ABSTRACT

Title of Document: EVALUATION OF ANTIBIOTIC-RESISTANT BACTERIA IN TERTIARY TREATED WASTEWATER, RECLAIMED WASTEWATER USED FOR SPRAY IRRIGATION, AND RESULTING OCCUPATIONAL EXPOSURES.

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Occupational exposures to vancomycin-resistant *Enterococcus* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) in reclaimed wastewater used for spray irrigation were evaluated. In 2009, wastewater was collected from a tertiary-treatment facility, and reclaimed wastewater, nasal and dermal swab samples from an irrigation site. Samples were evaluated for MRSA and VRE using standard methods, PCR, and susceptibility testing. MRSA and VRE were isolated from all wastewater samples except effluent. While wastewater MRSA isolates were multidrug resistant (98%), no MRSA was isolated in irrigation water or swabs. VRE was isolated in one irrigation water sample. Fewer irrigation workers were colonized with *S. aureus* (31%) compared to controls (46%), but they harbored more multidrug resistant *S. aureus*. This is the first study to 1) evaluate antibiotic-resistant bacteria (ARB) in U.S. reclaimed

wastewater and resulting occupational exposures, and 2) detect MRSA in U.S. wastewater. The findings suggested that tertiary wastewater treatment effectively reduced MRSA and VRE.

EVALUATION OF ANTIBIOTIC-RESISTANT BACTERIA IN TERTIARY TREATED WASTEWATER, RECLAIMED WASTEWATER USED FOR SPRAY IRRIGATION, AND RESULTING OCCUPATIONAL EXPOSURES.

By

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Dedication

For my mother, and my friend, Ellen Witte Rosenberg, who told me that I have the potential to do and be whatever I want.

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Chapter 1: Introduction

Water Use

Water is an essential component of the human body, a necessity for good health and agriculture, and if contaminated, a carrier of harmful xenobiotics. At the beginning of the twenty-first century, 70 percent of all water used from surface and ground water around the world was being used for agriculture (FAO, 2003; Kocaman, Yaganoglu, & Angin, 2007). The Food and Agriculture Organization of the United Nations anticipates that agricultural water use will increase by another 14 percent between 2000 and 2030 (FAO, 2003; Kocaman, et al., 2007). Consumption of water for drinking, industrial, and household uses has increased as well, globally and in the United States (Angin, Yaganoglu, & Turan, 2005; Dobbie, Henderson, & Stevens Jr., 2008; EPA, 2008b; Kocaman, et al., 2007; Levine & Asano, 2004; Rodriguez, et al., 2009; UN, 2003).

In the United States in 2005, approximately 410 billion gallons of water were withdrawn per day for use (Barber, 2009). Demand for public and domestic water uses has increased steadily since 1950 in the U.S. (Barber, 2009). Although national demand for water is increasing, the water supply is not, and it is in fact decreasing. According to the Government Accountability Office, 36 states are expected to experience water shortages by 2013, even in "normal, non-drought conditions" (GAO, 2003). As water use increases, and droughts and water shortages become more common, agricultural and landscaping applications across the United States increasingly use reclaimed wastewater for irrigation and source water for drinking water treatment plants (Levine & Asano,

2004). One of the first documented uses of reclaimed wastewater in the United States was Golden Gate Park in San Francisco, California in 1912 when reclaimed wastewater was used for lawn irrigation and to replenish ornamental lakes (Asano, 2001). Since this use, the number and type of applications of reclaimed wastewater in the United States have continued to grow.

Although there are many potential benefits from using reclaimed wastewater, including reduced cost and freshwater conservation, the potential public health implications of its use have not been fully explored. Previous studies have analyzed specific microbial and chemical contaminants in wastewater and treated wastewater, but no studies to date have quantified the prevalence of antibiotic-resistant bacteria in reclaimed wastewater used for spray irrigation in the United States or the public health implications of occupational exposure to this media (Borjesson, Melin, Matussek, & Lindgren; Börjesson, Melin, Matussek, & Lindgren, 2009; Caplin, Hanlon, & Taylor, 2008; de Zutter & van Hoof, 1984; Harwood, Brownell, Perusek, & Whitlock, 2001; Iwane, Urase, & Yamamoto, 2001; Miguel Ferreira da Silva, et al., 2006; Mispagel & Gray, 2005; Poole, et al., 2005; Rose, 2007; Schwartz, Kohnen, Jansen, & Obst, 2003; Volkmann, Schwartz, Bischoff, Kirchen, & Obst, 2004). This thesis seeks to contribute to the current limited body of knowledge on possible health risks, or lack thereof, from exposure to reclaimed wastewater. To be as cautious as possible, before continuing to expose the public to new sources of irrigation and drinking water, it is most prudent to assess and better understand possible health risks that could result from exposure to this medium.

Microbial Contaminants in Wastewater

Bacterial pathogens occur in large quantities in wastewater because they are excreted in the feces of colonized individuals. In addition to large concentrations of bacterial pathogens as a whole, wastewater also contains antibiotic-resistant bacteria (Garcia, et al., 2007; Nagulapally, et al., 2009). 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, Rhinehart, Jackson, Chiarello, & Committee, 2006; Zhang, Marrs, Simon, & Xi, 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).

Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. (VRE) are two of the leading causes of antibiotic-resistant bacterial infections in hospital settings, and rising causes of community-acquired infections (Siegel, et al., 2006; Sievert, et al., 2008; System, 2004). Because of their increasing occurrence in human infections and high rates of related mortality, MRSA and VRE are the focus of the Master's thesis research presented here.

To my knowledge, only one study has previously identified MRSA in wastewater in Europe and no studies have identified MRSA from wastewater or reclaimed wastewater in the United States (Börjesson, et al., 2009). VRE has been identified in wastewater in the United States in a limited number of studies, but has never been examined in treated wastewater used in downstream applications (Beier, et al., 2008; Garcia, et al., 2007; Harwood, et al., 2001; Nagulapally, et al., 2009; Poole, et al., 2005).

Public Health Risks

If bacterial pathogens and antibiotic-resistant bacteria survive the wastewater treatment process, there is the possibility for occupational exposure to these agents among wastewater treatment plant workers and employees engaged in the use of reclaimed wastewater (Nagulapally, et al., 2009). Spray irrigators using reclaimed wastewater have higher levels of exposure to reclaimed wastewater than the general public and could potentially be exposed to contaminants contained in the water, including antibiotic-resistant bacteria. The public health consequences of exposure to reclaimed wastewater, and specifically occupational exposure to reclaimed wastewater, have not been fully evaluated.

More research on the possible health risks of exposure to reclaimed wastewater is needed. In a world with increasing droughts and demands on water resources, even with improvements in water efficiency and conservation, we will most likely begin to rely more heavily on alternative water sources. To protect human health, additional research is needed on microbial contaminants that might remain in treated wastewater.

Research Rationale

The overall goal of the research conducted in the current Master's thesis project was to generate baseline information about the presence of antibiotic-resistant bacteria in wastewater and reclaimed wastewater and the accompanying public health risks from occupational exposure to treated wastewater. My Master's thesis evaluated potential occupational exposures to antibiotic-resistant bacteria in reclaimed wastewater among spray irrigation workers. My central hypothesis was that untreated municipal wastewater, and treated municipal wastewater used in spray irrigation activities would contain pathogenic, and antibiotic-resistant, bacteria. As a consequence, spray irrigation workers using reclaimed wastewater for irrigation would be exposed to these agents through both inhalation and dermal routes of exposure.

The specific aims of the study were as follows:

1. To determine the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. (VRE) at a tertiary municipal wastewater treatment plant.

2. To evaluate air and reclaimed wastewater samples collected during reclaimed wastewater irrigation activities for the presence of MRSA and VRE.

3. To determine exposure levels and exposure routes of MRSA and VRE among spray irrigation workers from nasal and dermal swab samples.

The findings contained in this Master's thesis will serve as the foundation for future epidemiological studies to comprehensively evaluate the public health risks associated with reuse of treated municipal wastewater.

Chapter 2: Background

Introduction

As the use of reclaimed wastewater continues and expands in applications in which occupational and general public exposures could occur, it would be helpful to have a holistic picture of human health risks from exposures to this alternative source of water. In particular, this Master's thesis focuses on possible occupational exposures to methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. (VRE) from reclaimed wastewater used in spray irrigation. This chapter provides background on MRSA, VRE, wastewater treatment in the United States, bacteria in wastewater, and reclaimed wastewater use in the United States.

Staphylococcus aureus

Genus Description

Staphylococci are one of the most common bacterial pathogens that cause disease in humans (Metzger, Bonotti, & Sawyer, 2009). Out of 32 species and 15 subspecies of *Staphylococcus*, *S. aureus* is one of the species most commonly associated with human disease (Murray, Rosenthal, Kobayashi, & Pfaller, 2002).

Species Description

S. aureus are round, often cluster in chain or grape-shaped formation, and are usually golden colored because of carotenoid pigments produced during the growth phase (Murray, et al., 2002). The name "aureus", the Latin word for golden, refers to the golden color of the organisms. These organisms are aerobic or facultatively anaerobic, which means that they are able to grow in both aerobic and anaerobic conditions (Murray, et al., 2002). *S. aureus* can also grow in environments with salt concentrations as high as 10% (Friis, 2007). The optimal temperature range for *S. aureus* is between 18 and 40°C, which spans the average internal temperature of the human body.

S. aureus are generally larger than 5 μm in aerodynamic diameter and thus more likely to settle quickly (Andersen, 1958; Bassetti, et al., 2005; Jensen, Todd, Davis, & Scarpino, 1992). This partially explains why *S. aureus* are often found in the nares of humans and animals, and not deeper in the respiratory system.

Several of *S. aureus*' physical properties help it to survive in a range of environments. *S. aureus* is a gram-positive bacterium, which means that it has a peptidogylycan layer that helps it exist in harsh environments. The peptidoglycan layer is a thick, rigid component of the cell wall and can stimulate immune responses in the body (Murray, et al., 2002).

S. aureus is catalase-positive, which means it can catalyze the breakdown of hydrogen peroxide into water and oxygen. Hydrogen peroxide can accumulate during bacterial metabolism and is toxic to the cell (Murray, et al., 2002). A polysaccharide, or slime, layer is thought to occur frequently in *S. aureus* that protects it from injury and death, as well as enabling adherence to synthetic surfaces. Researchers have identified 11

serotypes of *S. aureus* that have a polysaccaride capsule (Murray, et al., 2002). This physical characteristic could be important for the survival of *S. aureus* in the environment and the human body. *S. aureus* are also resistant to drying, are carried by airborne particles, and are frequent colonizers of the nasal mucosa (Lidwell, Brock, Shooter, Cooke, & Thomas, 1975).

In addition to possessing characteristics that help it survive in the environment, *S. aureus* can also 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, which produce antibodies. Protein A inhibits the production of antibodies and therefore the clearance of *S. aureus* from the body (Murray, et al., 2002). Once inside the body, *S. aureus* is able to survive partially due to 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 (Murray, et al., 2002). Some strains of *S. aureus* also produce a highly heat-stable protein toxin, or enterotoxin (FDA, 2009).

Ecological Habitat and Distribution

Staphylococci are found throughout the environment in humans and animals, air, dust, water, milk, and food, although humans and animals are the primary reservoirs (FDA, 2009). This bacterium can be found on the skin and mucous membranes of a large percentage of healthy humans and animals (Center for Disease Control and Prevention (CDC), 2006).

Humans

It has been estimated that *S. aureus* is present in the nose, throats, 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 (Murray, et al., 2002). Colonization with *S. aureus* can be either acute or chronic (Chambers, 2001; Sanford, Widmer, Bale, Jones, & Wenzel, 1994). Colonization with *S. aureus* is not homogeneous across subpopulations, but rather differs by gender, ethnicity, age, and other demographic factors. Based on National Health and Nutrition Examination Survey (NHANES) data, males, compared with females ($P < .001$), non-Hispanic whites and Mexican Americans, compared with non-Hispanic blacks ($P < .001$ and $P < .01$, respectively), obese individuals ($P < .01$, men; $P < .05$, women), and individuals less than 20 years old, compared with older persons ($P < .001$) were more likely to be colonized with *S. aureus* (Gorwitz, et al., 2008). One reason why children have higher colonization rates than adults, could be because of their frequent contact with respiratory secretions (Chambers, 2001; Ross, Rodroguez, Controni, & Khan, 1974). In neonates, *S. aureus* is often found in the oropharynx, umbilical stump, skin, and perineal area (Adcock, Pastor, Medley, Patterson, & Murphy, 1998). In adults, *S. aureus* is often found in moist skin folds, in the nasopharynx, gastrointestinal, and urogenital tract (Murray, et al., 2002). Although colonization is widespread among humans, the vast majority of individuals are asymptomatic rather than presenting for infection (Chambers, 2001).

An important subcategory of human sources of *S. aureus* is hospitals. Close inter-individual contact, contaminated surfaces, and bodily fluids, make the hospital an ideal environment for this organism (Lidwell, et al., 1975).

Animals

S. aureus can also be found in the nares, skin, genital, and gastrointestinal tracts of some warm-blooded animals (Rusin, Maxwell, Brooks, Gerba, & Pepper, 2003). Studies on the prevalence of *S. aureus* in animals are limited, but several studies have pointed to transmission of specific strains of *S. aureus* between animals and humans. A study conducted in Denmark found that 13 of 21 *S. aureus* infections in farm workers were among individuals who had been exposed to pigs with a similar strain of *S. aureus* (H. C. Lewis, et al., 2008). Additional studies conducted in France, the Netherlands, Canada, and the United States have also found evidence of transmission of *S. aureus* between humans and animals (D. Currie, 2008; H. C. Lewis, et al., 2008; Smith, et al., 2009).

Epidemiology and Pathogenicity

S. aureus is associated with a wide range of infections. As of 2005, *S. aureus* was the most frequently identified cause of hospital-acquired infections in the United States, responsible for 20% of surgical-site infections, 18% of pneumonias, and 13% of bloodstream infections (Bassetti, et al., 2005; Program, Diseases, Prevention, Service, & Services., 1999). *S. aureus* is also commonly found in the general human population. According to samples collected in the 2003–2004 NHANES, between 27.2% and 30% of the United States population, or 75 to 82.9 million individuals, were colonized with *S. aureus* (Gorwitz, et al., 2008). Twenty to 30% of the general population have nasal colonization of *S. aureus* (Bassetti, et al., 2005; John & Barg, 1999). *S. aureus* has been the subject of numerous studies, and has received media attention for the increasing

incidence of antibiotic-resistant strains that cause infections that cannot be treated with common antibiotics.

Methicillin Resistance

MRSA is a type of *S. aureus* that possesses genes that confer resistance to penicillinase-stable penicillins, which include methicillin, oxacillin, penicillin, and ampicillin. *mecA* is the most common gene that encodes resistance to methicillin among MRSA isolates (Araj, Talhouk, Simaan, & Maasad, 1999). The first strains of MRSA were observed in the early 1960s (Ornskov, et al., 2007). MRSA infections have historically occurred predominantly in hospitals and healthcare facilities among individuals with weakened immune systems and other risk factors, such as foreign objects in the body (CDC, 2009). MRSA infections are now being identified in otherwise healthy individuals outside of the healthcare setting. This type of infection is called a community-associated (or community-acquired) (CA-MRSA) infection. Both nosocomial and CA-MRSA infections are often associated with skin and soft tissue infections (SSTI) (CDC, 2009).

Using data from the 2001-2002 and 2003-2004 NHANES of the U.S. population, the prevalence of methicillin-susceptible *S. aureus* (MSSA) colonization in the anterior nares decreased, while the prevalence of MRSA colonization increased (Gorwitz, et al., 2008). Between 1997 and 1999 the number of both nosocomial and CA-MRSA infections increased (Diekema, et al., 2001). In 2005, MRSA infections were ultimately responsible for the death of approximately 18,650 individuals in the United States (Klevens, et al., 2007).

Nosocomial and CA-MRSA isolated in the United States through the SENTRY Antimicrobial Surveillance Program exhibited resistance to a median of three antimicrobial classes in addition to methicillin (Diekema, et al., 2001). Nearly 15% of MRSA isolates were also resistant to tetracycline (Diekema, et al., 2001). The Asia-Pacific region, including Taiwan, Singapore, Japan, and Hong Kong had the highest rates of methicillin resistance (greater than 60%) among *S. aureus* isolates identified through the SENTRY program (Diekema, et al., 2001).

Hospital acquired MRSA

By the end of 1998, the prevalence of MRSA isolates from intensive care units was almost 50% (Chambers, 2001). Most nosocomial strains of MRSA are resistant to several classes of antimicrobials including aminoglycosides, erythromycin, clindamycin, and tetracycline (Flynn & Cohen, 2008).

Community associated MRSA

The emergence of CA-MRSA is associated with infections among individuals who lack traditional risk factors, such as direct contact with healthcare workers, hospitalization, and previous antimicrobial treatment (Chambers, 2001). CA-MRSA spreads through skin-to-skin contact and is associated with SSTIs as well as severe systemic infections such as sepsis and necrotizing pneumonia (Malik, Vranken, Silio, Ratard, & Van Dyke, 2006; Ozaki, et al., 2009). CA-MRSA often exhibits multiple resistance to erythromycin (98%) and β-lactam agents (ampicillin, 99%; penicillin, 99%) (Bou, 2007). Most community-associated strains of MRSA are susceptible primarily to clindamycin and tetracyclines (Flynn & Cohen, 2008). Although genetic differences exist between typical HA-MRSA and CA-MRSA strains, CA-MRSA infections are

increasingly found in both community and healthcare settings, challenging the relevance of separating the two types of infections (Gorwitz, et al., 2008; Healy, Hulten, Palazzi, Campbell, & Baker, 2004; Kourbatova, et al., 2005; Miller & Diep, 2008; Saiman, O'Keefe, Graham, & al, 2003; Seybold, et al., 2006).

Enterococcus *spp.*

Genus Description

Previously classified as group D streptococci, enterococci are gram-positive, catalase-negative, cocci able to grow in the presence of high levels of bile and sodium chloride (Fisher & Phillips, 2009; Murray, et al., 2002; Spacek & Vinetz, 2009). *Enterococcus* spp. are facultatively anaerobic and more resistant to chlorination and other environmental factors than other types of coliforms (Fisher & Phillips, 2009; Maier, 2009). The temperature range for enterococci is between 5 and 50°C which encompasses the average human body temperature, 37°C (Fisher & Phillips, 2009). It is important to note that optimal temperature for enterococci is 35°C (Murray, et al., 2002).

Ecological habitat and distribution

Enterococci are part of the common flora of the human intestines and the female genital tract (Centers for Disease Control and Prevention (CDC), 2008; Murray, et al., 2002; Spacek & Vinetz, 2009). Because they are found in the animal and human gut, *Enterococcus* spp. are often used as indicators of fecal contamination ((Centers for Disease Control and Prevention (CDC), 2008)). Small numbers of *E. faecalis* and *E. facieum* have also been isolated in the upper respiratory tract (Murray, et al., 2002).

Enterococcus spp. can also be found in the environment in soil, water, and food (Spacek & Vinetz, 2009).

Epidemiology and Pathogenicity

Enterococci are commensal organisms that were historically classified as opportunistic pathogens. As such, they can cause urinary tract infections as well as infections of the bloodstream (bacteremia) and wounds, meningitis, and endocarditis (Centers for Disease Control and Prevention (CDC), 2008; Murray, et al., 2002; Spacek & Vinetz, 2009). Between 2006 and 2007, one out of every eight hospital infections was caused by *Enterococcus* spp. (Centers for Disease Control and Prevention (CDC), 2008). Approximately 30% of these infections were resistant to the antibiotic vancomycin (Centers for Disease Control and Prevention (CDC), 2008).

