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Occurrence of vancomycin-resistant and -susceptible *Enterococcus* spp. in reclaimed water used for spray irrigation

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Abstract

Reclaiming municipal wastewater for agricultural, environmental, and industrial purposes is increasing in the United States to combat dwindling freshwater supplies. However, there is a lack of data regarding the microbial quality of reclaimed water. In particular, no previous studies have evaluated the occurrence of vancomycin-resistant enterococci (VRE) in reclaimed water used at spray irrigation sites in the United States. To address this knowledge gap, we investigated the occurrence, concentration, and antimicrobial resistance patterns of VRE and vancomycin-susceptible enterococci at three U.S. spray irrigation sites that use reclaimed water. We collected 48 reclaimed water samples from one Mid-Atlantic and two Midwest spray irrigation sites, as well as their respective wastewater treatment plants, in 2009 and 2010. Samples were analyzed for total enterococci and VRE using standard membrane filtration. Isolates were purified and then confirmed using biochemical tests and PCR. Antimicrobial susceptibility testing was conducted using the Sensititre® microbroth dilution system. Data were analyzed by two-sample proportion tests and one-way analysis of variance. We detected total enterococci and VRE in 71% (34/48) and 4% (2/48) of reclaimed water samples, respectively. *E. faecalis* was the most common species identified. At the Mid-Atlantic spray irrigation site, UV radiation decreased total enterococci to undetectable levels; however, subsequent storage in an open-air pond at this site resulted in increased concentrations of enterococci. *E. faecalis* isolates recovered from the Mid-Atlantic spray irrigation site expressed intrinsic resistance to quinupristin/dalfopristin; however, non-*E. faecalis* isolates expressed resistance to quinupristin/dalfopristin (52% of isolates), vancomycin (4%), tetracycline (13%), penicillin (4%) and ciprofloxacin (17%). Our findings show that VRE are present in low numbers in reclaimed water at point-of-use at the sampled spray irrigation sites; however, resistance to other antimicrobial classes is more prevalent, particularly among non-*E. faecalis* isolates.

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Keywords

antibiotic-resistant bacteria; enterococci; vancomycin-resistant enterococci; wastewater; reclaimed water; spray irrigation

1. Introduction[1]

As the world population increases and global water use escalates, freshwater resources continue to dwindle. To alleviate pressures on freshwater resources, countries—including the United States—are reclaiming treated municipal wastewater for potable and nonpotable reuse (EPA, 2012). This reclaimed water has been defined as “municipal wastewater that has been treated to meet specific water quality criteria with the intent of being used for a range of purposes”(EPA, 2012). In the United States, reclaimed water is used in landscape irrigation, food crop irrigation, snowmaking, groundwater recharge, power production, and indirect and direct potable reuse (EPA, 2012). With increasing reclaimed water use, the potential public health impacts due to microbial contamination of reclaimed water need to be explored and addressed.

Previous studies have shown that a number of bacterial pathogens can survive wastewater treatment including methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, *Salmonella,* and enterococci (Levantesi et al., 2010; Nagulapally et al., 2009; Rosenberg Goldstein et al., 2012; Rosenberg Goldstein et al., 2014). Vancomycin-resistant enterococci (VRE), in particular, have recently been isolated from wastewater effluent (Garcia et al., 2007; Nagulapally et al., 2009; Rosenberg Goldstein et al., 2014) and could persist in distribution systems that supply reclaimed water to spray irrigation sites.

VRE are gram-positive, opportunistic human pathogens that are resistant to vancomycin (a drug of last resort) and can cause urinary tract infections, wound infections, bacteremia and endocarditis (CDC, 2009). Between 2006 and 2007, *Enterococcus* spp. was the third most commonly reported pathogen causing healthcare-acquired infections in the United States (Hidron et al., 2008). Twelve percent and 4% of pathogens recovered from healthcare-acquired infections were *Enterococcus* spp. and VRE, respectively (Hidron et al., 2008). And by 2010, *Enterococcus* spp. became the second leading cause of healthcare-acquired infections (Sievert et al., 2013). Enterococci, in general, are tolerant to an array of environmental stressors, including extreme temperatures (5–65°C), variable pH levels (4.5–10), and high NaCl concentrations (Fisher and Phillips, 2009). Due to the higher tolerance of enterococci to chlorination, these microorganisms can withstand wastewater treatment processes—including tertiary treatments involving chlorination—and persist in the environment (Castillo-Rojas et al., 2013; Varela et al., 2013).

