Presence of Antibiotic Resistant Bacteria and Antibiotic Resistance Genes in the Coastal Waters of Southeast Louisiana

Final Report Submitted to LA Sea Grant

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ABSTRACT

In the past few decades, the medical community has faced a rising problem in the spread of antibiotic-resistant bacteria (ARB) and the difficulty of treating related infections. The presence of these bacteria in high-traffic bodies of water, as well as the presence of the antibiotic resistance genes (ARG) floating freely in the water, pose the threat of antibiotic-resistant infections in individuals living and recreating in these areas of southeast Louisiana. Water samples from Chauvin, Louisiana and Port Fourchon, Louisiana were analyzed using chemical, microbial, and molecular methods to determine the presence of ARBs and ARGs. The main species analyzed include *E. coli*, *Klebsiella* spp., and *Enterobacter* spp., but a few other species were also isolated and analyzed. They were tested for resistance to carbapenem, monobactam, penicillin, sulfonamide, and cephalosporin antibiotics. Monthly samples were taken in triplicate for a 6-month testing period and tested for water quality standards including salinity, temperature; phosphate, nitrate, and ammonia concentration; dissolved oxygen, and total and fecal coliforms. Bacteria were isolated, identified using biochemical assays, and tested for antibiotic resistance using Kirby-Bauer assays. DNA isolation, polymerase chain reaction (PCR) and gel electrophoresis were used to identify ARGs. Significant numbers of ARBs were consistently found at both sites, and ARGs were found throughout testing. These numbers, as well as the chemical and coliform data, show that these high-traffic recreational bodies of water may be putting wildlife and humans at risk for antibiotic-resistant infections.

1. Introduction

In the modern healthcare industry, antibiotic resistant bacteria (ARB) infections have become one of the most insidious epidemics seen throughout the world. The need for solutions to the growing antibiotic resistance problem has been the basis of a large number of recent healthcare research projects, according to Allen (2017). The CDC (2017) estimates that each year, at least 2,000,000 people in the United States contract ARB infections, and at least 23,000 of those cases become directly fatal. This statistic also does not take into account the patients who were indirectly killed by the effects of these infections or never had their infection diagnosed. The methods necessary to treat these infections can be very costly and even harmful to the patient, according to the CDC (2017).

The spread of resistance is known to come about by the simple use of antibiotics, a practice that has dominated the medical field for the past 70 years since the development of penicillin. Up to 50% of antibiotic prescriptions are misuses that involve misdiagnosis or overdosing, according to the CDC (2017). The CDC also states that antibiotics given either to humans or to livestock kill normal flora indiscriminately, allowing for the remaining resistant bacteria to prosper in a wealth of resources. Both human contact and poorly handled food allow for the spread of these ARBs to new hosts that previously did not have the resistant bacteria. Antibiotic resistance genes (ARGs) are a part of the genetic information of ARBs, and they can be released into the environment and allow susceptible bacteria to integrate the ARGs into their genome and express antibiotic resistance.

In southern Louisiana, the bayous, waterways, and marshlands provide the location for many popular recreational activities. Hunting, fishing, boating, swimming, and other water-

based activities play a huge part in the culture of the area and have done so for centuries before. According to the Louisiana Department of Wildlife and Fisheries (LDWF) researchers Isaacs and Lavergne (2010), there are at least 316,593 active motorboat registrations in Louisiana, which roughly equates to one motorboat per every six Louisiana homes. This statistic still does not include boats that do not have motors or may be registered exclusively by the United States Coast Guard. It also goes without saying that Louisiana's industry relies heavily on seafood and other water-based activities. The LDWF (2013) also states that there were 71,631 total active commercial licenses as of 2012.

It also should also be noted that in homes further down the bayous toward the Gulf of Mexico, there tends to be no state-run sewage operation; instead, homes typically have outdated septic tanks that are rarely maintained and dump directly into the bayou water. As shown by Zhang et al. (2009), many antibiotics when taken are not fully metabolized in the body and can be excreted into the environment. These antibiotics in waste water can lead to either a proliferation of ARBs in waste treatment plants in the case of state-run sewage – as shown by Naquin et al. (2017) – or in the bayou water itself in the case of outdated septic tanks.

The sites in this experiment were chosen specifically for their location downstream from insufficient waste disposal and their high human activity. Site 1, the Louisiana Universities Marine Consortium site in Chauvin, LA, is down Bayou Terrebonne and close to an outlet that drains into marshlands. It is surrounded by docks with plenty of daily watercraft activity. Site 2 in Port Fourchon, LA, is near the mouth of Bayou Lafourche at the Gulf of Mexico. The area is known for water-based commerce, fishing charters, and recreational activities.

Chemical data was analyzed to verify that the environments of the water at the two sites were able to support microbial growth. Coliform data was collected to verify that the bacterial population was large enough to facilitate the spread of antibiotic resistance. Bacterial species were identified using biochemical assays. Kirby-Bauer assays were used to indicate the resistances of isolates after they were identified. Polymerase chain reaction (PCR) and gel electrophoresis were used to identify the presence of ARGs. The antibiotics tested for resistance were either commonly used in healthcare or closely related to drugs used in healthcare. The genes tested for are commonly tested genes for resistance to tetracycline and methicillin.

2. Materials and Methods

2.1 Collection of Samples

Water samples were collected from two coastal sites: the Louisiana Universities Marine Consortium (LUMCON) site in Chauvin, Louisiana (Site 1) and from the side of Old Highway 1 in Port Fourchon, Louisiana (Site 2). Satellite images of the sampling sites are shown in Figure 1 with corresponding GPS coordinates. Monthly samples were collected for six months from April to September of 2017. Samples were collected in sterile collection bottles in triplicate from both sites and transported back to Nicholls State University on ice. Samples were labeled as being from either Site 1 or Site 2 (S1, S2) and being Trial 1, 2, or 3 for that site (T1,T2,T3) Samples were stored at 4°C for the duration of that month's analysis.

