Resistance on the rise: Assessment of antibiotic-resistant indicator organisms in Shem Creek, Charleston, South Carolina

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ABSTRACT

Shem Creek is a Charleston waterway well-known for numerous recreational activities such as paddle boarding, fishing, and kayaking. However, Charleston WaterKeepers, a local organization, has consistently found high levels of coliform bacteria within Shem Creek. With antibiotic prescription rates surging and coastal flooding becoming an increasing concern, antibiotic resistant bacteria (ARBs) have become both a public health and environmental risk. ARBs can lead to the spread of antibiotic-resistant infections (ARIs) within populations. Precipitation influences bacterial concentrations in a body of water. Immediately after rainfall, the levels of bacteria rise tremendously. Runoff from livestock, sewage, and hospitals are known to contribute to the development of ARBs in bodies of water. Consistent water testing is essential to preventing the risk and spread of ARIs and determining what additional factors contribute to the development of ARBs in an aquatic environment. This pilot study found that precipitation was directly associated with the levels of bacteria found within Shem Creek and validated that ARBs are present within local waterways in Charleston, South Carolina.

1. Introduction

Antibiotics are defined as substances that are produced by living organisms and can prevent the growth of other organisms when in low concentrations [1]. Recently, antibiotic resistance, the decrease in the effectiveness of a specific antibiotic against bacteria, has become an urgent issue within the field of public health and environmental biology. Resistance genes in environmental bacteria have begun to transfer to human pathogens, contributing to the increased prevalence of antibiotic-resistant bacteria (ARBs). In addition to being a threat to natural ecosystems, ARBs are also a threat to human health via ingested food or water [2]. The Environmental Protection Agency (EPA) states that the two primary pathways of exposure to harmful water pollutants are through direct and accidental ingestion of water and consumption of fish and shellfish from the polluted waterway [3]. This could include drinking water or water used for recreational purposes such as boating, fishing, and swimming. Annual global deaths for antibiotic-resistant infections (ARIs) are predicted to increase from 700,000 in 2014 to approximately 10 million by 2050, costing $100 trillion [4]. High antibiotic use is credited to many factors including over-prescription and ease of accessibility through over-the-counter medication. Many bacterial species began to develop antibiotic resistance abilities preceding human usage for treatment of infectious disease. Additionally, every approved antibiotic class has been met by resistance in at least one of the pathogens they target

[5]. Antibiotic resistance is a result of the transfer of genetic material between humans, animals, and the environment. Polluted environments are more likely to contain the genetic elements involved in the transfer of antibiotic-resistant genes. The addition of fecal bacteria into the environment further enhances the evolution of antibiotic-resistant genes (ARGs) by providing genetic elements that specifically target ARGs [5]. Antibiotics are released into waterways from a variety of sources including human waste, healthcare facilities, and the livestock industry [2,6].

1.1. Antibiotic pollution and fecal coliforms

Antibiotics can be transferred into the environment through the urine and feces of both humans and domestic animals. Investigators have found that coliform bacteria can be found in the feces of warm-blooded animals, the intestines of cold-blooded animals, in sediment, and on the surface of various plants [5]. A variety of intestinal bacteria are known sources of genetic elements that can accelerate the transfer of ARGs to pathogens in the body [6]. Exposure levels from this mode of excretion depend on the proportion of the population using a certain antibiotic at a given time, the dosage of the antibiotic, and the metabolism rate of the humans and domesticated animals that make up that population [6]. Additionally, antibiotics in wastewater are not fully removed by water treatment procedures which contributes to the presence of fecal bacteria in the

environment. Residues of consumed drugs, antibiotics, and stimulants are excreted in urine and feces from households and hospitals and greatly contribute to the antibiotic compounds that are found in wastewater [7]. The main pathway that leads to antibiotic resistance is the fecal-oral route in which people ingest or consume contaminated food or water.

1.2. Primary health care

In 2020, healthcare providers prescribed 201.9 million outpatient antibiotic prescriptions. Primary care physicians were responsible for 64.1 million of those outpatient prescriptions [8]. Specifically, South Carolina has an outpatient prescription rate of 700/1,000 people [8]. Urinary tract infections (UTIs) and upper respiratory tract infections (URTIs) are the top reasons for prescribing antibiotics within primary health care [9]. As a result, these two infections are the most common ARIs in primary healthcare. Although most URTI cases are caused by viral infections that do not require an antibiotic prescription, physicians often prescribe antibiotics to prevent a secondary bacterial infection [10]. Patient pressure towards antibiotic prescription is an additional factor that contributes to the over-prescription of antibiotics in the primary healthcare setting. In a survey that evaluated physician knowledge of antibiotics, their attitudes, expectations, and their prescription practices, 75.4% of respondents reported feeling pressured to provide an antibiotic prescription if their patient expects one [11].

