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**THE EFFECT OF COMBINED SEWER OVERFLOWS ON THE ABUNDANCE OF
ANTIBIOTIC RESISTANCE GENES AND BACTERIA IN THE JAMES RIVER**

A thesis submitted in partial fulfillment of the requirements for the Degree of Master of
Science at Virginia Commonwealth University

by

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December, 2017

ACKNOWLEDGEMENTS

I would like to thank my friends and family for all of their support throughout my graduate studies. This project would not have been possible without the assistance of: the Bukaveckas Lab, specifically Paul Bukaveckas, William “Mac” Lee, and Spencer Tassone for their field work and environmental data analysis; Ella Balasa, Taylor Jones, and Imaan Muhammad for water sample processing, and the rest of the Franklin lab for their guidance; Sunauz Moezzi and Shira Lanyi for their lab assistance; Dr. David Hooper, Dr. Michael Strickland, and Dr. Brian Badgley for sharing their qPCR standards. I would like to extend a special thanks to VCU, my committee, and my advisor Dr. Rima Franklin for their immense patience and support over the past 2 years.

TABLE OF CONTENTS

The effect of combined sewer overflows on the abundance of antibiotic resistance genes and bacteria in the James river	i
Acknowledgements	ii
Table of Contents	iii
Abstract	v
1. Introduction	1
2. Methods	3
2.1. Site selection and sampling	3
2.2. DNA Extraction	4
2.3. Quantification of genes using qPCR	5
2.4. Multi-Drug Resistance (MDR) Screening	7
2.5. Environmental Data	8
2.6. Data Analysis	9
3. Results	11
3.1. Correlation of antibiotic resistance abundance with environmental data	11
3.2. Effect of CSO events on the abundance of resistance genes	12
3.3. Multi-drug Resistance of Isolates	12
4. Discussion	13
4.1. Linking abundance of resistance genes to environmental conditions and CSO events	14
4.2. Multi-drug resistance and CSO events	16
4.3. Conclusions and Future Work	19
Tables & Figures	i
Table 1. Primers and reaction conditions for qPCR assays.	i
Table 2. Antibiotic concentrations of disks used in multi-drug resistant screening (Oxoid™ Thermo Fisher Scientific, Waltham, Massachusetts).	ii
Table 3. Pearson correlations between water quality parameters, hydrologic data, or <i>E. coli</i> abundance and each antibiotic resistance gene copy ratio at the CSO site (n = 37-41).	iii
Figure 1. Site map. Image created with Google Maps.	iv
Figure 2. The <i>bla</i> _{TEM} to <i>16S rRNA</i> gene copy ratio and three day mean discharge (ft ³ sec ⁻¹) at the CSO (a) and HUG (b) sites during the summers of 2015 and 2016. Each point on the discharge line represents a sampling event and the bar	

is the corresponding qPCR value. Asterisk represents missing qPCR data. No bars represent qPCR results below the detection limit..... v

Figure 3. The *qnrA* to *16S rRNA* gene copy ratio (a) the *tetW* to *16S rRNA* gene copy ratio (b) and *E. coli* abundance at the CSO site during the summer of 2015 and 2016. Each point on the *E. coli* line represents a sampling event and the bar is the corresponding qPCR value. Asterisk represents missing qPCR data. No bars represent qPCR results below the detection limit.vi

Figure 4. Mean (+SE) gene abundance ratios of *bla_{TEM}* (a), *qnrA* (b), and *tetW* (c) during event and non-event conditions. Within each panel, different letters indicate statistically significant differences ($p < 0.05$) determined using Mann-Whitney test ($n = 12$ per group).....vii

Figure 5. Abundance of *E. coli* (a), abundance of culturable bacteria (b), and the fraction of bacteria resistant to each antibiotic (c) at the CSO site and the HUG site during an event and a non-event.viii

Figure 6. Fraction of isolates resistant to 1-10 antibiotics at the CSO site and the HUG site during an event and a non-event.ix

Figure 7. Fraction of isolates resistant to each of the 10 antibiotics from the CSO site and the HUG site during an event and a non-event..... x

Figure 8. Nonmetric multidimensional scaling (NMDS) ordination plot displaying the overall resistance patterns for the MDR isolates in the three different situations: CSO event, CSO non-event, and HUG (stress = 0.23) ($n = 126$).....xi

Work Citedxii

ABSTRACT

THE EFFECT OF COMBINED SEWER OVERFLOWS ON THE ABUNDANCE OF ANTIBIOTIC RESISTANCE GENES AND BACTERIA IN THE JAMES RIVER

By: Enjolie Levensgood

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University

Virginia Commonwealth University, 2017

Advisor: Dr. Rima B. Franklin, Associate Professor, VCU Department of Biology

Antibiotic resistance is a major threat to human health. Clinical situations are the main focus for antibiotic resistance research, but understanding the spread of resistance in the environment is also vital. A major contributor to this spread is wastewater from combined sewer overflow (CSO) events. The effect of CSO events on antibiotic resistance in the James River near Richmond, Virginia was studied using genomic and microbiological approaches. The abundance of genes associated with resistance to quinolones (*qnrA*) and tetracycline (*tetW*) was strongly correlated with the presence of fecal indicator bacteria (*E. coli* abundance) as well as total nitrogen and phosphorus loads, which suggests an anthropogenic source of these genes.

Abundance of the *bla*_{TEM} gene, which confers resistance to β -lactam antibiotics, was elevated during CSO events and increased with precipitation and river discharge.

Bacteria isolated during a CSO event were resistant to more antibiotics and had higher multi-drug resistance when compared to isolates from a non-event. This study demonstrated that CSO events are contributing to the spread of antibiotic resistance.

1. INTRODUCTION

Antibiotics are vital to human health. They have been used to treat bacterial infections worldwide since the discovery of antibiotics in the early 20th century. Unfortunately, the heavy use of antibiotics in the medical, veterinary, industrial, and agricultural fields has led to the increased natural selection of antibiotic resistant bacteria (Davies and Davies 2010). Antibiotic resistance originates from evolutionary changes towards naturally occurring antibiotics, but human use during the antibiotic era has exacerbated this effect (Aminov and Mackie 2007). This increased spread of resistance is largely due to the misuse and overuse of antibiotics in many medical practices, where antibiotic use and prescriptions go unmonitored and unregulated (Septimus and Owens 2011). Though antibiotic resistance is most often studied in clinical situations and hospitals, there is also a need to study the environmental dissemination of antibiotic resistance, particularly in urban wastewater and riverine systems.

The leading contributor to the spread of resistance in urban river systems is wastewater (Amos et al. 2014). There are two key factors that contribute to this. First, resistant bacteria are abundant in wastewater, and conditions in wastewater treatment plants are favorable for the proliferation of these organisms (Prestinaci et al. 2015). Second, wastewater treatment plants have been found to promote a process called horizontal gene transfer (Laht et al. 2014), which is the direct transfer of mobile genetic element from one organism to another. The genes that encode for antibiotic resistance are usually part of these mobile genetic elements and subject to especially high rates of gene transfer, which can rapidly spread resistance throughout the microbial community.

Of additional concern is the rise of multi-drug resistant (MDR) organisms, which can have a level of resistance ranging from two or more antibiotics to nearly every commercially available antibiotic. These MDR organisms have been shown to be more prevalent in urban riverine and wastewater systems than in pristine environments (Szczepanowski et al. 2009; Graham et al. 2011; Pruden et al. 2012; Korzeniewska et al. 2013; Ouyang et al. 2015).

One problematic wastewater system, used in over 750 cities in the US, is the Combined Sewer System (CSS) (EPA 2012). A CSS collects storm water and sewage for treatment at a single facility. Occasionally during heavy rain, the volume of water that needs to be treated exceeds the capacity of the facility and the combined untreated wastewater is released into the river in what is called a CSO (Combined Sewer Overflow) event. These CSO events and the released wastewater could be a contributing factor to the spread of antibiotic resistance in the environment.