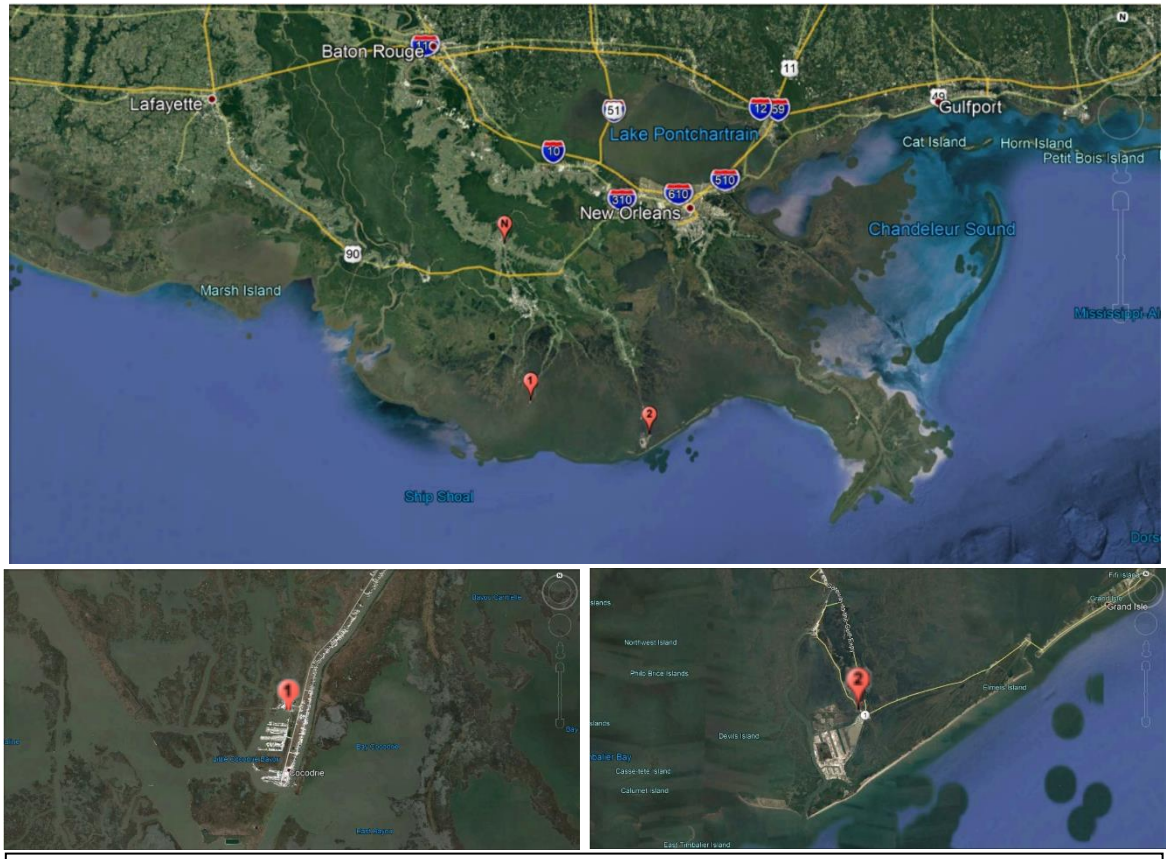

Figure 1. Satellite imaging of sampling sites.
(1) Site 1 in Chauvin, LA (29°15'13.69"N, 90°39'40.82"W).
(2) Site 2 in Port Fourchon, LA (29° 9'41.25"N, 90°10'58.35"W).
(N) Nicholls State University in Thibodaux, LA (29°47'22.26"N, 90°48'13.45"W)

2.2 Chemical Analysis

Water temperature and salinity were measured using a YSI 63 pH, salinity, conductivity, and temperature probe at the site when samples were collected. Samples were returned to the lab and first analyzed for chemical composition. Dissolved oxygen was measured using the YSI 5100 dissolved oxygen meter. Phosphate, nitrate, and ammonia concentrations were measured using the standard methods described by the APHA (1998) and the HACH DR 6000 spectrophotometer.

2.3 Bacterial Quantification, Isolation, and Identification

An estimated quantification of both fecal and total coliforms in each sample was measured using the fecal coliform and total coliform most probable number (MPN) method described by Everage et al. (2014), using 1:1 dilutions in phosphate-buffered saline (PBS) and culturing of varying sample-PBS solution volumes in A1 broth with inverted Durham tubes. Fecal coliforms were incubated at 44°C for 24 hours, and total coliforms were incubated at 37°C for 48 hours. MPN estimates were averaged out of the three trials per site. At least one pure culture isolate was collected from each fecal and total coliform set. Isolates were identified via biochemical assays as described by Everage et al. (2014), including eosin methylene blue (EMB) agar plates, triple sugar iron agar (TSIA) slants, and sulfide-indole-motility medium (SIM) deeps. Specific methods used to identify *Enterobacter* spp. are described by Delost (2014).

2.4 Antibiotic Resistance Assay

Antibiotic resistance was measured via the Kirby-Bauer method described by Brown (2005) and Delost (2014). After identification, each pure culture isolate was cultured in tryptic soy broth (TSB) and diluted to standard turbidity between 0.08 and 0.10 absorbance. Using sterile cotton swabs, a bacterial lawn of the diluted culture was streaked onto Mueller-Hinton (MH) agar plates as described by Everage et al. (2014). BD Sensi-Disc antibiotic discs containing aztreonam 30 mcg (ATM-30), ceftazidime/clavulanic acid 30/10 mcg (CAZ-CLA), imipenem 10 mcg (IPM-10), meropenem 10 mcg (MEM-10), piperacillin 100 mcg (PIP-100), and sulfamethizole 0.25 mg (TH-.25) were aseptically placed on the lawn using sterile forceps. Plates were incubated at 37°C for 24 hours, and zones of inhibition were then measured using standard laboratory calipers. The BD Sensi-Disc zone size chart was used to determine whether the size of each zone of inhibition indicated that the species was sensitive (S), intermediate (I), or resistant (R) to each specific antibiotic as described by Delost (2014).