1.3. Anthropogenic sources of antibiotic pollution

Internationally, the livestock industry accounts for over half of all antibiotic usage. In 2013 alone, 131,109 tons of antibiotics were used. This number is projected to increase to more than 200,000 tons in 2030 [4]. Antibiotics are used to prevent the spread of disease and as growth stimulants in livestock and other animals used as sources of food. Twenty-five to seventy-five percent of antibiotics given to animals are excreted into the environment [12]. Antibiotics are consistently detected in the gastrointestinal environments of livestock, increasing the selective pressure for bacteria in livestock digestive systems to acquire antibiotic resistant genes (ARGs) and increase the abundance of resistant populations [4]. Several studies have shown an increase in ARBs after the application of the manure from animals treated with antibiotics is added to farmland as fertilizer [6]. Studies have shown high levels of tetracyclines, fluoroquinolones, macrolides, and sulfonamides in livestock manure [12]. Livestock farms are often located near a water source for their animals. Due to this proximity, animal wastes runoff contaminates surface waters with antibiotic residues and fecal coliforms [7]. An expert workshop with the Food Agricultural Organization of the United Nations (FAO), the World Organization for Animal Health (OIE), and the World Health Organization recognized that ARBs were an international public and animal health concern that has been fueled using antibiotics in a variety of sectors. They emphasized that the types of antibiotics used within livestock were often the same, or very closely related, to those used in human healthcare. There is evidence that human health is threatened by ARBs by increasing the prevalence of infections, increasing severity of infections, and increased treatment failures [13]. Antibiotic resistance presents health risks for farmworkers and communities around the United States in the form of occupational hazards and potential food-borne illness [7]. A study found that 80.5% of feces samples collected from healthy individuals contained ARBs, with 98% of those bacteria being *E. coli.* The feces and intestinal flora of healthy individuals serve as reservoirs for multi-resistant bacteria strains. These bacteria enter the environment along with treated sewage after being treated at wastewater treatments plants [14]. Poor water quality is associated with stormwater runoff of human and animal feces and other bacterial sources into a waterway [14]. Another study found that *E. coli* strains from a sewage plant that treated both municipal sewage and hospital waste had the highest prevalence of ARBs [15].

1.4. Antibiotic significance

The WHO splits antimicrobial classes into three different categories: Critically Important, Highly Important, and Important. Categorization is determined based on three different criteria: Criterion 1: the specific antimicrobial class is the only, or one of the few available treatments for a severe bacterial infection; Criterion 2: the antimicrobial class is used to treat infections caused by bacteria that are from non-human sources or bacteria that may acquire resistance genes from non-human sources. Critically important antimicrobials meet both criteria, highly important antimicrobials meet either Criteria 1 or 2, and important microbes meet neither criterion. Vancomycin, azithromycin, erythromycin, ampicillin, ciprofloxacin, streptomycin, imipenem are categorized as critically important while chloramphenicol, doxycycline, and tetracycline are highly important [13]. Additionally, vancomycin-resistant *Enterococci* are considered a serious threat by the CDC [16]. Ampicillin was used as our control antibiotic because penicillins have historically been the most prescribed antibiotic in the United States. In 2020 alone, 23.2 million antibiotic prescriptions were for penicillins [8].

Antibiotics are organized by prioritization factors. Prioritization factor 1 (P1) signifies that a large portion of the community is affected by diseases that have limited antimicrobial treatment options. Prioritization factor 2 (P2) signifies high usage of that specific antimicrobial class, which favors the development of resistance. Prioritization factor 3 (P3) antibiotics are used to treat infections where resistant bacteria are already known originate from non-human sources. Aminoglycosides (streptomycin) are P2 and P3. Carbapenems (imipenem) and other carbapenems are P1, and P2. Glycopeptides (vancomycin) is P1, P2, and P3. Macrolides and ketolides (azithromycin, erythromycin are P1, P2, and P3. Penicillins (ampicillin is P2. Quinolones and fluoroquinolones (ciprofloxacin) are P1, P2, P3. Amphenicols (chloramphenicol) C2. Tetracyclines (doxycycline, tetracycline) is C1 [13].