Vancomycin Resistance

The first cases of enterococci with high levels of resistance to vancomycin were reported in the United Kingdom in the 1980s (Uttley, Collins, Naidoo, & George, 1988; Zirakzadeh & Patel, 2006). Vancomycin is typically considered a "last resort" drug to treat gram-positive bacterial infections (Nagulapally, et al., 2009; Wegener, Aarestrup, Jensen, Hammerum, & Bager, 1999). As of 2003, nearly 30% of all enterococci isolated from intensive care unit patients in the United States were resistant to vancomycin (Drees, et al., 2008; System, 2004). Many species of enterococci are also resistant to a number of other antibiotics, often in conjunction with vancomycin-resistance (Murray, Rosenthal, & Pfaller, 2009). Almost all *E. faecium* are resistant to ampicillin and as of 2009 the majority of clinical *E. faecalis* isolates were resistant to vancomycin (Murray, et al., 2009). Hanrahan et al. found that vancomycin and ampicillin are part of the same

large, transferable chromosomal element, causing resistance to these two antibiotics to occur often in the same organisms (Hanrahan, Hoyen, & Rice, 2000).

To date, it has been confirmed that seven different genes (*vanA-vanG)* can confer vancomycin resistance to *Enterococcus* spp., but the three most prevalent genes are *vanA, vanB,* and *vanC* (Fisher & Phillips, 2009). Both *vanA* and *vanB* are acquired, but *vanA* confers resistance to vancomycin and teicoplanin, while *vanB* only confers resistance to vancomycin (Patel, Uhl, Kohner, Hopkins, & Cockerill, 1997). *vanC* is an intrinsic resistance gene that most commonly occurs in *E. gallinarum, E. casseliflavus,* and *E. flavescens*, but it has also been identified in *E. faecalis* and *E. faecium* (Fisher & Phillips, 2009; Patel, et al., 1997). The resistance genes *vanA* and *vanB* are of particular concern in terms of horizontal gene transfer because they occur on plasmids and can be easily transferred to other gram-positive bacteria, including *S. aureus* (Fisher & Phillips, 2009; Garcia, et al., 2007; Giraffa, 2002; Patel, et al., 1997; Poole, et al., 2005; Sievert, et al., 2008; Zirakzadeh & Patel, 2006).

Co-colonization of patients with VRE and MRSA has been observed with increasing frequency (Warren, Nitin, Hill, Fraser, & Kollef, 2004; Zirakzadeh & Patel, 2006). 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 & Dalsgaard, 2004). VRE are able to survive in the environment for approximately one week (Zirakzadeh & Patel, 2006). VRE can also be transferred between animals and humans. Kuhn et al. found that horizontal transfer of *van*A genes could take place between animal strains and human strains (Kuhn, et al., 2005).

In addition to transfer of vancomycin resistance between strains of enterococci, vancomycin resistance can also be transferred from enterococci to other types of bacteria, including *S. aureus* (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). By 2008, only seven clinical cases of VRSA had been confirmed in the United States, but the incidence of VRSA infections could continue to rise (Sievert, et al., 2008).

When compared with other types of nosocomial bacterial infections, VRE was associated with increased mortality, length of hospital stay, admission to the intensive care unit, surgical procedures, and associated costs (Carmeli, Eliopoulos, Mozaffari, & Samore, 2002; Drees, et al., 2008; Siegel, et al., 2006).

Wastewater Treatment in the United States

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 incoming wastewater. Primary treatment usually includes screening of large objects, grit removal, and sedimentation (EPA, 2004b; Maier, 2009). Secondary treatment entails the use of biological processes to remove suspended solids and microorganisms from water that has already undergone primary treatment (Maier, 2009). The most common type of secondary treatment in the United States is the activated sludge process, which introduces nitrifying microorganisms and high levels of oxygen into wastewater to break down suspended organic materials (Maier, 2009). After the activated sludge process, the

wastewater is slowed down in secondary clarifiers to induce die-off in the introduced microbial population. The secondary clarifiers also allow for physical removal of remaining organic material. The secondary treatment processes can remove as much as 90% of organic matter from wastewater (EPA, 2004b). Secondary treatment is the minimum level of treatment required by the United States government through the Clean Water Act (EPA, 2004b). Tertiary treatment is any treatment beyond secondary treatment (Maier, 2009). Free chlorine is the most common disinfectant used in the tertiary treatment process, but chloramines, ultraviolet light, and ozone are also used (EPA, 2004a, 2004b). In addition to disinfection, filtration and other processes can be employed in tertiary treatment.

Microorganisms that pose a threat to human health are not necessarily completely removed during wastewater treatment. The primary goal of wastewater treatment is not to fully eradicate harmful contaminants that could affect human health, but to remove and degrade organic matter (Maier, 2009).

In 2009, more than 15,000 wastewater treatment plants in the United States treated approximately 150 billion liters of wastewater per day (Maier, 2009). Wastewater treatment facilities in the United States range from less than secondary to tertiary treatment facilities. In 2004, approximately 100 million individuals living in the United States were serviced by 9,261 secondary, or less than secondary, wastewater treatment facilities (EPA, 2004b, 2008a). Secondary treatment facilities comprise over half of the municipal wastewater treatment facilities in the United States (EPA, 2004b, 2008a). More than 30% of municipal wastewater treatment facilities go beyond secondary treatment (EPA, 2004b).

Bacteria in wastewater

Raw wastewater contains a mixture of many substances, including pathogenic and non-pathogenic microorganisms, and antimicrobials that could pose a risk to human health (Rose, 2007). Several previous studies have analyzed the bacterial content of wastewater and aerosols from wastewater at isolated locations (Caplin, et al., 2008; de Zutter & van Hoof, 1984; Fracchia, Pietronave, Rinaldi, & Giovanna Martinotti, 2006; Harwood, et al., 2001; Iwane, et al., 2001; Karra & Katsivela, 2007; Miguel Ferreira da Silva, et al., 2006; Mispagel & Gray, 2005; Poole, et al., 2005; Prazmo, et al., 2003; Schwartz, et al., 2003; Volkmann, et al., 2004). These studies, however, have not followed the path of wastewater from effluent to secondary use, i.e. as a source of irrigation water or source water for drinking water treatment plants in the United States.

Many of the organisms found in wastewater are mesophilic bacteria whose optimal temperature range is between 25-40°C, which includes the average temperature of the human body. The human body is a favorable habitat for these bacteria and therefore they pose a threat to human health. Individuals working with untreated and reclaimed wastewater might be exposed to these microorganisms in the form of aerosolized particles through inhalation or through direct contact. To be able to prevent adverse health outcomes from exposures to microbial contaminants, it is necessary to determine first the types of agents and their corresponding concentrations found in the water sources of interest. A study at a Polish wastewater treatment plant identified 34 species of bacteria and 14 species of fungi in influent 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 both untreated and treated wastewater (Prazmo, et al., 2003).

To address the issue of contaminants in incoming wastewater, wastewater treatment plants are designed to reduce the amount of organic matter, but reduction of enteric microorganisms can vary greatly and wastewater effluent often still contains detectable levels of these organisms (Koivunen, Siitonen, & Heinonen-Tanski, 2003; Rose, 2007). It may even be the case that the number of pathogenic microorganisms and percentage of antibiotic-resistant microorganisms increase at certain stages during the wastewater treatment process (de Zutter & van Hoof, 1984; Emparanza-Knorr & Torrella, 1995; Kayser, Boll, & Muller, 1987; Koivunen, et al., 2003; LeChevallier, Cawthon, & Lee, 1988). Prazmo et al. found that the concentration of total microorganisms analyzed was two to three times greater at the initial and intermediate treatment phases compared to the final treatment phase (Prazmo, et al., 2003).

Wastewater treatment plants use indicator organisms, such as total coliforms and *E. coli*, to determine whether treatment processes have reduced microorganisms to a level of acceptable risk for human health. However, the absence of indicator organisms in treated wastewater does not necessarily mean that all bacteria present in incoming wastewater have been killed (Levine & Asano, 2004). Providing measurements of indicator organisms alone, therefore, might not be representative of true human health risks from all contaminants in reclaimed wastewater.

Antibiotic-Resistant Bacteria in Wastewater

Along with total microbial concentration, the concentration of antibiotic-resistant bacteria varies throughout the wastewater treatment process. Several studies have

concluded that the percentage of antibiotic-resistant bacteria increases with secondary treatment, but decreases with tertiary treatments such as ultraviolet radiation or chlorination (Nakamura & Shirota, 1990; Prazmo, et al., 2003; Rose, 2007).

Tolba et al. found that MRSA can survive for as many as 14 days in aquatic environments, both saline and non-saline (2008). MRSA is better adapted to survive in seawater than river water because of its preference for higher salinity (Tolba, et al., 2008). Wastewater typically has high levels of salt, which suggest it would be a favorable environment for MRSA (Axelrad & Feinerman, 2009; Levine & Asano, 2004). However, some strains of MRSA are inhibited by high salt concentrations in the range of 7.5% (Smyth, Kahlmeter, Olsson Liljequist, & Hoffman, 2001). It might be difficult to isolate active microorganisms from a large volume of water when taking grab samples, but this does not rule out the possibility that those organisms are present. MRSA might also not be culturable or express resistance, but still be able to survive the transmission from hospital or home to wastewater treatment plant and the treatment process. If MRSA does survive wastewater treatment, it could come into contact with other environments or individuals. It also should be taken into consideration that at wastewater treatment plants, contaminants and microorganisms are concentrated, thereby increasing the risk of infection.

Börjesson et al. recently published the first study to report successfully isolating MRSA from wastewater (Börjesson, et al., 2009). Several types of antibiotic-resistant bacteria occur in higher numbers in wastewater than in the natural environment, but in general, *S. aureus* occurs at low levels in both surface water and wastewater (Börjesson, et al., 2009; Schwartz, et al., 2003). The wastewater treatment plant in Börjesson et al.'s

study had a hydraulic retention time of 8 hours and a solid retention time of 2 to 4 days in the activated sludge system (Börjesson, et al., 2009). Combining these retention times with the results from Tolba et al.'s study that MRSA can survive in river water up to 14 days, a plausible explanation appears for how MRSA can survive the wastewater treatment process and represent a risk for infection for those who come into contact with treated water or work in wastewater treatment plants (Börjesson, et al., 2009; Tolba, et al., 2008). MRSA was more prevalent in early, compared to final, treatment steps, but this could be due more to reduction of biomass than to effective removal of the microorganisms. Börjesson et al. identified an increase in the *mecA* gene concentration at the trickling filter. The association between high concentrations of antibiotic-resistant bacteria and trickling filters could be due to biofilms located on the filters' surfaces. The trickling filter is similar to the biological aerated filter and effluent filters at the tertiary treatment plant sampled in the current study. These similarities emphasize the importance of taking water, air, and swab samples from these locations (Börjesson, et al., 2009; Fracchia, et al., 2006; Schwartz, et al., 2003).

Aerosolization of Pathogens at Wastewater Treatment Plants

Although the current study focused on contaminants found in reclaimed wastewater, aerosols also present an important exposure pathway. Bacteria can become airborne when they attach to skin, lint, and dust and spread via air currents, possibly to be taken up into the respiratory system (Weese & Ji, 2007). The inhalation route of exposure is one of the main exposure routes for pathogenic microorganisms from spray irrigation operations using reclaimed wastewater (Xie, Hu, Guo, & Wu, 2009). Outbreaks of MRSA postoperative infections have been linked to airborne transmission (Weese & Ji,

2007). *S. aureus* has been isolated from air samples at wastewater treatment plants and has been found in high concentrations among individuals living in proximity to areas fertilized with treated wastewater. These findings suggest that wastewater treatment processes might not be effective at removing *S. aureus* from wastewater (Börjesson, et al., 2009).

Seasonal Variations

Enteric infections, caused by viruses and bacteria, have been found to vary seasonally (Gabriel, et al., 2010; Grassly & Fraser, 2006; Jung, Yoon, Jang, & Jeon, 2007; Maier, 2009). For example, enteric infections caused by *Campylobacter jejuni* consistently peak in summer (Gabriel, et al., 2010; Jore, et al.; Jung, et al., 2007; Maier, 2009). These seasonal peaks could be the result of changes in the survival rate of different agents in the environment during different seasons and/or the seasonal exchange between groundwater and surface water (Harwood, et al., 2001; Maier, 2009). In addition to the impact of temperature on water levels and the exchange of water between different reservoirs, temperature has also been studied as a limiting factor for the survival of culturable *Enterococcus* spp. in freshwater (Scott, Jenkins, Lukasik, & Rose, 2005). In terms of antibiotic-resistant bacteria, low temperatures and low flow were found to increase resistance among *Acinetobacter* spp. to a greater number of antibiotics (Zhang, et al., 2009). High water temperatures might accelerate degradation of antibiotics and high flows could reduce the concentration of antibiotics in water and thereby reduce selective pressure for antibiotic resistance (Zhang, et al., 2009).

Studies looking at aerosolized bacteria in wastewater treatment plants have noted significant associations between season and amount of bacteria, which have been

attributed to meteorological conditions (Fracchia, et al., 2006; Karra & Katsivela, 2007). Karra and Katsivela found that solar radiation, temperature, and relative humidity, all factors that are correlated with seasonal changes, were the most important factors associated with the concentration of aerosolized microorganisms (Karra & Katsivela, 2007).

Reclaimed Wastewater Use in the United States

Great potential exists for harnessing treated wastewater for reuse instead of discharging it into water bodies and withdrawing freshwater for use, which have been the historical patterns. Although publically-supplied water in the United States is treated to obtain potable levels, this level of treatment is not required for all uses, such as industrial and landscaping applications (EPA, 2004a). Reclaimed wastewater is already being used across the United States for a number of different activities. In 2004, 1.7 billion gallons of reclaimed wastewater were used per day in the United States (EPA, 2004a). Also in 2004, 15 states estimated needing $4.3 billion for reclaimed wastewater distribution (EPA, 2008a). California and Florida accounted for 84% of that figure (EPA, 2008a). The volume of reclaimed wastewater used is increasing at an approximate rate of 15% per year (EPA, 2004a). Reclaimed wastewater is used for a number of reasons including, but not limited to: replenishing groundwater supplies, water conservation, additional treatment of wastewater, and reduced cost (EPA, 2004a; Levine & Asano, 2004).

Reclaimed wastewater is used in both potable (sources for drinking water treatment plants) and non-potable (land application) projects. Land applications for reuse of wastewater can be categorized as low-rate irrigation, overland flow, and high-rate infiltration (groundwater recharge) (EPA, 2004a; Maier, 2009; C. Tien, 2007). The

choice of reuse method usually depends on site-specific conditions, level of wastewater treatment, and intended use of reclaimed wastewater (Maier, 2009; C.-T. Tien, 2010). The federal government and individual states are encouraging land application of wastewater as an alternative to the traditional discharge of wastewater into surface water. Amendments to the Federal Water Pollution Control Act require that during the planning phase for new wastewater treatment facilities, land application of wastewater be considered as an end use (MDE, 2009). The State of Maryland enacted the Annotated Code of Maryland in 2002 to encourage reclaimed wastewater use (MDE, 2009).

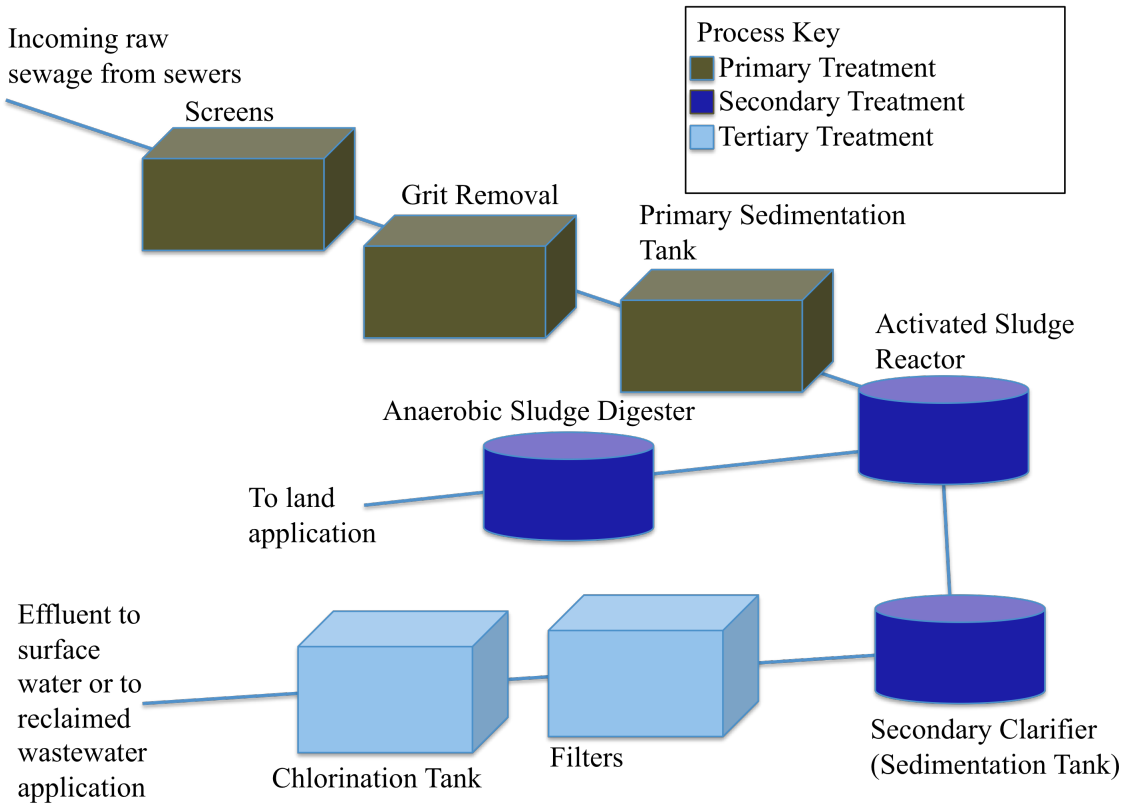
At this time, no federal regulations exist for wastewater reuse, only guidelines. Regulations are legally enforceable, whereas guidelines are suggestions for use, but cannot be enforced (EPA, 2004a). EPA allows states to determine whether to develop regulations or guidelines to oversee the use of reclaimed wastewater within their boundaries. As of 2002, 25 states had passed regulations concerning the use of reclaimed wastewater, 16 states had developed guidelines, and nine states had neither regulations nor guidelines (EPA, 2004a). By 2004, no reclaimed wastewater standards or guidelines in the United States were based on microbial risk assessments to determine the risk of infection from exposure to reclaimed wastewater (EPA, 2004a). In the past, secondary treatment and disinfection were considered standard for reusing treated wastewater (EPA, 2004a). Even when states do distinguish between effluent from different levels of wastewater treatment, they may still allow use of primary and secondary treated wastewater, but with additional requirements for buffer zones, as is the case in the State of Maryland (MDE, 2009).

Conclusion

As reclaimed wastewater becomes an increasingly common water source, assessing possible microbial risks from its use is becoming more crucial for public health. In particular, MRSA and VRE are bacteria linked with increased risk of mortality from infection and could be excreted into wastewater. If these organisms survive the wastewater treatment process, widespread occupational and community exposure could occur. Through the findings of my Master's thesis research, I plan to contribute knowledge that may be used for informing environmental management decisions and protecting the environment and human health. The detection of pathogenic microorganisms in reclaimed wastewater could serve as an impetus to evaluate possible improvements to treatment, distribution, and reuse processes. Such findings would also have implications for the health of wastewater treatment plant workers and spray irrigation workers, as well as other affected members of the community.

We are facing a future that includes a growing number of states experiencing drought conditions with increasing frequency. Reduction of water use and exploration of new water sources are both important as we consider irrigation and drinking water needs for the future. Reclaimed wastewater has the potential to be an important alternative source of water, but before its applications are further expanded, it is important to conduct a holistic analysis of the possible human health risks involved.

