Does Salinity Have an Impact on the Presence of Antibiotic Resistant Bacteria and Antibiotic Resistance Genes in the Marshes of Southeast Louisiana

Final Report Submitted to LA Seagrant

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Running Title: Antibiotic Resistance Genes in Fresh and Saltwater Marshes

ABSTRACT

One of the major public health problems facing the world today is the occurrence and spread of antibiotic resistant bacteria (ARB) in the environment. The main reservoir for ARB is the aquatic ecosystems. Culture based methods and qualitative molecular techniques were used to screen and determine the presence of antibiotic resistance genes (ARG) and ARB in three different salinity gradients of wetland marsh in the southeast Louisiana of USA. The bacteria of interest include *Enterobacter cloacae/aerogenes, Enterococci spp.* and *E. coli*. The antibiotic resistance genes of interest include *ermB, sul1, tetA, tetX, tetW,* and *mecA* that are responsible for resistance to erythromycin, sulfonamide, tetracycline, and methicillin antibiotics. The water salinity ranged from 0 to 12 parts per thousand (ppt). Monthly samples were taken for a six-month period and analyzed for the presence of ARB and ARGs along with carbon, nitrogen, and phosphorous levels in the water samples. The results indicated salinity did not have significant difference in the presence of ARB and ARGs in the wetlands. Significant numbers of ARB were found in all three salinity levels (0, 6, and 12 ppt) in the marshes of Southeast Louisiana. ARGs were more prevalent in site 2 with the salinity of 6 ppt followed by site 1 with the salinity of 12 ppt and site 3 with 0 ppt salinity. Bacterial load and the pollution load varied from month to month and among the three salinities. This study indicates the presence of ARB and ARGs in the wetland habitat is a cause for concern as the potential threat of the spread of ARGs into native bacteria and into fish and wildlife exists due to human activities even under high salinity habitat.

Keywords: Antibiotics, wetland, marshes, salinity, Sulfonamide, Tetracycline, Erythromycin, Antibiotic Resistance Genes (ARG).

1. **Introduction**

Antibiotic resistance is becoming a very large problem throughout the world. The spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment is a major public health issue. Aquatic ecosystem is a significant source for ARB and ARGs. The rise of antibiotic resistance has led to much discussion on the spread of antibiotic resistance genes and the future of antibiotic resistance on public health. Since the production of antibiotics there has been a noted impact with resistant bacteria. Each year there are over 23,000 deaths with at least 2 million people becoming infected with antibiotic resistant bacteria in the United States (CDC, 2015). Antibiotics are among the most commonly used and successful group of pharmaceuticals used for human medicine (Bouki et al, 2013). Rapid spread in resistance to these antibiotics has caused medical concerns to both public and health professionals. Resistance is a result of both the appropriate use of antibiotics, such as normal exposure due to usage, and inappropriate use, such as not finishing a prescription or over-use of the drugs. Other reasons include the selective pressure of antibiotic use in the human body and in the environment, as well as change in genome that enhance the transmission of resistant organisms. The goal of the medical professional is to slow down the rise in antibiotic resistance genes (ARGs) by implementing better hygiene, preventing infections, controlling the nosocomial transmission of organisms, treating the source of the causative agent, and changing and developing new treatment methods (Dzidic and Bedekovic, 2003). The general public also plays a key role in control and spread of antibiotic resistant bacteria in the environment through their prudent use of antibiotics and proper disposal of unused antibiotics and also ensuring their waste disposal system is functioning properly.

Some used antibiotics do not always get fully metabolized by the body and are mostly excreted in its original form into the environment (Zhang et al. 2009). There is a growing problem of discharge of antibiotic residues into the environment due to the common use of antibiotics (Zhang et al. 2009). Presence and spread of antibiotics into the environment have arisen antibiotic resistance in bacteria (Auerbach et al. 2007) especially in wastewater treatment plant, where there is high variety of antibiotics and bacterial densities, bacteria can easily acquire resistance against those antibiotics and release their

antibiotic resistance genes (ARGs) into the environment during their release from the treatment plant (Everage et al. 2014; Naquin et al. 2015). These released ARGs through genetic transformation can get easily be transferred to the environmental bacteria and pathogens, increasing risks and dangers to environment and human (Liu et al. 2012). Recent studies show that incomplete metabolism in humans and improper disposal of antibiotics to sewage treatment plants has been a main source of antibiotic release into the environment (Rizzo et al, 2013; Everage et al. 2014). This gives bacteria enough time and sufficient contact to shield themselves selecting for strains that have genes and cellular mechanisms, favoring their growth and reproduction (Galvin et al, 2010). These bacteria have the potential to infect the wildlife in nature, where the treated water is released.

Louisiana is known as "Sportsman's Paradise", and has over 300,000 registered boats, with approx. 41,500 of these belonging to southeast Louisiana (Louisiana Department of Wildlife and Fisheries, 2013). Recreational activities such as hunting, fishing, and boating are economically important to Louisiana and with so much physical interaction with the waterways and bayous, water quality becomes a major concern. There are various ways in which water can become polluted and fecal content in aquatic environments can increase, such as agricultural and storm runoff, the waste of animals, and human sewage. In southeast Louisiana, USA, most of the rural household is responsible for their own septic system to treat the wastewater. These systems are effective but require maintenance, are costly, and if not taken care of properly, can lead to water pollution. When waste is improperly disposed, the risk of antibiotic resistance increases. In this study, a site was chosen, where people reside near the wetlands, which include freshwater, brackish, and saltwater marshes and the waste disposal in these households is mainly individual septic tank. The effectiveness of these septic tanks is not always reliable leading to fecal contamination of wetlands. The purpose of this research is to test antibiotic resistance in three salinity gradients in southeast Louisiana in order to observe whether salinity affects fecal coliforms and their contribution to antibiotic resistant bacteria and antibiotic resistance genes to the environment.

