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Detection of vancomycin-resistant enterococci (VRE) at four U.S. wastewater treatment plants that provide effluent for reuse

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HIGHLIGHTS

- Vancomcyin-resistant enterococci (VRE) were detected at wastewater treatment plants.
- VRE were not seen in disinfected effluent, but were present in unchlorinated effluent.
- All unique VRE isolates were multidrug resistant.
- The majority of VRE isolates displayed high-level aminoglycoside resistance.
- WWTP workers and spray irrigators using reclaimed effluent could be exposed to VRE.

ARTICLE INFO

ABSTRACT

Vancomycin-resistant enterococci (VRE), a leading cause of hospital-acquired infections, can occur in wastewater. However, to date, no previous studies have evaluated the occurrence of VRE at wastewater treatment plants (WWTPs) that send their treated effluent to reuse sites. We evaluated the occurrence, concentration, and antimicrobial resistance patterns of VRE at U.S. WWTPs associated with reuse sites. We collected 44 wastewater samples, representing treatment steps from influent to effluent, from two Mid-Atlantic and two Midwest WWTPs between October 2009 and October 2010. Samples were analyzed for total enterococci and VRE using membrane filtration. Isolates were confirmed using biochemical tests and PCR. Antimicrobial susceptibility testing was performed by Sensititre® microbroth dilution. Data were analyzed by two-sample proportion tests and analysis of variance. We detected VRE in 27% (12/44) of all wastewater samples collected and VRE represented 3% of total enterococci detected at all WWTPs. More samples were VRE-positive from the Mid-Atlantic compared to the Midwest WWTPs ($p = 0.008$). VRE concentrations decreased as treatment progressed at all WWTPs, except at Mid-Atlantic WWTP1 where there was an increase in VRE concentrations in activated sludge reactor samples. VRE were not detected in chlorinated effluent, but were detected in one un-chlorinated effluent sample. All unique VRE isolates were multidrug resistant. Fifty-five percent (12/22) of the isolates displayed high-level aminoglycoside resistance. Our findings show that chlorination reduces the occurrence of VRE in wastewater. However, WWTP workers could be exposed to VRE during wastewater treatment. Our data also raise potential concerns about VRE exposure among individuals who come into contact with un-chlorinated reclaimed water.

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1. Introduction

The number of hospitalizations associated with antibiotic-resistant bacterial infections in the United States nearly quadrupled between 1997 and 2006 (Mainous et al., 2011). One antibiotic-resistant bacterial pathogen of particular concern is vancomycin-resistant enterococci (VRE), an opportunistic Gram-positive bacterium that is resistant to vancomycin – "a drug of last resort" – and can cause urinary tract infections, wound infections, septicemia, and endocarditis (CDC, 2008; Wegener et al., 1999). The first cases of infection with enterococci that expressed

high-level vancomycin resistance were reported in the United Kingdom in the 1980s (Uttley et al., 1988). As of 2008, VRE were the third leading cause of hospital-acquired infections in the U.S. (Hidron et al., 2008; Uttley et al., 1988).

In addition to VRE's ability to cause multiple types of severe infections, this bacterium is a significant public health concern because of its propensity to acquire and transfer mobile resistance genes (Hayakawa et al., 2012). Acquisition of vancomycin resistance genes can take place between strains of enterococci, but these genes can also be transferred from enterococci to other types of bacteria, including *Staphylococcus aureus* (NIAID, 2009; Sievert et al., 2008). The Michigan Department of Community Health reported the first clinical isolate of vancomycin-resistant *S. aureus* (VRSA) in 2002 (Sievert et al., 2008). VRSA is cause for concern because of the limited treatment options available for this type of infection. As of 2012, only 13 clinical cases of VRSA had been confirmed in the U.S., but the incidence of VRSA infections could continue to rise (CDC, 2012). Resistance genes can also be transferred between enterococci isolates originating from different settings, including clinical strains released into wastewater and community strains contained in wastewater (Guardabassi and Dalsgaard, 2004; Rizzo et al., 2013).

Previous studies have detected VRE at different stages in the wastewater treatment process, including treated effluent, suggesting that VRE present in wastewater effluent could be partially responsible for the dissemination of VRE into the environment and human communities (Araujo et al., 2010; Caplin et al., 2008; Harwood et al., 2001; Kotzamanidis et al., 2009; Luczkiewicz et al., 2010; Morris et al., 2012; Nagulapally et al., 2009; Poole et al., 2005; Shannon et al., 2007; Talebi et al., 2008). As drought conditions continue to stress freshwater resources in the U.S. and other countries, treated municipal wastewater effluent, or reclaimed water, is increasingly used for applications such as landscape and crop irrigation, groundwater recharge, and snowmaking (EPA, 2012). During these processes, individuals who apply, use, or come in contact with reclaimed water could possibly be exposed to VRE and other bacterial pathogens that may persist in this alternative water source.

To our knowledge, there are no published studies analyzing municipal wastewater intended for reuse for the presence of VRE. In this study, we evaluated the occurrence, concentration and antimicrobial susceptibilities of VRE at four wastewater treatment plants (WWTPs) located in the Mid-Atlantic and Midwest regions of the U.S. from which treated wastewater is reused at spray irrigation sites.