Hospital effluent discharged to municipal wastewater treatment plants has been identified as an important initial source of environmental contamination of VRE (Varela et al., 2013). Antibiotic-resistant enterococci has been recovered from treated municipal wastewater

[1]Abbreviations: Analysis of variance (ANOVA); Clinical and Laboratory Standards Institute (CLSI); minimal inhibitory concentration (MIC); vancomycin-resistant enterococci (VRE); wastewater treatment plant (WWTP)

effluent in the United States, China, and Portugal (Ferreira da Silva et al., 2006; Garcia et al., 2007; Huang et al., 2012; Martins da Costa et al., 2006), and VRE, specifically, has been isolated from treated wastewater effluent in the United States and the United Kingdom (Beier et al., 2008; Caplin et al., 2008; Rosenberg Goldstein et al., 2014).

However, to our knowledge, there are no published studies analyzing reclaimed water recovered from U.S. spray irrigation sites (at point-of-use) for the presence of VRE and total enterococci. In this study, we evaluated the occurrence, concentration, and antimicrobial susceptibilities of VRE and total enterococci recovered from reclaimed water used at three U.S. spray irrigation sites. We also evaluated the impact of storing reclaimed water in open-air ponds on levels of VRE and total enterococci.

2. Materials and Methods

2.1 Study Sites

We sampled three spray irrigation sites that use reclaimed water: one Mid-Atlantic site and two Midwest sites. All sites were chosen based on the willingness of the site operator to participate.

The Mid-Atlantic spray irrigation site (Mid-Atlantic SI1) receives wastewater effluent from a tertiary wastewater treatment plant (WWTP) in an urban area that has been described previously as Mid-Atlantic WWTP1 (Rosenberg Goldstein et al., 2012). Briefly, the raw wastewater influent (681,390 m^3/day) at this plant is comprised of domestic and hospital wastewater and the plant employs the following treatment steps: screens, primary clarifier, primary aeration tank, secondary aeration tank, secondary clarifier, multimedia filter, chlorination, dechlorination and discharge. The chlorination dose at this plant was 2–3 mg/L, followed by dechlorination with sodium bisulfite such that the chlorine residual in effluent is < 0.1 mg/L. This treated effluent is then piped to Mid-Atlantic SI1. Once it arrives at Mid-Atlantic SI1 the effluent passes through a double-walled aluminum screen and is then treated with 254 nanometer wavelength ultraviolet (UV) radiation bulbs that produce a minimum of 30,000 microwatt seconds per square centimeter. After UV treatment, the water is pumped into an open-air storage pond at a rate of 230,000 gallons per day with a peak capacity of 4 million gallons. The reclaimed water is then pumped from the storage pond to spray irrigation heads for use in landscaping (Figure 1).

Midwest spray irrigation site 1 (Midwest SI1) receives wastewater effluent from a tertiary WWTP in a rural area that has been described previously as Midwest WWTP1 (Rosenberg Goldstein et al., 2012). Briefly, the raw wastewater influent (1,363 m^3/day) at this plant is comprised of domestic wastewater and agriculturally influenced stormwater, and the plant employs the following treatment steps: screens, activated sludge lagoons, clarifiers, seasonal chlorination (and dechlorination), and discharge. Seasonal chlorination occurs at this plant in June, July, and August, and during these times the chlorination dose is 4 mg/L with a contact time to assure a chlorine residual of 0 mg/L in effluent. The effluent is then piped to Midwest SI1 where it undergoes no additional treatment, is stored in an open-air storage pond and is then pumped to spray irrigation heads for use in landscaping (Figure 1).

Midwest spray irrigation site 2 (Midwest SI2) receives wastewater effluent from a tertiary WWTP in a rural area that has been described previously as Midwest WWTP2 (Rosenberg Goldstein et al., 2012). Briefly, the raw wastewater influent (1,439 m^3/day) at this plant is comprised of domestic wastewater, wastewater from a food production facility, and agriculturally influenced stormwater, and the plant employs the following treatment steps: screens, sequencing batch reactor, lagoon cell A, lagoon cell B, lagoon cell C, lagoon cell D, lagoon cell E, and discharge. The unchlorinated effluent from this plant is piped to Midwest SI2 where it undergoes no additional treatment, is stored in an open-air storage pond and is then pumped to spray irrigation heads for use in landscaping and crop irrigation (Figure 1).

2.2 Sample collection

A total of 48 reclaimed water samples were included in this study (Table 1). All samples were collected between August 2009 and October 2010, and the timing of sample collection was determined by the site operators. Figure 1 indicates the specific locations where the samples were collected. All samples were collected in 1-L sterile polyethylene Nalgene® Wide Mouth Environmental Sample Bottles and transported to the laboratory at 4°C.