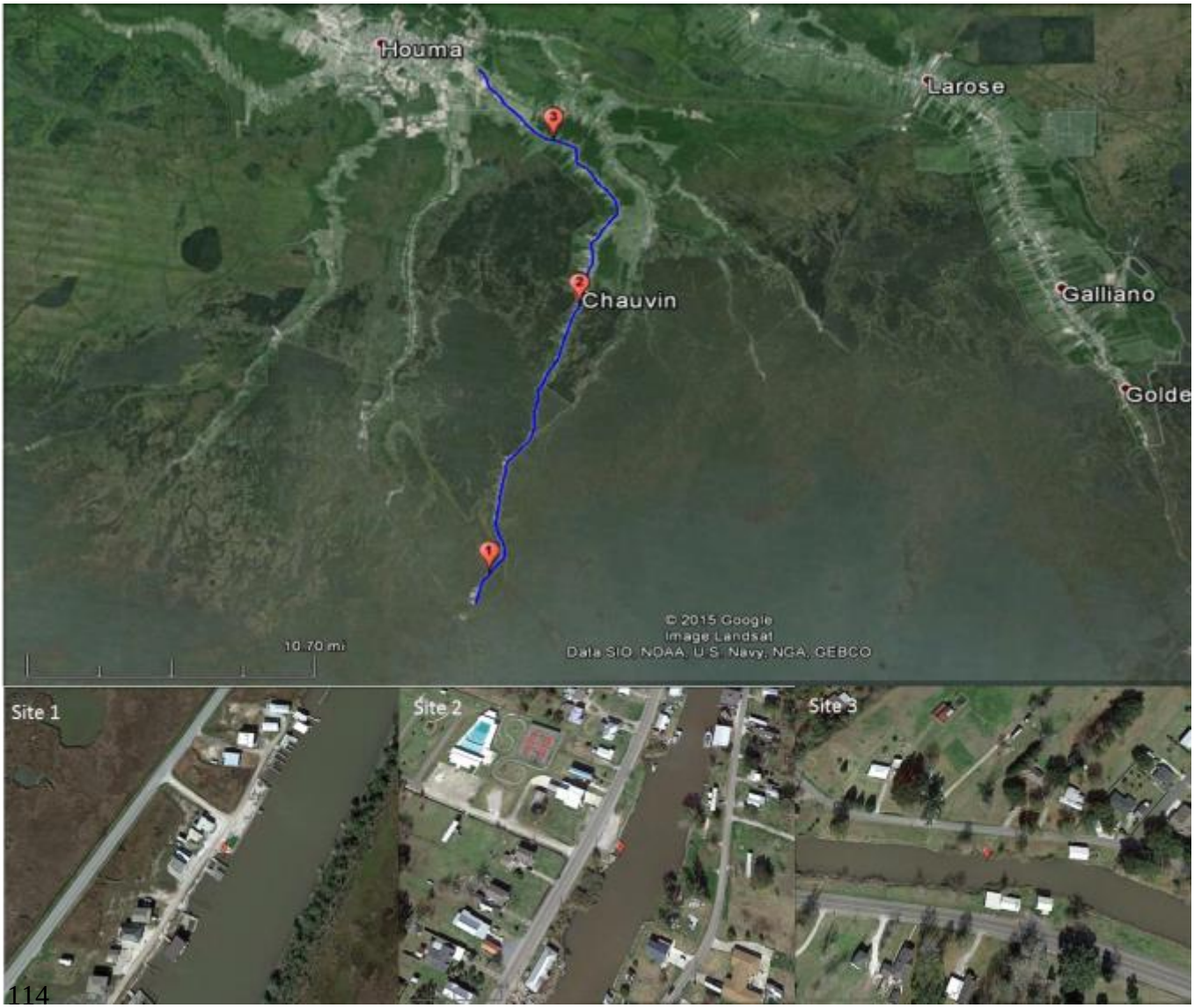


Figure 1. The sampling sites on Bayou Petit Caillou. Site 1 (12 ppt salinity) in Cocodrie, Louisiana (29°15'49.44"N, 90°39'9.81"W), site 2 in Chauvin (6 ppt salinity), Louisiana (29°25'53.24"N, 90°35'49.43"W), and site 3 in Houma (0 ppt salinity), Louisiana (29°32'5.75"N, 90°36'46.85"W).

2. Materials and Methods

2.1. Collection of Sample

Monthly water samples were collected from wetlands that are interconnected with a salinity gradient of 0 (site 3), 6 (site 2), and 12 (site 1) parts per thousand (ppt) in Bayou Petit Caillou in southeast Louisiana, USA. The sampling sites are shown in Figure 1 with GPS coordinance. The water samples were collected for six months from April to September in 2015. Duplicate samples were collected from the above-mentioned sites using sterile containers. Samples were transported back to the lab on ice, and stored at 4°C until analysis was completed.

2.2. Analysis of Sample

Once the samples were received in the lab, they were manually mixed by shaking the sample bottles. The pH was measured using a pH meter (Denver Instruments, Denver, CO). The organic carbon in terms of biological oxygen demand (BOD), nitrate, nitrite (Cadmium reduction method), and phosphate (Ascorbic acid method) in the sample was analyzed by the methods described in APHA (1995).

2.3. Bacterial Analysis

Total aerobic heterotrophic bacteria (pour plate method) and fecal coliform (Most Probable Number (MPN) method) were analyzed every month according to the method described by Everage et al. (2014). Various Pure cultures were isolated and identified using BIOLOG method and by various specific biochemical tests as described by Everage et al. (2014). *Enterobacter* spp., was identified using the method described by Delost (2014).

2.4 Antibiotic Resistance Test

Antibiotic resistance was determined using the Kirby-Bauer method (Brown, 2005; Delost, 2014). Different pure cultures isolated each month from different water samples were subjected to antibiotic resistant assay. A bacterial lawn of the sample was grown on Muller-Hinton (MH) agar plate, using sterile cotton swabs as described by Everage et al. (2014). After the sample was streaked onto the MH plates, the antibiotic

discs of erythromycin, tetracycline, neomycin, chloramphenicol, kanamycin, streptomycin, oxacillin, clindamycin, and vancomycin were placed using an automatic, hand-held disk dispenser. The plates were then incubated at 37°C for 24 hours. The zone of inhibition for each antibiotic was measured in millimeters with a standard laboratory caliper at the end of the 24-hour incubation period. The antibiotic resistance was consulted with the Kirby-Baur chart as described by Delost (2014).

2.5 *DNA Extraction*

One ml of water sample was incubated with tryptic soy broth (TSB) at 37°C for 24 hours. The sample was centrifuged at 3000 RPM for 15 minutes and the pellet was used for DNA extraction. Bacterial DNA was extracted from the pellet using the Fast ID DNA Extraction Kit according to manufacturer's instruction to extract the DNA. After the DNA was extracted, polymerase chain reaction (PCR) was used to amplify the DNA as described by Naquin et al. (2014; 2015) and Bergeron et al. (2015). The presence of various antibiotic resistance genes was analyzed using the well known primers for methicillin (*mecA* gene), erythromycin (*ermB* gene), sulfonamides (*sul1*gene), tetracycline (*tetA*, *tetW*, and *tetX* genes for efflux pump, ribosomal protection, and enzymatic modification respectively) as shown in Table 1 based on Burch et al. 2013. The presence of *mecA* gene in the water samples was analyzed using the *mecA* primer, (Table 1) as demonstrated by Suzuki et al. (1992). All primers were obtained from Sigma Aldrich Co. (St. Louis, MO). A 2% agarose gel with ethidium bromide was prepared and used to visualize the PCR samples. 10 µL PCR sample was mixed with 2 µL 6x loading dye and injected into each well. The gel was run at 100 V for an hour. The gel was visualized using FluorChem FC2 imaging system. Antibiotic resistant strains and primers served as a positive control and the DNA free water served as the negative control. A universal 16s rRNA gene was used as the housekeeping genes for the presence of bacteria and the bacterial DNA in the samples.

2.6. Statistical Analysis

All chemical data were subjected to an analysis of variance (ANOVA) test ($p \leq 0.05$) followed by a tukey "*post hoc"* analysis when needed (SAS).