Figure 1. Typical wastewater treatment plant process steps



Chapter 3: **Short form manuscript**: Reductions of Methicillin-resistant *Staphylococcus aureus* and Vancomycin-resistant *Enterococcus* spp. at a U.S. Tertiary Wastewater Treatment Plant

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. (VRE) were isolated from primary and secondary wastewater samples at a U.S. tertiary wastewater treatment plant, but not from the effluent. Ninety-seven percent of the MRSA isolates were multidrug resistant. These findings suggest that tertiary treatment effectively reduces MRSA and VRE.

Introduction

As the global, and United States, populations increase, so too do freshwater usage, water shortages, and droughts (Angin, et al., 2005; Dobbie, et al., 2008; EPA, 2008b; Kocaman, et al., 2007; Levine & Asano, 2004; Rodriguez, et al., 2009; UN, 2003). As water shortages become more common, alternative water sources, such as treated municipal wastewater, are increasingly being used for downstream applications including drinking water treatment plant sources and irrigation (Angin, et al., 2005; Axelrad & Feinerman, 2009; Friedel, Langer, Siebe, & Stahr, 2000; Kim & Aga, 2007; Levine & Asano, 2004). Because of this practice, individuals exposed to reclaimed wastewater, including wastewater and spray irrigation workers, could be exposed to antibiotic-resistant bacterial pathogens that may remain in treated wastewater. Also, bacteria possessing resistance genes to common antibiotics that survive the treatment process could transfer these genes to other bacteria (Kim & Aga, 2007; Martinez, 2006; Zhang, et

al., 2009). Antibiotic-resistant bacteria can cause life-threatening infections that have limited treatment options (Zhang, et al., 2009). To better understand the potential for exposure to antibiotic-resistant bacteria in treated wastewater, it is important to identify the presence or absence of these organisms throughout the wastewater treatment process. This study evaluates the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. (VRE) at a tertiary wastewater treatment plant located in the Mid-Atlantic region. We hypothesized that wastewater throughout the treatment process would contain both MRSA and VRE.

Methods

The wastewater treatment plant in this study processes 180 million gallons of wastewater per day and uses tertiary treatment, including sand filtration, chlorination, and de-chlorination. A total of 18 water samples from the primary (raw untreated) and secondary (screened) influent, activated sludge reactor, secondary clarifier, and effluent treatment steps were collected on August 3, August 17, October 22, and December 1, 2009. All samples were collected in 500 milliliter or 1 liter sterile Nalgene collection bottles as grab samples. Samples were stored at 4°C during sampling and transportation to the laboratory. All samples were processed within 24 hours of collection.

Standard membrane filtration was used to recover MRSA and VRE from water samples (EPA, 2002). Vancomycin-amended membrane-*Enterococcus* Indoxyl-β-D-Glucoside agar (Yan, et al.) (EMD Chemicals) was used to isolate VRE. *Enterococcus* spp. were confirmed biochemically by detection of pyrrolidonyl arylamidase activity and the ability of the bacterium to break down hydrogen peroxide by catalase.

Filtered samples were enriched for MRSA and total *S. aureus* using m Staphylococcus Broth (BD Diagnostic Systems, Franklin Lakes, NJ). Samples enriched for MRSA were plated on MRSASelect (Bio-Rad Laboratories, Hercules, CA). Samples enriched for total *S. aureus* were plated on Mannitol Salt Agar (BD Diagnostic Systems, Franklin Lakes, NJ) and Baird Parker Agar (BD Diagnostic Systems, Franklin Lakes, NJ). DNA from presumptive MRSA and *S. aureus* isolates was extracted using the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, CA). The presence of MRSA was confirmed by PCR amplification of the *mecA* gene and the *S. aureus*-specific *nuc* gene. The primers used to detect the *nuc* and *mecA* genes were NUC1 and NUC2, and MECA1 and MECA2 (Fang & Hedin, 2003). The conventional PCR program used for amplification was based on the PCR assay previously described by Fang and Hedin in 2003 (Fang & Hedin, 2003).

Antimicrobial susceptibility testing was performed on MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates using the Sensititre microbroth dilution system (Trek Diagnostic systems Inc., Cleveland, OH, USA). Samples were grown overnight on Trypticase Soy Agar with 5% Sheep Blood (BD Diagnostic Systems, Franklin Lakes, NJ). *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as controls for all susceptibility testing. Antimicrobial minimal inhibitory concentrations (MICs) were determined using general gram positive plates (GPN3F) (Trek Diagnostic systems Inc., Cleveland, OH, USA), and were recorded as the lowest concentration of an antimicrobial that inhibited the growth of the bacterial suspension in Mueller-Hinton Broth (Beier, et al., 2008). Resistance breakpoints were determined using

the Clinical and Laboratory Standards Institute (CLSI) standards for antimicrobial susceptibility testing (CLSI, 2010).

Results

MRSA and MSSA

A total of 100 MRSA isolates were confirmed from the wastewater samples. MRSA was isolated from 43% of influent, 100% of activated sludge reactor, and 67% of secondary clarifier samples. To our knowledge, this is the first study to identify MRSA at a municipal wastewater treatment plant in the U.S. The percentage of MRSA out of total *S. aureus* isolates grown on non-antibiotic-amended media was highest in the activated sludge (40% of isolates) and secondary clarifier (90% of isolates) samples. No MRSA was isolated from influent samples grown on non-antibiotic amended plates. No MRSA or MSSA was detected in effluent wastewater samples.

VRE and VSE

Eighty-seven VRE isolates were isolated from the wastewater samples. VRE was present throughout the wastewater treatment process in 100% of influent, activated sludge reactor, and secondary clarifier samples. The calculated number of VRE ranged from 5×10^{3} CFU/mL in primary influent to 1×10^{-1} CFU/mL in the secondary clarifier. No VRE was detected in effluent wastewater samples, however vancomycin-susceptible *Enterococcus* spp. (VSE), a fecal indicator, was isolated in the effluent (Figures 2 and 3).

VRE in the primary influent was higher on August 17, 2009 than samples collected at the same location on the other sampling dates by at least one order of magnitude (Figure 3). The concentration of VRE increased in the activated sludge reactor

in both the October and December samples and then decreased in the secondary clarifier and effluent. At least one VRE isolate from the primary influent and one isolate from the activated sludge reactor samples contained the *vanA* gene. Previous studies have found seasonal differences in the presence of VRE at wastewater treatment plants, with the absence of VRE more likely in winter than other seasons (Nagulapally, et al., 2009).

The concentration of VSE was greater in the raw influent in October than any other sampling date (August 3, August 17, and December 1, 2009), with the concentration in samples from December second highest. The concentration of VSE in the effluent was very low at each collection date, but was highest in the sample from August 17 (Figure 2).

Antimicrobial Susceptibility

The 100 MRSA isolates had a median oxacillin MIC of ≥16 μg/mL (range, 2 - ≥16 μg/mL) (Table 1). MRSA isolates also displayed resistance to a number of antibiotics approved by the U.S. Food and Drug Administration for treating MRSA infections, including linezolid, tetracycline, fluoroquinolones (ciprofloxacin, gatifloxacin, and levofloxacin), and clindamycin (Johnson & Decker, 2008). Ninety-seven percent (97 out of 100) of all MRSA isolates were resistant to ≥ 6 antimicrobial agents. Among isolates from the primary influent, 95% were multi-drug resistant, resistant to two or more classes of antibiotics (Table 2). One hundred percent of activated sludge reactor and secondary clarifier isolates were multi-drug resistant. The MIC distribution for isolates from all treatment steps was similar for erythromycin, the penicillins (ampicillin, penicillin, and oxacillin) and the fluorquinolones, although resistance to the

fluoroquinolones increased slightly as treatment progressed (Table 1). Resistance to tetracycline and clindamycin was identified only among activated sludge reactor and primary influent isolates respectively. The average number of antibiotics that isolates were resistant to was greater among the activated sludge reactor isolates than either primary influent or secondary clarifier isolates from both October and December 2009 (Figure 4).

Sixty-eight percent of the MSSA isolates from all treatment steps were multi-drug resistant (Tables 2 and 3). As the treatment process progressed from influent to activated sludge reactor to secondary clarifier, the MICs increased for the penicillinase-labile penicillins (penicillin and ampicillin), but decreased for the fluoroquinolones. One MSSA isolate was resistant to linezolid in the primary influent (Table 3).

The percentage of MSSA resistant to multiple classes of drugs was greater in isolates collected in October 2009 than in December 2009 (Table 2). In the December 2009 isolates, the percentage of multi-drug resistant (MDR) isolates increased as the treatment process progressed, but in the October isolates, the percentage of MDR isolates was slightly greater among the influent isolates than the activated sludge reactor isolates (Table 2).

Discussion

Linezolid and daptomycin are two antibiotics that recently have been considered important alternatives for treating severe invasive MRSA infections (Johnson & Decker, 2008; Kaka, et al., 2006; Loffler & MacDougall, 2007). Only 3% of all MRSA isolates were resistant to linezolid. Sixty-seven percent of linezolid-resistant MRSA isolates were from primary influent, and the remaining 33% from secondary clarifier. The only MSSA

isolate found to be resistant to linezolid was identified in the primary influent. No MRSA or MSSA isolates were found to be resistant to daptomycin. These findings suggest that as of yet, the wastewater treatment process decreases the number of isolates with resistance to these two more recent antibiotics used in the treatment of severe MRSA infections.

From previous studies it appears that microbial concentration and the concentration of antibiotic-resistant bacteria varies throughout the treatment process (Börjesson, et al., 2009; Iwane, et al., 2001; Nakamura & Shirota, 1990; Prazmo, et al., 2003; Rose, 2007). Several studies have concluded that the percentage of antibiotic-resistant bacteria increases with secondary treatment, peaking in the activated sludge reactor, and decreases or is eliminated with tertiary treatments such as ultraviolet radiation or chlorination (Börjesson, et al., 2009; Kim & Aga, 2007; Nagulapally, et al., 2009; Nakamura & Shirota, 1990; Prazmo, et al., 2003; Rose, 2007). Based on the results of the present study, and previous studies at wastewater treatment plants, this type of pattern seems to depend on the specific organism and antibiotic (Iwane, et al., 2001; Nagulapally, et al., 2009; Zhang, et al., 2009). The ratio of MRSA compared to total *S. aureus* did increase as treatment progressed, from 40% in the activated sludge reactor to 90% in the secondary clarifier. However, the average number of antibiotics that isolates were resistant to was greater among the activated sludge reactor isolates than either primary influent or secondary clarifier isolates (Figure 4). Further questioning the idea that resistance increases from primary to secondary treatment, resistance to tetracycline was identified only in primary influent isolates, not in any secondary treatment isolates. Among MSSA isolates, the MICs for the penicillinase-labile penicillins increased from

primary to secondary treatment, while MICs for fluoroquinolones decreased from primary to secondary treatment.

Because VRE were isolated from samples in the primary and secondary treatment stages (primary and secondary influent, activated sludge reactor, and secondary clarifier steps), it is possible that genes that confer resistance to vancomycin could still be present in the effluent and taken up by other bacteria (Courvalin, 2005). These genes and bacteria could then be released into a receiving water body or effluent used for reuse applications. DNA released from bacteria may persist in environmental samples for a period of time (Pote, et al., 2003; Volkmann, et al., 2004).

VRE could also be present in effluent, but in a viable but not culturable state (Signoretto & Canepari, 2008). Disinfection can reduce the number of culturable organisms by injuring cells, but does not always completely eradicate all of the organisms (Xi, et al., 2009). A study at a tertiary wastewater treatment plant conducted by Xi et al. found that although heterotrophic plate counts decreased by six orders of magnitude between raw influent and effluent, the occurrence of 16S rRNA decreased by only one order of magnitude, suggesting that injured or dead bacterial cells were still present (Xi, et al., 2009). Both *E. faecalis* and *E. hirae* have been found to able to resume cell reproduction after being in a viable but not culturable state upon entering a favorable environment (Lleò, et al., 2001; Signoretto & Canepari, 2008). Also, only approximately 5 to 10% of bacteria found in wastewater is culturable at all (Hiraishi, 1998; Xi, et al., 2009). Even the presence of total *Enterococcus* spp. in effluent samples is important, as they are indicator organisms used to determine the presence of other fecal pathogens. So, although VRE and MRSA were not identified in the effluent samples, this does not mean

that using tertiary treated wastewater in reuse applications has zero risk of exposing individuals to these pathogens and/or antibiotic resistance genes.

Although no MRSA or VRE were isolated from the effluent at the tertiary wastewater treatment plant, both MRSA and VRE were recovered in all other wastewater samples, including the secondary clarifier, which is the last step in the secondary treatment process. Secondary treatment is the highest level of treatment required of municipal wastewater treatment facilities by the United States Environmental Protection Agency (EPA) and consists of removing suspended organic solids through biological processes. Tertiary treatment is any type of treatment beyond secondary treatment. As of 2004, approximately 100 million individuals living in the United States were serviced by secondary, or less than secondary, wastewater treatment facilities (9,261 facilities). If the effluent from these facilities, which comprise over half of the municipal wastewater treatment facilities in the U.S., is reused in irrigation or other reuse activities, a large percentage of the U.S. population could be exposed to MRSA and VRE (USEPA, 2004, 2008).

Conclusions

In conclusion, MRSA and VRE were found throughout the secondary wastewater treatment process at a tertiary wastewater treatment facility in the Mid-Atlantic region. High levels of multi-drug resistance in MRSA and MSSA were prevalent throughout the treatment process, 97% and 68% respectively, but increased slightly from primary to secondary treatment. To our knowledge, this is the first time that MRSA has been isolated from a wastewater treatment plant in the United States. Because secondary wastewater treatment is the highest level of treatment at over half of the municipal

treatment facilities in the United States, the public health implications of exposure to, and reuse of, secondary treated wastewater could be significant. As the use of reclaimed wastewater accelerates across the United States, the risk of antibiotic-resistant bacterial infections from exposure to treated wastewater, from both secondary and tertiary facilities, deserves further attention.

Figure 2. Colony forming units (CFU) per milliliter (CFU/mL) of vancomycin-susceptible *Enterococcus* spp. found in selected wastewater samples

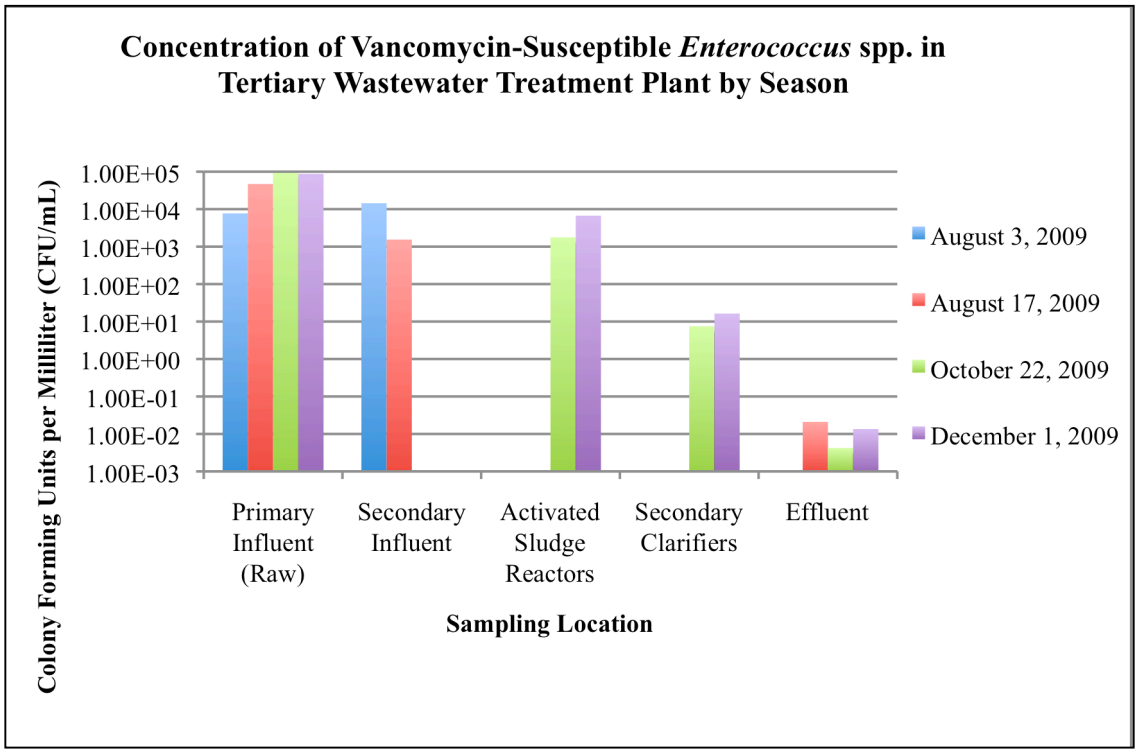


Figure 3. Colony forming units (CFU) per milliliter (CFU/mL) of vancomycin-resistant *Enterococcus* spp. found in selected wastewater samples

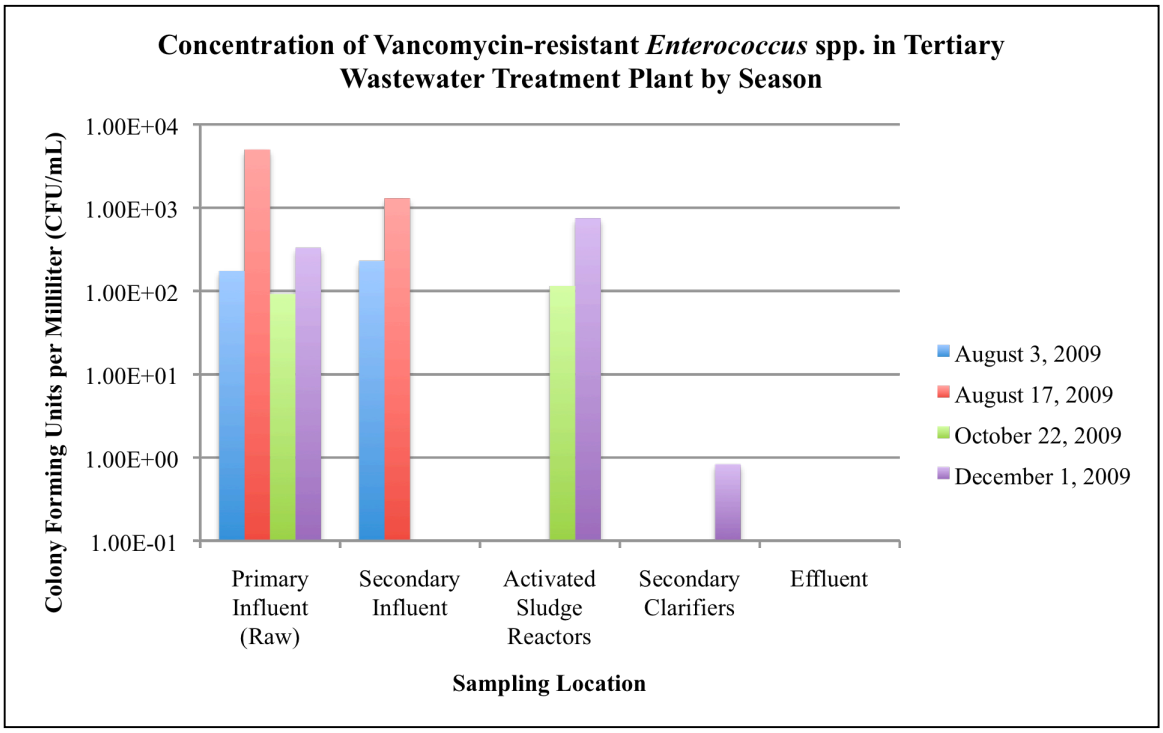
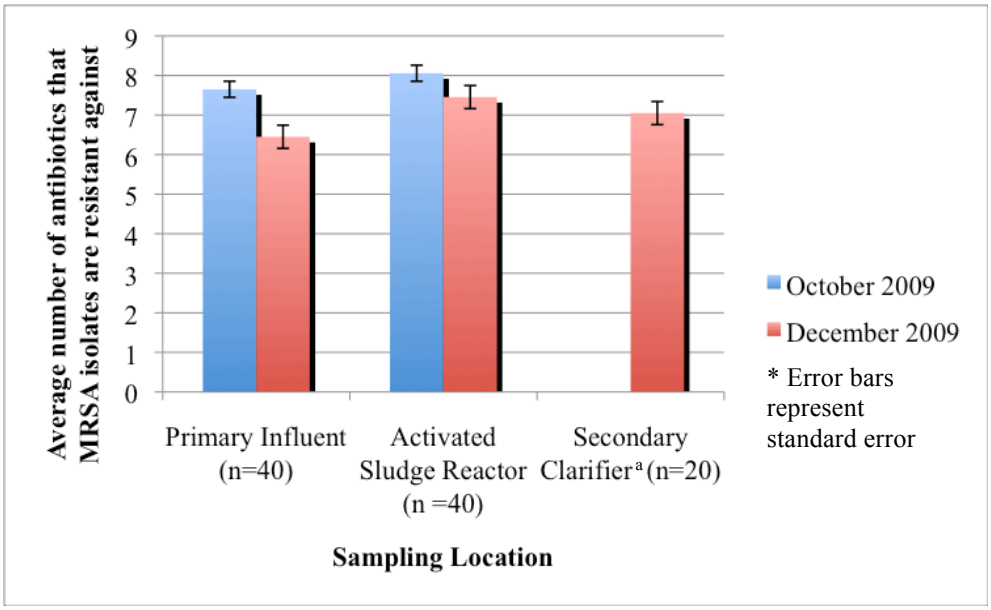


Figure 4. Average number of antibiotics that MRSA isolates display resistance to in different treatment steps of a tertiary wastewater treatment facility in the Mid-Atlantic Region (n = total number of isolates in sample type from both collection dates).



