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 to 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, beach sands and wildlife feces. However, VRE harboring *vanA* genes that confer high-level resistance have infrequently been found outside of clinical settings in the U.S. This study found culturable *Enterococcus faecium* harboring the *vanA* gene in water and sediment up to three days after a sewage spill, and the qPCR signal for *vanA* persisted for an additional week. Culturable enterococci levels in water exceeded recreational water guidelines for two weeks following the spill, declining about five orders of magnitude in sediments and two orders of magnitude in the water column over six weeks. Analysis of bacterial taxa via 16S rRNA gene sequencing showed changes in community structure through time following the sewage spill in both 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 examining the persistence, occurrence and survival of VRE in different environmental matrices.

Significance of the Study

Vancomycin-resistant enterococci (VRE) are harmful bacteria that are resistant to the powerful antibiotic vancomycin, 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.

Introduction

Antibiotic resistant bacteria (ARB) are a growing public health threat and economic burden globally. The Centers for Disease Control (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 of 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, antibiotic resistance genes (ARGs) and antimicrobial compounds, through both treated effluent and 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 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 *E. casseliflavus* and *E. gallinarum*, but is of less clinical concern than acquired, high-level vancomycin resistance ($\geq$32 $\mu g \cdot ml^{-1}$) (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 human VRE infections. *vanA* is usually carried on a plasmid-borne transposon (Tn1546) (19-21) and confers high-level resistance to vancomycin (>64 mg/L) (22).

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 has been predominantly in clinical cases and hospital sewage (30, 31). The monitoring of VRE and associated resistance genes outside of the hospital setting is necessary to better understand the spread of resistance and increased risk to public health (6). Previous studies in Europe and Australia have reported community spread of VRE and fecal colonization of non-hospitalized 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 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 U.S. (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, but 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 on 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* spp. isolates with *vanA* phenotypes in WWTP effluent and surface waters (56-59). One study in the United States isolated *E. faecium* carrying *vanA* genes on a recreational marine beach in Washington (52), but no other confirmation has been established outside of 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, Florida released more than 500,000 gallons of untreated sewage into a neighborhood drainage ditch beginning September 27, 2014. The line break was repaired with a bypass valve on September 30th, 2014, after the sewage leakage was diverted. The site was also washed down, vactored (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, consisting 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 as Figures S3A and S3B. Water and sediment samples were collected at the spill site, along the drainage ditch for a distance of 800 meters, and in adjacent receiving waters. Samples were collected seven times over the course of seven weeks after the spill (10/1/14-11/21/14), to determine persistence of sewage-associated microbes and VRE in the environment.

Six sites (NC-01, NC-02, NC-03, NC-04, NC-05, 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 as supplementary material (Figure S1). Site NC-01 became inaccessible after the first two 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 could 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 1-2 cm of sediments. All samples were transported on ice to the laboratory and processed within six hours. Enterococci were also quantified by the Pinellas County Water and Sewer Department staff at 16 sites (Figures S2A and S2B) 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 US 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- 300 mL) on each sampling date over the course of the

141 sampling period to account for variability in enterococci concentrations. Vancomycin stock
142 solution was prepared as an aqueous solution from sodium salt (Acros Organics/Thermo Fisher
143 Scientific, New Jersey USA) and sterile nuclease free water to a final concentration of 10
144 mg/mL, and filter sterilized. To detect culturable VRE, mEI agar (Becton Dickinson, Sparks,
145 MD) was prepared according to manufacturer's instructions. After the media cooled to 55°C,
146 vancomycin solution was added to a final concentration of 32 μg/mL, the breakpoint for full
147 resistance (14, 61). Sediment samples (30 g wet weight) were diluted 1:10 in phosphate buffered
148 saline (PBS) and hand shaken for two minutes to detach bacteria from particles (62). Sediment
149 samples of the diluted buffered solution were processed in volumes from 0.1-100 mL depending
150 on the sampling date and previous concentrations of enterococci. Multiple dilutions for both
151 water and sediment were processed on each date to obtain viable colony counts.

152 To confirm culturable VRE as enterococci harboring the *vanA* gene, colonies with blue halos
153 that grew on vancomycin-amended-mEI were transferred to enterococcosel broth (EB) using
154 sterile pipet tips or sterile toothpicks and grown 24 h. Wells that turned black were streaked for
155 isolation onto tryptic soy agar (TSA) (Becton Dickinson, Sparks, MD), then isolated again onto
156 vancomycin-amended mEI (32 $\mu g \cdot ml^{-1}$). Isolated colonies were grown overnight in 5 mL Luria
157 Bertani (LB) broth (Thermo Fisher Scientific, Waltham, MA) amended with 32 $\mu g \cdot ml^{-1}$
158 vancomycin. DNA was extracted from overnight cultures using a GenElute Bacterial Genomic
159 DNA Kit (Sigma-Aldrich, St. Louis, MO). Nucleic acid concentration was measured using a
160 NanoDrop spectrophotometer to confirm successful extraction and DNA was stored at -20°C in
161 aliquots. Quantitative PCR (qPCR) was carried out with an AB 7500 Real Time PCR system to
162 confirm isolates as *Enterococcus* spp. (63) carrying the *vanA* gene (64). Isolates were identified
163 to the species level by DNA sequencing of the 16S rRNA gene using universal bacterial primers

(8F, 1492R:) to amplify 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 level by using BLAST to reference the GenBank database (NCBI).