Though many studies have examined the connection between wastewater treatment and antibiotic resistance, the results are variable. Resistance towards antibiotics can prevail in certain areas when high amounts of specific antibiotics or a high abundance of resistant organisms enter the wastewater and river system (Korzeniewska et al. 2013). Resistant organisms persist in the environment, especially during wet weather events, though exposure to ultraviolet light (sunlight) may increase mortality (Engemann et al. 2008; West et al. 2011). Numerous studies have found that bacteria intrinsically-resistant to antibiotics and bacteria resistant to chemically modified antibiotics are common and widespread without the effect of wastewater (Rodríguez-Martínez et al. 2011, Ash et al. 2002). Additionally, increased resistance to specific

antibiotics and their respective genes can be region specific (Marston et al. 2016). Therefore, it is important to study the effects of CSOs on the occurrence of antibiotic resistance in the environment to better understand the spread of antibiotic resistance genes and the possible health effects they pose.

The city of Richmond (Virginia) is a moderately sized urban area that relies on a CSS, which often overflows and discharges untreated wastewater into the James River. In this study, we analyzed James River water samples to better understand the impact CSO events play on antibiotic resistance. Antibiotic resistance was studied using microbiological and genomic approaches. Relationships between antibiotic resistance, environmental parameters, and the dates of CSO events/non-events were used to address three major questions: (1) does the abundance of antibiotic resistance genes increase in association with CSO events (2) are antibiotic resistant organisms more prevalent during CSO events and, if so, (3) do those organisms harbor more resistance to multiple antibiotics?

2. METHODS

2.1. Site selection and sampling

This study compared two sites along the James River (Figure 1) as it flows through the city of Richmond, Virginia (USA). The first was a riverine site (HUG) located near the Huguenot Bridge (37.560471, -77.545801) which was selected to represent the quality of water entering the city. The watershed upstream of this site (between Lynchburg and Richmond) is forested and agricultural land, but is also impacted by over 150 industrial sites, more than 90 additional discharge sources, and runoff from over

700 km² of impervious surfaces (Furry 2011; Brown et al. 2015). The second was an estuarine site (CSO) near the outflow of the Shockoe CSO - 06 (37.529486, -77.429382) in downtown Richmond, which is the largest of the 29 CSO outfalls in the city.

Approximately every week during the summers of 2015 and 2016 (1st May through 15th October), surface water samples were collected from these sites using a bucket thrown from shore. Samples (1L) were transferred to sterile brown plastic bottles and sterile clear plastic bottles and stored on ice. All samples were returned to the lab within 2 hours at which time subsamples were removed from each brown bottle and immediately processed to determine *Escherichia coli* (*E. coli*) abundance using Modified mTEC agar (BD Difco™, Sparks, Maryland) following EPA guidelines Method #1603 (2009). In addition, an aliquot of water (~ 250 mL) was removed from each of these bottles and filtered using 0.2 µm pore-size mixed esters of cellulose filter membranes (Millipore, Molsheim, France). Filters were stored at -20°C until DNA extraction could be performed. Lastly, the water in the clear bottles were used to determine certain water quality and nutrient parameters (details below).

2.2. DNA Extraction

Sample DNA was extracted from archived filters using the PowerWater® DNA Isolation Kit (Mo Bio Laboratories, Inc. Carlsbad, California) with the following modifications to increase extraction efficiency. First, each membrane filter was torn into small pieces using sterile forceps prior to being inserted into PowerWater® Bead tube. To minimize DNA shearing, all vortex speeds were reduced to the lowest possible speed that still allowed for mixing. The incubation step for the removal of non-DNA

organic and inorganic matter was extended to 10 minutes. Lastly, the elution step, normally one 100 μ L elution with no incubation, was split into two elutions of 50 μ L with an additional room temperature 5 minute incubation before each centrifugation. Successful extraction was determined by checking for the presence of genomic DNA bands using agarose gel electrophoresis (1.5%) and ethidium bromide staining. All DNA concentrations were measured using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, Carlsbad, California).

2.3. Quantification of genes using qPCR

The following antibiotic resistance genes were assessed using quantitative PCR (qPCR): *bla*_{TEM}, *qnrA*, and *tetW*. The *bla*_{TEM} gene confers resistance to β -lactam antibiotics, such as penicillin, ampicillin, and cephalosporins, and is one of the most common β -lactam resistance genes due to it being transferred via plasmid or transposon (Bradford 2001). The *qnrA* gene is also plasmid mediated, and confers resistance to quinolones (Rodríguez-Martínez et al. 2011). The *tetW* gene confers resistance to tetracyclines on a chromosomal mobile genetic element (Scott et al. 1997) and has been shown to commonly occur in fecal contaminated waters (Xi et al. 2009). The *16S rRNA* gene was additionally quantified to determine overall bacterial abundance following Fierer et al. (2005).

Reaction mixtures (15 μ L) contained Bio-Rad SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, California), forward and reverse primers (Table 1), and 5 ng of template DNA. Each qPCR assay was performed in triplicate (*qnrA* performed in quadruplicate) using a CFX384™ Real-Time System (Bio-Rad, Hercules, California). The primers (purchased from Integrated DNA Technologies, Coralville,

lowa) and optimized reaction conditions for each gene are presented in Table 1. A melt curve and agarose gel electrophoresis were conducted to verify the specificity of the amplified products. Each qPCR run included an appropriate standard curve (discussed below) as well as two types of negative controls. The first was a “negative template control,” which contained nuclease-free water instead of the template DNA, and the second was a “resistance gene-free control,” which contained *Methanococcus voltae* (DSM #1537, DSMZ, Braunschweig, Germany) DNA instead of sample DNA.

For each antibiotic resistance gene, a standard curve was constructed using plasmid DNA extracted using the Zyppy™ Plasmid Miniprep Kit (Zymo Research Corp, Irvine, California). Sources were SpyTag-β-Lactamase-Spycatcher (pET28a) (for *bla_{TEM}*, Addgene, Cambridge, Massachusetts), pTrcHis+*qnrA* (provided by Dr. David Hooper, Harvard University) in *E. coli* J53 (Thermo Fisher Scientific, Waltham, Massachusetts) and pCR®2.1-TOPO+*tetW* (provided by Dr. Brian Badgley and Dr. Michael Strickland, Virginia Tech) in DH5α *E. coli* (Invitrogen, Carlsbad, California). For the *16S rRNA* gene standard curve, genomic DNA was extracted from *E. coli* (ATCC 11775, Manassas, Virginia) using the DNeasy UltraClean Microbial Kit (Quiagen, Germantown, Maryland).

Copy numbers for standard curves were calculated following Ritalahti et al. (2006), with the *E. coli* genome size being used for *16S rRNA* copy numbers and plasmid + gene insert size being used for the resistance genes. The gene copies were calculated using the mean starting quantity obtained from the Bio-Rad CFX Manager Version 3.1 (Hercules, California). The detection limit for the antibiotic resistance genes studied was determined to be a starting quantity of 120 copies per ng DNA. The

abundance of each antibiotic resistance gene was normalized based on *16S rRNA* gene abundance and these ratios were used for all subsequent analyses.

2.4. Multi-Drug Resistance (MDR) Screening

Antibiotic resistant bacteria were cultured, isolated, and assessed for multi-drug resistance for two sampling dates: June 28, 2016 (during a CSO event, both sites) and July 5, 2016 (non-event; CSO site only). Water samples were collected as previously described, diluted in sterile phosphate buffered saline (PBS) and plated onto four types of R2A agar (BD Difco™, Sparks, MD): (i) unamended, (ii) tetracycline amended at 50 µg/mL, (iii) streptomycin amended at 100 µg/mL, and (iv) ampicillin amended at 100 µg/mL (antibiotics purchased from Sigma Aldrich, St. Louis, Missouri). These antibiotic concentrations are the maximum of the ranges recommended by major antibiotic retailers (ATCC, Thermo Fisher Scientific, Sigma Aldrich) and represent high levels of exposure. The motivation for choosing this high concentration was to select for very resistant organisms and to limit growth environmental bacteria, which often have low levels of resistance due to exposure to sub-inhibitory levels of antibiotics (Sandegren 2014). A preliminary study used the full range of recommended concentrations of major antibiotic retailers and showed that the selected concentrations were the most effective (data not shown).

