

Vancomycin-Resistant Enterococci and Bacterial Community Structure following a Sewage Spill into an Aquatic Environment

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ABSTRACT

Sewage spills can release antibiotic-resistant bacteria into surface waters, contributing to environmental reservoirs and potentially impacting human health. Vancomycin-resistant enterococci (VRE) are nosocomial pathogens that have been detected in environmental habitats, including soil, water, and beach sands, as well as wildlife feces. However, VRE harboring *vanA* genes that confer high-level resistance have infrequently been found outside clinical settings in the United States. This study found culturable *Enterococcus faecium* harboring the *vanA* gene in water and sediment for up to 3 days after a sewage spill, and the quantitative PCR (qPCR) signal for *vanA* persisted for an additional week. Culturable levels of enterococci in water exceeded recreational water guidelines for 2 weeks following the spill, declining about five orders of magnitude in sediments and two orders of magnitude in the water column over 6 weeks. Analysis of bacterial taxa via 16S rRNA gene sequencing showed changes in community structure through time following the sewage spill in sediment and water. The spread of opportunistic pathogens harboring high-level vancomycin resistance genes beyond hospitals and into the broader community and associated habitats is a potential threat to public health, requiring further studies that examine the persistence, occurrence, and survival of VRE in different environmental matrices.

IMPORTANCE

Vancomycin-resistant enterococci (VRE) are harmful bacteria that are resistant to the powerful antibiotic vancomycin, which is used as a last resort against many infections. This study followed the release of VRE in a major sewage spill and their persistence over time. Such events can act as a means of spreading vancomycin-resistant bacteria in the environment, which can eventually impact human health.

Antibiotic-resistant bacteria (ARB) are a growing public health threat and an economic burden globally. The Centers for Disease Control and Prevention (CDC) in the United States has placed a high priority on addressing antibiotic resistance because of rising rates of ARB infection and associated disease burden and health care costs (1, 2). Most infections caused by ARB are nosocomial transmissions (i.e., originating in a hospital), but the role of environmental reservoirs in spreading ARB outside clinical settings is poorly understood. Studies have emphasized the role of environmental reservoirs in the spread of antibiotic resistance for decades, but more field and laboratory studies are necessary to address the specific mechanisms and conditions under which ARB survive and antibiotic resistance genes (ARGs) persist or can be transferred (3–5). Wastewater treatment plants (WWTPs) are sources of ARB, ARGs, and antimicrobial compounds through both treated effluent and the unplanned release of raw sewage to surface waters (6–9). ARB, ARGs, and antibiotics can be released into aquatic environments through human and agricultural waste, establishing routes of human exposure and threats to ecosystem health.

Vancomycin is a glycopeptide antibiotic that is used to treat infections caused by Gram-positive bacteria. It is considered a drug of last resort because of its historical success with the most recalcitrant infections caused by Gram-positive bacteria (10, 11). When vancomycin is rendered ineffective (i.e., when target bacteria are resistant), therapeutic treatment may fail and infections can be fatal (12, 13). Intrinsic, low-level resistance to vancomycin is characteristic of *Enterococcus casseliflavus* and *Enterococcus gal-*

linarum but is of less clinical concern than acquired, high-level vancomycin resistance ($\geq 32 \mu\text{g} \cdot \text{ml}^{-1}$) (14). Acquired vancomycin resistance can occur through the transfer of mobilizable genetic elements (15–17). Nine genes that confer vancomycin resistance in enterococci have been described, eight of which can be acquired (18). The most concerning from a public health perspective is the *vanA* gene, which is linked to most infections with human vancomycin-resistant enterococci (VRE). *vanA* is usually carried on a plasmid-borne transposon (Tn1546) (19–21) and confers high-level resistance to vancomycin ($>64 \mu\text{g} \cdot \text{ml}^{-1}$) (22).

The use of the glycopeptide avoparcin in animal agriculture in Europe has been linked to clinical vancomycin resistance (23–25). Although glycopeptides have not been used in animals in the United States, clinical incidence of VRE has steadily increased in past decades (2, 18, 26–29). Detection of VRE in the United States

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has been predominantly in clinical cases and hospital sewage (30, 31). The monitoring of VRE and associated resistance genes outside the hospital setting is necessary to better understand the spread of resistance and the increased risk to public health (6). Previous studies in Europe and Australia have reported community spread of VRE and fecal colonization of nonhospitalized individuals, but this has not been shown in the United States (32–35).