[a]Did not isolate any MRSA from secondary clarifier October 2009 samples.

Table 1. MIC distributions at different sampling locations for 12 antibiotics observed in methicillin-resistant *Staphylococcus aureus* collected from a tertiary wastewater treatment plant

	Number of methicillin-resistant *Staphylococcus aureus* isolates with the following MICs (μg/mL)													
Sampling location, antibiotic	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**≥128**	**%S**	**%I**	**%R**
PRIMARY INFLUENT (n=40)														
Erythromycin		1		1			38[a]					5	0	95
Clindamycin	35	4										100	0	0
Vancomycin				40								100	0	0
Tetracycline					26	2			12[c]			70	0	30
Ampicillin		1		1				6	32[c]			3	0	98
Levofloxacin		3					26	11[d]				0	0	100
Linezolid					14	23	2					95	0	5
Penicillin	1	1						38[d]				3	0	98
Ciprofloxacin			3			37[b]						8	0	93
Ceftriaxone							14	22	3	1		35	62.5	3
Gatifloxacin				3			30	6	1[d]			0	8	93
Oxacillin+2%NaCL						12	4	24[d]				0	0	100
ACTIVATED SLUDGE REACTOR (n=40)														
Erythromycin							40[a]					0	0	100
Clindamycin	15	3				22[b]						45	0	55
Vancomycin				38	2							100	0	0
Tetracycline					36	4						100	0	0
Ampicillin						7	1	5	27[c]			0	0	100
Levofloxacin						3	19	18[d]				0	0	100
Linezolid					18	22						100	0	0
Penicillin						4	4	32[d]				0	0	100
Ciprofloxacin						40[b]						0	0	100
Ceftriaxone							7	23	3	1	6	18	65	18
Gatifloxacin					4	18	17	1[d]				0	0	100
Oxacillin+2%NaCL					1	1	6	32[d]				3	0	98
SECONDARY CLARIFIER (n=20)														
Erythromycin							20[a]					0	0	100
Clindamycin	19	1										100	0	0
Vancomycin				20								100	0	0
Tetracycline					20							100	0	0
Ampicillin									20[c]			0	0	100
Levofloxacin						7	13					0	0	100
Linezolid					9	10		1[d]				95	0	5
Penicillin								20[d]				0	0	100
Ciprofloxacin						20[b]						0	0	100
Ceftriaxone								15	4			0	100	0
Gatifloxacin					1	18	1					0	0	100
Oxacillin+2%NaCL							2	18[d]				0	0	100

[a]MIC is ≥8
[b]MIC is ≥4
[c]MIC is ≥32
[d]MIC is ≥16

Table 2. Percentage of multidrug resistant methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) (fraction of total isolates per season)

	October 2009	December 2009
Primary Influent		
MRSA	100 (20/20)	90 (18/20)
MSSA	100 (5/5)	43 (3/7)
Activated Sludge Reactor		
MRSA	100 (18/18)	100 (22/22)
MSSA	90 (9/10)	75 (6/8)
Secondary Clarifier		
MRSA	0 (0/0)	100 (20/20)
MSSA	0 (0/0)	100 (1/1)

Table 3. MIC distributions at different sampling locations for 12 antibiotics observed in methicillin-susceptible *Staphylococcus aureus* collected from a tertiary wastewater treatment plant

	Number of methicillin-susceptible *Staphylococcus aureus* isolates with the following MICs (µg/mL)												
Sampling location, antibiotic	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**≥32**	**%S**	**%I**	**%R**
PRIMARY INFLUENT (n=12)													
Erythromycin			2	4				5[a]			55	0	45
Ampicillin		3							1	8	25	0	75
Levofloxacin			4				2	6			33	0	67
Linezolid							11	1			92	0	8
Penicillin	3								9[b]		25	0	75
Ciprofloxacin				3	1		8[c]				33	0	67
Gatifloxacin					4	3	5				0	33	67
Oxacillin+2%NaCL			6	6							100	0	0
ACTIVATED SLUDGE REACTOR (n=18)													
Erythromycin			2	3				12[a]			29	0	71
Ampicillin		1	3		1	4				8	24	0	76
Levofloxacin			10	1			2	3	1[b]		61	0	33
Linezolid							17	1			94	0	6
Penicillin	3			1		2	3		8[b]		18	0	82
Ciprofloxacin				11	1		6				67	0	33
Gatifloxacin					11	1	5	1			0	61	39
Oxacillin+2%NaCL			11	6		1					100	0	0
SECONDARY CLARIFIER													
Erythromycin								1[a]			0	0	100
Ampicillin										1	0	0	100
Levofloxacin			1								100	0	0
Linezolid						1					100	0	0
Penicillin									1[b]		0	0	100
Ciprofloxacin				1							100	0	0
Gatifloxacin					1						0	100	0
Oxacillin+2%NaCL					1						100	0	0

[a]MIC is ≥8
[b]MIC is ≥16
[d]MIC is ≥4

Chapter 4: Antibiotic-Resistant Bacteria Present in Reclaimed Wastewater and Occupational Exposure at a Spray Irrigation Site

Abstract

As water shortages become more common, reclaimed wastewater is increasingly being used for irrigation activities. Because of this practice, spray irrigation workers could be exposed to pathogens and antibiotic-resistant bacteria that may remain in treated wastewater. This study evaluated the presence of vancomycin-resistant *Enterococcus* spp. (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) in reclaimed wastewater, air, and nasal and dermal swabs of employees at a spray irrigation site in the Mid-Atlantic region of the United States. Water, air, and swab samples were processed for MRSA and VRE using centrifugation, standard membrane filtration, and direct plating. Vancomycin-amended membrane-Enterococcus Indoxyl-β-D-Glucoside agar (m EI) and MRSASelect were used to isolate VRE and MRSA, respectively. The presence of MRSA and *S. aureus* was confirmed by PCR amplification of the *mecA* gene and the *S. aureus*-specific *nuc* gene. VRE was confirmed biochemically. Antimicrobial susceptibility testing was performed using the Sensititre microbroth dilution system. *Enterococcus* spp. was isolated in all water samples except after on-site ultraviolet radiation treatment. VRE was identified in a sample from a storage pond at the point of the inlet to the pumphouse that distributes the reclaimed wastewater to the irrigation system. No MRSA or methicillin-susceptible *S. aureus* (MSSA) was identified in any of the water samples. No MRSA, MSSA, VRE, or *Enterococcus* spp. were identified in air samples. Spray irrigation workers had a lower overall prevalence of *S. aureus* nasal

colonization (31%) when compared to a control group (46%), however a greater percentage of isolates from spray irrigation workers were multidrug resistant (25% vs. 17%). Our findings suggest that UV treatment effectively reduces *Enterococcus* spp. found in reclaimed wastewater but that open-air storage of UV-treated reclaimed wastewater can lead to recontamination with these organisms. The higher percentage of multidrug resistant *S. aureus* among spray irrigation workers compared to controls could be cause for concern and deserves additional research to evaluate whether there is a significant association with exposure to reclaimed wastewater.

Introduction

Spray irrigation is increasingly relying on reclaimed wastewater, resulting in potential exposures among spray irrigation workers to water that could contain biological contaminants as well as antibiotics (Angin, et al., 2005; Kocaman, et al., 2007). As droughts and water shortages become more common across the United States and the world, cities are using reclaimed wastewater for spray irrigation applications, as well as source water for drinking water treatment plants. *Staphylococcus aureus* and *Enterococcus* spp. are organisms that are commonly found on the skin and in the intestinal tracts of animals and humans that under certain conditions can cause potentially fatal diseases (Centers for Disease Control and Prevention (CDC), 2008; FDA, 2009; Murray, et al., 2002; Rusin, et al., 2003; Spacek & Vinetz, 2009). The emergence of antibiotic-resistant strains of *S. aureus* and *Enterococcus* spp. means that there is a risk that the illnesses caused by these bacteria might no longer be treatable with traditional antibiotics, posing significant risks to public health. If pathogens and antibiotic-resistant bacteria, such as *S. aureus* and *Enterococcus* spp., survive the wastewater treatment

process, spray irrigation workers using reclaimed wastewater could be exposed to these harmful agents.

Goals

One goal of our study was to evaluate the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. (VRE) in reclaimed wastewater used for spray irrigation and air samples from the spray irrigation site. We also sought to determine the prevalence of MRSA, VRE, *S. aureus,* and *Enterococcus* spp. in nasal and dermal swab samples collected from spray irrigation workers, an occupational group with high levels of exposure to reclaimed wastewater.

Hypothesis: Antibiotic-resistant *S. aureus* and *Enterococcus* spp. will be present in water, air, and dermal and nasal swab samples collected during wastewater irrigation activities.

The results of this study have implications not only for occupational exposures among spray irrigation workers, but also for the general public whose recreation areas might be irrigated with reclaimed wastewater or who consume drinking water that originated from treated wastewater.

Methods

Site Description

Samples were collected from a spray irrigation site located in the Mid-Atlantic region of the United States. The spray irrigation site receives treated wastewater from a municipal tertiary wastewater treatment plant. A discharge pipeline was built to distribute

treated wastewater from the treatment plant to a nearby steel mill. The spray irrigation site uses a polyvinyl chloride pipe connection to divert a segment of the treated wastewater from the discharge pipeline to the spray irrigation site before it reaches the steel mill. Once the treated wastewater reaches the spray irrigation site, it passes through an approximately two-foot long, double-walled aluminum screen and is then treated with ultraviolet (UV) radiation (Figure 5). After UV treatment, the water is pumped into an open-air pond at a rate of 230,000 gallons per day (Figure 5). The storage pond can hold up to 4 million gallons of water. Based on irrigation needs, water is then pumped from the holding pond to a shed that contains a pump to distribute the water to the appropriate spray heads (Figure 5). The spray irrigation site employs eight full-time employees and approximately 22 seasonal employees each year.

Sample Collection

Spray irrigation water and nasal and dermal swab samples were collected in August and October 2009. Air samples were collected in August, but not October 2009, because of precipitation that interfered with sample collection. Meteorological data were collected during each sampling trip.

Water

A total of eight water samples from before and after on-site UV radiation treatment ("Before UV" and "After UV"), input to the storage pond ("Input to Pond"), and inlet to the pumphouse from the pond ("Inlet to Pumphouse") were collected on August 17 and October 27, 2009 (Figure 5). All samples were collected in 500 mL or 1 L Nalgene collection bottles as grab samples. Samples were stored at 4°C during sampling

and transportation to the laboratory. All samples were processed within 24 hours of collection.

Air

On August 17, 2009 two air samples each were collected in the range of spray heads and at a control site that is not irrigated with reclaimed wastewater. Samples were collected using SKC BioSamplers (SKC, Eighty Four, PA). The BioSamplers were filled with 20 mL of phosphate buffered saline (PBS) and run for one hour at each sampling location. Thirty minutes into sampling, the BioSamplers were turned off, the volume of PBS in the collection vessel was measured, and distilled water was added until the volume was 20 mL again. Samples were stored at 4°C during sampling and transportation to the laboratory. Air samples were processed within 24 hours of collection.

Nasal and Dermal Swabs

Nasal and dermal swab samples were collected from a convenience sample of 13 spray irrigation workers who were available on August 17, 2009 at the time of sample collection. On October 27, 2009, nasal and dermal swabs were collected for a second time from three available participants, spray irrigation workers 1, 2, and 4. Swabs were collected to evaluate inhalation and dermal exposures to pathogens from reclaimed wastewater. Between September and November 2009, nasal and dermal swabs were also collected from a control group of office workers from a site that is not exposed to reclaimed wastewater. Office worker controls were age- and gender-matched to the spray irrigation study population.

Nasal swabs were collected using a rayon-tipped plastic applicator pre-moistened in Stuart's transport media (Copan, Italy). The swab was inserted approximately 1.25

centimeters into the participant's right nostril and gently rotated five times on the inside wall of the nostril.

Dermal swabs were also collected using a rayon-tipped plastic applicator pre-moistened in Stuart's transport media (Copan, Italy). An approximately five-by-five centimeter area of the participant's right forearm was swabbed with the rayon-tipped applicator by rolling the swab back and forth 15 times (Kullander, Forslund, & Dillner, 2009).

After sample collection, nasal and dermal swabs were inserted into Stuart's transport media, stored at 4°C during transportation to the lab, and processed within 24 hours of collection.

Questionnaire

A questionnaire was provided to all study participants to collect demographic and occupational information (Appendix B). Participants also were asked about risk factors that could increase the likelihood of *S. aureus* or MRSA colonization, such as whether they had ever personally worked in a healthcare setting and if anyone in their household had ever worked in a healthcare setting (Table 5).

Isolation

Isolation of S. aureus and MRSA from Water Samples

August Samples

Water samples collected in August were isolated based on a technique used by Börjesson et al. (2009) to recover *S. aureus* and MRSA from wastewater samples (Börjesson, et al., 2009). From each sample, 150 mL were centrifuged at 8000x*g* for 30

minutes. The resulting pellet was enriched in either Luria-Bertani Miller (LB Miller) Broth (BD Diagnostic Systems, Franklin Lakes, NJ) amended with cefoxitin and aztreonam or m Staphylococcus Broth (BD Diagnostic Systems, Franklin Lakes, NJ) to isolate MRSA and *S. aureus*, respectively. Enriched samples were incubated at 37°C for 24 hours. Samples enriched in amended LB Miller Broth were plated on MRSASelect (Bio-Rad Laboratories, Hercules, CA). Samples enriched in m Staphylococcus Broth were plated on Mannitol Salt Agar (MSA) (BD Diagnostic Systems, Franklin Lakes, NJ).

October Samples

Standard membrane filtration was used to isolate MRSA and *S. aureus* from October 27, 2009 water samples. 300 mL of each sample were filtered through 0.45 µm cellulose filters (Millipore, Billerica, MA). Filtered samples were enriched for MRSA and *S. aureus* in 40 mL of LB Miller Broth amended with cefoxitin and aztreonam and m Staphylococcus Broth. Enriched samples were then incubated at 37°C for 24 hours. Samples enriched in amended LB Miller Broth were streaked onto MRSASelect with a calibrated 10 µL loop. Samples enriched in m Staphylococcus Broth were streaked onto MSA, Baird Parker Agar (BD Diagnostic Systems, Franklin Lakes, NJ), and MRSASelect with a calibrated 10 µL loop.

Isolation and Enumeration of Enterococcus spp. from water

Standard membrane filtration, based on U.S. Environmental Protection Agency (EPA) Method 1600, was used to isolate VRE and other *Enterococcus* spp. from water samples ((EPA), 2002). One liter, 300 mL, and 100 mL of Before UV, After UV, and Input to Pond samples, and 200, 100, and 10 mL of the Inlet to Pumphouse samples were

filtered through 0.45 μm cellulose filters (Millipore, Billerica, MA). Filters were then placed on membrane-*Enterococcus* Indoxyl-β-D-Glucoside agar (m EI) (EMD Chemicals) and mEI amended with 16 μg/mL vancomycin to isolate *Enterococcus* spp. and VRE respectively and incubated for 24 hours in a circulating water bath set at 41°C.

Colonies with a blue halo, regardless of the actual colony color, were presumptively identified as *Enterococcus* spp. Using plates with between 30 and 300 colony forming units, enumeration of the concentration of *Enterococcus* spp. and VRE per 1 mL of water was calculated.

Isolation of S. aureus and Enterococcus spp. from nasal and dermal swabs

Nasal and dermal swabs were streaked onto MSA and Baird Parker Agar for isolation of *S. aureus* and onto Enterococcosal Agar (BD Diagnostic Systems, Franklin Lakes, NJ) for isolation of *Enterococcus* spp. MSA and Baird Parker Agar plates were incubated at 37°C for 24 hours while the Enterococcosal Agar plates were incubated at 41°C for 24 hours.

Isolation of S. aureus and Enterococcus spp. from air samples

Standard membrane filtration was used to filter 10 mL of each air sample through 0.45 μm cellulose filters. Filters were then enriched in m Staphylococcal Broth and Enterococcosel Broth (BD Diagnostic Systems, Franklin Lakes, NJ) to enrich for *S. aureus* and *Enterococcus* spp. respectively. m Staphylococcal Broth was incubated for 24 hours at 37°C while Enterococcosel Broth was incubated at 41°C for 24 hours. A 10 μL loop of m Staphylococcal Broth was streaked onto MSA and incubated for 24 hours at

37°C. A 10 µL loop of Enterococcosel broth was streaked onto EA and incubated at 41°C for 24 hours.

Identification

Identification of S. aureus from water, air, and swab samples

Presumptive *S. aureus* isolates were identified as yellow colonies with yellow precipitate on MSA and shiny black colonies with a clear halo on Baird Parker Agar. Presumptive MRSA isolates were identified as bright pink colonies on MRSASelect. Presumptive colonies underwent a series of biochemical tests to further confirm their identification as *S. aureus* or MRSA. Gram staining, catalase testing, and coagulase testing were performed on all presumptive *S. aureus* and MRSA isolates. All isolates appearing as Gram-positive cocci, with positive catalase and coagulase tests were confirmed as presumptive *S. aureus* and MRSA.

DNA from presumptive MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates was extracted using the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, CA). The presence of MRSA and MSSA was confirmed by PCR amplification of the *mecA* gene and the *S. aureus*-specific *nuc* gene. The primers used to detect the *nuc* and *mecA* genes were NUC1 and NUC2, and MECA1 and MECA2 (Fang & Hedin, 2003). An internal control of a 16S rRNA gene was added to the PCR duplex. The conventional PCR program used for amplification was based on the PCR assay previously described by Fang and Hedin in 2003 (Fang & Hedin, 2003).