Sequencing and molecular analysis of environmental DNA. Water (500mL) 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 MoBio PowerWater from 0.45 um filters. DNA from environmental sediment samples was extracted using MoBio PowerSoil kits directly from 0.3 g samples of sediment (MoBio 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 that included sequencer adapter sequences for Illumina sequencing (67, 68). The forward primer also contained a 12bp barcode sequence unique to each sample. Each 25 µL PCR reaction contained 12 µL of PCR Water (MO BIO Laboratories, Carlsbad, CA, USA), 10 µL of 2.5X 5 Prime HotMasterMix (5 Prime, Gaithersburg, MD), 1 µL of each of the primers (5 µM), and 1 µL of template DNA. The conditions for PCR were as follows: 94 °C for 3 minutes, 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 then pooled in equimolar ratios. This pool was cleaned up using the UltraClean® PCR Clean-Up Kit (MO BIO), and sequenced in an Illumina Miseq run (2×150bp) at Argonne National Laboratory. Sequencing reads were processed QIIME (69) and USEARCH (70). The forward and reverse reads were merged, and then the merged reads were demultiplexed and filtered with a minimum

187 Phred quality score of 20. Filtering resulted in about 388,000 high quality reads averaging about
188 28,000 reads per sample. Those reads were then clustered into 1,685 operational taxonomical
189 units (OTUs) with a 97% similarity threshold. Chimeric sequences were identified with
190 UCHIME and removed from OTUs (71) The taxonomy of OTUs was assigned with RDP
191 classifier against the SILVA databases (72, 73). For all downstream analysis, 10,000 reads were
192 randomly selected per sample to correct for differences in sequencing depth. Sequences were
193 deposited in the NCBI BioProject database (available with BioProject accession number
194 SRP075690).

195 Quantitative PCR (qPCR) was carried out with an AB 7500 Real Time PCR system, based on
196 a previously published protocol for the *vanA* gene (64). Targets in environmental DNA were
197 amplified using the following mastermix composition, per 25 µL reaction: 12.5 µL TaqMan
198 Environmental MasterMix 2.0 (Thermo Fisher Scientific, Waltham, MA); 3 µL primer/probe
199 mix (composed of 74.5 µL each primer at 100 µM; 6 µL of target probe at 100 µM); 2.5 µL BSA
200 (2 mg/mL); 2 µL sterile nuclease-free water; 5 µL template DNA. Temperature cycling
201 consisted of 2 minutes at 50°C, 10 minutes at 95°C, then 40 cycles of 15 seconds at 95°C and 1
202 min at 60°C. The lower limit of quantification (LLOQ) for the qPCR assay was 2.5 gene copies
203 · reaction based on successful amplification in 50% of replicates of the lowest concentration on
204 the standard curve (74). Sample LLOQ was 1.67×10^4 gene copies · $100\ g^{-1}$ for sediment
205 samples and 10 gene copies · $100\ mL^{-1}$ for water samples. Blanks containing sterile nanopure
206 water in place of a sample were processed as negative control ("no template control" or "NTC").
207 No blank amplified in any *vanA* qPCR assay. When quantification cycle (Cq) for both replicates
208 were greater than the Cq for the LLOQ, results were reported as "DNQ", or "detected but not
209 quantified". Samples where neither replicate amplified or when samples did not successfully

amplify in both replicate qPCR reactions (amplified in 1 of 2), were reported as "ND", or "not detected". 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. Reactions contained 4 µL of DNA sample and 1 µL of *V. vulnificus* DNA (20,000 copies), and were compared to a control reaction containing 4 µL of nuclease-free water and 1 µL of *V. vulnificus* DNA (20,000 copies) using previously published cycling conditions and primers (76) .

Results

Culturable enterococci concentrations in water were high at the site of the spill (NC-03) immediately after the event (4.2×10^3 CFU · 100 mL^{-1}), exceeding US EPA sample threshold values (STV) standards for recreational waters of 1.3×10^2 CFU · 100 mL^{-1} (77) (Figure 1). Levels decreased over time but did not fall below 1.3×10^2 CFU · 100 mL^{-1} at NC-03 until 10/30/2014, more than one month after the event (Figure 1). Enterococci 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 5-22 CFU · 100 mL^{-1}. Enterococci levels were 2-3 orders of magnitude higher in sediment than in water at NC-03 and also decreased over time (Figure 1).

Enterococci were also monitored by Pinellas County at eight surface water sites ranging from 1-9km away from the spill (B, C, F, G, H, I, J, Q) for 12 consecutive days following the spill (Figure S2A and S2B). Four sites within 4.5 km (from near to far: B, C, J, Q) displayed enterococci levels that exceeded recreational water quality standards (130 CFU · 100mL^{-1}) (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; Site Q for 1 day. The maximum enterococci level was recorded 6 days after the spill at Site B (2100 CFU · $100mL^{-1}$), and 1 day after the spill at Site C (210 CFU · $100mL^{-1}$), Site J (160 CFU · $100mL^{-1}$) and Site Q (170 CFU · $100mL^{-1}$). 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, I), all in Boca Ciega Bay, a water body 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 two and three days after the spill ceased at NC-01, NC-02 and NC-03 (10/1/2014 and 10/2/2014), but could not be confirmed in water or sediment on subsequent dates (Figure 1). A subset of putative VRE isolates from water sampled on 10/1/2014 and 10/2/2014 (11 of 15) were identified as *E. faecium* by 16S rRNA gene sequencing. The qPCR assay for *vanA* also confirmed that all eleven isolates identified as *E. faecium* carried the *vanA* gene. The other four putative VRE isolates were identified as *Pediococcus* spp. by16S rRNA sequencing. Colonies that grew on mEI amended with 32 $\mu g \cdot ml^{-1}$ vancomycin but could not be isolated and confirmed with molecular analyses were detected in water up to 10/30/2014 and in sediment up to 10/16/2014.

The *vanA* gene was detected in environmental DNA samples extracted from both water and sediment up to 12 days after the spill (10/9/14), at the sites within 800 meters of the spill (NC-01, NC-02, NC-03) (Figure 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 100g (wet weight) versus per 100mL. In water, the maximum for *vanA* gene copies was 2.2 $\log_{10}$ gene copies · 100 mL^{-1} (at site NC-01 on 10/9/14) and the average was 1.9 $\log_{10}$ gene copies· 100 mL^{-1}. In sediment, the maximum for *vanA* gene copies was 5.0 $\log_{10}$ gene copies · 100 g^{-1} (at site NC-03 on 10/2/14) and the average was 3.9 $\log_{10}$ gene copies· 100 g^{-1}.

