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List of Terms and Abbreviations

Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA)

Limit of detection (LOD)

Membrane-Enterococcus Indoxyl-β-D-Glucoside (mEI)

Methicillin-resistant *Staphylococcus aureus* (MRSA)

Methicillin-susceptible *Staphylococcus aureus* (MSSA)

Minimal inhibitory concentration (MIC)

Multidrug resistance (MDR)

Polymerase chain reaction (PCR)

Pulsed field gel electrophoresis (PFGE)

Pyrrolidonyl peptidase (pyr)

Spray irrigation site (SI)

Vancomycin-intermediate *Enterococcus* spp. (VIE)

Vancomycin-resistant *Enterococcus* spp. (VRE)

Vancomycin-susceptible *Enterococcus* spp. (VSE)

Wastewater treatment plants (WWTPs)

Abstract

Spray irrigation of reclaimed wastewater is an attractive alternative for wastewater disposal and an economic method of irrigation. However, as reclaimed wastewater use expands, it is necessary to evaluate the extent of potential human exposures to emerging wastewater contaminants such as antibiotic-resistant bacteria and antimicrobials, particularly among wastewater irrigation workers. Thus, the goal of our study was to advance understanding of wastewater irrigation workers' exposures to antibiotic-resistant bacteria and antimicrobials in the Mid-Atlantic and Midwest regions of the U.S. To accomplish this goal, we studied four wastewater treatment plants and four of their associated reclaimed wastewater spray irrigation sites. We collected environmental samples from each site in 2009 and 2010. Nostril and skin swab samples were also collected from spray irrigation workers and office workers (control group). All samples were tested for antibiotic-resistant bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomyin-resistant *Enterococcus* (VRE). Wastewater samples were also tested for antimicrobial residues, including ciprofloxacin and tetracycline. We detected MRSA in 50% of wastewater samples from the four treatment plants and 93% of MRSA isolates were multidrug resistant (resistant to ≥2 classes of antibiotics). We also detected VRE in 32% of wastewater samples from the treatment plants. While both MRSA and VRE were detected in a few of the unchlorinated effluent samples (finished water coming out of the plants), MRSA was not detected at any of the wastewater spray irrigation sites, and VRE was detected in only one sample from one spray irrigation site. Similarly, we did not detect MRSA or VRE in nostril or skin swabs from any of the spray irrigation workers. However, we did detect *S. aureus* in nostril swabs from 26% of spray irrigation workers and 29% of office workers. A greater percentage of spray irrigation workers carried multidrug resistant *S. aureus* in their nostrils compared to office workers. Moreover, a range of antimicrobial residues, including azithromycin, linezolid and ciprofloxacin, were detected in raw wastewater, effluent and reclaimed wastewater used at spray irrigation sites. Levels of antimicrobials declined throughout the wastewater treatment process; however, concentrations were still elevated in wastewater used at spray irrigation sites. To our knowledge, this is the first report of the detection of MRSA at wastewater treatment plants in the U.S. It is also the first study to 1) analyze reclaimed wastewater at reuse sites in the U.S. for antibiotic-resistant bacteria and antimicrobial residues; and 2) assess the rate of *S. aureus* carriage among spray irrigation workers. Our findings suggest that implementing tertiary treatments (e.g. chlorination, filtration) for wastewater intended for reuse applications could reduce the potential risk of exposures to antibiotic-resistant bacteria and antimicrobial residues among individuals working with reclaimed wastewater. However, reclaimed wastewater spray irrigation workers, as well as wastewater treatment plant workers, could still potentially be exposed to these emerging contaminants through skin contact and inhalation. Encouraging frequent handwashing and the use of gloves, as well as changing clothes before going home, could reduce the potential risks associated with these exposures among spray irrigation workers and wastewater treatment plant workers.

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Section 1

Significant (Key) Findings:

In this study, we sought to advance understanding of reclaimed wastewater irrigation workers' exposures to antibiotic-resistant bacteria and antimicrobial residues that may remain in municipal wastewater after treatment. Four wastewater treatment plants and four of their associated reclaimed wastewater spray irrigation sites—located in the Mid-Atlantic and Midwest regions of the United States—were included in the study.

For the first time, we identified that methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and other antibiotic-resistant bacteria are prevalent in municipal wastewater at U.S. wastewater treatment plants. However, tertiary wastewater treatment (e.g. the use of chlorination and/or filtration) seems to effectively reduce levels of MRSA and other antibiotic-resistant bacteria to very low levels in wastewater effluent (treated wastewater that leaves the plant) that is subsequently reclaimed for spray irrigation activities.

As a result, we did not detect MRSA at any of the reclaimed wastewater spray irrigation sites included in the study. We did, however, detect low levels of other types of antibiotic-resistant bacteria in a few samples recovered from the spray irrigation sites. These findings suggest that, although few antibiotic-resistant bacteria might survive distribution to a wastewater reuse site, unprotected exposure to the environment such as storage in an open air pond could either resuscitate injured antibiotic-resistant bacteria or introduce antibiotic-resistant bacteria from the environment into water being used for spray irrigation.

In our analysis of workers, we observed that 26% of spray irrigation workers, compared with 29% of office worker controls, were colonized with *S. aureus* in their noses. These percentages of colonization fall within the range that is typically observed in the U.S. population (20-30%). Nonetheless, we found that a greater percentage of spray irrigation workers were nasally colonized with multidrug resistant (resistant to ≥ 2 classes of antibiotics) *S. aureus* compared to office worker controls.

In terms of antimicrobial residues, we detected nearly all of the tested antibiotics in raw municipal wastewater (influent), wastewater effluent, and reclaimed wastewater recovered from spray irrigation sites. Concentrations of antibiotics decreased from influent to effluent samples, and then again in reclaimed wastewater samples; however, reclaimed wastewater samples still had elevated concentrations of antibiotics. Therefore, irrigation workers using reclaimed wastewater could potentially be exposed to these antibiotics through skin exposures and/or inhaling aerosolized reclaimed wastewater.

Translation of Findings:

Our findings indicate that reclaimed wastewater spray irrigation workers, as well as wastewater treatment plant workers, could potentially be exposed to antibiotic-resistant bacteria and antimicrobial residues that remain in wastewater. Wastewater treatment plant workers (which were not the focus of this study) could be exposed to higher levels of these emerging contaminants, while spray irrigation workers are likely exposed to very low levels of resistant bacteria and antimicrobials. Encouraging frequent handwashing and the use of gloves, as well as implementing rules about changing clothes before going home, could reduce the potential risks associated with these exposures among both spray irrigation workers and wastewater treatment plant workers. These suggestions could easily be adopted at reclaimed wastewater irrigation sites and wastewater treatment plants. Our findings also suggest that implementing tertiary treatments for wastewater intended for reuse applications could reduce the potential risk of exposures to antibiotic-resistant bacteria and antimicrobial residues among reclaimed wastewater spray irrigation workers, as well as their close contacts.

Outcomes/Impact:

The first manuscript published from this study described our detection of MRSA at U.S. wastewater treatment plants. Since it was the first study of its kind, we issued a press release of the findings which were subsequently broadly disseminated by a variety of sources including the nightly news. News broadcasters emphasized the message of frequent handwashing and the use of gloves among wastewater treatment plants workers. To our delight, during our next visit to a local wastewater treatment plant, several of the workers (who were not aware that we were the researchers who had conducted this study) emphasized that they had heard news reports about antibiotic-resistant bacteria in wastewater and were now taking extra precautions (e.g. more frequent hand washing, wearing gloves) to reduce their potential risk of exposure to both antibiotic-resistant bacteria and antimicrobials. This is one anecdotal example of how this project has directly led to improvements in occupational safety and health among (at least a sampling of) wastewater treatment plant workers.

On a broader scale, in terms of wastewater reuse, our findings can guide both future research and future legislative activities. Concerning research, our findings suggest that further study is likely needed to evaluate a broader array of pathogenic bacteria and antimicrobials that may remain in treated wastewater utilized by spray irrigation workers. The findings of this current study may represent only the tip of the iceberg with regard to potential microbial and chemical exposures that may be experienced by individuals working with (and/or exposed to) reclaimed wastewater. Additional studies that utilize high-throughput, next generation sequencing approaches are necessary to fully evaluate the total bacterial diversity of reclaimed wastewater, as well as the possible impacts of this potential water resource on the microbiome (the total bacterial communities) of spray irrigation workers and other exposed populations. At the same time, it may be prudent to begin to advance legislative actions while research is ongoing. Currently, no federal regulations exist for wastewater reuse, although the U.S. Environmental Protection Agency (EPA) has issued water reuse guidelines. Meanwhile, the use of reclaimed wastewater continues to increase. As alluded to above, our findings suggest that implementing federal regulations dictating the use of tertiary treatments for wastewater intended for reuse could reduce the potential risk of exposures to MRSA, other antibiotic-resistant bacteria, and antimicrobials among individuals who are working on properties sprayed with reclaimed wastewater.