Antibiotic resistance can spread in bacterial habitats in the external environment, where antibiotics, ARB, and ARG enter water and sediments (6). The influx of sewage-associated microbes and other allochthonous bacteria into an aquatic environment can have ecological impacts, affecting community structure, nutrient cycling, and other ecosystem processes (36–38). In addition, the dynamics of gene exchange in microbial communities can be altered, and transfer of resistance genes may occur (39, 40). VRE and vancomycin resistance genes have been detected globally in the feces of agricultural and wild animals (30, 41–44), surface waters (45–47), WWTPs (48), domestic (community) sewage (49), and hospital sewage (30, 46, 50). Clinically relevant strains and *vanA* genes have rarely been reported in the environment in the United States (51, 52). The prevalence of genes encoding vancomycin resistance in the environment may increase the frequency of transfer to other Gram-positive pathogens (53), including the opportunistic pathogen *Staphylococcus aureus* (54). The incidence of vancomycin-resistant *S. aureus* (VRSA) in hospitals is low; however, 13 incidences have been reported in the United States as of 2014 (55), and the emerging threat is a concern for public health.

Relatively little information is available about the prevalence of clinically relevant VRE and *vanA* genes in aquatic environments, but many studies that have attempted to detect them have failed to find them in relatively pristine environments. Studies around the world have infrequently and inconsistently detected *vanA* genes and *Enterococcus* species isolates with *vanA* phenotypes in WWTP effluent and surface waters (56–59). One study in the United States isolated *Enterococcus faecium* carrying *vanA* genes on a recreational marine beach in Washington (52), but no other confirmation has been established outside hospital settings. In this field study, culturable VRE and/or *vanA* genes were detected in sediment and water samples after a sewage spill released more than 500,000 gallons of untreated sewage in a residential neighborhood. Illumina next-generation sequencing (NGS) of environmental DNA from sediment and water revealed the temporal changes in the microbial community after a major influx of untreated sewage.

MATERIALS AND METHODS

Sample collection. A sewer line break in Pinellas County, FL, released more than 500,000 gallons of untreated sewage into a neighborhood drainage ditch beginning 27 September 2014. The line break was repaired with a bypass valve on 30 September 2014 after the sewage leakage was diverted. The site was also washed down, vacuumed, and disinfected with lime. A well-point system was also installed at the site to dewater, which resulted in groundwater discharge. Well-point systems are commonly used in engineering and construction and consist of a series of vacuum pumps designed to draw water up out of the ground. The ditch is connected to estuarine waters through wetlands. Photos of the site are included in Fig. S3A and B in the supplemental material. Water and sediment samples were collected at the spill site, along the drainage ditch for a distance of 800 m, and in adjacent receiving waters. Samples were col-

lected seven times over the course of 7 weeks after the spill (1 October 2014 to 21 November 2014), to determine the persistence of sewage-associated microbes and VRE in the environment.

Six sites (NC-01, NC-02, NC-03, NC-04, NC-05, and NC-06) were selected for spatial assessment, but the majority of reported results are limited to one site that was sampled on all dates, NC-03. The additional sites where early sampling occurred are noted in the maps provided in Fig. S1 in the supplemental material. Site NC-01 became inaccessible after the first 2 weeks of sampling because it was filled in by construction crews. We were not able to collect sediment at the boat ramp in any instance because the site was a dock surrounded by mangroves. The boat ramp was included to represent recreational waters that may have been impacted by the spill. Water samples were collected in 500-ml sterile containers. Sediment samples were collected using a 50-ml sterile, screw-cap tube to scoop up the top 1 to 2 cm of sediments. All samples were transported on ice to the laboratory and processed within 6 h. Enterococci were also quantified by the Pinellas County Water and Sewer Department staff at 16 sites (see Fig. S2A and B in the supplemental material) near the point of the line break for 12 days using standard methods (ASTM D6503-99).