Identification of Enterococcus spp. from water, air, and swab samples

Enterococcus spp. and VRE were confirmed biochemically by Gram staining, detection of pyrrolidonyl arylamidase activity with a PYR test, and the ability of the bacterium to break down hydrogen peroxide by catalase. All isolates appearing as Gram-positive cocci, with negative catalase, and positive PYR tests were confirmed as *Enterococcus* spp. and VRE.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on MRSA and MSSA isolates using the Sensititre microbroth dilution system (Trek Diagnostic systems Inc., Cleveland, OH, USA). Samples were grown overnight on Trypticase Soy Agar with 5% Sheep Blood (BD Diagnostic Systems, Franklin Lakes, NJ). *Enterococcus faecalis* 29212 and *S. aureus* 29213 were used as controls for all susceptibility testing. Antibiotic minimal inhibitory concentrations (MICs) were determined using general gram-positive plates (GPN3F) (Trek Diagnostic systems Inc., Cleveland, OH, USA), and were recorded as the lowest concentration of an antimicrobial that inhibited the growth of the bacterial suspension in Mueller-Hinton Broth (Beier, et al., 2008). Resistance breakpoints were determined using the Clinical and Laboratory Standards Institute (CLSI) standards for antimicrobial susceptibility testing (CLSI, 2010).

Results

Water

We did not identify MSSA or MRSA in any of the spray irrigation water samples. *Enterococcus* spp. was isolated from Before UV, Input to Pond, and Inlet to Pumphouse samples. The concentration of *Enterococcus* spp. was undetectable in the After UV samples in both August and October. The concentration of *Enterococcus* spp. increased between Input to Pond and Inlet to Pumphouse samples by three orders of magnitude in August and October (Figure 6). A concentration of 1×10^{-2} colony forming units/mL of VRE was detected in a sample taken from the Inlet to the Pumphouse in October (Figure 6). No VRE was isolated from any samples in August. At this time, we have not identified whether the *Enterococcus* spp. or VRE we found in the spray irrigation water samples can be traced to human or animal strains.

The levels of *Enterococcus* spp. in the Before UV samples, which came directly from the wastewater effluent, were highest in the samples collected on August 17, 2009. The concentration of *Enterococcus* spp. in the treated municipal wastewater effluent was also higher in August than October 2009 (refer to Chapter 3). The concentrations of *Enterococcus* spp. in the Input to Pond and Inlet to Pumphouse samples were slightly higher on October 27 compared to August 17.

Air

No MRSA, VRE, MSSA, or *Enterococcus* spp. was isolated from air samples taken at the spray irrigation area or at the control site.

Nasal and Dermal Swabs

Thirty-one percent of spray irrigation workers exposed to reclaimed wastewater were colonized with MSSA in their right naris. Of the three spray irrigation workers swabbed on both August 17 and October 27, 2009, worker 1 was not colonized with *S. aureus* on either occasion, worker 2 was colonized in August but not October, and worker 4 was colonized on both dates (Table 4). None of the spray irrigation workers' dermal swabs were positive for *S. aureus.* Among the control group of office workers, there was a 46% rate of nasal colonization with MSSA. A dermal swab isolate from one office worker control (7.7%) was positive for MSSA. Based on the PCR duplex for the *mecA* and *nuc* genes, none of the nasal or dermal swab isolates from spray irrigation workers or office workers were identified as MRSA. However, one isolate from spray irrigation worker 13 possessed the *mecA* gene and was identified as methicillin-resistant coagulase negative *S. aureus* (MR-CoNS) (Table 4). None of the nasal or dermal swabs from the spray irrigation workers or office workers were positive for VRE or *Enterococcus* spp.

The spray irrigation and office workers had a similar percentage of MSSA nasal isolates that were resistant to ampicillin and pencillin, but differed in resistance to erythromycin, tetracycline, and linezolid (Figure 7). From the spray irrigation workers, 75% of the MSSA nasal swab isolates were resistant to at least one antibiotic and 25% of isolates (one out of four) were multidrug resistant, resistant to two or more classes of antibiotics (Table 4). Sixty-seven percent of office worker nasal swab MSSA isolates were resistant to one or more antibiotic (Table 4). Seventeen percent of MSSA nasal swab isolates from office workers, one out of six MSSA isolates, were multidrug resistant. The average number of antibiotics that isolates were resistant to, was 1.75 and

1.33 among the spray irrigation worker and office worker nasal swab isolates respectively.

The MSSA isolate from office worker 7's dermal swab was multidrug resistant to high concentrations of erythromycin, ampicillin, and penicillin (Table 4).

The MR-CoNS isolate from spray irrigation worker 13 was multidrug resistant to oxacillin, penicillin, and tetracycline (Table 4).

The odds ratio of being colonized with MSSA as a spray irrigation worker versus an office worker control was 0.52 (95% CI, 0.10-2.60), meaning that the office workers in this study were 1.93 times more likely to be colonized with MSSA as the spray irrigation workers, but this association was not statistically significant.

Both of the spray irrigation workers (n=2) who reported having a household member who had ever worked in a healthcare setting had MSSA positive nasal swabs. However, only 60% of office worker controls (three out of five) who reported having a household member who had ever worked in a healthcare setting had MSSA positive nasal swabs (Table 5).

Discussion

Water

In August and October 2009, we analyzed reclaimed wastewater used for spray irrigation, air, and nasal and dermal swab samples at a site in the Mid-Atlantic region for the presence of MRSA, MSSA, VRE, and *Enterococcus* spp. To our knowledge, this is the first study to evaluate the presence of antibiotic-resistant bacteria in reclaimed wastewater used for spray irrigation and resulting occupational exposures in the United

States. Our results suggest that concentrations of gram-positive antibiotic-resistant bacteria present in wastewater are significantly reduced through disinfection, but can still survive in varying degrees in reclaimed wastewater used for spray irrigation.

The finding that *Enterococcus* spp. were isolated in Before UV but not After UV samples suggests that UV treatment successfully reduced the amount of *Enterococcus* spp. to an undetectable level. In terms of MRSA, Tolba et al. found that a free chlorination concentration of 2.9 ppm killed both hospital- and community-acquired MRSA (Tolba, et al., 2008). The wastewater treatment plant supplying treated wastewater to the spray irrigation site uses chlorine as a method for disinfection. Bleach is added to the wastewater in the chlorination step to give a concentration of chlorine of 2 or 3 ppm. The concentration of chlorine used could have been high enough to eradicate all viable *S. aureus* from the reclaimed wastewater. Also, depending on how long it takes the treated wastewater to travel from the wastewater treatment plant to the spray irrigation site, there could be die-off of any residual *S. aureus* in the wastewater effluent in the distribution system. Tolba et al. found that there is significant die-off of MRSA after 14 days in both fresh and saline water (Tolba, et al., 2008). *Enterococcus* spp., however, can survive between 14 and 50 days in water, which could explain the presence of *Enterococcus* spp. but not *S. aureus* in the Before UV samples (Signoretto & Canepari, 2008).

The spike in the concentration of *Enterococcus* spp. in the Inlet to Pumphouse samples, which were collected from the open-air storage pond, suggests that recontamination of the reclaimed wastewater could had occurred. *Enterococcus* spp. is a common inhabitant of human, animal, and avian intestinal tracks and could have entered

the storage pond from animal or avian fecal matter. If animal or avian sources were the cause of the increase of *Enterococcus* spp. concentrations in the Inlet to Pumphouse samples, covering the storage pond would protect reclaimed wastewater supplies from exposure to animals and runoff, which might also contain fecal contaminants. Pathogens, including antibiotic-resistant bacteria, introduced into the storage pond by animals could pose a risk of infection to spray irrigation workers and other individuals at the spray irrigation site. Antibiotic-resistant bacteria, including MRSA and VRE, can move from animals to the environment and from environmental media to humans (Kuhn, et al., 2005; Moodley, et al., 2008; Smith, et al., 2009; Van Belkum, et al., 2008).

However, the possibility also exists that *Enterococcus* spp. were reduced to a viable but not culturable state after UV treatment, and the pond environment allowed them to recover and reproduce (Signoretto & Canepari, 2008). Previous studies have found that only 5 to 10% of bacteria found in wastewater are culturable (Hiraishi, 1998; Xi, et al., 2009). Disinfection might reduce the number of culturable organisms by injuring cells, but not completely kill the organisms (Xi, et al., 2009). Although heterotrophic plate counts decreased by six orders of magnitude between influent raw wastewater and disinfected effluent in one study, the occurrence of 16S rRNA, a type of RNA that encodes for protein production in prokaryotes, decreased by only one order of magnitude (Xi, et al., 2009). *E. faecalis* and *E. hirae* have been shown to be able to resume cell reproduction after being in a viable but not culturable state upon entering a favorable environment (Lleò, et al., 2001; Signoretto & Canepari, 2008).

The finding that *S. aureus* was not identified in any of the spray irrigation samples could be explained by a number of factors. First, *S. aureus* might have been effectively

killed in the tertiary treatment step (chlorination, de-chlorination, and filtration) at the wastewater treatment plant where the wastewater originated. In Chapter 3 of the present thesis, MRSA and *S. aureus* were identified in all wastewater samples except for effluent. Also, because *S. aureus* typically occurs in low concentrations in water, and because we collected only eight grab samples from the spray irrigation site, by chance we might have collected samples that did not contain *S. aureus*, even if it was present (Tolba, et al., 2008). Also, although many studies have compared various isolation techniques for MRSA, the majority of the existing protocols for isolating MRSA were developed for clinical swab specimens. Protocols for culturing clinical specimens were taken into account when developing the protocol for the present study, but organisms behave differently in different environments. The protocol used to isolate MRSA and *S. aureus* in August was not successful, even with influent wastewater samples that on other sampling occasions, with the protocol followed in October, were always positive for MRSA and MSSA. Also, although MSA has previously been found to work well in low prevalence areas, we experienced overgrowth (Smyth, et al., 2001). The overgrowth observed on MSA could have been the manifestation of out competition of *S. aureus* and MRSA by other bacteria. The inhibitory influence of other bacteria on the growth of MRSA has been documented previously (Grmek-Kosnik, et al., 2005).

Although we did not identify MRSA or VRE in reclaimed wastewater samples at the spray irrigation site through culture methods, there could be free antibiotic resistance genes in the reclaimed wastewater piped to the irrigation site. DNA released from bacteria may remain in water without degrading for varying amounts of time (Pote, et al., 2003; Volkmann, et al., 2004). Total DNA extraction of water samples to determine the

presence of the *mecA* and vancomycin resistance *van* genes in reclaimed wastewater spray irrigation samples is an area for future investigations.

Air

The absence of *S. aureus* and *Enterococcus* spp. from the air samples collected in this study could be more the result of the type of sampling equipment used than an actual lack of these organisms in the air at the spray irrigation site. In a study by Chang et al. (2001), although *Staphylococcus and Micrococcus* were the dominant aerosolized bacterial species in their samples, the vigorous scrubbing that takes place in impingers was suspected of breaking up bacterial clusters and reducing the number of viable organisms (Chang, Chung, Huang, & Su, 2001; Thorne, Kiekhaefer, Whitten, & Donham, 1992). In low concentration situations, such as the open-air spray irrigation site, impactors are usually more efficient than impingers (Lundholm, 1982; Thorne, et al., 1992). A study by Thorne et al. found that for collecting aerosolized enteric bacteria, only multi-stage impactors consistently recovered the bacteria compared to impingers and filters (73% data yield) (Thorne, et al., 1992). In future iterations of this study, spray irrigation site air samples should be collected using impactors instead of impingers.

Nasal and Dermal Swabs

S. aureus prevalence among the spray irrigation workers in our study was 31%, slightly higher than the prevalence recorded in the general population (20-30%) (Bassetti, et al.; John & Barg, 1999). Although the total prevalence of MSSA was higher in our control population (46%) than in our spray irrigation worker study population, a greater percentage of nasal swab isolates from spray irrigation workers were multidrug resistant, 25% versus 17%, and resistant to one or more antibiotics, 75% compared to 67%. The

average number of antibiotics that isolates were resistant to, was slightly higher among the spray irrigation worker (1.75) than the office worker (1.33) isolates. A direct association between the level of antibiotic resistance and multidrug resistance patterns among MSSA isolates and exposure to reclaimed wastewater cannot be determined in the current study. However, these results suggest that additional sampling efforts should be conducted and a greater number of samples should be collected to determine if the higher percentage of multidrug resistant MSSA isolates among spray irrigation workers is statistically significant and if it is associated with exposure to reclaimed wastewater.

The subject population in the current study was mostly young to middle-aged (median age = 32 years) Caucasian males that are concurrently exposed to chemical pesticides and fertilizers. Although previous studies have found that male gender and young age are risk factors for *S. aureus* nasal colonization, the study population's exposure to chemical substances and water might interact with pathogens in the body with varying results (Gorwitz, et al., 2008; Halablab, Hijazi, Fawzi, & Araj, 2009). A study by Halablab et al. found that washing the nasal passages with water more than twice daily was significantly associated with lower *S. aureus* nasal colonization rates (Halablab, et al., 2009). Halablab et al. explains this association by hypothesizing that water may interfere with adherence of *S. aureus* in the nasal passages (Beachey, 1981; Halablab, et al., 2009). As spray irrigation workers are by definition routinely exposed to water, this could help explain why the prevalence of MSSA was lower in the spray irrigation workers than the office worker controls.

The frequent application of chemical substances at the study site could also have an effect on the resident microbial population and likelihood of infection. Exposure to

chemicals could cause irritation of skin and mucous membranes, hindering the body's ability to protect against bacterial infections (D. Lewis, Gattie, Novak, Sanchez, & Pumphrey, 2002). *S. aureus* in particular has been noted to preferentially invade irritated tissue (Brook, 1992; D. Lewis, et al., 2002; Leyden & Kligman, 1978). Although the prevalence of *S. aureus* in this study was not higher among spray irrigation workers than office worker controls, additional research into the interaction between chemicals and colonization rates at reclaimed wastewater application sites should be further investigated.

Limitations

General

To provide a more complete picture of exposure to antibiotic-resistant bacteria from reclaimed wastewater used for spray irrigation we would need to collect additional samples at the site used in the current study, as well as samples from additional spray irrigation sites. In the current study we collected swab samples only once from the entire study population, and twice from three participants. Additional swab samples would help to capture seasonal variations, and determine if participants colonized with *S. aureus* are continuous or intermittent carriers. Additional sampling trips would also help us evaluate seasonal variability in MRSA and VRE isolated from water samples. Wastewater treatment plants across the United States apply different levels of treatment (secondary or tertiary) and use a variety of tertiary treatment techniques (filtration, UV radiation, chlorination, etc.). Differing levels of wastewater treatment could affect the concentration

of antibiotic-resistant bacteria in effluent wastewater transported to spray irrigation site, as well as occupational exposure levels.

Water

In this study, we did not collect measurements on water parameters such as temperature, dissolved oxygen, pH, and salinity. All of these environmental parameters could have affected the survival of the pathogens of interest.

Some of the *Enterococcus* spp. isolated on non-vancomycin-amended mEI, could be VRE. To identify additional VRE isolates, and determine antimicrobial susceptibility patterns, a combination of PCR, to identify possession of *van* genes, and antimicrobial susceptibility testing would be needed.

To estimate the source of *Enterococcus* spp. and VRE isolated at the spray irrigation site, identification of the isolates to the species, and potentially strain, level is needed. To determine if the vancomycin resistance genes possessed by the VRE isolates found in the Inlet to the Pumphouse samples are acquired or intrinsic, the specific *van* genes need to be identified.

Nasal and Dermal Swabs

The small sample size of the spray irrigation worker population was a major limitation in the current study. Previous studies have found low carriage rates of MRSA in the community, for instance in the range of 0.70-3.9% among veterinary professionals and 0.6% in a religious community (Coronado, et al., 2007; Moodley, et al., 2008). Because our sample size was so small, it would have been largely due to chance to collect swabs from a spray irrigation or office worker that was colonized with MRSA. The small sample size also limited our ability to report statistically significant findings.

The nares are the most common body site to screen for *S. aureus* colonization, but they are not the only common sites of colonization. The throat, perineum, axilla, and groin are also important reservoirs of *S. aureus* in, or on, the body (Bassetti, et al., 2005; Gorwitz, et al., 2008; Grmek-Kosnik, et al., 2005; Mertz, et al., 2007). Several studies have found that screening for *S. aureus* from only one body site, including the nares, can lead to false negative results (A. Currie, et al., 2008; Grmek-Kosnik, et al., 2005; Mertz, et al., 2007; Meurman, Routamaa, & Peltonen, 2005). By only swabbing the right nostril and right forearm of spray irrigation and office workers, we could be underestimating the prevalence of *S. aureus* in both populations. At a minimum, nasal swabs are usually collected from both nares, however, our approved Institutional Review Board Human Subjects Testing document only allowed us to sample from the right nare and right forearm (Bassetti, et al., 2005; Gorwitz, et al., 2008; Ohara-Nemoto, Haraga, Kimura, & Nemoto, 2008; Schuenck, et al., 2006).

Although the percentage of multidrug resistant MSSA isolates was higher among spray irrigation workers than the office worker controls, the spray irrigators could be exposed to *S. aureus* from a source other than reclaimed wastewater. Although they are a direct measure of exposure levels, samples such as nasal and dermal swabs do not provide information on the source of exposure. Without evaluating the presence of resistance genes in spray irrigation water samples with a total DNA extraction, and without determining the specific strains of *S. aureus* in nasal swab samples, we are unable to determine if reclaimed wastewater used for spray irrigation was the source of resistance in *S. aureus* isolated from nasal swabs. Even using these methods, it could be difficult to determine the origin of these isolates conclusively.

Conclusions

The low concentrations of VRE found in the reclaimed wastewater spray irrigation samples and the absence of MRSA from these samples, suggests that reclaimed wastewater from tertiary treatment, with UV treatment and covered storage, can be used as a source of spray irrigation with a low risk of exposure to gram-positive, antibiotic-resistant bacteria. However, as this is the first study of its kind, the public health risks from exposure to reclaimed wastewater need to be explored further. Based on the increase in *Enterococcus* spp. concentration and detection of VRE in the open-air storage pond used to store reclaimed wastewater for spray irrigation, it seems prudent to store the water in a covered location. Covering the reclaimed wastewater could help prevent possible fecal contamination from wildlife and the addition of other contaminants from surface runoff into the pond.

The occurrence of multidrug resistant MSSA among spray irrigation workers could be cause for concern. Additional research is needed to determine whether the higher percentage of multidrug resistant MSSA among spray irrigation workers compared to office worker controls is associated with exposure to reclaimed wastewater.