2.5 DNA Extraction and Gel Electrophoresis

1 ml of sample was cultured in TSB at 37°C for 24 hours. 1.5 ml of this culture was transferred to a microcentrifuge tube, centrifuged at 4000rpm for 15 minutes, and decanted. The pellet was used for DNA extraction. The Fast ID DNA Extraction Kit was used to isolate DNA from the pellet according to the manufacturer's instructions included in the kit. Polymerase chain reaction (PCR) was used to amplify the extracted data according to the methods described by Naquin et al. (2014; 2015) and Bergeron et al. (2015). Primers for resistance to tetracycline (*tetA*, *tetW*, *tetX*) as described by Burch et al. (2013) and methicillin (*mecA*) as described by Suzuki et al. (1992) were used to identify antibiotic resistance genes

(ARG) in the bacteria and the water itself. Sigma-Aldrich Co. (St. Louis, MO) produced all primers used (Table 1). Positive controls were made for each gene using known positive sewage treatment plant water samples, and negative controls were made using sterile DI water in place of environmental samples. 10 µl of each PCR sample was mixed with 2 µl of loading dye and loaded into a 2% agarose gel made with 5 µl of ethidium bromide for visualization. Gels were run for at 100 V for 1 hour and visualized via the Alpha Innotech FluorChem FC2 imaging system.

Table 1. List of targeted antibiotic resistance genes and their resistance mechanisms, primer sequences, size, and PCR annealing temperature, adapted from Burch et al. (2013).

Gene target	Resistance mechanism	Primer sequence (5' → 3')	Size (bp)	Annealing temp (°C)
TetA	Efflux	F: GCT ACA TCC TGC TTG CCT TC R: CAT AGA TCG CCG TGA AGA GG	210	60
TetW	Ribosomal protection	F: GAG AGC CTG CTA TAT GCC AGC R: GGG CGT ATC CAC AAT GTT AAC	168	60
TetX	Enzymatic modification	F: AGC CTT ACC AAT GGG TGT AAA R: TTC TTA CCT TGG ACA TCC CG	278	60
MecA	β-lactam binding protein	F: ATGCGCTATAGATTGAAAGGAT R: TACGCGATATCTAACTTTCCTA	163	60

2.6 Statistical Analysis

Data was compiled and analyzed by averaging the findings of all 3 trials from each site for each month. Standard deviation was also calculated.

3. Results and Discussion

3.1 Chemical Analysis

The salinity of site 1 was found to average approximately 5 ppt with little variation; the salinity of site 2 was found to average approximately 23 ppt, ranging anywhere from 20.3 to 25.8 ppt, as shown in Figure 2A. Water temperature was very consistent between the two sites, and there is a clear increase in temperature into the summer months, as shown in Figure 2B. Dissolved oxygen varied monthly, but there seemed to be little to no correlation between the two sites, as shown in Figure 2C. Phosphate concentration showed a comparable variation and lack of correlation between sites; however the standard deviation among trials from one site seemed to be rather large, as shown in Figure 2D. Nitrate concentration, though still varying every month, seemed to follow a similar trend between sites, as show in Figure 2E. Ammonia concentration stayed relatively consistent throughout all six months; however, ammonia concentration was found to be up to 10 times in higher in site 2, as shown in Figure 2F. From the chemical data, it can be concluded that the chemical components needed for abundant bacterial growth and development were adequately present, allowing for the propagation of antimicrobial resistance among present microbes.

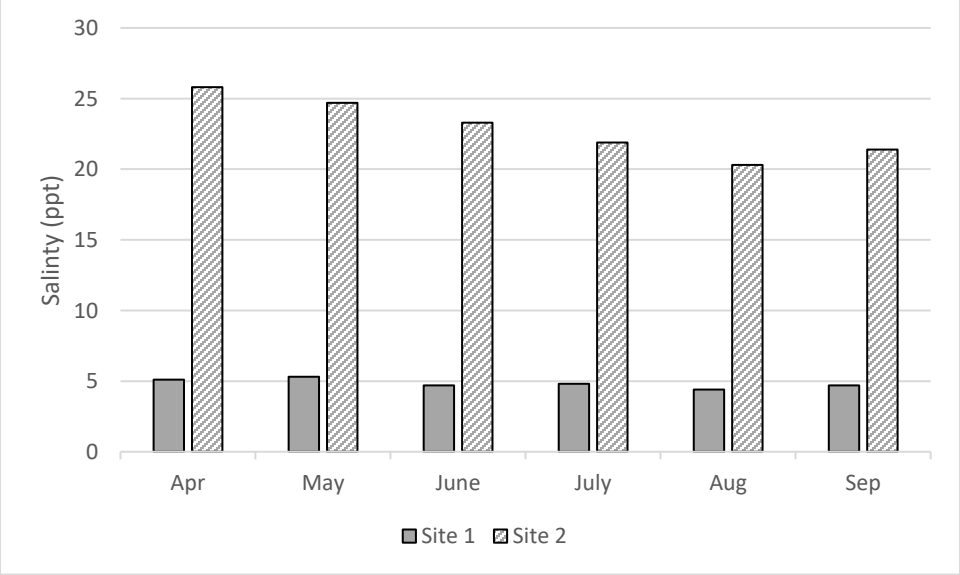

Figure 2A. Salinity (ppt) of Sites 1 and 2 over a 6-month period.

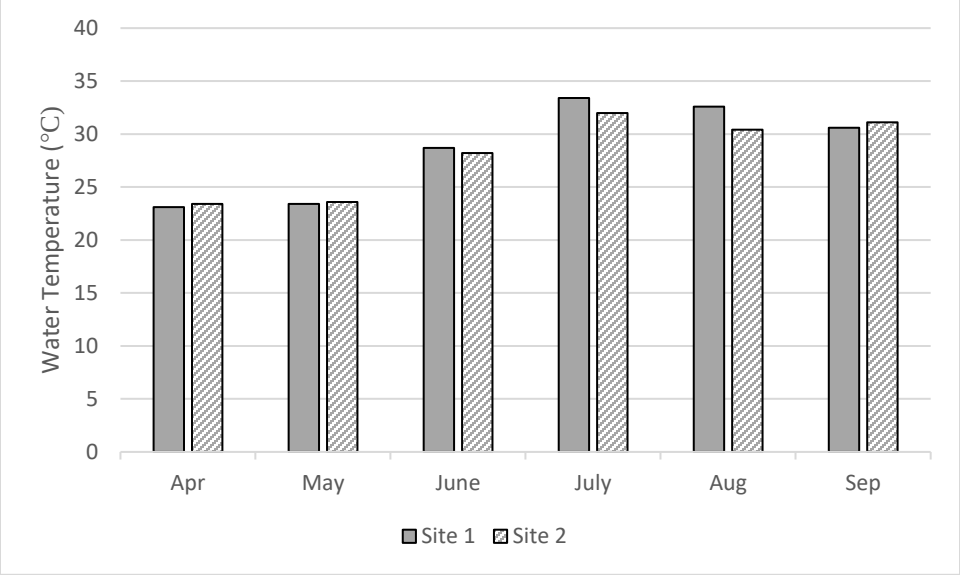

Figure 2B. Water temperatures (°C) of Sites 1 and 2 over a 6-month period.