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 (10/2 and 10/9 for sediment and water, plus 10/16 for water) were distinctly separate from those in the later sampling weeks (Figure 2). The trend shown by these data suggests that the sediment and water at this site took approximately 2-3 weeks to return to a stable structure following the spill. The change in community composition is supported by the similar time frame of noticeable sewage impacts on fecal indicator bacteria and VRE (Figure 1).

Six bacterial families shown to be highly prevalent in domestic sewage in the U.S. (*Bacteroidaceae, Ruminococcaceae, Lachnospiraceae, Porphyromonadaceae, Veillonellaceae, Prevotellaceae*) (78) decreased in frequency with respect to total 16S rRNA sequences over time at NC-03 in both sediment and water (Figure 3). OTUs identified to family level represented 85-95% of total OTUs with the exception of sediment on 10/2/2014, where 43% were identified to the family level. One of the sewage associated families, *Porphyromonadaceae* was found in the dominant taxa (top ten 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 (Figures 1-3). Dominant families in sediment were different than dominant families in water (Figure 4). *Nesseriaceae*, 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 distinction between microbial communities in water and sediment were observed in taxonomic diversity based on phyla (Figure S4A and S4B). Families containing common pathogens (*Enterobacteriaceae* and *Enterococcaceae*) were present at low levels in water and sediment throughout the study (Figure S5A and S5B), and 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 two 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 enterococci levels exceeded recreational water quality standards at the site closest to the spill (Site B) but decreased after 8 days. Sites downstream of the spill where enterococci levels were high decreased after 1-2 days. Flow rates,

302 temperature and other environmental conditions may impact the persistence and reach of
303 contamination, but these factors were beyond the scope of this study.

304 The transfer of resistance through mechanisms such as horizontal gene transfer, demonstrated
305 by the detection of the mobile *vanA* gene, can impact human health and the spread of resistance
306 in the environment. This study is the first to demonstrate the release of potentially pathogenic
307 VRE and *vanA* genes into surface waters by sanitary sewer overflow in the United States. High
308 level VRE and *vanA* genes have been found in sewage from a hospital in Florida, but were not
309 found in other sewage samples that were not directly associated with a hospital (30). The spill in
310 this study was not in close proximity to any hospital; the closest is 2.6 miles from the site of the
311 sewer line break, and sewage from the hospital flows away from the break site. Previous studies
312 have also investigated VRE in aquatic ecosystems, sanitary sewage and WWTPs (48, 79-81) but
313 community sewage (not associated with a hospital) has not been explicitly linked to *vanA* genes
314 or highly resistant VRE in the United States. Results confirmed that untreated residential sewage
315 released into aquatic environments can potentially be a route of human exposure to ARB, and
316 contribute to environmental reservoirs of ARB and ARGs.

317 Colonies that resembled VRE were detected in water samples through 10/30/2014 and in
318 sediment samples through 10/16/2014; however, putative VRE colonies observed after
319 10/2/2014 could not be isolated based on methods described above for confirmation. In all
320 probability, they were either *Enterococcus* species or members of other genera that could
321 "struggle" at 32 $\mu g \cdot ml^{-1}$ vancomycin on a crowded plate, but did not possess *vanA* and so could
322 not grow when subcultured on vancomycin. This observation reemphasizes the inaccuracy
323 implicit in reporting VRE solely based on culture methods, as further evidenced by the
324 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 two weeks. Abundance of sewage associated families declined on a similar timescale 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 FIB (*Enterococcaceae, Enterobacteriaceae*) were represented throughout the sampling period.

Differences in community structure in sediment vs. water were evident. Dominant phyla in water were consistent with those found in a study of ten 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 later dates, compared to consistent low levels in Mississippi River samples. 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 to weeks after the initial introduction. Although the study area was flushed with water immediately after the spill, the sewage signal, as measured by enterococci levels, persisted for two 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* 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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References

1. **Centers for Disease Control.** 2011. Antimicrobial Resistance Posing Growing Health Threat.
2. **Centers for Disease Control.** 2013. Antibiotic Resistance Threats in the United States. U.S. Department of Health and Human Services,
3. **Walsh F.** 2013. Investigating antibiotic resistance in non-clinical environments. Front Microbiol **4:**19.
4. **Levy SB, Fitzgerald GB, Macone AB.** 1976. Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. N Engl J Med **295:**583-588.
5. **McLain JE, Cyntryn E, Durso LM, Young S.** 2016. Culture-based Methods for Detection of Antibiotic Resistance in Agroecosystems: Advantages, Challenges, and Gaps in Knowledge. Journal of Environmental Quality **45:**432-440.
6. **Baquero F, Martinez JL, Canton R.** 2008. Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol **19:**260-265.
7. **Michael I, Rizzo L, McArdell CS, Manaia CM, Merlin C, Schwartz T, Dagot C, Fatta-Kassinos D.** 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. Water Res **47:**957-995.
8. **Schwartz T, Kohnen W, Jansen B, Obst U.** 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiol Ecol **43:**325-335.
9. **Young S, Juhl A, O'Mullan GD.** 2013. Antibiotic-resistant bacteria in the Hudson River Estuary linked to wet weather sewage contamination. J Water Health **11:**297-310.
10. **Bassetti M, Ginocchio F, Giacobbe DR.** 2011. New approaches for empiric therapy in Gram-positive sepsis. Minerva Anestesiol **77:**821-827.
11. **Bode C, Muenster S, Diedrich B, Jahnert S, Weisheit C, Steinhagen F, Boehm O, Hoeft A, Meyer R, Baumgarten G.** 2015. Linezolid, vancomycin and daptomycin modulate cytokine production, Toll-like receptors and phagocytosis in a human in vitro model of sepsis. J Antibiot (Tokyo) doi:10.1038/ja.2015.18.
12. **Edmond MB, Ober JF, Dawson JD, Weinbaum DL, Wenzel RP.** 1996. Vancomycin-resistant enterococcal bacteremia: natural history and attributable mortality. Clin Infect Dis **23:**1234-1239.
13. **Cetinkaya Y, Falk P, Mayhall CG.** 2000. Vancomycin-resistant enterococci. Clin Microbiol Rev **13:**686-707.
14. **(CLSI) CLSI.** 2010. *Performance standards for antimicrobial susceptibility testing,* 20th Informational Supplement M100-20. Clinical Laboratory Standards Institute, Wayne, PA.
15. **Arthur M, Courvalin P.** 1993. Genetics and mechanisms of glycopeptide resistance in enterococci. Antimicrob Agents Chemother **37:**1563-1571.
16. **Hayakawa K, Marchaim D, Martin ET, Tiwari N, Yousuf A, Sunkara B, Pulluru H, Kotra H, Hasan A, Bheemreddy S, Sheth P, Lee DW, Kamatam S, Bathina P, Nanjireddy P, Chalana IK, Patel S, Kumar S, Vahia A, Ku K, Yee V, Swan J, Pogue JM, Lephart PR, Rybak MJ, Kaye KS.** 2012. Comparison of the Clinical Characteristics and Outcomes Associated with Vancomycin-Resistant Enterococcus faecalis and Vancomycin-Resistant E. faecium Bacteremia. Antimicrobial Agents and Chemotherapy **56:**2452-2458.
17. **Murray BE.** 2000. Vancomycin-resistant enterococcal infections. N Engl J Med **342:**710-721.
18. **O'Driscoll T, Crank CW.** 2015. Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. Infect Drug Resist **8:**217-230.