The purpose of this study was to determine if confirmed high levels of bacteria have a direct relationship with the presence of ARBs. We hypothesized that due to the high levels of *enterococci* found within Shem Creek by Charleston WaterKeepers, we would find a higher-than-normal number of ARBs as well. The information found through the completion of this experiment is an important contribution to data that shows national and international trends in antibiotic resistance.

1.5. Experiment structure

Little is known about the effect of other factors, such as tidal movement and rainfall levels, on the prevalence of ARBs in recreational waters. This project will directly build upon the data that Charleston Waterkeepers has been collecting since 2009 at one of their sampling locations within Charleston, South Carolina. Specifically, this project focused on the consistent high levels of fecal coliforms found by WaterKeepers at Shem Creek Boat Launch. This project was testing the susceptibility of the bacteria found in Shem Creek to several commonly used or critically important antibiotics. Understanding the influence that tides and rainfall have on ARBs will help develop better methods for predicting high levels of bacteria and ARBs within recreational waters around Charleston. Increasing the awareness of increasing levels of bacteria will aid in preventing the spread of ARIs within the surrounding communities. Additionally, environmental information such as air temperature, water temperature, and water salinity were collected along with water samples. These factors are suspected to also contribute to the transmission of both resistant and non-resistant bacterial strains in an environment [6].

2. Materials and methods

2.1. Sampling

The first portion of this project consisted of water sampling and water filtration for isolation of bacteria. Water was collected from Shem Creek

Boat Landing twice a day for two weeks, once during an incoming tide and once during an outgoing tide. Sampling times were based off the South Carolina's Department of Health and Environmental Control's (SCDHEC) Tide Tables for June 2021. Sampling was conducted at the Shem Creek boat landing during incoming and outgoing tides to determine the total number of fecal coliforms present (see Supplemental Table #1 for exact sampling times). Subsequently, stock cultures were prepared and tested for antibiotic resistance to six commonly used antibiotics using the Mueller-Hinton antibiotic disk diffusion assay method. Along with collection of water samples, environmental factors including water temperature and salinity were measured using a YSIProplus (YSI, Yellow Springs, Ohio). The height of the tides and the air temperature for Cooper River Entrance were recorded at time of sampling using data from [17]. Two water samples were collected in sterile collection tubes, placed directly onto ice, and transported back to the lab within thirty minutes of collection. Samples were collected as far down the dock as possible and submerged until the entire collection tube was filled. Once in the lab, vacuum filtration was used to filter the water samples. Sample water was filtered in volumes of 1mL, 2mL, 5mL, or 10mL to calculate the total fecal coliforms on MacConkey agar. The 5mL and 10mL samples were consistently too many to count (TMTC), while 1mL and 2mL were within the countable range of 20-80 colonies. Agar plates with filters were incubated for 18-24 hours at 37°C. After the incubation period, the number of bacterial colonies was counted and recorded for each volume. Total coliforms per 100mL of water was then calculated using the formula [(# of colonies x 100)/(sample volume)].

2.2. Bacterial colony isolation

Medium-colored, dark pink, dark purple, and metallic green colonies were subcultured on EMB plates, while colonies that were lighter than the medium, yellow, tan, or translucent were subcultured on hektown enteric (HE) agar plates for 18-24 hours at 37°C. Approximately five different-colored colonies were selected for isolation per water sample. In total, two-hundred and twelve total colonies were isolated on either EMB or HE plates. Approximately five different-colored colonies were selected for isolation per water sample. EMB agar is formulated for the isolation of fecal coliforms due to two dyes: Eoin Y and methylene blue. These dyes are inhibitory for gram-positive organisms and indicate a low pH when lactose is fermented [18]. HE agar is formulated to differentiate *Salmonella* and *Shigella* organisms from other gram-negative rods. Gram-positive organisms are inhibited by bile salts, bromthymol blue, and acid fuchsin [19]. Ferric ammonium citrate causes a black precipitate to form when hydrogen sulfide is produced [19]. *Escherichia coli* (*E. coli*) can be differentiated from other lactose fermenters based on its metallic-green sheen when grown on EMB [18]. Therefore, each different colored colony suggested different levels of lactose fermentation and a different species.