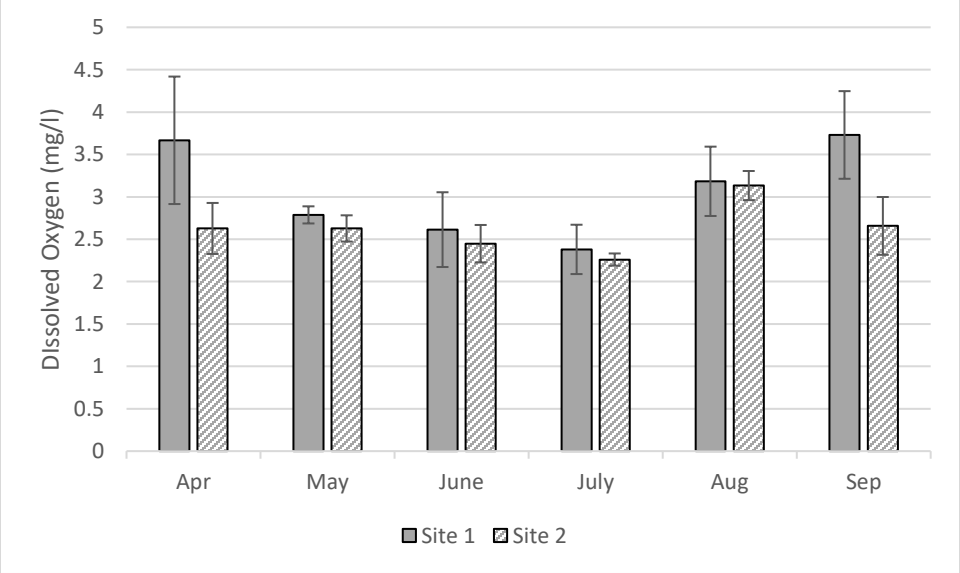

Figure 2C. Dissolved oxygen (mg/l) of Sites 1 and 2 over a 6-month period.

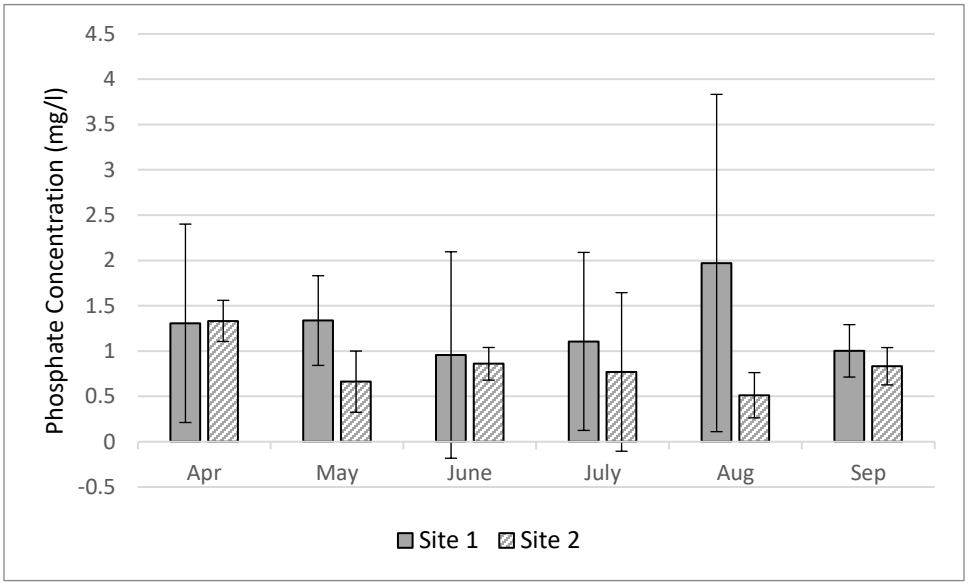

Figure 2D. Phosphate concentration (mg/l) of Sites 1 and 2 over a 6-month period.

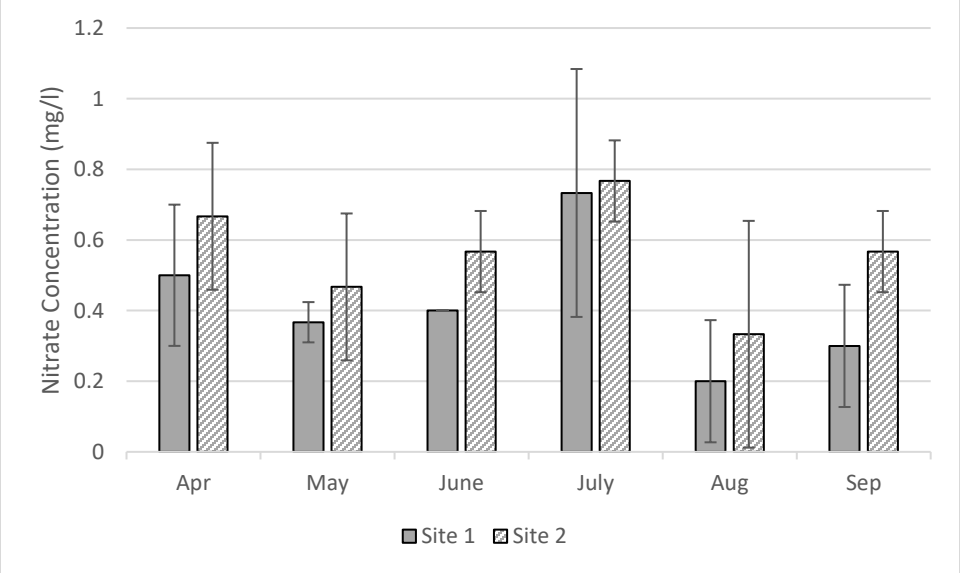

Figure 2E. Nitrate concentration (mg/l) of Sites 1 and 2 over a 6-month period.

