water distribution systems even when the same bacteria had been nondetectable, or detected in low quantities, in effluent from WWTPs (Jjemba et al. 2010). Among the bacteria analyzed, the bacteria with the highest increases in growth in the distribution

systems were– *Aeromonas*, *Legionella*, and *Mycobacterium*– all human pathogens (2010). The WHO suggested monitoring reclaimed water used for agriculture and aquaculture for *E. coli* or parasites in guidelines published in 2006 (WHO 2006). However, testing for a single indicator organism in reclaimed water is not enough to determine the risk for the presence of human pathogens because there is no significant correlation between indicators organisms and most pathogens in this type of water (Harwood et al. 2005; Jjemba et al. 2010; Levine et al. 2008).

*Exposure pathways for microbial pathogens originating from reclaimed water*

Inhalation, dermal contact, and accidental ingestion of wastewater-saturated soil have all been suggested as possible routes of exposure to pathogens present in reclaimed water (Blumenthal et al. 2001; Camann et al. 1983; Camann et al. 1988; Crook 2005; Mara et al. 2007; O'Toole et al. 2008). Because the infective dose for some pathogens is lower for respiratory infections than GI infections, it is especially important to consider aersolization of pathogens and inhalation as a route of exposure (Crook 2005; Sobsey 1978). Bacteria and viruses have both been detected in aerosols collected at reclaimed water spray irrigation sites (Camann and Moore 1987; Camann et al. 1988; Crook 2005; Teltsch et al. 1980). Total coliforms were detected in reclaimed water bioaerosols at a spray irrigation reuse site in concentrations ranging from 18-1,076 CFU/$m^3$ (Teltsch et al. 1980). A study of secondary treated wastewater reused for spray irrigation in Texas found fecal coliform levels in air samples downwind from the reuse site that were significantly higher than background levels, although longer retention times in reservoirs reduced observed concentrations (Camann et al. 1988).

Both bacteria of interest for the three projects described in this dissertation—MRSA and VRE— have previously been detected in aerosols from a variety of settings. Enterococci have been isolated from air samples taken from WWTPs (Fracchia et al. 2006; Korzeniewska et al. 2009). Enterococci could be breathed in and colonize either the upper respiratory tract, or be swallowed and colonize the GI tract. In a bioaerosol study at a confined swine operation in Canada, *Enterococcus* spp. were detected in 17 of 18 air samples and in 17 of 35 nasal swabs collected from the hog producers (Létourneau et al. 2010). Eighty-seven percent of the enterococci isolated from air samples at the confined swine operation also contained tetracycline-resistance genes (Létourneau et al. 2010).

MSSA and MRSA can be aerosolized from water and have been detected in air samples from a number of environments, including WWTPs (Fracchia et al. 2006; Gandara et al. 2006; Gibbs et al. 2006; Schulz et al. 2012). However, no *S. aureus* was isolated from air samples collected at biosolids application sites in the U.S. in a study by Rusin et al. (2003). Direct, dermal contact is the main exposure route for *S. aureus*, however because *S. aureus* can become airborne, human exposure can also occur through inhalation (Bassetti et al. 2005).

To reduce exposure to bioaerosols at reclaimed water spray irrigation sites, recommendations generally focus on design features such as buffer zones and setbacks from areas with unrestricted use (Crook 2005). However, for spray irrigation workers hand applying reclaimed water, these design features would not reduce their contact with reclaimed water and its bioaersols. It has been estimated that about 10% of bioaerosols are actually ingested after inhalation (Tanner et al. 2008). Rose et al. (1996) estimated that 100 mL/day would be the maximum amount of reclaimed water an individual would ingest, if exposed to reclaimed water through spray irrigation (Rose et al. 1996).

*Epidemiological evidence for microbial pathogen exposure from reclaimed water use*

Few epidemiological studies have been undertaken to explore exposure to, and health risks from, treated wastewater reuse (Crook 2005; Shuval 1991). No infectious disease outbreaks have been linked to exposure to reclaimed water used for landscape irrigation in the U.S., however, it is difficult to confirm causes of sporadic illnesses (Crook 2005). Raw consumption of crops irrigated with untreated wastewater has been found to increase the risk of parasitic infections and cholera (Feachem 1982; Shuval 1991). Occupational exposure to raw wastewater used for agricultural irrigation has also been associated with increased risk of parasitic and cholera infections (Krishnamoorthi et al. 1973; Shuval 1991). Previous epidemiological studies looking at the risk of infections among workers exposed to treated wastewater have produced conflicting results (Blumenthal et al. 2001; Durand and Schwebach 1989; Linnemann et al. 1984; Shuval 1991). A study by Blumenthal et al. (2001) in Mexican agricultural communities found increased odds of parasitic infections from exposure to wastewater stored in one reservoir (Blumenthal et al. 2001). Individuals exposed to wastewater stored in a reservoir used for agricultural irrigation had higher odds of infection with *Ascaris lumbricoides* compared to controls (Blumenthal et al. 2001). Wastewater retention in multiple reservoirs for longer periods of time reduced the odds of infection with *A. lumbricoides* and resulting diarrheal disease (Blumenthal et al. 2001). In contrast to the increased risk of infection detected in by Blumenthal et al. (2001), a study by Linnemann et al. (1984) in the United States found no significant difference between reported illness or viral antibody levels (polio, coxsackie, or echoviruses) between spray irrigation workers exposed to secondary treated wastewater and a group of unexposed controls (Linnemann et al. 1984). However, the

Linnemann et al. (1984) study relied on a small sample size (35 spray irrigation workers, 41 controls) and did not evaluate bacterial colonization among the participants, which could have underestimated the true public health risks among those exposed to reclaimed water (Linnemann et al. 1984). Durand and Schwebach (1989) found no increased odds of reported GI illness among those recreationally exposed to parks irrigated with reclaimed water (Durand and Schwebach 1989). However, they did find that a greater proportion of individuals exposed to wet grass conditions reported GI symptoms (Durand and Schwebach 1989). As part of their jobs, reclaimed water spray irrigation workers are routinely working in wet grass conditions. A Texas-based study analyzing the risk of viral infections among individuals residentially exposed to secondary treated wastewater reused for agricultural spray irrigation found an increased risk of 1.5-1.8 compared to those not exposed to reclaimed water (Camann et al. 1988).

*Antibiotic-resistant bacteria in wastewater and reclaimed water*

Several studies have identified the presence of antibiotic-resistant bacteria, including MRSA and VRE, in wastewater throughout the treatment process (Araujo et al. 2010; Börjesson et al. 2009; Garcia et al. 2007; Iwane et al. 2001a; Koivunen et al. 2003; Kotzamanidis et al. 2009; Nagulapally et al. 2009). The concentration of antibiotics released into wastewater are often high enough to exert selective pressures to favor the proliferation of resistant strains of microorganisms (Garcia et al. 2007; Kummerer 2001). The combination of high concentrations of microorganisms, nutrients, and antibiotics found in wastewater makes it a favorable environment for bacterial growth and horizontal transfer of resistance genes (Garcia, et al., 2007; Lorenz & Wackemagel, 1994; Mezrioui & Baleux, 1994; Nagulapally, et al., 2009). Antibiotic-

resistant *E. coli* and *Salmonella* have been isolated from secondary-treated wastewater effluent in Japan and Finland, respectively (Iwane et al. 2001a; Koivunen et al. 2003).

Previous studies conducted in Sweden have detected MRSA in wastewater, but reported a decline in MRSA as wastewater treatment progressed. Specifically, Börjesson et al. (2009) showed that the concentration of MRSA as measured by real-time PCR assays decreased as treatment progressed from approximately $6x10^3$ to $5x10^2$ *mec*A genes 100 $ml^{-1}$ from inlet to outlet, except for a peak in activated sludge reactor samples of $5x10^5$ mecA genes 100 $ml^{-1}$ (Börjesson et al. 2009).

VRE has also been detected in all wastewater treatment steps, including effluent samples (Araujo et al. 2010; Caplin et al. 2008; Kotzamanidis et al. 2009; Morris et al. 2012). The majority of studies assessing the presence of VRE in wastewater have been conducted in Europe and found VRE prevalence ranging from 2-52% (Kotzamanidis et al. 2009; Luczkiewicz et al. 2010; Morris et al. 2012). To my knowledge, Nagulapally et al. (2009) is the only published study to have detected VRE at a U.S. municipal WWTP (Nagulapally et al. 2009). Nagulapally et al. (2009) isolated VRE from influent and secondary clarifier (biologically treated) samples, but not from UV-disinfected effluent samples (Nagulapally et al. 2009). Many previous studies have also concluded that VRE concentrations decreased as wastewater treatment progressed; however VRE has been detected in effluent including disinfected effluent in Europe (Kotzamanidis et al. 2009; Morris et al. 2012; Nagulapally et al. 2009).

Studies by Harwood et al. (2005) and Jjemba et al. (2010) both identified bacteria in reclaimed water, but neither performed antimicrobial susceptibility testing on the bacteria that were isolated (Harwood et al. 2005; Jjemba et al. 2010). A study by Negreanu et al. (2012) that

analyzed tertiary treated wastewater used for agricultural irrigation in Israel for the presence of antibiotic resistance genes (ARG) detected genes that confer resistance to fluroquinolone, sulfonamide, tetracycline, macrolide, lincosamide, and streptogramin antibiotics in these samples (Negreanu et al. 2012). However, the ARG detected by Negreanu et al. (2012) could have been from nonviable bacteria.

*Unanswered questions about antibiotic-resistant bacteria originating from wastewater and reclaimed water*

In summary, antibiotic-resistant bacteria pose a major threat to human health. The high number of MRSA and VRE infections inside healthcare settings, and the growing number of these infections in community settings are concerning. It is important to determine if there are environment reservoirs for these bacteria that are leading to exposure in community settings. MRSA and VRE have both been identified in wastewater in Europe, pointing to a possible exposure pathway for these bacteria among WWTP workers and the general population after wastewater discharge (Araujo et al. 2010; Börjesson et al. 2009; Garcia et al. 2007; Iwane et al. 2001a; Koivunen et al. 2003; Kotzamanidis et al. 2009; Nagulapally et al. 2009). However, to my knowledge the presence of MRSA in wastewater has never been evaluated in the U.S. and only one study at one Midwest WWTP has analyzed wastewater for VRE in the U.S. (Nagulapally et al. 2009). Also, at a time when wastewater reuse is expanding across the U.S., little information still exists about the presence of pathogens and public health risks from exposure to reclaimed water. Although some reports and reviews on the safety of reclaimed water have concluded that bacterial pathogens do not pose a risk to human health from reclaimed water use, the presence of several important human bacterial pathogens has never been evaluated

(Crook 2005). To my knowledge, no previous studies have assessed the presence of viable antibiotic-resistant bacteria in reclaimed water. The three papers included in this dissertation seek to evaluate wastewater intended for reuse for important human pathogens and provide more epidemiological evidence about exposure to human pathogens from reclaimed water reuse.

# Chapter 3: Methicillin-Resistant *Staphylococcus aureus* (MRSA) Detected at Four U.S. Wastewater Treatment Plants

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*Abbreviations*

| | |
|---|---|
| AMP | Ampicillin |
| AXO | Ceftriaxone |
| CA-MRSA | Community-acquired methicillin-resistant *Staphylococcus aureus* |
| CIP | Ciprofloxacin |
| CLI | Clindamycin |
| DAP | Daptomycin |
| ERY | Erythromycin |
| GAF | Gatifloxacin |
| GEN | Gentamicin |
| HA-MRSA | Hospital-acquired methicillin-resistant *Staphylococcus aureus* |

| | |
|---|---|
| LEVO | Levofloxacin |
| LZD | Linezolid |
| MIC | Minimal inhibitory concentration |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| MSSA | Methicillin-susceptible *Staphylococcus aureus* |
| OXA+ | Oxacillin+2%NaCl |
| PCR | Polymerase chain reaction |
| PEN | Penicillin |
| PFGE | Pulsed field gel electrophoresis |
| PVL | Panton valentine leukocidin toxin |
| RIF | Rifampin |
| SCC*mec* | Staphylococcal cassette chromosome *mec* |
| STR | Streptomycin |
| SXT | Trimethoprim/sulfamethoxazole |
| SYN | Quinupristin/dalfopristin |
| TET | Tetracycline |
| VAN | Vancomycin |
| WWTP | Wastewater treatment plant |

*Abstract*

Background

The incidence of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections is increasing in the United States, and it is possible that municipal wastewater could be a reservoir of this microorganism. To date, no U.S. studies have evaluated the occurrence of MRSA in wastewater.

Objective

We examined the occurrence of MRSA and methicillin-susceptible *S. aureus* (MSSA) at U.S. wastewater treatment plants.

Methods

We collected wastewater samples from two Mid-Atlantic and two Midwest wastewater treatment plants between October 2009 and October 2010. Samples were analyzed for MRSA and MSSA using membrane filtration. Isolates were confirmed using biochemical tests and PCR (polymerase chain reaction). Antimicrobial susceptibility testing was performed by Sensititre® microbroth dilution. Staphylococcal cassette chromosome mec (SCC*mec*) typing, Panton-Valentine leucocidin (PVL) screening, and pulsed field gel electrophoresis (PFGE) were performed to further characterize the strains. Data were analyzed by two-sample proportion tests and analysis of variance.

Results

We detected MRSA (n=240) and MSSA (n=119) in 22 out of 44 (50%) and 24 out of 44 (55%) wastewater samples, respectively. The odds of samples being MRSA-positive decreased as treatment progressed: 10 out of 12 (83%) influent samples were MRSA-positive, while only one out of 12 (8%) effluent samples was MRSA-positive. Ninety-three percent and 29% of unique MRSA and MSSA isolates were multidrug-resistant, respectively. SCC*mec* types II and IV, the *pvl* gene, and USA types 100, 300, and 700 were identified among the MRSA isolates.

Conclusions

Our findings raise potential public health concerns for wastewater treatment plant workers and individuals exposed to reclaimed wastewater. As reclaimed wastewater use accelerates, the risk of exposure to antibiotic-resistant bacteria in treated wastewater deserves further attention.

*Introduction*

*Staphylococcus aureus* is a bacterial pathogen associated with a wide range of human infections including skin infections, pneumonia, and septicemia (Bassetti et al. 2009). Infections with this microorganism can be difficult to treat because the strains are often resistant to one or more antibiotics including methicillin. Methicillin-resistant *Staphylococcus aureus* (MRSA) was first isolated in 1960 and for the past four decades MRSA infections have been largely associated with hospital environments and referred to as hospital-acquired MRSA (HA-MRSA) (Bassetti et al. 2009; Gorwitz et al. 2008). However, in the late 1990s, community-acquired MRSA (CA-MRSA) infections began to appear in otherwise healthy people who had no known risk factors for these infections (Bassetti et al. 2009; Gorak et al. 1999). The incidence of CA-MRSA has continued to increase in the United States, and while outbreaks of CA-MRSA have occurred among individuals sharing close contact with others in schools, prisons, and locker rooms, other possible environmental reservoirs of MRSA have yet to be comprehensively explored (Diekema et al. 2001).

Identifying environmental reservoirs of MRSA in the community, however, is critical if the spread of CA-MRSA infections is to be controlled. Among other potential environmental reservoirs, wastewater has been identified as a possible source of exposure to MRSA in the community (Börjesson 2009; Börjesson et al. 2010; Plano et al. 2011). Colonized humans shed MRSA from the nose, feces, and skin; therefore, MRSA can end up in municipal wastewater streams (Börjesson 2009; Börjesson et al. 2010; Plano et al. 2011). Börjesson et al. (2009) recently detected MRSA resistance genes in all treatment steps at a Swedish municipal wastewater treatment plant. This group also cultured MRSA from influent samples in their 2009

study, as well as influent and activated sludge samples in a subsequent study (Börjesson et al., 2010; Börjesson et al., 2009). Currently, as water shortages expand, treated municipal wastewater is increasingly used for applications including landscape and crop irrigation, groundwater recharge, and snowmaking (Levine and Asano 2004; Tonkovic and Jeffcoat 2002). During these activities, individuals applying, using, or coming in contact with reclaimed wastewater could potentially be exposed to MRSA and other bacteria that may remain in treated wastewater (Iwane et al. 2001b).

However, to our knowledge, no studies have demonstrated the occurrence of MRSA in wastewater in the United States. In this study, we evaluated the occurrence of MRSA and methicillin-susceptible *Staphylococcus aureus* (MSSA) at four wastewater treatment plants (WWTPs) located in two different regions of the United States: the Mid-Atlantic and the Midwest. To further assess the MRSA strains, isolates were characterized by staphylococcal cassette chromosome *mec* (SCC*mec*) typing and pulsed field gel electrophoresis (PFGE), and screened for Panton-Valentine leucocidin (PVL)—an exotoxin often associated with virulent strains of *S. aureus.*

*Materials and methods*

Study sites

Four WWTPs were included in this study – two in the Mid-Atlantic and two in the Midwest. The treatment steps and sampling locations at each of the treatment plants are illustrated in Figure 1.

Mid-Atlantic WWTP1 (Figure 1a) is a tertiary WWTP in an urban area that processes 681,390 cubic meters per day ($m^3$/d) of wastewater with a peak capacity of 1.51 million $m^3$/d. Mid-Atlantic WWTP2 (Figure 1b) is a tertiary WWTP in a suburban area that processes 7,570 $m^3$/d of wastewater with a peak capacity of 45,425 $m^3$/d. Tertiary wastewater treatment includes primary treatment (physical removal of solids), secondary treatment (biological treatment), and additional treatment that can include, but is not limited to, chlorination, UV radiation, or filtration. The incoming wastewater at both Mid-Atlantic plants includes domestic and hospital wastewater, and effluent from both Mid-Atlantic plants is piped to landscaping sites for reuse in spray irrigation.

Midwest WWTP1 (Figure 1c) is a tertiary WWTP in a rural area that processes 1,363 $m^3$/d of wastewater with a peak capacity of 10,978 $m^3$/d. The incoming water includes domestic wastewater and agriculturally influenced stormwater. Seasonal chlorination occurs in June, July and August and chlorinated effluent is piped to a landscaping site for reuse in spray irrigation. Midwest WWTP2 (Figure 1d) is a secondary WWTP (with no on-site disinfection) in a rural area that processes 1,439 $m^3$/d with a peak capacity of 7,571 $m^3$/d. Secondary wastewater treatment includes only primary treatment (physical removal of solids) and secondary treatment (biological treatment). The incoming water at this plant includes domestic wastewater, wastewater from a

food production facility, and agriculturally influenced stormwater. Unchlorinated effluent is piped to an agricultural site for crop irrigation.

### Sample collection

A total of 44 grab samples were collected between October 2009 and October 2010: 12 samples from Mid-Atlantic WWTP1; 8 samples from Mid-Atlantic WWTP2; 12 samples from Midwest WWTP1; and 12 samples from Midwest WWTP2. The timing of each sampling event was determined by the availability and schedule of the WWTP operators. The sampling time schedule and specific sampling locations for each plant are indicated in Tables 1 and 2 and Figure 1. Samples were collected in 1L sterile polyethylene Nalgene® Wide Mouth Environmental Sample Bottles (Nalgene, Lima, OH), labeled, and transported to the laboratory at 4 °C. All samples were processed within 24 h.

### Isolation

Membrane filtration was used to recover *S. aureus* and MRSA from wastewater samples. Briefly, 300 ml of each sample were vacuum filtered through a 0.45 µm, 47 mm mixed cellulose ester filter (Millipore, Billerica, MA). Filters were then enriched in 40 ml of m Staphylococcus broth (Becton, Dickinson and Company, Franklin Lakes, NJ), vortexed, and incubated at 37 °C for 24 h. A 10 µl loopful of each enrichment was then plated in duplicate on MRSASelect (Bio-Rad Laboratories, Hercules, CA) and Baird Parker agar (Becton, Dickinson and Company) for the isolation of MRSA and total *S. aureus,* respectively. Plates were incubated at 37 °C for 24 h. Resulting black colonies with halos on Baird Parker and hot pink colonies on MRSASelect were considered presumptive *S. aureus* and MRSA, respectively. These colonies were purified on Brain Heart Infusion (BHI) agar (Becton, Dickinson and Company) and archived in Brucella broth (Becton, Dickinson and Company) with 15% glycerol at -80 °C. *S. aureus* ATCC 43300

was used as a positive control and phosphate buffered saline was used as a negative control throughout the isolation process for quality control and quality assurance.

### Identification

*S. aureus* and MRSA were confirmed using the Gram stain, the coagulase test (Becton, Dickinson and Company), the catalase test, and PCR. DNA extraction was carried out using the MoBio UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) per the manufacturer's recommendations. For confirmation of *S. aureus*, PCR amplification of the *S. aureus*-specific *nuc* gene was carried out using the NUC1 and NUC2 primers (Fang and Hedin, 2003). For MRSA differentiation, PCR amplification targeting the *mecA* gene, which encodes for methicillin resistance, was performed using the MECA1 and MECA2 primers, both as previously described by Fang and Hedin (Brakstad et al. 1992; Fang and Hedin 2003; Smyth et al. 2001) The method was modified by including an internal control, using primers targeting the 16S rDNA genes, in a multiplex PCR assay (Edwards et al. 1989). PCR amplification consisted of an initial denaturing step of 95 °C for 3 min, followed by 34 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min.

### Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Sensititre® microbroth dilution system in accordance with the manufacturer's instructions on all PCR-confirmed MRSA (n=240) and MSSA (n=119) isolates (Trek Diagnostic Systems Inc., Cleveland, OH). Overnight cultures were transferred to sterile demineralized water (Trek Diagnostic Systems, Westlake, OH) to achieve a 0.5 McFarland standard. Then, 30 µL of each suspension was transferred to sterile cation-adjusted Mueller Hinton broth (Trek Diagnostic Systems, Westlake, OH), and 50

µL of the broth solution was then dispensed into GPN3F minimal inhibitory concentration (MIC) plates (Trek Diagnostic Systems Inc.) with the following antibiotics (range of concentrations in µg/ml): erythromycin (ERY; 0.25-4), clindamycin (CLI; 0.12-2), quinupristin/dalfopristin (SYN; 0.12-4), daptomycin (DAP; 0.25-8), vancomycin (VAN; 1-128), tetracycline (TET; 2-16), ampicillin (AMP; 0.12-16), gentamicin (GEN; 2-16, 500), levofloxacin (LEVO; 0.25-8), linezolid (LZD; 0.5-8), ceftriaxone (AXO; 8-64), streptomycin (STR; 1000), penicillin (PEN; 0.06-8), rifampin (RIF; 0.5-4), gatifloxacin (GAT; 1-8), ciprofloxacin (CIP; 0.5-2), trimethoprim/sulfamethoxazole (SXT; 1/19-4/76), and oxacillin+2%NaCl (OXA+; 0.25-8). *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were used as quality control strains. MICs were recorded as the lowest concentration of an antimicrobial that completely inhibited bacterial growth (CLSI 2010). Resistance breakpoints published by the Clinical and Laboratory Standards Institute were used (CLSI 2010). Multidrug resistance (MDR) was defined as resistance to two or more classes of antibiotics.

SCC*mec* typing

A multiplex PCR assay developed by Milheiriço et al. (2007) was used to characterize the MRSA isolates (n=240) by SCC*mec* type (Milheiriço et al. 2007; Oliveira and de Lencastre 2002). SCC*mec* strains COL (type I), BK2464 (type II), ANS46 (type III), MW2 (type IVa), HAR22 (type IVh), and HDE288 (type VI) were used as positive controls for SCC*mec* typing.

PVL screening

All MRSA isolates, confirmed by possession of the *nuc* and *mecA* genes by PCR and an identifiable SCC*mec* type (n=236), were screened for PVL by PCR of the *pvl* gene according to Strommenger et al. (2008). *S. aureus* ATCC strain 25923 was used as a positive control.

### Pulsed field gel electrophoresis

PFGE was performed on a subset of 22 MRSA isolates. To ensure a diverse, representative subset, isolates were selected using the following criteria: treatment plant, sampling date, SCC*mec* type, and each sampling location that had a positive sample. PFGE was based on the Centers for Disease Control and Prevention (CDC) Laboratory Protocol for Molecular Typing of *S. aureus* by PFGE (www.cdc.gov/pulsenet). *Sma*I (Promega, Madison, WI) was used to digest genomic DNA. Digested samples were run in 1% SeaKem® Gold agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME) gels in 0.5X TBE using a CHEF Mapper (Bio-Rad) for 18.5-19 h at the following settings: voltage of 200 V, temperature of 14° C, and initial and final switch of 5 and 40 seconds. Cluster analysis was performed using BioNumerics software v5.10 (Applied Maths Scientific Software Development, Saint-Martens-Latem, Belgium) using Dice coefficient and the unweighted pair-group method (UPGMA). Optimization settings for dendrograms were 1.0% with a position tolerance of 0.95%. Based on the similarity of the control strains, isolates were considered clones if similarity was ≥ 88%. *Salmonella* serotype Braenderup strain H9812 was used as the standard.

### Statistical analyses

Descriptive statistics were reported for the percentages of wastewater samples that were positive for MRSA and MSSA by WWTP. Statistical analyses of antibiotic resistance data were limited to MRSA (n=84) and MSSA (n=58) isolates expressing unique phenotypic profiles to reduce bias that could be introduced by including clones, since PFGE was not performed on all isolates. Two-sample tests of proportions were performed between MRSA and MSSA isolates

with respect to the percent resistance of each group of isolates to each of the 18 tested antibiotics. ANOVA was then used to compare the average numbers of antibiotics against which MRSA and MSSA isolates were resistant. In all cases, $p$-values of $\leq 0.05$ were defined as statistically significant. All statistical analyses were performed using Stata/IC 10 (StatCorp LP, College Station, TX) and SAS 9.2 (SAS Inc., Cary, NC).

*Results*

Occurrence of MRSA

MRSA were detected at all WWTPs in this study. The distribution of MRSA-positive samples differed by WWTP, sampling date, and sampling location (Table 1). Across all treatment plants sampled, 50% (22/44) of wastewater samples were positive for MRSA: 60% (12/20) of samples from Mid-Atlantic WWTPs; and 42% (10/24) of samples from Midwest WWTPs. Eighty-three percent (10/12) of influent samples from all WWTPs were MRSA-positive; 100% (5/5) from Mid-Atlantic WWTPs and 71% (5/7) from Midwest WWTPs. No MRSA were detected in any tertiary-treated (chlorinated) effluent samples (Table 1). However, MRSA was detected in one effluent sample from Midwest WWTP1 in October 2010 when chlorination was not taking place. Overall, Midwest WWTP2 had the lowest percentage of MRSA-positive wastewater samples with MSRA detected only in the influent (Table 1). This plant is the only WWTP in the study that does not use an activated sludge reactor step; instead, it uses a system of lagoons for biological treatment.

Occurrence of MSSA

MSSA were also detected at all WWTPs in this study. The distribution of MSSA-positive samples differed by WWTP, sampling date, and sampling location (Table 2). Across all treatment plants sampled, 55% (24/44) of wastewater samples were positive for MSSA: 60% (12/20) of samples from Mid-Atlantic WWTPs; and 50% (12/24) of samples from Midwest WWTPs. Eighty-three percent (10/12) of influent samples from all WWTPs were MSSA-positive; 100% from Mid-Atlantic WWTPs and 71% from Midwest WWTPs. No MSSA were

detected in tertiary-treated (chlorinated) effluent samples (Table 2). However, MSSA was detected in two effluent samples from Midwest WWTP1 in September and October 2010 when chlorination was not taking place. Overall, Midwest WWTP2 had the lowest percentage of MSSA-positive wastewater samples of all four WWTPs with MSSA detected only in the influent.

### Antibiotic resistance patterns

In total, 240 MRSA isolates were isolated from all WWTPs. However, as noted above, the statistical analyses concerning antibiotic resistance patterns among these isolates were limited to those that could be confirmed as unique (n=84) using phenotypic analyses, since PFGE was not performed on all isolates. The unique MRSA isolates had a median oxacillin MIC of ≥16 μg/ml (range, 4 to ≥16 μg/ml) and expressed resistance to several antibiotics approved by the U.S. Food and Drug Administration for treating MRSA infections, including TET, CIP, LEVO, GAT, and CLI, as well as LZD and DAP (Figure 2) which are important alternatives to older antibiotics for treating severe MRSA infections (Johnson and Decker 2008).

Antimicrobial resistance patterns among unique MRSA isolates varied by WWTP and sampling location (Figure 2). In general, at both Mid-Atlantic WWTPs and Midwest WWTP1, the percentage of isolates resistant to individual antibiotics increased or stayed the same as treatment progressed (Figures 2a-2c). At Midwest WWTP2, only influent samples were positive for MRSA and the majority of these isolates were resistant to most of the tested antibiotics (Figure 2d).

In total, 119 MSSA isolates were isolated from all WWTPs. Similar to our statistical analyses of MRSA isolates, our analyses of antimicrobial resistance patterns among MSSA isolates were limited to those isolates that could be confirmed as unique (n=58) using phenotypic analyses. Antimicrobial resistance patterns among unique MSSA isolates also varied by WWTP (Figure 3). The percentages of ERY-, AMP- and PEN-resistant unique MSSA isolates at Mid-Atlantic WWTP1 increased as treatment progressed, whereas the percentages of isolates resistant to the fluoroquinolones (LEVO, CIP, and GAT) decreased from influent to activated sludge reactor samples (Figure 3a). At Mid-Atlantic WWTP2, the percentages of ERY-, AMP-, PEN- and GAT-resistant MSSA isolates increased from influent to activated sludge reactor samples (Figure 3b). Similarly, among Midwest WWTP1 and Midwest WWTP2 MSSA, resistance to AMP and PEN increased as treatment progressed (Figure 3c and 3d).

In terms of percent resistance among the groups of isolates, a greater percentage of MRSA isolates compared to MSSA isolates were resistant to the following 14 antibiotics: ERY, CLI, STR, SYN, DAP, TET, AMP, RIF, LEVO, PEN, CIP, AXO, GAT, and OXA+ (Table 3). MRSA isolates were also resistant to more antimicrobials (on average 6.94) than MSSA isolates (on average 2.26) ($p < 0.001$).

### Multi-drug resistance

Ninety-three percent (78/84) of phenotypically unique MRSA isolates from all WWTPs were MDR, while 29% (17/58) of unique MSSA isolates from all WWTPs were MDR. The summary of percent MDR MRSA and MSSA by sampling location (across all plants) is shown in Figure 4.

SCC*mec* typing

SCC*mec* types II and IV were identified among the MRSA isolates (Table 4). Overall, 83% (199/240) of the MRSA isolates were type IV and 15% (37/240) were type II. For all WWTPs, except Mid-Atlantic WWTP1, only one SCC*mec* type was identified at each plant (Table 4). Four isolates (2%) displayed resistance to oxacillin in antimicrobial susceptibility testing, but did not have the *mecA* band in the Fang and Hedin PCR multiplex or the *mecA* band in the SCC*mec* PCR multiplex.

PVL screening

Among our total MRSA isolates where SCC*mec* type could be confirmed, 68% (161/236) were positive for the *pvl* gene: 72% at Mid-Atlantic WWTP1, 75% at Mid-Atlantic WWTP2, 83% at Midwest WWTP1 and 0% at Midwest WWTP2 (Table 4).

PFGE

Clusters based on $\geq$ 88% similarity resulted in 12 unique types among our subset of 22 isolates, suggesting a heterogeneous population among MRSA from U.S. WWTPs (Figure 5). Three different USA types, 100, 300, and 700, were identified. Nine isolates did not match any of the USA types.

*Discussion*

MRSA and MSSA occurrence in U.S. wastewater

Although MRSA has been identified in WWTPs in Sweden (Börjesson et al. 2009; Börjesson et al. 2010), to our knowledge, this is the first report of the detection of MRSA at municipal wastewater treatment plants in the United States. Fifty percent of total wastewater samples were positive for MRSA, while 55% of total samples were positive for MSSA. Yet, the odds of samples being MRSA-positive decreased as treatment progressed. For example, 10 out of 12 (83%) influent samples were MRSA-positive, while only one out of 12 (8%) effluent samples was MRSA-positive (Table 1). Based on these findings, wastewater treatment seems to reduce the number of MRSA and MSSA isolates released in effluent. However, the few isolates that do survive in effluent might be more likely to be multidrug resistant and virulent isolates.

Previous studies conducted in Sweden have also reported a decline in MRSA as wastewater treatment progressed. Specifically, Börjesson et al. (2009) showed that the concentration of MRSA as measured by real-time PCR assays decreased as treatment progressed from approximately $6x10^3$ to $5x10^2$ *mecA* genes 100 $ml^{-1}$ from inlet to outlet, except for a peak in activated sludge reactor samples of $5x10^5$ *mecA* genes 100 $ml^{-1}$ (Börjesson et al. 2009). Based on these findings, we might also expect to see an overall decrease in MRSA concentrations throughout the wastewater treatment process in the U.S., except for perhaps a peak in activated sludge. It is also interesting to note that at Midwest WWTP2, the only WWTP in the study that does not employ an activated sludge step, MRSA was detected only in the influent. The lack of MRSA detected beyond influent at Midwest WWTP2 could be due to the effectiveness of an anaerobic step in the sequencing batch reactor (Figure 1) (Minnigh H, personal communication).

Cycling of MRSA between humans and the environment

Our findings also provide evidence that municipal wastewater could serve as a medium for the cycling of CA-MRSA strains between humans and the environment. MRSA has been found at concentrations between $10^4$–$10^8$ CFU/g of fecal material (Wada et al. 2010). PVL-positive strains, SCC*mec* type IV, and USA 300, all of which characterize the majority of the MRSA isolated from wastewater in this study, have traditionally been associated with CA-MRSA (Gorwitz et al. 2008; Seybold et al. 2006). The high prevalence of PVL-positive CA-MRSA in the U.S. population as compared to other countries could explain the high percentage of PVL-positive MRSA isolates in wastewater in this study (Seybold et al., 2006; Tristan et al., 2007). The association of PVL-positive MRSA and CA-MRSA with skin infections could also explain the occurrence of PVL-positive MRSA isolates in wastewater samples in this study, as MRSA could be shed in showers at concentrations of approximately $1.4 \times 10^4 - 1.0 \times 10^5$ CFU/person (Lina et al. 1999). The large cluster of MRSA isolates recovered in this study that were PVL-positive and showed similarity to USA 300 is concerning, as USA 300 strains—which are typically resistant to erythromycin and β-lactam antibiotics—and the *pvl* gene are associated with increased virulence, severe bloodstream infections, and necrotizing pneumonia (Gorwitz et al. 2008; Lina et al. 1999).

Moreover, the abundance of SCC*mec* type IV among the recovered MRSA isolates could be indicative of superior survival characteristics, namely the lower energy cost of SCC*mec* type IV carriage (Börjesson et al. 2010). SCC*mec* type IV strains recovered in this study appeared to persist longer in the wastewater treatment process than type II strains. However, this phenomenon warrants further investigation as our results are based on only one WWTP (Mid-

Atlantic WWTP1) and a previous study found that SCC*mec* type was not significantly associated with MRSA survival (Levin-Edens et al. 2011).

Four isolates that did not have the *mecA* band in SCC*mec* typing but were found to be oxacillin-resistant through antimicrobial susceptibility testing could have the novel *mecA* homologue, MRSA-LGA 251, as identified by García-Álvarez et al. (García-Álvarez et al. 2011). Interestingly, three of these four isolates were from Midwest WWTP1, which is surrounded by animal production facilities. García-Álvarez detected the novel *mecA* homologue in bovine MRSA, although the original source of MRSA-LGA 251 is still under investigation (García-Álvarez et al. 2011). Because traditional *mecA* primers do not detect this homologue, there could be an even greater number of wastewater samples containing MRSA than was detected in this study (García-Álvarez et al. 2011). However, it was beyond the scope of the current study to further assess the wastewater samples for the presence of MRSA-LGA 251.

### Public health implications

Our findings raise potential public health concerns for wastewater treatment plant workers and individuals exposed to reclaimed wastewater. Wastewater treatment plant workers could potentially be exposed to MRSA and MSSA through several exposure pathways, including dermal, and inhalation exposures. However, very few studies have evaluated microbial exposures among wastewater workers. Mulloy et al. (2001) published a review article summarizing findings of exposures to *Leptospira*, Hepatitis A, bacterial enterotoxins and endotoxins among WWTP workers (Mulloy 2001). Yet, to our knowledge, no studies have evaluated MRSA or MSSA carriage rates among these populations. Encouraging frequent

handwashing and the use of gloves among WWTP workers could reduce the potential risks associated with possible MRSA exposures.

Beyond wastewater workers, individuals who are exposed to reclaimed secondary wastewater, including spray irrigators and people living near spray irrigation sites, could be potentially exposed to MRSA and MSSA. No federal regulations exist for wastewater reuse from either secondary or tertiary facilities, although EPA has issued water reuse guidelines (EPA 2004b). States determine whether to develop regulations or guidelines to oversee the use of reclaimed wastewater within their boundaries, and most state guidelines allow secondary effluent to be used for certain reuse applications, including spray irrigation of golf courses, public parks, and agricultural areas (EPA 2004b). In this study, we detected MRSA and MSSA in unchlorinated effluent from Midwest WWTP1, a WWTP with only seasonal chlorination (that could be defined as a secondary treatment plant during periods where chlorine is not applied). Our findings suggest that implementing tertiary treatments for wastewater that is intended for reuse applications could reduce the potential risk of MRSA exposures among individuals who are working on or living by properties sprayed with reclaimed wastewater.

### Limitations

There are some notable limitations of this study. The number and timing of sampling events and samples collected at each WWTP was not the same due to access issues at some of the plants. Also, enriching the samples preempted our ability to report concentrations of MRSA and MSSA in wastewater. Meanwhile, since PFGE was performed on a representative subset of all MRSA isolates, the true heterogeneity of the MRSA isolates contained in the wastewater samples may have been underestimated. On the other hand, MRSA strains have evolved from a

small number of clonal strains, so the likelihood of isolating MRSA with phenotypic and genetic similarities during our isolation procedure was high (Enright et al. 2002; Fang and Hedin 2003; Oliveira et al. 2002). However, the goal of this study was to evaluate the occurrence of MRSA at WWTPs in the U.S. and even if clones were selected, the findings concerning the presence and types of MRSA at the four WWTPs are still accurate.

*Conclusions*

To our knowledge, our study is the first to demonstrate the occurrence of MRSA in U.S. municipal wastewater. While tertiary wastewater treatment may effectively reduce MRSA in wastewater, secondary-treated wastewater (unchlorinated) could be a potential source of exposure to these bacteria in occupational settings and reuse applications. As reclaimed wastewater use accelerates, the risk of antibiotic-resistant bacterial infections from exposure to treated wastewater deserves further attention.

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*Tables*

**Table 1:** Distribution of methicillin-resistant *Staphylococcus aureus* positive wastewater samples at all WWTPs, sampling events and sampling locations.

| Sampling location (total # of samples collected) | Distribution of positive samples at each WWTP | | | | | | | | | | | | Total Positive Samples (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mid-Atlantic WWTP 1 (n=12) | | | Mid-Atlantic WWTP 2 (n=8) | | Midwest WWTP 1 (n=12) | | | Midwest WWTP 2 (n=12) | | | | |
| | Oct 2009 | Dec 2009a | Dec 2009b | Oct 2010a | Oct 2010b | July 2010 | Sept 2010 | Oct 2010 | July 2010 | Aug 2010 | Sept 2010 | Oct 2010 | |
| Influent (n=12) | Pos | Pos | Pos | Pos | Pos | Neg | Pos | Pos | Pos | Pos | Neg | Pos | 10/12(83) |
| Activated sludge reactor (n=5) | Pos | Pos | Pos | Pos | Pos | – | – | – | – | – | – | – | 5/5(100) |
| Post aeration (n=3) | – | – | – | – | – | Neg | Pos | Pos | – | – | – | – | 2/3(67) |
| Cell B (n=4) | – | – | – | – | – | – | – | – | Neg | Neg | Neg | Neg | 0/4(0) |
| Secondary clarifier (n=8) | Neg | Pos | Pos | Neg | Neg | Pos | Neg | Pos | – | – | – | – | 4/8(50) |
| Effluent (n=12) | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Pos[a] | Neg | Neg | Neg | Neg | 1/12(8) |
| Total Positive Samples (%) | 2/4(50) | 3/4(75) | 3/4(75) | 2/4(50) | 2/4(50) | 1/4(25) | 2/4(50) | 4/4(100) | 1/3(33) | 1/3(33) | 0/3(0) | 1/3(33) | 22/44(50) |

Pos = positive sample
Neg = negative sample
WWTP = wastewater treatment plant
[a]Sample was collected in October 2010 when chlorination of effluent was not taking place.

**Table 2:** Distribution of methicillin-susceptible *Staphylococcus aureus* positive wastewater samples at all WWTPs, sampling events and sampling locations.

| Sampling location (total # of samples collected) | Distribution of positive samples at each WWTP | | | | | | | | | | | | Total Positive Samples (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mid-Atlantic WWTP 1 (n=12) | | | Mid-Atlantic WWTP 2 (n=8) | | Midwest WWTP 1 (n=12) | | | Midwest WWTP 2 (n=12) | | | | |
| | Oct 2009 | Dec 2009a | Dec 2009b | Oct 2010a | Oct 2010b | July 2010 | Sept 2010 | Oct 2010 | July 2010 | Aug 2010 | Sept 2010 | Oct 2010 | |
| Influent (n=12) | Pos | Pos | Pos | Pos | Pos | Pos | Neg | Pos | Pos | Pos | Neg | Pos | 10/12(83) |
| Activated sludge reactor (n=5) | Pos | Pos | Pos | Pos | Pos | – | – | – | – | – | – | – | 5/5(100) |
| Post aeration (n=3) | – | – | – | – | – | Pos | Pos | Pos | – | – | – | – | 3/3(100) |
| Cell B (n=4) | – | – | – | – | – | – | – | – | Pos | Neg | Neg | Neg | 1/4(25) |
| Secondary clarifier (n=8) | Neg | Pos | Pos | Neg | Neg | Pos | Neg | Pos | – | – | – | – | 4/8(50) |
| Effluent (n=12) | Neg | Neg | Neg | Neg | Neg | Neg | Pos[a] | Pos[a] | Neg | Neg | Neg | Neg | 2/12(17) |
| Total Positive Samples (%) | 2/4(50) | 3/4(75) | 3/4(75) | 2/4(50) | 2/4(50) | 3/4(75) | 2/4(50) | 4/4(100) | 2/3(67) | 1/3(33) | 0/3(0) | 1/3(33) | 24/44(55) |

Pos = positive sample
Neg = negative sample
WWTP = wastewater treatment plant
[a]Samples were collected in September and October 2010 when seasonal chlorination was not taking place.