Section 2

Scientific Report:

Background:

Spray and subsurface irrigation of reclaimed wastewater on agricultural lands continue to be attractive alternatives for wastewater disposal, as well as economic methods of irrigation, particularly during heavy droughts[1]. Currently, an estimated 1.7 billion gallons per day of treated wastewater is used in the United States, and reclaimed wastewater use is growing at an estimated rate of 15 percent per year [1]. As weather and climate patterns continue to change, water shortages persist, and populations swell, it is anticipated that spray and subsurface irrigation of wastewater will continue to grow as viable options to meet increasing demands for water and maximize existing water resources [1]. However, as the use of reclaimed wastewater expands, it is necessary to identify and evaluate the extent of human exposures to emerging wastewater contaminants such as antibiotic-resistant bacteria and antimicrobials, particularly among the highest exposed occupational groups such as reclaimed wastewater irrigation workers.

Microbial Exposures Among Irrigation Workers: Over the past four decades, several researchers have investigated microbial exposures that may be experienced by reclaimed wastewater irrigation workers and their families. Most of the studies have focused on exposures to enteric viruses, enteric bacteria (e.g. fecal coliforms, *Escherichia coli*, enterococci) and bacteriophage [2-9], and the findings have been mixed. For example, Katzenelson et al. (1976) conducted the first study of the incidence of enteric diseases in agricultural communities in Israel that were using partially-treated wastewater for spray irrigation, and found that the incidence of salmonellosis, shigellosis, typhoid fever and hepatitis was two to four-fold higher in communities practicing spray irrigation. In another study, the same research group found no differences in the incidence of enteric disease in communities exposed to wastewater aerosols from spray irrigation compared to unexposed communities [5]. More recently, Devaux et al. (2001) observed an increased incidence of rashes, itchy skin and cuts among wastewater spray irrigation workers [10]. Nonetheless, to our knowledge, no research groups have investigated exposures to antibiotic-resistant bacteria among wastewater irrigation workers despite studies that have shown that levels of resistant enteric bacteria found in both untreated and treated wastewater are significantly higher than those found in control surface waters [11-16]. And yet, the development and spread of antibiotic-resistant bacteria remains one of the most serious infectious disease risks in both agricultural and other populations [17]. In particular, the recent increase in community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections has been identified as one of the most important emerging occupational and public health risks worldwide [18;19].

CA-MRSA infections most commonly present as skin and soft tissue infections; yet, increasing numbers of cases are progressing to bacteremias, invasive tissue infections and deaths [19;20]. Because of the increasingly severe nature of CA-MRSA infections [20], it is necessary to understand the various environmental exposure pathways that could lead to these infections. Some investigators have begun to explore food pathways of exposure to CA-MRSA [21-23]; however, there have been limited investigations that have explored other environmental sources [24;25]. Those studies that have been performed have focused largely on the role of healthcare environments in the spread of this organism. To our knowledge, no groups have tested U.S. reclaimed wastewater for MRSA, nor sought to understand whether spray irrigators are being exposed to this important emerging community-acquired pathogen.

Pharmaceutical Exposures Among Irrigation Workers: In addition to potential exposures to antibiotic-resistant bacteria, wastewater irrigation workers also could be exposed to numerous pharmaceuticals that remain in reclaimed water [16]. In particular, workers could be exposed to a variety of commonly used antimicrobials including amoxicillin, oxacillin, penicillin, azithromycin and ciprofloxacin to name a few [16;26-28]. Studies conducted in the United States and Europe have identified concentrations of these (and other) antimicrobials ranging from 1 to 150 ppm in both untreated and treated wastewater [29;30], as well as surface waters that receive treated effluent[27]. Some studies have shown that certain wastewater treatment processes, including the use of activated sludge, can reduce or eliminate some parent compounds. Nonvolatile, hydrophobic antimicrobials, such as ciprofloxacin and tetracycline, can partition onto activated sludge [16;31]; however, more hydrophilic compounds, including trimethoprim, sulfamethoxazole, and hydrophilic metabolites of parent compounds can remain in treated wastewater [31;32].

To date, the few human health risk assessments that have been performed concerning the issue of pharmaceuticals in the environment indicate that low levels of antimicrobials present in the environment may not be a major risk to human health [33;34]. Yet, in these and other related risk assessments, the focus has been on exposure to a single compound over a lifetime, and the effects associated with exposures to mixed pharmaceuticals—as observed in treated wastewater—have not been adequately addressed. Moreover, these studies have evaluated risk associated with low levels of antimicrobials present in environmental media such as surface waters impacted by treated wastewater where a certain degree of dilution has already taken place. To our knowledge, no groups have studied potentially higher environmental exposures to antimicrobials that may be experienced by spray irrigation workers who have direct contact with reclaimed wastewater.

Specific Aims:

In light of the research gaps summarized above, the long-term goal of this proposed study was to advance understanding of reclaimed wastewater irrigation workers' exposures to antibiotic-resistant bacteria and antimicrobials in two U.S. regions (the Mid-Atlantic and Midwest) that are characterized by different meteorological conditions (e.g. rainfall, humidity, temperature and wind speed). Our specific aims were as follows:

Specific Aim 1: To evaluate air, wastewater, dermal swab and nasal swab samples collected during wastewater irrigation activities at sites in Maryland and Nebraska for the presence of antibiotic-resistant *Staphylococcus aureus* and *Enterococcus* spp.

Hypothesis 1: Antibiotic-resistant Staphylococcus aureus and Enterococcus spp. will be present in air, wastewater, dermal swab and nasal swab samples collected during wastewater irrigation activities in both Maryland and Texas.

Specific Aim 2: To evaluate air and wastewater samples for the presence of residues from commonly used antimicrobials, including oxacillin, tetracyclines, and triclocarban.

Hypothesis 2: Detectable levels of antimicrobial residues will be present in all air and wastewater samples.

Methodology:

Study Sites (Aims 1 & 2): Four wastewater treatment plants (WWTPs) (two in Maryland which are referred to as the Mid-Atlantic sites, and two in Nebraska which are referred to as the Midwest sites) were included in the study. The treatment steps and sampling locations at each of the treatment plants are illustrated in Figure 1 [35]. Four wastewater spray irrigation sites that are associated with the WWTPs were also included in the study and served as the focus of the project: Mid-Atlantic Spray Irrigation Site 1 (Mid-Atlantic SI 1) received wastewater effluent from Mid-Atlantic WWTP1; Midwest Spray Irrigation Site 1 (Midwest SI 2) received wastewater effluent from Midwest WWTP1; and Midwest Spray Irrigation Sites 2 and 3 (Midwest SI 2 and Midwest SI 3) received wastewater from Midwest WWTP2.

Environmental sample collection at the WWTPs (Aims 1 & 2): A total of 44 grab samples were collected between October 2009 and October 2010 from the 4 WWTPs included in the study: 12 samples from Mid-Atlantic WWTP1; 8 from Mid-Atlantic WWTP2; 12 from Midwest WWTP1; and 12 from Midwest WWTP2. The timing of each sampling event was determined by the availability and

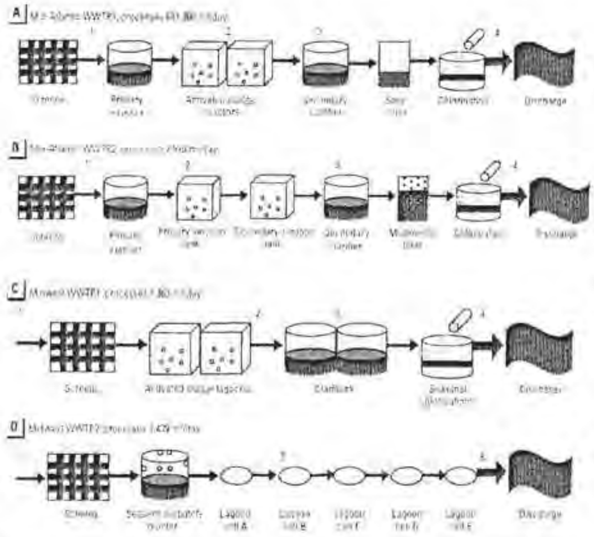

Figure 1. Schematic of wastewater treatment processes at four wastewater treatment plants in the Mid-Atlantic and Midwest regions of the United States. For Mid-Atlantic WWTP1 (*A*) and Mid-Atlantic WWTP2 (*B*), 1 = influent, 2 = activated sludge reactor, 3 = post aeration, and 4 = effluent. (*C*) For Midwest WWTP1, 1 = influent, 2 = post aeration, 3 = secondary clarifier, and 4 = effluent. (*D*) For Midwest WWTP2, 1 = influent, 2 = cell B, and 3 = effluent.
*Seasonal chlorination takes place in June, July, and August.

schedule of the WWTP operators. Samples were collected in 1-L sterile polyethylene Nalgene® Wide Mouth Environmental Sample Bottles (Nalgene, Lima, OH), labeled, and transported to the laboratory at 4°C. All samples were processed within 24 hr.