Isolation of and confirmation of VRE. Water and sediment samples were processed using membrane filtration according to U.S. Environmental Protection Agency (EPA) Method 1600 for culturable enterococci (60), with modifications for the detection of VRE. Water samples were processed in multiple volumes (1 to 300 ml) on each sampling date over the course of the sampling period to account for variability in enterococcal concentrations. Vancomycin stock solution was prepared as an aqueous solution from sodium salt (Acros Organics/Thermo Fisher Scientific, NJ, USA) and sterile nuclease-free water to a final concentration of $10 \text{ mg} \cdot \text{ml}^{-1}$ and filter sterilized. To detect culturable VRE, *Enterococcus* indoxyl- β -D-glucoside (mEI) agar (Becton Dickinson, Sparks, MD) was prepared according to the manufacturer's recommendations. After the medium cooled to 55°C , the vancomycin solution was added to a final concentration of $32 \mu\text{g} \cdot \text{ml}^{-1}$, the breakpoint for full resistance (14, 61). Sediment samples (30 g wet weight) were diluted 1:10 in phosphate-buffered saline (PBS) and hand shaken for 2 min to detach bacteria from particles (62). Sediment samples of the diluted buffered solution were processed in volumes from 0.1 to 100 ml depending on the sampling date and on previous concentrations of enterococci. Multiple dilutions for water and sediment were processed on each date to obtain viable colony counts.

To confirm culturable VRE as enterococci harboring the *vanA* gene, colonies with blue halos that grew on vancomycin-amended mEI were transferred to enterococcosel broth (EB) using sterile pipet tips or sterile toothpicks and were grown for 24 h. Wells that turned black were streaked for isolation onto tryptic soy agar (TSA) (Becton Dickinson, Sparks, MD) and then isolated again onto vancomycin-amended mEI ($32 \mu\text{g} \cdot \text{ml}^{-1}$). Isolated colonies were grown overnight in 5 ml Luria-Bertani (LB) broth (Thermo Fisher Scientific, Waltham, MA) that was amended with $32 \mu\text{g} \cdot \text{ml}^{-1}$ vancomycin. DNA was extracted from overnight cultures using a GenElute bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO). Nucleic acid concentration was measured using a NanoDrop spectrophotometer to confirm successful extraction, and DNA was stored at -20°C in aliquots. Quantitative PCR (qPCR) was carried out with an Applied Biosystems 7500 real-time PCR system to confirm isolates as *Enterococcus* spp. (63) carrying the *vanA* gene (64). Isolates were identified to the species level by DNA sequencing of the 16S rRNA gene using universal bacterial primers (8F, 1492R) to amplify the 16S rRNA (65, 66); the PCR product was then purified using a GeneJet PCR purification kit (Thermo Fisher Scientific, Waltham, MA), sequenced by Eurofins Genomics (Huntsville, AL), and identified to the genus and species levels by using BLAST to reference the GenBank database (NCBI).

Sequencing and molecular analysis of environmental DNA. Water (500 ml) was also filtered to obtain environmental DNA, and filters were stored at -80°C for DNA extraction. Sediment samples were also stored for DNA extraction. DNA from environmental water and samples was extracted and purified using the Mo Bio PowerWater kit from $0.45\text{-}\mu\text{m}$

filters. DNA from environmental sediment samples was extracted using Mo Bio PowerSoil kits directly from 0.3-g samples of sediment (Mo Bio Laboratories, Carlsbad, CA). Bacterial communities in those samples were characterized by sequencing the V4 region of the 16S rRNA gene. PCR was carried out to amplify the V4 region with the 515F and 806R primer pair, which included sequencer adapter sequences for Illumina sequencing (67, 68). The forward primer also contained a 12-bp barcode sequence unique to each sample. Each 25- μ l PCR mixture contained 12 μ l of PCR Water (Mo Bio Laboratories, Carlsbad, CA, USA), 10 μ l of 2.5 \times 5 Prime HotMasterMix (Gaithersburg, MD), 1 μ l of each of the primers (5 μ M), and 1 μ l of template DNA. The conditions for PCR were as follows: 94°C for 3 min, 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and a final extension of 10 min at 72°C. Amplicons were quantified using PicoGreen (Invitrogen) and a plate reader (Infinite 200 Pro; Tecan) and were then pooled in equimolar ratios. This pool was cleaned using the UltraClean PCR clean-up kit (Mo Bio) and sequenced in an Illumina MiSeq run (2 \times 150 bp) at Argonne National Laboratory. Sequencing reads were processed using QIIME (69) and USEARCH (70). The forward and reverse reads were merged, and then the merged reads were demultiplexed and filtered with a minimum Phred quality score of 20. Filtering resulted in about 388,000 high-quality reads, averaging about 28,000 reads per sample. Those reads were then clustered into 1,685 operational taxonomical units (OTUs) with a 97% similarity threshold. Chimeric sequences were identified with UCHIME and removed from OTUs (71). The taxonomy of the OTUs was assigned an RDP classifier against the SILVA databases (72, 73). For all downstream analyses, 10,000 reads were randomly selected per sample to correct for differences in sequencing depth.