**Table 3:** Differences in percentage of MRSA and MSSA isolates resistant to each tested antibiotic, compared using two-sample tests of proportions.

| | **Percentage of Resistant Isolates** | | |
|---|---|---|---|
| **Antibiotic** | **MRSA** | **MSSA** | ***p*-value (one-sided)** |
| Erythromycin | 82.14% (69/84) | 28.57% (16/56) | <0.0001 |
| Clindamycin | 27.38% (23/84) | 1.72% (1/58) | <0.0001 |
| Gentamicin | 10.84% (9/83) | 3.45% (2/58) | 0.0537 |
| Streptomycin | 4.76% (4/84) | 0% (0/58) | 0.0459 |
| Quinupristin/dalfopristin | 7.14% (6/84) | 0% (0/58) | 0.0188 |
| Daptomycin | 16.67% (14/84) | 0% (0/58) | 0.0005 |
| Vancomycin | 0% (0/83) | 0% (0/57) | - |
| Tetracycline | 14.29% (12/84) | 0% (0/58) | 0.0013 |
| Ampicillin | 98.81% (83/84) | 68.97% (40/58) | <0.0001 |
| Rifampicin | 9.76% (8/82) | 0% (0/58) | 0.0071 |
| Levofloxacin | 63.41% (52/82) | 15.79% (9/57) | <0.0001 |
| Linezolid | 5.95% (5/84) | 3.45% (2/58) | 0.2494 |
| Penicillin | 98.81% (83/84) | 73.21% (41/56) | <0.0001 |
| Ciprofloxacin | 63.10% (53/84) | 15.79% (9/57) | <0.0001 |
| Trimethoprim/ sulfamethoxazole | 2.38% (2/84) | 0% (0/58) | 0.1184 |
| Ceftriaxone | 30.49% (25/82) | 0% (0/58) | <0.0001 |
| Gatifloxacin | 62.65% (52/83) | 18.97% (11/58) | <0.0001 |
| Oxacillin+2%NaCl | 98.81% (83/84) | 0% (0/58) | <0.0001 |

**Table 4:** Number (%) of MRSA isolates recovered from wastewater by SCC*mec* type and by possession of the *pvl* gene[a]

| Sampling Location | SCC*mec Type* | | | PVL +[b] |
|---|---|---|---|---|
| | Type II | Type IV | No *mecA* | |
| Mid-Atlantic 1 (n=100) | | | | |
| Influent (n=40) | 0(0) | 40(100) | 0(0) | 28(70) |
| Activated sludge reactor (n=40) | 13(33) | 27(68) | 0(0) | 25(63) |
| Secondary clarifier (n=20) | 0(0) | 19(95) | 1(5) | 18(95) |
| Effluent (n=0) | 0(0) | 0(0) | 0(0) | 0(0) |
| Total (n=100) | 13(13) | 86(86) | 1(1) | 71(72) |
| Mid-Atlantic 2 (n=47) | | | | |
| Influent (n=20) | 0(0) | 20(100) | 0(0) | 9(45) |
| Activated sludge reactor (n=27) | 0(0) | 27(100) | 0(0) | 26(96) |
| Secondary clarifier (n=0) | 0(0) | 0(0) | 0(0) | 0(0) |
| Effluent (n=0) | 0(0) | 0(0) | 0(0) | 0(0) |
| Total (n=47) | 0(0) | 47(100) | 0(0) | 35(75) |
| Midwest 1 (n=69) | | | | |
| Influent (n=22) | 0(0) | 19(86) | 3(14) | 9(47) |
| Post aeration (n=21) | 0(0) | 21(100) | 0(0) | 20(95) |
| Secondary clarifier (n=13) | 0(0) | 13(100) | 0(0) | 13(100) |
| Effluent (n=13) | 0(0) | 13(100) | 0(0) | 13(100) |
| Total (n=69) | 0(0) | 66(96) | 3(4) | 55(83) |
| Midwest 2 (n=24) | | | | |
| Influent (n=24 ) | 24(100) | 0(0) | 0(0) | 0(0) |
| Cell B (n=0) | 0(0) | 0(0) | 0(0) | 0(0) |
| Effluent (n=0) | 0(0) | 0(0) | 0(0) | 0(0) |
| Total (n=24) | 24(100) | 0(0) | 0(0) | 0(0) |

[a]SCC*mec* types I, III, V, and VI were not identified in any sample.

[b]The PVL PCR was performed only on isolates that were found to contain the *mecA* gene.

*Figures*

**Figure 1** (Reproduced with permission from *Environmental Health Perspectives*)**:** Schematic of wastewater treatment processes at four wastewater treatment plants in the Mid-Atlantic and Midwest regions of the United States. Sampling locations are indicated with numbers. Numbers correspond to the following sampling locations: Mid-Atlantic WWTP1 and Mid-Atlantic WWTP2: 1=Influent, 2=Activated sludge reactor, 3=Post aeration, 4=Effluent; Midwest WWTP1: 1=Influent, 2=Post aeration, 3=Secondary clarifier, 4=Effluent; Midwest WWTP2: 1=Influent, 2=Cell B, 3=Effluent.

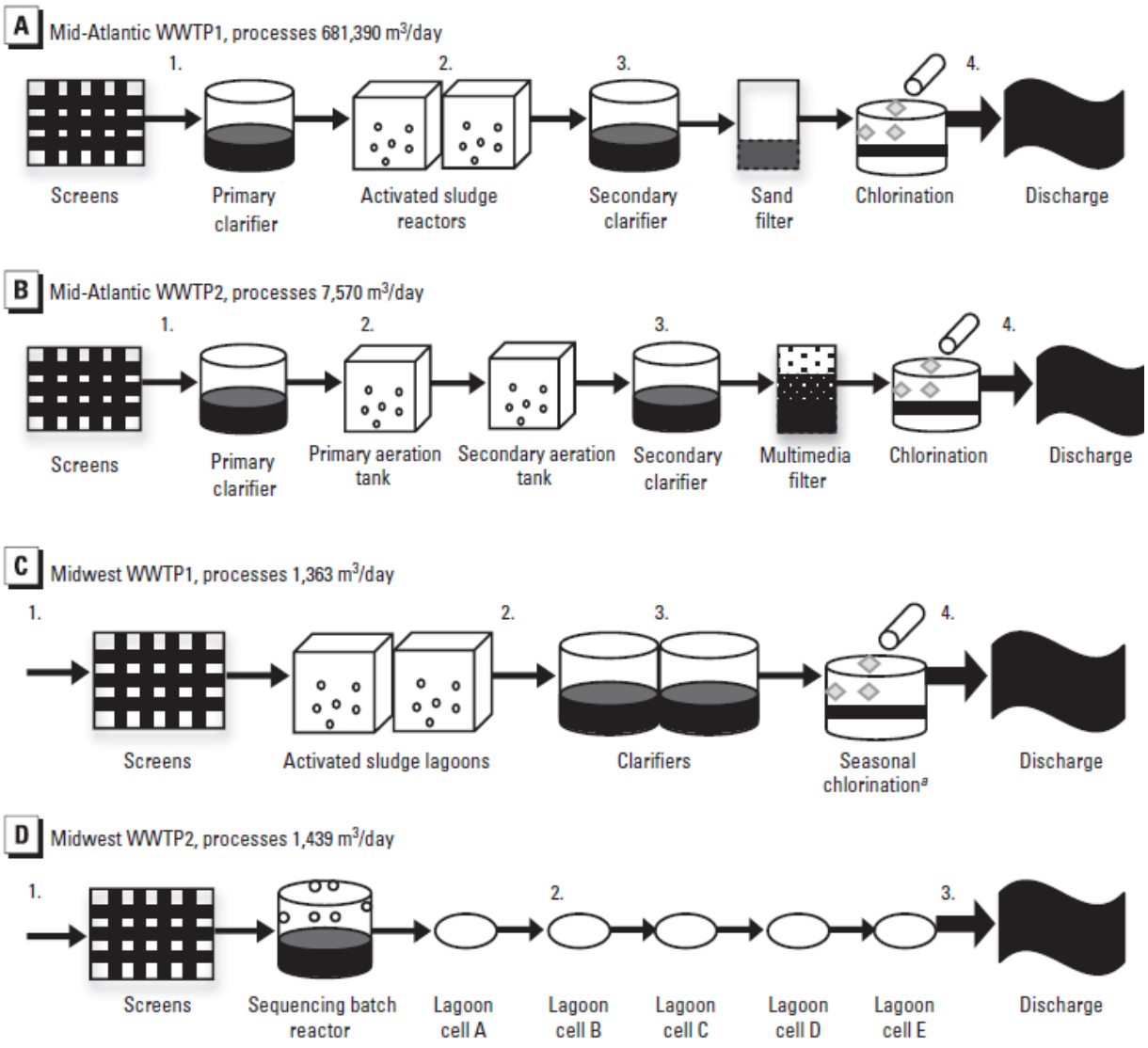


**Figure 2**: Resistance to antimicrobial agents detected among MRSA isolates at (a) Mid-Atlantic WWTP1, (b) Mid-Atlantic WWTP2, (c) Midwest WWTP1, and (d) Midwest WWTP2.

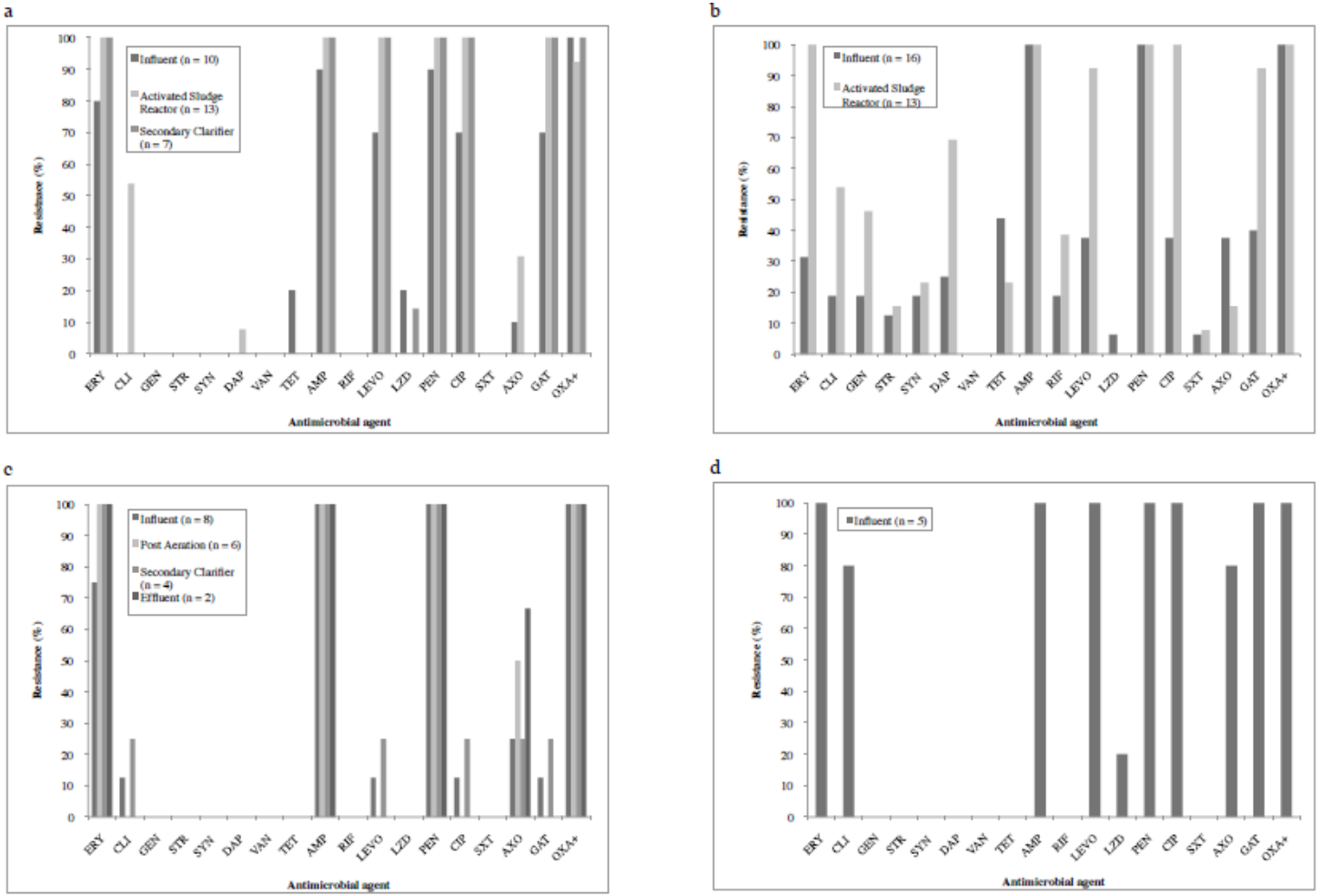


**Figure 3:** Resistance to antimicrobial agents detected among MSSA isolates at (a) Mid-Atlantic WWTP1, (b) Mid-Atlantic WWTP2, (c) Midwest WWTP1, and (d) Midwest WWTP2.

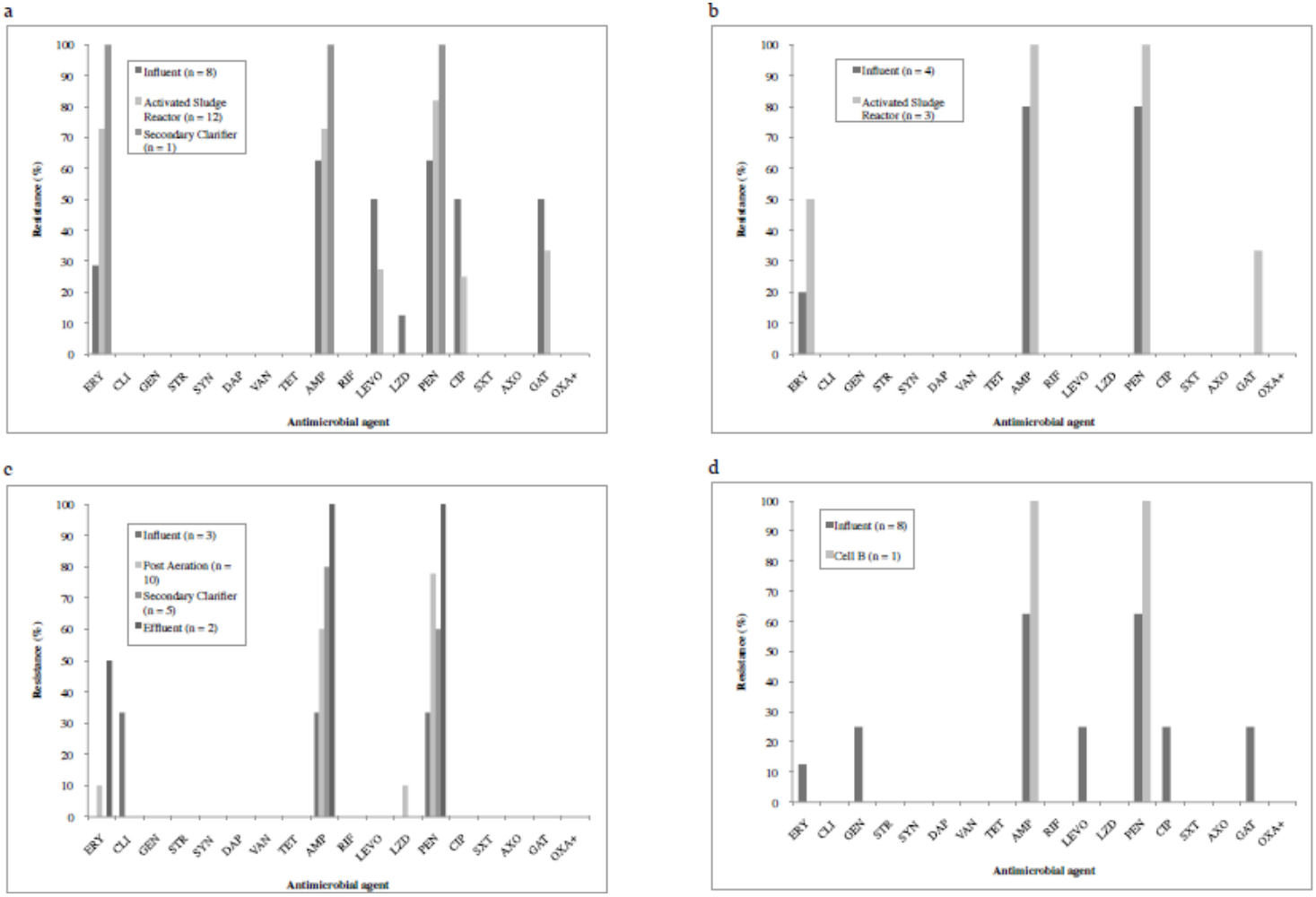


**Figure 4:** Percentage of multidrug-resistant (resistant to two or more classes of antibiotics) MRSA and MSSA isolates from all WWTPs, by wastewater treatment step.

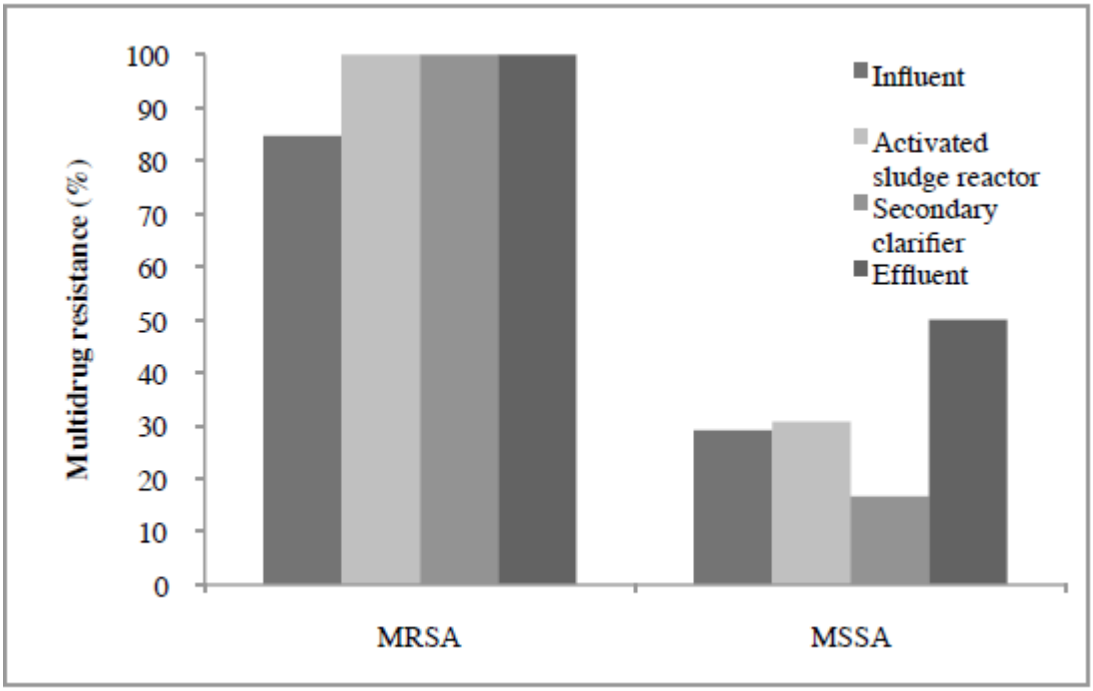
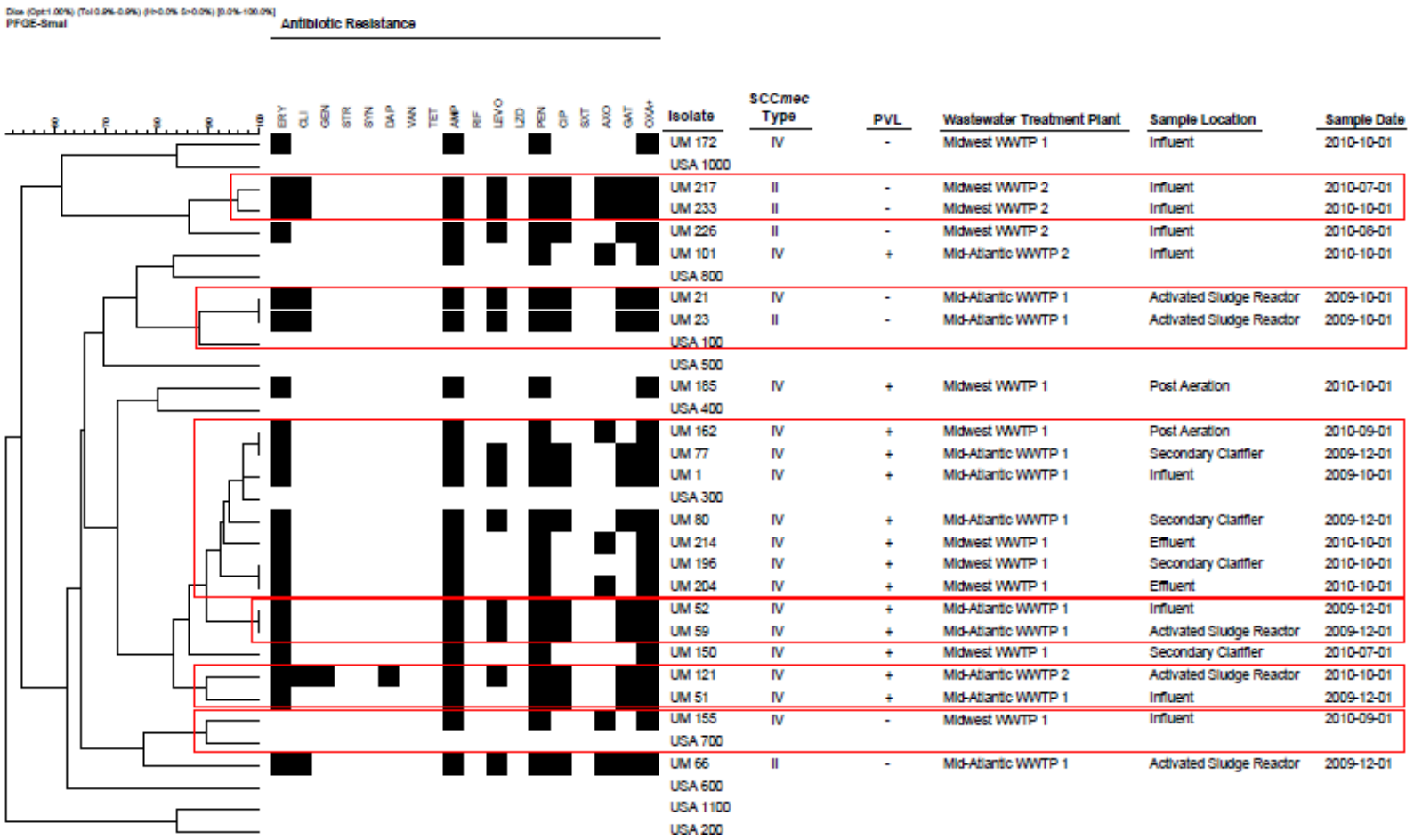