Figure 5. Spray irrigation site: reclaimed wastewater treatment and distribution process

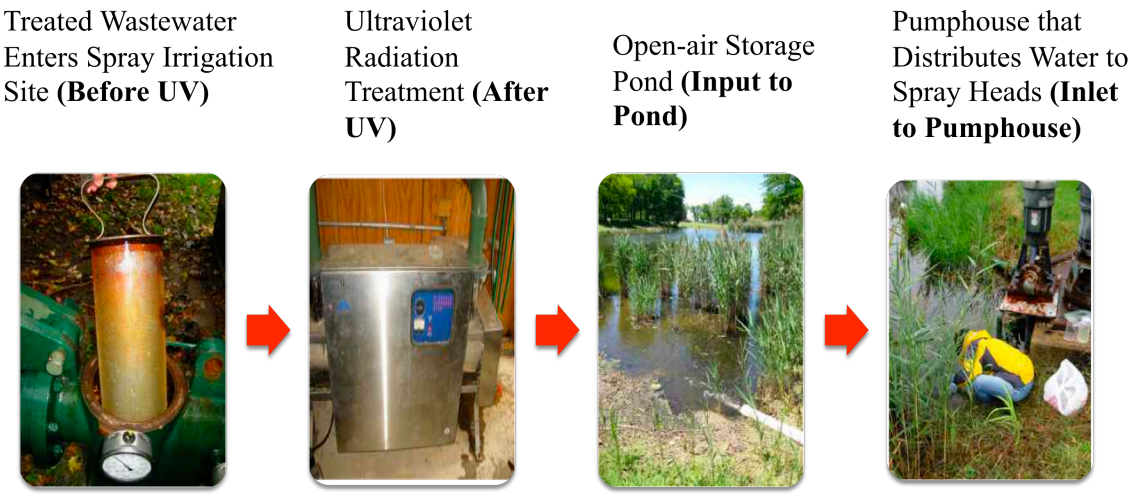


Figure 6. Concentration of *Enterococcus* spp. and vancomycin-resistant *Enterococcus* spp. in reclaimed wastewater used for spray irrigation samples

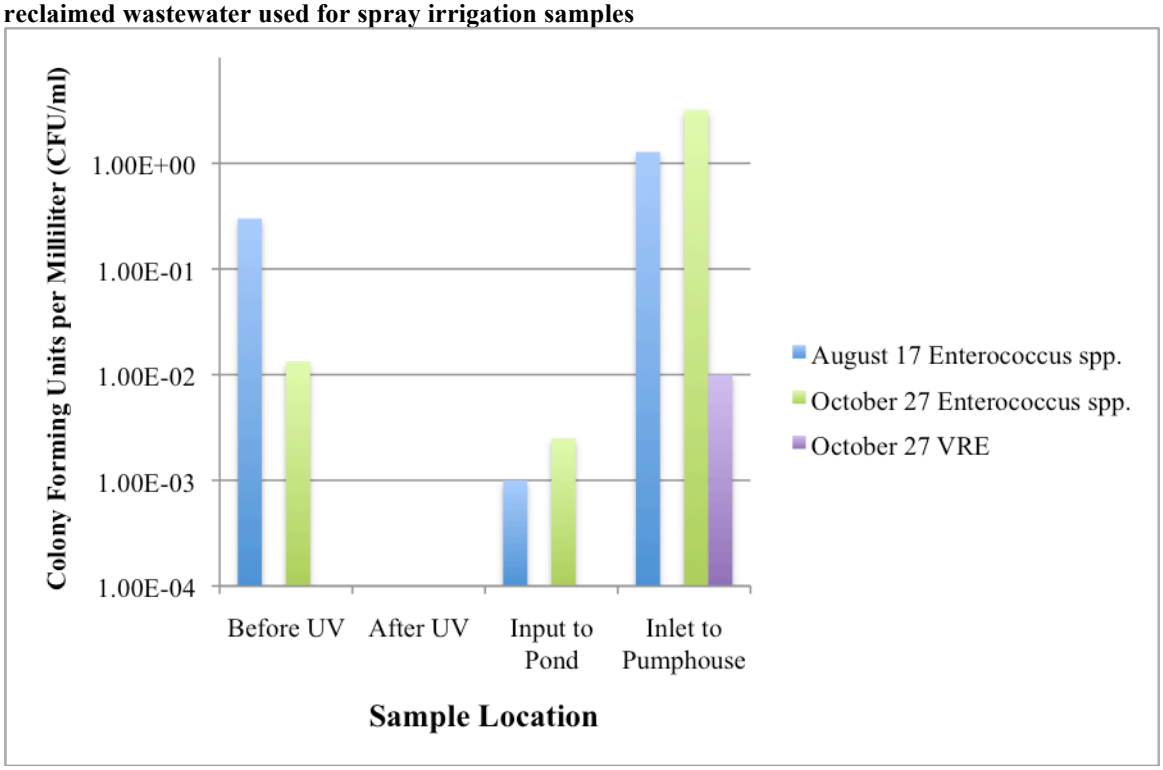


Figure 7. Percentage of methicillin-susceptible *Staphylococcus aureus* nasal swab isolates from spray irrigation worker and office worker controls expressing resistance to common gram-positive antibiotics

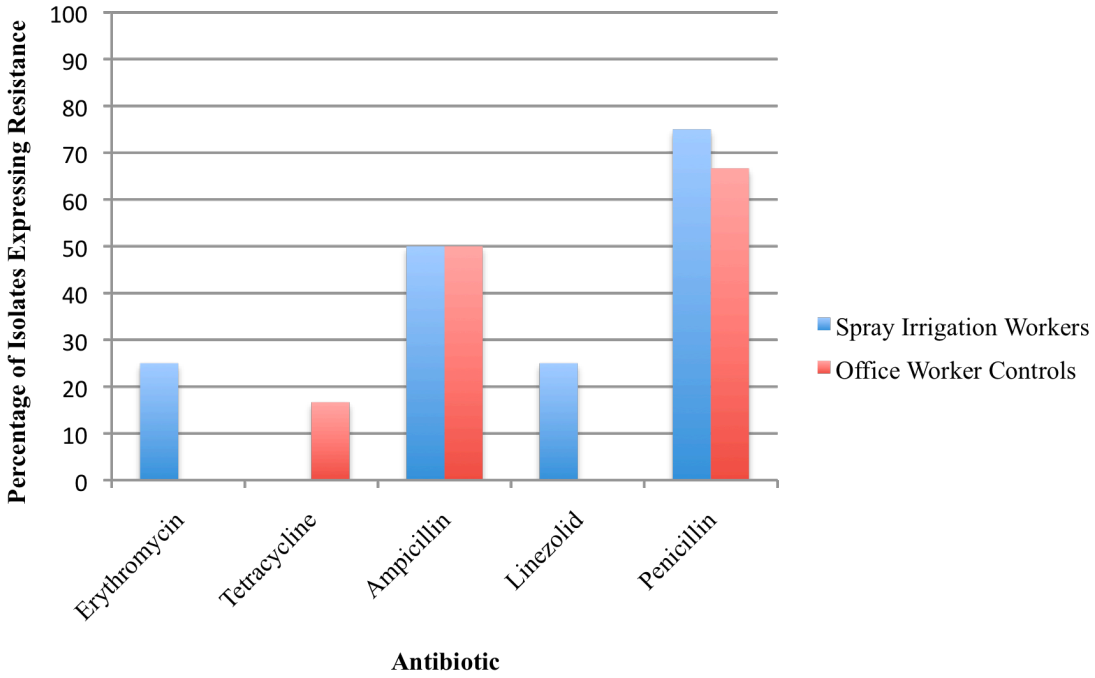


Table 4. Minimum inhibitory concentrations (MICs) of select *Staphylococcus aureus* and methicillin-resistant coagulase negative *Staphylococcus* isolates from spray irrigation and office workers' nasal and dermal swabs

	MIC (µg/ml), Interpretation					
Subject ID, swab type	**Erythromycin**	**Tetracycline**	**Ampicillin**	**Linezolid**	**Penicillin**	**Oxacillin**
Spray Irrigation Worker2, nasal[a]	0.5, S	2, S	16, R	4, S	≥16, R	0.25, S
Spray Irrigation Worker4, nasal[a]	4, S	2, S	0.12, S	4, S	0.06, S	0.25, S
Spray Irrigation Worker4_II, nasal[a,c]	≥8, R	4, S	0.12, S	4, S	0.06, S	0.25, S
Spray Irrigation Worker5, nasal[a]	0.5, S	2, S	0.12, S	4, S	0.25, R	0.25, S
Spray Irrigation Worker11, nasal[a]	≥8, R	2, S	8, R	8, R	≥16, R	0.25, S
Spray Irrigation Worker13, nasal[b]	0.25, S	≥32, R	0.12, S	2, S	0.25, R	0.5, R[d]
Office Worker1, nasal[a]	0.5, S	≥32, R	4, R	4, S	8, R	0.25, S
Office Worker6, nasal[a]	0.5, S	2, S	0.12, S	4, S	0.06, S	0.25, S
Office Worker7, nasal[a]	0.5, S	2, S	0.25, S	4, S	1, R	0.25, S
Office Worker7, dermal[a]	≥8, R	2, S	16, R	4, S	≥16, R	0.5, S
Office Worker9, nasal[a]	0.5, S	2, S	0.12, S	4, S	0.06, S	0.25, S
Office Worker10, nasal[a]	0.25, S	2, S	16, R	4, S	≥16, R	0.25, S
Office Worker13, nasal[a]	0.5, S	2, S	≥32, R	4, S	≥16, R	1, S

[a]Methicillin-susceptible *Staphylococcus aureus.*
[b]Methicillin-resistant coagulase negative *Staphylococcus* spp. (MR-CoNS) isolate.
[c]Nasal swab taken from Spray Irrigation Worker4 in October. Swabs were taken from only Worker1, 2, and 4 in both August and October. Worker4 was the only participant with persistant colonization.
[d]The MIC for coagulase negative *Staphylococcus* spp. to oxacillin is 0.5 µg/ml.

Table 5: Demographic characteristics and risk factors of spray irrigation workers and office worker controls

Subject ID	Gender	Age	Race	Education	Duration in Job (years;months)	Days/Week in Job	Hours/Day in job	Do you wear Personal Protective Equipment? (Y/N)	Yearly Income ($1000s)	Personally Worked in Healthcare Setting? (Y/N)	Anyone in Household worked in Healthcare? (Y/N)	Ever diagnosed with MRSA? (Y/N)	Smoked at least 100 cigarettes in past 6 mos? (Y/N)	Do you smoke now? (Y/N)
SPRAY IRRIGATION WORKERS														
SI001	Male	41	Caucasian	College	10; 8	6	8	N		Y	N	N	N	N
SI002	Male	26	Caucasian	College	4; 2	7	8	N	25-35	N	N	N	Y	Y
SI003	Male	40	Caucasian	High School	5; 7	7	8	Y	25-35	N	N	N	Y	N
SI004	Male	30	Caucasian	College	4; 5	7	8	N	35-50	N	N	N	Y	Y
SI005	Male	54	Other	High School	2; 4	14	48	N	Less than 15	N	Y	N	N	Y
SI006	Male	25	Caucasian	High School	1; 7	6	8	N	15-25	N	N	N	Y	Y
SI007	Male	18	Caucasian	College	1; 3	7	8	N	Less than 15	N	N	I don't know	N	N
SI008	Male	49	Caucasian	High School	15; 0	7	40	N		N	N	N	N	Y
SI009	Female	41	Hispanic	High School	0; 6	6	8	N		N	N	N	N	N
SI010	Male	32	Caucasian	High School	0; 10	6	8	NA	35-50	N	N	N	N	N
SI011	Male	17	Caucasian	High School	3; 0	5	8	N	Less than 15	N	Y	I don't know	N	N
SI012	Male	17	Caucasian	High School	0; 2	5	8	N	Less than 15	N	N	I don't know	N	N
SI013	Male	32	Caucasian	High School	0; 5	7	8	N	Less than 15	N	N	N	Y	Y
OFFICE WORKER CONTROLS														
OW001	Male	25	Caucasian	College	0;2	5	6	NA	15-25	N	N	I don't know	N	N
OW002	Male	26	Caucasian	College	0;2	5	12	NA	15-25	N	N	N	N	N
OW003	Male	28	Caucasian	College	1;3	5	9	NA	15-25	N	N	N	N	N
OW004	Male	24	Caucasian	College	2;0	5	8	N	15-25	N	Y	N	N	Y
OW005	Male	19	Caucasian	College				NA	Less than 15	N	N	N	N	N
OW006	Male	31	Caucasian	College	5;2	5	10	NA	>50	N	Y	I don't know	N	Y
OW007	Male	40	Caucasian	College	5;0	5	8	N	>50	N	N	I don't know	N	N
OW008	Male	36	Caucasian	College	7;0	5	10	NA	>50	N	N	N	N	N
OW009	Male	35	Caucasian	College	2;11	6	10	NA	35-50	N	N	I don't know	N	N
OW010	Male	20	Caucasian	College	4;0	5	4	NA	Less than 15	N	Y	N	N	
OW011	Male	36	Caucasian	College	3;4	5	6	N	>50	Y	Y	N	N	N
OW012	Male	21	Caucasian	College				NA	Less than 15	N	N	N	N	N
OW013	Male	55	Caucasian	College	20; 4	5	9	NA	>50	Y	Y	N	N	N

Chapter 5: Overall Conclusions and Public Health Implications

Summary

Reclaimed wastewater is already being used across the United States and around the world, but limited studies have been conducted on the potential public health risks from exposure to this source of water (Rose, 2007; Rose, et al., 1999). In terms of water conservation, reuse of treated wastewater has enormous potential and could be a boon for protecting against degradation and drawdown of surface water sources. However, before the number of wastewater reuse applications continues to grow, to be cautious in order to avoid possible human health risks, additional research is needed on the types and concentrations of contaminants in reclaimed wastewater.

The research described in this thesis sought to begin the process of evaluating possible health risks associated with exposure to antibiotic-resistant bacteria that may be present in reclaimed wastewater. This study only analyzed reclaimed wastewater at a spray irrigation site being used for landscaping purposes. It should be noted that reclaimed wastewater is also currently being used to irrigate agricultural fields and as a source for drinking water treatment plants, among other applications (EPA, 2004a; Levine & Asano, 2004). Although this thesis research evaluated occupational exposure to reclaimed wastewater, because of its wide use, individuals in the general public are also exposed to reclaimed wastewater.

In conclusion, the results of this thesis project suggest that tertiary treatment reduces the concentration of VRE and MRSA to undetectable levels in effluent and most

reclaimed wastewater samples used for spray irrigation that have undergone additional on-site UV treatment. MRSA and VRE were, however, isolated from influent, activated sludge, and secondary clarifier wastewater samples. Our findings suggest that secondary treatment is not effective at eradicating MRSA and VRE in wastewater effluent that ultimately could be used in downstream applications. If this is the case, then approximately 100 million individuals in the United States, serviced by secondary treatment, or less than secondary treatment, wastewater facilities (9,261 facilities) could be exposed to antibiotic-resistant bacteria if reclaimed wastewater from secondary treatment facilities is allowed for irrigation applications in their state (Figure 8) (EPA, 2008a).

This was the first published study to isolate MRSA from a wastewater treatment plant in the United States. The isolation methods for *S. aureus* and *MRSA* from water are still in their infancy, so our results could be underestimating the total *S. aureus* and MRSA present in wastewater and spray irrigation water samples (Börjesson, et al., 2009). Even low concentrations of MRSA in treated wastewater could present health risks to individuals with high sensitivity or high levels of exposure, i.e. wastewater treatment plant and spray irrigation workers. The presence of MRSA in wastewater samples also represents a risk for transfer of resistance genes to other bacteria that are better adapted to aquatic environments (Börjesson, et al., 2009; Pote, et al., 2003; Schwartz, et al., 2003; Volkmann, et al., 2004). Additional studies exploring different tertiary treatments are necessary to determine what types of treatment can successfully reduce antibiotic-resistant bacteria to non-detectable levels and what types of treatment can successfully reduce the risk of transfer of resistance genes.

No spray irrigation workers exposed to reclaimed wastewater in our study had nasal or dermal colonization of MRSA or VRE. Methicillin-susceptible *S. aureus* (MSSA) nasal colonization rates among the spray irrigation workers agreed with rates reported in the general population (31%), however 25% of the MSSA isolates were multidrug resistant. A higher percentage of MSSA nasal swab isolates were multidrug resistant from spray irrigation workers exposed to reclaimed wastewater compared to office worker controls (17%). To account for possible seasonal and regional variations, samples should be collected throughout the year and from multiple geographic regions.

Public Health Implications

A lack of epidemiological data limits the ability to determine the risk of infection from specific concentrations of microorganisms in environmental media (EPA, 1986a; Maier, 2009). Current national and state standards for water quality are based on studies conducted over twenty years ago and focused primarily on gastrointestinal illness outcomes (EPA, 1986a). However, the most commonly reported illnesses associated with water exposure are skin, throat, and upper respiratory tract (Charoenca & Fujioka, 1993; Favero, 1985; Hunter, Beach, Fleming, Teunis, & Wade, 2007; Maier, 2009). As recently as 2007, an expert scientific board suggested that EPA update its microbial water quality standards, but this has yet to take place (Hunter, et al., 2007). The findings from the current study will hopefully lay the foundation for future studies to determine the risk of infection from certain concentrations of MRSA, MSSA, VRE, and VSE isolated from wastewater and reclaimed wastewater irrigation samples.

Federal water quality standards, including criteria for acceptable bacterial concentrations, are only suggestions and not legally enforceable (EPA, 1986b; Maier,

2009; Salzman & Thompson, 2007). Legal authority for controlling ambient water quality degradation rests with the states (Houck, 2002; Salzman & Thompson, 2007). For drinking water, the only bacterial concentrations contained in these regulations are for heterotrophic plate counts, coliforms, fecal coliforms, mycobacteria, and *Legionella* (EPA, 2009). However, it has been noted in numerous studies that enterococci are a better indicator of fecal contamination and have a stronger correlation with the occurrence of gastrointestinal illnesses than coliforms (EPA, 1986a; Favero, 1985; Maier, 2009). Enterococci, along with *E. coli*, are currently used as water quality indicators for recreational water (EPA, 1986a). The current EPA guidelines for recreational water quality standards are 33 CFU/100 mL of enterococci in freshwater and 35 CFU/100 mL in marine waters (EPA, 1986a; Maier, 2009). The EPA standard is based on an acceptable risk of 8 gastrointestinal illnesses per 1000 swimmers in freshwater and 19 gastrointestinal illnesses per 1000 swimmers in marine waters (EPA, 1986a). When freshwater is lightly or infrequently used for full body contact, the allowable CFUs/mL is even greater (107 and 151 CFU/mL) (EPA, 1986a). The concentration of enterococci isolated in the Inlet to Pumphouse samples at the spray irrigation in this study on August 17 and October 27, 2009, were 128 and 320 CFU/100 mL respectively, one order of magnitude above EPA standards for enterococci in freshwater recreation waters with full body contact. The concentration of enterococci isolated in the Input to Pumphouse sample on August 17 was above the allowable levels for recreational water when lightly used for full body contact. The concentration of enterococci isolated in the October 27 Input to Pumphouse sample was above the allowable levels for recreational water for both lightly and infrequently used for full body contact. Also, a review of EPA

recreational water quality standards by an expert scientific panel, concluded that the acceptable risk and the corresponding water quality standards had been set somewhat arbitrarily, do not take into account sensitive subpopulations, and might not be applicable to current water conditions and population characteristics (Hunter, et al., 2007). Based on these critiques and the results of the present study, the concentrations of *Enterococcus* spp. and VRE found in wastewater effluent and reclaimed wastewater samples might present a greater risk for gastrointestinal infection from exposure to reclaimed wastewater.

Because skin, eye, ear, and upper respiratory infections are more common from contact with recreational water than gastrointestinal illnesses, it has been suggested that *S. aureus* might be a better indicator organism than coliforms, *E. coli*, or enterococci (Charoenca & Fujioka, 1993; Favero, 1985; Maier, 2009). Because the samples for *S. aureus* in the current study were enriched, we cannot determine the concentration of *S. aureus* in the wastewater samples and therefore cannot determine whether these levels could ultimately result in human infection. Also, the only infective dose for *S. aureus* that, to our knowledge, currently exists is for the enterotoxin produced by *S. aureus* that causes food poisoning. The infective dose of *S. aureus* toxin is reached when the number of organisms per milliliter (or gram) is greater than 100,000 (FDA, 2009). This dose roughly corresponds to 1 ug of toxin (FDA, 2009). There is no infective dose that has been calculated for the number of *S. aureus* that lead to other health outcomes, such as SSTIs, wound and bloodstream infections.