Quantitative PCR (qPCR) was carried out with an Applied Biosystems 7500 real-time PCR system based on a previously published protocol for the *vanA* gene (64). Targets in environmental DNA were amplified using the following master mix composition per 25- μ l reaction mixture: 12.5 μ l TaqMan environmental master mix 2.0 (Thermo Fisher Scientific, Waltham, MA), 3 μ l primer/probe mix (composed of 74.5 μ l of each primer at 100 μ M and 6 μ l of target probe at 100 μ M), 2.5 μ l bovine serum albumin (BSA) (2 mg \cdot ml⁻¹), 2 μ l sterile nuclease-free water, and 5 μ l template DNA. Temperature cycling consisted of 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. The lower limit of quantification (LLOQ) for the qPCR assay was 2.5 gene copies per reaction based on successful amplification in 50% of replicates of the lowest concentration on the standard curve (74). Sample LLOQ was 1.67 \times 10⁴ gene copies per 100 g for sediment samples and 10 gene copies per 100 ml for water samples. Blanks containing sterile Nanopure water in place of a sample were processed as negative controls (no-template controls [NTCs]). No blank amplified in any *vanA* qPCR assay. When the quantification cycle (C_q) values for the two replicates were greater than the C_q values for the LLOQ, results were reported as detected but not quantified (DNQ). Samples where neither replicate amplified and samples that did not successfully amplify in the two replicate qPCRs (amplified in 1 of 2) were reported as not detected (ND). The standard curve for *vanA* was constructed using a synthetic plasmid (IDT, Coralville, IA), containing the target sequence of the pIP816 *vanA* plasmid as previously published (NCBI accession number X56895) (64). Inhibition of amplification in environmental samples was tested using a qPCR SYBR green assay for the *vvhA* gene of *Vibrio vulnificus* (75). *V. vulnificus* is an autochthonous marine bacterium that does not grow in freshwater environments. Reaction mixtures contained 4 μ l of DNA sample and 1 μ l of *V. vulnificus* DNA (20,000 copies) and were compared to a control reaction mixture containing 4 μ l of nuclease-free water and 1 μ l of *V. vulnificus* DNA (20,000 copies) using previously published cycling conditions and primers (76).

Accession number(s). Sequences were deposited in the NCBI BioProject database under BioProject accession number PRJNA322710.

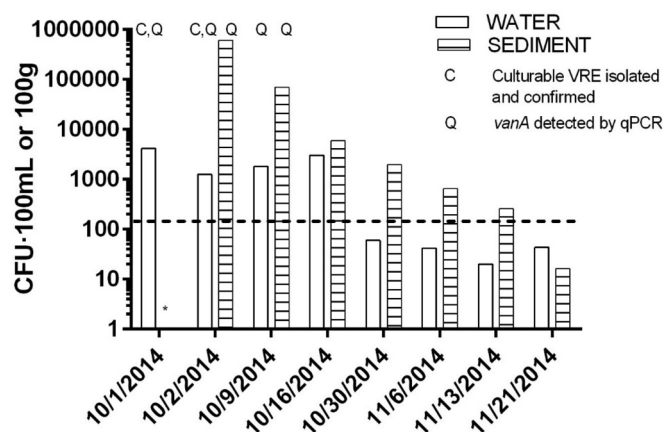


FIG 1 Culturable enterococci in water and sediment at site NC-03, near the site of the sewage spill. The dashed line represents the EPA standard for a single-sample maximum of enterococci in recreational water (130 CFU per 100 ml). Letters indicate where VRE were cultured (C) and where *vanA* was detected within 800 m of the spill (NC-01, NC-02, and NC-03). Note that on 1 October 2014, no sediment was collected or processed (indicated by *).