Environmental sample collection at the spray irrigation sites (Aims 1 & 2): A total of 40 reclaimed wastewater samples were collected between August 2009 and October 2010 from the 4 spray irrigation sites included in the study. These samples were also collected in 1-L sterile polyethylene Nalgene® Wide Mouth Environmental Sample Bottles (Nalgene, Lima, OH), labeled, and transported to the laboratory at 4°C, where they were processed within 24 hr. During wastewater irrigation activities, air samples were also collected using SKC biosamplers (SKC Inc., Eight Four, PA) or two-stage impactors (Tisch Environmental Inc., Cleves, OH). Briefly, in August 2009, air samples were collected for a period of 30 min using duplicate biosamplers at calibrated flow rates of 12.5 liters per minute. For samples collected from October 2009 through October 2010, tryptic soy agar (TSA) plates were placed in each of the two stages of duplicate impactors and samples were collected for one, five, and ten minute intervals. Temperature and relative humidity was monitored throughout the air sampling periods. Control air samples were collected at nearby upwind locations.

Nasal and Dermal Swab Sampling (Aim 1): After reclaimed wastewater irrigation activities at Mid-Atlantic SI 1, dermal swab samples and nasal swab samples were collected from all wastewater irrigation workers that were present at the site and willing to participate in the study (IRB approval was obtained from institutional review boards at the University of Maryland College Park and the University of Nebraska Medical Center, and informed consent was obtained from each participant). 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 cm 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 cm area of the participant's right forearm was swabbed by rolling the swab back and forth 15 times. An equal number of dermal swab and nasal swab samples were recovered from a convenience sample of office workers at the University of Maryland College Park. These office workers were matched by age (+/- 2 years), sex and race to the wastewater irrigation workers included in the study. Samples from the office workers served as controls. After collection, all nasal and dermal swabs samples were inserted into Stuart's transport media, labeled, and transported to the laboratory at 4°C. All samples were processed within 24 hr.

Questionnaire (Aim 1): During nasal swab and dermal swab collection, study participants were also asked to complete a short questionnaire. Questions included those on demographics, smoking status, previous MRSA diagnoses (if any), and previous work experience in a healthcare setting (if any). Each of these factors could influence carriage rates of MRSA and other antibiotic-resistant bacteria. Questionnaire data were input into an Excel database, linked with the exposure data, and cleaned using

Stata/IC 10 (StataCorp LP, College Station, TX) where descriptive and inferential statistics were performed.

Isolation (Aim 1): Membrane filtration was used to recover *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *Enterococcus* spp., and vancomycin-resistant *Enterococcus* spp. (VRE) from wastewater and air samples. Streak plating was used to recover *S. aureus*, MRSA, *Enterococcus* spp., and VRE from nasal and dermal swab samples. m Staphylococcus broth (Becton, Dickinson and Company, Franklin Lakes, NJ), MRSASelect (Bio-Rad Laboratories, Hercules, CA) and Baird Parker (Becton, Dickinson and Company) were the media used for the isolation of MRSA and total *S. aureus*. Enterococcosel agar (BD Diagnostic Systems), membrane-Enterococcus Indoxyl-β-D-Glucoside (mEI) agar (EMD Millipore, Darmstadt, Germany), and vancomycin-amended mEI agar (EMD Millipore, Darmstadt, Germany) were the media used for the isolation of *Enterococcus* spp. and VRE. Resulting colonies were purified on Brain Heart Infusion (BHI) agar (Becton, Dickinson and Company) and archived in Brucella broth (Becton, Dickinson and Company) with 15% glycerol at –80°C. For quality control and quality assurance throughout the isolation process, *S. aureus* ATCC 43300 and *Enterococcus faecalis* ATCC 29212 were used as positive controls and phosphate-buffered saline was used as a negative control.

Confirmation (Aim 1): S. aureus was confirmed using the gram stain, the coagulase test (Becton, Dickinson and Company), the catalase test, and PCR. DNA extraction was carried out using the MoBio UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) per the manufacturer's recommendations. For confirmation of *S. aureus* and MRSA differentiation, PCR amplification of the *S. aureus*-specific *nuc* gene, the *mec*A gene, and a 16S rDNA internal control was performed. Enterococci were confirmed using the gram stain, the catalase test, and by detection of pyrrolidonyl peptidase (pyr) activity (Remel, Lenexa, KS, U.S.). A multiplex PCR assay was also used to identify enterococci to the species level.

Antimicrobial susceptibility testing (Aim 1): Antimicrobial susceptibility testing was performed using the Sensititre® microbroth dilution system with GPN3F and CMV5ACDC minimal inhibitory concentration (MIC) plates (that each included 18 test antibiotics) in accordance with the manufacturer's instructions on all PCR-confirmed MRSA, methicillin-susceptible *S. aureus* (MSSA), VRE, and vancomycin-susceptible enterococci (VSE) (Trek Diagnostic Systems Inc., Cleveland, OH). *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were used as quality control strains. MICs were recorded as the lowest concentration of an antimicrobial that completely inhibited bacterial growth[36]. Resistance breakpoints published by the Clinical and Laboratory Standards Institute were used[36]. Multidrug resistance (MDR) was defined as resistance to two or more classes of antibiotics.

MRSA characterization (Aim 1): Several additional methods (above and beyond those proposed in the grant proposal) were used to characterize the MRSA isolates including SCC*mec* typing, PVL screening and pulsed field gel electrophoresis (PFGE). We used a multiplex PCR assay developed by Milheiriço et al. (2007) to characterize the MRSA isolates by SCC*mec* type. SCC*mec* strains COL (type I), BK2464 (type II), ANS46 (type III), MW2 (type IVa), HAR22 (type IVh), and HDE288 (type VI) were used as positive controls for SCC*mec* typing. All MRSA isolates, confirmed by possession of the *nuc* and *mec*A genes by PCR and an identifiable SCC*mec* type, were screened for PVL by PCR of the *pvl* gene according to Strommenger et al. (2008). *S. aureus* ATCC strain 25923 was used as a positive control. For PFGE, we used *Sma*I (Promega, Madison, WI) to digest genomic DNA. Digested samples were run in 1% SeaKem® Gold agarose gels (Cambrex Bio Science Rockland Inc., Rockland, ME) in 0.5X TBE (tris-borate- EDTA) using a CHEF Mapper (Bio-Rad) for 18.5–19 hr at 200 V, 14°C, and initial and final switch of 5 and 40 sec. Cluster analysis was performed using BioNumerics software v5.10 (Applied Maths Scientific Software Development, Saint-Martens-Latem, Belgium) using Dice coefficient and the unweighted pair-group method. Optimization settings for dendrograms were 1.0% with a position tolerance of 0.95%. Based on the similarity of the control strains, isolates were considered clones if similarity was ≥ 88%. *Salmonella* serotype Braenderup strain H9812 was used as the standard. PFGE strain types were compared with USA types (100, 200, 300, 400, 500, 600, 700, 800, 1000, and 1100).