2. Materials and methods

2.1. Study sites

We sampled four U.S. WWTPs that distribute treated effluent to reuse sites: two WWTPs in the Mid-Atlantic region and two WWTPs in the Midwest region (Rosenberg Goldstein et al., 2012). Sites in the Mid-Atlantic and Midwest were chosen in order to compare WWTPs in two climatically different regions of the United States. Schematics of each WWTP are published in Rosenberg Goldstein et al. (2012).

Mid-Atlantic WWTP1 is a tertiary WWTP in an urban area that processes 681,390 m³/day of wastewater, with a peak capacity of 1.51 million m³/day. Mid-Atlantic WWTP2 is a tertiary WWTP in a suburban area that processes 7570 m³/day of wastewater and has a peak capacity of 45,425 m³/day. Tertiary wastewater treatment is defined as any treatment beyond primary treatment (physical removal of solids) and secondary treatment (biological treatment) that provides a final treatment step to further improve effluent quality. Tertiary treatments can include filtration, disinfection and lagooning. The incoming wastewater (influent) at both Mid-Atlantic plants includes domestic and hospital wastewater. At Mid-Atlantic WWTP1, the following treatment steps are employed: screens, primary clarifier, activated sludge reactors, secondary clarifier, sand filters, chlorination, dechlorination and discharge. At Mid-Atlantic WWTP2, the following treatment steps

are employed: screens, primary clarifier, primary aeration tank, secondary aeration tank, secondary clarifier, multimedia filter, chlorination, dechlorination and discharge. At both Mid-Atlantic plants, the chlorination dose was 2–3 mg/L, followed by dechlorination with sodium bisulfite such that the chlorine residual in effluent is <0.1 mg/L. The effluent (discharge) from both Mid-Atlantic plants is piped to landscaping sites for reuse in spray irrigation.

Midwest WWTP1 is a tertiary WWTP in a rural area that processes 1363 m³/day of wastewater, with a peak capacity of 10,978 m³/day. The incoming wastewater includes domestic wastewater and agriculturally influenced stormwater. At Midwest WWTP1, the following treatment steps are employed: screens, activated sludge lagoons, clarifiers, seasonal chlorination (and dechlorination), and discharge. Seasonal chlorination occurs 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. This effluent is then piped to a landscaping site for reuse in spray irrigation. Midwest WWTP2 is a tertiary WWTP (with no on-site disinfection) in a rural area that processes 1439 m³/day and has a peak capacity of 7571 m³/day. The incoming wastewater at this plant includes domestic wastewater, wastewater from a food production facility, and agriculturally influenced stormwater. At Midwest WWTP2, the following treatment steps are employed: screens, sequencing batch reactor, lagoon cell A, lagoon cell B, lagoon cell C, lagoon cell D, lagoon cell E, and discharge. Unchlorinated effluent from this plant is piped to an agricultural site for crop irrigation.

2.2. Sample collection

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

2.3. Isolation

Standard membrane filtration was used to recover total enterococci and VRE from the water samples (EPA, 2002). Briefly, ten-fold serial dilutions in the range of 1 to 0.001 mL for influent, activated sludge, and post aeration samples; and 100 to 1 mL for secondary clarifier and cell B samples were prepared using sterilized phosphate buffered saline (PBS) and filtered through 0.45 μm, 47 mm mixed cellulose ester filters (Millipore, Billerica, MA). One liter of each non-diluted effluent sample was also filtered in the same fashion. Filters were then plated in duplicate on membrane-Enterococcus Indoxyl-β-D-Glucoside (mEI) agar (EMD Millipore, Billerica, MA) to isolate total enterococci and mEI agar amended with 16 μg/mL of vancomycin to isolate VRE. Plates were incubated at 41 °C for 24 h. Resulting colonies with blue halos were considered presumptive total enterococci and VRE. These colonies were purified on Brain Heart Infusion (BHI) agar (Becton, Dickinson and Company, Franklin Lakes, NJ) and archived in Brucella broth (Becton, Dickinson and Company) with 15% glycerol at −80 °C. *Enterococcus faecalis* ATCC 29212 was used as a positive control and PBS was used as a negative control throughout the isolation process.

2.4. Identification

VRE were confirmed using the Gram stain, the catalase test, and by detection of pyrrolidonyl peptidase activity (Remel, Lenexa, KS). For confirmation, a multiplex PCR assay developed by Micallef et al. (2013) was used. Genomic DNA from VRE 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 Enterococcus faecium, the vancomycin resistance-encoding vanC1 and vanC2/3 genes of Enterococcus gallinarum and Enterococcus 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.