2.3. Subculturing and antibiotic susceptibility testing

One-hundred and thirteen colonies were isolated onto EMB agar and ninety-nine colonies were isolated onto HE agar. Thirty-four (24.3%) HE samples and forty-two (37.2%) EMB samples grew on their designated plates and were tested for antibiotic resistance using methodology based off the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol described by [20]. Mueller-Hinton plates were prepared using dehydrated media according to manufacturer's instructions. A sterile cotton swab was immersed in a bacterial colony resuspension set at a standard density for inoculation of the MH agar plates. The antibiotic discs were placed on each labeled MH plate using a Self-Tamping Sensi-Disc Dispenser.

The six antibiotics used to test susceptibility in this experiment were doxycycline, imipenem, ampicillin, azithromycin, ciprofloxacin, and vancomycin. These antibiotics were chosen based on factors such as high prescription levels, antibiotic class, and threat severity of antibiotic-resistant organisms based on data from the Center for Disease Control. Azithromycin and doxycycline are included in the top five prescribed antibiotics in the

United States according to the CDC [16]. If an organism was resistant or intermediately resistant to three or more of these antibiotics, then they were also tested, using the same protocol, for resistance against streptomycin, tetracycline, and chloramphenicol. Resistance was determined by measuring the diameter of the zones of inhibition (ZI) to the nearest millimeter. National Committee for Clinical Laboratory Standards (NCCLS) guidelines were used to determine the resistance of the organisms to vancomycin, azithromycin, and doxycycline. Ampicillin, tetracycline, ciprofloxacin, streptomycin, and chloramphenicol susceptibility was measured using methodology from published studies [34–36]. Zone of Inhibition values can be seen in Supplemental Table #2. Organisms were determined to be either resistant (R), intermediately resistant (IR), or susceptible (S) to the antibiotics. If an organism was resistant or intermediately resistant to three or more antibiotics, it was considered a multi-resistant bacterium (MRB) and sent off to be sequenced and identified. Supplemental Table #2 shows the ZIs for each antibiotic.

2.4. Isolate identification

Isolate sequences were inputted in MEGA11 to align the sequences and cut them down to a maximum of 750 nucleotides. Once the sequences were shortened and aligned, they were entered into the U.S. National Library of Medicine's National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) for identification [21]. The two NCBI nearest species are listed in Supplemental Table #3. Isolates were classified by Silva High Quality Ribosomal RNA Databases to determine the family of the bacteria [22].

2.5. Statistical analysis

Statistical analysis of study data was performed using SPSS software. Correlations were assessed to determine the relationship between average colony-forming units (CFUs) and precipitation. Three correlations were performed: Kendall's tau, Spearman's Rho, and Pearson correlations. A Microsoft Excel spreadsheet was created to include average CFUs, precipitation levels, tidal movements for each sampling time. The average CFUs and precipitation levels from this document were entered into SPSS and each correlation was conducted separately using bivariate correlation settings.

A Pearson correlation measure the relationship between two different variables and ranges between -1.00 and +1.00. For a Pearson correlation to be valid, the data must be normally distributed. Once normal distribution has been confirmed, data analysis can continue. Experimental data was confirmed to fit a normal distribution by using the Kolmogorov-Smirnov (KS) test of Normality via Social Science Statistic's webpage [23]. Two-tailed was selected if the relationship between the two variables was unknown and one-tail was selected if the relationship between the two variables was predicted to be either positive or negative [24]. A value of 0.00 means that there is no correlation between the two variables. A value closer to -1.00 signifies an inverse relationship and a value closer to +1.00 signifies a direct relationship between the two variables. Significance level values were calculated simultaneously. A significance value below 0.05 is considered statistically significant [25].

3. Results

3.1. Colony-forming units

If every volume for a specific sample was TMTC, a maximum value of 12,000 was given as the average CFUs for that sample time. The highest average CFUs for the incoming samples was 12,000 and the lowest was 1,060. For the outgoing samples, the highest average CFU count was 12,000 and the lowest was 536.67. On the fifteenth day, there was an average CFUs value of 12,000, the highest out of all the incoming samples. For the outgoing samples, the fifteenth day had the second highest average CFUs value (5366.67) compared to Day 3 which was 12,000.