RESULTS

Concentrations of culturable enterococci in water were high at the site of the spill (NC-03) immediately after the event (4.2×10^3 CFU per 100 ml), exceeding the U.S. EPA sample threshold value (STV) standards for recreational waters of 1.3×10^2 CFU per 100 ml (77) (Fig. 1). Levels decreased over time but did not fall below 1.3×10^2 CFU per 100 ml at NC-03 until 30 October 2014, more than 1 month after the event (Fig. 1). Enterococcal levels at the boat ramp in receiving marine waters approximately 3 km from the spill were within regulatory limits at each sampling date, ranging from 5 to 22 CFU per 100 ml. Enterococcal levels were 2 to 3 orders of magnitude higher in sediment than in water at NC-03 and also decreased over time (Fig. 1).

Enterococci were also monitored by Pinellas County at eight surface water sites ranging from 1 to 9 km away from the spill (B, C, F, G, H, I, J, and Q) for 12 consecutive days following the spill (see Fig. S2A and B in the supplemental material). Four sites within 4.5 km (from near to far, B, C, J, and Q) displayed enterococcal levels that exceeded recreational water quality standards (130 CFU per 100 ml) (77) for some duration after the spill. Exceedances were recorded at site B for 8 days, site C for 1 day, site J for 2 days, and site Q for 1 day. Maximum enterococcal levels were recorded 6 days after the spill at site B (2,100 CFU per 100 ml) and 1 day after the spill at site C (210 CFU per 100 ml), site J (160 CFU per 100 ml), and site Q (170 CFU per 100 ml). These sites were in the receiving waters directly adjacent to the site of the spill, Long Bayou and Cross Bayou, with the exception of site Q, which was in Boca Ciega Bay. Recreational water quality standards were not exceeded at the other four sites where enterococci were measured (F, G, H, and I) in Boca Ciega Bay, a body of water that mixes with the Gulf of Mexico and that is more than 5 km away from the spill.

VRE were detected by culture and confirmed as *Enterococcus faecium* in water collected 2 and 3 days after the spill ceased at NC-01, NC-02, and NC-03 (1 October 2014 and 2 October 2014) but could not be confirmed in water or sediment on subsequent dates (Fig. 1). A subset of putative VRE isolates from water sampled on 1 October 2014 and 2 October 2014 (11 of 15) was iden-

TABLE 1 Detection and levels of *vanA* measured by qPCR in water and sediment at three sites near the origin (within 800 m) of the sewage spill over eight sampling dates^a

Sample type	Site	Days postspill							
		1	2	9	16	30	36	43	51
Water (log ₁₀ gene copies per 100 ml)	NC-01	1.96	1.79	2.21	—	—	—	—	—
	NC-02	ND ^b	1.54	1.84	—	—	ND	ND	ND
	NC-03	1.92	ND	ND	ND	ND	ND	ND	ND
Sediment (log ₁₀ gene copies per 100 g)	NC-01	— ^c	ND	DNQ ^d	—	—	—	—	—
	NC-02	—	4.24	ND	—	—	ND	ND	ND
	NC-03	—	4.95	4.54	ND	ND	ND	ND	ND

^a Sample limits of detection were 4 gene copies per 100 ml water and 6.7×10^3 gene copies per 100 g sediment. Day 1 postspill is considered to be 1 October 2014. Note that access issues prevented sampling at all sites on all dates; data analysis focuses on site NC-03 where samples were collected on each sampling date.

^b ND, not detected.

^c —, not measured.

^d DNQ, detected but not quantifiable.

tified as *E. faecium* by 16S rRNA gene sequencing. The qPCR assay for *vanA* also confirmed that all 11 isolates identified as *E. faecium* carried the *vanA* gene. The other four putative VRE isolates were identified as *Pedococcus* spp. by 16S rRNA sequencing. Colonies that grew on mEI amended with $32 \mu\text{g} \cdot \text{ml}^{-1}$ vancomycin but could not be isolated and confirmed with molecular analyses were detected in water until 30 October 2014 and in sediment until 16 October 2014.

The *vanA* gene was detected in environmental DNA samples extracted from water and sediment up to 12 days after the spill (9 October 2014) at the sites within 800 m of the spill (NC-01, NC-02, and NC-03) (Fig. 1; Table 1) but not at later dates. Concentrations of *vanA* gene copies were approximately two orders of magnitude higher in sediment than in water (Table 1) but were reported per 100 g (wet weight) versus per 100 ml. In water, the maximum for *vanA* gene copies was $2.2 \log_{10}$ gene copies per 100 ml (at site NC-01 on 9 October 2014), and the average was $1.9 \log_{10}$ gene copies per 100 ml. In sediment, the maximum for *vanA* gene copies was $5.0 \log_{10}$ gene copies per 100 g (at site NC-03 on 2 October 2014), and the average was $3.9 \log_{10}$ gene copies per 100 g.