2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Sensititre® microbroth dilution system in accordance with the manufacturer's instructions on all confirmed VRE (n = 34) and vancomycin-intermediate enterococci (VIE) (enterococci that express intermediate, and not complete, resistance to vancomycin) (n = 22) isolates (Trek Diagnostic Systems Inc., Cleveland, OH). Overnight cultures were transferred to sterile demineralized water (Trek Diagnostic Systems) to achieve a 0.5 McFarland standard. Then, 50 μL of each suspension was transferred to sterile cation-adjusted Mueller Hinton broth (Trek Diagnostic Systems), and 50 μL of the broth solution was then dispensed into GPN3F or custom designed CMV5ACDC minimal inhibitory concentration plates (Trek Diagnostic Systems) that included the following antibiotics (range of concentrations in μg/mL): erythromycin (0.25–8), quinupristin/dalfopristin (synercid) (0.12–32), vancomycin (1–128), tetracycline (2–32), gentamicin (2–16, 128–1024), linezolid (0.5–8), streptomycin (512–2048), penicillin (0.06–16), and ciprofloxacin (0.5–4). E. faecalis ATCC 29212 and S. aureus ATCC 29213 were used as quality control strains. Minimal inhibitory concentrations were recorded as the lowest concentration of an antimicrobial that completely inhibited bacterial growth (CLSI, 2013). Resistance breakpoints published by the Clinical and Laboratory Standards Institute

were used (CLSI, 2013). Multidrug resistance was defined as resistance to two or more classes of antibiotics.

2.6. Statistical analyses

Descriptive statistics included the percentage of wastewater samples that were positive for VRE and the percentage of VRE out of total enterococci by WWTP. A two-sample test of binomial proportions was used to compare the percentage of positive VRE samples between the Mid-Atlantic and the Midwest WWTPs. One-way analysis of variance (ANOVA) was then used to compare the average log concentration of presumptive VRE by treatment step for each WWTP. ANOVA was followed by linear contrasts as a post-hoc test to compare concentrations of presumptive VRE between each specific treatment step. In all cases, p-values of ≤ 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. Presence and concentration of VRE

VRE were detected at all WWTPs in this study and made up 3% of the total enterococci recovered (Table 1). Total enterococci were detected at all WWTPs in all treatment steps, including chlorinated effluent. Across all treatment plants sampled, 27% (12/44) of wastewater samples were positive for VRE; 45% (9/20) of samples from the Mid-Atlantic WWTPs; and 13% (3/24) of samples from the Midwest WWTPs ($p = 0.008$). Thirty-three percent (4/12) of influent samples from all WWTPs were VRE-positive; 60% (3/5) from the Mid-Atlantic WWTPs and 14% (1/7) from the Midwest WWTPs. The percentage of VRE out of total enterococci increased in the activated sludge reactor step at both Mid-Atlantic WWTP1 and Mid-Atlantic WWTP2 but decreased to undetectable levels in the effluent (Table 1). At Midwest WWTP1, the percentage of VRE out of total enterococci increased as treatment progressed, with the highest percentage of VRE present in the effluent (Table 1). No confirmed VRE were detected in any tertiary-treated (chlorinated) effluent samples. However, VRE were detected in one effluent sample from Midwest WWTP1 in October 2010 when chlorination was not being used — the same sample that methicillin-resistant Staphylococcus aureus (MRSA) was isolated from as described in Rosenberg Goldstein et al. (2012).

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

	Total enterococci (CFU/100 mL)	VRE (CFU/100 mL)	Percentage of VRE
Mid-Atlantic WWTP1			
Influent (n = 3)	7.17×10^6	1.93×10^4	0.3
Activated sludge reactor (n = 3)	4.64×10^5	1.89×10^5	40.7
Secondary clarifier (n = 3)	1.49×10^3	9.60×10^1	6.5
Effluent (n = 3)	1.04	0	0.0
Mid-Atlantic WWTP2			
Influent (n = 2)	1.73×10^6	8.56×10^4	4.9
Activated sludge reactor (n = 2)	2.19×10^5	1.32×10^4	6.0
Secondary clarifier (n = 2)	1.86×10^4	8.46×10^2	4.6
Effluent (n = 2)	5.00×10^{-2}	0	0.0
Midwest WWTP1			
Influent (n = 3)	1.21×10^6	4.03×10^4	3.3
Post aeration (n = 3)	6.34×10^4	8.55×10^2	1.3
Secondary clarifier (n = 3)	6.63×10^3	1.03×10^3	15.5
Effluent (n = 3)	1.56×10^1	3.29[a]	21.1
Midwest WWTP2			
Influent (n = 4)	5.30×10^5	2.52×10^3	0.5
Cell B (n = 4)	5.08×10^2	0	0.0
Effluent (n = 4)	6.05×10^1	0	0.0
Total	1.14×10^7	3.52×10^5	3.1