3.2. Precipitation

Throughout the two-week sampling period, there was a total of 4.02 inches of precipitation. The highest levels of precipitation occurred on the fifteenth day of sampling (1.67 inches), and there were six days that experienced no rainfall (0 inches). Precipitation was most directly related to average CFUs when there was a lag of 0 days. This means that the levels of bacteria within the samples was most influenced by the rain that had occurred within the same day of sample collection. Fig. 1 shows the correlation between the number of average CFUs and the level of precipitation in inches. According to the lines of best fit, there is a positive correlation between rainfall and average CFUs during incoming tides, but there is a negative correlation between rainfall and average CFUs during outgoing tides. The highest level of rain experienced was on Day #13. Precipitation levels were determined using NOAA's Climate Data Online Search [26]. The relationship between the average CFUs for both sampling times of each day and the daily precipitation is shown by Fig. 2. Additionally, Supplemental Table #1 summarizes the precipitation and CFU data for the sampling period.

3.3. Antibiotic susceptibility and resistance

Of seventy-six isolates tested for antibiotic resistance, two isolates (2.63%) were resistant to zero antibiotics, twenty isolates (26.3%) were resistant to one antibiotic, and thirty-two (42.1%) isolates were resistant to two antibiotics. One isolate (1.32%) was found to be R/IR to six antibiotics, three isolates (3.95%) were R/IR to five antibiotics, nine isolates (11.8%) were R/IR to four antibiotics, and seven isolates (9.21%) were R/IR to three antibiotics. All isolates were susceptible to CIP 5. All the isolates were also susceptible to IPM 10 except for three isolates. Approximately 28.9% of tested isolates were IR/R to AZM 15 and 3.95% to D 30. At the conclusion of the testing, twenty MRBs were identified. MRBs are defined as a sample that is intermediately resistant (IR) and/or resistant (R) to three or more antibiotics and show significant threat to public health. Two of the samples were found to be duplicate isolates, resulting in only nineteen samples being tested with additional antibiotics. Of the nineteen isolates that were tested with the three additional antibiotics due to their multi-resistance properties, approximately 63.2% were IR/R to C30, 10.5% to S10, and 0% to TE 30.

3.4. Species identification

Supplemental Table #3 presents the sequence number, family, NCBI two nearest identifications, and the specific antibiotic susceptibility of each of the sequenced samples. Fourteen of the twenty samples (70.0%) were a part of the *Pseudomonadaceae* family, four (20.0%) belonged to the *Enterobacteriaceae* family, one (5.00%) belonged to the *Xanthomonadaceae* family, and two (10.0%) isolates were labeled as Unclassified by Silva. Therefore, it is concluded that *Pseudomonas* species showed the highest rate of resistance to the selected antibiotics. *Pseudomonas protegens* strain CHA0 was the only sample that was IR/R to six different antibiotics. Twenty-five percent of the *Pseudomonas* isolates were IR/R to three antibiotics, 50.0% were resistant to four antibiotics, and 16.7% were resistant to five antibiotics.

Of the nineteen multi-resistant isolates, there were three samples that we IR/R to five antibiotics: *Pseudomonas protegens* strain CHA0, *Stenotrophomonas maltophilia* strain IAM, and *Pseudomonas aeruginosa* strain DSM 50071. The remaining samples were IR/R to four or less antibiotics and can be read about in detail in Supplemental Table #3.

3.5. Association between precipitation and CFU concentration

Within two of the three correlations calculated using SPSS, statistically significant values were calculated. The statistical correlation examined were Pearson's correlation due to the normal distribution of the data. As seen in Table 1, the Pearson correlation coefficient between average CFUs and precipitation was 0.449, which was indicated as significant by SPSS. The significance level calculated by SPSS was $p = 0.008$ between the two variables.