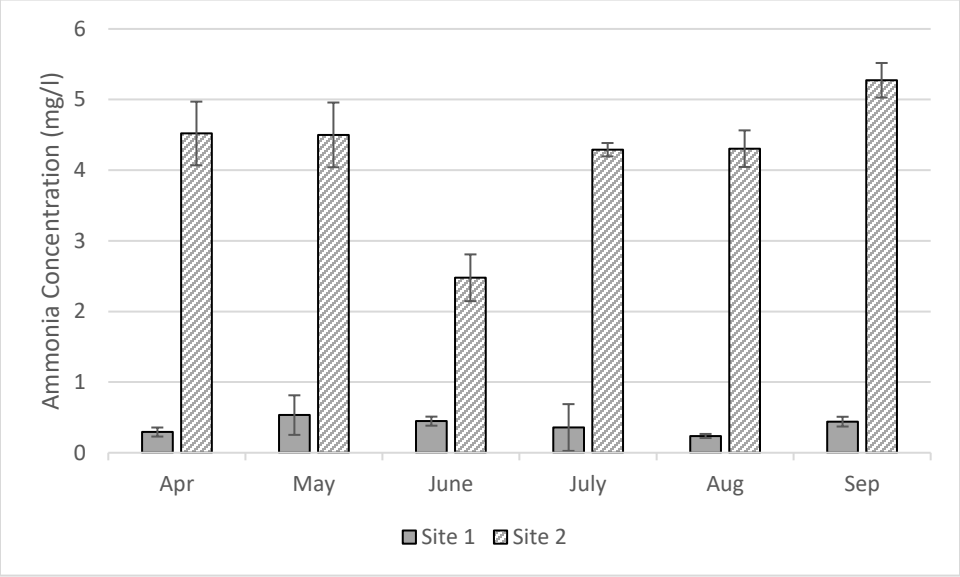

Figure 2F. Ammonia concentration (mg/l) of Sites 1 and 2 over a 6-month period.

3.2 Bacterial Quantification, Isolation, and Identification

As shown in Figures 3A and 3B, fecal and total coliform data showed rather unpredictable patterns, though Site 1 was generally found to have more microbial life overall. Site 1's fecal coliforms varied anywhere from single digits to thousands of CFU/100 ml, while Site 2's fecal coliforms general stayed around 10 CFU/100 ml. Similarly, total coliforms in Site 1 greatly varied from month to month, while total coliforms in Site 2 generally stayed in the 10 to 100 CFU/100 ml range. There was no clear correlation between coliforms in one site and coliforms in the other; however, the fecal coliforms in Site 1 did seem to follow a very similar trend to the total coliforms in Site 1. This data illustrates that there are certainly enough bacteria present to facilitate the spread of antibiotic resistance. It should be noted that Site 1's total coliforms in both May and June showed all tubes returning

positive, meaning that the MPN was 2400 or more CFU/100 ml. In this case, 2400 was used for the sake of calculations and graphical representation.

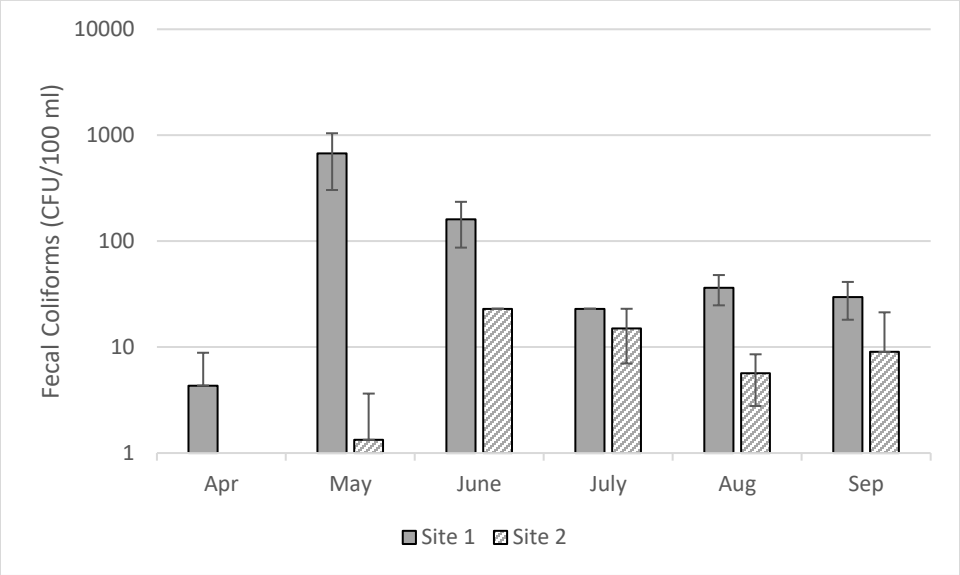

Figure 3A. Fecal coliforms (CFU/100 ml) of Sites 1 and 2 over a 6-month period.

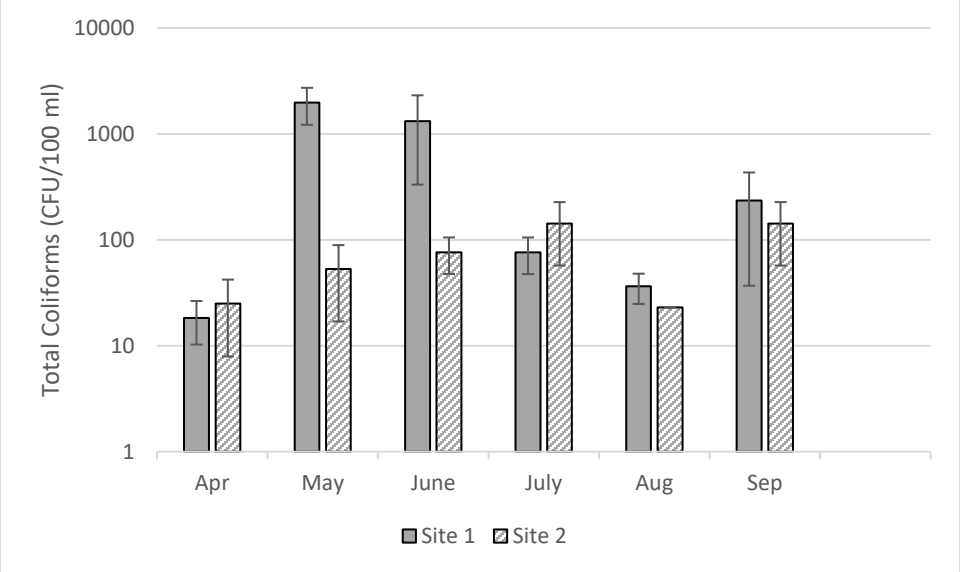

Figure 3B. Total coliforms (CFU/100 ml) of Sites 1 and 2 over a 6-month period.