2.3 Isolation

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

2.4 Identification

Total enterococci and VRE were confirmed and identified using the Gram stain, the catalase test, detection of pyrrolidonyl peptidase (pyr) activity (Remel, Lenexa, KS), and a multiplex PCR assay developed by Micallef et al. (2013). Genomic DNA was extracted by heat lysis as described previously (Micallef et al., 2013). Briefly, the PCR reaction targeted the D-alanine:D-alanine ligase (*ddl*) genes of *E. faecalis* and *E. faecium*, the vancomycin resistance-encoding *van*C1 and *van*C2/3 genes of *E. gallinarum* and *E. casseliflavus,* respectively, and an internal control targeting a 350 base pair portion of the 16S rRNA gene. PCR amplification consisted of an initial denaturing step of 95°C for 3 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. Positive controls used for PCR amplification were *E. faecalis* ATCC 51299, *E. faecium* ATCC 51559, *E. casseliflavus* ATCC 25788, and *E. gallinarum* ATCC 49573. Molecular grade water was used as a negative control for PCR amplification.

2.5 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on all PCR-confirmed *Enterococcus* isolates ($n = 41$) using the Sensititre® microbroth dilution system (Trek Diagnostic Systems Inc., Cleveland, OH) following the manufacturer's recommendations. Cultures incubated overnight were transferred to sterile, demineralized water (Trek Diagnostic Systems) to achieve a 0.5 McFarland standard. Then, 50 μL of each suspension was transferred to sterile cation-adjusted Mueller Hinton broth (Trek Diagnostic Systems), and 50 μL of the broth solution was then dispensed into minimal inhibitory concentration (MIC) plates (Trek Diagnostic Systems) that included the following antibiotics: erythromycin, quinupristin/dalfopristin, vancomycin, tetracycline, gentamicin, linezolid, streptomycin, penicillin, and ciprofloxacin. *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 strains were used for quality control. All plates were read manually. MICs were recorded as the lowest concentration of an antimicrobial that completely inhibited bacterial growth. Resistance break points published by the CLSI were used (CLSI, 2012). Multidrug resistance was defined as resistance to two or more classes of antibiotics.

2.6 Statistical analyses

Descriptive statistics included the percentage of reclaimed water samples that were positive for total enterococci and VRE, as well as the antimicrobial susceptibility patterns of all tested isolates. A two-sample proportion test was performed to compare the percentages of enterococci-positive samples between the Mid-Atlantic spray irrigation sites and the Midwest spray irrigation sites. One-way analysis of variance (ANOVA) was performed to compare average log concentrations of total enterococci by treatment step for each spray irrigation site and its corresponding wastewater treatment plant. For the Mid-Atlantic spray irrigation site, ANOVA was followed by linear contrasts as a post-hoc test to compare average log concentrations of total enterococci between specific treatment/storage steps. 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).

3. Results

3.1 Occurrence of total enterococci and VRE

Enterococcus spp. were detected at all spray irrigation sites (Table 1) and in the majority of reclaimed water samples. Specifically, 71% (34/48) of reclaimed water samples were positive for enterococci: 56% (18/32) of samples from Mid-Atlantic SI1; 100% (2/2) of samples from Mid-Atlantic WWTP1; 100% (3/3) of samples from Midwest SI1; 100% (3/3) of samples from Midwest WWTP1; 100% (5/5) of samples from Midwest SI2; and 100% (3/3) of samples from Midwest WWTP2. The percentage of enterococci-positive samples collected from the Midwest region (100%; 14/14) was greater than that of the Mid-Atlantic region (59%; 20/34) (p=0.002).

VRE were detected at 2 out of 3 of the spray irrigation sites. Specifically, 4% (2/48) of reclaimed water samples were positive for VRE: 3% (1/32) of samples from Mid-Atlantic SI1; 0% (0/2) of samples from Mid-Atlantic WWTP1; 0% (0/3) of samples from Midwest

SI1; and 33% (1/3) of samples from Midwest WWTP1. No samples recovered from Midwest SI2 or its associated WWTP were positive for VRE.