<u>*Antimicrobial residue analysis (Aim 2)*</u>:

Extraction of reclaimed wastewater samples: Aliquots (200 mL) of the reclaimed wastewater samples were extracted for antimicrobial analyses following our previously published methods with some modifications [27]. In brief, a 10 µL aliquot of a methanol stock solution containing 10 µg/mL of the surrogate standard (Linezolid-d3, Toronto Research Chemical Inc, Toronto Canada, Cat # L466502) was added to all samples. Following thorough mixing and equilibration, samples were extracted using Oasis HLB (60 mg) cartridges (Waters Corp; Milford MA), pre-equilibrated with 3mL methanol followed by 3mL water. Samples were loaded under a vacuum using Visiprep 12-port Vacuum Manifolds (Sigma-Aldrich, St. Louis, MO). Following loading of the sample, cartridges were washed with 1mL water containing 5% methanol by volume, and analytes were eluted with 6 mL of Acetonitrile with 0.2% formic acid followed by 3 mL of Methanol:Acetone mix (50:50) at a flow rate of 0.5 mL/min. Extracts were dried under nitrogen at 40°C and reconstituted in 1 mL of Acetonitrile: 0.1% Formic Acid mix (50:50). A 10 ul aliquot of 10µg/mL Internal Standard (OxolinicAcid-d5, Toronto Research Chemical Inc, Toronto, Canada) was added to each sample.

Antimicrobial analysis: Antimicrobials that are commonly used in the United States and have been previously detected in wastewater samples were analyzed using our previously described ID-LC-MS/MS method [27]. The list of antibiotics included in the analysis, along with their limits of detection, is provided in Box 1. The analytical instrumentation consisted of an Applied Biosystem ABI3000 tandem mass spectrometer with positive electrospray ionization. Chromatographic separation was achieved using an Xterra MS C18 2.5um, 2.1x50mm column (Waters Corporation, Milford, MA) with a pre-column filter (Phenomenex, Torrance CA).

Box 1: List of antibiotics Included in the Analysis

Antibiotics	Parent Ion	Daughter Ion	LOD (ng/L)
Ampicillin	366.7	206.9	24.2
Azithromycin	375.0	113.1	9.2
Ciprofloxacin	331.5	287.4	13.1
Linezolid	337.5	295.4	21.7
Oxacillin	402.0	158.2	20.1
Oxolinic Acid	261.1	243.0	21.3
Penicillin G	334.6	158.2	30.8
Pipemidic Acid	303.4	215.9	27.9
Tetracycline	445.0	409.9	10.7

<u>*Statistical Analyses (Aims 1 & 2):*</u> Descriptive statistics included the percentages of samples positive for MRSA, MSSA, VRE and VSE by WWTP and spray irrigation site. Two-sample tests of proportions were performed between MRSA and MSSA isolates with respect to the percent resistance of each group of isolates to each of the 18 tested antibiotics. Analysis of variance was used to compare the average numbers of antibiotics against which MRSA and MSSA isolates were resistant. Analysis of variance also was used to compare the concentration of antibiotics across influent, effluent and spray irrigation sites. Antibiotic concentrations between Mid Atlantic and Midwest sites were compared using the student t-test. In all cases, p-values ≤ 0.05 were defined as statistically significant. All statistical analyses were performed using Stata/IC 10 (StataCorp LP, College Station, TX) and SAS 9.2 (SAS Institute Inc., Cary, NC).

<u>Results and Discussion for Specific Aim 1:</u>

<u>*Occurrence of antibiotic-resistant S. aureus at U.S. WWTPs:*</u> We detected MRSA at all four WWTPs in this study. The distribution of MRSA-positive samples differed by WWTP, sampling date, and sampling location (Table 1)[35]. Across all treatment plants sampled, 50% (22/44) of wastewater samples were positive for MRSA. Eighty-three percent (10/12) of influent samples from all WWTPs were MRSA-positive; 100% (5/5) from Mid-Atlantic WWTPs and 71% (5/7) from Midwest WWTPs. MRSA was not detected in any tertiary-treated (chlorinated) effluent samples (Table 1). <u>However, MRSA was detected</u>

in one effluent sample from Midwest WWTP1 in October 2010 when chlorination was not taking place. Overall, Midwest WWTP2 had the lowest percentage of MRSA-positive wastewater samples, with MSRA detected only in the influent (Table 1). This plant is the only WWTP in the present study that does not use an activated sludge reactor step; instead, it uses a system of lagoons for biological treatment.

Table 1. Distribution of MRSA-positive and -negative wastewater samples at all WWTPs by sampling event and sampling location.

	Mid-Atlantic WWTP1 (*n* = 12)			Mid-Atlantic WWTP2 (*n* = 8)		Midwest WWTP1 (*n* = 12)			Midwest WWTP2 (*n* = 12)				
Sampling location (total samples collected)	Oct 2009	Dec 2009A	Dec 2009B	Oct 2010A	Oct 2010B	Jul 2010	Sep 2010	Oct 2010	Jul 2010	Aug 2010	Sep 2010	Oct 2010	Total positive samples (%)
Influent (*n* = 12)	Pos	Pos	Pos	Pos	Pos	Neg	Pos	Pos	Pos	Pos	Neg	Pos	10/12 (83)
Activated sludge reactor (*n* = 5)	Pos	Pos	Pos	Pos	Pos	—	—	—	—	—	—	—	5/5 (100)
Post aeration (*n* = 3)	—	—	—	—	—	Neg	Pos	Pos	—	—	—	—	2/3 (67)
Cell B (*n* = 4)	—	—	—	—	—	—	—	—	Neg	Neg	Neg	Neg	0/4 (0)
Secondary clarifier (*n* = 8)	Neg	Pos	Pos	Neg	Neg	Pos	Neg	Pos	—	—	—	—	4/8 (50)
Effluent (*n* = 12)	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos[a]	Neg	Neg	Neg	Neg	1/12 (8)
Total positive samples (%)	2/4 (50)	3/4 (75)	3/4 (75)	2/4 (50)	2/4 (50)	1/4 (25)	2/4 (50)	4/4 (100)	1/3 (33)	1/3 (33)	0/3 (0)	1/3 (33)	22/44 (50)

Abbreviations: Neg, negative sample; Pos, positive sample. Samples were collected twice during December 2009 at Mid-Atlantic WWTP1 (A and B) and twice during October 2010 at Mid-Atlantic WWTP2 (A and B).
[a]Sample was collected when chlorination of effluent was not taking place.

MSSA was also detected at all four WWTPs in this study. The distribution of MSSA-positive samples differed by WWTP, sampling date, and sampling location (Table 2)[35]. Across all treatment plants sampled, 55% (24/44) of wastewater samples were positive for MSSA. Eighty-three percent (10/12) of influent samples from all WWTPs were MSSA-positive; 100% from Mid-Atlantic WWTPs and 71% from Midwest WWTPs. MSSA was not detected in tertiary-treated (chlorinated) effluent samples (Table 2). However, MSSA was detected in two effluent samples from Midwest WWTP1 in September and October 2010 when chlorination was not taking place. Of all four WWTPs, Midwest WWTP2 had the lowest percentage of MSSA-positive wastewater samples, and MSSA was detected only in the influent.

Table 2. Distribution of MSSA-positive and -negative wastewater samples at all WWTPs by sampling event and sampling location.

	Mid-Atlantic WWTP1 (*n* = 12)			Mid-Atlantic WWTP2 (*n* = 8)		Midwest WWTP1 (*n* = 12)			Midwest WWTP2 (*n* = 12)				
Sampling location (total samples collected)	Oct 2009	Dec 2009A	Dec 2009B	Oct 2010A	Oct 2010B	Jul 2010	Sep 2010	Oct 2010	Jul 2010	Aug 2010	Sep 2010	Oct 2010	Total positive samples (%)
Influent (*n* = 12)	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Pos	Pos	Pos	Neg	Pos	10/12 (83)
Activated sludge reactor (*n* = 5)	Pos	Pos	Pos	Pos	Pos	—	—	—	—	—	—	—	5/5 (100)
Post aeration (*n* = 3)	—	—	—	—	—	Pos	Pos	Pos	—	—	—	—	3/3 (100)
Cell B (*n* = 4)	—	—	—	—	—	—	—	—	Pos	Neg	Neg	Neg	1/4 (25)
Secondary clarifier (*n* = 8)	Neg	Pos	Pos	Neg	Neg	Pos	Neg	Pos	—	—	—	—	4/8 (50)
Effluent (*n* = 12)	Neg	Neg	Neg	Neg	Neg	Neg	Pos[a]	Pos[a]	Neg	Neg	Neg	Neg	2/12 (17)
Total positive samples (%)	2/4 (50)	3/4 (75)	3/4 (75)	2/4 (50)	2/4 (50)	3/4 (75)	2/4 (50)	4/4 (100)	2/3 (67)	1/3 (33)	0/3 (0)	1/3 (33)	24/44 (55)

Abbreviations: Neg, negative sample; Pos, positive sample. Samples were collected twice during December 2009 at Mid-Atlantic WWTP1 (A and B) and twice during October 2010 at Mid-Atlantic WWTP2 (A and B).
[a]Samples were collected when seasonal chlorination was not taking place.