Triplicate plates were prepared for each dilution and each agar type and then incubated in the dark at 27°C for 72 hours. Plates containing 10-200 colony forming units (CFU) were counted, stored at 4°C (<2 weeks) and then isolated. For each antibiotic type, 25-35 morphologically distinct colonies were selected and streaked on to antibiotic-amended R2A agar plates of the same type from which they were isolated.

Plates were again incubated for 72 hours at 27°C. Isolates were transferred to R2A broth and incubated at 27°C for 72 hours. A small aliquot of each liquid culture was then archived (15% glycerol, stored at -80°C) until MDR testing could be performed using antibiotic susceptibility disk tests (Table 2).

Each isolate was determined to be resistant or susceptible to each antibiotic following methods in the Manual of Antimicrobial Susceptibility Testing (American Society for Microbiology 2005). Archived isolates were tested using direct colony suspension, with the inoculum reaching the 0.5 McFarland turbidity standard in no longer than 24 hours. Once cultures reached the 0.5 McFarland standard, they were spread on plates and incubated in the dark at 27°C for 16-18 hours after which time the zone of inhibition surrounding each antibiotic disk was measured. *E. coli* (ATCC 25922, Manassas, Virginia) was used as a control and tested with each batch of samples.

Some isolates did not reach the standard turbidity within 24 hours and a second attempt was made to regrow them from the archived cultures. Any that still did not have adequate growth after this second attempt were excluded from the MDR testing. This created an unequal sample size, with different numbers of isolates from each site and each original antibiotic type. To adjust for this in the data analyses, a random subset of 9-15 isolates was selected from each combination for final comparison (total n=126).

2.5. Environmental Data

Data for CSO event dates were obtained from the open access monthly reports posted by the City of Richmond Department of Public Utilities (Combined Sewer Overflow Project). Specific conductance, temperature, and pH were measured directly using a water quality sonde (YSI 6600, YSI Environmental, Inc., Yellow Springs, Ohio).

Total suspended solids (TSS) were determined gravimetrically using pre-weighed, pre-combusted filters (APHA 1998). Samples for total nitrogen (TN) and total phosphorus (TP) were being collected as part of a long-term monitoring program and analytical methods are described in greater detail in Bukaveckas et al. (2011). Discharge data for the James River were obtained from the USGS gaging station at site 02037500 (37.563055, -77.547222), which is near the HUG sampling site. Precipitation data provided by National Climatic Data Center, using the Richmond International Airport, "KRIC".

2.6. Data Analysis

Correlation of environmental data with abundance of antibiotic resistance:

Pearson correlations were used to compare antibiotic resistance gene ratios to *E. coli* abundance and environmental data (TN, TP, TSS, specific conductance, pH, temperature, discharge, precipitation) across all sampling dates. Correlations with river discharge considered three different time frames: mean daily discharge for the day of sampling ("Sampling day"), the difference in the mean discharge for the day before and the day after sampling ("Prior minus following") to reflect whether the sampling event was during a period of increasing or decreasing flow, and the mean discharge for the day before, day of, and day after sampling ("Three day mean"). Correlations with precipitation also considered several timeframes: "day of sampling" (cumulative rainfall from midnight on sampling day until sampling time (usually ~12 hours), "1 day prior" (rainfall that fell between 12 and 36 hours prior to sampling), "2 days prior" (between 36 and 60 hours), "3 days prior" (between 60 and 84 hours), and "4 days prior" (between

108 and 84 hours). Because of the large number of analyses this created, α was adjusted to 0.01.

Effect of CSO events on the abundance of resistance genes: To specifically determine the effect of CSO events on gene abundance ratios, two categories were defined: “event” (a CSO overflow was reported by the City of Richmond during sampling or in the three days immediately prior) and “non-event” (no CSO overflow reported in at least seven days prior to sampling). Of the 88 total sample dates considered in this study, 18 and 17 dates, respectively, met these criteria. From this list of possible dates, 12 full data sets were selected from each category and used for statistical analyses. Because a Shapiro-Wilk test indicated these data were not normally distributed, a non-parametric Kruskal-Wallis test was used to compare resistance gene ratios during these “events” and “non-events.” Post hoc pairwise comparisons were performed using the Mann-Whitney test. Data from both sites was considered, though it is important to note that HUG is upstream of the CSO discharge point. Thus, any change in resistance gene ratios during an “event” at HUG likely reflects discharge from upstream of the city and/or increased runoff from precipitation.

Multi-drug resistance of isolates: Each of the isolates that were cultured during the summer of 2016 (see section 2.4.) were classified as resistant (1) or not resistant (0) to 10 different antibiotics (Table 2). From this data matrix, we calculated the total number of antibiotics to which each isolate was resistant and fraction of isolates resistant to each individual antibiotic. The Jaccard coefficient was used to calculate similarity between each isolate based on their resistance profiles. Results were visualized using nonmetric multidimensional scaling (NMDS) ordination, and a

permutational multivariate analysis of variance (PERMANOVA, 9999 permutations) was used to test if the origin of the isolate (i.e., obtained from CSO event, CSO non-event, or HUG) had a significant effect on its resistance profile.

All statistical tests were performed using PAST Version 3.16 (Hammer et al. 2001). All graphs were visualized using SigmaPlot Version 13.0.

3. RESULTS

3.1. Correlation of antibiotic resistance abundance with environmental data

None of the water chemistry, river discharge, precipitation or *E. coli* data were significantly correlated with any of the antibiotic resistance gene ratios for the HUG site (data not presented, all $|r| < 0.26$ and $p > 0.04$). Additionally, temperature and pH were not significantly correlated with any of the antibiotic resistance gene ratios for the HUG or CSO site (data not presented). In contrast, several environmental parameters were correlated with antibiotic resistance gene ratios at the CSO site (Table 3). For *bla*_{TEM}, discharge on the day of sampling and the “three day mean” were positively correlated. In cases where discharge was increasing around the sampling day (i.e., the difference in discharge the day before and the day after sampling was high), *bla*_{TEM} was also high. For *qnrA* and *tetW*, *E. coli* abundance, TN, and TP were the best predictors of increased gene ratios.

The tendency for *bla*_{TEM} to increase with the discharge of the three day mean can also be seen over time at both sites (Figure 2). Similarly, the correlation of *E. coli* abundance with the *qnrA* ratio and the *tetW* ratio at the CSO site is also consistent with

the tendency for spikes in *E. coli* counts to be accompanied by spikes in the respective antibiotic resistance genes over time (Figure 3).

3.2. Effect of CSO events on the abundance of resistance genes

For *bla*_{TEM}, there was an overall difference in mean abundance ratios across sites and event categories ($p = 0.05$), though only the CSO event was significantly different than the CSO non-event in the post-hoc comparisons ($p = 0.02$) (Figure 4a). There were no significant differences in *qnrA* copies ($p = 0.84$) (Figure 4b). For *tetW*, there is no significant difference between event and non-events ($p < 0.96$), but *tetW* copies are significantly greater at the CSO site ($p \leq 0.02$) (Figure 4c).

3.3. Multi-drug Resistance of Isolates

A focused study of the CSO site during a non-event and during an event and at the HUG site was performed. Discharge was low during the non-event sampling (5,750 ft³ sec⁻¹), with only a trace of precipitation (0.06 inches) in the previous 12 hours. During the CSO event, discharge was much higher (12,900 ft³ sec⁻¹) and the previous 12 hours of precipitation was 0.98 inches. *E. coli* counts at the CSO site were elevated and exceeded the Virginia State law of primary contact (Figure 5a). In addition to *E. coli* counts, the total abundance of culturable bacteria was measured and we found that the CSO was approximately an order of magnitude greater than the HUG site (Figure 5b). We then quantified the fraction of organisms resistant to ampicillin, streptomycin, and tetracycline and found that across all samples, the greatest percent of organisms were resistant to ampicillin, followed by streptomycin, then by tetracycline (Figure 5c).