19. **Arthur M, Molinas C, Depardieu F, Courvalin P.** 1993. Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in Enterococcus faecium BM4147. J Bacteriol **175:**117-127.
20. **Guardabassi L, Dalsgaard A.** 2004. Occurrence, structure, and mobility of Tn1546-like elements in environmental isolates of vancomycin-resistant enterococci. Appl Environ Microbiol **70:**984-990.
21. **Werner G, Strommenger B, Witte W.** 2008. Acquired vancomycin resistance in clinically relevant pathogens. Future Microbiol **3:**547-562.
22. **Courvalin P.** 2006. Vancomycin resistance in gram-positive cocci. Clin Infect Dis **42 Suppl 1:**S25-34.
23. **Bager F, Aarestrup FM, Madsen M, Wegener HC.** 1999. Glycopeptide resistance in Enterococcus faecium from broilers and pigs following discontinued use of avoparcin. Microb Drug Resist **5:**53-56.
24. **Klare I, Badstubner D, Konstabel C, Bohme G, Claus H, Witte W.** 1999. Decreased incidence of VanA-type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. Microb Drug Resist **5:**45-52.
25. **Nilsson O.** 2012. Vancomycin resistant enterococci in farm animals - occurrence and importance. Infect Ecol Epidemiol **2**.
26. **Martone WJ.** 1998. Spread of vancomycin-resistant enterococci: why did it happen in the United States? Infect Control Hosp Epidemiol **19:**539-545.
27. **Rubinstein E, Keynan Y.** 2013. Vancomycin-resistant enterococci. Crit Care Clin **29:**841-852.
28. **Kirst HA, Thompson DG, Nicas TI.** 1998. Historical yearly usage of vancomycin. Antimicrob Agents Chemother **42:**1303-1304.
29. **Deshpande LM, Fritsche TR, Moet GJ, Biedenbach DJ, Jones RN.** 2007. Antimicrobial resistance and molecular epidemiology of vancomycin-resistant enterococci from North America and Europe: a report from the SENTRY antimicrobial surveillance program. Diagn Microbiol Infect Dis **58:**163-170.
30. **Harwood VJ, Brownell M, Perusek W, Whitlock JE.** 2001. Vancomycin-resistant Enterococcus spp. isolated from wastewater and chicken feces in the United States. Appl Environ Microbiol **67:**4930-4933.
31. **Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK, National Healthcare Safety Network T, Participating National Healthcare Safety Network F.** 2008. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infect Control Hosp Epidemiol **29:**996-1011.
32. **Stevenson KB, Searle K, Stoddard GJ, Samore M.** 2005. Methicillin-resistant Staphylococcus aureus and vancomycin-resistant Enterococci in rural communities, western United States. Emerg Infect Dis **11:**895-903.
33. **Padiglione AA, Grabsch EA, Olden D, Hellard M, Sinclair MI, Fairley CK, Grayson ML.** 2000. Fecal colonization with vancomycin-resistant enterococci in Australia. Emerg Infect Dis **6:**534-536.
34. **Endtz HP, van den Braak N, van Belkum A, Kluytmans JA, Koeleman JG, Spanjaard L, Voss A, Weersink AJ, Vandenbroucke-Grauls CM, Buiting AG, van Duin A, Verbrugh HA.** 1997. Fecal carriage of vancomycin-resistant enterococci in hospitalized patients and those living in the community in The Netherlands. J Clin Microbiol **35:**3026-3031.