In terms of antibiotic resistance, 93% and 29% of unique MRSA and MSSA isolates, respectively, were multidrug resistant (MDR) (Figure 2)[35]. In particular, the MRSA isolates expressed resistance to several antibiotics approved by the U.S. Food and Drug Administration for treating MRSA infections, including tetracycline, ciprofloxacin, levofloxacin, gatifloxacin, and clindamycin, as well as linezolid and daptomycin (Figure 3)[35], which are important alternatives to older antibiotics for treating severe MRSA infections. Among the MRSA isolates, SCC*mec* types II and IV, the pvl gene, and USA types 100, 300, and 700 (PFGE strain types commonly found in the United States) were identified. The large cluster of MRSA isolates that were PVL-positive and showed similarity to USA 300 is concerning because both USA 300 strains and the *pvl* gene are associated with increased virulence, severe bloodstream infections, and necrotizing pneumonia [37]. To our knowledge, these are the first

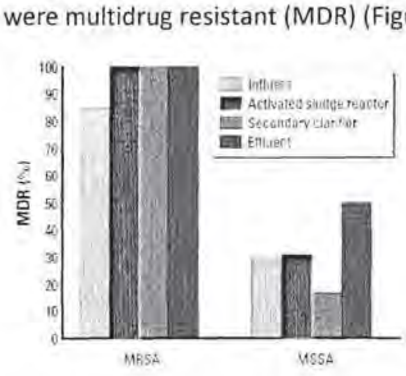

Figure 2. Percentage of multidrug-resistant (resistant to two or more classes of antibiotics) MRSA and MSSA isolates from all WWTPs, by wastewater treatment step.

data to show that both MRSA and MSSA are prevalent at U.S. WWTPs. Encouragingly, the odds of samples being positive decreased as treatment progressed. For example, 10 of 12 (83%) influent samples were MRSA-positive, but only 1 of 12 (8%) effluent samples was MRSA-positive (Table 1). Based on these findings, wastewater treatment seems to reduce the number of MRSA and MSSA isolates released in effluent that is subsequently reclaimed for spray irrigation activities. However, the few isolates that do survive in effluent might be more likely to be MDR and virulent isolates.

Occurrence of antibiotic-resistant Enterococcus spp. at U.S. WWTPs: We also detected VRE at all four WWTPs included in the study (Table 3). The distribution of VRE-positive samples differed by WWTP, sampling date, and sampling location. Across all treatment plants sampled, 32% (16/50) of wastewater samples were positive for VRE: 50% (13/26) of samples from Mid-Atlantic WWTPs; and 13% (3/24) of samples from Midwest WWTPs. Forty-three percent (6/14) of influent samples from all WWTPs were VRE-positive; 71% (5/7) from Mid-Atlantic WWTPs and 14% (1/7) from Midwest WWTPs. No VRE were detected in any tertiary-treated (chlorinated) effluent samples (Table 3).

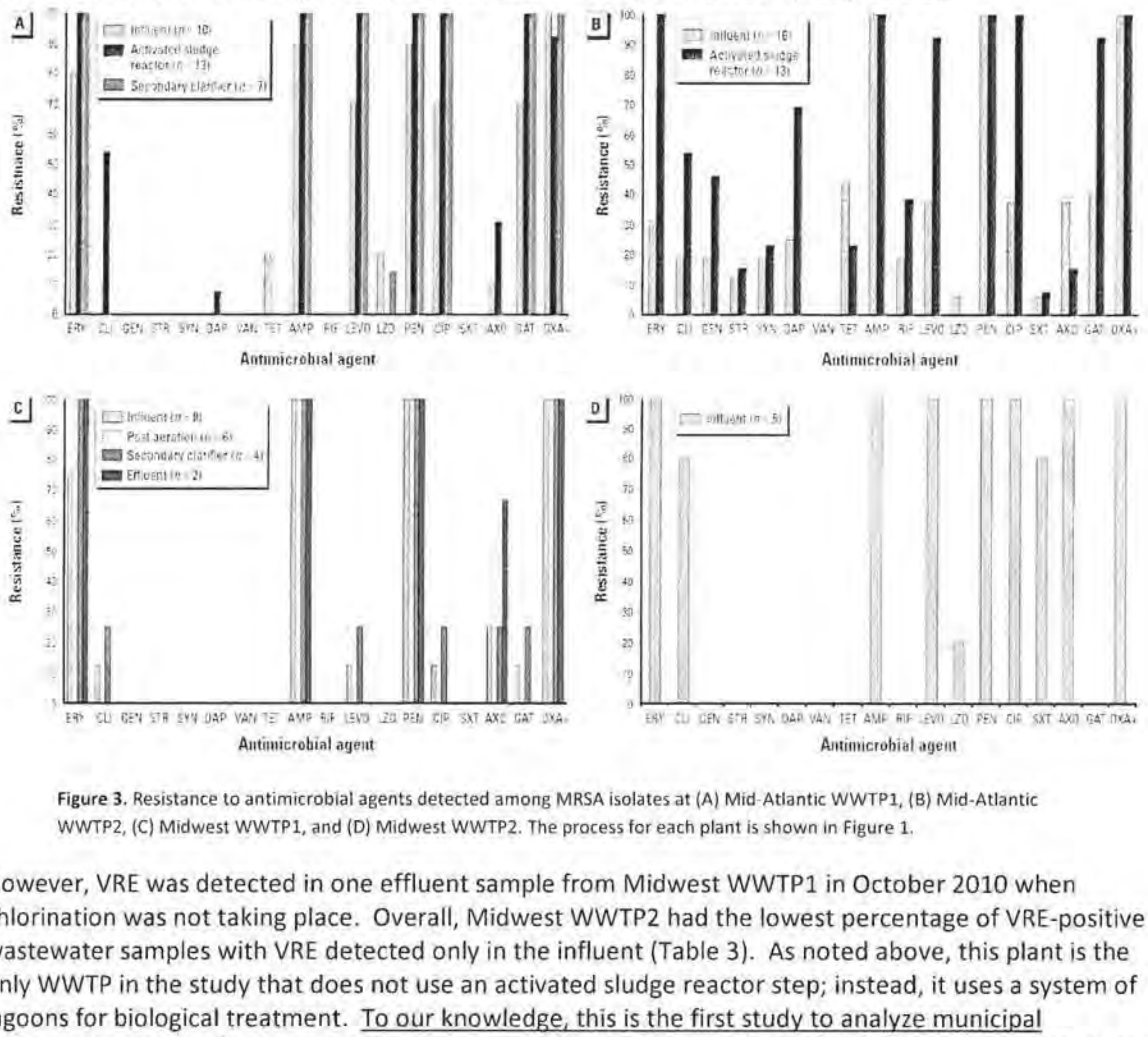

Figure 3. Resistance to antimicrobial agents detected among MRSA isolates at (A) Mid-Atlantic WWTP1, (B) Mid-Atlantic WWTP2, (C) Midwest WWTP1, and (D) Midwest WWTP2. The process for each plant is shown in Figure 1.

However, VRE was detected in one effluent sample from Midwest WWTP1 in October 2010 when chlorination was not taking place. Overall, Midwest WWTP2 had the lowest percentage of VRE-positive wastewater samples with VRE detected only in the influent (Table 3). As noted above, this plant is the only WWTP in the study that does not use an activated sludge reactor step; instead, it uses a system of lagoons for biological treatment. To our knowledge, this is the first study to analyze municipal wastewater planned for reuse for the presence of VRE. Also, to our knowledge, this is the first study to evaluate wastewater from the U.S. Mid-Atlantic region for the presence of VRE. Our findings suggest that tertiary wastewater treatment effectively reduces the occurrence of VRE in discharged effluent.

Table 3: Distribution of vancomycin-resistant *Enterococcus* spp. positive wastewater samples at all WWTPs, sampling events and sampling locations.