4. Discussion

Fecal coliform levels have been measured within Charleston, South Carolina throughout the last decade; however, antibiotic resistance has not been a focal point. This data sets forth a procedure that can be used to continue testing Shem Creek and other popular recreational waters for the presence of ARBs. There is little data for ARB levels within any waterways in Charleston and this data can serve as the basis of new programs that work to monitor the presence and development of dangerous ARBs. A British study examined fecal swabs from surfers who were considered more likely to ingest seawater than non-surfers and were found to be more at risk of carrying cephalosporin-resistant *E. coli*. Another study found that recreational swimming may be a risk factor for UTIs and other bacterial infections [6]. These results emphasize the needs for extensive testing for ARBs and fecal coliforms of both fresh-water environments as well as other recreational bodies of water, such as beaches, that may experience runoff from the surrounding environment. In a recent study, ciprofloxacin was found in sewage sludge at a concentration of one milligram per kilogram. However,
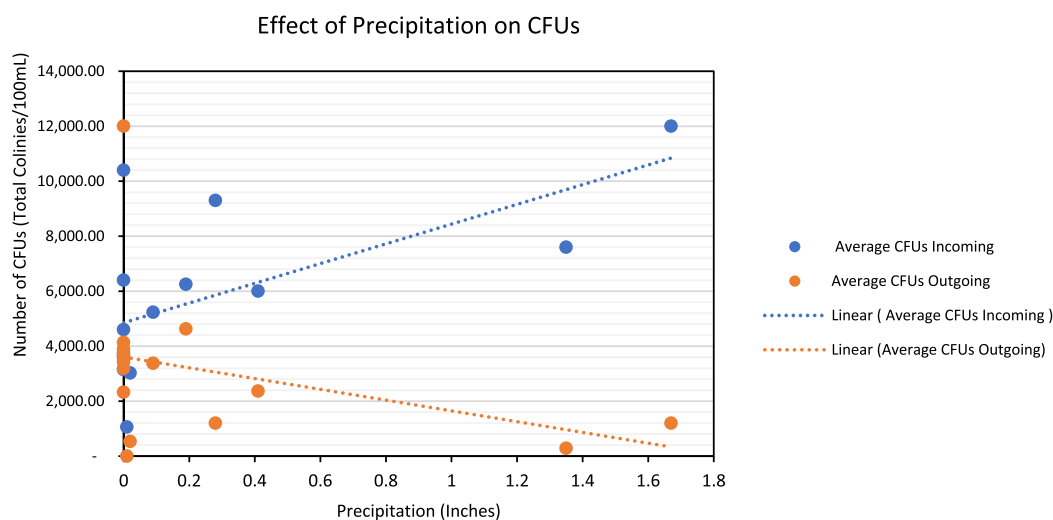

Fig. 1. Linear correlation of precipitation and average CFUs with line of best fit
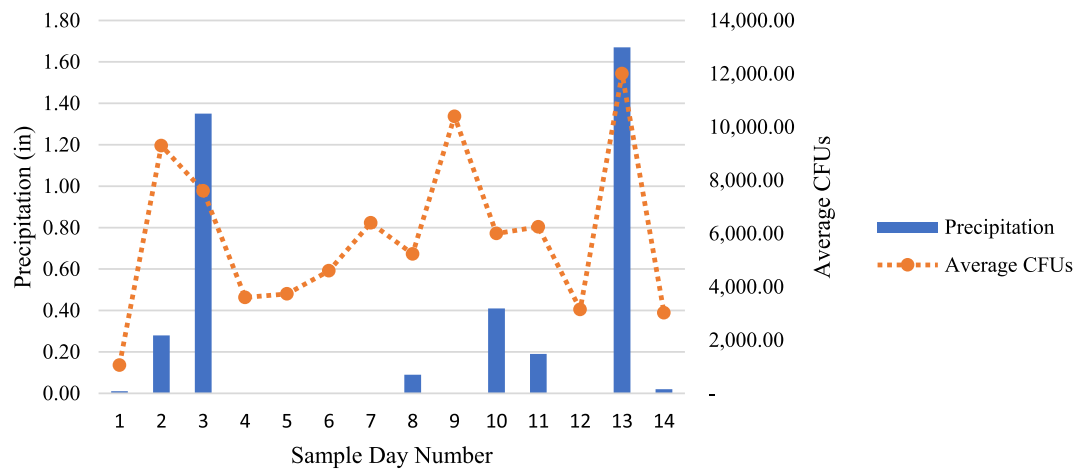
Average Daily CFUs vs. Daily Precipitation

Fig. 2. Average daily CFUs relating to daily precipitation

Table 1
SPSS statistical analysis.

Correlations			
		Precipitation	CFUs
Precipitation	Pearson Correlation	1	0.449**
	Sig. (1-tailed)		0.008
	N	28	28
CFUs	Pearson Correlation	0.449**	1
	Sig. (1-tailed)	0.008	
	N	28	28

* Significant correlation.

ciprofloxacin-sensitive strains remained common [6]. This remained true for the samples collected from Shem Creek as every sample tested showed susceptibility to ciprofloxacin.