From the resistant isolates, multi-drug tests were performed. At the CSO site, the fraction of isolates conferring resistance to 6 or more antibiotics was higher than the

HUG site, and during a CSO event it is even greater (Figure 6). Over half of the CSO event isolates were resistant to 6 or more antibiotics, whereas during a non-event, about a quarter were resistant to 6 or more of the tested antibiotics, and at the HUG site, less than 10% were resistant to 6 or more antibiotics. Additionally, resistance varied depending on the antibiotic (Figure 7). For all but one antibiotic (Bactrim), the fraction of isolates resistant was greatest at the CSO site and highest during an event. However, in some cases, the difference between the CSO event and non-event sampling was not great (vancomycin, augmentin). In all but one case (ampicillin), the fraction of resistant isolates was lowest at HUG during the non-event sampling. For streptomycin, cefepime, and ciprofloxacin, the CSO non-event and HUG were about equal. For ampicillin, all isolates were resistant for the CSO event, but a slightly greater fraction of isolates was resistant at HUG than the CSO non-event. Lastly, ciprofloxacin had about an equal amount of resistant isolates in all samples and the lowest fraction of resistant isolates was observed for this antibiotic overall.

The isolates from the three different situations (CSO event, CSO non-event, and HUG) have significantly different MDR patterns (PERMANOVA $F = 2.6$, $p = 0.008$), with the CSO event being significantly different than both the CSO non-event ($p = 0.030$) and HUG ($p = 0.004$). This difference in MDR patterns is consistent with the pattern of separation of points in the NMDS plot (Figure 8).

4. DISCUSSION

Antibiotic resistance is a rapidly spreading health threat. Since resistant bacteria can infect humans and many bacteria can transfer their resistance to human pathogens,

the environmental presence of resistance is key to fully understanding the spread of antibiotic resistance. One possible contributor to the spread of antibiotic resistance is CSO events. This study addresses the effect of CSO events on the abundance of antibiotic resistance genes and the prevalence of MDR organisms.

4.1. Linking abundance of resistance genes to environmental conditions and CSO events

The HUG site was selected to represent the water that is entering the James River near Richmond. In this water, a diversity of upstream inputs of genes and bacteria have been homogenized. For this reason, we did not necessarily expect to see any correlation of antibiotic resistance gene abundances with environmental conditions measured in Richmond. While we did detect resistance genes at HUG, none of the resistance gene ratios correlated with water chemistry, river discharge, or precipitation. In contrast, all three antibiotic resistance gene ratios were correlated to various environmental parameters at the CSO site.

The *bla_{TEM}* gene was significantly increased at the CSO site during an event (Figure 4a), indicating a wastewater influence on the gene, consistent with previous studies (Narcisco-da-Rocha et al. 2014, Laht et al. 2014, Hsu et al. 2015). Additionally, *bla_{TEM}* was correlated with river discharge and precipitation, but not with *E. coli*, TN, or TP, as with *qnrA* and *tetW* (Table 3). *bla_{TEM}* is one of the more prevalent and wide-spread resistance genes (Matthew 1979, Jacoby and Bush 2005), but this correlation could be indicative of an upstream input of treated water and additional environmental inputs, which have been shown to harbor increased amounts of *bla_{TEM}* genes (Lachmayr et al. 2009). The correlation of *bla_{TEM}* to discharge and precipitation could

be due to the sediment retention of the gene and water column persistence (Knapp et al. 2012).

The gene ratios of *qnrA* and *tetW* were both correlated with *E. coli*, TN, and TP at the CSO site (Table 1), which is suggestive of wastewater and anthropogenic urban influences. *E. coli* counts are used by the EPA as an indicator for fecal and wastewater contaminated waters (Figure 5a), and high loads of TN and TP are commonly derived from anthropogenic point sources, such as industrial and wastewater treatment plant effluent and sewage (Smith 2009). In the James specifically, TN has been correlated with point sources and TP with runoff (Bukaveckas and Isenberg 2013). The correlation between these environmental parameters and *E. coli* counts suggests a wastewater-related influence. That being said, *qnrA* was not significantly increased during a CSO event at the CSO site (Figure 4b), contrary to what may be expected. This contradiction may be due to the combined effect of the *qnrA* gene being highly associated with *Enterobacteriaceae* (Jacoby 2005, Robicsek et al. 2006), but at the same time, it has been found to be rare (Cummings et al. 2011, Marti and Balcázar 2013, Poirel et al. 2012).

On the other hand, *tetW* gene ratios were significantly increased during a CSO event at the CSO site (Figure 4c). These results are similar to those of Xu et al. (2015), who found increased concentrations of tetracycline resistance genes (including *tetW*) in wastewater and lesser concentrations in wastewater effluent. Additionally, both *bla_{TEM}* and *tetW* gene ratios were found at similar ratios as other related resistance genes (Laht et al. 2014).

It is important to note *tetW* was not detected at the HUG site. It has been shown that exposure to light and the distance from the source input rapidly decreases the amount of *tet* genes (Engemann et al. 2008, Tamminen et al 2011), so it is possible that some *tetW* genes are released from sources upstream of Richmond but are simply not detectable by the time the water reached the sampling site. The lack of *tetW* at the HUG site but the presence at the CSO site suggests an active point source of *tetW* between the two sites and in close proximity to the city. Despite non-detection of *tetW* at HUG, we still were able to isolate tetracycline resistant organisms (Figure 5c). This may be due to our microbiological approach, which can detect a single resistant organism that would normally be an outlier when compared to the larger number of unculturable bacterial community. Additionally, some tetracycline resistances are due to multi-drug efflux pumps or another tetracycline resistance genes, such as *tetO* or *tetM* (Warburton et al. 2016). Therefore, the lack of *tetW* may not be a deficit of that antibiotic resistance but rather that specific gene (Chopra and Roberts 2001).

4.2. Multi-drug resistance and CSO events

In urban river systems, one of the largest contributors to the spread of MDR is horizontal gene transfer (HGT) of multiple resistance genes or MDR efflux pumps occurring in wastewater treatment plant effluent (Laht et al. 2014). In this study, organisms were first isolated in ampicillin, tetracycline, or streptomycin. These are all antibiotics to which bacteria are commonly resistant; but since these were the originally isolated from these antibiotics, there may be a selection bias towards these organisms and not towards organisms resistant to only one of the subsequently tested antibiotics.

Both the number of resistant organisms (Figure 5c) and the number of drugs to which each isolate was resistant (Figure 6) were linked to CSO events. This increase in MDR may be reflective of an increased exposure to antibiotics, other resistant organisms, and antibiotic resistance-associated genetic mobile elements, which have been shown to be in greater abundance in wastewater (O'Brien 2002, Tennstedt et al. 2003, von Wintersdorff et al. 2016, Kim and Aga 2007, Alekshun and Levy 2007). Though the CSO and HUG non-event results were expected to be similar, the slightly increased amount of resistant isolates may be due to a tidal effect at the CSO site bringing in possible wastewater treatment plant effluent from downstream. These mechanisms can explain the greater abundance of resistant organisms found at the CSO site during a CSO event: more than 50% of the isolates from the CSO site during a CSO event were highly resistant (i.e., 6 or more antibiotics) in contrast to 25% at the CSO site during a non-event, and only 10% at the HUG site (Figure 6). Furthermore, a greater amount of antibiotic resistance genes, specifically *tetW* and *bla_{TEM}*, were found during a CSO event (Figure 4). Therefore, overall resistance is increasing during CSO events.

The antibiotics that were most affected by CSO events were ampicillin, cefotaxime, tetracycline, streptomycin, and cefepime (Figure 7). Of the greatest concern are cefotaxime and cefepime, which are both used to treat severe infections that are usually already multi-drug resistant (Kim et al. 2016). Environmental resistance to cefotaxime and cefepime is rare, so the relatively high frequency of resistant isolates is further evidence of an anthropogenic input of resistance during CSO events. On the other hand, ampicillin, streptomycin, and tetracycline are all widely used in the medical,

veterinary, and aquaculture fields. Though they increase with CSO events, resistance has also been shown to be prevalent in the environment (Amos et al. 2014, Popowska et al. 2012, and West et al. 2011). This is shown best with ampicillin, which was slightly greater at the HUG site than the CSO non-event. This may be due to a ubiquitous amount of resistant environmental bacteria.