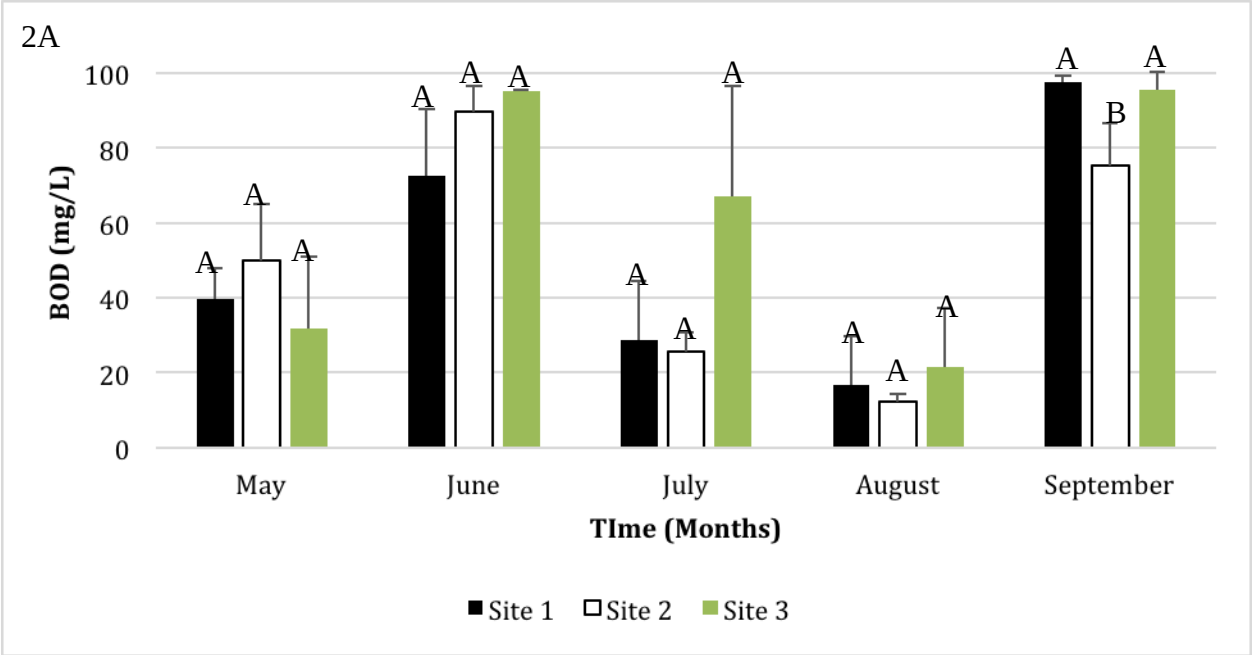
3. Results and Discussion

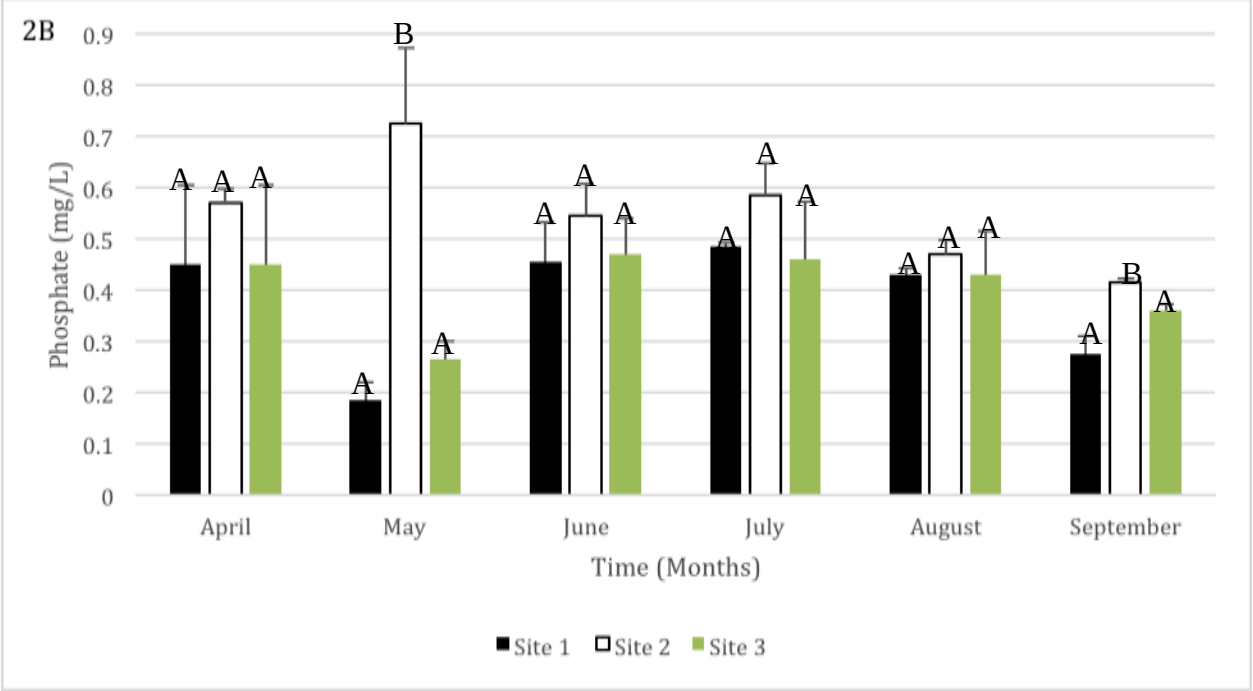
3.1. Water Chemistry Analysis

The salinity in site 1 was always around 12 ppt and this is consisdered as brackish marsh. Site 2 had intermediate salinity of 6 ppt and site 3 is a freshwater marsh with the salinity reading of 0 ppt (data not shown). The total organic carbon in the form of BOD was analyzed for six months and the results are presented in Fig. 2A. There was variation in BOD from month to month and also among different sites. Similar trend was observed for nitrate and phosphate levels in water samples (Figure 2B and C). The pH in water samples did not vary much and it ranged from 7.6 to 8.2 in all samples with site 1 always showed higher pH than other sites due to high salinity (Fig. 2D). The dissolved oxgyen (DO) in the water sample is presented in Fig. 2E and the water was aerobic during the sampling period with freshwater site consistently showed higher DO in most of the study period. The DO was not statistically different among the three sites except for April 2015 sampling. These water quality parameters showed that there are plenty of carbon, nitrogen, and phosphorous in the water to support microbial acitivities and the water was aerobic with optimum pH for enteric bacteria to thrive. These sites have rural population with individual septic tanks and treatment plants in the households, which let the wastewater into the bayous and marshes are the major sources of pollution. These water bodies are interconnencted and there are ample sources of fecal coliform and enteric bacteria to inhabit these habitats (Naquin et al. 2015).