Average concentrations of total enterococci and VRE, as well as the percentage of VRE out of total recovered isolates, are shown in Table 1. At the Mid-Atlantic SI1 and Midwest SI1, average concentrations of enterococci were lower in the WWTP effluent samples collected directly from their respective WWTPs compared to the first reclaimed water samples tested at the sites ("Before UV" sample at Mid-Atlantic SI1, and "Pond" sample at Midwest SI1) (Table 1). In contrast, at the Midwest SI2 site, average concentrations of enterococci were higher in the WWTP effluent samples collected directly from the WWTP compared to the first reclaimed water samples tested at the spray irrigation site ("Hose" samples at Midwest SI2) (Table 1). However, these differences were not statistically significant based on one-way ANOVA and post-hoc analyses. At Mid-Atlantic SI1, the concentration of total enterococci in reclaimed water decreased to undetectable levels after on-site UV treatment, but then increased after the water was pumped to and stored in the open-air pond (Table 1; Figure 2). Levels of total enterococci in samples collected from the "Inlet to Pumphouse" at Mid-Atlantic SI1 were statistically significantly higher than levels of total enterococci detected in "Before UV" ($p = 0.02$), "After UV" ($p = 0.005$), and "Pond" ($p = 0.005$) samples (Table 1; Figure 2).

In total, 41 enterococci isolates were recovered from all sampled sites. The majority of these isolates were identified as *E. faecalis* (44%), followed by *E. faecium* (27%), *E. casseliflavus* (12%), *E. gallinarum* (5%) and other (12%) (Table 2).

3.2 Antibiotic resistance patterns

Few isolates were recovered from Midwest SI1 and Midwest SI2; therefore, antibiotic resistance patterns are presented only for isolates recovered from Mid-Atlantic SI1 (n=36). *E. faecalis* recovered from Mid-Atlantic S11 (n=13) were intrinsically resistant to quinupristin/dalfopristin as expected, but did not express resistance to erythromycin, vancomycin, tetracycline, gentamicin, linezolid, streptomycin, penicillin or ciprofloxacin. In contrast, non-*E. faecalis* isolates recovered from Mid-Atlantic SI1 (n=23) expressed resistance to several antibiotics: 52% (12/23) of isolates were resistant to quinupristin/dalfopristin; 4% (1/23) were resistant to vancomycin; 13% (3/23) were resistant to tetracycline; 4% (1/23) were resistant to penicillin; and 17% (4/23) were resistant to ciprofloxacin. Figure 3 depicts the antibiotic resistance patterns of non-*E. faecalis* recovered from each sampling location of Mid-Atlantic SI1.

Three out of 23 (13%) non-*E. faecalis* isolates from Mid-Atlantic SI1 expressed multidrug resistance. One *E. faecium* isolate expressed resistance to quinupristin/dalfopristin, tetracycline and penicillin; one *Enterococcus* spp. isolate that could not be identified to the species level expressed resistance to vancomycin, tetracycline and ciprofloxacin; and one other *Enterococcus* spp. that could not be identified to the species level expressed resistance to quinupristin/dalfopristin and tetracycline.

4. Discussion

4.1 Occurrence of Enterococcus spp.

To our knowledge, this is the first report of the occurrence of enterococci, VRE and other antibiotic-resistant enterococci in reclaimed water recovered from point-of-use at spray irrigation sites in the United States. Previous studies primarily focused on the detection of VRE and *Enterococcus* spp. at wastewater treatment plants and in treated effluent (Beier et al., 2008; Caplin et al., 2008; Ferreira da Silva et al., 2006; Garcia et al., 2007; Huang et al., 2012; Martins da Costa et al., 2006; Rosenberg Goldstein et al., 2014). Other studies have identified the presence of enteric bacteria and fecal indicators (in general) in reclaimed water at point-of-use (Abreu-Acosta and Vera, 2011; Bahri et al., 2001; Ryu et al., 2005). Building upon these previous studies, we detected antibiotic-resistant and -susceptible enterococci in the effluent delivered from wastewater treatment plants to spray irrigation sites, reclaimed water stored at these sites, and reclaimed water at point-of-use.

At the Mid-Atlantic spray irrigation site, where the treated wastewater effluent was further disinfected through on-site UV radiation treatment, total enterococci were significantly reduced to an undetectable level after UV treatment. This finding is consistent with previous studies that have identified UV radiation as a successful disinfectant for enterococci (Conner-Kerr et al., 1998; Luczkiewicz et al., 2011; Nagulapally et al., 2009; Ryu et al., 2005). Of particular note, Nagulapally, et al. (2009) determined that VRE were eliminated to undetectable levels in WWTP effluent after UV disinfection. However, at the Mid-Atlantic spray irrigation site, the benefits of UV-disinfection were negated once the treated water was piped to an open-air pond, where concentrations of total enterococci subsequently increased, possibly due to contamination associated with precipitation/run-off events and wildlife. These data suggest that future guidelines regarding the safe use of reclaimed water at spray irrigation sites need to include water storage recommendations in addition to any on-site treatment requirements.