Vancomycin, Bactrim, and ciprofloxacin had similar fractions of resistant isolates in all situations (Figure 7). Overall levels of resistance to vancomycin were high, which may be due to increased resistant organisms and the presence of the environmental bacteria from which the antibiotic originated (Larsen et al. 2012, Ouyang et al. 2015). Bactrim and ciprofloxacin had low fractions of resistance, which is due to their limited use and specific qualities. Bactrim is often used in combination with other drugs and therefore organisms are less commonly resistant (Kim et al. 2016). Ciprofloxacin has not often been shown to have cross resistances, so organisms are also less commonly resistant (Kim et al. 2016).

Lastly, the CSO site had a greater fraction of resistant isolates than the HUG site for augmentin and piperacillin, but the event and non-event were similar (Figure 7); since both of these antibiotics are used in clinical situations, this could be indicative of resistance that persists through wastewater treatment or foregoes treatment. For augmentin, it could be due to runoff from Richmond and its use in the veterinary field (pet waste); for piperacillin, the increased fraction may be due to the common use of this antibiotic with other drugs against multi-bacterial infections (Kim et al. 2016).

Overall, isolates impacted by a CSO event had a different pattern of resistance than isolates during a non-event or at the HUG site (Figure 8). This pattern tended to

be more isolates which were resistant to a greater number of antibiotics during a CSO event.

4.3. Conclusions and Future Work

This study demonstrated that CSO events significantly contributed to the environmental pool of antibiotic resistance in the James River; there was both an increase in the abundance of antibiotic resistance genes and antibiotic resistant isolates during CSO events. Additional research is needed to determine whether any of the organisms are pathogenic or HGT-susceptible organisms, which will help assess the direct threat these bacteria have on human health. Preliminary DNA sequencing of ~30 of these isolates revealed several clinically-relevant genera including *Staphylococcus*, *Pseudomonas*, and *Sphingomonas* (Moezzi and Lanyi, personal communication).

Future work should also consider additional resistance genes, such as *ermB* or *ampC*, to cover a greater scope of antibiotic classes and mechanisms (Alexander et al. 2015). *ermB* confers resistance to erythromycin and is found on mobile genetic elements, especially in water sources (Rodriguez-Mozaz et al. 2015, Böckelmann et al. 2009). *ampC* confers resistance to β -lactam antibiotics and is both chromosomal and plasmid-mediated but less ubiquitous than other similar β -lactamase genes (Hawkey and Jones 2009). Additionally, a longer timescale would help us understand if there has been a steady increase of CSO events and corresponding antibiotic resistances.

Though all the intricacies of the spread of antibiotic resistance have yet to be fully understood, one contributing method – CSO events – has been found, and hopefully a change in treatment facility infrastructure may help mitigate the amount of resistance being spread to the environment. If the proliferation of antibiotic resistance continues

unabated, those deadly infections could rise from the currently estimated 700,000 annual deaths worldwide to 10 million worldwide in 2050 (O'Neill 2014). Given the slow pace of research and development for new antibiotics (Coates et al. 2011), decreasing the amount of resistant bacteria released into the environment is crucial to reduce the possibility of a rapidly increasing human death rate from antibiotic resistant and MDR infections.

TABLES & FIGURES

Table 1. Primers and reaction conditions for qPCR assays.

Antibiotic Class	Gene	Primer name and sequence	Primer conc. (μM)	Thermal cycling	Modified from
β -lactams	<i>bla_{TEM}</i>	bla-TEM, FX: GCKGCCAACTTACTTCTGACAACG	0.3	95 °C for 5 minutes; 95 °C for 15 seconds, 61 °C for 30 seconds, 72 °C for 30 seconds, (40 cycles)	Marti et al. 2014
		bla-TEM, RX: CTTTATCCGCCTCCATCCAGTCTA	0.3		
Quinolones	<i>qnrA</i>	qnrAf-RT: ATTTCTCACGCCAGGATTTG	0.1	95 °C for 3 minutes; 95 °C for 15 seconds, 59.9 °C for 20 seconds, (45 cycles)	Marti et al. 2013
		qnrAr-RT: GCAGATCGGCATAGCTGAAG	0.1		
Tetracyclines	<i>tetW</i>	<i>tetW</i> -F: GAGAGCCTGCTATATGCCAGC	0.1	95 °C for 4 minutes; 95 °C for 30 seconds, 57.4 °C for 15 seconds, 72 °C for 15 seconds, (40 cycles)	Thames et al. 2012
		<i>tetW</i> -R: GGGCGTATCCACAATGTAAAC	0.1		
	<i>16S rRNA</i>	Eub338: ATTTCTCACGCCAGGATTTG	0.15	95 °C for 4 minutes; 95 °C for 30 seconds, 55.5 °C for 30 seconds, 72 °C for 1 minute (40 cycles); 95 °C for 1 minute	Fierer et al. 2005
		Eub518: GCAGATCGGCATAGCTGAAG	0.15		

Table 2. Antibiotic concentrations of disks used in multi-drug resistant screening (Oxoid™ Thermo Fisher Scientific, Waltham, Massachusetts).

Antibiotic	Concentration (µg)
Ampicillin	10
Augmentin (amoxicillin/clavulanic acid)	20/10
Bactrim (sulfamethoxazole/trimethoprim)	1.25/23.75
Cefepime	30
Cefotaxime	30
Ciprofloxacin	5
Piperacillin	100
Streptomycin	10
Tetracycline	30
Vancomycin	30

Table 3. Pearson correlations between water quality parameters, hydrologic data, or *E. coli* abundance and each antibiotic resistance gene copy ratio at the CSO site (n = 37-41).

	<i>bla</i> _{TEM}	<i>qnrA</i>	<i>tetW</i>
Water Chemistry			
Specific Conductance ($\mu\text{S cm}^{-1}$)	-0.22	0.19	0.25
TSS (mg L^{-1})	0.36	-0.14	-0.08
TN (mg L^{-1})	0.39	0.60 **	0.74 **
TP (mg L^{-1})	0.18	0.58 **	0.65 **
River Discharge ($\text{ft}^3 \text{sec}^{-1}$)			
Sampling day	0.41 *	-0.13	-0.10
Prior minus following	0.62 **	0.17	0.14
Three day mean	0.45 **	-0.13	-0.10
Precipitation (inches)			
Sampling day	-0.07	-0.08	-0.03
1 day prior to sampling	0.13	0.26	0.33
2 days prior to sampling	0.02	-0.05	-0.10
3 days prior to sampling	-0.08	-0.14	-0.08
4 days prior to sampling	0.67 **	-0.12	-0.07
<i>E. coli</i> abundance ($\text{CFU } 100 \text{ mL}^{-1}$)	0.18	0.88 **	0.97 **

* $0.001 < p \leq 0.01$

** $0.001 < p$



Figure 1. Site map. Image created with Google Maps.