**Figure 5:** Pulsed field gel electrophoresis (PFGE)-based dendrogram, antimicrobial resistance profile, SCC*mec* type, PVL status, and source of a representative subset of MRSA isolates recovered from wastewater. The dendrogram is based on PFGE analysis from BioNumerics software. Clusters were based on ≥88% similarity and are outlined with boxes. For antimicrobial resistance phenotypes, black indicates resistance and white indicates intermediate or susceptible.




| Isolate | SCCmec Type | PVL | Wastewater Treatment Plant | Sample Location | Sample Date |
|---|---|---|---|---|---|
| UM 172 | IV | - | Midwest WWTP 1 | Influent | 2010-10-01 |
| USA 1000 | | | | | |
| UM 217 | II | - | Midwest WWTP 2 | Influent | 2010-07-01 |
| UM 233 | II | - | Midwest WWTP 2 | Influent | 2010-10-01 |
| UM 226 | II | - | Midwest WWTP 2 | Influent | 2010-08-01 |
| UM 101 | IV | + | Mid-Atlantic WWTP 2 | Influent | 2010-10-01 |
| USA 800 | | | | | |
| UM 21 | IV | - | Mid-Atlantic WWTP 1 | Activated Sludge Reactor | 2009-10-01 |
| UM 23 | II | - | Mid-Atlantic WWTP 1 | Activated Sludge Reactor | 2009-10-01 |
| USA 100 | | | | | |
| USA 500 | | | | | |
| UM 185 | IV | + | Midwest WWTP 1 | Post Aeration | 2010-10-01 |
| USA 400 | | | | | |
| UM 162 | IV | + | Midwest WWTP 1 | Post Aeration | 2010-09-01 |
| UM 77 | IV | + | Mid-Atlantic WWTP 1 | Secondary Clarifier | 2009-12-01 |
| UM 1 | IV | + | Mid-Atlantic WWTP 1 | Influent | 2009-10-01 |
| USA 300 | | | | | |
| UM 80 | IV | + | Mid-Atlantic WWTP 1 | Secondary Clarifier | 2009-12-01 |
| UM 214 | IV | + | Midwest WWTP 1 | Effluent | 2010-10-01 |
| UM 196 | IV | + | Midwest WWTP 1 | Secondary Clarifier | 2010-10-01 |
| UM 204 | IV | + | Midwest WWTP 1 | Effluent | 2010-10-01 |
| UM 52 | IV | + | Mid-Atlantic WWTP 1 | Influent | 2009-12-01 |
| UM 59 | IV | + | Mid-Atlantic WWTP 1 | Activated Sludge Reactor | 2009-12-01 |
| UM 150 | IV | + | Midwest WWTP 1 | Secondary Clarifier | 2010-07-01 |
| UM 121 | IV | + | Mid-Atlantic WWTP 2 | Activated Sludge Reactor | 2010-10-01 |
| UM 51 | IV | + | Mid-Atlantic WWTP 1 | Influent | 2009-12-01 |
| UM 155 | IV | - | Midwest WWTP 1 | Influent | 2010-09-01 |
| USA 700 | | | | | |
| UM 66 | II | - | Mid-Atlantic WWTP 1 | Activated Sludge Reactor | 2009-12-01 |
| USA 600 | | | | | |
| USA 1100 | | | | | |
| USA 200 | | | | | |

# Chapter 4: Occurrence of vancomycin-resistant enterococci at four U.S. wastewater treatment plants

(Rosenberg Goldstein RE, Micallef SA, Gibbs SG, George A, Claye E, Sapkota A, Joseph SW, and Sapkota AR. Occurrence of vancomycin-resistant enterococci at four U.S. wastewater treatment plants. [Submitted March 2013])

*Acknowledgements*

We thank the operators at the wastewater treatment plants for their participation and assistance. We thank D. Jack, V. Long, and B. Pasturel for performing laboratory analyses. This work was supported by R03 Small Grants Program, Grant #1-R03-OH009598-01 from the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention (CDC). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC. The Maryland Water Resources Research Center also provided a summer fellowship to Rachel E. Rosenberg Goldstein that supported this work.

*Abbreviations*

| | |
|---|---|
| CIP | ciprofloxacin |
| ERY | erythromycin |
| GEN | gentamicin |
| GI | gastrointestinal |
| HLAR | high-level aminoglycoside resistance |
| LZD | linezolid |
| MDR | multidrug resistance |

| | |
|---|---|
| MEI | membrane-Enterococcus Indoxyl-β-D-Glucoside |
| MIC | minimal inhibitory concentration |
| PBS | phosphate buffered saline |
| PEN | penicillin |
| PYR | pyrrolidonyl peptidase |
| STR | streptomycin |
| SYN | quinupristin/dalfopristin |
| TET | tetracycline |
| VAN | vancomycin |
| VBNC | viable but non-culturable |
| VIE | vancomycin-intermediate enterococci |
| VRE | vancomycin-resistant enterococci |
| WWTP | wastewater treatment plant |

*Abstract*

Background

Vancomycin-resistant enterococci (VRE) are a leading cause of hospital-acquired infections and are associated with increased mortality rates. VRE has been detected in wastewater throughout the treatment process, but to our knowledge no previous studies have analyzed the presence of VRE at wastewater treatment plants (WWTPs) that send their treated effluent to reuse sites. As wastewater reuse becomes more common in the U.S. it becomes even more important to determine whether VRE and other important human pathogens could survive wastewater treatment and be distributed in reclaimed water. In this study we evaluated the presence, concentration, and antimicrobial resistance patterns of VRE at four U.S. municipal WWTPs.

Methods

We collected 44 wastewater samples from four WWTPs – two in the Mid-Atlantic and two in the Midwest region of the U.S. Samples were analyzed for total enterococci and VRE using standard membrane filtration. Isolates were confirmed using biochemical tests and polymerase chain reaction. Antimicrobial susceptibility testing was performed by Sensititre® microbroth dilution. Data were analyzed by two-sample proportion tests and analysis of variance.

Results

Twenty-seven percent of all wastewater samples collected were positive for VRE. VRE made up 3% of the total enterococci detected at all WWTPs. More

samples were VRE-positive from the Mid-Atlantic compared to the Midwest WWTPs ($p = 0.008$). In general, the concentration of VRE decreased as treatment progressed at all WWTPs, except at Mid-Atlantic WWTP1 where there was an increase in VRE concentrations in samples from the activated sludge reactor. VRE was not detected in disinfected effluent, but was detected in one un-chlorinated effluent sample. All phenotypically unique VRE isolates from all WWTPs were multidrug resistant. Fifty-five percent (12/22) of the isolates displayed high-level aminoglycoside resistance.

## Conclusions

Chlorination seems to effectively reduce the occurrence of VRE in wastewater. However, WWTP workers could be exposed to VRE during the treatment process. Our data also show that individuals who come into contact with un-chlorinated reclaimed water could also be exposed to VRE.

*Background*

The number of hospitalizations associated with antibiotic-resistant infections in the United States nearly quadrupled between 1997 and 2006 (Mainous et al. 2011). One antibiotic-resistant pathogen of particular concern is vancomycin-resistant enterococci (VRE), an opportunistic gram-positive bacterium resistant to vancomycin – "a last resort drug" – that can cause urinary tract infections, as well as wound infections, septicemia, meningitis, and endocarditis (CDC 2008; Wegener et al. 1999). The first cases of enterococci with high levels of resistance to vancomycin were reported in the United Kingdom in the 1980s, and as of 2008 VRE was the third leading cause of hospital acquired infections in the U.S. (Hidron et al. 2008; Uttley et al. 1988).

In addition to the ability of VRE to cause multiple types of severe infections, this bacterium is a major public health concern because of its propensity to acquire and transfer mobile resistance genes (Hayakawa et al. 2012). Acquisition of vancomycin resistance genes can take place between strains of enterococci, but vancomycin resistance genes can also be transferred from enterococci to other types of bacteria, including *Staphylococcus aureus* (NIAID 2009; Sievert et al. 2008). The Michigan Department of Community Health reported the first clinical isolate of vancomycin-resistant *S. aureus* (VRSA) in 2002 (Sievert et al. 2008). VRSA is cause for concern because of the limited treatment options for this type of infection. As of 2012, only 13 clinical cases of VRSA had been confirmed in the United States, but the incidence of VRSA infections could continue to rise (CDC 2012). Resistance

genes can also be transferred among isolates originating from different environmental and clinical settings, such as from clinical strains released into wastewater to community strains contained in the wastewater (Guardabassi and Dalsgaard 2004). Because of the ability of enterococci to survive for long periods of time in water (18-30 days at room temperature), it could be cycled between clinical and environmental settings, and cause re-exposure among humans (Lleò et al. 2005; NIAID 2009; Talebi et al. 2008). Previous studies have detected VRE at different stages in the wastewater treatment process, including in treated effluent, suggesting that VRE present in wastewater effluent could be partially responsible for the dissemination of VRE in human communities (Araujo et al. 2010; Caplin et al. 2008; Harwood et al. 2001; Kotzamanidis et al. 2009; Luczkiewicz et al. 2010; Morris et al. 2012; Nagulapally et al. 2009; Poole et al. 2005; Shannon et al. 2007; Talebi et al. 2008). In light of the growing number of antibiotic-resistant infections and the increase in reclaimed water usage in the U.S. (Miller 2011), it is important to assess whether VRE are able to survive treatment at wastewater treatment plants (WWTPs) that supply effluent for reuse applications.

To our knowledge, there are no published studies analyzing municipal wastewater intended for reuse for the presence of VRE. Also, to our knowledge, no studies have specifically evaluated wastewater from the U.S. Mid-Atlantic region for the presence of VRE. A study by Nagulapally et al. (2009) is the only study to evaluate wastewater for VRE from a WWTP in the U.S. Midwest region. In the present study, we evaluated the occurrence and concentration of VRE at four WWTPs

located in the Mid-Atlantic and the Midwest regions of the United States from which treated wastewater is reused at spray irrigation sites. We also analyzed the antimicrobial resistance patterns among VRE isolates collected.

## *Methods*

### Study sites

Four U.S. WWTPs that distribute treated effluent to reuse sites were included in this study: two in the Mid-Atlantic region and two in the Midwest (Rosenberg Goldstein et al. 2012). Sites in the Mid-Atlantic and Midwest were chosen to be able to compare WWTPs in two climatically different regions of the United States. The WWTPs were previously described in detail in Rosenberg Goldstein et al. (2012) (Rosenberg Goldstein et al. 2012). Briefly, Mid-Atlantic WWTP1 is a tertiary WWTP in an urban area. Mid-Atlantic WWTP2, is a tertiary WWTP in a suburban area. Tertiary wastewater treatment includes primary treatment (physical removal of solids), secondary treatment (biological treatment), and additional treatment that can include, but is not limited to, chlorination, ultraviolet (UV) radiation, or filtration. The incoming wastewater (influent) at both Mid-Atlantic plants includes domestic and hospital wastewater, and effluent (discharge) from both Mid-Atlantic plants is piped to landscaping sites for reuse in spray irrigation. Midwest WWTP1 is a tertiary WWTP in a rural area. The incoming water includes domestic wastewater and agriculturally influenced stormwater. Seasonal chlorination occurs in June, July, and August, and chlorinated effluent is piped to a landscaping site for reuse in spray irrigation. Midwest WWTP2, is a secondary WWTP (with no on-site disinfection) in a rural area. Secondary wastewater treatment includes only primary treatment (physical removal of solids) and secondary treatment (biological treatment). The incoming water at this plant includes domestic wastewater, wastewater from a food

production facility, and agriculturally influenced stormwater. Unchlorinated effluent is piped to an agricultural site for crop irrigation.

### Sample collection

Samples were collected throughout the treatment process at all four WWTPs to determine whether certain treatment steps cause the concentration of culturable VRE to increase or decrease, as previous studies have suggested that the concentration of antibiotic-resistant bacteria differs depending on the treatment step sampled (Börjesson et al. 2009; Kim and Aga 2007; Nakamura and Shirota 1990). A total of 44 grab samples were collected between October 2009 and October 2010: 12 samples from Mid-Atlantic WWTP1; 8 samples from Mid-Atlantic WWTP2; 12 samples from Midwest WWTP1; and 12 samples from Midwest WWTP2 (Rosenberg Goldstein et al. 2012). The timing of each sampling event was dependent on the availability and schedule of the WWTP operators. Samples were collected in 1‑L sterile polyethylene Nalgene® Wide Mouth Environmental Sample Bottles (Nalgene, Lima, OH), labeled, and transported to the laboratory at $4^0$C within 24 hr for processing.

### Isolation

Standard membrane filtration was used to recover total enterococci and VRE from the water samples (EPA 2002). Briefly, ten-fold serial dilutions in the range of 1 to 0.001 ml for influent, activated sludge, and post aeration samples; and 100 to 1

ml for secondary clarifier and cell B samples were prepared using sterilized phosphate buffered saline (PBS) and filtered through 0.45 µm, 47 mm mixed cellulose ester filters (Millipore, Billerica, MA). One liter of non-diluted effluent samples was also filtered in the same fashion. 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 amended with 16 µg/mL of vancomycin to isolate VRE. Plates were incubated at 41°C for 24 hr. Resulting 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. *Enterococcus faecalis* ATCC 29212 was used as a positive control and PBS was used as a negative control throughout the isolation process for quality control and quality assurance.

### Identification

VRE was 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. was used (Micallef et al. 2013). Genomic DNA from VRE was extracted by heat lysis as described by Micallef et al. (Micallef et al. 2013). Briefly, the PCR reaction targeted the D-alanine:D-alanine ligase (*ddl*) genes of *E. faecalis* and *E. faecium*, the vancomycin resistance-encoding *van*C1 and *van*C2/3 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.

### Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Sensititre® microbroth dilution system in accordance with the manufacturer's instructions on all confirmed VRE (n=34) and vancomycin-intermediate enterococci (VIE) (n=22) isolates (Trek Diagnostic Systems Inc., Cleveland, OH). Overnight cultures 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 or custom designed CMV5ACDC minimal inhibitory concentration (MIC) plates (Trek Diagnostic Systems) that included the following antibiotics (range of concentrations in µg/ml): erythromycin (ERY; 0.25-8), quinupristin/dalfopristin (SYN; 0.12-32), vancomycin (VAN; 1-128), tetracycline (TET; 2-32), gentamicin (GEN; 2-16, 128-1024), linezolid (LZD; 0.5-8), streptomycin (STR; 512-2048), penicillin (PEN; 0.06-16), and ciprofloxacin (CIP; 0.5-4). *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were used as quality control strains. MICs were recorded as the lowest concentration of an antimicrobial that completely inhibited bacterial growth (CLSI 2013). Resistance breakpoints

published by the Clinical and Laboratory Standards Institute were used (CLSI 2013). Multidrug resistance (MDR) was defined as resistance to two or more classes of antibiotics.

### Statistical analyses

Descriptive statistics included the percentage of wastewater samples that were positive for VRE and the percentage of VRE species by WWTP. A two-sample test of binomial proportions was used to compare the percentage of positive VRE samples between the Mid-Atlantic and the Midwest WWTPs. To describe total enterococci and VRE concentrations, both CFU/100 ml and log CFU/100 ml were calculated. One-way analysis of variance (ANOVA) was then used to compare the average log concentration of presumptive VRE by sampling location for each WWTP. In all cases, p-values of $\leq 0.05$ were defined as statistically significant. All statistical analyses were performed using Stata/IC 10 (StatCorp LP, College Station, TX).

*Results*

Presence and concentration of VRE

VRE were detected at all WWTPs in this study and made up 3% of the total enterococci (Table 1). Total enterococci were detected at all WWTPs in all treatment steps, including chlorinated effluent. Across all treatment plants sampled, 27% (12/44) of the wastewater samples were positive for VRE: 45% (9/20) of samples from the Mid-Atlantic WWTPs; and 13% (3/24) of samples from the Midwest WWTPs ($p = 0.008$). Thirty-three percent (4/12) of influent samples from all of the WWTPs were VRE-positive; 60% (3/5) from the Mid-Atlantic WWTPs and 14% (1/7) from the Midwest WWTPs. The percentage of VRE increased in the activated sludge reactor step at both Mid-Atlantic WWTP1 and Mid-Atlantic WWTP2 but decreased to undetectable levels in the effluent. At Midwest WWP1 the percentage of VRE increased as treatment progressed, with the highest percentage of VRE present in the effluent (Table1). No confirmed VRE were detected in any tertiary-treated (chlorinated) effluent samples. However, VRE was detected in one effluent sample from Midwest WWTP1 in October 2010 when chlorination was not being used.

In general, the average concentration of presumptive VRE at each WWTP decreased as treatment progressed (Figure 1). Looking at all sampling dates combined, there were significant differences in the VRE concentrations between treatment steps at all WWTPs, except at Midwest WWTP 2 where VRE was only detected in one influent sample (Mid-Atlantic WWTP1 $p \leq 0.001$; Mid-Atlantic

WWTP2 $p = 0.001$; Midwest WWTP1 $p \leq 0.001$). At Mid-Atlantic WWTP1, assessing all sampling dates, there was a slight increase in presumptive VRE concentrations in the activated sludge reactor from $1.9 \times 10^4$ CFU/100 mls in influent to $1.9 \times 10^5$ CFU/100 mls in the activated sludge reactor. At all WWTPs, the lowest concentration of VRE was detected in the effluent samples, with no VRE identified in the effluent at Mid-Atlantic WWTP1, Mid-Atlantic WWTP 2, or Midwest WWTP2.

The majority of VRE isolates from all WWTPs were identified as *E. faecium* (78.26%), followed by *E. faecalis* (17.39%), and *E. gallinarium* (4.35%) (Table 2).

### Presence of VIE

VIE were detected at all WWTPs in this study except for Mid-Atlantic WWTP1. No VIE were detected in any of the tertiary-treated (chlorinated) effluent samples. However, similar to VRE, VIE was detected in one effluent sample from Midwest WWTP1 in October 2010 when chlorination was not taking place. VIE isolates from all of the WWTPs were identified as *E. gallinarium* (78.95%) and *E. casseliflavus* (21.05%) (Table 3).

### Antibiotic resistance patterns

In total, 34 VRE isolates were isolated from the four WWTPs. However, only 22 isolates that could be identified as unique using phenotypic analyses were included in the analysis of antibiotic resistance patterns in order to eliminate bias that would have resulted from including clones. All phenotypically unique VRE isolates, of all

species, from all WWTPs were MDR. The resistance patterns among unique isolates varied by WWTP.

The VR *E. faecium* isolates (n=13) from Mid-Atlantic WWTP1 were resistant to multiple antibiotics used to treated enterococci infections including penicillin, ciprofloxacin, and streptomycin (Figure 2a). The percentage of resistant VR *E. faecium* isolates were mostly constant throughout the treatment process at this particular WWTP, except for an increasing percentage of isolates displaying resistance to streptomycin progressing through the treatment process and a spike in the percentage of isolates resistant to tetracycline in the activated sludge reactor (Figure 2a). Four VR *E. faecalis* isolates were detected at Mid-Atlantic WWTP1 (one from influent, one from the activated sludge reactor, and two from the secondary clarifier) (Figure 2b). All of the VR *E. faecalis* isolates were also resistant to erythromycin, quinupristin/dalfopristin, tetracycline, vancomycin, and ciprofloxacin. Three out of four VR *E. faecalis* isolates from Mid-Atlantic WWTP1 were resistant to gentamicin (one from the activated sludge reactor and two from the secondary clarifier), two of the four isolates were resistant to streptomycin (both from the secondary clarifier), and the one isolate from the influent was resistant to penicillin (Figure 2b).

At Mid-Atlantic WWTP2 the one unique VR *E. gallinarum* isolate was resistant to quinupristin/dalfopristin in addition to vancomycin. The three unique VR *E. faecium* isolates from Midwest WWTP 1 (one from the secondary clarifier, two

from the effluent) were all resistant to erythromycin, penicillin, vancomycin, and ciprofloxacin. From Midwest WWTP2, the one VR *E. faecium* isolate from the influent expressed resistance to erythromycin, penicillin, tetracycline, vancomycin, ciprofloxacin, and streptomycin.

In total, 22 VIE isolates were isolated from Mid-Atlantic WWTP2, Midwest WWTP1, and Midwest WWTP2, however only 19 isolates that could be identified as phenotypically unique were included in the analysis of antibiotic resistance patterns. The isolates were resistant or intermediately resistant to a number of clinically relevant antibiotics including erythromycin, tetracycline, and ciprofloxacin, although patterns differed by treatment plant (Figure 3). At Mid-Atlantic WWTP2 and Midwest WWTP1 the percentage of resistant or intermediately resistant isolates generally remained the same or increased as treatment progressed (Figure 3). At Midwest WWTP2, VIE were only isolated from influent samples.