Sampling location (total number of samples collected)	Mid-Atlantic WWTP 1 (n=12)			Mid-Atlantic WWTP 2 (n=8)		Midwest WWTP 1 (n=12)			Midwest WWTP 2 (n=12)				Total (%)
	Oct-12	Dec 09a	Dec 09b	Oct 10a	Oct 10b	Jul-12	Sep-12	Oct-12	Jul-12	Aug-12	Sep-12	Oct-12	
Influent (n=14)	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	4/12(33)
Activated sludge reactor (n=5)	Neg	Pos	Pos	Neg	Neg	–	–	–	–	–	–	–	2/5(40)
Post aeration (n=3)	–	–	–	–	–	Neg	Neg	Neg	–	–	–	–	0/3(0)
Cell B (n=4)	–	–	–	–	–	–	–	–	Neg	Neg	Neg	Neg	0/4(0)
Secondary clarifier (n=8)	Pos	Pos	Pos	Neg	Pos	Pos	Neg	Neg	–	–	–	–	5/8(63)
Effluent (n=14)	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos[a]	Neg	Neg	Neg	Neg	1/12(8)
Total (n=44) (%)	2/4 (50)	3/4 (75)	3/4 (75)	0/4 (0)	1/4 (25)	1/4 (25)	0/4 (0)	1/4 (25)	0/3 (0)	1/3 (33)	0/3 (0)	0/3 (0)	12/44(27%)

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

Figure 4. Percent resistance of total *Enterococcus* isolates recovered from Mid-Atlantic Spray Irrigation Site 1.

__*Occurrence of antibiotic-resistant S. aureus and Enterococcus spp. at U.S. wastewater spray irrigation sites:*__ MRSA was not detected at any of the reclaimed wastewater spray irrigation sites sampled. We did, however, recover MSSA isolates from one reclaimed wastewater sample from Midwest SI1. Although not resistant to methicillin, the MSSA isolates from the storage pond at Midwest SI1 were all resistant to erythromycin, ampicillin, and penicillin. VRE was detected in one sample (from the "Before UV" sampling location) from Mid-Atlantic SI1 and vancomycin-intermediate *Enterococcus* spp. (VIE) was detected in three samples (from the "Input to Pumphouse" sampling location) from Mid-Atlantic SI1 (Table 4). Other *Enterococcus* spp. isolates were not resistant to vancomycin, but expressed resistance to several other classes of antibiotics. Among total *Enterococcus* spp. recovered from Mid-Atlantic SI1, resistance to the following antibiotics was observed: synercid, vancomycin, tetracycline, penicillin and ciprofloxacin (Figure 4). <u>To our knowledge, this is the first study to analyze reclaimed wastewater samples at reuse sites in the United States for the presence of antibiotic-resistant bacteria.</u> Our findings suggest that although few antibiotic-resistant bacteria might survive distribution to a reuse site, storage in an open air pond could either resuscitate injured antibiotic-resistant bacteria or introduce antibiotic-resistant bacteria from the environment into the water being used for spray irrigation.

Table 4. Distribution of vancomycin-resistant *Enterococcus* (VRE) and vancomycin-intermediate *Enterococcus* (VIE) positive samples at each spray irrigation site.

Sampling location (total number of samples	Mid-Atlantic SI 1 (n=32)								Midwest SI 1 (n=3)			Midwest SI 2 (n=4)				Midwest SI 3 (n=1)	Total (%)
	Aug-09	Oct 09a	June 09a	June 09b	July 09a	July 09b	Sept 09a	Sept 09b	Jul-12	Sep-12	Oct-12	Jul-12	Aug-12	Sep-12	Oct-12	Aug-12	
Before U.V. (n=8)	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	–	–	–	–	–	–	–	–	1/8 (13)
After U.V. (n=8)	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	–	–	–	–	–	–	–	–	0/8 (0)
Storage Pond (n=11)	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	–	–	–	–	–	0/11 (0)
Input to Pumphouse (n=9)	Pos[a]	Pos[a]	Neg	Pos[a]	Neg	Neg	Neg	Neg	–	–	–	–	–	–	–	Neg	3/9 (33)
Hose (n=4)	–	–	–	–	–	–	–	–	–	–	–	Neg	Neg	Neg	Neg	–	0/4 (0)
Total (n=40) (%)	2/4 (50)	1/4 (25)	0/4 (0)	1/4 (25)	0/4 (0)	0/4 (0)	0/4 (0)	0/4 (0)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)	0/1	4/40 (10)

Pos = positive sample
Neg = negative sample
SI = spray irrigation site

Carriage of antibiotic-resistant and susceptible S. aureus and Enterococcus spp. in wastewater spray irrigation workers: We did not detect any MRSA or VRE in any of the nasal or dermal swabs collected from the wastewater spray irrigation workers, or the office worker controls. However, we did detect MSSA in nasal swabs from five (26%) spray irrigation workers and seven (29%) office workers (Figure 5). Based on an NHANES study which found that 20-30% of the general U.S. population is nasally colonized with *S. aureus*, the prevalence of MSSA in nasal swabs among both groups is in line with expected prevalence[37]. Nonetheless, we found that a greater percentage of spray irrigation workers were nasally colonized with MDR MSSA (Figure 5) and a greater percentage of MSSA isolates from spray irrigation worker nasal swabs were resistant to erythromycin, linezolid, and penicillin (Figure 6). VIE and vancomycin-susceptible *Enterococcus* spp. (VSE) were each isolated from one spray irrigation worker nasal swab (VIE 4%; VSE 4%). To our knowledge, this is the first study to evaluate the nasal and dermal carriage of antibiotic-resistant bacteria among reclaimed wastewater spray irrigation workers. Our findings suggest that spray irrigation workers using reclaimed wastewater might be colonized with organisms displaying greater resistance to a number of clinically relevant antibiotics compared to office workers who are not routinely exposed to reclaimed wastewater.

Figure 5. Percentage of reclaimed wastewater spray irrigation workers ever colonized with *Staphylococcus aureus* and multi-drug resistant *S. aureus*.

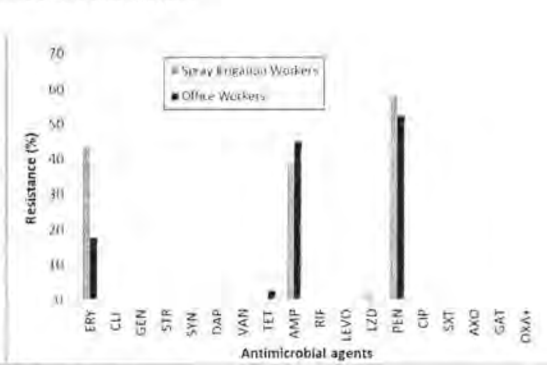

Figure 6. Percent resistance of methicillin-susceptible *S. aureus* recovered from reclaimed wastewater spray irrigation workers and office worker controls.

Results and Discussion for Specific Aim 2:

Occurrence of antimicrobial residues at U.S. WWTPs and spray irrigation sites: The majority of WWTP influent samples had detectable levels of all tested antibiotics except for the following: penicillin G (28% of samples were below the limit of detection (LOD)); ampicillin (21% of samples were below the LOD); as well as oxacillin and tetracycline (18% of samples were below the LOD). Likewise, 70% and 60% of effluent samples were below the LOD for oxacillin and penicillin G, respectively.

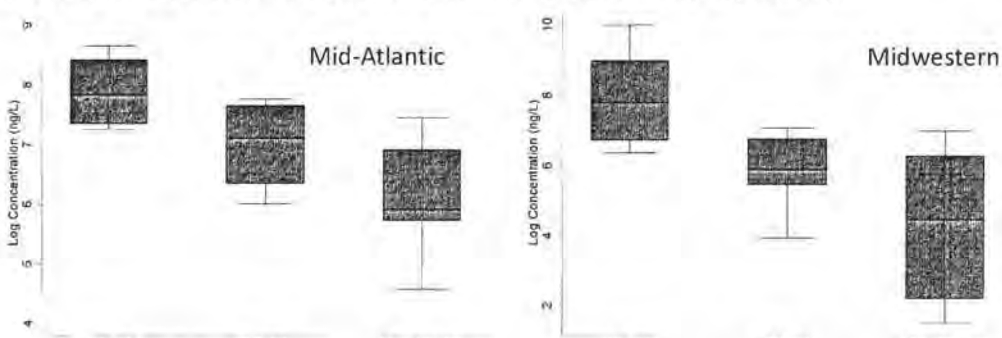

Figure 7. Concentration of azithromycin in influent, effluent and spray irrigation site samples from the Mid-Atlantic and Midwest region of the U.S.