35. **Wendt C, Krause C, Xander LU, Loffler D, Floss H.** 1999. Prevalence of colonization with vancomycin-resistant enterococci in various population groups in Berlin, Germany. J Hosp Infect **42:**193-200.
36. **Costanzo SD, Murby J, Bates J.** 2005. Ecosystem response to antibiotics entering the aquatic environment. Mar Pollut Bull **51:**218-223.
37. **Mallin MA, Cahoon LB, Toothman BR, Parsons DC, McIver MR, Ortwine ML, Harrington RN.** 2007. Impacts of a raw sewage spill on water and sediment quality in an urbanized estuary. Mar Pollut Bull **54:**81-88.
38. **Barcina I, Lebaron P, VivesRego J.** 1997. Survival of allochthonous bacteria in aquatic systems: A biological approach. Fems Microbiology Ecology **23:**1-9.
39. **Martinez JL.** 2008. Antibiotics and antibiotic resistance genes in natural environments. Science **321:**365-367.
40. **Nazaret S, Aminov R.** 2014. Role and prevalence of antibiosis and the related resistance genes in the environment. Front Microbiol **5:**520.
41. **Klibi N, Ben Amor I, Rahmouni M, Dziri R, Douja G, Ben Said L, Lozano C, Boudabous A, Ben Slama K, Mansouri R, Torres C.** 2015. Diversity of species and antibiotic resistance among fecal enterococci from wild birds in Tunisia. Detection of vanA-containing Enterococcus faecium isolates. European Journal of Wildlife Research **61:**319-323.
42. **Lozano C, Gonzalez-Barrio D, Garcia JT, Ceballos S, Olea PP, Ruiz-Fons F, Torres C.** 2015. Detection of vancomycin-resistant Enterococcus faecalis ST6-vanB2 and E. faecium ST915-vanA in faecal samples of wild Rattus rattus in Spain. Veterinary Microbiology **177:**168-174.
43. **Oravcova V, Zurek L, Townsend A, Clark AB, Ellis JC, Cizek A, Literak I.** 2014. American crows as carriers of vancomycin-resistant enterococci with vanA gene. Environmental Microbiology **16:**939-949.
44. **Goncalves A, Igrejas G, Radhouani H, Correia S, Pacheco R, Santos T, Monteiro R, Guerra A, Petrucci-Fonseca F, Brito F, Torres C, Poeta P.** 2013. Antimicrobial resistance in faecal enterococci and Escherichia coli isolates recovered from Iberian wolf. Lett Appl Microbiol **56:**268-274.
45. **Ben Said L, Klibi N, Lozano C, Dziri R, Ben Slama K, Boudabous A, Torres C.** 2015. Diversity of enterococcal species and characterization of high-level aminoglycoside resistant enterococci of samples of wastewater and surface water in Tunisia. Sci Total Environ **530-531C:**11-17.
46. **Novais C, Coque TM, Ferreira H, Sousa JC, Peixe L.** 2005. Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal. Appl Environ Microbiol **71:**3364-3368.
47. **Nishiyama M, Iguchi A, Suzuki Y.** 2015. Identification of Enterococcus faecium and Enterococcus faecalis as vanC-type Vancomycin-Resistant Enterococci (VRE) from sewage and river water in the provincial city of Miyazaki, Japan. J Environ Sci Health A Tox Hazard Subst Environ Eng **50:**16-25.
48. **Goldstein RER, Micallef SA, Gibbs SG, George A, Claye E, Sapkota A, Joseph SW, Sapkota AR.** 2014. Detection of vancomycin-resistant enterococci (VRE) at four US wastewater treatment plants that provide effluent for reuse. Science of the Total Environment **466:**404-411.
49. **Lanthier M, Scott A, Lapen DR, Zhang Y, Topp E.** 2010. Frequency of virulence genes and antibiotic resistances in Enterococcus spp. isolates from wastewater and feces of domesticated mammals and birds, and wildlife. Can J Microbiol **56:**715-729.
50. **Iversen A, Kuhn I, Franklin A, Mollby R.** 2002. High prevalence of vancomycin-resistant enterococci in Swedish sewage. Appl Environ Microbiol **68:**2838-2842.
51. **Roberts MC, No DB, Marzluff JM, Delap JH, Turner R.** 2016. Vancomycin resistant Enterococcus spp. from crows and their environment in metropolitan Washington State, USA: Is there a

correlation between VRE positive crows and the environment? Vet Microbiol doi:10.1016/j.vetmic.2016.01.022.

52. **Roberts MC, Soge OO, Giardino MA, Mazengia E, Ma G, Meschke JS.** 2009. Vancomycin-resistant Enterococcus spp. in marine environments from the West Coast of the USA. J Appl Microbiol **107:**300-307.

53. **Davison J.** 1999. Genetic exchange between bacteria in the environment. Plasmid **42:**73-91.

54. **de Niederhausern S, Bondi M, Messi P, Iseppi R, Sabia C, Manicardi G, Anacarso I.** 2011. Vancomycin-resistance transferability from VanA enterococci to Staphylococcus aureus. Curr Microbiol **62:**1363-1367.

55. **Limbago BM, Kallen AJ, Zhu W, Eggers P, McDougal LK, Albrecht VS.** 2014. Report of the 13th vancomycin-resistant Staphylococcus aureus isolate from the United States. J Clin Microbiol **52:**998-1002.

56. **Alexander J, Bollmann A, Seitz W, Schwartz T.** 2015. Microbiological characterization of aquatic microbiomes targeting taxonomical marker genes and antibiotic resistance genes of opportunistic bacteria. Sci Total Environ **512-513:**316-325.

57. **Bessa LJ, Barbosa-Vasconcelos A, Mendes A, Vaz-Pires P, Martins da Costa P.** 2014. High prevalence of multidrug-resistant Escherichia coli and Enterococcus spp. in river water, upstream and downstream of a wastewater treatment plant. J Water Health **12:**426-435.

58. **Nam S, Kim MJ, Park C, Park JG, Maeng PJ, Lee GC.** 2013. Detection and genotyping of vancomycin-resistant Enterococcus spp. by multiplex polymerase chain reaction in Korean aquatic environmental samples. Int J Hyg Environ Health **216:**421-427.

59. **Santiago-Rodriguez TM, Rivera JI, Coradin M, Toranzos GA.** 2013. Antibiotic-resistance and virulence genes in Enterococcus isolated from tropical recreational waters. J Water Health **11:**387-396.

60. **USEPA.** 2002. Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI) **EPA-821-R-02-022**.

61. **Eliopoulos GM, Gold HS.** 2001. Vancomycin-Resistant Enterococci: Mechanisms and Clinical Observations. Clinical Infectious Diseases **33:**210-219.