All coliform data was represented on a logarithmic scale for ease of visualization and interpretation.

3.3 Antibiotic Resistance Assay

Isolates were identified by species using biochemical and assays, and grouped into categories based on speciation. The number of each isolate that read as susceptible, intermediate, or resistant according to the Kirby-Bauer assay is shown in Table 2. The data indicates very few instances of resistance to the carbapenems, ceftazidime w/ clavulanic acid, and aztreonam; however, each of these drugs had at least one isolate with intermediate resistance. Many more

Table 2. Kirby-Bauer assay results for each antibiotic used. Isolates were identified and grouped for ease of representation. "S" indicates that isolates were susceptible to the drug. "I" indicates that isolates had an intermediate level of resistance to the drug. "R" indicates that isolates were completely resistant to the drug.

		ATM	CAZ	IPM	MEM	PIP	TH
E. coli	S	23	28	28	28	24	20
n = 28	I	3	0	0	0	2	5
	R	0	0	0	0	2	3
Klebsiella spp.	S	28	30	29	30	21	20
n = 30	I	2	0	1	0	5	6
	R	0	0	0	0	4	4
Enterobacter spp.	S	8	9	9	9	8	6
n = 10	I	2	2	1	1	1	2
	R	0	0	0	0	1	2
Other	S	5	6	5	6	4	1
n = 6	I	1	0	1	0	1	0
	R	0	0	0	0	1	5

cases of both intermediate resistance and complete resistance to piperacillin and sulfamethizole were present. Resistance seemed to be rather evenly spread among the species of bacteria present.

3.4 DNA Extraction and Gel Electrophoresis

After viewing the Kirby-Bauer assay data, it was surprising that the gel electrophoresis results gave significantly less positive results. However, the genes tested in gel electrophoresis were for tetracycline and methicillin resistance, which were not tested in the Kirby-Bauer assay. Perhaps if the primers for *KPC* and *Oxa* worked more consistently, there could have been carbapenem resistance genes found, seeing that the Kirby-Bauer assay showed some intermediate resistance to carbapenems; however, these primers were not used after the first month due to the inconsistency of results. Also, interestingly enough, the sample lanes for *TetA* would often produce an unexpected band of varying bp length that never matched the positive control for *TetA*. This has yet to be explained, but could be due to user error in PCR. In furthering this project, more primers for genes relative to the drugs tested could be used to truly identify if the genes themselves are free in the water source. Gel electrophoresis results are shown in Figure 4.

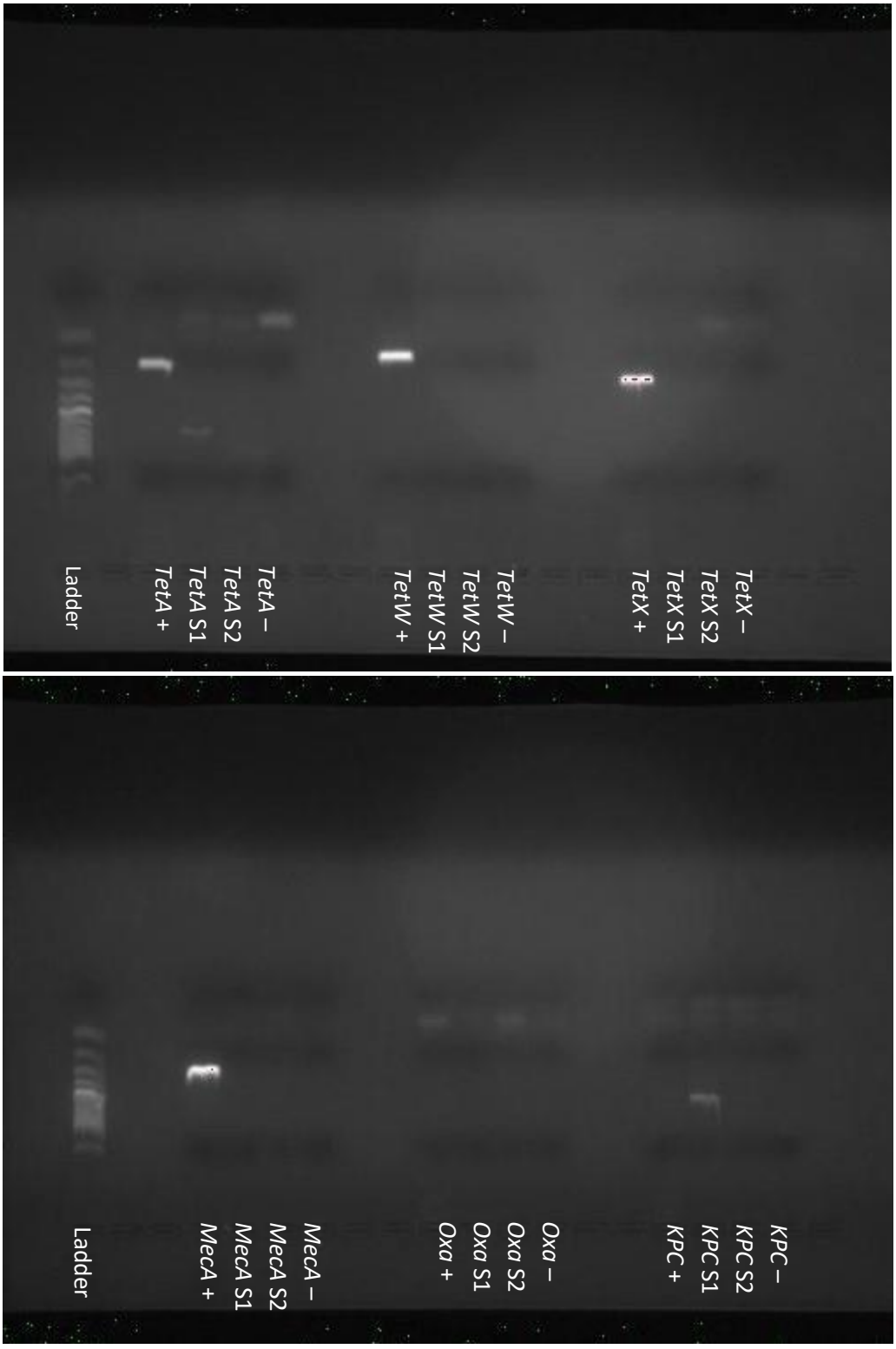

Figure 4A. Gel electrophoresis results for April.