In wastewater samples from both the Mid-Atlantic and the Midwest, azithromycin had the highest concentration (Table 5). Interestingly, the concentration of azithromycin decreased significantly from influent to effluent samples, and then again in reclaimed wastewater samples that were collected from the spray irrigation sites (Figure 7). This observation was consistent across the two regions and was consistent across most antibiotics that were tested. For example, the concentrations of ciprofloxacin

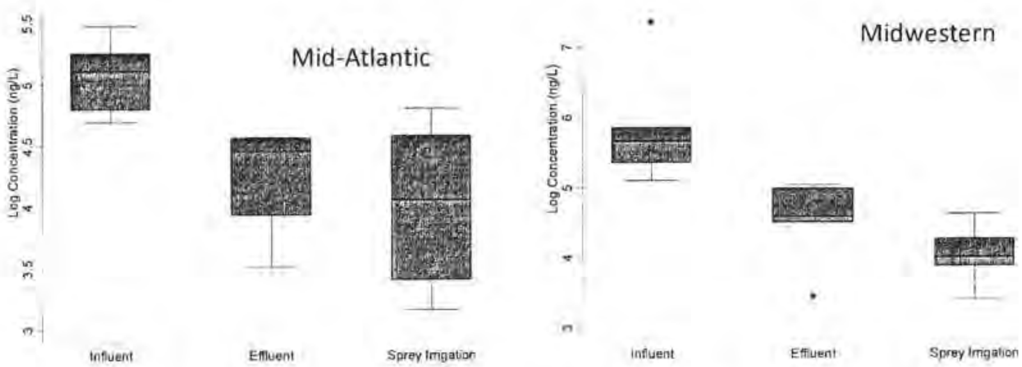

Figure 8. Concentrations of ciprofloxacin in influent, effluent and spray irrigation site samples from the Mid-Atlantic and Midwest regions of the U.S.

across influent, effluent and spray irrigation site samples were similar to those observed for azithromycin (Figure 8). As wastewater treatment proceeded, concentrations declined; and concentrations were the lowest in samples recovered from spray irrigation sites (Figure 8).

The detailed findings for all 9 tested antibiotics across different sampling sites are presented in Table 5. As noted above, azithromycin had the highest concentration among the influent samples (mean 2877 ng/L and 5466ng/L in Mid-Atlantic and Midwest samples, respectively). This was followed by linezolid. This was true for the spray irrigation sites as well, although there was a significant reduction in concentration of both antibiotics in the samples from the spray irrigation sites, compared to the influent samples. While the reduction in concentration was significant for most of the antibiotics measured, there was no appreciable reduction in the concentrations of ampicillin across both study regions (Table 5).

These findings suggest that processes at WWTPs considerably reduce the levels of antibiotics in wastewater. However, the effluent samples from WWTPs still contain elevated levels of antibiotics that are commonly used in human medicine, some of which are approved by the U.S. Food and Drug Administration for treating MRSA infections including tetracycline, ciprofloxacin, and linezolid. Moreover, the antibiotics seem to persist in the treated effluent that is ultimately used as reclaimed wastewater at spray irrigation sites where spray irrigation workers could encounter both dermal and inhalation exposures to these antimicrobials. The potential public health implications of these findings warrant further investigation.

Antibiotics	Influent (ng/L)					Effluent (ng/L)					Spray Irrigation Site (ng/L)				
	N	Mean	SD	Median	P95	N	Mean	SD	Median	P95	N	Mean	SD	Median	P95
Mid Atlantic															
Ampicillin	7	275.0	200.9	335.1	486.8	4	148.9	47.0	164.4	186.5	18	208.1	122.4	204.4	440.6
Azithromycin	7	2877.2	1648.7	2520.2	5683.5	4	1358.3	912.7	1338.4	2350.7	18	672.0	524.2	368.1	1708.7
Ciprofloxacin	7	163.7	44.3	166.0	239.0	4	75.7	29.5	86.5	96.2	18	61.3	32.7	58.4	122.2
Linezolid	7	656.5	473.6	588.7	1525.2	4	595.5	428.1	632.3	1062.5	18	348.1	333.8	248.3	952.2
Oxacillin	7	242.5	146.9	279.9	447.2	4	10.5	0.0	10.5	10.5	18	73.5	84.2	10.5	220.9
Oxolinic Acid	7	330.8	86.5	334.4	428.1	4	175.5	42.0	184.4	216.3	18	186.4	63.6	201.2	276.7
Penicillin G	7	162.2	129.0	186.5	293.2	4	65.8	74.3	38.8	171.5	18	46.4	51.4	14.0	184.8
Pipemidic Acid	7	352.5	204.6	328.4	762.6	4	135.0	69.1	150.3	199.3	18	120.7	72.8	113.6	242.7
Tetracycline	7	298.3	273.4	243.6	872.8	4	83.0	45.5	103.1	110.9	18	83.4	94.1	15.0	357.7
Midwestern															
Ampicillin	7	445.8	403.5	319.4	1069.6	6	459.5	412.3	379.5	930.7	8	470.3	323.1	533.9	929.5
Azithromycin	7	5466.3	7730.2	2409.6	22047.2	6	506.3	421.4	361.3	1170.1	8	318.4	407.0	148.7	1076.3
Ciprofloxacin	7	454.1	494.2	288.3	1565.3	6	102.7	44.7	97.5	154.5	8	60.0	21.7	55.0	101.4
Linezolid	7	1032.4	1060.2	805.7	3171.8	6	245.8	211.6	240.0	471.3	8	148.5	148.3	85.2	434.0
Oxacillin	7	262.3	262.3	222.5	754.6	6	107.1	140.7	23.2	306.4	8	55.0	71.5	10.5	180.9
Oxolinic Acid	7	279.4	132.5	244.1	519.0	6	282.1	156.5	283.8	492.7	8	231.5	84.2	188.9	348.0
Penicillin G	7	335.7	455.9	139.8	1266.0	6	119.3	163.4	14.0	342.2	8	197.3	164.4	149.9	447.1
Pipemidic Acid	7	290.4	120.5	277.8	466.5	6	104.9	82.9	97.7	211.9	8	104.5	42.7	94.4	178.8
Tetracycline	7	290.4	120.5	277.8	466.5	6	181.4	142.2	148.9	415.0	8	190.7	96.9	157.9	367.8

Table 5: Concentration (ng/L) of selected antibiotics in influent, effluent and spray irrigation site samples from the Mid-Atlantic and Midwest regions of the U.S.

Conclusions:

Throughout this research, we achieved each of the aims that we set out to accomplish, and generated novel antibiotic resistance and antimicrobial residue data that provide new insights into infectious disease and other occupational health implications associated with exposures to reclaimed wastewater. To our knowledge, our study is the first to evaluate 1) the occurrence of MRSA in U.S. municipal wastewater; 2) the occurrence of MRSA in treated effluent that is ultimately used for spray irrigation; 3) the occurrence of antibiotic-resistant bacteria at wastewater reuse sites in the U.S.; and 4) the nasal and dermal carriage of susceptible and antibiotic-resistant bacteria among reclaimed wastewater spray irrigation workers. This is also the first study to report on levels of antimicrobial residues in reclaimed wastewater used in reuse applications at U.S. spray irrigation sites.

Our findings show that, although tertiary wastewater treatment may effectively reduce MRSA and other resistant bacteria in wastewater, secondary-treated wastewater (unchlorinated) could be a potential source of exposure to antibiotic-resistant bacteria in occupational settings and reuse applications. Moreover, our findings suggest that spray irrigation workers using reclaimed wastewater might be colonized with organisms displaying greater resistance to a number of clinically relevant antibiotics compared to office workers who are not routinely exposed to reclaimed wastewater. Beyond antibiotic-resistant bacteria, our data suggest that WWTP processes considerably reduce levels of antibiotics in wastewater. Yet, effluent samples and spray irrigation site samples still contain elevated levels of antimicrobial residues which may present a potential exposure risk among spray irrigation workers.

Because of increasing use of reclaimed wastewater, further study is needed to evaluate a broader array of pathogenic bacteria and antimicrobials that may remain in treated wastewater utilized by spray irrigation workers. The findings of this current study may represent only the tip of the iceberg with regard to potential microbial and chemical exposures that may be experienced by individuals working with (and/or exposed to) reclaimed wastewater. In the future, we aim to continue this work, incorporating the application of culture-independent, high-throughput next generation sequencing approaches that would enable the evaluation of total bacterial diversity of reclaimed wastewater, as well as the impacts of reclaimed wastewater exposures on the microbiome of spray irrigation workers.