4.2 Enterococcus Species Diversity

Out of the enterococci isolates that could be identified to the species level, 71% were identified as either *E. faecium* or *E. faecalis*. These findings are in line with our previous study of the wastewater treatment plants that supplied the reclaimed water, where nearly all of the characterized VRE isolates were *E. faecium* or *E. faecalis* (Rosenberg Goldstein et al., 2014). These data are also similar to the recent findings of Ben Said et al. (2015) that demonstrated the predominance of *E. faecium* and *E. faecalis* in both wastewater and surface water samples recovered from Tunisia (Ben Said et al., 2015). These two species are the predominant enterococci species found in the human gastrointestinal tract (Silva et al., 2011); therefore, it is not surprising to detect them in significant numbers in municipal wastewater and reclaimed water (Fisher and Phillips, 2009; Murray, 1990). *E. faecalis is* also the most common species isolated from human clinical enterococcal infections (Fisher and Phillips, 2009; Murray, 1990; Silva et al., 2011) and generally expresses more virulence traits compared to *E. faecium* (Banerjee and Anupurba, 2015). Thus, the common occurrence of *E. faecalis* in reclaimed water samples tested in this study may represent a potential public health concern among spray irrigation workers who are heavily exposed to

this water source, especially if these individuals are immuno-compromised. However, the fact that the infectious dose of enterococci remains unknown hampers our ability to estimate the potential risk of enterococcal infections among spray irrigators or others exposed to reclaimed water.

4.3 Antibiotic Resistance Patterns

While *E. faecalis* was the most predominant enterococcal species isolated in this study, these isolates expressed only intrinsic resistance to quinupristin/dalfopristin, as expected. In contrast, the non-*E. faecalis* isolates recovered from the Mid-Atlantic spray irrigation site expressed resistance to several antibiotics, including quinupristin/dalfopristin (52%), vancomycin (4%), tetracycline (13%), penicillin (4%) and ciprofloxacin (17%) (Figure 3). These resistance rates were considerably lower than those associated with vancomycin-resistant *E. faecium* isolates that were recovered directly from the wastewater treatment plant that supplied reclaimed water to this site (Rosenberg Goldstein et al., 2014), suggesting that the tertiary wastewater treatment process that was employed at Mid-Atlantic WWTP1 resulted in reduced percentages of antibiotic-resistant enterococci exiting the plant. However, our finding that 52% of non-*E. faecalis* isolates (including *E. faecium*) recovered from reclaimed water at point-of-use were resistant to quinupristin/dalfopristin is concerning. Currently, quinupristin/dalfopristin is one of only two chemotherapeutic agents approved by the U.S. Food and Drug Administration for the treatment of vancomycin-resistant *E. faecium* infections (Arias et al., 2012); therefore, the release of quinupristin/dalfopristin-resistant *E. faecium* strains into the environment through reclaimed water could potentially have implications for the successful treatment of VRE infections. Nevertheless, additional studies are needed to better understand the linkages between rates of resistant bacteria detected in environmental media (such as reclaimed water) and resistant bacterial infections occurring in clinical settings.

4.5 Limitations

As with any field study, there were limitations to our work. Our study was based on culture-dependent methods; therefore, if VRE were present in viable but non-culturable states within our reclaimed water samples they would not have been detected by our protocols, resulting in possible underestimations of the prevalence and concentrations of VRE in the tested reclaimed water (Lleo et al., 2001). In addition, because we aimed to generate data on concentrations of enterococci and VRE in reclaimed water samples, we refrained from using enrichment steps in our protocols, which resulted in relatively low numbers of recovered isolates. Finally, because only three spray irrigation sites located in two U.S. regions could be included in the study, our findings are not representative of all spray irrigation sites in the U.S. that are utilizing reclaimed water as an alternative freshwater source.

5. Conclusions

To our knowledge, our study is the first to demonstrate the occurrence, concentration, and antimicrobial susceptibility of enterococci and VRE in reclaimed water at point-of-use at U.S. spray irrigation sites. In summary, total enterococci were recovered from the majority of reclaimed water samples and the prevalence of VRE within these samples was low.

However, resistance to antibiotics other than vancomycin among enterococci recovered from reclaimed water was prevalent. In addition, we showed that UV radiation of reclaimed water at a spray irrigation site was effective in reducing levels of enterococci to undetectable levels; yet, subsequent storage of the reclaimed water in an open-air pond resulted in concentrations of enterococci that were higher than those before UV treatment. Future regulations regarding the use of reclaimed water in spray irrigation activities should include recommendations concerning additional onsite treatment of the water as well as appropriate storage conditions that would limit increases in bacterial growth.