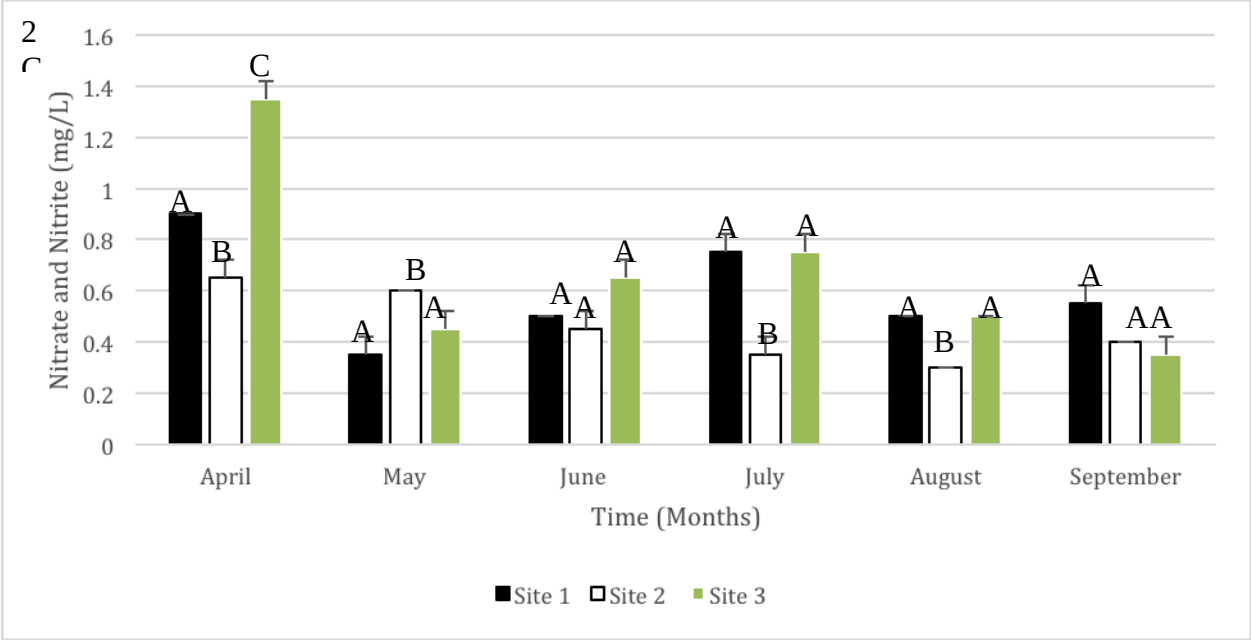
3.1. Fecal Coliform and Heterotrophic Bacteria

Figure 3A shows the fecal coliform numbers in the water samples. The resutls indicated that the fecal coliform bacteria were present in all three sites during the six months of the sampling period. This indicates that the water is contaminated with fecal matter and this is mainly from two sources including local households and treateed sewage from sewage treatment plants of nearby towns. The houshlods near the water have individual sewage treatment plant or septic tanks that discharge into this water body and these individaul treatment system are not maintained properly leading to fecal


2A
BOD (mg/L)
100
80
60
40
20
0
May
June
July
August
September
TIme (Months)
Site 1
Site 2
Site 3


2B
Phosphate (mg/L)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
April
May
June
July
August
September
Time (Months)
Site 1
Site 2
Site 3

2
C



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224
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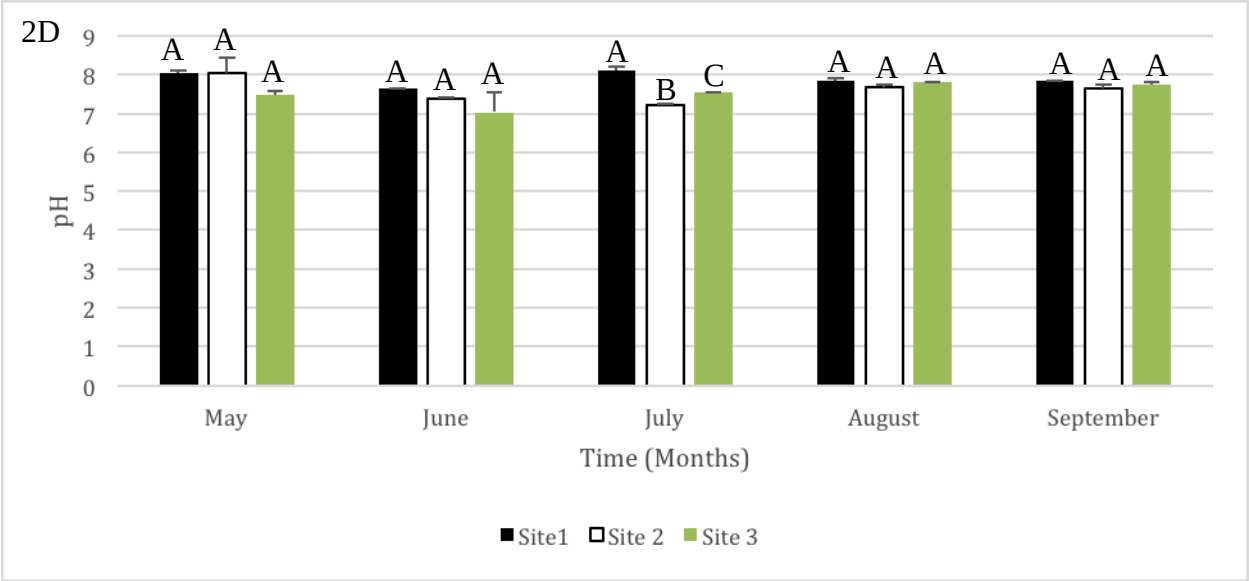
2D