<u>Publications:</u> (†denotes corresponding author; [#]denotes advised undergraduate students; *denotes advised graduate students; **denotes advised post-doctoral students)

<u>Journal Articles</u>

Rosenberg Goldstein RE*, Micallef SA**, Gibbs SG, Davis JA, He X, George A[#], Kleinfelter LM[#], Schreiber NA[#], Mukherjee S, Sapkota A, Joseph SW, and Sapkota AR†: [2012] Methicillin-resistant *Staphylococcus aureus* (MRSA) detected at four U.S. wastewater treatment plants. *Environmental Health Perspectives.* 120:1551–1558. **Impact Factor: 7.04**.

> This manuscript was selected by EHP as one of its Science Selections for the November 2012 issue: "Superbug Hideout: Finding MRSA in U.S. Wastewater Treatment Plants" <u>http://ehp.niehs.nih.gov/2012/11/120-a437a/</u>.
>
> We also issued a press release concerning the manuscript and received widespread media coverage (e.g., internet; radio and T.V. news) in both the United States and abroad.
>
> <u>NOTE:</u> The data reported in this manuscript are relevant to **<u>Aim 1</u>** which included the assessment of antibiotic-resistant *Staphylococcus aureus* in wastewater samples. Each of the wastewater treatment plants included in this paper supplied reclaimed wastewater to the spray irrigation sites that were tested in this study.

Rosenberg Goldstein RE*, Gibbs SG, Micallef SA**, George A[#], Kleinfelter LM[#], Sapkota A, Joseph SW, and Sapkota AR†. Vancomycin-resistant enterococci (VRE) detected at four U.S. wastewater treatment plants [In preparation]. <u>NOTE:</u> The data reported in this manuscript are relevant to **<u>Aim 1</u>** which included the assessment of antibiotic-resistant *Enterococcus* spp. in wastewater samples. Each of the wastewater treatment plants included in this paper supplied reclaimed wastewater to the spray irrigation sites that were tested in this study.

Sapkota A, Rosenberg Goldstein RE*, Gibbs SG, Joseph SW, and Sapkota AR†. Antimicrobial residues detected in municipal and reclaimed wastewater in the United States [In preparation]. <u>NOTE:</u> The data reported in this manuscript are relevant to **<u>Aim 2</u>,** which included the assessment of antimicrobial residues in wastewater.

Rosenberg Goldstein RE*, Micallef SA**, Gibbs SG, Sapkota A, Joseph SW, and Sapkota AR†. Occupational exposure to *Staphylococcus aureus* and *Enterococcus* spp. among reclaimed wastewater spray irrigation workers in the Mid-Atlantic region of the United States [In preparation]. <u>NOTE:</u> The data reported in this manuscript are relevant to **<u>Aim 1</u>,** which included the assessment of antibiotic-resistant *S. aureus* and *Enterococcus* spp. in nasal and dermal samples collected during wastewater irrigation activities.

Sapkota AR†, Gibbs SG, Rosenberg Goldstein RE*, Micallef SA**, Sapkota A, Joseph SW. Occurrence of antibiotic-resistant bacteria and fecal indicators at reclaimed wastewater spray irrigation sites. [In preparation]. <u>NOTE</u>: The data reported in this manuscript are relevant to **<u>Aim 1</u>**, which included the assessment of antibiotic-resistant *S. aureus* and *Enterococcus* spp. in air and wastewater during wastewater irrigation activities.

Sapkota A, Rosenberg Goldstein RE*, Gibbs SG, Joseph SW, and Sapkota AR†. Association between antimicrobial residues and antibiotic-resistant bacteria detected in municipal and reclaimed wastewater in the United States [In preparation]. NOTE: The data reported in this manuscript are relevant to **Aims 1 & 2.**

Thesis

Rosenberg Goldstein RE. Evaluation of antibiotic-resistant bacteria in tertiary treated wastewater, reclaimed wastewater used for spray irrigation, and resulting occupational exposures. Thesis. Master of Public Health in Environmental Health, 2010. NOTE: The data included in this thesis are relevant to **Aim 1.**

Rosenberg Goldstein RE. Antibiotic-resistant bacteria in wastewater and potential human exposure through wastewater reuse. Dissertation. Doctor of Philosophy in Environmental Health. [In preparation.] NOTE: The data included in this dissertation are relevant to **Aim 1.**

Inclusion of Gender and Minority Study Subjects:

Two female, hispanic women were included in the study out of a total of 48 participants. All other participants were white males. Please see the attached Inclusion Enrollment Report for details.

Inclusion of Children:

Children were not enrolled in this study.

Materials Available for Other Investigators:

The methods that we utilized for recovering methicillin-resistant *S. aureus* (MRSA) from environmental samples were developed by a doctoral student, Rachel Rosenberg Goldstein, in my research group. The methods may be accessed in the following peer-reviewed, open-access manuscript: Rosenberg Goldstein RE*, Micallef SA**, Gibbs SG, Davis JA, He X, George A[#], Kleinfelter LM[#], Schreiber NA[#], Mukherjee S, Sapkota A, Joseph SW, and Sapkota AR†: [2012] Methicillin-resistant *Staphylococcus aureus* (MRSA) detected at four U.S. wastewater treatment plants. *Environmental Health Perspectives*. 120:1551–1558.

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Program Director/Principal Investigator (Last, First, Middle): Sapkota, Amy Rebecca

Inclusion Enrollment Report

This report format should NOT be used for data collection from study participants.

Study Title: Irrigation worker's exposures to antibiotic-resistant bacteria and antimicrobials

Total Enrollment: 48 **Protocol Number:** 09-0211

Grant Number: 5R03OH009598-02 REVISED

PART A. TOTAL ENROLLMENT REPORT: Number of Subjects Enrolled to Date (Cumulative) by Ethnicity and Race

Ethnic Category	Females	Males	Sex/Gender Unknown or Not Reported	Total
Hispanic or Latino	2	0	0	2 **
Not Hispanic or Latino	0	45	0	45
Unknown (individuals not reporting ethnicity)	0	1	0	1
Ethnic Category: Total of All Subjects*	2	46	0	48 *
Racial Categories				
American Indian/Alaska Native	0	0	0	0
Asian	0	0	0	0
Native Hawaiian or Other Pacific Islander	0	0	0	0
Black or African American	0	0	0	0
White	0	45	0	45
More Than One Race	0	0	0	0
Unknown or Not Reported	2	1	0	3
Racial Categories: Total of All Subjects*	2	46	0	48 *

PART B. HISPANIC ENROLLMENT REPORT: Number of Hispanics or Latinos Enrolled to Date (Cumulative)

Racial Categories	Females	Males	Sex/Gender Unknown or Not Reported	Total
American Indian or Alaska Native	0	0	0	0
Asian	0	0	0	0
Native Hawaiian or Other Pacific Islander	0	0	0	0
Black or African American	0	0	0	0
White	0	0	0	0
More Than One Race	0	0	0	0
Unknown or Not Reported	2	0	0	2
Racial Categories: Total of Hispanics or Latinos**	2	0	0	2 **

* These totals must agree.
** These totals must agree.

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Form Approved Through 06/30/2012
OMB No. 0925-0001

Department of Health and Human Services

Final Invention Statement and Certification

(For Grant or Award)

DHHS Grant or Award No

R03OH009598

A. We hereby certify that, to the best of our knowledge and belief, all inventions are listed below which were conceived and/or first actually reduced to practice during the course of work under the above-referenced DHHS grant or award for the period

09/01/2009	through	08/31/2012
original effective date		*date of termination*

B. Inventions (Note: If no inventions have been made under the grant or award, insert the word *"NONE"* under Title below.)

NAME OF INVENTOR	TITLE OF INVENTION	DATE REPORTED TO DHHS
N/A	N/A	
(Use continuation sheet if necessary)		

C. Signature — This block *must* be signed by an official authorized to sign on behalf of the institution.

Title	Name and Mailing Address of Institution
Director	University of Maryland Office of Research Administration 3112 Lee Building College Park, MD 20742 (301) 405-6269 oraa@umd.edu
Typed Name: Antoinette Lawson	
Signature: Antoinette Lawson — Date: 12/17/2012	

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