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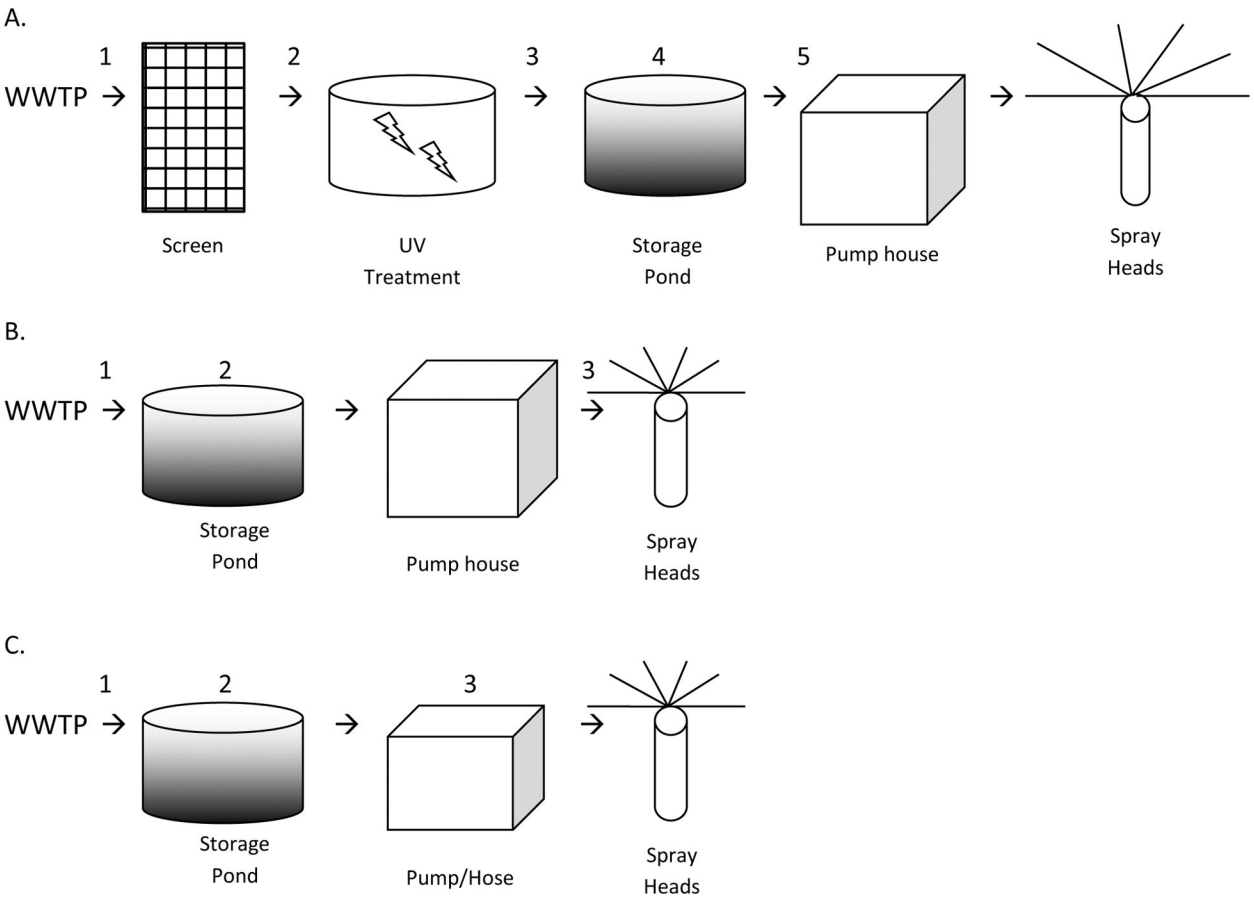

Figure 1.

Treatment and/or storage processes at the spray irrigation sites: A) Mid-Atlantic SI1, B) Midwest SI1, and C) Midwest SI2. Numbers depict sampling locations at each site: A) 1=Effluent, 2=Before UV, 3=After UV, 4=Pond, 5=Inlet to Pumphouse; B) and C) 1=Effluent, 2=Storage Pond, 3=Pump/Hose.