## *Discussion*

### Occurrence of VRE

Previous studies have detected VRE in all wastewater treatment steps, including effluent samples, however to our knowledge this is the first study to evaluate the occurrence of VRE at a WWTP in the Mid-Atlantic region of the U.S. and to evaluate wastewater intended for reuse (Araujo et al. 2010; Caplin et al. 2008; Kotzamanidis et al. 2009; Morris et al. 2012). We identified VRE in 27% of all samples from all WWTPs, including an un-chlorinated effluent sample from Midwest WWTP1 (a WWTP that chlorinates only in summer). The majority of studies assessing the presence of VRE in wastewater have been conducted in Europe and found VRE prevalence ranging from 2-52% (Kotzamanidis et al. 2009; Luczkiewicz et al. 2010; Morris et al. 2012). Interestingly, VRE was isolated in 2-3% of samples from European secondary WWTPs (Luczkiewicz et al. 2010; Morris et al. 2012), and in 52% of wastewater samples from a tertiary WWTP using chlorination (Kotzamanidis et al. 2009). To our knowledge, Nagulapally et al. (2009) is the only published study to have detected VRE at a U.S. municipal WWTP (Nagulapally et al. 2009). Similar to our findings of VRE in influent and biologically treated samples (from the activated sludge reactors, post aeration, and secondary clarifiers) but not in tertiary-treated effluent, Nagulapally et al. (2009) isolated VRE from influent and secondary clarifier samples, but not from UV-disinfected effluent samples (Nagulapally et al. 2009).

Many prior studies have also concluded that VRE concentrations decreased as wastewater treatment progressed; however VRE has been detected in effluent including disinfected effluent (Kotzamanidis et al. 2009; Morris et al. 2012; Nagulapally et al. 2009). In the current study we found that the concentration of VRE significantly decreased as treatment progressed at most WWTPs where samples were collected (Figure 1). At Mid-Atlantic WWTP1, there was a slight increase in the concentration of VRE in the activated sludge reactor step. Also at Mid-Atlantic WWTP1 and Mid-Atlantic WWTP2 the proportion of VRE out of total enterococci was highest in the activated sludge reactor step (Table 1). Previous studies have also found an increase in the concentration or percentage of antibiotic-resistant bacteria in the secondary treatment step, and specifically activated sludge reactors, which decreased with tertiary treatments (Börjesson et al. 2009; Luczkiewicz et al. 2010; Nakamura and Shirota 1990).

The un-chlorinated effluent sample from Midwest WWTP1 that contained a confirmed VRE isolate is from the same WWTP and sampling date (October 2010) where MRSA was detected in a previous study by our group (Rosenberg Goldstein et al. 2012), suggesting that chlorination is important for reducing the presence of multiple types of antibiotic-resistant bacteria in wastewater.

### Regional differences

The different VRE prevalence rates between WWTPs in the Mid-Atlantic (45%) and Midwest (13%) ($p = 0.008$) in this study could be a reflection of

geographic differences in human VRE infection rates (Bouchillon et al. 2005; CDDEP 2013). Since 2004, more VRE has been isolated in the Mid-Atlantic region of the U.S. compared to the Midwest region (Bouchillon et al. 2005; CDDEP 2013). Assessing VR *E. faecalis* and VR *E. faecium* individually, regional differences are still apparent although less pronounced for VR *E. faecalis* (CDDEP 2013). The regional differences in clinically confirmed VRE in the U.S. could be due, in part, to aggressive intervention and control efforts undertaken in the late 1990s at acute and long-term care facilities in Iowa, Nebraska, and South Dakota, including isolation of patients colonized with VRE and greater emphasis on handwashing and cleaning (Low et al. 2001; Ostrowsky et al. 2001). The prevalence of VRE in facilities that participated in the intervention program in this region decreased from 2.2% to 0.5% between 1997 and 1999 (Ostrowsky et al. 2001). In addition to differences in human VRE infection rates between the Midwest and Mid-Atlantic regions, the WWTPs sampled from these regions differed by surrounding land uses and types of influent received. The Mid-Atlantic WWTPs are located in urban and suburban settings and receive domestic and hospital influent, whereas both Midwest WWTPs are located in rural areas and receive agriculturally influenced stormwater in addition to domestic wastewater (Rosenberg Goldstein et al. 2012).

### Species diversity

*E. faecium* was the dominant species of VRE identified in wastewater samples in this study (78.26%). Because wastewater contains human excreta, and *E. faecium* and *E. faecalis* are the two species of enterococci that most commonly cause human

infections, our findings on species diversity verified our expectations (Table 1) (Hayakawa et al. 2012). Several previous studies in Europe and Iran that have analyzed species diversity among VRE isolated from wastewater also found a greater percentage of VRE *faecium* than VRE *faecalis* in this type of water (Araujo et al. 2010; Kotzamanidis et al. 2009; Luczkiewicz et al. 2010; Morris et al. 2012; Talebi et al. 2008). In addition, acquired antibiotic resistance is generally more common among *E. faecium* than *E. faecalis* because of its greater ability to acquire resistance genes (Araujo et al. 2010; Fisher and Phillips 2009; Giraffa 2002). In a study of clinical VRE species diversity, Deshpande et al. (2007) found that 92.8% of VRE isolates from 26 U.S. sites were *E. faecium* (Deshpande et al. 2007). However, the study by Nagulapally et al. (2009) that detected VRE at one U.S. municipal WWTP in the Midwest region found a greater percentage of *E. faecalis* among VRE isolates than any other species (Nagulapally et al. 2009).

### Antibiotic resistance patterns

The presence of high-level aminoglycoside resistance (HLAR) in more than half (54.5%) of the enterococci isolated in this study is cause for concern given that the current international guidelines for treatment of *Enterococcus* infections recommend a combination of ampicillin or penicillin and an aminoglycoside antibiotic (including gentamicin, streptomycin and kanamycin) (Fernández-Hidalgo et al. 2013). Fifty percent (9/18) of VR *E. faecium* isolates from all of the WWTPs sampled in this study were resistant to streptomycin, and none were resistant to gentamicin. Among the four VR *E. faecalis* isolates detected at Mid-Atlantic

WWTP1, 25% (1/4) were streptomycin-resistant and 75% (3/4) were gentamicin-resistant. Gentamicin-resistance has previously been found to be more prevalent among clinical VRE *faecalis* isolates compared to VRE *faecium* (Deshpande et al. 2007). Previous studies have also isolated HLAR enterococci and VRE from wastewater samples and found a wide range of the prevalence of VRE displaying HLAR (Kotzamanidis et al. 2009; Luczkiewicz et al. 2010; Rice et al. 1995; Talebi et al. 2008; Tejedor Junco et al. 2001). Rice et al. (1995) detected enterococci resistant to both gentamicin and streptomycin at U.S. secondary WWTPs, although gentamicin-resistant *Enterococcus* spp. were only isolated from a treatment plant receiving both hospital and domestic sewage, as opposed to only domestic waste (Rice et al. 1995). One *E. faecalis* isolate resistant to gentamicin, streptomycin, and kanamycin was identified in secondary-treated wastewater effluent in a study by Tejedor Junco et al. (2001) in Spain (Tejedor Junco et al. 2001). Luczkiewicz et al. (2010) found high-level resistance to gentamicin and streptomycin among 4.5% and 6.3% of *E. faecium* and 2.3% and 6.8% of *E. faecalis* isolates, respectively, from wastewater samples (Luczkiewicz et al. 2010). Among VR *E. faecalis* isolates collected from municipal wastewater, Kotzamanidis et al. (2009) reported that 5% had high-level gentamicin resistance and 95% had high-level streptomycin-resistance (Kotzamanidis et al. 2009). Twenty-five percent and 75% of VR *E. faecium* isolates, respectively from wastewater and sludge from Portuguese WWTPs were resistant to gentamicin and streptomycin (Araujo et al. 2010). Talebi et al. (2008) found that 95% of VRE isolates from a WWTP in Tehran were gentamicin-resistant (Talebi et al. 2008).

### Multidrug resistance

Our finding that 100% of the VRE isolates detected in this study were MDR is consistent with findings from previous studies analyzing VRE from wastewater. Talebi et al. (2008), Kotzamanidis et al. (2009), and Araújo et al. (2010) found that 100% of VRE *faecium* isolates detected in samples collected from municipal WWTPs were MDR (Araujo et al. 2010; Kotzamanidis et al. 2009; Talebi et al. 2008). Because multidrug resistance further limits treatment options for infections, this finding is concerning considering occupational exposure to wastewater among WWTP workers.

### Public health implications

Our study raises possible public health concerns for WWTP workers exposed to wastewater and for individuals exposed to treated wastewater when it is reused. Based on data from numerous studies, WWTP workers tend to report higher levels of gastrointestinal (GI) and respiratory disease symptoms compared to the general population, although there is conflicting evidence about increased risks of infection due to specific pathogens identified in wastewater (Glas et al. 2001; Khuder et al. 1998; Seuri et al. 2005; Thorn and Kerekes 2001). A review of the literature through 2001 by Thorn and Kerekes (2001) found that several studies identified WWTP workers as more likely to report GI and respiratory symptoms and more likely to have antibodies against hepatitis A (Thorn and Kerekes 2001). Few of the studies analyzed in the review by Thorn and Kerekes (2001) evaluated the risk of specific bacterial

infections among WWTP workers (Thorn and Kerekes 2001). Khuder et al. (1998) found that a greater proportion of WWTP workers reported gastroenteritis than controls, but again no specific microbial agents were identified as causing these symptoms (Khuder et al. 1998). A Finnish study interested in determining whether the increased risk of diarrheal disease among wastewater treatment plant workers was attributable to *Salmonella* found that although more GI symptoms were reported by WWTP workers than controls, there was no significant difference in *Salmonella* antibody levels between the two groups (Seuri et al. 2005). Because VRE was identified at all WWTPs included in the current study, enterococci, and VRE in particular, could potentially cause infections among WWTP workers. Also, because VRE was identified in the final effluent at Midwest WWTP1, and the effluent from this WWTP is sent to a reuse site for irrigation, individuals exposed to reclaimed water could also be at risk for exposure to VRE. Interventions aimed at reducing VRE exposures and infections in clinical facilities including education and increased handwashing have been found to be effective (Low et al. 2001; Ostrowsky et al. 2001). Improving awareness about VRE, and other human pathogens, at WWTPs and encouraging handwashing could be important interventions to explore to reduce potential exposure among WWTP workers.

### Limitations

As with all field studies, there are limitations to this study. If VRE were injured or otherwise present in a viable but nonculturable state, they would not have grown on the vancomycin-amended mEI plates, which are a less hospitable media,

possibly resulting in underestimations of VRE occurrence and concentrations (Lleò et al. 2005; Lleò et al. 2001). The concentrations of VRE could also have been underestimated because of the use of grab samples in this study. Also, the generalizability of our results is limited because we sampled only four WWTPs in two regions of the U.S.

*Conclusions*

VRE was detected for the first time in wastewater at four U.S. WWTPs whose effluent is intended for reuse and at Mid-Atlantic WWTPs. The concentration of VRE decreased as treatment progressed, although at both Mid-Atlantic WWTPs the proportion of VRE out of total enterococci increased in the activated sludge step. VRE was only isolated in one effluent sample at a treatment plant during a month when chlorination was not taking place. Because the WWTP where VRE was found in effluent sends its treated wastewater to a reuse site, individuals exposed to reclaimed water at that site could be potentially exposed to VRE. All phenotypically unique VRE isolates from all WWTPs sampled in this study were MDR, and specifically resistant to a number of antibiotics used to treat VRE infections, including the aminoglycosides gentamicin and streptomycin. WWTP workers and those exposed to reclaimed water could potentially be exposed to VRE.

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*Tables*

Table 1. Average concentration of total enterococci and vancomycin-resistant enterococci (VRE) by wastewater treatment plant and treatment step across all sample collection dates

| | Total Enterococci (CFU/100 mls) | VRE (CFU/100 mls) | Percentage of VRE |
|---|---|---|---|
| Mid-Atlantic WWTP1 | | | |
| Influent | 7.17E+06 | 1.93E+04 | 0.3 |
| Activated Sludge Reactor | 4.64E+05 | 1.89E+05 | 40.7 |
| Secondary Clarifier | 1.49E+03 | 9.60E+01 | 6.5 |
| Effluent | 1.04E+00 | 0.00E+00 | 0.0 |
| Mid-Atlantic WWTP2 | | | |
| Influent | 1.73E+06 | 8.56E+04 | 4.9 |
| Activated Sludge Reactor | 2.19E+05 | 1.32E+04 | 6.0 |
| Secondary Clarifier | 1.86E+04 | 8.46E+02 | 4.6 |
| Effluent | 5.00E-02 | 0.00E+00 | 0.0 |
| Midwest WWTP1 | | | |
| Influent | 1.21E+06 | 4.03E+04 | 3.3 |
| Post Aeration | 6.34E+04 | 8.55E+02 | 1.3 |
| Secondary Clarifier | 6.63E+03 | 1.03E+03 | 15.5 |
| Effluent | 1.56E+01 | 3.29E+00[a] | 21.1 |
| Midwest WWTP2 | | | |
| Influent | 5.30E+05 | 2.52E+03 | 0.5 |
| Cell B | 5.08E+02 | 0.00E+00 | 0.0 |
| Effluent | 6.05E+01 | 0.00E+00 | 0.0 |
| Total | 1.14E+07 | 3.52E+05 | 3.1 |

[a] Sample collected in October 2010 when chlorination was not taking place.

Table 2. Number and percentage of vancomycin-resistant enterococci (VRE) isolates by species and wastewater treatment plant

| | Number of Isolates (%) | | | | |
|---|---|---|---|---|---|
| *Enterococcus* species | Mid-Atlantic WWTP1 | Mid-Atlantic WWTP2 | Midwest WWTP1 | Midwest WWTP2 | Total |
| *E. faecium* | 14 (77.78) | 0 (0) | 3 (100) | 1 (100) | 18 (78.26) |
| *E. faecalis* | 4 (22.22) | 0 (0) | 0 (0) | 0 (0) | 4 (17.39) |
| *E. gallinarum* | 0 (0) | 1 (100) | 0 (0) | 0 (0) | 1 (4.35) |

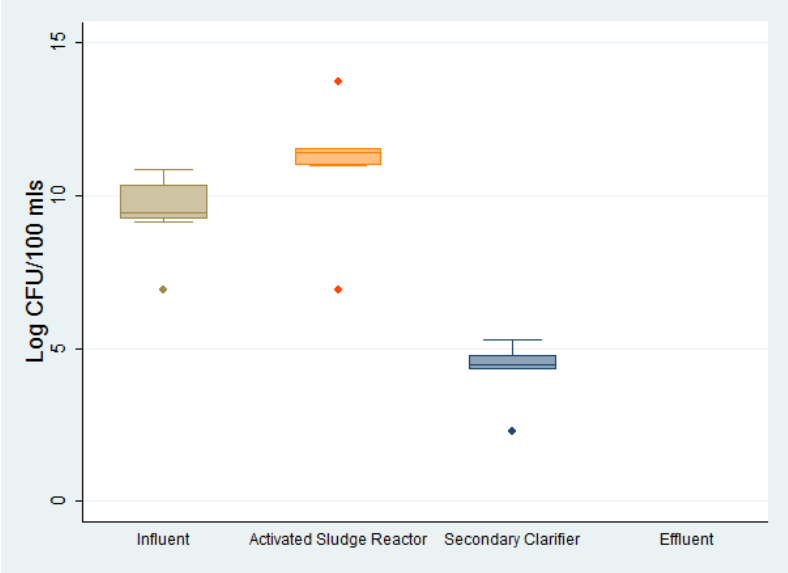
Table 3. Number and percentage of vancomycin-intermediate enterococci (VIE) isolates by species and wastewater treatment plant

| | Number of Isolates (%) | | | | |
|---|---|---|---|---|---|
| *Enterococcus* species | Mid-Atlantic WWTP1 | Mid-Atlantic WWTP2 | Midwest WWTP1 | Midwest WWTP2 | Total |
| *E. gallinarum* | 0 (0) | 5 (83.33) | 6 (75) | 4 (80) | 15 (78.95) |
| *E. casseliflavus* | 0 (0) | 1 (16.67) | 2 (25) | 1 (20) | 4 (21.05) |

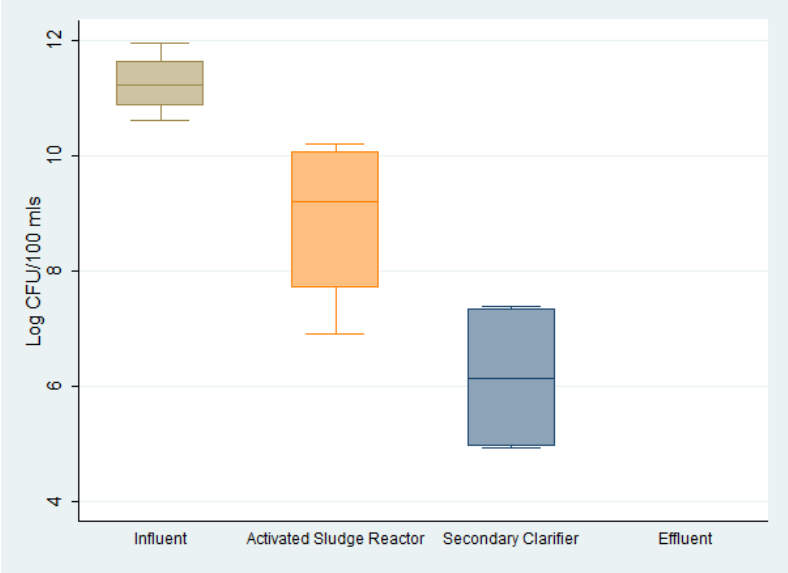
*Figures*

**Figure 1**: Concentration of presumptive vancomycin-resistant enterococci (VRE) by wastewater treatment plant and sampling location.

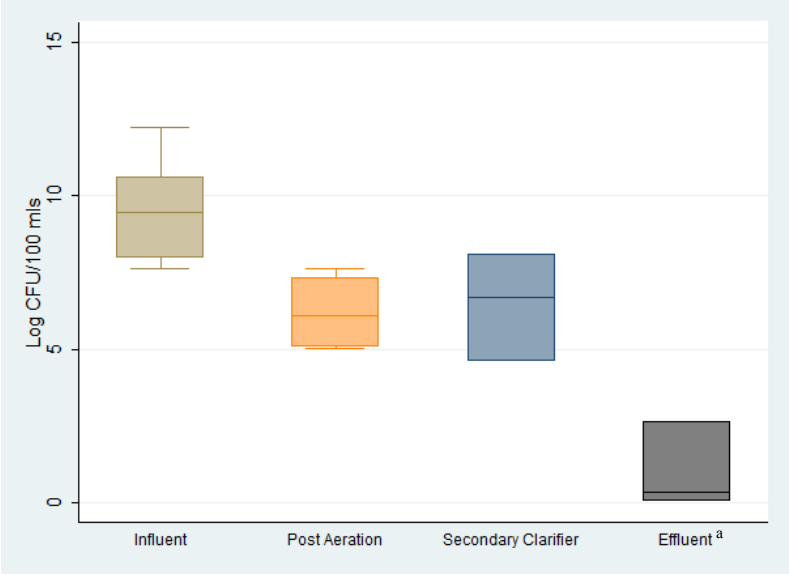
A. Mid-Atlantic WWTP1



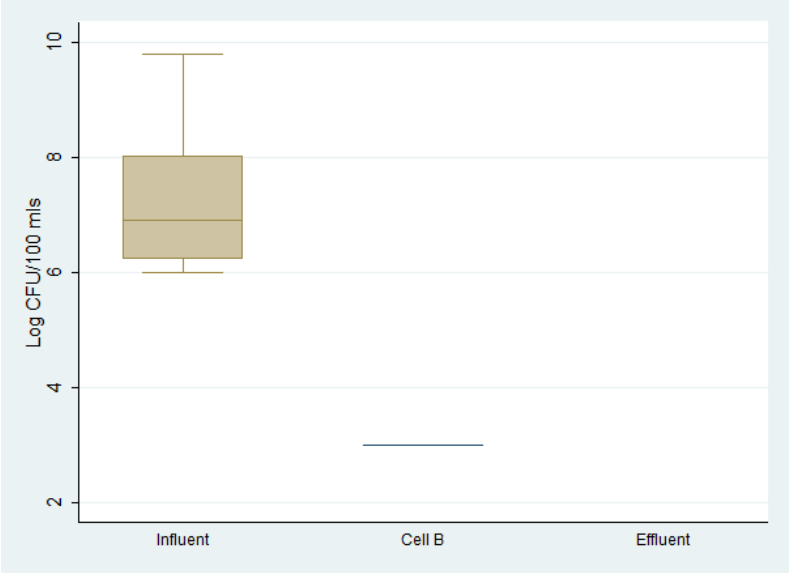

B. Mid-Atlantic WWTP2



C. Midwest WWTP



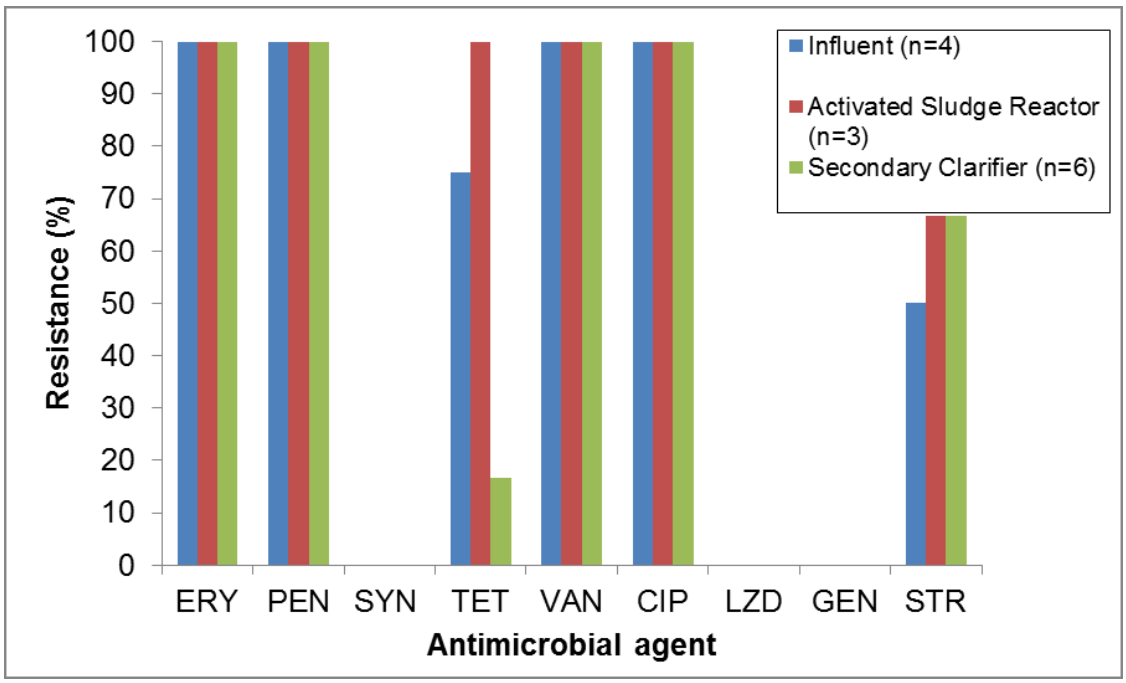

D. Midwest WWTP2




[a] Sample collected in October 2010 when chlorination as not taking place.