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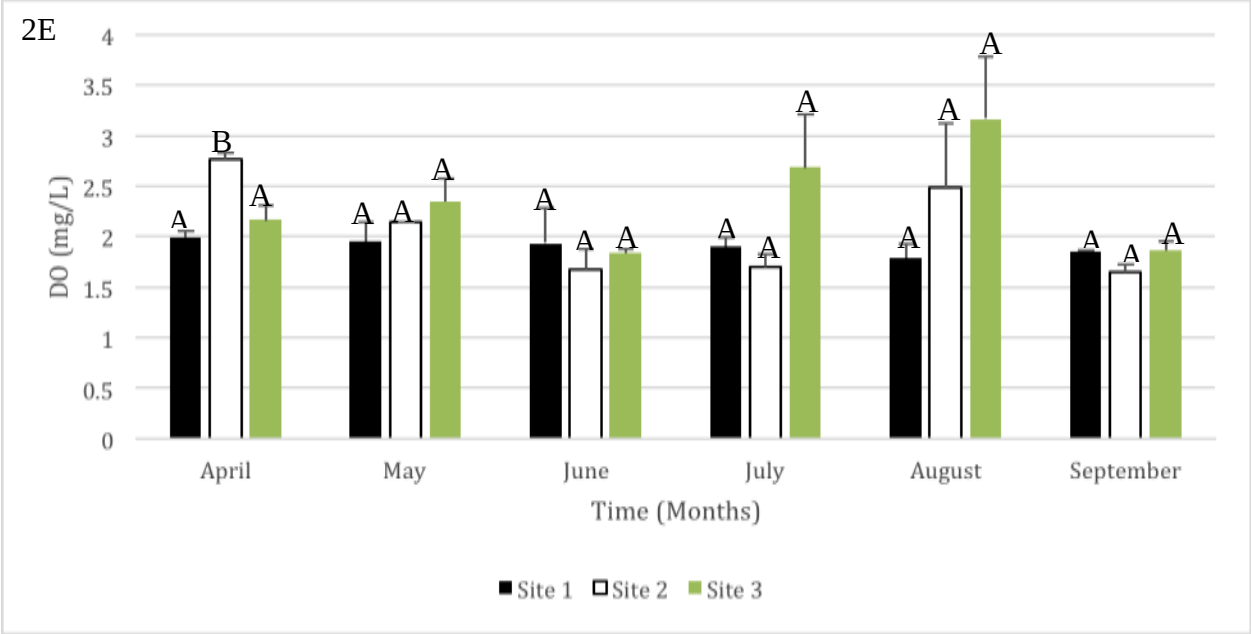
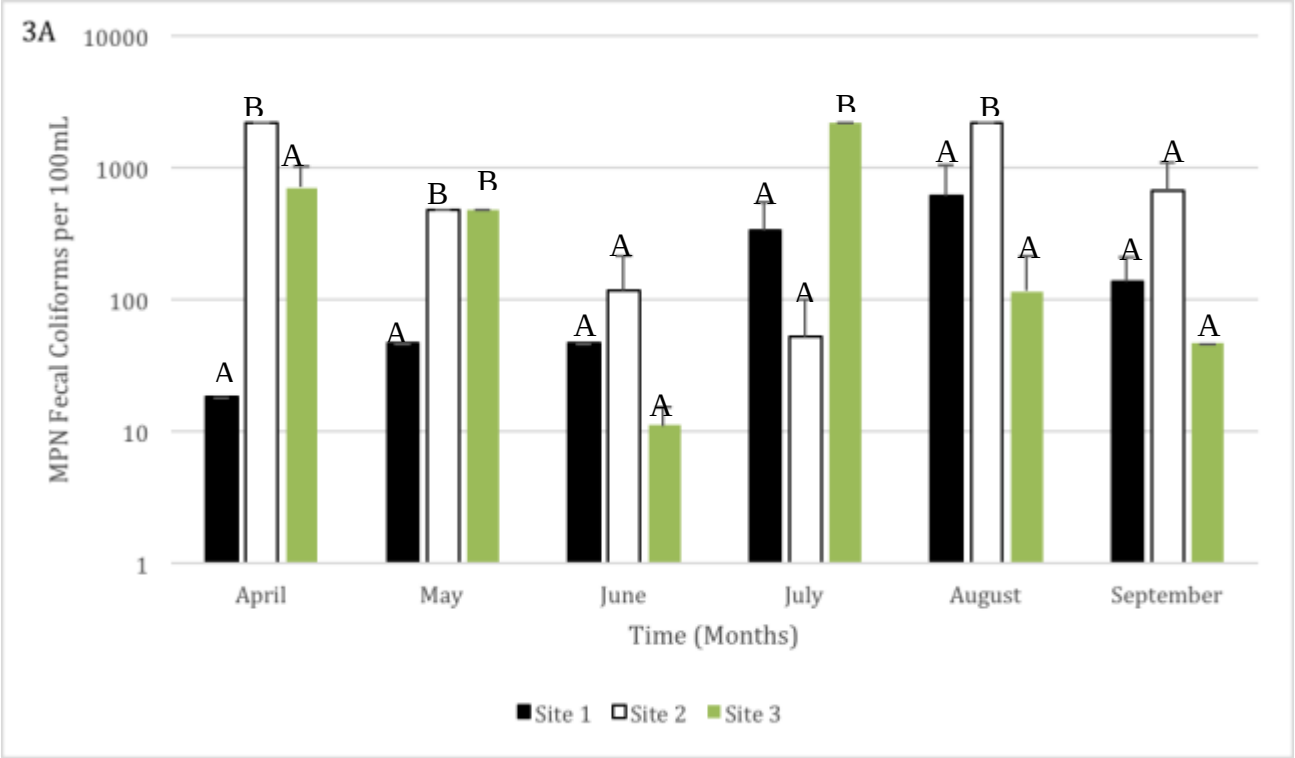
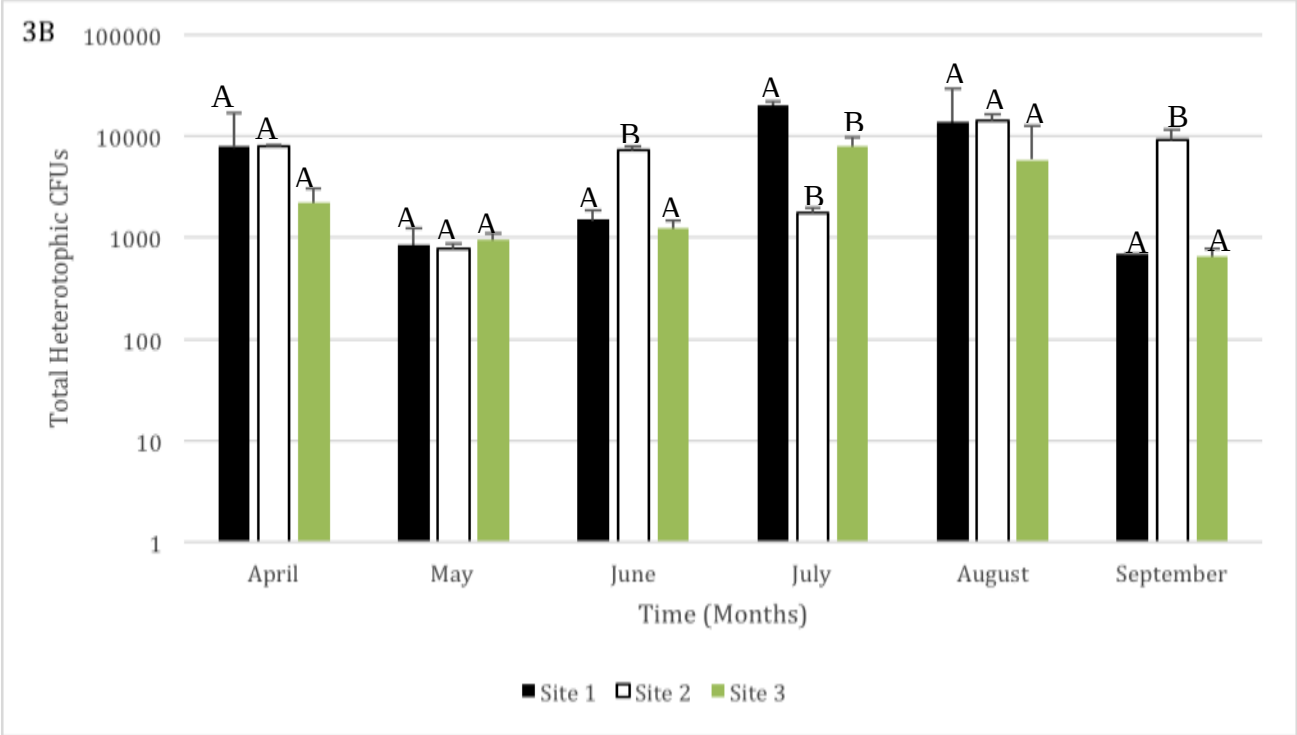


Figure 2. Water chemistry data for different salinity water samples with standard deviation. 2A, BOD; 2B, Phosphate; 2C, Nitrate; 2D, pH, and 2E, Dissolved oxygen. Different letters denote statistical difference between sites during each month ($p \leq 0.05$).

pollution in the water. Another source of pollution is from nearby towns of Houma and Thibodaux as the treated sewage from these towns end in the marshes and ultimately reach the Gulf of Mexico as these waterbodies are interconnected. Site 1 has high salinity and it showed less number of fecal counts most of the time compared to sites 2, and 3. Total heterotrophic bacteria were monitored in the water samples and the results are given in Fig. 3B. Heterotrophic bacteria were consistently present in the water and most of the time there was no significant difference among the three sites with diferent salinities. There are plenty of organic carbon, nitrgoen and phosphorous present in the water samples in all three sites to support the growth of fecal coliform and heterotrophic bacteria.