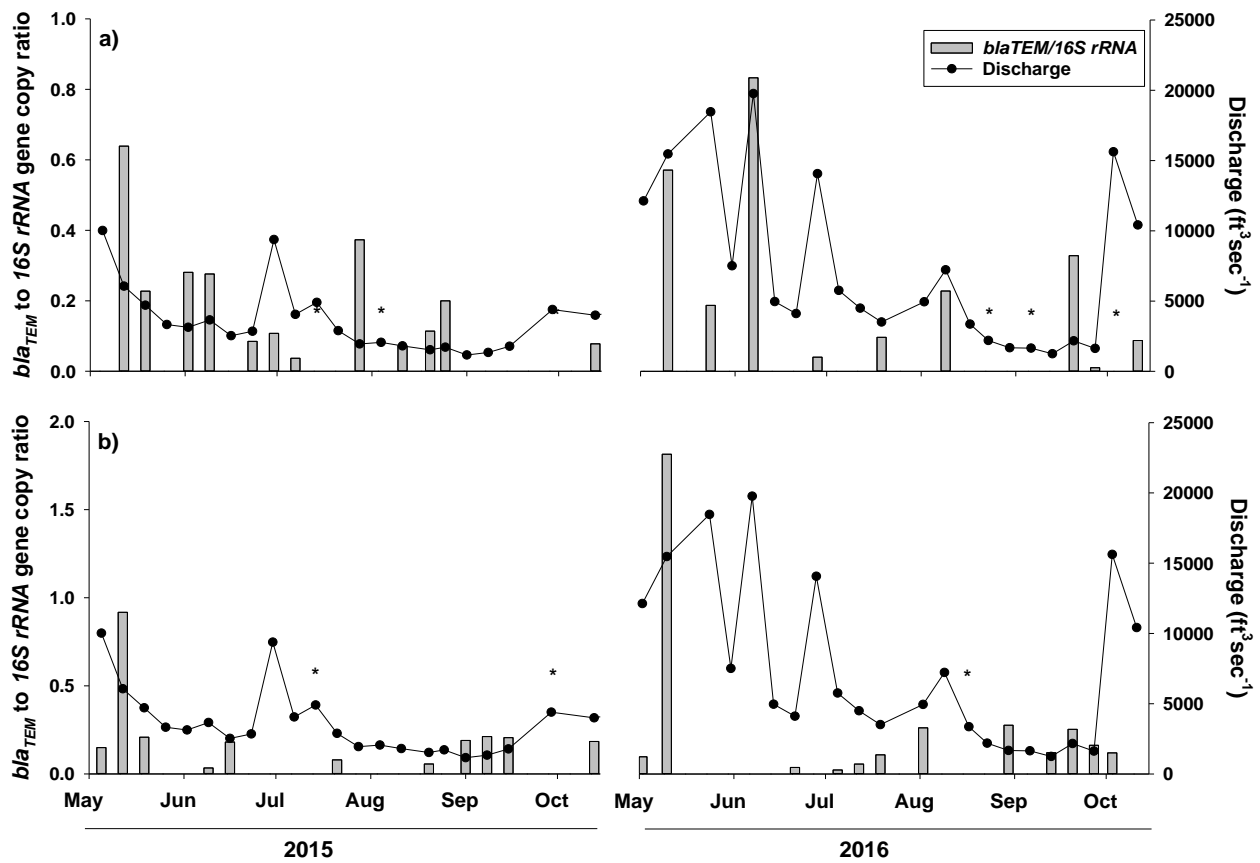


Figure 2. The *bla*_{TEM} to 16S rRNA gene copy ratio and three day mean discharge (ft³ sec⁻¹) at the CSO (a) and HUG (b) sites during the summers of 2015 and 2016. Each point on the discharge line represents a sampling event and the bar is the corresponding qPCR value. Asterisk represents missing qPCR data. No bars represent qPCR results below the detection limit.

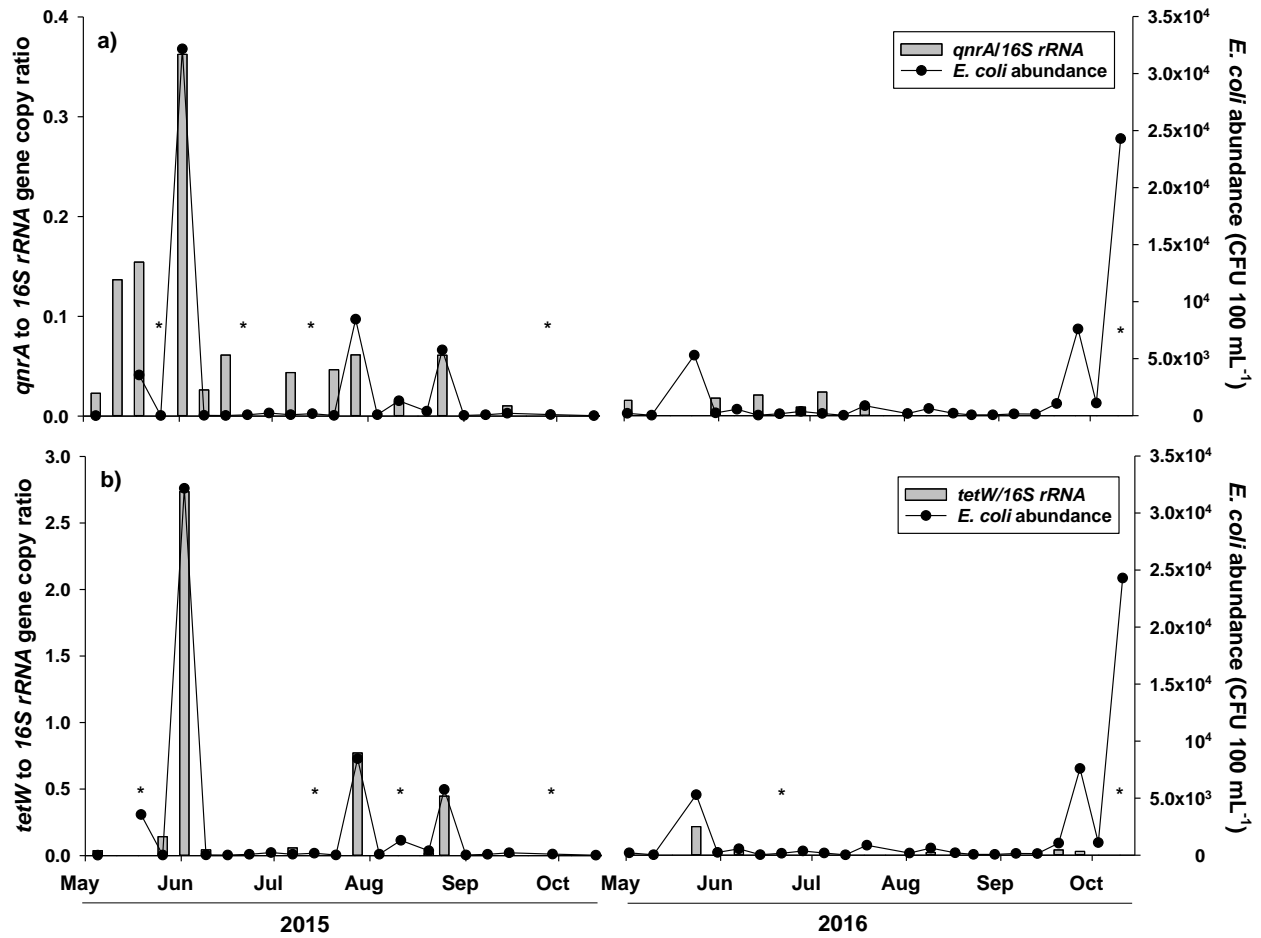


Figure 3. The *qnrA* to 16S rRNA gene copy ratio (a) the *tetW* to 16S rRNA gene copy ratio (b) and *E. coli* abundance at the CSO site during the summer of 2015 and 2016. Each point on the *E. coli* line represents a sampling event and the bar is the corresponding qPCR value. Asterisk represents missing qPCR data. No bars represent qPCR results below the detection limit.