**Figure 2**: Resistance to antimicrobial agents detected at Mid-Atlantic WWTP1 among (a) vancomycin-resistant *Enterococcus* (VRE) *faecium* and (b) VR *E. faecalis* isolates.

A. VR *E. faecium*




B. VR *E. faecalis*

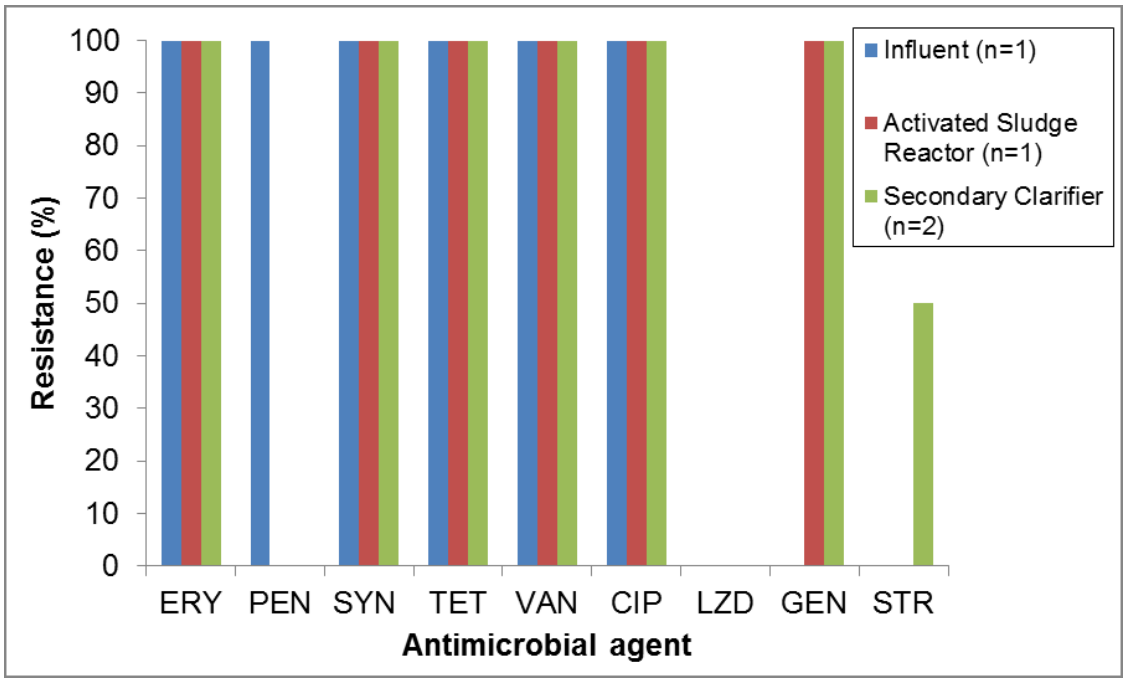
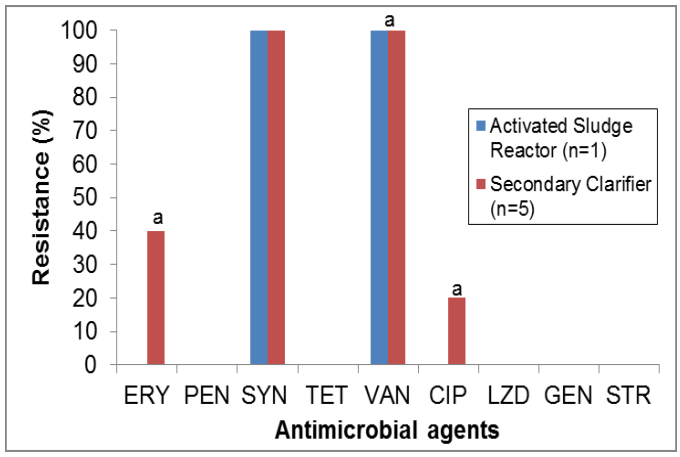
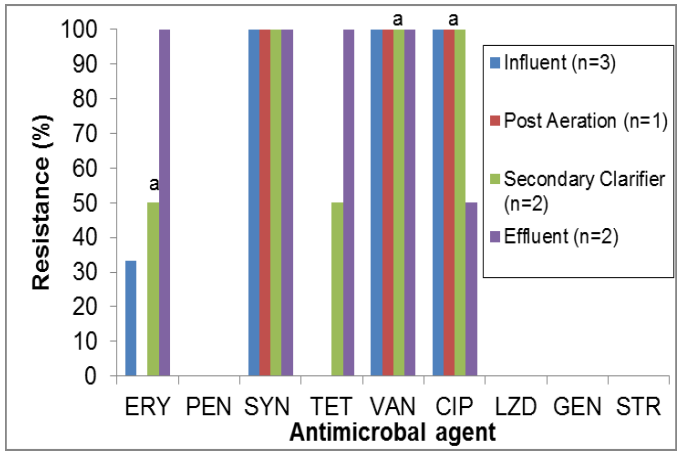


**Figure 3**: Resistance and intermediate resistance to antimicrobial agents detected among vancomycin-intermediate enterococci (VIE) isolates at (a) Mid-Atlantic WWTP2, (b) Midwest WWTP1, and (c) Midwest WWTP2.

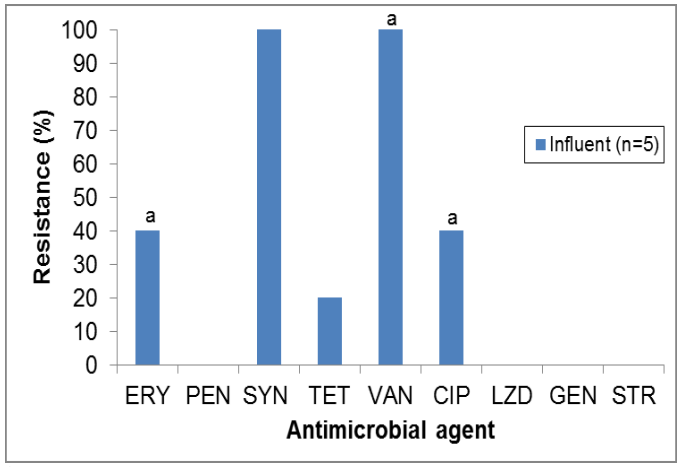
A. Mid-Atlantic WWTP2




B. Midwest WWTP1




C. Midwest WWTP2




[a] Intermediately resistant

# Chapter 5: Occupational exposure to *Staphylococcus aureus* and *Enterococcus* spp. among spray irrigation workers using reclaimed water

(Rosenberg Goldstein RE, Micallef SA, Gibbs SG, He X, George A, Sapkota A, Joseph SW, and Sapkota AR. Occupational exposure to *Staphylococcus aureus* and *Enterococcus* spp. among spray irrigation workers using reclaimed water. [Submitted March 2013])

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*Abstract*

Objectives

As reclaimed water use expands, it is important to evaluate potential health risks from exposure to this water source. We compared odds of colonization with methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *S. aureus* (MSSA), vancomycin-resistant enterococci (VRE), and vancomycin-susceptible enterococci (VSE) between reclaimed water spray irrigation workers and controls.

Methods

Nasal and dermal swabs from 19 spray irrigation workers and 24 controls were collected and analyzed for MRSA, MSSA, VRE, and VSE. Isolates were confirmed and antimicrobial susceptibility testing was performed by microbroth dilution. Data were analyzed by two-sample proportion, chi-square, and Fisher's exact tests, and logistic regression.

Results

No MRSA or VRE were detected in any samples. MSSA was detected in 26% and 29% of spray irrigation workers and controls. VSE was detected in 11% and 0% of spray irrigation workers and controls. The odds of MSSA, multidrug-resistant MSSA, and either MSSA or VSE colonization were not significantly different between spray irrigators and controls.

## Conclusions

Reclaimed water spray irrigation workers may be occupationally exposed to human pathogens based on the detection of pathogens in wastewater intended for reuse in previous studies. Future studies with larger sample sizes are needed to further evaluate this relationship.

*Introduction*

Between 5-6% of municipal wastewater effluent, approximately 2.22 billion gallons per day, is reclaimed and reused in the United States (Miller 2011). Landscape irrigation is one of the most common uses of reclaimed water in the U.S., making up 18% of all water reused across the country (EPA 2012; NRC 2012). Although irrigation with reclaimed water is growing, limited data exists on pathogens present in reclaimed water, as well as health risks from exposure to this water source (Blumenthal et al. 2001; Devaux et al. 2001; Durand and Schwebach 1989; Harwood et al. 2005; Jjemba et al. 2010). Some studies have identified several bacterial and parasitic species in wastewater intended for reuse (Blumenthal et al. 2001; Harwood et al. 2005; Jjemba et al. 2010; Rosenberg Goldstein et al. 2012). Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) have both been identified in wastewater throughout the treatment process, including final effluent in the U.S. and Europe (Araujo et al. 2010; Börjesson et al. 2009; Börjesson et al. 2010; Kotzamanidis et al. 2009; Nagulapally et al. 2009; Rosenberg Goldstein et al. 2012). Several studies have suggested that wastewater, and the reuse of wastewater, could be a source of exposure to these resistant bacteria, as well as other human pathogens, in the community (Börjesson et al. 2009; Plano et al. 2011; Rosenberg Goldstein et al. 2012). As rates of antibiotic-resistant bacterial infections in hospitals and the community continue to rise (Mainous et al. 2011), including infections with MRSA and VRE, it is important to evaluate whether reclaimed water could serve as a potential source of exposure to these particular microorganisms.

*Staphylococcus aureus* is a bacterial pathogen that colonizes multiple body sites, most commonly the nostrils, and causes a number of infections, including dermal and soft tissue infections, pneumonia, and septicemia (Bassetti et al. 2009). MRSA and methicillin-susceptible *S. aureus* (MSSA) have been detected in air samples from a number of environments, including wastewater treatment plants (Fracchia et al. 2006; Gandara et al. 2006; Gibbs et al. 2006; Schulz et al. 2012). Since these bacteria can be aerosolized from water and are capable of colonizing skin and soft tissues, exposure through inhalation is of concern, particularly among workers at wastewater treatment plants and spray irrigation sites using the reclaimed water.

Enterococci are opportunistic pathogens that can cause urinary tract infections, bacteremia, and endocarditis (CDC 2008; P Murray et al. 2002; Spacek and Vinetz 2009). Between 2006 and 2007, 13% of hospital infections were caused by enterococci, and approximately 30% of these infections were VRE (Hidron et al. 2008). VRE and vancomycin-susceptible enterococci (VSE) have been detected in wastewater in both raw influent and treated effluent, as well as wastewater bioaerosols (Araujo et al. 2010; Caplin et al. 2008; Fracchia et al. 2006; Korzeniewska et al. 2009; Kotzamanidis et al. 2009; Luczkiewicz et al. 2010; Morris et al. 2012; Nagulapally et al. 2009; Talebi et al. 2008). Although the exact sources of community-acquired VRE and VSE infections remain unclear, animals, food, and

wastewater have been suggested as important environmental reservoirs (McDonald 1997; Poole et al. 2005).

If *S. aureus* or enterococci survive wastewater treatment and distribution to reuse sites, spray irrigation workers could be exposed to these organisms through dermal contact or inhalation (Bassetti et al. 2005). To our knowledge, no previous studies have evaluated the risk for occupational exposure to antibiotic-resistant bacteria from reclaimed water (Devaux et al. 2001; NRC 2012). In the present study we compared MRSA, MSSA, VRE, and VSE colonization among reclaimed water spray irrigation workers and office worker controls in the Mid-Atlantic region of the United States.

*Methods*

Study Site

The spray irrigation reuse site described in this study is located in the Mid-Atlantic region of the U.S. The site was chosen based on the willingness of the site operator to participate in the study. This site receives treated wastewater from a tertiary wastewater treatment plant in the Mid-Atlantic region (Mid-Atlantic WWTP1) (Rosenberg Goldstein et al. 2012). Once the treated wastewater reaches the reuse site, it passes through an 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. Based on turf irrigation needs, water is then pumped from the storage pond to a pump that distributes the water to spray heads. Spray irrigation workers also carry backpack spray systems to irrigate additional areas. The reuse site employs eight full-time employees and approximately 22 seasonal employees each year.

Subject Selection

A total of 43 subjects were enrolled in the study; 19 spray irrigation workers from the spray irrigation site who were occupationally exposed to reclaimed water, and 24 office workers controls from an academic work setting who were not exposed to reclaimed water or healthcare settings on the job. Study subjects were selected through a convenience sample based on employment status. Office worker controls were matched by age (+/- 2 years), sex, and race to the spray irrigation workers and

recruited into the study in person and over email. Individuals were excluded from participation if they reported a nosebleed three days prior to sample collection to avoid dislodging blood clots.

### Survey

Participants were asked to complete a short survey containing questions related to sociodemographics, as well as questions related to risk factors associated with MRSA colonization and previous MRSA diagnosis. The survey also asked participants about previous work in healthcare facilities and household members who work in healthcare facilities, because *S. aureus* nasal colonization rates among healthcare workers is greater than the 20-30% colonization rate found in the general population (FDA 2009; Gorwitz et al. 2008; P Murray et al. 2002). The survey was filled out by participants on site at each sampling event.

### Sample Collection

A total of 94 nasal and 94 dermal swab samples were collected between August 2009 and February 2011. All participants were sampled multiple times when possible. Nasal swabs were collected using, Liquid Stuart Medium Transport swabs (Copan, Italy). The swab was inserted approximately 1.25 cm into the participant's right nostril and gently rotated five times on the inside wall of the nostril (Bassetti et al. 2005; Ochei and Kolhatkar 2000; Vegunta et al. 2009). Dermal swabs were also collected using the same type of swabs. An approximately five-by-five cm area of the participant's right forearm was swabbed by rolling the swab back and forth 15

times (Kullander et al. 2009). All samples were transported to the laboratory at 4°C and processed within 24 hr.

### Isolation

All media was obtained from Becton, Dickinson and Company (Franklin Lakes, NJ). Nasal and dermal swabs were streaked onto Baird Parker agar for isolation of total *S. aureus* and Enterococcosel agar for isolation of total *Enterococcus* spp. Baird Parker agar plates were incubated at 37°C for 24 hr, while Enterococcosel agar plates were incubated at 41°C for 24 hr. Resulting black colonies with halos on Baird Parker, and colonies with a black precipitate on Enterococcosel agar were considered presumptive *S. aureus* and enterococci, respectively. These colonies were purified on Brain Heart Infusion agar and archived in Brucella broth with 15% glycerol at -80°C. *S. aureus* ATCC 43300 and *Enterococcus faecalis* ATCC 29212 were used as positive controls and phosphate buffered saline was used as a negative control throughout the isolation process.

### Confirmation

*S. aureus* were identified using the Gram stain, the coagulase test (Becton, Dickinson and Company), and the catalase test. For confirmation of *S. aureus* and MRSA differentiation, the *S. aureus*-specific *nuc* gene, the MRSA-specific *mecA* gene, and a 16S rDNA internal control were PCR amplified in a multiplex reaction as described previously (Brakstad et al. 1992; Edwards et al. 1989; Fang and Hedin 2003; Rosenberg Goldstein et al. 2012; Smyth et al. 2001). DNA extraction was

performed using the MoBio UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) per the manufacturer's recommendations. Briefly, PCR amplification consisted of an initial denaturing step of 95°C for 3 min, followed by 34 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. *S. aureus* ATCC 43300 was used as a positive control for PCR amplification of *nuc* and *mecA* genes.

Enterococci were presumptively identified using the Gram stain, the catalase test, and by detection of pyrrolidonyl peptidase (pyr) activity (Remel, Lenexa, KS). Confirmation was accomplished using a modified multiplex PCR assay previously described by Micallef et al. (2013). Genomic DNA from enterococci was extracted by heat lysis as described previously (Micallef et al. 2013). Briefly, the PCR reaction targeted the *ddl* genes of *Enterococcus faecalis* and *E. faecium*, the vanC1 and vanC2/3 genes of *E. gallinarum* and *E. casseliflavus*, and a 16S rDNA internal control (Micallef et al. 2013). 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.

Colonization among study participants was defined as MRSA, MSSA, VRE, or VSE isolated and confirmed from any swab sample (dermal or nasal).

### Antimicrobial Susceptibility Testing

We performed antimicrobial susceptibility testing using the Sensititre® microbroth dilution system with GPN3F minimum inhibitory concentration (MIC) plates (Trek Diagnostic Systems Inc., Cleveland, OH) in accordance with the manufacturer's instructions on all PCR-confirmed *S. aureus* (n=97) and *Enterococcus* spp. (n=20) isolates. Details on specific antibiotics and concentrations tested are included in Appendix A. *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were used as quality control strains. MICs were recorded as the lowest concentration of an antimicrobial that completely inhibited bacterial growth (CLSI 2013). Resistance breakpoints published by the Clinical and Laboratory Standards Institute were used (CLSI 2013). Multidrug resistance (MDR) was defined as resistance to two or more classes of antibiotics.

### Statistical Analyses

Descriptive statistics were reported including the percentages of nasal and dermal swab samples positive for MSSA and VSE by worker classification. Differences in sociodemographic variables between spray irrigation workers and controls were compared using the chi-square or Fisher's exact test. Two-sample tests of binomial proportions were used to compare the percentage of spray irrigation workers and office worker controls who were colonized with each of the microorganisms of interest. Statistical analyses of antibiotic resistance data were limited to MSSA (n=32) and VSE (n=3) isolates expressing unique antimicrobial

resistance profiles; this allowed us to reduce bias that could be introduced by including possible clones. A two-sample *t*-test was used to compare the number of antibiotics isolates were resistant to. Logistic regression models were used 1) to determine if the odds of ever being colonized with the bacteria of interest were different for spray irrigation workers compared to controls, while controlling for other factors; and 2) to conduct analyses of spray irrigation worker odds of colonization. A generalized linear mixed effects model (GLMM) was used to evaluate the odds of being colonized with *S. aureus* over time by occupational status. In all cases, p-values of $\leq 0.05$ were defined as statistically significant. All statistical analyses were performed using Stata/IC 10 (StatCorp LP, College Station, TX) and SAS 9.2 (SAS Inc., Cary, NC).

*Results*

The participation rate for the study was 88% (43/49). Participants ranged in age from 17 to 66 years and both spray irrigation workers and office worker controls were composed largely of Caucasian males (Table 1). The mean age of the spray irrigation workers and controls was 34 and 33 years, respectively. None of the participants in either group reported previous diagnoses of MRSA. Of the sociodemographic variables collected from participants, there were a few significant differences between spray irrigation workers and controls. Education levels and yearly income differed by exposure group ($p$ <0.001; $p$=0.011). Spray irrigation workers reported "currently smoking" ($p$=0.002) and "smoking more than 100 cigarettes in the past six months" ($p$ <0.001) more than the controls (Table 1). Slightly more controls reported either personally having worked in a healthcare setting or having a household member who had worked in a healthcare setting, but these differences were not statistically significant.

Presence of MRSA and MSSA

No MRSA was detected in any of the nasal or dermal swabs collected from the spray irrigation workers or controls. MSSA was recovered from 28% (12/43) of all study participants. Twenty-six percent (5/19) of spray irrigation workers had nasal swabs that were positive for MSSA during at least one sampling event (Figure 1). Among controls, 29% (7/24) were positive for MSSA in nasal swabs during at least one sampling event. We did not detect MSSA in any spray irrigation worker dermal

swab samples, but 8% (2/24) of controls were MSSA-positive based on dermal swab samples alone. The probability of ever having been colonized with MSSA from either sample type was not significantly different between spray irrigation workers and controls ($p = 0.836$).

Presence of VRE and VSE

VRE was not detected in any nasal or dermal swab samples from the spray irrigation workers or controls (Figure 1). A greater proportion of spray irrigation workers were colonized with VSE compared to controls, but this was not statistically significant (11% vs. 0%; $p$=0.189).