+ indicates positive control. - indicates negative control. S1 indicates Site 1. S2 indicates Site 2.

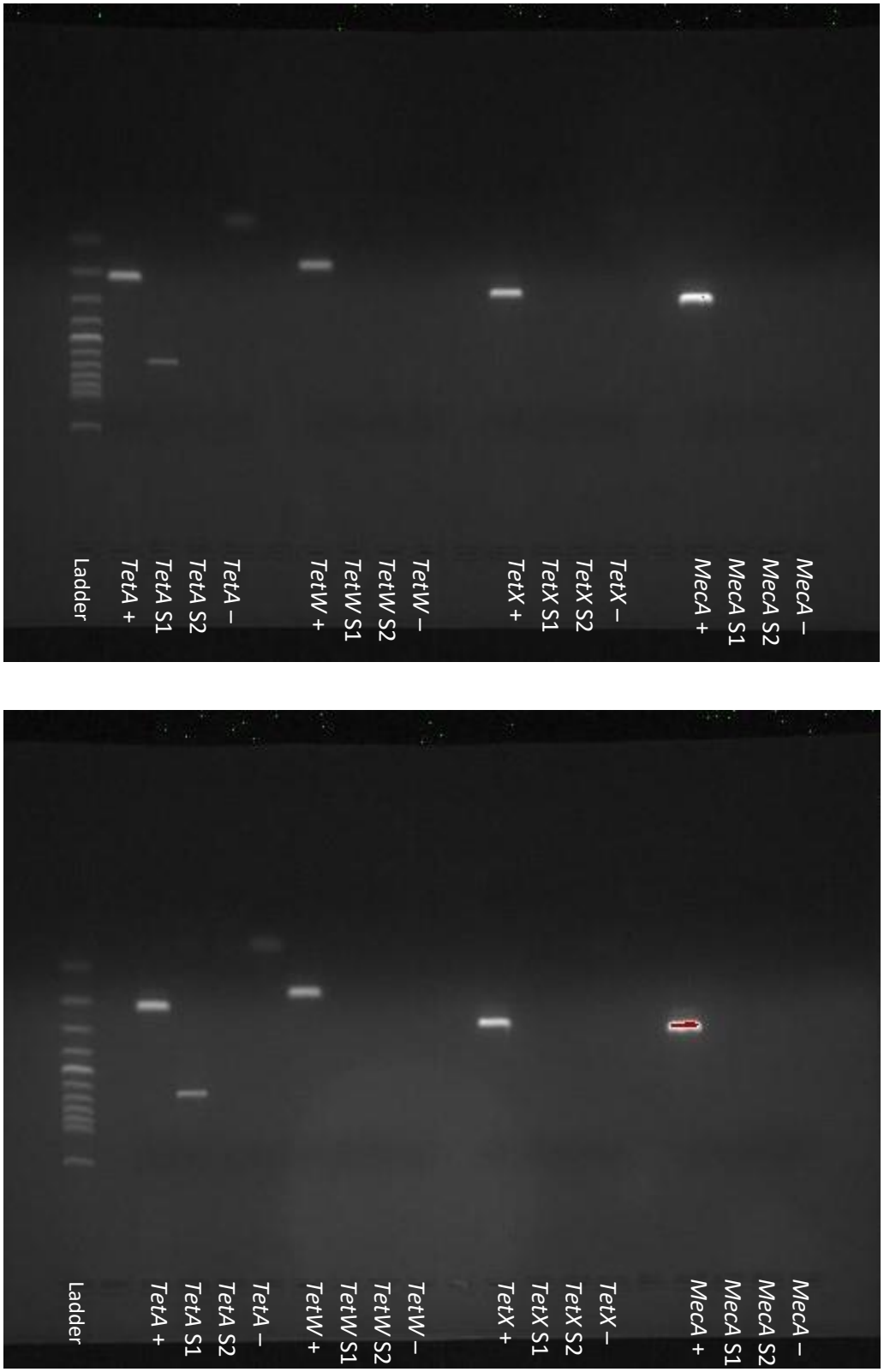

Figure 4B. Gel electrophoresis results for May (top) and June (bottom).

+ indicates positive control. - indicates negative control. S1 indicates Site 1. S2 indicates Site 2.