Sequencing results from environmental DNA on seven sampling dates where both sediment and water were collected showed distinct bacterial communities in water and sediment samples. In both matrices, dates closest to the spill (2 October 2014 and 9 October 2014 for sediment and water plus 16 October 2014 for water) were distinctly separate from those in the later sampling weeks (Fig. 2). The trend shown by these data suggests that the sediment and water at this site took approximately 2 to 3 weeks to return to a stable structure following the spill. The change in community composition is supported by a similar time frame of noticeable sewage impacts on fecal indicator bacteria and VRE (Fig. 1).

Six bacterial families shown to be highly prevalent in domestic sewage in the United States (*Bacteroidaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Porphyromonadaceae*, *Veillonellaceae*, and *Prevotellaceae*) (78) decreased in frequency with respect to total 16S rRNA sequences over time at NC-03 in sediment and water (Fig. 3). OTUs identified to the family level represented 85% to 95% of the total OTUs, with the exception of sediment on 2 October 2014, where 43% were identified to the family level. One of the sewage-

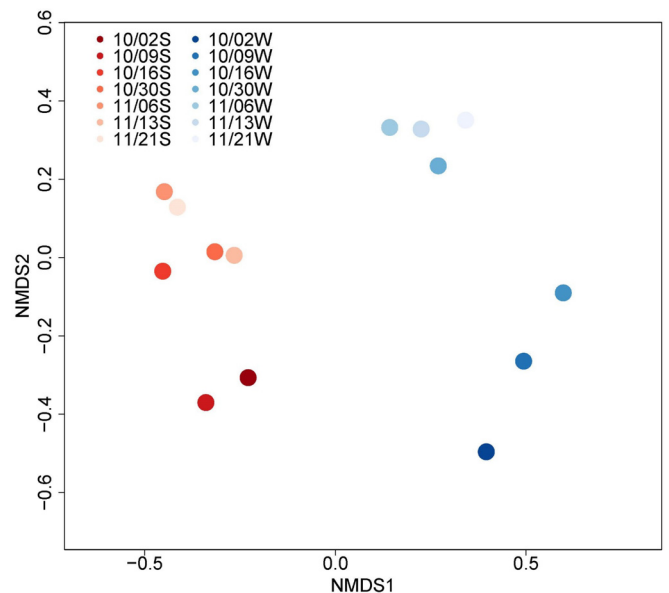


FIG 2 Analysis of DNA sequencing of the 16S rRNA gene in water and sediment on seven dates at site NC-03. The blue gradient represents water samples, and the red gradient represents sediment samples; the color gradient represents different time points in either water (W) or sediment (S) where darker shades are immediately after the spill or earlier in time.

associated families, *Porphyromonadaceae*, was found in the dominant taxa (top 10 most abundant) on the first sampling date and not at any later dates. Alpha diversity did not reveal temporal trends during the course of the sampling. The temporal trend of sewage-associated families also aligns with trends demonstrated in enterococci, VRE, and community structure (Fig. 1 to 3). Dominant families in sediment were different from dominant families in water (Fig. 4). *Neisseriaceae*, a family containing many genera associated with the gut flora of mammals, and *Comamonadaceae*, a family containing common environmental denitrifiers, had the greatest decline in relative abundance in water from the first to later sampling dates. Similar trends in the distinction between microbial communities in water and sediment were observed in taxonomic diversity based on phyla (see Fig. S4A and B in the supplemental material). Families containing common pathogens

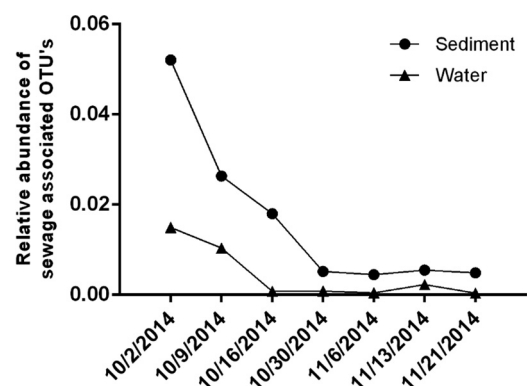


FIG 3 Relative abundance of select sewage-associated OTUs (*Bacteroidaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Porphyromonadaceae*, *Veillonellaceae*, and *Prevotellaceae*) at site NC-03 in water and sediment samples.