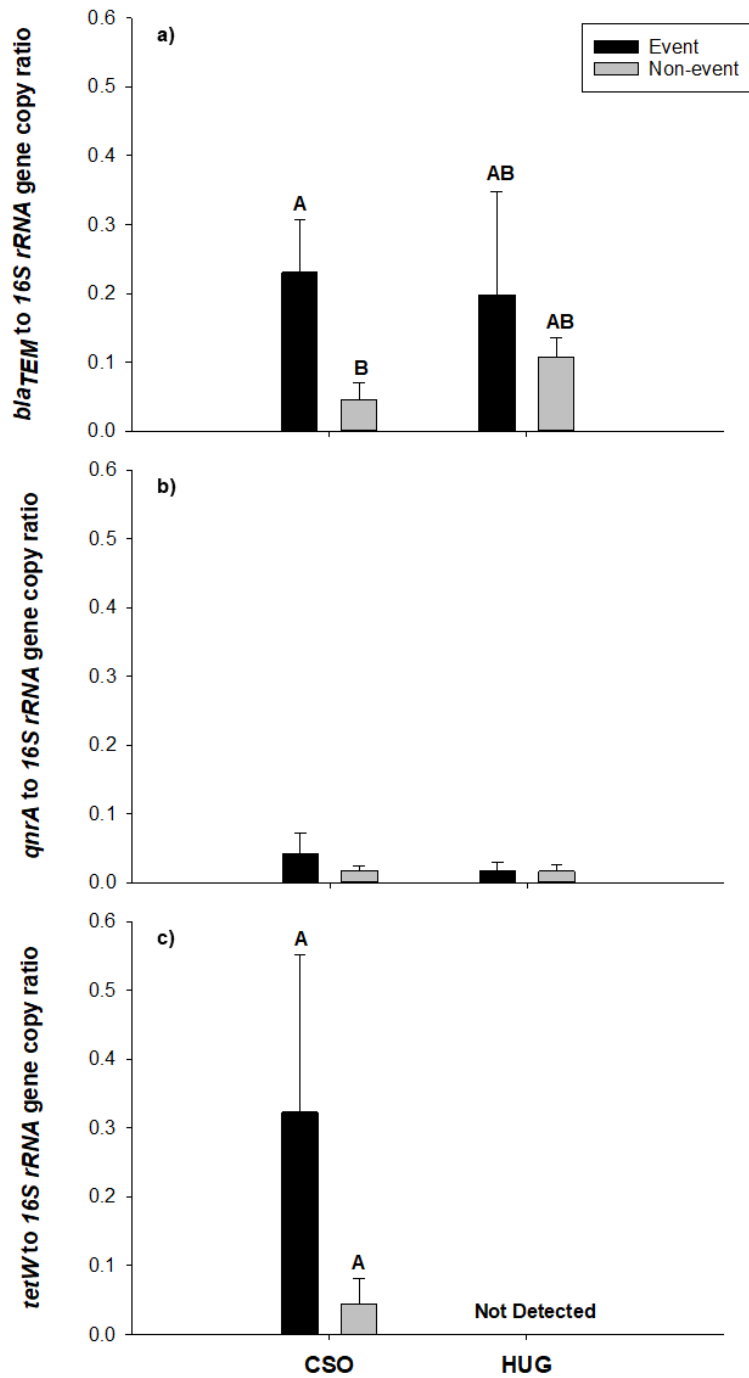


Figure 4. Mean (+SE) gene abundance ratios of *bla_{TEM}* (a), *qnrA* (b), and *tetW* (c) during event and non-event conditions. Within each panel, different letters indicate statistically significant differences ($p < 0.05$) determined using Mann-Whitney test ($n = 12$ per group).

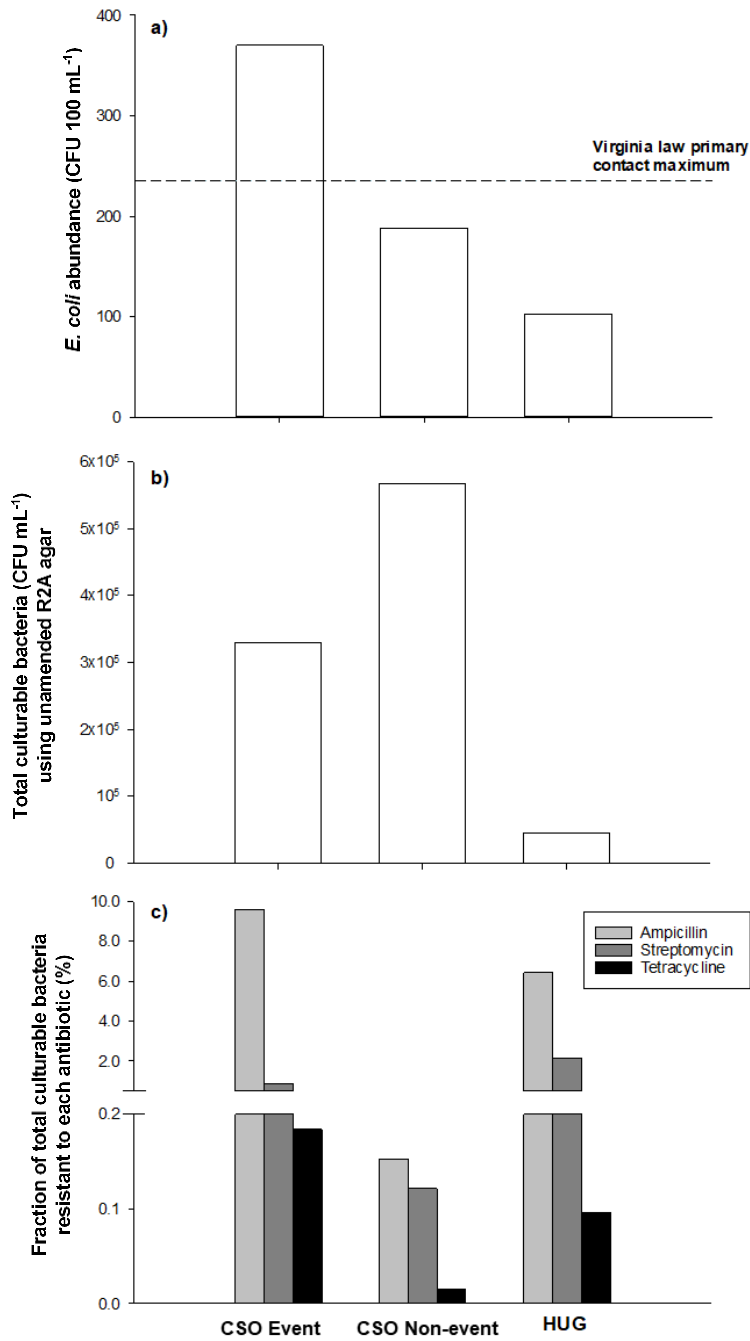


Figure 5. Abundance of *E. coli* (a), abundance of culturable bacteria (b), and the fraction of bacteria resistant to each antibiotic (c) at the CSO site and the HUG site during an event and a non-event.

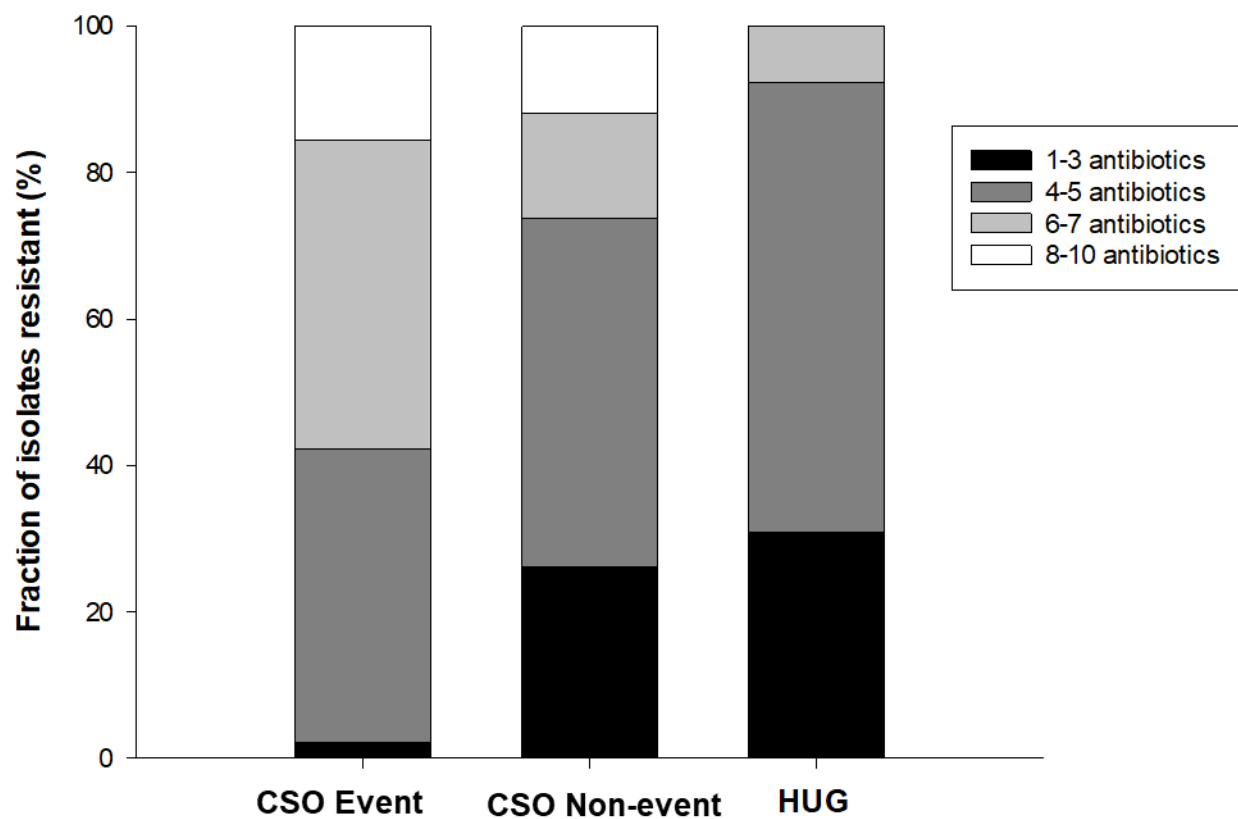


Figure 6. Fraction of isolates resistant to 1-10 antibiotics at the CSO site and the HUG site during an event and a non-event.