Presence of either of the target bacteria in swab samples

Thirty-three percent (14/43) of all participants were colonized with either MSSA or VSE. A greater proportion of spray irrigation workers compared to controls were colonized with at least one of the bacteria of interest, but this was not statistically significant ($p$=0.2969) (Figure 1).

Antibiotic resistance patterns

In total, 97 MSSA isolates were recovered from nasal and dermal swabs: 57 isolates from spray irrigation workers, and 40 from controls. However, statistical analyses concerning antibiotic resistance patterns among these isolates were limited to 32 isolates that could be confirmed as unique using phenotypic analyses (15 from

spray irrigation workers; 17 from controls). Isolates were resistant to a variety of the 18 antibiotics tested. A greater percentage of spray irrigation workers compared to controls were colonized with MDR MSSA (11% vs. 8.3%) ($p$=0.4029) (Figure 1) and a greater percentage of MSSA isolates from spray irrigation worker swabs were resistant to erythromycin ($p$=0.1260) and linezolid ($p$=0.1313) (Figure 2). A greater percentage of MSSA isolates from office worker control swabs compared to spray irrigation worker swabs were resistant to tetracycline ($p$=0.1699) and ampicillin ($p$=0.2458) (Figure 2). MSSA isolates from spray irrigation workers' nasal swabs were resistant to an average of 1.6 antibiotics compared to 1.47 antibiotics among control nasal swab MSSA isolates ($p$=0.3702).

In total, 20 VSE isolates were isolated from two spray irrigation worker nasal swabs. Of the three isolates that were phenotypically unique, all three were resistant to rifampicin and all three were either resistant or intermediately resistant to quinupristin/dalfopristin.

### Impact of occupational exposure on colonization

Our univariate logistic regression models indicated that the odds of being colonized with MSSA, MDR MSSA, or either MSSA or VSE did not differ significantly between the spray irrigation workers occupationally exposed to reclaimed water and our control group of office workers (Table 2). Our adjusted, multivariate logistic regression model also indicated that the odds of being colonized with MSSA, MDR MSSA, or either MSSA or VSE did not differ significantly

between the spray irrigation workers and controls (Table 2). Specifically, the odds of being colonized with MDR MSSA or either MSSA or VSE were greater among the spray irrigation workers compared to the controls in both the unadjusted and adjusted models, however, the differences were not statistically significant (Table 2). In the adjusted model, the odds of being colonized with MSSA were also greater among spray irrigation workers compared to controls but the difference was not statistically significant (Table 2). After adjusting for changes over time in addition to the other predictor variables using the GLMM, there were no significant differences in the odds of colonization with any of the bacteria of interest ($p$=0.7486) (data not shown).

### Factors impacting MSSA and VSE colonization among spray irrigation workers

The results of our logistic regression models focused only on spray irrigation workers showed that most of the variables used in our model did not have statistically significant effects on the odds of the spray irrigation workers being colonized with MSSA, MDR MSSA, VSE, or either MSSA or VSE (data not shown). However, spray irrigation workers, who reported either personally having worked in a healthcare setting or having a household member who had worked in a healthcare setting (n=8) tended to be more likely to be colonized with either MSSA or VSE compared to those who did not report this type of exposure (OR=7.50; 95% CI 0.9214 to 61.0472).

*Discussion*

MRSA and MSSA prevalence

Based on the 2003–2004 National Health and Nutrition Examination Survey (NHANES), between 27.2% and 30% of the U.S. population were colonized with *S. aureus* (Gorwitz et al. 2008). It has also been estimated that 20-30% of the general population is colonized in the nostrils with *S. aureus* (FDA 2009; John and Barg 1999). Twenty-eight percent of all study participants in this study were nasally colonized with MSSA, which falls within the expected range of MSSA prevalence. Previous studies have identified low carriage rates of MRSA in the community, including a study by Gorwitz et al. (2008) that found that the prevalence of MRSA colonization in the U.S. is approximately 1.5% based on the 2003-2004 NHANES data (Gorwitz et al. 2008). Therefore, the finding that MRSA was not detected in any samples in the current study could be a factor of our small sample size. Also, the lack of any statistically significant difference in the odds of MSSA colonization between spray irrigation workers and office worker controls could also be due to our small sample size. To be able to detect a 1.5 difference in the odds of MSSA colonization between spray irrigation workers and controls with 80% power, we would have needed to enroll approximately 878 participants. Our experience, and the anecdotal experience of others, however, has shown that gaining access to wastewater reuse sites for research purposes is difficult, limiting the number of possible study participants (Crook 2005).

VRE and VSE prevalence

Community-associated VRE (CA-VRE) (defined as no previous hospital stay reported) is rarely reported in the U.S. (McDonald 1997; Stevenson 2005), however, the introduction of VRE into hospital settings from outside environments has been documented in both the U.S. and internationally (Anzar et al. 2004; Raja et al. 2005; Stevenson 2005). In a study by Stevenson et al. (2005) of rural U.S. hospitals, 22% of patients with positive VRE cultures had been in the hospital for under 48 hr or were outpatients, prompting classification as a CA-VRE infection (Stevenson 2005). A 1999 study in Germany found a 0.9% prevalence of VRE among a "healthy" student population, not admitted to a hospital for infection with VRE (Wendt et al. 1999). Therefore, the fact that VRE was not detected in any of the current study's participants could also be a factor of our small sample size.

*E. faecalis* and *E. faecium* have previously been isolated in small numbers from the upper respiratory tract; however, in a large-scale hospital study of VRE and MRSA colonization by Warren et al. (2004), the majority of VRE-positive specimens were recovered from stool or rectal samples (87%) compared to respiratory (0%) and soft tissue and wound samples (2%) (PR Murray et al. 2002; Warren et al. 2004). Yet, in a study by Hendrix et al. (2001), 13% of all hospital patients who provided an oropharyngeal (back of the oral cavity) culture had VRE-positive results (Hendrix et al. 2001). Rectal swabs are often used to detect VRE, however to increase participation among our study population we did not use this method. Using only nasal and dermal swabs to detect VRE and VSE from participants in the current study

could have underestimated the true prevalence of VRE and VSE among the study population. To have been able to detect a difference in the VSE colonization of our two groups with 80% power, we would have had to enroll 1,644 participants.

### Public health implications

Although we found no statistically significant differences between the odds of reclaimed water spray irrigation workers and office worker controls being colonized with *S. aureus* or enterococci, results from previous studies from our research group that detected MRSA and VRE in wastewater intended for reuse raises potential public health concerns for those working with or otherwise exposed to reclaimed water (Rosenberg Goldstein et al. 2012). Several previous studies have analyzed the risk of different types of infections among individuals occupationally, recreationally, or residentially exposed to wastewater that has undergone various levels of treatment and found conflicting results (Blumenthal et al. 2001; Devaux et al. 2001; Shuval 1991). A study in Mexican agricultural communities found increased odds of parasitic infections and associated diarrheal disease among individuals exposed to wastewater stored in one reservoir (which could be categorized as secondary (biologically) treated wastewater) and used for agricultural irrigation (Blumenthal et al. 2001). With additional time or storage in more than one reservoir, there was no difference in the odds of infection between exposed and unexposed groups (Blumenthal et al. 2001). Durand and Schwebach (1989) examined whether individuals recreationally exposed to turf irrigated with reclaimed water in Colorado were more likely to report gastrointestinal (GI) illnesses than those exposed to turf

irrigated with potable water (Durand and Schwebach 1989). They found no difference in the number of reported illnesses between the exposed and unexposed groups. However, their study did show that exposure to wet grass was associated with more reported GI symptoms (Durand and Schwebach 1989). Because reclaimed water irrigation workers routinely work in wet grass as they are spraying, they could be at a greater risk for experiencing GI symptoms. Similarly, a study by Devaux et al. (2001) in France identified that farmers exposed to wastewater treated in stabilization ponds reported more respiratory and GI symptoms compared to a non-exposed group of controls (Devaux et al. 2001). A Texas-based study found that individuals exposed to secondary treated wastewater reused for agricultural spray irrigation had an increased risk of viral infections 1.5-1.8 times that of individuals not exposed to reclaimed water (Camann et al. 1988).

To our knowledge, the current study is the first to evaluate occupational exposure to *S. aureus* and enterococci in reclaimed water in the United States. Although the differences between odds of MSSA, MDR MSSA, and VSE colonization in this study were not statistically significant, the data still provide evidence of potential human health issues that should be further investigated with larger samples sizes across the U.S. This is particularly important with regard to potential exposures to MRSA and VRE–leading causes of hospital-acquired infections, as well as microorganisms associated with a growing number of community-acquired infections–because it has been detected in treated U.S. wastewater that is used in reclamation activities (Rosenberg Goldstein et al. 2012).

Similarly, more work is needed to evaluate potential exposures to other human pathogens among spray irrigation workers including *Legionella* spp. and *Aeromonas* spp. since these microorganisms have also been isolated from reclaimed water (Brissaud et al. 2008; Jjemba et al. 2010; Palmer et al. 1995).

## Conclusions

Our findings are the first to evaluate and detect MSSA and VSE among spray irrigation workers using reclaimed water. As reclaimed water use continues to grow, additional studies with larger samples sizes are needed to further evaluate occupational exposures to human pathogens originating from this water source.

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*Tables*

**Table 1**: Comparison of participant characteristics between spray irrigation workers and office worker controls [a]

| Variable | n (%) | | *p*-value |
|---|---|---|---|
| | Spray Irrigation Workers | Office Worker Controls | |
| Age (years) | | | 0.406 |
| ≤ 17 | 3 (16) | 0 (0) | |
| 18-19 | 2 (11) | 2 (8) | |
| 20-30 | 4 (21) | 10 (42) | |
| 31-41 | 5 (26) | 6 (25) | |
| 42-56 | 4 (21) | 5 (21) | |
| >56 | 1 (5) | 1 (4) | |
| Gender | | | 1.00 |
| Male | 18 (95) | 23 (96) | |
| Female | 1 (5) | 1 (4) | |
| Race | | | 0.575 |
| Caucasian | 17 (90) | 23 (96) | |
| Other | 2 (10) | 1 (4) | |
| Education | | | < 0.001 |
| Less than high school | 1 (5) | 0 (0) | |
| High school | 12 (63) | 0 (0) | |
| Associate | 2 (11) | 0 (0) | |
| College | 4 (21) | 24 (100) | |
| Yearly income ($1000s) | | | 0.011 |
| < 15 | 10 (56) | 5 (21) | |
| 15-25 | 3 (17) | 6 (25) | |
| 25-35 | 2 (11) | 1 (4) | |
| 35-50 | 2 (11) | 1 (4) | |
| > 50 | 1 (6) | 11 (46) | |
| Duration in job | | | 0.221 |
| ≤ 1 month | 2 (10.5) | 1 (4) | |

| | | | |
|---|---|---|---|
| > 1 month – ≤ 6 months | 6 (31.5) | 3 (12.5) | |
| > 6 months – ≤ 2 years | 3 (16) | 5 (21) | |
| > 2 – ≤ 5 years | 5 (26) | 5 (21) | |
| > 5 – ≤ 20 years | 3 (16) | 5 (21) | |
| ≥ 20 years | 0 (0) | 5 (21) | |
| Currently smoke | | | 0.002 |
| Yes | 10 (53) | 2 (8) | |
| No | 9 (47) | 22 (92) | |
| Smoke more than 100 cigarettes in past 6 months | | | <0.001 |
| Yes | 9 (47) | 0 (0) | |
| No | 10 (53) | 24 (100) | |
| Personally worked in healthcare setting | | | 1.000 |
| Yes | 3 (16) | 4 (17) | |
| No | 16 (84) | 20 (83) | |
| Household member worked in healthcare setting | | | 0.686 |
| Yes | 6 (32) | 9 (37.5) | |
| No | 13 (68) | 15 (62.5) | |

[a] Differences between spray irrigation workers and controls were analyzed using Fisher's exact test for all analyses except "Household Member Worked in Healthcare Setting" which was analyzed using the chi-square test.

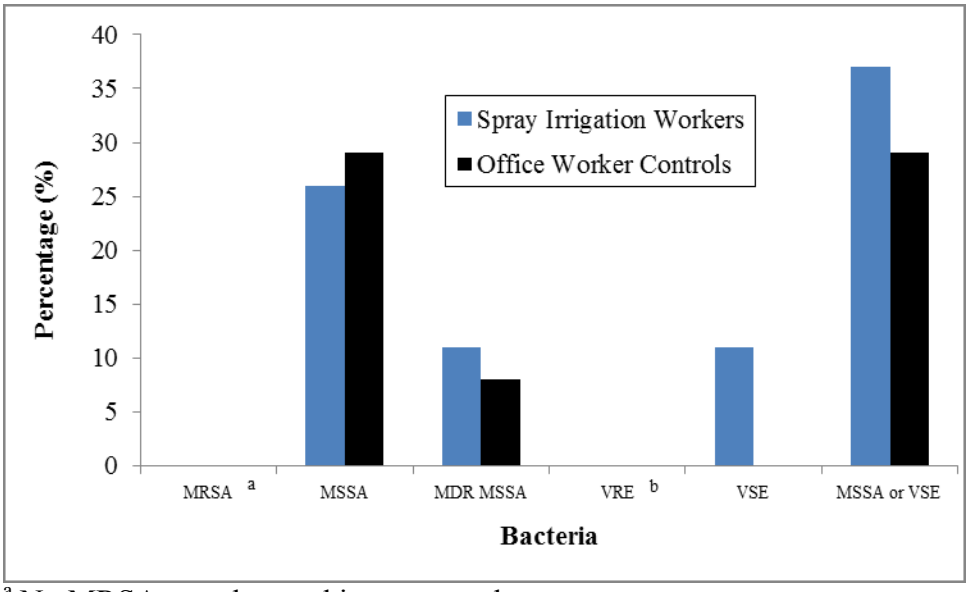
**Table 2**: Estimated odds ratios of ever being colonized with MSSA, MDR MSSA, or either MSSA or VSE, by occupational status.

| | Unadjusted OR | 95% CI | Adjusted OR[a] | 95% CI |
|---|---|---|---|---|
| MSSA | | | | |
| Spray irrigation worker | 0.87 | 0.23, 3.34 | 1.4 | 0.09, 22.4 |
| Office worker control | — | | | |
| MDR MSSA | | | | |
| Spray irrigation worker | 1.29 | 0.17, 10.15 | 7.01 | 0.13, 367.77 |
| Office worker control | — | | | |
| MSSA or VSE | | | | |
| Spray irrigation worker | 1.42 | 0.39, 5.11 | 2.55 | 0.15, 44.15 |
| Office worker control | — | | | |

[a] Confounders included in the adjusted model were: education, duration of job, yearly income, and current smoking status. Confounders were defined as a change in OR of ≥ 10%.

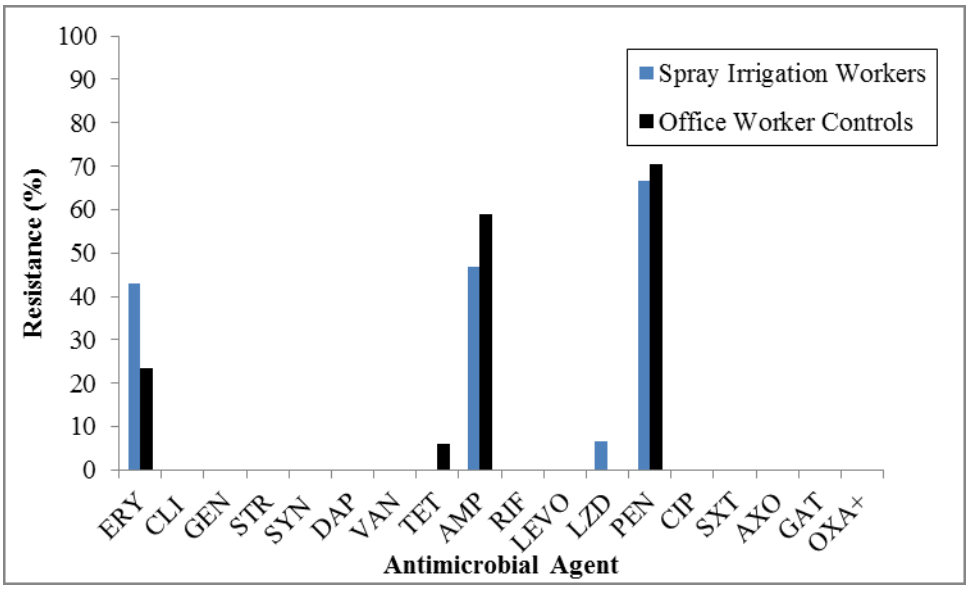
*Figures*

**Figure 1**: Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *S. aureus* (MSSA), multidrug-resistant (MDR) MSSA, vancomycin-resistant enterococci (VRE), vancomycin-susceptible enterococci (VSE), and MSSA or VSE among spray irrigation workers and office worker controls.




[a] No MRSA was detected in any samples.
[b] No VRE was detected in any samples.

**Figure 2**: Percent resistance to antimicrobial agents observed among MSSA isolates recovered from spray irrigation worker and office worker control nasal and dermal swabs. ERY= erythromycin; CLI=clindamycin; SYN=quinupristin/dalfopristin; DAP=daptomycin; VAN=vancomycin; TET=tetracycline; AMP=ampicillin; GEN=gentamicin; LEVO=levofloxacin; LZD=linezolid (LZD;0.5–8 µg/mL), AXO=ceftriaxone; STR=streptomycin; PEN=penicillin; RIF=rifampin; GAT=gatifloxacin; CIP=ciprofloxacin; SXT=trimethoprim/sulfamethoxazole; OXA+=oxacillin.



*Appendix A*

Antimicrobial Susceptibility Testing Detailed Methods

The following methods were used to perform antimicrobial susceptibility testing on all PCR-confirmed *S. aureus* (n=97) and *Enterococcus* spp. (n=20) isolates using the Sensititre® microbroth dilution system (Trek Diagnostic Systems Inc., Cleveland, OH) in accordance with the manufacturer's instructions. Overnight cultures were transferred to sterile demineralized water (Trek Diagnostic Systems) to achieve a 0.5 McFarland standard. Then, 30 µL of the suspension for *S. aureus* and 50 µL of the suspension for enterococci 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 Inc.) with the following antibiotics: erythromycin (ERY; 0.25–4µg/mL), clindamycin (CLI;0.12–2µg/mL), quinupristin/dalfopristin (SYN; 0.12–4 µg/mL), daptomycin (DAP;0.25–8 µg/mL), vancomycin (VAN; 1–128 µg/mL), tetracycline (TET; 2–16µg/mL), ampicillin (AMP; 0.12–16µg/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). Plates were incubated at 37°C and read after 18-24 hours.

# Chapter 6: Conclusions & Public Health Significance

The need for alternative water sources because of increased freshwater demand combined with increased water shortages has spurred the growth of reclaimed water applications in the United States. Although around 2.22 billion gallons of reclaimed water are being used in the U.S. per day, there are no federal regulations governing the treatment or use of reclaimed water (Miller 2011). EPA has recently updated their Guidelines for Water Reuse, but each state determines whether or not to adopt water reuse regulations or guidelines (EPA 2012).

There are a wide range of permitted uses for reclaimed water among different U.S. states (EPA 2012). State oversight of wastewater reuse has created a patchwork of rules that could have unintended consequences for the health of certain states' populations if they are exposed to reclaimed water that contains microbial or chemical contaminants not removed by wastewater treatment (EPA 2012; NRC 2012; WHO 2006). Although antibiotic-resistant bacterial infections are a growing public health problem, little research has been conducted into the presence of, and exposure to, antibiotic-resistant bacteria in reclaimed water. According to EPA's 2012 Guidelines, "Where reclaimed water is intended for nonpotable reuse, the major priority in design, construction, and operation of a reclaimed water distribution system is the prevention of cross-connections [,]" not water quality (EPA 2012). Although I agree that it is important to protect water intended for consumption from

reclaimed water contamination, human exposure to nonpotable reclaimed water in planned applications could also have health consequences from inhalation or dermal exposure, or even inadvertent ingestion from hand-to-mouth behavior.

It is not logistically or economically feasible to routinely monitor wastewater or reclaimed water for all possible contaminants that could impact human health. However, the public health and water reuse communities have a responsibility to fully explore contaminants known to be contained in human waste and wastewater that could cause serious human health consequences. The 2012 EPA Guidelines for Water Reuse suggest total or fecal coliforms, both indicator organisms, as the only microorganisms to monitor for reuse applications (EPA 2012). However, numerous studies have identified a weak correlation between any single indicator organism and most human pathogens (Harwood et al. 2005; Jjemba et al. 2010).

My dissertation research focused on examining the public health risks of antibiotic- resistant bacteria surviving wastewater treatment and causing exposure during water reuse applications. The first two articles of this dissertation explored whether or not two of the leading causes of antibiotic-resistant bacterial infections, MRSA and VRE, could survive the wastewater treatment process. MRSA and VRE were detected at all WWTPs sampled as part of this dissertation research. The percentage of MRSA-positive samples and concentration of VRE decreased as treatment progressed at all WWTPs. Neither MRSA nor VRE were ever identified in tertiary-treated (chlorinated) samples, but both were identified in an effluent sample at a Midwest WWTP during a time when chlorination was not taking place

(secondary-treated effluent). Secondary treatment is the highest level of wastewater treatment required in the U.S. In addition to identifying the mere presence of MRSA and VRE in wastewater, the majority of both MRSA and VRE isolates (93% and 100%, respectively) were multidrug resistant (MDR), resistant to two or more classes of clinically relevant antibiotics tested. These results could have major public health implications for WWTP workers in the U.S. as well as those at water reuse sites if these organisms, and other human pathogens, survive the distribution process and any further treatment at reuse sites. MDR bacteria limit treatment options for those infected with this type of bacteria. This was the first report of MRSA at U.S. WWTPs and the first report of VRE at WWTPs that discharge effluent intended for reuse and at Mid-Atlantic WWTPs.