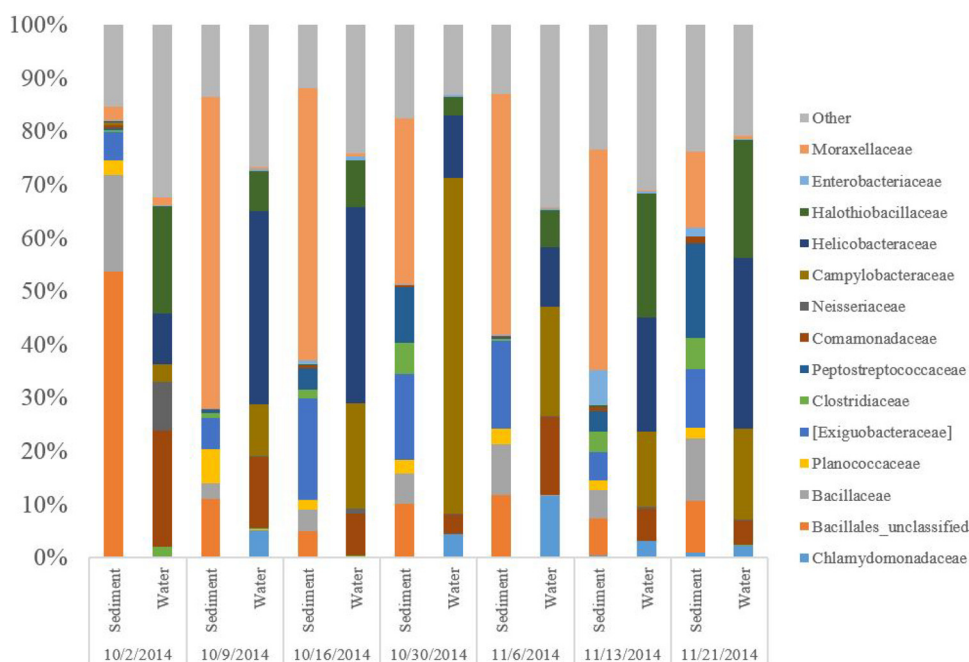


FIG 4 Dominant families in sediment and water at site NC-03 on all sampling dates.

(*Enterobacteriaceae* and *Enterococcaceae*) were present at low levels in water and sediment throughout the study (see Fig. S5A and B in the supplemental material) and were combined to represent an average of 0.41% and 1.4% of sequence reads over time in water and sediment, respectively.

DISCUSSION

The sewage spill that we studied corresponded with elevated levels of enterococci, VRE, and *vanA* genes in water and sediment, indicating their release into the environment. All of these levels diminished steadily over the 2 weeks following the spill. No *vanA* genes were detected in environmental samples after 12 days at the site of the spill. This observation, and the fact that high-level VRE have been infrequently observed in uncontaminated surface waters (30), indicates that their presence in the environment before the spill is unlikely and that these contaminants were sewage associated (i.e., no background levels of *vanA* or VRE would be expected in the environment). The mitigation measures taken after the spill (vacuum pumping, washing out, lime treatment) probably decreased levels of microorganisms from sewage but left high levels of enterococci that slowly diminished over time in the area directly adjacent to the spill. The plume of the sewage spill was also indicated by the broader sampling effort in the region (as processed by Pinellas County), where enterococcal levels exceeded recreational water quality standards at the site closest to the spill (site B) but decreased after 8 days. Sites downstream from the spill where enterococcal levels were high decreased after 1 to 2 days. Flow rates, temperature, and other environmental conditions may impact the persistence and reach of contamination, but these factors were beyond the scope of this study.