62. **Boehm AB, Griffith J, McGee C, Edge TA, Solo-Gabriele HM, Whitman R, Cao Y, Getrich M, Jay JA, Ferguson D, Goodwin KD, Lee CM, Madison M, Weisberg SB.** 2009. Faecal indicator bacteria enumeration in beach sand: a comparison study of extraction methods in medium to coarse sands. J Appl Microbiol **107:**1740-1750.

63. **USEPA.** 2015. Method 1611.1: Enterococci in Water by TaqMan Quantitative Polymerase Chain Reaction (qPCR). **EPA-820-R-15-008**.

64. **Volkmann H, Schwartz T, Bischoff P, Kirchen S, Obst U.** 2004. Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). Journal of Microbiological Methods **56:**277-286.

65. **Weisburg WG, Barns SM, Pelletier DA, Lane DJ.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol **173:**697-703.

66. **Baker GC, Smith JJ, Cowan DA.** 2003. Review and re-analysis of domain-specific 16S primers. J Microbiol Methods **55:**541-555.

67. **Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R.** 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J **6:**1621-1624.

68. **Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R.** 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci U S A **108 Suppl 1:**4516-4522.

69. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods **7:**335-336.

70. **Edgar RC.** 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics **26:**2460-2461.

71. **Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R.** 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics **27:**2194-2200.

72. **Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO.** 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res **41:**D590-596.

73. **Wang Q, Garrity GM, Tiedje JM, Cole JR.** 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol **73:**5261-5267.

74. **Layton BA, Cao Y, Ebentier DL, Hanley K, Balleste E, Brandao J, Byappanahalli M, Converse R, Farnleitner AH, Gentry-Shields J, Gidley ML, Gourmelon M, Lee CS, Lee J, Lozach S, Madi T, Meijer WG, Noble R, Peed L, Reischer GH, Rodrigues R, Rose JB, Schriewer A, Sinigalliano C, Srinivasan S, Stewart J, Van De Werfhorst LC, Wang D, Whitman R, Wuertz S, Jay J, Holden PA, Boehm AB, Shanks O, Griffith JF.** 2013. Performance of human fecal anaerobe-associated PCR-based assays in a multi-laboratory method evaluation study. Water Res **47:**6897-6908.

75. **Yamamoto K, Wright AC, Kaper JB, Morris JG, Jr.** 1990. The cytolysin gene of Vibrio vulnificus: sequence and relationship to the Vibrio cholerae E1 Tor hemolysin gene. Infect Immun **58:**2706-2709.

76. **Wright AC, Garrido V, Debuex G, Farrell-Evans M, Mudbidri AA, Otwell WS.** 2007. Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of Vibrio vulnificus bacteria. Appl Environ Microbiol **73:**7477-7481.

77. **USEPA.** 2012. Recreational Water Quality Criteria. **Office of Water 820-F-12-058**.

78. **Newton RJ, McLellan SL, Dila DK, Vineis JH, Morrison HG, Eren AM, Sogin ML.** 2015. Sewage reflects the microbiomes of human populations. MBio **6:**e02574.

79. **Furukawa T, Hashimoto R, Mekata T.** 2015. Quantification of vancomycin-resistant enterococci and corresponding resistance genes in a sewage treatment plant. J Environ Sci Health A Tox Hazard Subst Environ Eng **50:**989-995.

80. **Morris D, Galvin S, Boyle F, Hickey P, Mulligan M, Cormican M.** 2012. Enterococcus faecium of the vanA genotype in rural drinking water, effluent, and the aqueous environment. Appl Environ Microbiol **78:**596-598.

81. **Torres C, Reguera JA, Sanmartin MJ, Perez-Diaz JC, Baquero F.** 1994. vanA-mediated vancomycin-resistant Enterococcus spp. in sewage. J Antimicrob Chemother **33:**553-561.

82. **Nayak BS, Badgley B, Harwood VJ.** 2011. Comparison of genotypic and phylogenetic relationships of environmental Enterococcus isolates by BOX-PCR typing and 16S rRNA gene sequencing. Appl Environ Microbiol **77:**5050-5055.

83. **McLellan SL, Huse SM, Mueller-Spitz SR, Andreishcheva EN, Sogin ML.** 2010. Diversity and population structure of sewage-derived microorganisms in wastewater treatment plant influent. Environ Microbiol **12:**378-392.

84. **Staley C, Unno T, Gould TJ, Jarvis B, Phillips J, Cotner JB, Sadowsky MJ.** 2013. Application of Illumina next-generation sequencing to characterize the bacterial community of the Upper Mississippi River. J Appl Microbiol **115:**1147-1158.

85. **Janssen PH.** 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl Environ Microbiol **72:**1719-1728.

86. **Badgley BD, Nayak BS, Harwood VJ.** 2010. The importance of sediment and submerged aquatic vegetation as potential habitats for persistent strains of enterococci in a subtropical watershed. Water Res **44:**5857-5866.

87. **Anderson KL, Whitlock JE, Harwood VJ.** 2005. Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. Appl Environ Microbiol **71:**3041-3048.

88. **Ferguson CM, Coote BG, Ashbolt NJ, Stevenson IM.** 1996. Relationships between indicators, pathogens and water quality in an estuarine system. Water Research **30:**2045-2054.

89. **Burton GA, Gunnison D, Lanza GR.** 1987. Survival of Pathogenic Bacteria in Various Fresh-Water Sediments. Applied and Environmental Microbiology **53:**633-638.

90. **Luczkiewicz A, Jankowska K, Kurlenda J, Olanczuk-Neyman K.** 2010. Identification and antimicrobial resistance of Enterococcus spp. isolated from surface water. Water Sci Technol **62:**466-473.

628 **Legends for Figures and Tables**

629 **Figure 1.** Culturable enterococci in water and sediment at Site NC-03, near the site of the
630 sewage spill. The dashed line represents the EPA standard for single sample maximum of
631 enterococci in recreational water (130 CFU · 100mL-1). (▲) VRE were cultured. within 800
632 meters of the spill (NC-01, NC-02 and NC-03). (○) The *vanA* gene was detected at sites within
633 800 meters of the spill. Note that on 10/1/2014, no sediment was collected or processed
634 (indicated by *).