[a] Sample collected in October 2010 when chlorination was not taking place.
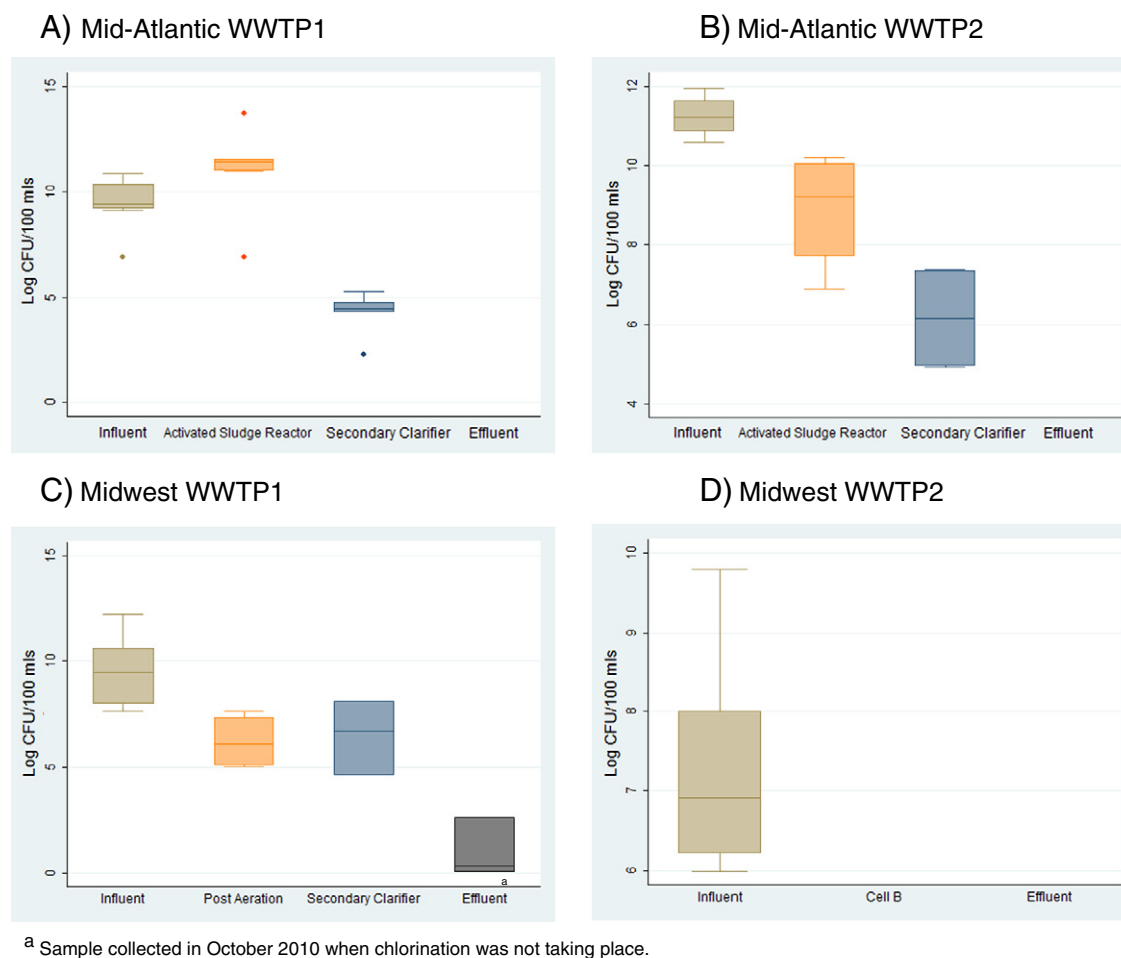
Fig. 1. Average concentration of presumptive vancomycin-resistant enterococci (VRE) by wastewater treatment plant and sampling location. Whiskers are drawn from the 75th percentile to the upper adjacent value and from the 25th percentile to the lower adjacent value, the mid-line is the median, and the points represent outliers.

In general, the average concentration of presumptive VRE at each WWTP decreased as treatment progressed (Fig. 1). Specifically, the ANOVA showed that there were significant differences in VRE concentrations between treatment steps at all WWTPs, except at Midwest WWTP2 where VRE were only detected in one influent sample (Mid-Atlantic WWTP1 $p \leq 0.001$; Mid-Atlantic WWTP2 $p = 0.001$; Midwest WWTP1 $p \leq 0.001$). At Mid-Atlantic WWTP1 and Mid-Atlantic WWTP2, this statistical significance was sustained when comparing each treatment step to one another using linear contrasts (all p-values were less than 0.05). At Midwest WWTP1, the statistical significance achieved by ANOVA was sustained for all linear contrasts between all treatment steps (all p-values were less than 0.05), except when comparing the activated sludge reactor step with post aeration ($p = 0.83$). At Mid-Atlantic WWTP1, there was a slight increase in presumptive VRE concentrations in the activated sludge reactor from 1.9×10^4 CFU/100 mL in influent to 1.9×10^5 CFU/100 mL in the activated sludge reactor. At all WWTPs,

the lowest concentration of VRE was detected in the effluent samples, with no VRE identified in the effluent at Mid-Atlantic WWTP1, Mid-Atlantic WWTP2, or Midwest WWTP2.

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

3.2. Presence of VIE

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

3.3. Antibiotic resistance patterns

In total, 34 VRE isolates were recovered from the four WWTPs. However, only 23 isolates that could be identified as unique using phenotypic analyses were included in the analysis of antibiotic resistance patterns in order to eliminate bias that would have resulted from including clones. All phenotypically unique VRE isolates, of all species, from all WWTPs were multidrug resistant. The resistance patterns among unique isolates varied by WWTP.