Concluding Thoughts

As water shortages occur more frequently and federal and state government agencies continue to encourage wastewater reuse, we can expect reclaimed wastewater reuse applications to continue to proliferate (MDE, 2009). The goal of this thesis was not to discourage the use of reclaimed wastewater, or to cause undue concern, but to evaluate the human health risks associated with its use, to be able to identify areas for improvement in terms of treatment and use, and to inform policy decisions in this area. Until we better understand the types of pathogens that remain in treated wastewater and the health risks from exposure to reclaimed wastewater, we cannot improve its use.

Additional studies are necessary to determine if the patterns of multidrug resistant MSSA among spray irrigation workers identified in this study are statistically significant and relevant to other populations exposed to reclaimed wastewater. It is our hope that the results of this study encourage additional research and help inform decision makers about possible human health risks when crafting policies related to the use of reclaimed wastewater.

Figure 8. Number of Treatment Facilities and Population Served per State by Level of Treatment for Year 2004

State	Number of Facilities Providing Listed Effluent Level[a]				Population Served by Listed Effluent Level[a]			
	Less than Secondary[b]	Secondary	Greater than Secondary	No Discharge[c]	Less than Secondary[b]	Secondary	Greater than Secondary	No Discharge[c]
Alabama	0	131	129	7	0	751,759	1,996,926	6,651
Alaska	5	30	0	9	207,994	108,879	0	21,920
Arizona	0	16	19	74	0	116,384	3,257,943	1,551,600
Arkansas	0	118	221	9	0	725,025	852,736	12,155
California	3	186	84	334	1,942,488	17,829,141	8,731,071	3,876,394
Colorado	0	221	42	36	0	1,333,330	2,303,870	14,437
Connecticut	0	43	42	6	0	1,062,280	1,010,189	2,645
Delaware	0	2	13	4	0	8,822	666,349	25,444
District of Columbia[d]	0	0	1	0	0	0	1,298,601	0
Florida	0	0	111	209	0	0	6,586,411	6,309,507
Georgia	0	203	96	41	0	1,594,624	2,881,293	114,309
Hawaii	1	5	3	12	344,706	139,609	207,958	89,512
Idaho	0	113	7	59	0	583,756	299,893	56,724
Illinois	0	417	297	2	0	707,927	10,077,113	2,257
Indiana	0	127	274	0	0	389,859	3,654,009	0
Iowa	0	716	10	1	0	2,092,494	194,071	209
Kansas	0	356	79	196	0	670,941	1,255,624	101,710
Kentucky	0	149	91	1	0	1,566,266	912,458	435
Louisiana	0	173	173	1	0	2,248,137	971,231	207
Maine	12	115	2	10	9,303	626,778	16,038	7,215
Maryland	0	75	73	5	0	949,514	2,040,001	3,920
Massachusetts	1	77	35	10	19,762	4,372,683	859,775	25,025
Michigan	0	204	120	68	0	1,277,066	6,227,896	108,121
Minnesota	1	425	80	4	25	1,166,010	2,219,811	3,513
Mississippi	0	208	84	3	0	1,132,063	641,674	1,272
Missouri	0	629	79	19	0	3,694,485	431,110	2,482
Montana	0	108	6	83	0	399,771	92,390	57,617
Nebraska	0	255	23	188	0	947,956	206,946	82,587
Nevada	0	8	3	44	0	245,905	916,572	300,957
New Hampshire	1	67	4	11	20,617	590,051	34,878	6,838
New Jersey	0	91	57	1	0	6,553,273	1,209,075	34,307
New Mexico	1	39	3	19	1,626	894,678	7,150	216,866
New York	0	387	156	31	0	12,140,214	3,133,991	110,284
North Carolina	0	270	142	37	0	914,904	2,697,631	118,428

North Dakota	0	254	1	27	0	559,545	21,531	5,909
Ohio	0	172	604	3	0	1,267,225	7,454,278	1,074
Oklahoma	0	238	54	200	0	1,661,004	818,547	149,803
Oregon	0	133	31	48	0	1,822,176	977,731	105,085
Pennsylvania	0	419	420	7	0	5,871,941	4,156,749	9,371
Rhode Island	0	18	2	0	0	700,818	10,184	0
South Carolina	1	105	51	11	4,000	1,700,794	696,221	50,361
South Dakota	0	234	9	29	0	271,567	168,006	13,002
Tennessee	0	157	78	7	0	2,007,226	1,519,925	10,646
Texas	2	506	667	182	1,070	2,509,633	16,761,753	679,461
Utah	0	47	5	49	0	1,800,130	200,925	161,259
Vermont	0	49	31	4	0	99,181	208,843	1,792
Virginia	0	154	63	1	0	2,360,084	2,506,387	1,067
Washington	0	198	7	31	0	3,683,763	1,054,599	47,319
West Virginia	2	143	64	2	861	623,922	375,042	1,117
Wisconsin	0	292	265	37	0	692,285	3,452,096	42,691
Wyoming	0	89	3	14	0	306,246	84,439	8,037
American Samoa	2	0	0	0	5,511	0	0	0
Guam	2	2	0	2	62,639	9,236	0	4,275
N. Mariana Islands	0	2	0	0	0	0	0	0
Puerto Rico	5	35	1	0	666,788	630,056	146,477	0
Virgin Islands	1	10	1	0	19,531	58,294	50	0
Total[e]	**40**	**9,221**	**4,916**	**2,188**	**3,306,921**	**96,469,710**	**108,506,467**	**14,557,817**

Source: EPA (2008). Clean Watersheds Needs Survey 2004.
Report to Congress

Appendices

A. Reclaimed Wastewater Spray Irrigation Project Protocol

Purpose: Presence/absence and enumeration experiments for presumptively identifying and archiving methicillin-susceptible, and -resistant *Staphylococcus aureus* (MRSA) and vancomycin-susceptible and -resistant *Enterococcus spp.* (VRE) from water, air, and nasal and dermal swab samples from wastewater treatment plant and spray irrigation sites. These experiments use MRSASelect chromogenic agar, Baird Parker agar (BP), Mannitol Salt Agar (MSA), Phosphate Buffered Saline (PBS), m Staphylococcus Broth, membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (Yan, et al.), mEI amended with vancomycin, Enterococcosel Agar, and BHI agar. For more details on materials, please refer to Appendix A (page 15).

Sampling Preparation (Week Before Sampling Scheduled)

1. Calculate the amount of media needed for sample processing.

2. Prepare vancomycin stock solution (*Unless stock solution is already in -80°C freezer).
    a. Measure about 12 mg vancomycin (Sigma Aldrich, St. Louis, MO) into weigh boat.
    b. Use the following equation to determine about of solvent/diluent needed:
        i. [(Y mg) x (X µg/ml)]/ (Z µg/ml) = W ml

        Where : Y = amount of antibiotic weighed on balance (do this first !)
        X = 1000 µg/ml = potency factor (*double check specific lot using MSDS sheet)
        Z = working stock desired concentration (usually 1000 µg/ml)
        W = amount of solvent/diluent to dissolve antibiotic into
    c. Vortex.
    d. Store at 4°C if using in next few days, then freeze at -80°C.

3. Prepare mEI agar amended with vancomycin
    a. Suspend 72 g of the mEI powder (EMD, Gibbsontown, NJ) in a 1 L of dH_20.
    b. Mix thoroughly, heat and boil for 1 min to completely dissolve the powder.

c. Autoclave at 121˚C for 30 minutes.
d. Cool to 50 °C in water bath.
e. Prepare a solution of 0.48g of Naladixic acid (Acros Organics, NJ, USA) in 10 ml of dH_20.
    i. Add 0.4 mls of 10 N NaOH to dissolve.
    ii. Filter sterilize.
    iii. Add 5.2 ml solution to 1 L of mEI medium in hood.
f. Prepare TTC.
    i. Add 0.1g of 2,3,5-triphenyltetrazolium chloride (TTC) (MP Biomedicals, Illkirch, Franch) to 10 ml dH_20.
    ii. Warm to dissolve.
    iii. Filter sterilize
    iv. Add 2 mL TTC to 1 L mEI in hood and mix well.
g. Prepare vancomycin solution.
    i. Use equation: $c_1v_1 = c_2v_2$
    Where c_1 = concentration of antibiotic stock solution (usually 1000 g/ml)
    v_1 = volume of antibiotic stock solution to add to agar
    c_2 = desired concentration in each plate = 16 μg/ml
    v_2 = volume of agar
    ii. Filter sterilize v_1 of vancomycin stock solution.
    iii. Add to mEI in hood.
h. Dispense 5-10 ml of agar into 15 × 60 mm plates and allow to solidify.
i. Place in the refrigerator in lower "crisper" bin at 4°C for later use.

4. Prepare MSA
    a. Suspend 111 g of the powder (Difco, Sparks, MD) in 1 L of ultrapure water.
    b. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
    c. Autoclave at 121°C for 15 minutes.
    d. Pour into 15 x 150 mm Petri dishes.
    e. Place in the refrigerator at 4°C for later use.

5. Prepare Enterococcosel Agar
    a. Suspend 56 g of powder (Difco, Sparks, MD) in 1 L of dH20.
    b. Mix thoroughly , heat and boil for 1 min to completely dissolve the powder.
    c. Autoclave at 121C for 30 min
    d. Cool to 50 °C
    e. Pour into 100 x 15mm Petri dishes and store.
    f. Place in the refrigerator at 4°C for later use.

6. Prepare m Staphylococcus Broth
    a. Dissolve 104 g of the powder (Difco, Sparks, MD) in 1 L of purified water.

b. Warm slightly to completely dissolve the powder.
c. Autoclave at 121°C for 15 minutes.
d. Cool to 50 °C
e. Place in the refrigerator at 4°C for later use.

7. Prepare BHI Agar
    a. Suspend 52 g of powder (Difco, Sparks, MD) in 1 L of dH20.
    b. Mix thoroughly, heat and boil for 1 min to completely dissolve the powder.
    c. Autoclave at 121°C for 30 min
    d. Cool to 50 °C
    e. Pour into 15 x 150 mm Petri dishes.
    f. Place in the refrigerator at 4°C for later use.

8. Prepare Phosphate Buffered Saline
    a. Dissolve 0.58g sodium dihydrogen phosphate, 2.5g sodium monohydrogen phosphate, and 8.5g sodium chloride in 1 L dH20.
    b. Autoclave at 121°C for 15 min
    c. Cool to 50 °C
    d. Final pH should be 7.4 ± 0.2.

9. Prepare Water Membrane Filtration System
    a. Wash funnel system (Nalgene)
    b. Spray manifold with ethanol, turn upside down, and let dry on paper towels.
    c. Wrap funnel system in paper and autoclave using "Gravity" cycle.

10. Prepare Tisch Impactor
    a. Wipe down impactor parts with 70% ethanol.
    b. Wrap top and inlets with aluminum foil.
    c. Autoclave on "Gravity" cycle to sterilize.
    d. Pack assembled impactor in pelican case.

Day 1: **Sampling, Streak Nasal and Dermal Swabs**

1. Sampling:

I. Nasal Samples
    a. Gently tilt participant's head back and steady chin.
    b. Moisten cotton-tipped swab by swirling in Stuart's transport media (Copan, Italy).
    c. Insert cotton-tipped swab approximately one-half inch into participant's right nostril and gently rotate the swab five times over inside of nostril.
    d. Remove swab from participant's nostril and immediately insert into Stuart's transport media tube.

e. Place cap on tube and label with unique coded identifier (Ex. SI1_03-10_N001 to label a sample from spray irrigation site 1 taken in March 2010 from participant 1's nostril).
f. Offer participant tissue to blow his nose.
g. Use ethanol to clean hands between each subject.

II. Dermal Samples
a. Ask participant to pull up shirt sleeve of his right arm (if applicable).
b. Moisten cotton-tipped swab in Stuart's transport media (Copan, Italy).
c. Gently swab a 2x2 inch area of participant's forearm with cotton-tipped swab by rolling swab back and forth 15 times.
d. Immediately insert the swab into Stuart's transport media tube.
e. Place cap on tube and label with unique coded identifier (Ex. SI1_03-10_D001 to label a sample from spray irrigation site 1 taken in March 2010 from participant 1's forearm).
f. Use ethanol to clean hands between each subject.

III. Wastewater and Irrigation Water Samples
a. Collect wastewater and irrigation water samples in 1-L and 500-mL Nalgene, polypropylene, wide-mouthed environmental sampling bottles.

IV. Air Samples
a. Warm-up portable generator for at least one-minute before connecting to the sampling train.
b. Place pre-labeled TSA plates (ex. WW1_12-09_Effluent _stage1 10 min) in both stages of impactor.
c. When ready to sample connect to the outlet of the generator and immediately record start time.
d. Sample for the following times:
1. All wastewater and spray irrigation samples: 1, 5, and 10 minutes
2. Control: 1 and 5 minutes
e. Turn off pump and remove TSA plates.
f. Wipe down all parts of impactor after each sampling time with 70% ethanol.
g. Re-start pump and sample for next time period.
h. After sampling is complete, switch off pump.
i. Place samples in cooler and transport to lab at 4°C.
j. Incubate plates for 24 hours at 37°C.

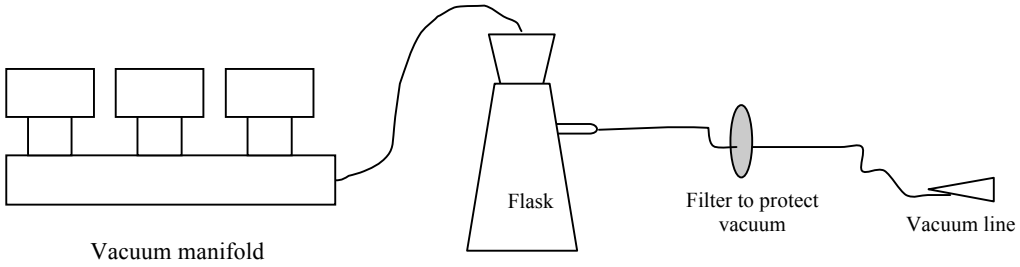
2. Nasal and Dermal Swabs

1. Direct plating
   a. Label MSA and Baird Parker plates with date and unique coded identifier (Ex. SI1_08-09_N001).
   b. Streak MSA and Baird Parker plates each with one side of cotton-tipped swab. Incubate overnight at 37°C.

c. Streak a (+) *S. aureus* control and (-) control plate on both MSA and Baird Parker and incubate overnight at 37°C.
d. Label EA plates with date and unique coded identifier.
e. Streak EA plate with second side of cotton-tipped swab. Incubate overnight at 41°C.
f. Streak a (+) *Enterococcus spp.* control and (-) control plate and incubate overnight at 41°C.

Day 2: **Water Membrane Filtration, Air Sample Plate Replication, Nasal and Dermal Swab Subculturing**

1. **Water Membrane Filtration (IMPORTANT: Must be within 24 hours of sample collection)**
    a. Label all plates with the appropriate sample media code and dilution (Example: WW1_10-09_1° influent_10 mls).
    b. Aseptically assemble the vacuum manifold/flask system, making sure that a trap is placed between the flask and the pump vacuum line to protect the vacuum line from contamination.
    c. Place ~ 10ml of chlorine bleach in the flask.



    d. Using aseptic technique, prepare serial dilutions of your sample, taking into consideration any rain events that may have occurred before sample.
        i. Dilutions for Influent Wastewater and Activated Sludge Reactor Samples: 1, 0.1, 0.01, 0.001 mls.
        ii. Secondary Clarifier dilutions: 100, 10, 1 mls
        iii. Effluent: 1 L (or as much close to 1 L as possible, record exact volume filtered)
        iv. Spray Irrigation Samples:
            1. Before UV, After UV, and Input to Pond: 1 L (if possible), 300 mls, 100 mls
            2. Inlet to Pumphouse: 200,100, and 10 mls
    e. Using sterilized tweezers, place a Millipore mixed cellulose filter (Millipore Corp., Billerica, MA) on the filter funnel, grid side up.
    f. Secure funnel and starting with the lowest dilution, pipette 10mls of sample dilution into funnel, filter, and follow with 10mls PBS to rinse.

g. Turn off suction, carefully remove filter with tweezers and place on 60x15mm mEI plate. Double bag in whirlpack bags and incubate at 41.5°C for 24-48 hours in water bath.
h. Place a new cellulose filter on funnel, secure funnel and pipette same dilution, this time placing on 60x15mm mEI+van plate. Double bag in whirlpack bags and incubate at 41.5°C for 24-48 hours in water bath.
i. Place a new cellulose filter on funnel, secure funnel and pipette your next dilution.
j. Place filters on mEI and mEI+van plates.
k. After 3 samples, soak filter funnels with 70% ethanol, let air dry, and apply UV light under hood for 5-10 minutes.
l. Set up a positive (+) and negative (-) control plates.
    i. For positive control, suspend known *Enterococcus* colony in PBS.
    ii. For negative control, filter 10 mls of PBS.
m. For *S. aureus* and MRSA enrichment:
    i. Label 50 ml Falcon tube with sample location, date, and initials (Example: WW1_10-09_ASR_mStaphB).
    ii. Filter 300 mls of influent wastewater (use more than one filter if necessary).
    iii. Add filter(s) to 40 mls of m Staph Broth in 50 ml Falcon tube.
    iv. Vortex
    v. Incubate at 37°C for 24 hours.

2. *Air Samples – Plate Replication*

1. Take TSA plates out of incubator and record presence/absence of growth. If there is no growth, leave in incubator for an additional 24 hours.
2. For TSA plates with growth, use wooden replicator.
3. Cover wooden replicator with velvet pads.
4. Label plates with sample name and time, initials, and date.
5. Press velvet pad covered replicator into Baird Parker, MSA, Chapman Stone, mEI, mEI +vancomycin, and TSA plates lined up in a row.
6. Press firmly enough to pick up all colonies, but not so hard that plate moves or colonies are squashed. Feel "jiggle."
7. Incubate overnight at 37°C.

3. *Nasal and Dermal Swabs: Subculturing*

Target colony (EA): Colonies with black precipitate; Take up to 10 target organisms from each sample and subculture onto vancomycin-amended EA to determine if they are vancomycin-resistant Enterococcus spp.

1. Label plates with Initials, Date, and sample ID (Example: WW1_10-09_N001).
2. With a marker, divide each plate into three sections.
3. Select 10 target colonies and streak each colony onto a section of the EA+van plate.
4. Incubate overnight at 41°C.

4. Nasal and Dermal Swabs: Prepare for Coagulase Testing

Target colonies:
- *MSA: Yellow colonies with yellow precipitate*
- *BP: Black shiny colonies with clear halo*

1. Label plates with Initials, Date, and sample ID (Example: WW1_10-09_N001).
2. With a marker, divide each plate into three sections.
3. Take up to 10 target organisms from each sample.
4. Streak for isolation onto a TSA plate.
5. Incubate for 24 hours at 37°C.

Day 3: **Isolation from Water Samples, Prepare Air Samples for Biochemical Testing, Biochemical Testing on Nasal and Dermal Swabs**

1. *S. aureus* from Water Samples
1. Label 2 MSA and Baird Parker plates for each sample with Initials, Date, and sample ID (Example: WW1_10-09_Inf1).
2. Take m Staph B out of incubator.
3. Using calibrated10 µl loop, streak 10 µl of m Staph B onto 2 MSA and Baird Parker plates.
4. Incubate at 37°C for 24 hours.

2. *MRSA* from Water Samples
1. With marker, divide each MRSASelect plate in half.
2. Label MRSASelect plates with Initials, Date, and sample ID (Example: WW1_10-09_Inf1).
3. Take m Staph B out of incubator.
4. Using calibrated 10 µl loop, streak 10 µl of m Staph B onto both halves of a MRSASelect plate.
5. Incubate at 37°C for 24 hours.

3. Water Samples - *Enterococcus* - Colony Count
1. Obtain your mEI plate from the incubator.
2. Use counter pen in Prep Lab for colony count.
3. Record colony forming units for each plate.