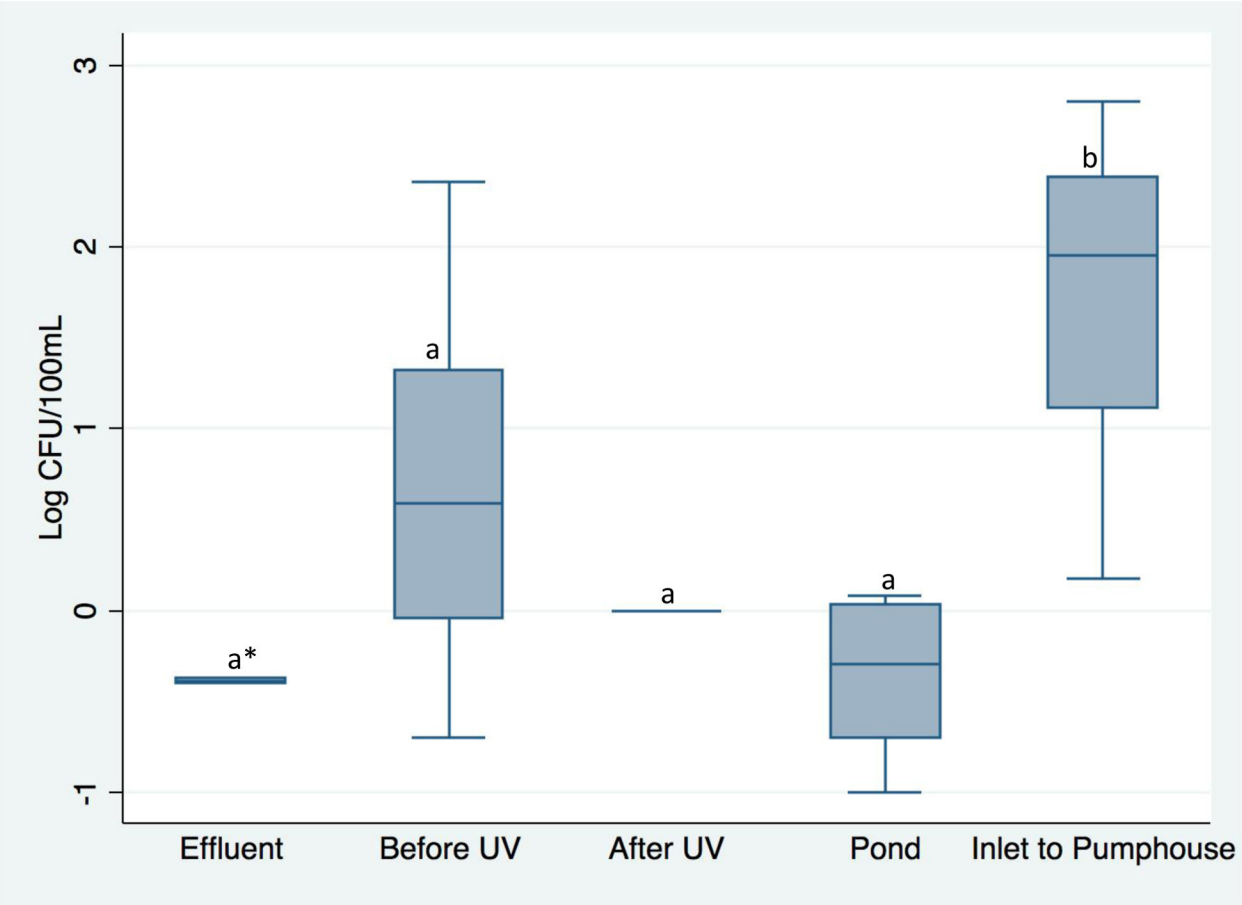

Figure 2.

Average log concentrations of total enterococci (Log CFU/100mL) as reclaimed water flows through treatment and storage locations at Mid-Atlantic Spray Irrigation Site 1. Whiskers are drawn from the 75th percentile to the upper adjacent value and from the 25th percentile to the lower adjacent value, the mid-line is the median, letters indicate statistical significance based on post-hoc analyses ($p \leq 0.05$), and * indicates that the difference between the effluent and inlet to pumphouse samples was marginally significant ($p \leq 0.06$).