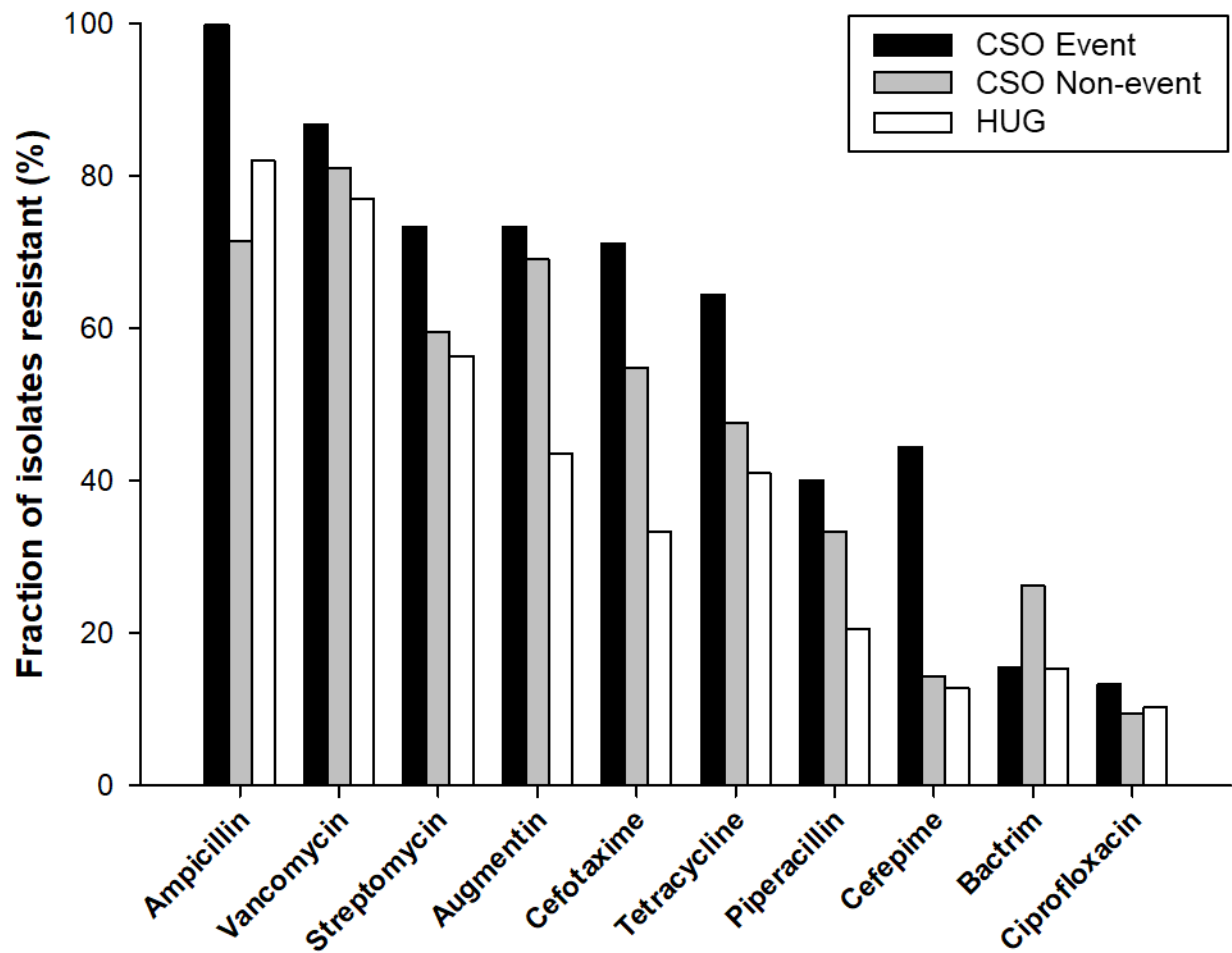


Figure 7. Fraction of isolates resistant to each of the 10 antibiotics from the CSO site and the HUG site during an event and a non-event.

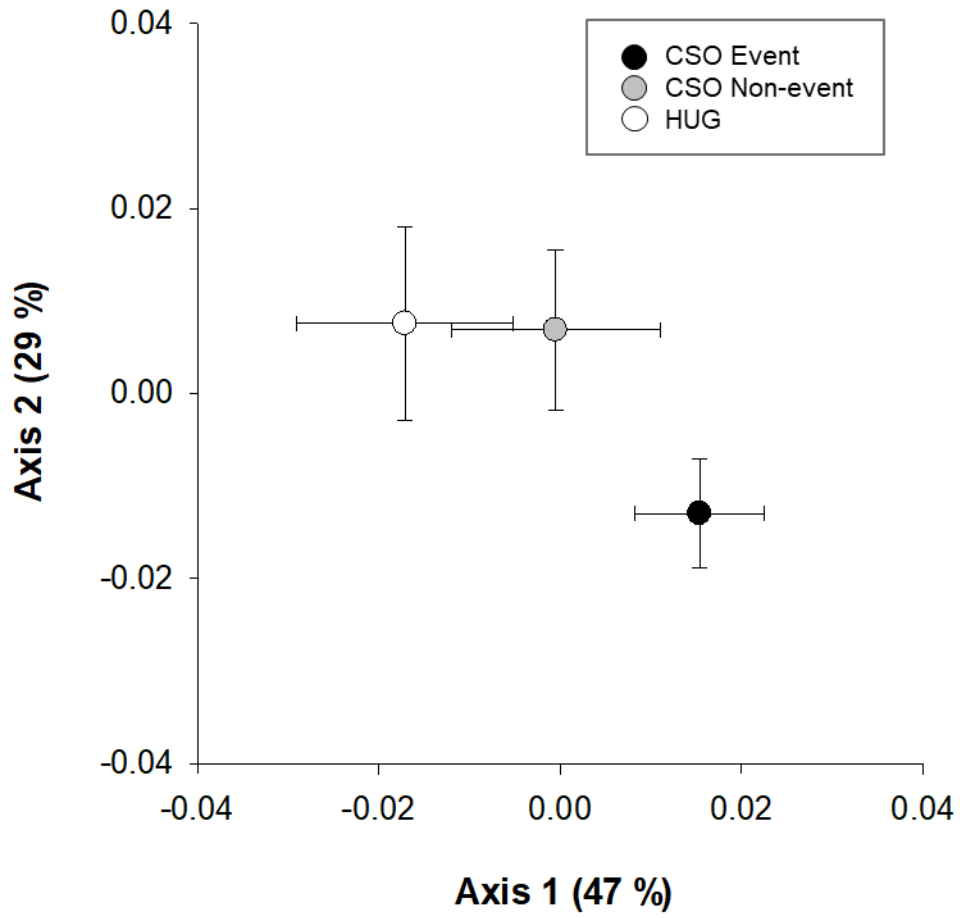


Figure 8. Nonmetric multidimensional scaling (NMDS) ordination plot displaying the overall resistance patterns for the MDR isolates in the three different situations: CSO event, CSO non-event, and HUG (stress = 0.23) (n = 126).

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