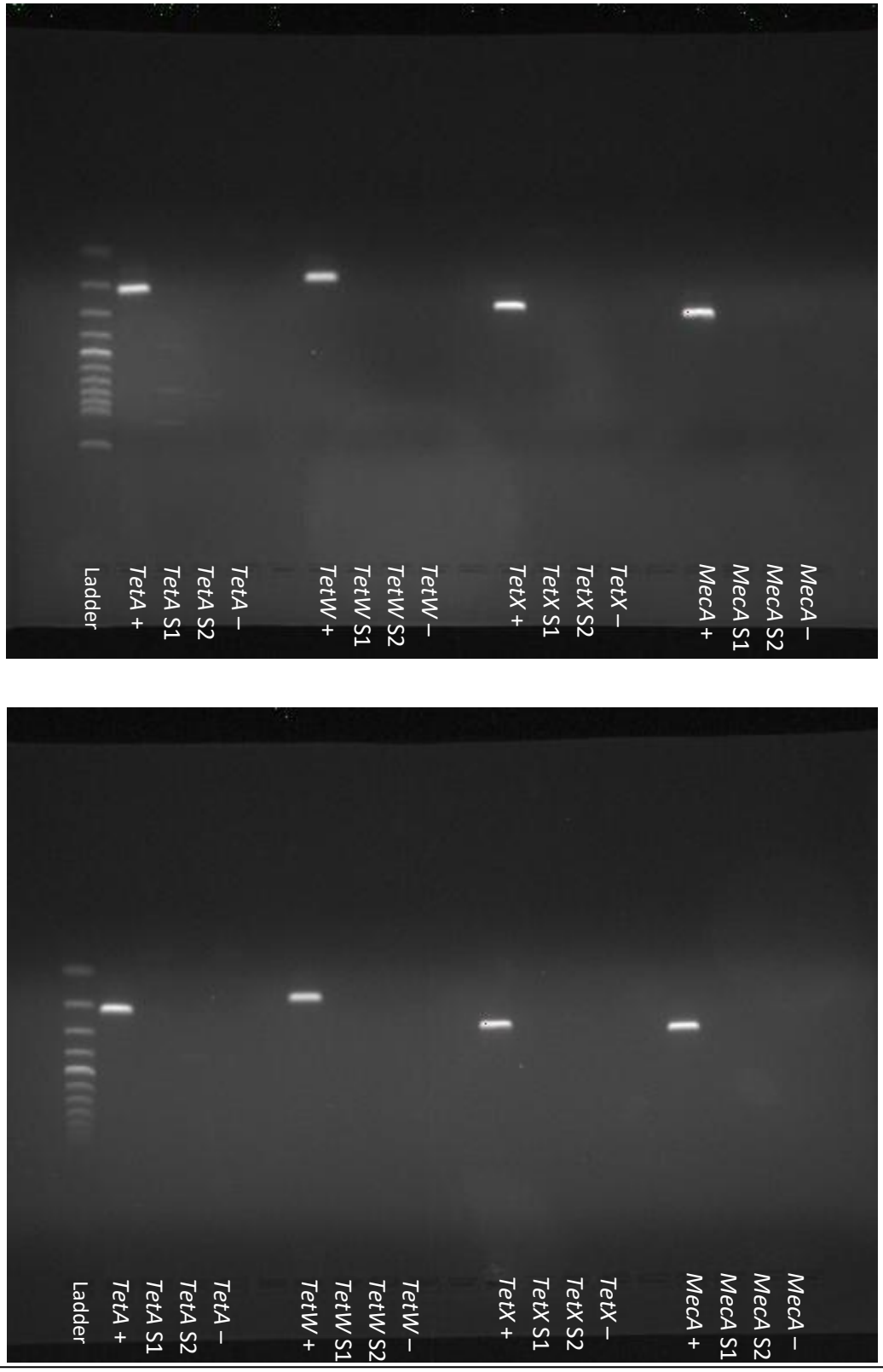

Figure 4C. Gel electrophoresis results for July (top) and August (bottom).

+ indicates positive control. - indicates negative control. S1 indicates Site 1. S2 indicates Site 2.

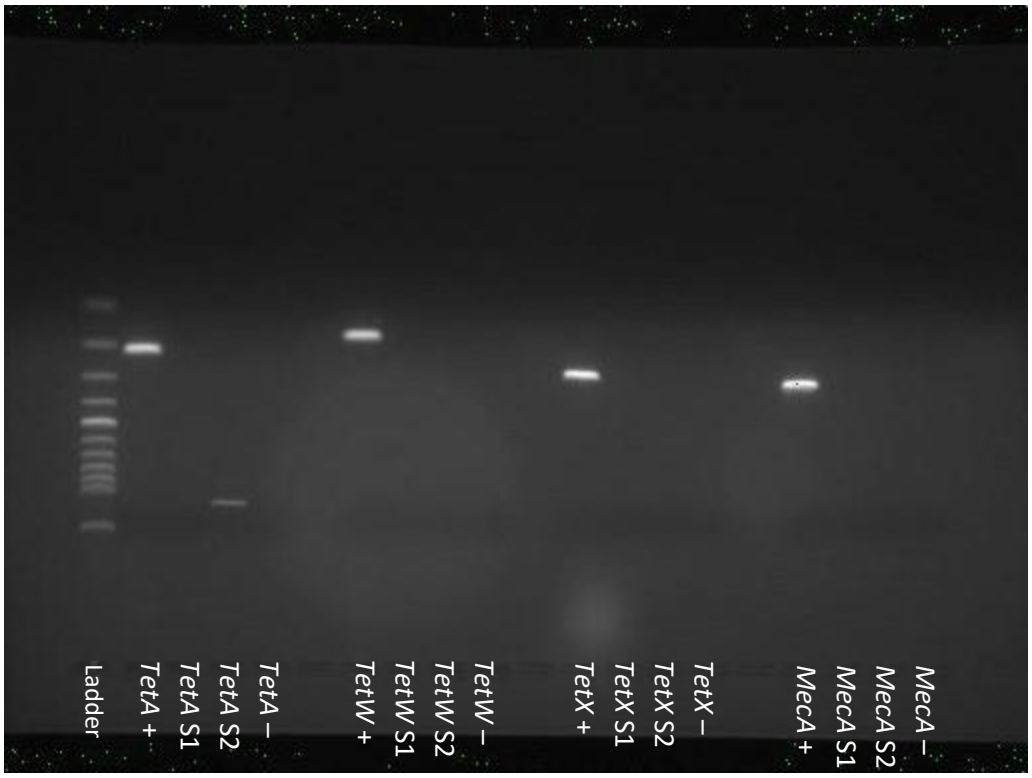

Figure 4D. Gel electrophoresis results for September.
+ indicates positive control. - indicates negative control. S1 indicates Site 1. S2 indicates Site 2.

4. Conclusions

Upon review of all data, three conclusions could be derived. First, the environmental conditions of both sites seemed to allow for prosperous bacterial growth. The two sites had significantly different salinity levels, but this only allowed for more variety of species between sites. Chemical data showed that nutrient concentrations were found to be more than sufficient for abundant bacterial growth. Fecal and total coliform data confirmed that microbial life was plentiful in both sites. Kirby-Bauer assay data showed a considerable number of drug-resistant isolates, with up to one third of some species being resistant to the same drug. Though it is not reflected in Table 1, some isolates were resistant to two or even

three of the antibiotics used. Resistance was distributed rather evenly among species, and all species tended to show more resistance to TH-.25 and PIP-100 than to the four other drugs. DNA isolation and amplification data were inconclusive, but this could be improved with more modern PCR techniques and improved primer quality. Overall, significant amounts of microbial growth were found, and antibiotic resistance is present with the possibility of more to spread.

5. Acknowledgements

This project was financially supported by the Undergraduate Research Opportunity Program (UROP) of Louisiana Sea Grant. Further thanks to Dr. Raj Boopathy for mentorship, Scott Bergeron for instruction in methods, Richard Grabert for assistance in the lab, and LUMCON for use of the collection site.

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