635 **Figure 2.** Analysis of DNA sequencing of the 16S rRNA gene in water and sediment on seven
636 dates at Site NC-03. Blue gradient represents water samples and red gradient represents
637 sediment samples; the color gradient represents different time points in either water (W) or
638 sediment (S) where darker shades are immediately after the spill or earlier in time.

639 **Figure 3**. Relative abundance of select sewage-associated OTU's (*Bacteroidaceae,*
640 *Ruminococcaceae, Lachnospiraceae, Porphyromonadaceae, Veillonellaceae, Prevotellaceae)* at
641 Site NC-03 in water (▲) and sediment (•) samples.

642 **Figure 4**. Dominant families in sediment and water at Site NC-03 on all sampling dates
643
644 **Table 1.** Detection and levels of *vanA* measured by qPCR in water and sediment.
645

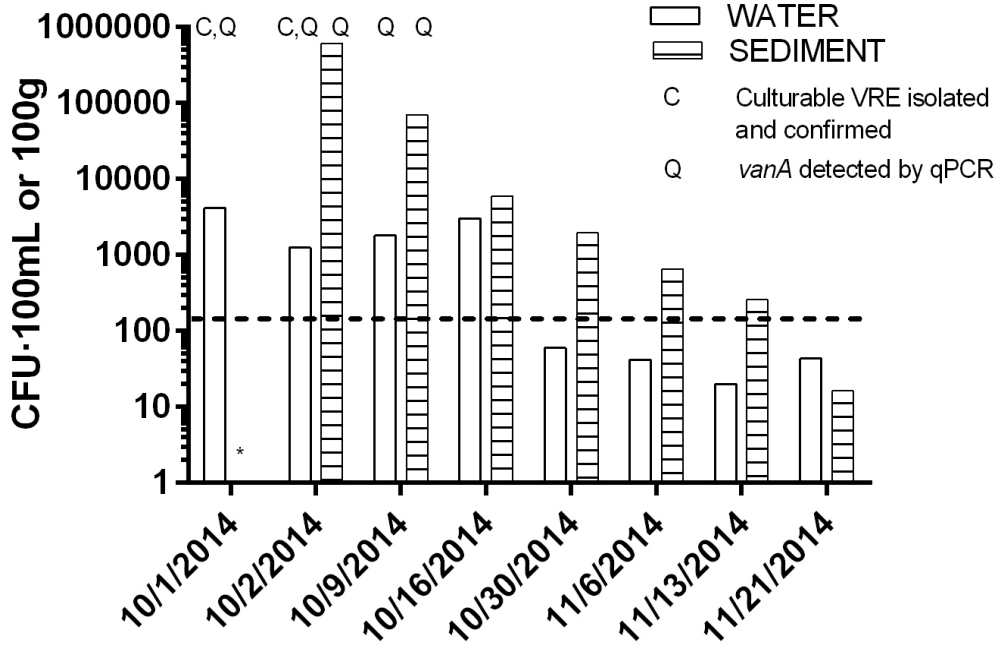

Figure 1. Culturable enterococci in water and sediment at Site NC-03, near the site of the sewage spill. The dashed line represents the EPA standard for single sample maximum of enterococci in recreational water (130 CFU · $100mL^{-1}$). Letters indicate where VRE were cultured (C) and where *vanA* was detected within 800 meters of the spill (NC-01, NC-02 and NC-03). Note that on 10/1/2014, no sediment was collected or processed (indicated by *).

666 **Table 1**. Detection and levels of *vanA* measured by qPCR in water and sediment at three sites
667 near the origin (within 800 meters) of the sewage spill over eight sampling dates. Sample limits
668 of detection were 4 gene copies · 100 mL^{-1} water and 6.7 x 10^3 gene copies · 100 g^{-1} sediment.
669 10/1 is considered Day 1 post-spill. Note that access issues prevented sampling at all sites on all
670 dates; data analysis focuses on Site NC-03 where samples were collected on each sampling date.

671

	Site	**Days Post-Spill**							
		1	2	9	16	30	36	43	51
Water (log10 gene copies· 100 mL)	NC-01	1.96	1.79	2.21	--[c]	--[c]	--[c]	--[c]	--[c]
	NC-02	ND[b]	1.54	1.84	--[c]	--[c]	ND[b]	ND[b]	ND[b]
	NC-03	1.92	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
Sediment (log 10 gene copies· 100 g)	NC-01	--[c]	ND[b]	DNQ[a]	--[c]	--[c]	--[c]	--[c]	--[c]
	NC-02	--[c]	4.24	ND[b]	--[c]	--[c]	ND[b]	ND[b]	ND[b]
	NC-03	--[c]	4.95	4.54	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]