4. **Water Samples - *Enterococcus* – Prepare for Biochemical Tests**
Target Colonies:
mEI: Colonies with blue halo (regardless of colony color)
mEI+van: Colonies with blue halo (regardless of colony color)

1. Label your BHI plate with Initials, Date, and sample ID (Example: WW1_10-09_Inf1).
2. With a marker, divide each plate into three sections.

3. Select up to 10 target colonies from each sample (you do not need to take 10 colonies from each dilution, only 10 total for each sample location).
4. Streak for purification on BHI agar.
5. Incubate at 41°C for 24 hours.
6. Streak positive and negative control plates and incubate overnight at 41°C.

5. Air Samples – Prepare presumptive isolates for Biochemical Tests

Target colonies:

1. *MRSASelect: Pink colonies*
2. *MSA: Yellow colonies with yellow precipitate*
3. *BP: Black shiny colonies with clear halo*
4. *mEI: Colonies with blue halo (regardless of colony color)*
5. *mEI+van: Colonies with blue halo (regardless of colony color)*

1. For Presumptive *Enterococcus* spp. and VRE:
    i. Label your BHI plate with Initials, Date, and sample ID (Example: WW1_10-09_Inf1).
    ii. With a marker, divide each plate into three sections.
    iii. Select up to 10 target colonies from each sample (you do not need to take 10 colonies from each dilution, only 10 total for each sample location).
    iv. Streak for purification on BHI agar.
    v. Incubate at 41°C for 24 hours.
    vi. Streak positive and negative control plates and incubate overnight at 41°C.
2. For Presumptive *S.aureus* and MRSA:

Target colonies:
-CSA: white colonies with clear halo.
-MSA: Yellow colonies with yellow precipitate
-BP: Black shiny colonies with clear halo

    i. Label your TSA plate with Initials, Date, and sample ID (Example: WW1_10-09_Inf1).
    ii. With a marker, divide each plate into three sections.
    iii. Select 10 target colonies and streak each colony onto a section of the TSA plate for purification.
    iv. Incubate for 24 hours at 37°C.
    v. Streak positive and negative control plates and incubate overnight at 37°C.

6. Biochemical Tests - Nasal and Dermal Swabs - *Presumptive S. aureus and MRSA*

Using Gram Stain, catalase test, and coagulase test, you will be able to presumptively identify S. aureus and MRSA from your positive nasal and dermal swab samples.

- The Gram stain will confirm that you have a pure culture and it will also confirm that you have a Gram-positive coccus (morphology and gram reaction for *S. aureus*).
- The catalase test examines the ability to breakdown hydrogen peroxide by catalase. Those organisms possessing the catalase enzyme will break down hydrogen peroxide into water and oxygen. The oxygen causes bubbles to form within, seconds, indicating a positive test. The absence of bubbles is considered a negative test. *S. aureus* is catalase positive.
- The Coagulase Plasma, Rabbit with EDTA (Difco, Sparks, MD) detects coagulase (both bound and free) to identify *S. aureus* and separate it from other species of staphylococci. Any degree of clotting is considered positive.

Obtain your TSA plates from the incubator and record results. Make sure that you have a pure (and NOT mixed) culture.

I. Gram Stain
   a. Prepare smear.
   b. Perform Gram Stain as directed.
   c. Record observations.

II. Catalase Test
   a. Collect an empty Petri dish and place one drop of 3 % hydrogen peroxide/per sample on to surface of Petri dish.
   b. Take small swab from each sample and place into the 3 % hydrogen peroxide.
   c. Examine plates for bubbles. Presence of bubbles= positive result; Absence of bubbles =negative result.
   d. Record observations.

III. Coagulase Test
   a. Take out coagulase test from refrigerator.
   b. Rehydrate plasma by adding 3 ml of sterile dionized water to bottle.
   c. Mix by rotating end over end.
   d. Add 300 µl rehydrated plasma to a glass tube.
   e. Add 4-5 colonies of presumptive *S.aureus* from a non-selective agar plate.
   f. Vortex.
   g. Incubate at 37°C for up to four hours.
   h. Check tubes every hour for up to four hours, and then again after 24 hours.
   i. Partial or complete coagulation=positive result. No coagulation=negative result.
   j. Record observations.

** For isolates with positive gram stain (+), catalase (+), and Coagulase tests (+), store in 4°C refrigerator until purification on Day 5 and archiving on Day 6. These isolates can be presumptively identified as *S. aureus* or MRSA.

Day 4: **Prepare Water Samples for Biochemical Testing, Biochemical Testing on Air Samples and *Enterococcus* from Water Samples**

1. *S. aureus* and MRSA from Water: Prepare for Biochemical Testing

Target colonies:
-MRSASelect: Hot pink colonies
-MSA: Yellow colonies with yellow precipitate
-BP: Black shiny colonies with clear halo

a. Label your TSA plate (Initials, Date, presumptive organism, source, etc.).
b. With a marker, divide each plate into three sections.
c. Select 10 target colonies and streak each colony onto a section of the TSA plate for purification.
d. Incubate for 24 hours at 37°C.
e. Streak positive and negative control plates and incubate overnight at 37°C.

2. Water and Air Samples – Biochemical Tests - *Enterococcus* spp. and *VRE*

Using Gram Stain, catalase test, and PYR test, you will be able to presumptively identify *Enterococcus* from your positive water samples.

- The Gram stain will confirm that you have a pure culture and it will also confirm that you have a Gram positive coccus (morphology and gram reaction for *Enterococcus*).
- The PYR test (Remel, Lenexa, KS) is a rapid, colorimetric test recommended for use in qualitative procedures for the detection of pyrrolidonyl arylamidase activity for presumptive identification of enterococcoci, group A streptococcoci, and Escherichia coli.
- The catalase test examines the ability to breakdown hydrogen peroxide by catalase. Those organisms possessing the catalase enzyme will break down hydrogen peroxide into water and oxygen. The oxygen causes bubbles to form within, seconds, indicating a positive test. The absence of bubbles is considered a negative test. Enterococcus is catalase negative (or very weakly positive).

Obtain your BHI purification plates from the incubator and record results. Make sure that you have a pure (and NOT mixed) culture.

IV. Gram Stain
a. Prepare smear.
b. Perform Gram Stain as directed.
c. Record observations.

V. PYR Test
a. Test isolates should be 18-24 hours old and taken from non-selective media, such as BHI.

b. Using forceps, place the disk on a clean microscope slide or in the lid of a Petri dish free from excess moisture.
c. Moisten the disk slightly with 5-10 ul of demineralized water using a micropipette or a 10 uL inoculating loop. DO NOT OVERSATURATE.
d. Remove a visible "paste" of the test isolate using a sterile loop.
e. Rub the inoculums gently into a small area of the disk.
f. Add one (1) drop of PYR Reagent to the disk.
g. Allow up to one minute for a color change.
    i. Positive test= pink to red color development w/in 1 min of applying PYR reagent
    ii. Negative test= Cream, yellow, or no color change within one minute of applying PYR Reagent

VI. Catalase Test

a. Collect an empty Petri dish and place one drop of 3 % hydrogen peroxide/per sample on to surface of Petri dish.
b. Take small swab from each sample and place into the 3 % hydrogen peroxide.
c. Examine plates for bubbles. Presence of bubbles= positive result; Absence of bubbles =negative result.
d. Record observations.

** For isolates with positive gram stain (+), negative catalase (-), and positive PYR tests(+), set aside for purification on Day 5 and archiving on Day 6. These isolates can be presumptively identified as *Enterococcus* spp. and VRE.

3. Air Samples – Biochemical Tests – *S. aureus* and MRSA

Using Gram Stain, catalase test, and coagulase test, you will be able to presumptively identify S. aureus and MRSA from your positive nasal and dermal swab samples.

- The Gram stain will confirm that you have a pure culture and it will also confirm that you have a Gram-positive coccus (morphology and gram reaction for *S. aureus*).
- The catalase test examines the ability to breakdown hydrogen peroxide by catalase. Those organisms possessing the catalase enzyme will break down hydrogen peroxide into water and oxygen. The oxygen causes bubbles to form within, seconds, indicating a positive test. The absence of bubbles is considered a negative test. *S. aureus* is catalase positive.
- The Coagulase Plasma, Rabbit with EDTA (Difco, Sparks, MD) detects coagulase (both bound and free) to identify *S. aureus* and separate it from other species of staphylococci. Any degree of clotting is considered positive.

Obtain your TSA plates from the incubator and record results. Make sure that you have a pure (and NOT mixed) culture.

I. Gram Stain
   a. Prepare smear.
   b. Perform Gram Stain as directed.
   c. Record observations.

II. Catalase Test
   a. Collect an empty Petri dish and place one drop of 3 % hydrogen peroxide/per sample on to surface of Petri dish.
   b. Take small swab from each sample and place into the 3 % hydrogen peroxide.
   c. Examine plates for bubbles. Presence of bubbles= positive result; Absence of bubbles =negative result.
   d. Record observations.

III. Coagulase Test
   a. Take out coagulase test from refrigerator.
   b. Rehydrate plasma by adding 3 ml of sterile dionized water to bottle.
   c. Mix by rotating end over end.
   d. Add 300 µl rehydrated plasma to a glass tube.
   e. Add 4-5 colonies of presumptive *S.aureus* from a non-selective agar plate.
   f. Vortex.
   g. Incubate at 37°C for up to four hours.
   h. Check tubes every hour for up to four hours, and then again after 24 hours.
   i. Partial or complete coagulation=positive result. No coagulation=negative result.
   j. Record observations.

** For isolates with positive gram stain (+), catalase (+), and Coagulase tests (+), store in 4°C refrigerator until purification on Day 5 and archiving on Day 6. These isolates can be presumptively identified as *S. aureus* or MRSA.

Day 5: **Biochemical Testing of *S. aureus* and MRSA from Water Samples, Purification for All Samples**

1. **Water Samples – Biochemical Tests - Presumptive *S. aureus and MRSA***

Using Gram Stain, catalase test, and coagulase test, you will be able to presumptively identify S. aureus and MRSA from your positive samples.

- The Gram stain will confirm that you have a pure culture and it will also confirm that you have a Gram-positive coccus (morphology and gram reaction for *S. aureus*).
- The catalase test examines the ability to breakdown hydrogen peroxide by catalase. Those organisms possessing the catalase enzyme will break down hydrogen peroxide into water and oxygen. The oxygen causes bubbles to form

within, seconds, indicating a positive test. The absence of bubbles is considered a negative test. *S. aureus* is catalase positive.

- The Coagulase Plasma, Rabbit with EDTA (Difco, Sparks, MD) detects coagulase (both bound and free) to identify *S. aureus* and separate it from other species of staphylococci. Any degree of clotting is considered positive.

Obtain your TSA purification plates from the incubator and record results. Make sure that you have a pure (and NOT mixed) culture.

I. Gram Stain
   a. Prepare smear.
   b. Perform Gram Stain as directed.
   c. Record observations.

II. Catalase Test
   a. Collect an empty Petri dish and place one drop of 3 % hydrogen peroxide/per sample on to surface of Petri dish.
   b. Take small swab from each sample and place into the 3 % hydrogen peroxide.
   c. Examine plates for bubbles. Presence of bubbles= positive result; Absence of bubbles =negative result.
   d. Record observations.

III. Coagulase Test
   a. Take out coagulase test from refrigerator.
   b. Rehydrate plasma by adding 3 ml of sterile dionized water to bottle.
   c. Mix by rotating end over end.
   d. Add 300 µl rehydrated plasma to a glass tube.
   e. Add 4-5 colonies of presumptive *S.aureus* from a non-selective agar plate.
   f. Vortex.
   g. Incubate at 37°C for up to four hours.
   h. Check tubes every hour for up to four hours, and then again after 24 hours.
   i. Partial or complete coagulation=positive result. No coagulation=negative result.
   j. Record observations.

IMPORTANT: From presumptive MRSA: if you have pink colonies, (+) gram stain, (+) coagulase test, and (+) catalase test, then purify the isolate as follows:

2. **Purification – From All Sample Types (Nasal, Dermal, Air, and Water)**
Take up to 10 target organisms from each sample and streak for purification onto BHI.

*Presumptive MRSA and *S. aureus*: positive gram stain, catalase, and Coagulase tests.

*Presumptive VRE and *Enterococcus* spp.: positive gram stain, negative catalase, and positive PYR tests.

a. Label your BHI plate (Initials, Date, presumptive organism, source, etc.).
b. With a marker, divide each plate into three sections.
c. Select 10 target colonies and streak each colony onto a section of the BHI plate for purification. Incubate overnight at 37°C (presumptive MRSA and *S. aureus*) or 41°C (presumptive VRE and *Enterococcus* spp).
d. Streak positive and negative control plates and incubate overnight at 37°C or 41°C.

Day 6: **Archiving of Sample Isolates**

Obtain your BHI purification plates from the incubator. Also gather Brucella Broth and glycerol.

1. Prepare 15% glycerol and Brucella Broth solution.
2. Observe and record the results of your BHI plate. Compare your plate to the control plate and make sure that you have a pure (and NOT mixed) culture.
3. Add 1 mL of 15% glycerol and Brucella Broth to a 1.5 mL cryogenic tube.
4. Label your 15% glycerol with Brucella Broth tube using the labeling machine with the following information: Date Archived, Sample Location, Date of sampling, Type of organism, Isolate number (Example: 12-08-09; WW1_12-09_MRSA1).
5. Record labeling scheme in lab notebook.
6. Using a sterile loop, collect one single colony from BHI plate. Place into the Brucella Broth and gently swirl in order to get remainder off of loop.
7. Vortex.
8. Place isolates in the -80°C freezer in box clearly labeled with sampling date, type of isolate, date of archiving, and initials.

Materials

Name	Manufacturer	Location of Manufacturer	Distributor	Catalogue/ Reference Number
ANTIBIOTICS				
Vancomycin Hydrochloride Hydrate	Aldrich Chemistry	St. Louis, MO, USA	Sigma Aldrich	861987-250MG
MEDIA				
Baird-Parker Agar Base	Difco/ BD Diagnostics	Sparks, MD	VWR	90003-974
Egg Yolk Tellurite Solution	Difco/ BD Diagnostics	Sparks, MD	VWR	90000-620
membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (Yan, et al.)	Difco/ BD Diagnostics	Sparks, MD	VWR	90000-790
Mannitol Salt Agar (MSA)	Difco	Sparks, MD	VWR	211407
Enterococcosel Agar	Difco	Sparks, MD	VWR	90000-528
m Staphylococcus Broth	Difco	Sparks, MD	VWR	264920
Brain Heart Infusion (BHI) Agar	Difco	Sparks, MD	VWR	211065
Tryptic Soy Agar	Difco	Sparks, MD	VWR	211043
Transystem LQ Stuart Plastic Applicator Rayon Tipped	Copan Innovation, Venturi Transystem	Italy	VWR	141C.USE
MRSASelect	BioRad		Biorad	
REAGENTS				
Nalidixic Acid	Acros Organics	NJ, USA	Thermo-Fisher Scientific	169901000
2,3,5-Triphenyltetrazolium Chloride	MP Biomedicals, LLC	Illkirch, Franch		103126
EQUIPMENT				
Water Membrane Filtration System	Nalgene			
2-stage Impactor	Tisch Environmental			
0.45 μm 47 mm	Millipore	Billerica, MA	VWR	HAWG047

mixed cellulose filters	Corporation			56
BIOCHEMICAL TESTS				
Coagulase Plasma, Rabbit with EDTA	Difco	Sparks, MD	VWR	90003-152
PYR Discs	Remel	Lenexa, KS	Fisher Scientific	22674073

B. Participant Questionnaire

SPRAY IRRIGATION WORKER STUDY	
1.1) Interviewer Name	
1.2) Date of Interview	
1.3) Subject ID Number	|__|__|__|__|
1.4) Gender (Circle one)	(1) Male (2) Female
1.5) Age	|__|__|
1.6) Race (Circle one)	(1) Caucasian (2) Black (3) Hispanic (4) Asian (5) Other
1.7) Education (Circle one)	(1) Less than high school (2) High School (3) Associate (4) College
1.8) Occupation (Circle one):	(1) Irrigation Worker (2) Office Worker
1.9) How long have you had this job?	|__|__| Years
1.10) How many days a week do you spend in this job?	|__|__| Days
1.11) How many hours a day do spend in this job?	|__|__| Hours
1.12) Do you wear personal protective equipment when spraying water at this job?	(1) Yes (2) No

1.13) Yearly Income (Circle one)	(1) Less than $15,000 (2) $15,000-$25,000 (3) $25,000-$35,000 (4) $35,000-$50,000 (5) >$50,000
1.14) Have you ever worked in a healthcare setting?	(1) Yes (2) No
1.15) Has anyone in your household ever worked in a healthcare setting?	(1) Yes (2) No
1.16) Have you had a nosebleed within the last three days?	(1) Yes (2) No
1.17) Have you ever been diagnosed with methicillin-resistant *Staphylococcus aureus* (MRSA)?	(1) Yes (2) No (3) I don't know
1.18) Have you smoked at least 100 cigarettes in the past 6 months?	(1) Yes (2) No
1.19) Do you smoke now?	(1) Yes (2) No

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Rachel Elizabeth Rosenberg Goldstein

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EDUCATION

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

- Relevant Coursework Includes: Nonpoint Source Pollution Assessment Techniques, Epidemiology, Health Behaviors, Biostatistics I, Toxicology, Environmental Policy, Biological Contaminants, 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: Environment and Society, Earth and Climate, Environmental Advocacy, Environmental Science, World Food Supply, Physics I & II, Estuarine Processes, Watershed Systems, Environmental Communication, Introduction to GIS, Energy and Material Processes

PROFESSIONAL EXPERIENCE

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

Graduate Assistant (September 2008 – Present)

- Conducting literature review and microbiology laboratory work for research projects on the prevalence and pathway of salmonella on tomato farms, and irrigation workers' exposures to antimicrobial-resistant bacteria and antimicrobials present in reclaimed wastewater.
- Collaborated on poster about antibiotic use among Nigerian women to treat menstrual symptoms.

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.

Camp Ramah Darom, Clayton, GA

Organic Farming Specialist (Summer 2005)

- Taught environmental stewardship and organic farming to high school students.

College of William & Mary, Research Experience for Undergraduates
Interdisciplinary Watershed Program, 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".

Ellerbe Creek Watershed Association, Durham, NC

Intern (August 2003 – December 2003)

- Streamlined descriptions of ECWA activities into an educational brochure and outreach tool.
- Featured in association's publication for "enthusiasm and excellent work".

INVITED LECTURES

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

The Clark School Engineering Sustainability Workshop (April 2010)
University of Maryland, College Park

- Oral presentation, "Antibiotic-Resistant Bacteria in Wastewater and Resulting Occupational Exposures."

Maryland Pathogen Research Institute (April 2010)
University of Maryland, College Park

- Oral presentation, "Evaluation of Methicillin-resistant *Staphylococcus aureus* and Vancomycin-resistant *Enterococcus* spp. in Tertiary Treated Wastewater, Reclaimed Wastewater used for Spray Irrigation, and Resulting Occupational Exposures."

University of Maryland Bioscience & Technology Review Day (November 2009)

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

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."

HONORS

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)

RELEVANT EXPERIENCE

Casey Tree Foundation, Washington, DC
Citizen Forester, (May 2006 - Present)

National Council for Science and the Environment,Washington, DC
EnvironMentor, (October 2006 – May 2007)

- Supervised high school junior's project on the ecological effects of pollution in the Anacostia River.

Hebrew Immigrant Aid Society, Washington, DC
Co-chair of Young Leaders Advocacy Committee, (February 2007 – July 2008)

- Coordinated and facilitated monthly meetings and educational events.

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.

SKILLS

- Proficient in Microsoft Word, Excel, Access, and PowerPoint.
- Experience in Dreamweaver and Stata.