Although I did detect MRSA and VRE in at least one sample from each WWTP sampled for this dissertation research, I could have underestimated the true prevalence of these organisms by relying on culture-based detection techniques as only 1% of bacteria are culturable. To provide a more accurate estimation of MRSA and VRE in U.S. wastewater, it would be important for future studies to pair culture-based techniques with molecular techniques to detect organism- and antibiotic-resistance-specific genes. I could have also used metagenomics to explore the bacterial diversity of wastewater samples in addition to evaluating the presence of specific microorganisms of interest.

While the first portion of this dissertation explored the presence of MRSA and VRE in wastewater and antibiotic resistance patterns among isolates, the second portion of this research evaluated potential human exposure to MRSA and VRE in reclaimed water. Although irrigation with reclaimed water is growing, limited data exists on pathogens present in reclaimed water, as well as health risks from exposure to this water source (Blumenthal et al. 2001; Devaux et al. 2001; Durand and Schwebach 1989; Harwood et al. 2005; Jjemba et al. 2010). Several previous studies have analyzed the risk of different types of infections among individuals occupationally, recreationally, or residentially exposed to wastewater that has undergone various levels of treatment and found conflicting results (Blumenthal et al. 2001; Devaux et al. 2001; Shuval 1991). In two separate studies, one in Mexico and one in the U.S., the risk of parasitic and viral infections were found to be greater among individuals exposed to secondary treated wastewater than controls (Blumenthal et al. 2001; Camann et al. 1988). When considering reported symptoms, a study of those recreationally exposed to turf irrigated with reclaimed water in Colorado found no difference in the number of reported illnesses between the exposed and unexposed groups (Durand and Schwebach 1989). However, this study did show that exposure to wet grass was associated with more reported GI symptoms (Durand and Schwebach 1989). Because reclaimed water irrigation workers routinely work in wet grass as they are spraying, they could be at a greater risk for experiencing GI symptoms. Similarly, a study by Devaux et al. (2001) in France identified that farmers exposed to wastewater treated in stabilization ponds reported more respiratory and GI symptoms compared to a non-exposed group of controls (Devaux

et al. 2001). However, no published studies have ever evaluated the risk for occupational exposure to antibiotic-resistant bacteria from reclaimed water.

To address this lack of exposure data, in the third article included in this dissertation, I collected nasal and dermal swabs from 19 reclaimed water spray irrigation workers and 24 office worker controls and analyzed them for MRSA, MSSA, VRE, and VSE and compared the odds of colonization with these four bacteria between the spray irrigators and controls. MRSA and VRE were not detected in any nasal or dermal swab samples from reclaimed water spray irrigation workers or controls. MSSA was detected in 26% and 29% of spray irrigation workers and controls, respectively. VSE was detected in 11% and 0% of spray irrigation workers and controls, respectively. The odds of MSSA, MDR MSSA, and either MSSA or VSE colonization were not significantly different between spray irrigators and controls. Although our findings were not statistically significant, which could be a factor of our limited study population size, our findings are still important because they begin to fill in a knowledge gap about public health implications from occupational exposure to reclaimed water. Because individuals are also recreationally and residentially exposed to reclaimed water, future research should also evaluate MRSA, MSSA, VRE, and VSE colonization among individuals living in proximity to reuse sites with varying levels of treated wastewater.

Also, more work is needed to evaluate potential exposures to other human pathogens among those exposed to reclaimed water including *Legionella* spp. and

*Aeromonas* spp. since these microorganisms have previously been isolated from reclaimed water (Brissaud et al. 2008; Jjemba et al. 2010; Palmer et al. 1995). As with the wastewater samples, to be able to better capture bacterial diversity in the nasal and dermal microbiomes of individuals occupationally exposed to reclaimed water, metagenomics could be included in future studies.

In summary, this dissertation research provides additional scientific evidence that antibiotic-resistant bacteria can survive secondary wastewater treatment and might cause an increased risk of infection among individuals exposed to reclaimed water. These findings can be used to support environmental and public health policy efforts to safely use reclaimed water in the United States. Ideally these findings would also help bolster efforts to curb the over-prescription and misuse of antimicrobials among humans and animals, in agriculture, and in personal care products. However because of the widescale and multi-industry nature of this issue, it is important to also consider alternative, more immediate, ways to reduce human exposure to pathogens that may remain in wastewater and reclaimed water through means such as health education, engineering controls, and the use of personal protective equipment.

Health education and specifically hygiene promotion have been suggested as important paths to explore to protect the health of those occupationally exposed to reclaimed water (Blumenthal et al. 2000). In the past decade there have been significant increases in theory-based research on the handwashing behavior of

healthcare workers. Previous studies have found that factors such as perceived ability and attitude about health behavior are more reactive to program design and promotion than demographic variables which have been the focus of traditional education campaigns (Apodaca et al. 1997). Whitby and McLaws (2007) emphasized the behavioral components of handwashing and proposed that the impact of the physical environment and perceptions of the environment are less important than traditionally thought (Whitby and McLaws 2007). In a study by Vernon et al. (2003), it was also concluded that the sink-to-bed ratio was not significantly associated with observed adherence to handwashing among healthcare workers (Vernon et al. 2003). Vernon hypothesized that other determinants, namely lack of time and physical damage to skin, are better predictors of healthcare workers' actual hand washing behavior (Vernon, 2003). Vernon asserted that the perception of large amounts of time required to wash hands, a behavioral belief, could be reduced with the introduction of alcohol-based hand sanitizers.

However, based on Whitby and McLaw's (2007) research, unless an individual perceives contact as presenting a risk to him or herself, s/he will not feel that it is important to wash his/her hands at all, whether it is time efficient or not (Whitby and McLaws 2007). Therefore a combination of educational and practical interventions might be needed, such as emphasizing the personal risk for infection as well as making handwashing and personal protective equipment available. A VRE outbreak was controlled in a neonatal intensive care unit in Washington, DC through

the use of interventions that included both the use of waterless disinfectants for handwashing and mandatory in-service education (Sherer 2005).

In addition to education, the use of engineering controls could reduce exposure to contaminants in reclaimed water. In Australia, some states have a four hour "withholding period" between reclaimed water application and use to reduce exposure to contaminants potentially contained in the water (O'Toole et al. 2008). A study by O'Toole et al. (2008) found that levels of *E. coli* were significantly reduced after the four hour period and suggested that risk of enteric bacterial infections could be reduced with withholding periods (O'Toole et al. 2008). However, for reclaimed water spray irrigators, this type of withholding period might not be feasible.

Wearing personal protective equipment (PPE) could be another possible control to reduce occupational exposure to pathogens in reclaimed water among spray irrigation workers. Blumenthal et al. (2000) suggested that the use of protective equipment including footwear for farmers and gloves for crop handlers working with reclaimed water could effectively reduce exposures to contaminants contained in the water (Blumenthal et al. 2000). In a study by Weldon et al. (2000) among WWTP workers, never wearing face protection was significantly associated with hepatitis A colonization (Weldon et al. 2000). Similarly, in a study of hog producers, a smaller percentage of workers who wore N95 masks during work activities (40%) were colonized with human pathogens or positive for tetracycline-resistance genes compared to workers who did not wear the masks (56.8%) (Létourneau et al. 2010).

Leedom Larson et al. (2012) explored a multipronged approach for interventions to reduce MRSA colonization among pork producers; their suggestions are outlined in Table 1 below (Leedom Larson et al. 2012). The authors found that seven pork production shower facilities that had previously tested positive for MRSA had no detectable MRSA after implementation of the interventions listed in Table 1 (Leedom Larson et al. 2012). However, they also found that four previously MRSA-free shower facilities were positive for MRSA after the intervention (Leedom Larson et al. 2012). They suggested that the interventions used in their study could have been improved with more clearly worded instructions (Leedom Larson et al. 2012). Using the interventions suggested by Leedom Larson et al. (2012) as a basis for interventions with reclaimed water spray irrigation workers, with an emphasis on health literacy issues, could be an important next step in efforts to protect this group of workers.

**Table 1**. Adapted methods to prevent MRSA infection to pork producers. Source: Leedom Larson et al., 2012.

| Category | Methods |
|---|---|
| Hygiene | Educate employees about good hand hygiene.<br>Encourage use of alcohol-based hand sanitizers when hands not visibly dirty.<br>Educate employees about general infection control.<br>Educate employees about MRSA transmission. |
| Wounds | Report cuts or wounds to supervisor immediately.<br>Clean wounds or abrasions immediately and cover with a clean, dry bandage.<br>Restrict worker to a single shower stall and disinfect after each use if wound cannot be covered.<br>Encourage proper treatment of non-healing wounds by a healthcare professional. |
| Showers | Advise workers to use warm water and soap.<br>Use liquid soap dispensers instead of bar soap.<br>Provide separate, clean towels for each employee.<br>Discourage sharing of personal items including soap and razors. |
| Clothing and laundry | Provide separate boots for each worker.<br>Provide separate, clean coveralls for each worker.<br>Wash soiled coveralls separately from other clothing and towels.<br>Wash all coveralls, clothing (including underwear and socks), and towels with hot water and soap after each use.<br>Machine dry every article of clothing and towel after washing. |
| Environment | Develop a protocol for cleaning and disinfection of showers.<br>Include removal of visible dirt, proper dilution of disinfectant, and contact time.<br>Use dilute bleach (1/4 cup bleach in 1 gal water) or EPA-approved disinfectant.<br>Develop a routine schedule for cleaning and disinfection of showers.<br>Provide a separate kitchen space for eating. |

Reclaimed water might very well be a safe alternative water source, but it is important to evaluate the human health risks associated with its use to be able to identify areas for improvement in terms of treatment and application, and to inform related policy. Also, although alternative sources of freshwater are becoming a necessity because of current demand and dwindling supplies, the focus should not exclusively be on harvesting new sources of water, but instead include encouraging water efficiency and conservation of available water resources. Until we better understand the types of pathogens that remain in treated wastewater and the health risks from exposure to reclaimed water, we cannot improve its use. It is my hope that the results of this dissertation research will be able to encourage additional research and help inform decision makers about possible human health risks when crafting policies related to reclaimed water.

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# Rachel Elizabeth Rosenberg Goldstein, MPH

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## EDUCATION

**University of Maryland, College Park**
Doctoral Program in Toxicology, Expected Graduation May 2013
GPA 4.0/4.0

- Member and past-president, Gamma Zeta Chapter of Delta Omega, Honorary Society in Public Health
- Relevant Coursework Included: Categorical Data Analysis, Intermediate Epidemiology, Infectious Disease Epidemiology, Water Resources, Diseases in the Chesapeake Bay

**University of Maryland, College Park**
Masters of Public Health, Environmental Health Sciences, May 2010
GPA 4.0/4.0

- Relevant Coursework Included: Nonpoint Source Pollution Assessment Techniques, Biological Contaminants, Epidemiology, Health Behaviors, Biostatistics, Toxicology, and Risk Assessment

**University of North Carolina at Chapel Hill**
Environmental Studies, Bachelor of Arts, May 2005
GPA 3.8/4.0

- Member, Phi Beta Kappa, National Scholars Honor Society
- Relevant Coursework Included: Environmental Science, Physics I & II, Estuarine Processes, Watershed Systems, Environmental Communication, Introduction to GIS

## PROFESSIONAL EXPERIENCE

**Maryland Institute for Applied Environmental Health -** University of Maryland, College Park, MD

*Graduate Assistant* (September 2008 – Present)

- Conducting environmental microbiology laboratory work for research projects on the prevalence and pathway of *Salmonella* on tomato farms, irrigation workers' exposures to antimicrobial-resistant bacteria present in reclaimed wastewater, and microbiological and chemical contaminants in private well water.

**Eastern Research Group, Inc.**, Arlington, VA

*Environmental Communications Specialist* (Fall 2005 – May 2008)

- Provided support to U.S. Environmental Protection Agency voluntary partnership programs.
- Developed outreach materials and managed communications with program partners.

**NSF REU Interdisciplinary Watershed Program***,* College of William & Mary, Williamsburg, VA

*Intern* (Summer 2003)

- Researched the ecological benefits of intermittent streams and the effect of comprehensive plans on watersheds; produced paper titled "Growth Management Plans in Virginia and the People Who Shape Them".

## INVITED LECTURES

**University of Maryland, College Park**
*Biological Contaminants* (November 2012)
- Lectured about wastewater treatment, contaminants, and related research projects.

**University of Maryland, College Park**
*Honors Seminar: Public Health Perspectives: How Does the Environment affect Human Health* (March 2012)
- Lectured about possible bacterial contaminants in reclaimed municipal wastewater.

**University of Maryland, College Park**
*Foundations of Environmental Health* (February 2011)
- Lectured about basic hydrological concepts, water quality, and water supply issues.

**University of North Carolina at Chapel Hill**
*Water Resource Management and Human Rights* (February 2010)
- Lectured about biological and chemical contaminants in U.S. surface waters.

**University of Maryland, College Park**
*Honors Seminar: Public Health Perspectives: How Does the Environment affect Human Health* (February 2010)
- Lectured about possible exposure to antibiotic-resistant bacteria in reclaimed municipal wastewater.

## PRESENTATIONS

**Maryland Water Monitoring Council 18th Annual Conference** (December 2012)
*Linthicum Heights, MD*
- Poster presentation, "Analysis of Private Well Water Quality and Well Owner Education Program in Maryland: A Pilot Project."

**Maryland Groundwater Symposium** (September 2012)
*Baltimore, MD*
- Oral presentation, "Analysis of Private Well Water Quality and Well Owner Education Program in Maryland: A Pilot Project."

**Priester National Extension Health Conference** (April 2012)
*Washington, DC*
- Oral presentation, "Well Water Education Program: A Collaborative Project Between Extension Educators and University of Maryland Public Health Researchers."

**Water and Health: Where Science Meets Policy 2011 Conference** (October 2011)
- Oral presentation, "Assessing an alternative water source: Evaluation of methicillin-resistant *Staphylococcus aureus* in secondary treated wastewater used for spray irrigation in Nebraska"

**American Society for Microbiology 111th General Meeting** (May 2011)
- Poster presentation "Survival of Methicillin-resistant *Staphylococcus aureus* in Secondary Treated Wastewater."

**Water and Health: Where Science Meets Policy 2010 Conference** (October 2010)
*University of North Carolina at Chapel Hill*

• Oral presentation, "Evaluating occupational exposures to antibiotic-resistant bacteria from wastewater reuse."

**American Society for Microbiology 110th General Meeting** (May 2010)

• Poster presentation "Reductions of Methicillin-resistant *Staphylococcus aureus* and Vancomycin-resistant *Enterococcus* spp. at a U.S. Tertiary Wastewater Treatment Plant."

**American Public Health Association 137th Annual Meeting & Expo** (November 2009)

• Poster presentation "Irrigation workers' exposures to antimicrobial-resistant bacteria and antimicrobials present in reclaimed wastewater."

## PUBLICATIONS

Shirley A. Micallef, **Rachel E. Rosenberg Goldstein**, Ashish George, Laura Ewing, Ben D. Tall, Marc S. Boyer, Sam W. Joseph, Amy R. Sapkota. 2013. *Diversity, Distribution and Antibiotic Resistance of* Enterococcus *spp. Recovered from Tomatoes, Leaves, Water and Soil on Mid-Atlantic Farms*. Food Microbiol. [SUBMITTED].

Barbara Z. Pasturel, Raul Cruz-Cano, **Rachel E. Rosenberg Goldstein**, Amanda Palmer, David Blythe, Pat Ryan, Brenna Hogan, Carrianne Jung, Sam W. Joseph, Min Qi Wang, Mei-Ling Ting Lee, Robin Puett, and Amy R. Sapkota. 2013. *Rurality, Presence of Broiler Operations, and Community Socioeconomic Factors Influence the Risk of Campylobacteriosis in Maryland*. AJPH. [ACCEPTED]

**Rachel E Rosenberg Goldstein**, Shirley A Micallef, Shawn G. Gibbs, Johnnie A. Davis, Ashish George, Lara M. Kleinfelter, Nicole A. Schreiber, Sampa Mukherjee, Amir Sapkota, Sam W. Joseph, and Amy R. Sapkota. 2012. *Methicillin-Resistant* Staphylococcus aureus *Detected At Four U.S. Wastewater Treatment Plants*. Environ Health Perspect. 2012 Nov; 120(11): 1551-1558.

Jean-Gilles Beaubrun J, Cheng C, Chen K, Ewing L, Wang H, Agpaoa MC, Huang MJ, Dickey E, Du JM, Williams-Hill DM, Hamilton B, Micallef SA, **Rosenberg Goldstein RE**, George A, Joseph SW, Sapkota AR, Jacobson AP, Tall BD, Kothary MH, Dudley K and Hanes DE. 2012. *The evaluation of a PCR-based method for identification of* Salmonella enterica *serotypes from environmental samples and various food matrices*. Food Microbiol. 2012 Sep;31(2):199-209.

Shirley A Micallef, **Rachel E Rosenberg Goldstein**, Ashish George, Lara Kleinfelter, Marc S Boyer, Cristina R McLaughlin, Andrew Estrin, Laura Ewing, Junia Jean-Gilles Beaubrun, Darcy E Hanes, Mahendra H Kothary, Ben D Tall, Jafar H Razeq, Sam W Joseph, Amy R. Sapkota. *Occurrence and Antibiotic Resistance of Multiple* Salmonella *Serotypes Recovered from Water, Sediment and Soil on Mid-Atlantic Tomato Farms*. Environ Res. 2012 Apr;114:31-9. Epub 2012 Mar 8.

Amy R. Sapkota, Morenike E. Coker, **Rachel E. Rosenberg Goldstein**, Nancy L. Atkinson, Shauna J. Sweet, Priscilla O. Sopeju, Modupe T. Ojo, Elizabeth Otivhia, Olayemi O. Ayepola, Olufunmiso O. Olajuyigbe, Laura Shireman, Paul S. Pottinger, Kayode K. Ojo. *Self-medication with antibiotics for the treatment of menstrual symptoms in southwest Nigeria: a cross-sectional study*. BMC Public Health 2010, 10:610.

## HONORS

**University of Maryland Bioscience & Technology Review Day Family, Community and Public Health Poster Winner** (November 2012)

- Poster presentation "Analysis of Private Well Water Quality and Well Owner Education Program in Maryland: A Pilot Project."

**"Engaged Me" Award, Priester National Extension Health Conference** (April 2012)

- Oral presentation, "Well Water Education Program: A Collaborative Project Between Extension Educators and University of Maryland Public Health Researchers."

**Student Scholarship, Priester National Extension Health Conference** (April 2012)

**Maryland Institute for Applied Environmental Health Fellowship** (November 2011 – Present)

**University of Maryland Bioscience & Technology Review Day Water Quality and Management Poster Winner** (November 2011)

- Poster presentation "Survival of Methicillin-resistant *Staphylococcus aureus* in Secondary Treated Wastewater."

**APHA Environment Section Student Scholarship** (October 2011)

**University of Maryland, College Park School of Public Health, Student Research Interaction Day Poster Award** (September 2011)

**University of Maryland Graduate Research Interaction Day Health Presentation Award (2nd place)** (April 2011)

- Oral presentation, **"**Survival of Methicillin-resistant Staphylococcus aureus in Secondary Treated Wastewater."

**University of Maryland Graduate Student Summer Research Fellowship** (Fall 2010 – Present)

**Dean's Fellowship** (Fall 2010 – Present)

**Maryland Water Resources Research Center 2010 Summer Fellowship** (May-August, 2010)

**Dean's Graduate Scholar** (March 2010)

**University of Maryland Bioscience & Technology Review Day Family, Community and Public Health Poster Award** (November 2009)

- Poster titled "Irrigation workers' exposures to antimicrobial-resistant bacteria and antimicrobials present in reclaimed wastewater."

**American Public Health Association Environment Section's Student Achievement Poster Award** (November 2009)

- Poster titled "Irrigation workers' exposures to antimicrobial-resistant bacteria and antimicrobials present in reclaimed wastewater."

**Jacob K. Goldhaber Travel Grant** (August 2009)

## SERVICE

**"Climbing Up and Reaching Back (CURB)": Ladder of Support for Research Careers in Biomedical and Behavioral Research**, College Park, MD
*Graduate Student Presenter* (December 2011 – April 2012)

- Prepared and presented lecture and interactive experience related to environmental health for high school students.

**Delta Omega, Gamma Zeta Chapter**, College Park, MD
*Inaugural President,* (November 2011 – September 2012)

- Coordinated meetings, service activities, nomination process, and induction ceremony.

**Public Health Garden**, College Park, MD
*Co-founder and Secretary Treasurer* (January 2011 – June 2012)

- Developed constitution for club and collaborated with stakeholders on campus to establish an edible garden.

**Dean's Student Advisory Committee**, University of Maryland School of Public Health
*Student Representative for Maryland Institute for Applied Environmental Health* (October 2009-May 2012)

- Proposed Public Health Garden idea.
- Provided feedback to Dean of School of Public Health on School and University issues.

**Accreditation Committee,** University of Maryland School of Public Health
*Student Representative for Maryland Institute for Applied Environmental Health* (August – October 2009)

- Represented the Institute and the School during the site visit for the accreditation of the School of Public Health by the Council on Education for Public Health (CEPH).

**United Nations Commission on Sustainable Development (CSD-16),** New York, NY
*Youth Delegate,* (May 2008)

- Drafted policy documents, participated in meetings, and presented action items on drought, desertification, and water sanitation.

## PROFESSIONAL MEMBERSHIPS

**American Public Health Association**
**American Society for Microbiology**
**American Water Resources Association**
**American Water Works Association**
**Delta Omega**