The VR *E. faecium* isolates ($n = 14$) from Mid-Atlantic WWTP1 were resistant to multiple antibiotics used to treated enterococci infections including penicillin, ciprofloxacin, and streptomycin (Fig. 2A). The

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

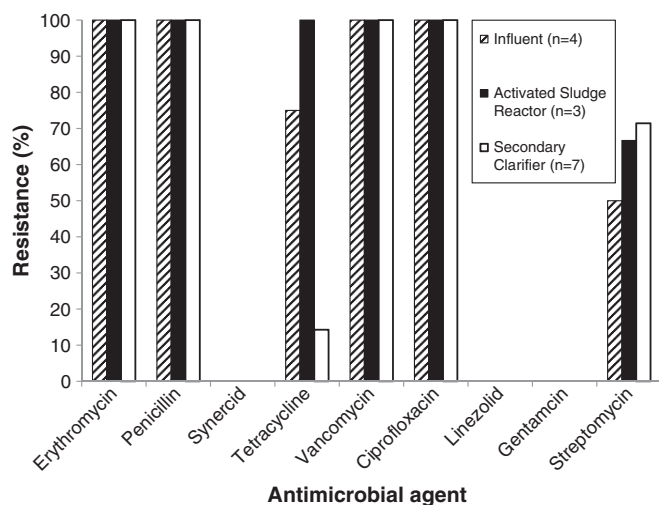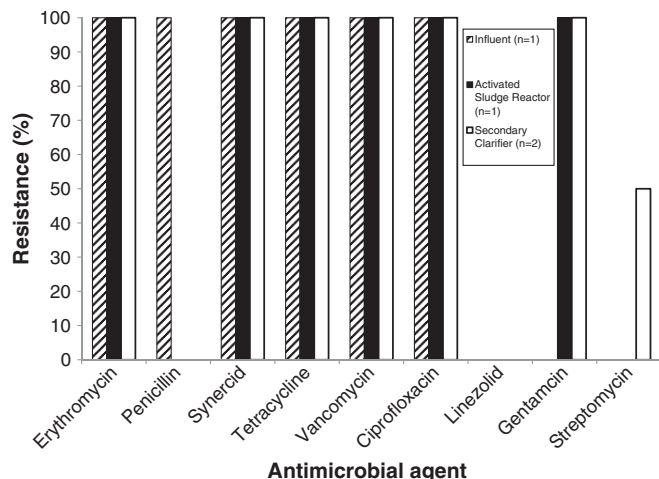| *Enterococcus* species	Number of isolates (%)				
	Mid-Atlantic WWTP1	Mid-Atlantic WWTP2	Midwest WWTP1	Midwest WWTP2	Total
E. faecium	14(77.8)	0(0)	3(100)	1(100)	18(78.3)
E. faecalis	4(22.2)	0(0)	0(0)	0(0)	4(17.4)
E. gallinarum	0(0)	1(100)	0(0)	0(0)	1(4.4)

A. VR *E. faecium*

B. VR *E. faecalis*

Fig. 2. Antimicrobial resistance patterns among (A) vancomycin-resistant *Enterococcus* (VRE) *faecium* and (B) VR *E. faecalis* isolates recovered from Mid-Atlantic WWTP1.

percentage of resistant VR *E. faecium* isolates were mostly constant throughout the treatment process at this WWTP, except for an increasing percentage of isolates displaying resistance to streptomycin as the treatment process progressed and a spike in the percentage of isolates resistant to tetracycline in the activated sludge reactor (Fig. 2A). Four VR *E. faecalis* isolates were detected at Mid-Atlantic WWTP1 (one from influent, one from the activated sludge reactor, and two from the secondary clarifier) (Fig. 2B). All of these VR *E. faecalis* isolates were resistant to erythromycin, quinupristin/dalfopristin (synercid), tetracycline, vancomycin, and ciprofloxacin. Quinupristin/dalfopristin (synercid) resistance observed among VR *E. faecalis* represents intrinsic antibiotic resistance. Three out of four VR *E. faecalis* isolates from Mid-Atlantic WWTP1 were resistant to gentamicin (one from the activated sludge reactor and two from the secondary clarifier), one of the isolates from the secondary clarifier was resistant to streptomycin, and the one isolate from the influent was resistant to penicillin (Fig. 2B).

At Mid-Atlantic WWTP2 the one unique VR *E. gallinarum* isolate was resistant to quinupristin/dalfopristin (synercid) in addition to vancomycin. The three unique VR *E. faecium* isolates from Midwest WWTP1 (one from the secondary clarifier, two from the effluent) were all resistant to erythromycin, penicillin, vancomycin, and ciprofloxacin. From Midwest WWTP2, the one VR *E. faecium* isolate from the influent

expressed resistance to erythromycin, penicillin, tetracycline, vancomycin, ciprofloxacin, and streptomycin.