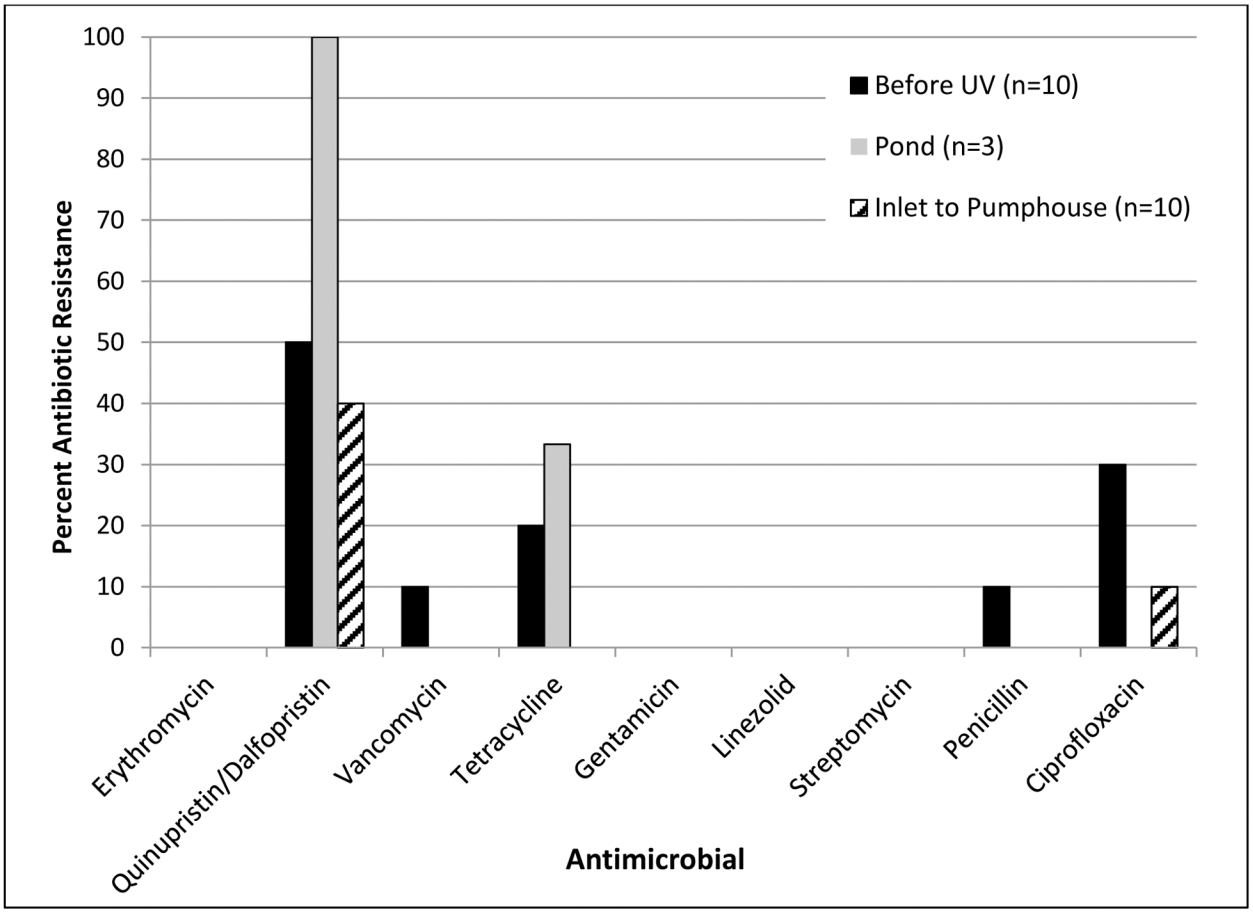

Figure 3:

Antimicrobial resistance patterns among non-*E. faecalis* isolates (*E. faecium, E. casseliflavus, E. gallinarum,* other) recovered from different sampling locations at Mid-Atlantic SI1.

Table 1.

Average concentrations of total enterococci and vancomycin-resistant enterococci (VRE) and percentage of VRE out of total recovered isolates by spray irrigation site and treatment or storage step across all sample collection dates.

Sampling Location (# of samples)	Total Enterococci (CFU/100mL)	VRE (CFU/100mL)	Percentage of VRE
Mid-Atlantic SI1			
Mid-Atlantic WWTP1 Effluent (n=2)	0.41	0.0005	0.1%
Before UV (n=8)	35.78	0.039	0.1%
After UV (n=8)	0	0	0%
Pond (n=8)	0.39	0	0%
Inlet to Pumphouse (n=8)	173.63	0.13	0.07%
Midwest SI1			
Midwest WWTP1 Effluent (n=3)	12.08	5.14	42.6%
Pond (n=3)	120.5	0.67	0.6%
Midwest SI2			
Midwest WWTP2 Effluent (n=4)	56.08	0	0
Hose (n=4)	30.88	0	0

Table 2.

Number and percentage of total enterococci isolates by species and spray irrigation site

	Number of Isolates (%)			
Enterococcus species	**Mid-Atlantic SI1 (n=36)**	**Midwest SI1 (n=4)**	**Midwest SI2 (n=1)**	**Total (n=41)**
E. faecalis	13 (36)	4 (100)	1 (100)	18 (44)
E. faecium	11 (31)	0 (0)	0 (0)	11 (27)
E. casseliflavus	5 (14)	0 (0)	0 (0)	5 (12)
E. gallinarum	2 (6)	0 (0)	0 (0)	2 (5)
Other	5 (14)	0 (0)	0 (0)	5 (12)