3A
10000
1000
100
10
1
MPN Fecal Coliforms per 100mL
A B A
A B B
A A A
A A A
A B A
A A A
April
May
June
July
August
September
Time (Months)
Site 1
Site 2
Site 3


3B
100000
10000
1000
100
10
1
Total Heterotophic CFUs
A A A
A A A
A B A
A B B
A A A
A A B
April
May
June
July
August
September
Time (Months)
Site 1
Site 2
Site 3

3.2. Antibiotic Resistant Bacteria in the Water Samples

Several pure cultures were isolated and identified and the bacteria that were present consistently every month in the water sample were *E. coli, Enterobacter cloacae/aerogenes*, and *Enterococcus* spp. The antibiotic resistant of these bacteria were tested using Kriby-Baur assay as described in the methods section and the results are given in Figure 4. Every month of sampling period, several ARB were found in all three sites with varying salinities. Bacteria were resistant to some of the common antibiotics such as ampicillin, erythromycin, neomycin, chloroamphenicol, tetracycline, kanamycin, gentamycin, and streptomycin. Some of these bacteria were gram negative as shown in Fig.4A and 4B and some were gram positive as indicated in Fig. 4C. A number of previous studies have reported ARB are common in water including raw sewage, treated sewage, and drinking water (Xi et al. 2009; Armstrong et al. 1981; 1982; Pathak and Gopal, 2008; Ramteke et al. 1990; Schwartz et al. 2003; Shrivastava et al. 2004; Pei et al. 2006; Everage et al. 2014; Bergeron et al. 2015). Interestingly, in this study ARB were found in the marshes with three different salinities of 0, 6, and 12 ppt. Higher salinities did not have any adverse effect on the presence of ARB in the wetland and marsh samples.

3.3. Antibiotic Resistance Genes in the Water Samples

Presence of various antibiotic resistance genes in the water samples of marshes with three different salinities was analyzed every month as described in the method section. The results indicated the presence of 16s rRNA, a common housekeeping gene in bacteria in all three sites in every sampling event and on the other hand, the *Sul1* gene for sulfonamide resistance was never found in these sites. (Table 2). Sulfonamides act as competitive inhibitors of the enzyme dihydropteroate synthase in the folic acid pathway. The gene *sul1* encodes alternative sulfonamide-resistant dihydropteroate synthases in gram-negative clinical bacteria (Huovinen et al. 1995) and this gene is commonly present in sulfisoxazole-resistant gram-negative bacteria. The *ermB* gene that codes for resistance to erythromycin was found in site 2 and 3 on three occasions. The erythromycin gene *ermB* was used because it codes rRNA methyltransferease that confers resistance to macrolides, lincosamides, and streptogramin B as reported by

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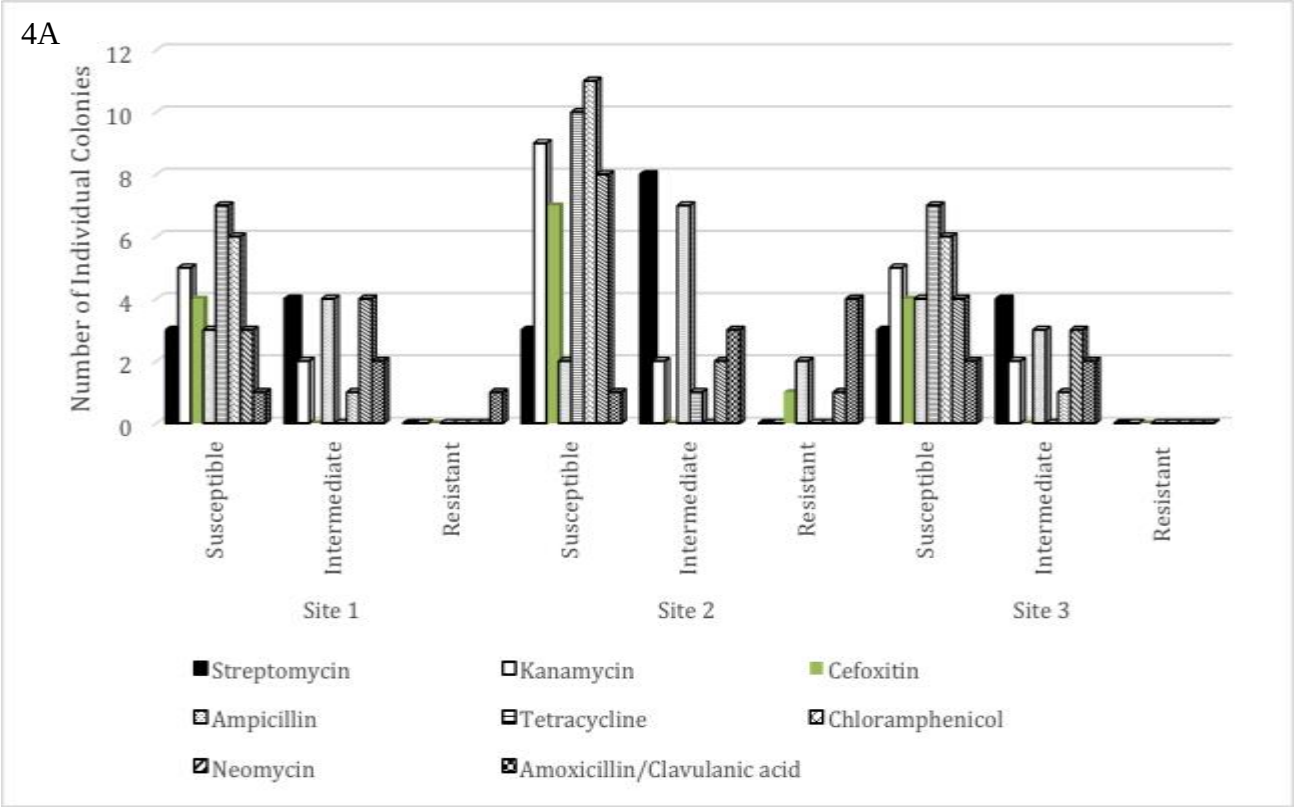
4A