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

A. Mid-Atlantic WWTP2
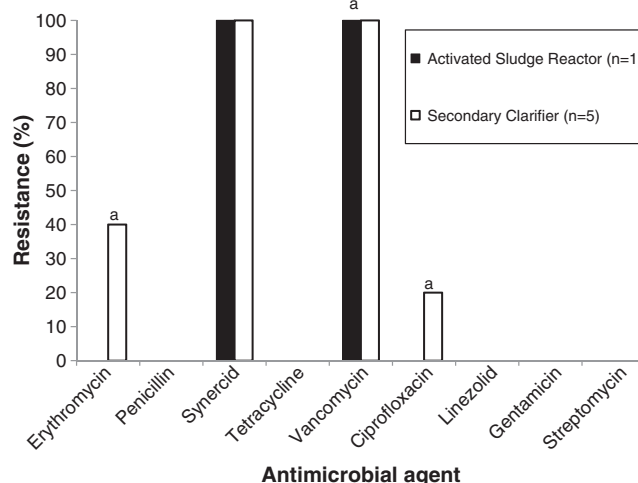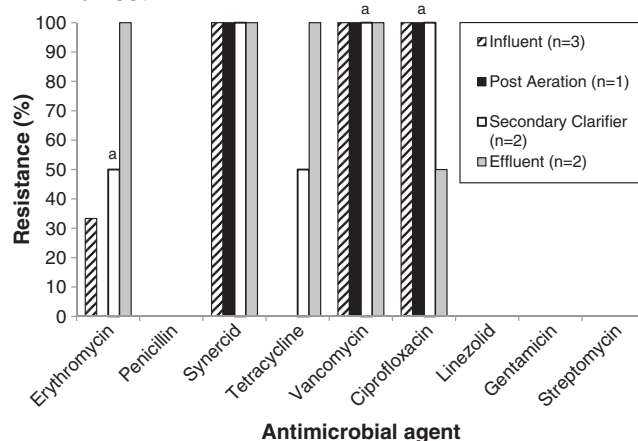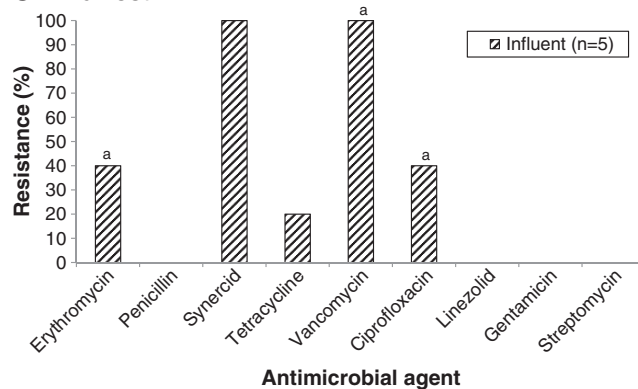

B. Midwest WWTP1

C. Midwest WWTP2

Fig. 3. Antimicrobial resistance patterns among vancomycin-intermediate enterococci (VIE) isolates recovered from (A) Mid-Atlantic WWTP2, (B) Midwest WWTP1, and (C) Midwest WWTP2.

isolates generally remained the same or increased as treatment progressed. At Midwest WWTP2 (Fig. 3C), VIE were only isolated from influent samples.

4. Discussion

4.1. Occurrence of VRE

Previous studies have detected VRE in wastewater, including effluent samples (Araujo et al., 2010; Caplin et al., 2008; Kotzamanidis et al., 2009; Morris et al., 2012). However, to our knowledge, this is the first study to evaluate the occurrence of VRE at U.S. WWTPs from which treated effluent is reclaimed and reused at spray irrigation sites. We identified VRE in 27% of all samples from all WWTPs, including an un-chlorinated effluent sample from Midwest WWTP1 (a WWTP that chlorinates only in summer). On average, the concentration of VRE at each WWTP decreased as treatment progressed, indicating that wastewater treatment seems to be effective in reducing the numbers of VRE released in final effluent.

The majority of studies assessing the presence of VRE in wastewater have been conducted in Europe and have found VRE prevalence ranging from 2 to 52% (Kotzamanidis et al., 2009; Luczkiewicz et al., 2010; Morris et al., 2012). Interestingly, VRE were isolated in 2–3% of samples from European secondary WWTPs (Luczkiewicz et al., 2010; Morris et al., 2012), and in 52% of wastewater samples from a tertiary WWTP that uses chlorination (Kotzamanidis et al., 2009). To our knowledge, Nagulapally et al. (2009) is the only published study to have detected VRE at a U.S. municipal WWTP. Nagulapally et al. (2009) isolated VRE from influent and secondary clarifier samples, but not from UV-disinfected effluent samples (Nagulapally et al., 2009). The lack of VRE in UV-disinfected effluent samples in the Nagulapally et al. (2009) study is interesting given the findings of Luczkiewicz et al. (2011) that showed that UV irradiation actually increased levels of antibiotic-resistant enterococci in treated water (Luczkiewicz et al., 2011). In our study we also identified VRE in influent and biologically treated samples (from the activated sludge reactors, post aeration, and secondary clarifiers), but not in tertiary-treated effluent. These findings provide evidence that tertiary treatments involving chlorination (Mid-Atlantic WWTP1 and Mid-Atlantic WWTP2) or lagooning (Midwest WWTP2) are effective in reducing numbers of VRE to undetectable levels.