4.1. Public health and environmental significance of ARBs

The following bacteria were isolated from the samples and are known to have an impact on the environment and/or public health: *Pseudomonas putida* strain NBRC 14164, *Stenotrophomonas maltophilia* strain IAM 12423, *Yokenella regensburgei* strain CIP 105435, *Klebsiella oxytoca* strain JCM 1665, and *Enterobacter bugandensis* strain 247BMC. The genus *Pseudomonas* is one of the most diverse and significant groups of bacteria. Specifically, the bacteria in this genus can withstand various forms of stress including physical, chemical, and antibacterial substances [13]. *Pseudomonas* species have been prioritized by the WHO as bacteria that requires the development of new, effective drugs [16]. *Pseudomonas* species are urgent public health threats for four reasons: the produce a variety of beta-lactamases that promote resistance to certain antibiotics; they act as pathogens that infect those who are immunocompromised; and they are associated with sepsis, wound infections, UTIs, and some forms of pneumonia [13] The overexpression of efflux pump systems contributes to the development of resistance to a wide range of drugs in *Pseudomonas* species [27]. Supplemental Table #3 presents the sequence number, family, NCBI nearest identification, and antibiotic susceptibility of each of the sequenced samples. Outbreaks of *Enterobacter* and *Klebsiella* infections in a healthcare setting may be caused by a variety of things including contaminations of medications, intravenous fluids, and medical equipment [28]. Fourteen percent of inpatient bloodstream infections (BSIs) were caused by gram-negative bacteria according to the National Nosocomial Infections Surveillance System from 1992 to 1999. Outbreaks of *Enterobacter* and *Klebsiella*

infections in a healthcare setting may be caused by a variety of things including contaminations of medications, intravenous fluids, and medical equipment. All but one of sample was a gram-negative organism. Additionally, gram-negative organisms are known to be a cause of catheter-related BSIs associated with contaminated fluids [28].

4.1.1. Pseudomonas putida

Pseudomonas putida has been found to serve as an exchange platform for antibiotic-resistant genes (ARGs) and is considered a serious threat for antibiotics resistant infections in hospital settings. This species of bacteria harbors metallo-beta-lactamase genes that can increase the likelihood of resistance to beta-lactams such as carbapenems. This specific class of drugs are considered "last-resort" drugs for critically ill patients. In a hospital setting, *P. putida* often originates from contaminated fluid and hospital wastewater systems [29].

4.1.2. Stenotrophomonas maltophilia

Stenotrophomonas maltophilia is a known cause of hospital-acquired infections that occurs mostly in immunocompromised individuals. *S. maltophilia* is considered an environmental bacterium, but its sources and reservoirs are not always naturally available [30]. Transmission from person-to-person is infrequent outside of the healthcare setting. Therefore, hospital environments often act as the reservoir for the development and growth of this specific bacteria. The majority of *S. maltophilia* strains are characterized by their resistance to antibiotics in the carbapenem class which is supported by the data shown above. Isolate 9 showed resistance to both carbapenems tested in this procedure (IPM 10, VA 5). S. maltophilia is often recovered from wet environments such as sink traps, bottled water, rivers, and wells. It has also been found in a variety of foods and animals such as frogs, lizards, raw cow's milk [30]. This supports the prediction that runoff from both livestock and hospital environments may influence the development of ARBs in Shem Creek.

4.1.3. Yokenella regensburgei

Isolate 18 was identified as *Yokenella regensburgei*. *Y. regensburgei* is the sole species of *Yokenella* genus. It has previously been isolated from the upper respiratory tract, wounds, knee fluid, urine, sputum, and feces [31]. Considering that *Y. regensburgei* is a known fecal coliform, it is possible that this bacteria was transferred into Shem Creek due to leakage in sewage systems or runoff from flooding and precipitation. It is also found in intestinal tracts of insects and insect-feeding animals such as bats which could also explain its presence in the sampling waters. Infections with *Y. regenburgei* are rare, but are possible and unpredictable. A study testing the susceptibility patterns of *Y. regensburgei* strains found that all strains

were susceptible to various beta-lactams, all aminoglycosides (including streptomycin), chloramphenicol, quinolones (ciprofloxacin), and tetracyclines (tetracycline, doxycycline). Strains were found to be either resistant or intermediately resistant to erythromycin and azithromycin [31]. However, isolate 18 only showed resistance to VA 5, AZM 15, and AM 10. This may be because *Y. regensburgei* was the second species listed on NCBI BLAST [21].

4.1.4. Klebsiella oxytoca

Isolate 4 was identified as *Klebsiella oxytoca*. *K. oxytoca* is a known cause of community and hospital-acquired infections that resemble those caused by *Klebsiella pneumonia*. The most common regions of the body that *K. oxytoca* are found are the gastrointestinal and respiratory tract. Resistant *K. oxytoca* outbreaks have been seen largely in hospital environments. Environmental reservoirs have been found to play a role in infection with *K. oxytoca* [32].