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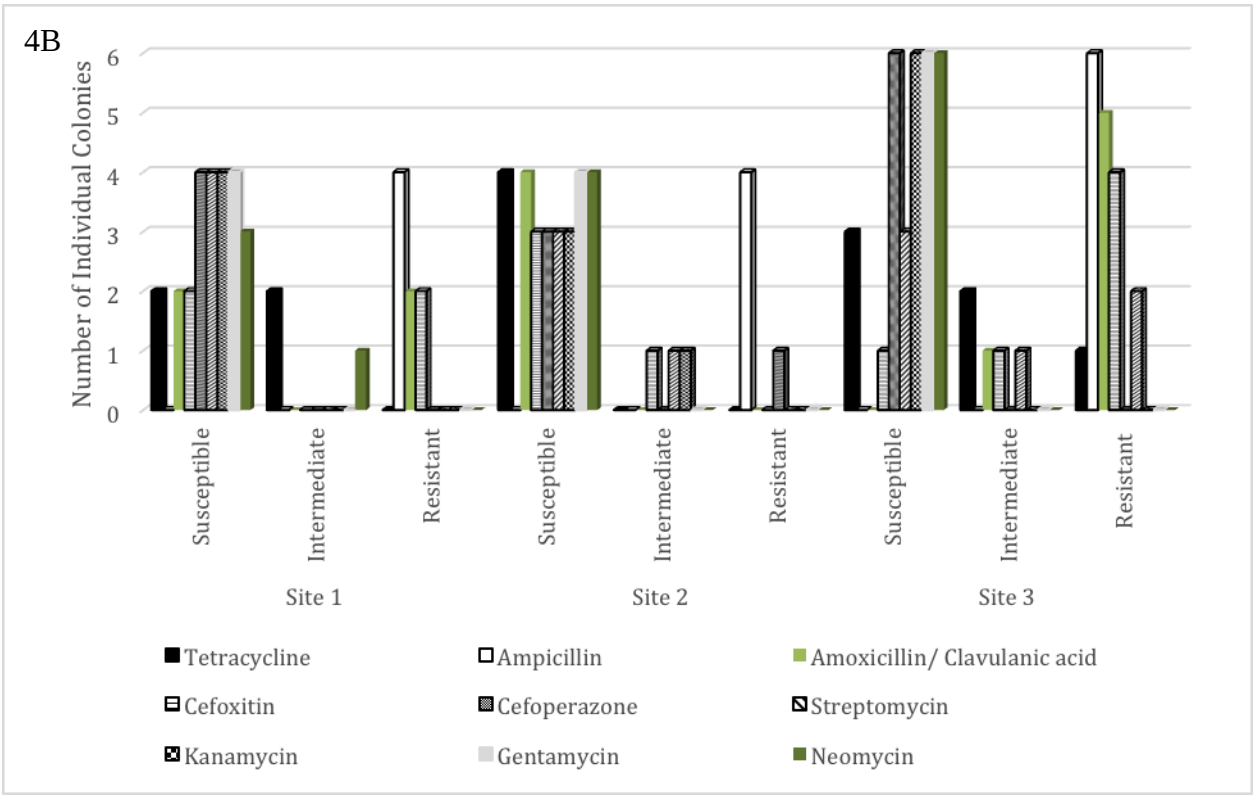
4B



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4C

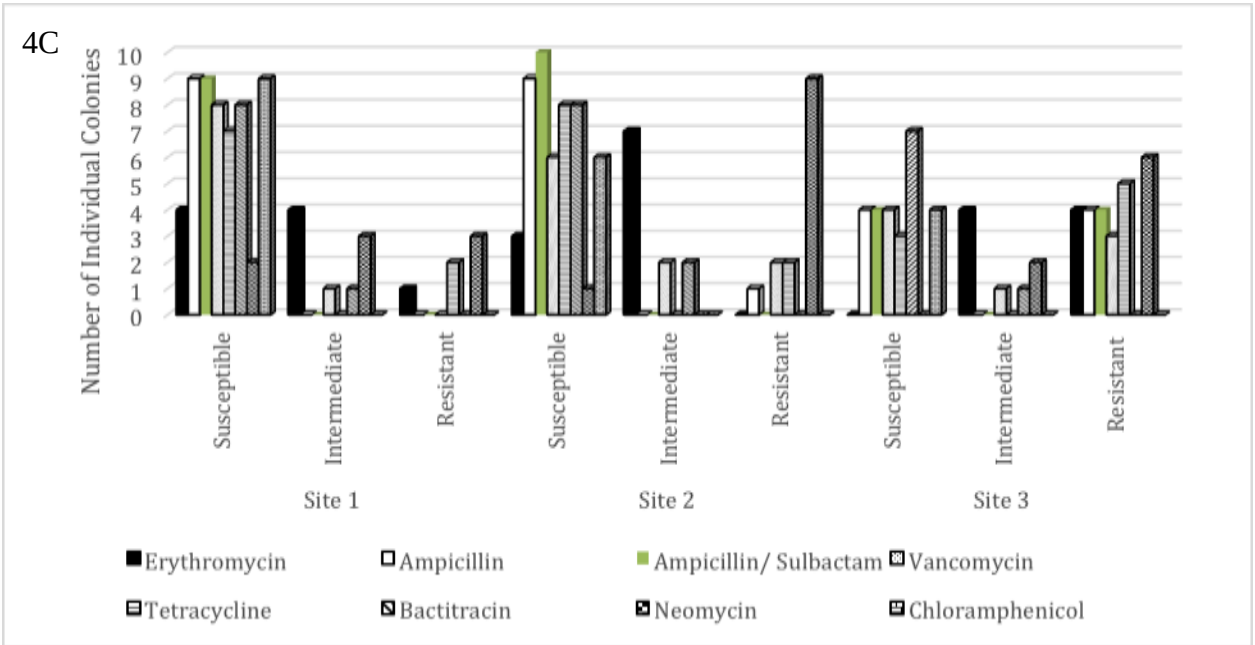

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Figure 4. Antibiotic resistance of various gram-negative and gram positive bacteria present in water samples. 4A, *E.coli*; 4B, *Enterobacter cloacae/aerogenes* and 4C, *Enterococcus* spp.

Roberts et al. (1999). Tetracycline resistance genes were most commonly found in all three sites. The three tetracycline resistance genes represent each of the three known mechanisms of tetracycline resistance, namely, efflux pumps modification, ribosomal protection, and enzymatic modification (Levy et al. 1999; Burch et al. 2013). Molecular analysis was done to study the presence of these genes that encode resistance to tetracycline, namely, *tetA, tetW,* and *tetX* and the results are presented in Figure. 5 and Table 2. In this study, all three tetracycline resistance genes were found in the waters of all three salinities. The *tetA* gene, which codes for efflux pump modification was consistently present in site 2, whereas in site 1 and 3, it was present on three and two sampling events respectively (Table 2). The *tetW* gene that codes resistance to tetracycline via ribosomal protection proteins was found in site 1 with the highest salinity and it was not found in other sites. The gene *tetX* responsible for enzyme modification to confer tetracycline resistance was observed in sites 2 and 3 on couple of occasions. The *mecA* gene was observed in site 1 on one sampling period. Suzuki et al. (1992) showed the presence of *mecA* gene in *S. aureus* and also in *S. epidermidis*. Genetic material that confers methicillin resistance may be passed from one organism to another through a process known as transformation in which free DNA from a dead organism is taken up by a live organism and as a result develop antibiotic resistance. Chlorination kills most bacteria and at the same time may promote the release of free DNA into the water. The free DNA may survive in the water up to 96 hours before it disintegrates in the environment (Naquin et al. 2015). Bacteria may inherit resistance to some antibiotics or can develop resistance via spontaneous mutation or the acquisition of resistant genes via genetic transformation from the environment as demonstrated by Everage et al. 2014 and Naquin et al. 2015. The acquisition of a resistant gene via horizontal transfer is the common way for bacteria to develop antibiotic resistance in the environment (Salyers et al. 2004).