672

673 [a] detected but not quantifiable; [b]not detected; [c]not measured

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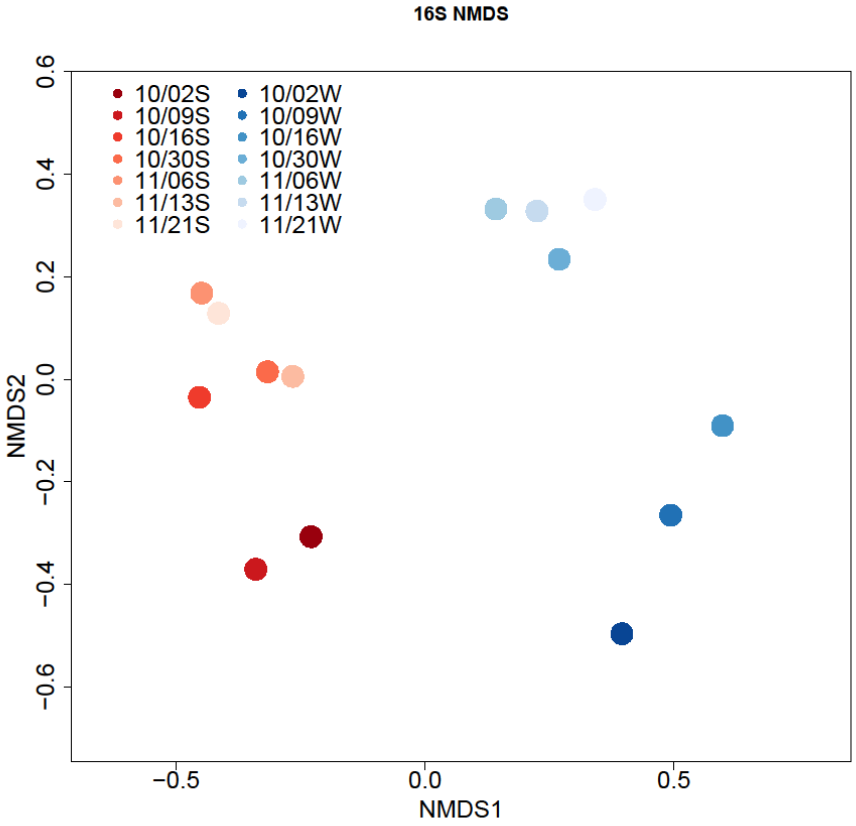

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678 **Figure 2.** Analysis of DNA sequencing of the 16S rRNA gene in water and sediment on seven

679 dates at site NC-03. Blue gradient represents water samples and red gradient represents sediment

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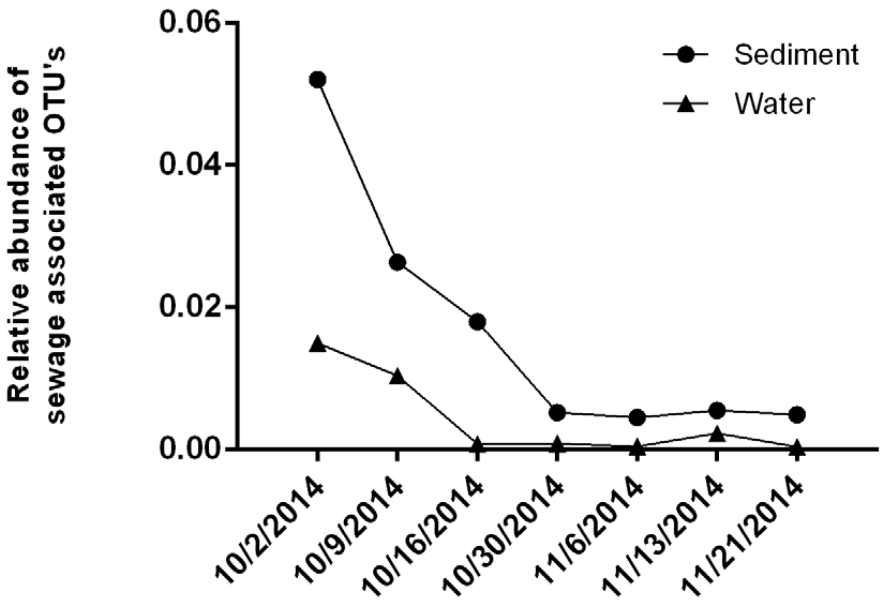

685

686 **Figure 3.** Relative abundance of select sewage-associated OTU's (*Bacteroidaceae,*

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688 Site NC-03 in water (▲) and sediment (•) samples.

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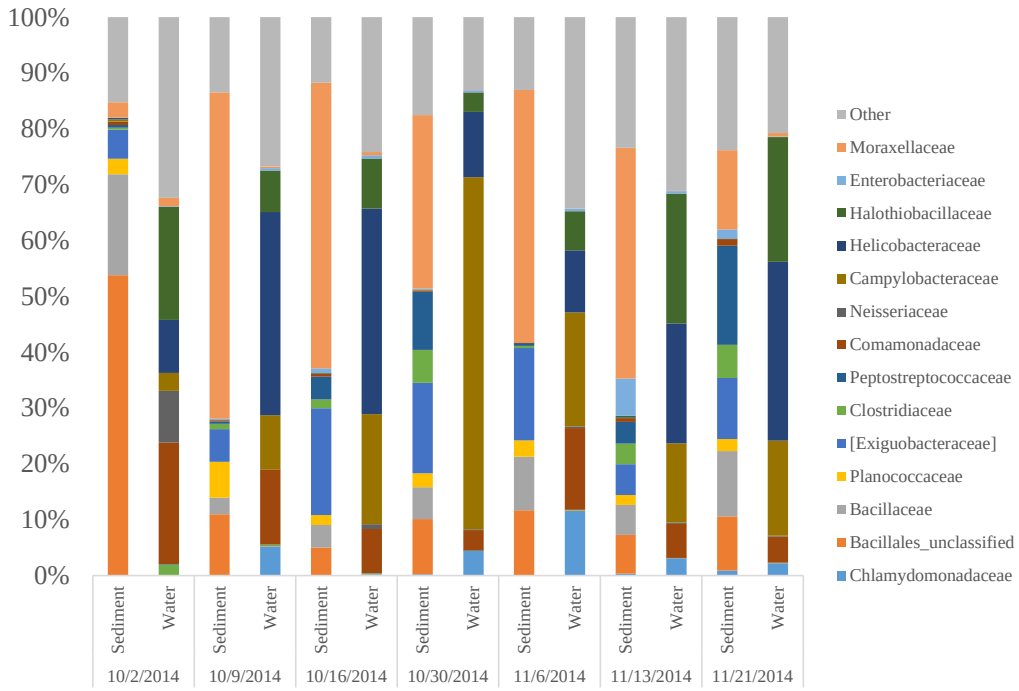

695

696 Figure 4. Dominant families in sediment and water at Site NC-03 on all sampling dates.

697