Prior studies have also shown that VRE concentrations decrease as wastewater treatment progresses (Kotzamanidis et al., 2009; Morris et al., 2012; Nagulapally et al., 2009). In the current study, we also found that the concentration of VRE decreased as treatment progressed at most WWTPs where samples were collected (Fig. 1). Yet, at Mid-Atlantic WWTP1, there was a slight increase in the concentration of VRE in the activated sludge reactor step. Also, at Mid-Atlantic WWTP1 and Mid-Atlantic WWTP2 the proportion of VRE out of total enterococci was highest in the activated sludge reactor step (Table 1). Previous studies have identified an increase in the concentration or percentage of antibiotic-resistant bacteria in the secondary treatment step, and specifically in activated sludge reactors, which decreased with tertiary treatments (Börjesson et al., 2009; Luczkiewicz et al., 2010; Nakamura and Shirota, 1990). The combination of high concentrations of microorganisms, nutrients, oxygen, and antibiotics found in the activated sludge reactor step could both encourage bacterial growth and select for antibiotic-resistant bacteria.

Interestingly, the un-chlorinated effluent sample from Midwest WWTP1 that contained a confirmed VRE isolate is from the same WWTP and sampling date (October 2010) where MRSA was detected in a previous study by our group (Rosenberg Goldstein et al., 2012), suggesting that chlorination plays an important role in reducing the presence of multiple types of antibiotic-resistant bacteria in wastewater. On the other hand, it is important to note that both VRE and MRSA (Rosenberg Goldstein et al., 2012) were only isolated from influent samples at Midwest WWTP2. Although this plant does not utilize

chlorination or filtration, the sequencing batch reactor at this plant, along with its series of five lagoons seems to effectively eliminate both VRE and MRSA without the use of disinfection or filtration (Fig. 1).

Beyond VRE specifically, it is important to note that vancomycin-susceptible enterococci were isolated from the effluent of all tested treatment plants (Table 1), including those that used chlorination. The survival of enterococci after chlorination during wastewater treatment can be explained by previous studies that have shown enterococci to be extremely hardy microorganisms that not only can survive for long periods of time on environmental fomites but also can tolerate excessive heat, chlorine and certain alcohol concentrations (Arias and Murray, 2012; Bradley and Fraise, 1996). The presence of vancomcyin-susceptible enterococci in treated wastewater effluent has both public health implications for users of reclaimed water and ecological implications for receiving waters.

4.2. Regional differences

The different VRE prevalence rates between WWTPs in the Mid-Atlantic (45%) and Midwest (13%) ($p = 0.008$) observed in this study could be a reflection of geographic differences in human VRE infection rates (Bouchillon et al., 2005; CDDEP, 2013). Since 2004, more VRE have been isolated from humans in the Mid-Atlantic region of the U.S. compared to the Midwest (Bouchillon et al., 2005; CDDEP, 2013). The regional differences in clinically confirmed VRE in the U.S. could be due, in part, to aggressive intervention and control efforts undertaken in the late 1990s at acute and long-term care facilities in Iowa, Nebraska, and South Dakota, including the isolation of patients colonized with VRE and a greater emphasis on hand washing and cleaning (Low et al., 2001; Ostrowsky et al., 2001). The prevalence of VRE in facilities that participated in the intervention program in this region decreased from 2.2% to 0.5% between 1997 and 1999 (Ostrowsky et al., 2001). Beyond regional differences in human VRE infection rates, the Mid-Atlantic and Midwest WWTPs differed by surrounding land uses and types of influent received, which also could help explain the varying VRE prevalence rates observed in this study. The Mid-Atlantic WWTPs are located in urban and suburban settings and receive domestic and hospital influent, whereas both Midwest WWTPs are located in rural areas and receive agriculturally influenced stormwater in addition to domestic wastewater (Rosenberg Goldstein et al., 2012).

4.3. Species diversity

E. faecium was the dominant species of VRE identified in wastewater samples in this study (78.26%). Because wastewater contains human excreta, and *E. faecium* and *E. faecalis* are the two species of enterococci that most commonly cause human infections, our findings on species diversity verified our expectations (Table 1) (Hayakawa et al., 2012). Several previous studies in Europe and Iran that have analyzed species diversity among VRE isolated from wastewater also found a greater percentage of VR *E. faecium* than VR *E. faecalis* in wastewater (Araujo et al., 2010; Kotzamanidis et al., 2009; Luczkiewicz et al., 2010; Morris et al., 2012; Talebi et al., 2008). In a study of clinical VRE species diversity, Deshpande et al. (2007) also found that 92.8% of VRE isolates from 26 U.S. sites were *E. faecium* (Deshpande et al., 2007). However, the study by Nagulapally et al. (2009) that detected VRE at one U.S. municipal WWTP in the Midwest region found a greater percentage of *E. faecalis* among VRE isolates than any other species (Nagulapally et al., 2009).