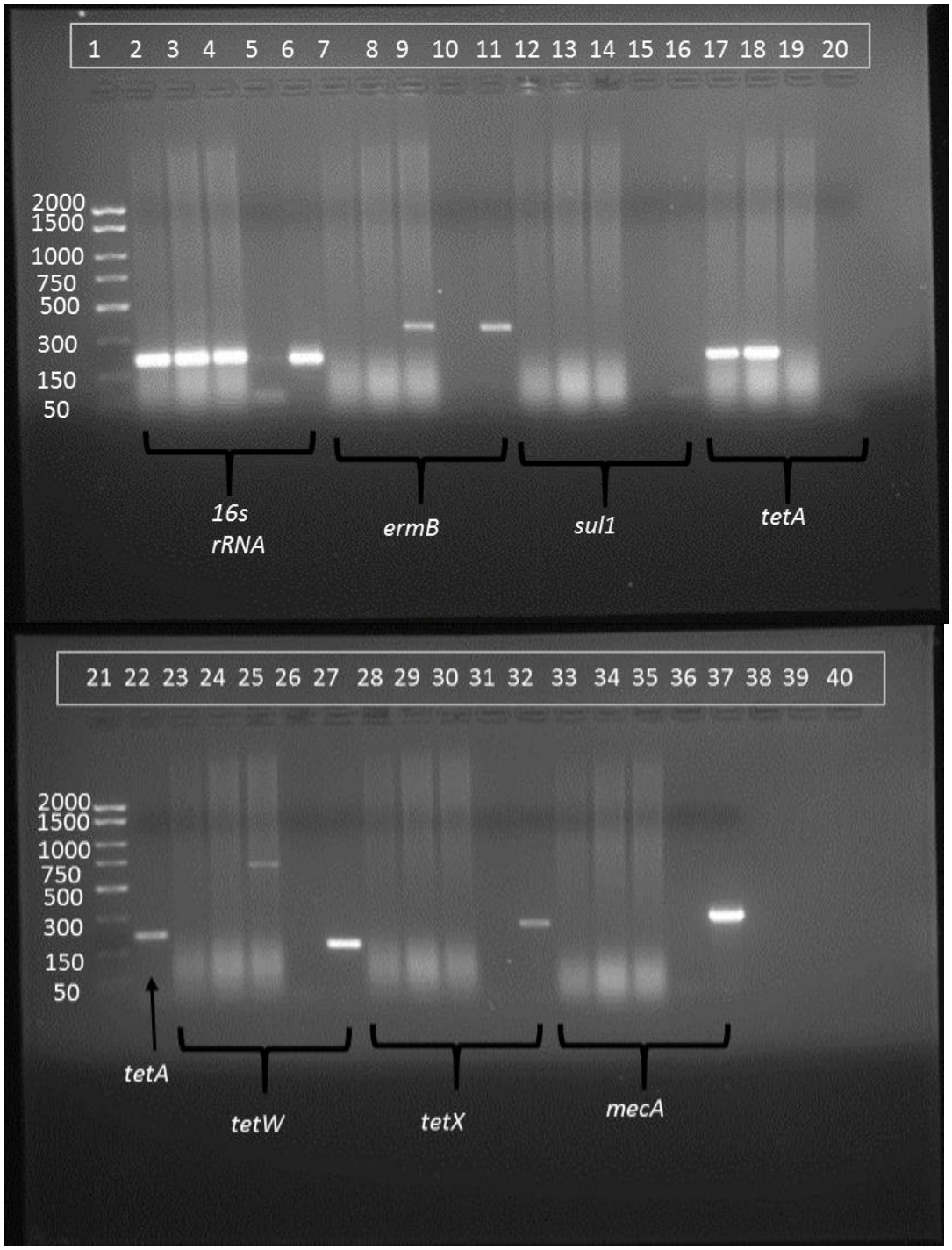

Figure. 5. Molecular analysis of the presence of ARGs in different water samples for the month of July 2015. Lane 1 is the DNA ladder and lanes 2-6 represent 16s rRNA in the following sample order: site 1, site 2, site 3, negative control, and positive control. Lanes 7-11 represent *ermB* gene in the following sample order: site 1, site 2, site 3, negative control, and positive control. Lanes 12-16 represent *Sul1* gene in the following sample order: site 1, site 2, site 3, negative control, and positive control. Lanes 17-22 include *tetA* gene with the following sample order: site 1, site 2, site 3, negative control, and positive control. Lanes 23-27 include *tetW* gene with the following sample order: site 1,

site 2, site 3, negative control, and positive control. Lanes 28-32 include *tetX* gene with the following sample order: site 1, site 2, site 3, negative control, and positive control. Lanes 33-37 include *mecA* gene with the following sample order: site 1, site 2, site 3, negative control, and positive control.

The gene *tetW* confers resistance to tetracycline via ribosomal protection proteins. This gene is commonly present in intestinal and rumen environments (Scott et al. 2000), thus their presence may indicate fecal contamination (Pei et al. 2006). All three sites with low to high salinities were contaminated with fecal matter as indicated by the presence of *tetW* gene as well as the fecal coliform bacteria. The high salinity of 12 ppt did have some adverse effect on fecal coliform bacteria for few sampling events, but it did not have any adverse effect on antibiotic resistance genes. The water with intermediate salinity (site 2) showed the abundance of most of the antibiotic resistance genes compared to brackish and freshwater samples in this study.

This study showed the presence of several gram negative and gram-positive bacteria in the raw source water that are highly resistant to many commonly used antibiotics. The water also contained significant number of fecal coliform and heterotrophic aerobic bacteria at three different salinities. Salinity did not have any effect on the bacteria and antibiotic resistance genes. Recent studies show that incomplete metabolism in humans and improper disposal of antibiotics to sewage treatment plants has been a main source of antibiotic release into the environment (Rizzo et al, 2013). This gives bacteria enough time and sufficient contact to shield themselves by altering their genes and cellular mechanisms, favoring their growth and reproduction (Galvin et al, 2010). These genes can go on to infect the wildlife in the estuaries when the treatment plants discharge their treated wastewater. Since 2007, over 3 million hunting and fishing licenses have been sold in Louisiana (Naquin et al. 2015). This has the potential to spread to humans that come into contact and consume the wildlife here in the wetland, where the sewage is discharged. Antibiotics are among the most commonly used and successful group of pharmaceuticals used for human medicine (Bouki et al, 2013). Therefore, rapid spread in resistance to these antibiotics has caused concerns to both public and health professionals. This study demonstrated the presence of ARB and ARGs in all three-study

sites and the salinities of water whether high or low did not have any major effect on them. Other studies have also shown that wastewater treatment plants are a common source of resistance genes (LaPara et al. 2011; Everage et al. 2014; Naquin et al. 2015) to the natural environment.

4. Conclusions

This study clearly demonstrated the prevalence of ARB and ARGs in the water samples of wetlands of southeast Louisiana. The salinity of water whether it is high (12 ppt), low (0 ppt) or intermediate (6 ppt) did not have any major adverse effect on the presence of ARB and ARGs, but the intermediate salinity showed higher frequencies of ARGs. Bacterial load including fecal coliform and heterotrophic bacteria was consistently present in all salinities. The presence of organic carbon, nitrate and phosphate in all three sites facilitated the presence and growth of bacteria in these aquatic systems. Sewage treatment plants are not designed to remove antibiotic resistance genes and hence ARGs are spreading in the environment mainly through aquatic systems. This is an emerging problem and should be addressed by public health officials. The water with antibiotics may be exerting selection pressure and select for bacterial strains that have developed resistant to many antibiotics. Future work should identify the presence of selection pressure for antibiotic resistance and develop methods to reduce the ARGs in wetlands.

5. Acknowledgements

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