4.1.5. Enterobacter bugandensis

Isolate 17 and 18 both identified as *Enterobacter bugandensis*. *E. bugandensis* is known to be associated with neonate sepsis and dangerous bloodstream infections. Other *enterobacter* infections can include bacteremia, lower respiratory tract infections, skin and soft-tissue infections, UTIs, septic arthritis, osteomyelitis, and more. This species can survive on skin, on dry surfaces, and in contaminated fluids, making it common in a hospital setting. *E. bugandensis* is known to show resistance to ampicillin, amoxicillin/clavulanic acid, ciprofloxacin, tetracycline, and various other antibiotics [33]. In this experiment, Isolate 17 and 18 showed resistance to VA 5, AZM 15, and AM 10.

4.2. Statistical correlation of average CFUs and precipitation

A Pearson correlation was utilized due to the normality of the data. Normal distribution fit was determined using the KS test of Normality via Social Science Statistic's webpage [23]. The correlation coefficient between average CFUs and precipitation for the Pearson correlation was 0.499, which was marked as significant by SPSS. Since this value approaches +1.00, a positive relationship can be concluded between the two variables and a direct relationship can be confirmed between average CFUs and precipitation. The significance value for the Pearson correlation was 0.008 as seen in Table #1.

4.3. Limitations

This study only determined ARB levels at a single location. A more accurate understanding of the causes and influential factors of the development of ARBs in waterways could be gathered through the testing of multiple sites. The prevalence of specific antibiotics in the water at the sampling location was not determined which also limits our understanding as to why certain antibiotics showed more or less resistance compared to others tested. The subculturing methods used resulted in only a specific type of resistant bacteria. By utilizing a more inclusive subculturing method, it would be possible to determine the different types of ARBs present in that specific waterway. On the other hand, by utilizing a more specific subculturing method, a more accurate conclusion could be made relating to the resistance of fecal coliforms in that waterway. According to the South Carolina Department of Health and Environmental Control (SCDHEC), *E. coli* and *Enterococcus* bacteria are a better indicator of pathogenic bacteria compared to fecal coliforms. Specifically, *E. coli* is suggested for use in fresh waters and *Enterococci* for marine water [31]. Therefore, a more *E. coli*-specific subculturing method would be more accurate in determining the risk of infection from pathogenic bacteria in a fresh water recreational body of water. In relation to statistical analysis, statistically significant results are more likely when the sample size is larger. Therefore, a larger sample size may also increase the strength of the relationships between environmental factors, such as precipitation, and the levels of bacteria within waterways.

5. Conclusion

The prevalence of ARBs in Shem Creek suggest that high levels of fecal coliforms are associated with an increased presence of ARBs. It can be concluded that areas with high antibiotic usage rates may have high prevalence of ARBs in their waterways due to runoff from a variety of sources such as livestock facilities, hospitals, and sewage treatment plants. It can also be concluded that precipitation has a direct relationship with the number of average CFUs due to the positive relationship between average CFUs and precipitation. As precipitation increases, the amount of runoff that enters waterways also increases. In frequently-flooding areas, increased precipitation may lead to sewer overflow and an influx of sewage and waste into local waterways. It is important to increase the frequency of water testing in frequently used and recreational waterways so that community leaders can develop strategies to prevent the spread of antibiotic-resistant bacterial infections within their populations. These findings will allow community members to become increasingly aware of the health risks of ARBs and emphasize the negative effects of over-prescribing antibiotics on both the environment and public health. Specifically for Charleston, it is important to monitor the antibiotic prescription rate as well as local precipitation levels. As of 2020, South Carolina had the 9th highest antibiotic prescription rate in the United States at 700 prescriptions per 1000 persons [8]. According to the results of this study, South Carolina's high prescription rates, and Charleston's susceptibility to flooding, Charleston waterways are at risk for increased levels of ARBs due to antibiotic runoff from local hospitals, residential and commercial sewage, and farmland. The methodology of this project has never been utilized in Charleston before the completion of this study and can be used to enhance the efforts to decrease the levels of antibiotic runoff into the environment and raise awareness to the health risks of ARBs and ARIs. If more funds were available for use, this study would be expanded to other waterways around Charleston to more accurately determine the effect that geographical location and environmental surroundings have on the levels of ARBs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dialog.2022.100063.

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