4.4. Antibiotic resistance patterns

The presence of high-level aminoglycoside resistance in more than half (54.5%) of the enterococci isolated in this study is cause for concern given that the current international guidelines for treatment of *Enterococcus* infections recommend a combination of ampicillin and an aminoglycoside antibiotic (including gentamicin, streptomycin, and

kanamycin) (Fernández-Hidalgo et al., 2013). Fifty percent (9/18) of VR *E. faecium* isolates from all WWTPs sampled in this study were resistant to streptomycin, and none were resistant to gentamicin. Among the four VR *E. faecalis* isolates detected at Mid-Atlantic WWTP1, 25% (1/4) were streptomycin-resistant and 75% (3/4) were gentamicin-resistant. Gentamicin-resistance has previously been found to be more prevalent among clinical VR *E. faecalis* isolates compared to VR *E. faecium* (Deshpande et al., 2007). Previous studies have also isolated high-level aminoglycoside-resistant enterococci and VRE from wastewater samples and found a wide range of the prevalence of VRE displaying high-level aminoglycoside resistance (Kotzamanidis et al., 2009; Luczkiewicz et al., 2010; Rice et al., 1995; Talebi et al., 2008; Tejedor Junco et al., 2001). Among VR *E. faecalis* isolates collected from municipal wastewater, Kotzamanidis et al. (2009) reported that 5% had high-level gentamicin resistance and 95% had high-level streptomycin-resistance (Kotzamanidis et al., 2009). Twenty-five percent and 75% of VR *E. faecium* isolates, respectively, from wastewater and sludge from Portuguese WWTPs were resistant to gentamicin and streptomycin (Araujo et al., 2010).

4.5. Multidrug resistance

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

4.6. Public health and ecological implications

Our study raises possible public health concerns for WWTP workers and for individuals exposed to treated wastewater during reuse. Based on data from numerous studies, WWTP workers report higher levels of gastrointestinal (GI) and respiratory disease symptoms compared to the general population, although there is conflicting evidence about increased infection risks due to specific pathogens identified in wastewater (Glas et al., 2001; Khuder et al., 1998; Seuri et al., 2005; Thorn and Kerekes, 2001). Because VRE were identified at all WWTPs in the current study, WWTP workers could be exposed to both enterococci and VRE through inhalation, dermal, and accidental ingestion exposure routes. The same workers could be exposed to MRSA (Rosenberg Goldstein et al., 2012), as well as *Legionella* spp. and *Aeromonas* spp., two important human pathogens more commonly associated with waterborne disease that have previously been isolated from reclaimed water (Brissaud et al., 2008; Jjemba et al., 2010; Palmer et al., 1995). These exposures could potentially result in infections among these workers.

Moreover, because both VRE and MRSA (Rosenberg Goldstein et al., 2012) were identified in the final effluent at Midwest WWTP1, and the effluent from this WWTP is sent to a reuse site for irrigation, individuals exposed to reclaimed water could also be at risk for exposure to both of these microorganisms, as well as other human pathogens that survive the distribution process and persist in reclaimed water. Interventions aimed at reducing VRE exposures and infections in clinical facilities including education and increased hand washing have been found to be effective (Low et al., 2001; Ostrowsky et al., 2001). Improving awareness about VRE, and other human pathogens, at WWTPs and reuse sites and encouraging hand washing could be important interventions to explore to reduce potential exposures among WWTP workers and spray irrigation workers.

Beyond human health impacts, our study findings raise ecological concerns with regard to surface waters that receive treated wastewater effluent (Lata et al., 2009). The release of treated wastewater containing antibiotic-resistant enterococci can result in the dissemination and persistence of bacterial antimicrobial resistance in water and the surrounding ecosystem (Lata et al., 2009). Specifically, antibiotic-resistant enterococci released into the water column can be transmitted to aquatic macrophytes, sediment and soil (Mundt, 1961, 1963), subsequently impacting the surrounding ecosystem through the transmission of these microorganisms by animals, fish, insects, wind and rain (Lata et al., 2009). This resulting environmental reservoir of antimicrobial resistance could then serve as a medium for the cycling of antibiotic-resistant strains, such as VRE, between the environment, the food chain and humans (Blanch et al., 2003).

4.7. Limitations

As with all field studies, there are some limitations to this study. If VRE were injured or otherwise present in a viable but nonculturable state, they would not have grown on the vancomycin-amended mEI plates, which are a less hospitable media, possibly resulting in underestimations of VRE occurrence and concentrations (Lleò et al., 2005, 2001). The concentrations of VRE could also have been underestimated because of the use of grab samples in this study. Moreover, because we sought to generate data on concentrations of enterococci and VRE throughout the wastewater treatment process we did not use enrichment methods to culture the isolates. As a result, the total number of isolates that were recovered in this study was relatively low. Finally, the generalizability of our results is limited because we sampled only four WWTPs in two U.S. regions.

5. Conclusions

To our knowledge, our study is the first to demonstrate the occurrence of VRE at U.S. WWTPs from which effluent is reused at spray irrigation sites. Although the concentration of VRE decreased as treatment progressed at the WWTPs sampled, VRE were isolated in one secondary-treated (un-chlorinated) effluent sample. All unique VRE isolates from all WWTPs sampled in this study were multidrug resistant, and were specifically resistant to a number of antibiotics used to treat VRE infections, including the aminoglycosides gentamicin and streptomycin. WWTP workers and those exposed to secondary-treated (un-chlorinated) reclaimed water, a growing alternative freshwater source, could potentially be exposed to VRE that persist in this water.

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