The transfer of resistance through mechanisms such as horizontal gene transfer, demonstrated by the detection of the mobile *vanA* gene, can impact human health and the spread of resistance in the environment. This study has demonstrated the release of

potentially pathogenic VRE and *vanA* genes into surface waters by sanitary sewer overflow in the United States. High-level VRE and *vanA* genes have been found in sewage from a hospital in Florida but were not found in other sewage samples that were not directly associated with a hospital (30). The spill in this study was not in close proximity to any hospital; the closest is 2.6 miles from the site of the sewer line break, and sewage from the hospital flows away from the break site. Previous studies have also investigated VRE in aquatic ecosystems, sanitary sewage, and WWTPs (48, 79–81), but community sewage (not associated with a hospital) has not been explicitly linked to *vanA* genes or highly resistant VRE in the United States. Results confirmed that untreated residential sewage released into aquatic environments can potentially be a route of human exposure to ARB and contribute to environmental reservoirs of ARB and ARGs.

Colonies that resembled VRE were detected in water samples through 30 October 2014 and in sediment samples through 16 October 2014; however, putative VRE colonies observed after 2 October 2014 could not be isolated based on the methods described above for confirmation. In all probability, they were either *Enterococcus* species or members of other genera that could “struggle” at $32 \mu\text{g} \cdot \text{ml}^{-1}$ vancomycin on a crowded plate but did not possess *vanA* and so could not grow when subcultured on vancomycin. This observation reemphasizes the inaccuracy implicit in reporting VRE solely based on culture methods as further evidenced by the identification of *Pediococcus* spp. in this study. Other studies have demonstrated the isolation of a small percentage of genera other than *Enterococcus* on mEI (47, 82). The addition of vancomycin in the screening step tends to exacerbate the issue, as selection for intrinsically resistant genera, such as *Pediococcus*, *Weissella*, and *Leuconostoc*, also occurs (30).

DNA sequencing analysis has explored the dominant microbial taxa associated with sewage and human feces (78, 83), but the microbial community in waters impacted by sewage has received

less attention. The advantages of this site included limited water input following the initial flushing so that changes in the community could be followed over time without the dilution effect that would occur in a large water body. The influx of sewage at this site produced a bacterial community with a prominent component of sewage- and fecal-associated bacteria that was detectable at the site for at least 2 weeks. The abundance of sewage-associated families declined on a similar time scale to enterococci, but the fate (i.e., death, transport, consumption by predators) of these bacteria and other pathogens was not determined. Some families containing pathogenic members and fecal indicator bacteria (FIB) (*Enterococcaceae*, *Enterobacteriaceae*) were represented throughout the sampling period.

Differences in community structure in sediment versus water were evident. The dominant phyla in water were consistent with those found in a study of 10 sites in the Mississippi River (*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, and *Verrucomicrobia* accounting for approximately 94% of sequences) (84). However, *Firmicutes* (containing pathogen taxa) were more prevalent in water on the days immediately after the sewage spill than at later dates compared to the consistent low levels in the Mississippi River samples. The dominant taxa in sediment were consistent with published research where *Proteobacteria* and *Firmicutes* are prevalent phyla (85). It is interesting to note that the communities in water and sediment changed over approximately the same time frame and that they also remained distinct from one another. The relative rate of change in various environmental habitats bears further exploration, particularly given the extensive literature discussion about the potential role of sediments as environmental reservoirs for microbial pathogens and indicators (86–89).

This study confirms that potentially pathogenic ARB and associated ARGs can be released into the environment through untreated sewage and can persist for days or weeks after the initial introduction. Although the study area was flushed with water immediately after the spill, the sewage signal, as measured by enterococcal levels, persisted for 2 weeks after the event. This study supports the need for more mechanistic, empirical studies to address the role of environmental variability in the survival of ARB and ARGs, including parameters such as temperature and flow rates. Later sampling events, when no *vanA* genes were detected and no VRE were detected, support the previous studies suggesting their sewage association and absence of environmental background levels (30, 50, 90). While this study lacks a “before” sampling date for this site, the temporal sampling and the current literature support the idea that the *vanA* genes and VRE were derived from sewage. Immediately following the spill, *E. faecium* isolates harboring the *vanA* gene were identified in water samples at the site. The probability of human exposure outside of the cleanup crew was minimal in this case study, but sewage contamination events that occur at popular beaches and recreational areas may put more people at risk of exposure to antibiotic-resistant pathogens. In this study, antibiotic-resistant, opportunistic pathogens (VRE) associated with sewage entered the environment through a contamination event and persisted, potentially contributing to the spread of antibiotic resistance in the environment. Environmental reservoirs of ARB need further research and should be considered in frameworks designed to assess the spread of antibiotic resistance.

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