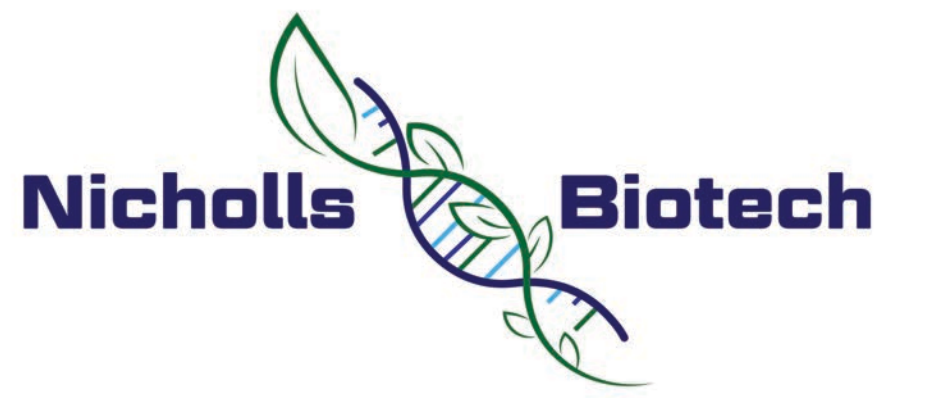# Presence of multi-drug resistant pathogens and antibiotic resistance genes in waterways and seafood populations of rural Southeastern Louisiana, USA

Ethan Naquin, J.P. Diagle, and Dr. Raj Boopathy, Biological Sciences, Nicholls State University

Introduction

Antibiotic resistance in bacteria is a natural phenomenon arising from the selective pressure due to exposure to clinical antibiotics and is also caused by misuse and overuse of antibiotics. This can cause considerable public health problems. Within the past decade alone, the Center for Disease Control has identified various bacteria as posing serious, urgent, and concerning threats to the US healthcare system due to its resistance to antibiotics (CDC, 2013). Improper usage and disposal of antibiotics by consumers, hospitals, industries, and sewage treatment plants has furthered the emergence of antibiotic resistance in the waterways of Southeast Louisiana, namely Bayou Lafourche and Bayou Terrebonne, a main source of drinking water impacting over 300,000 individuals that live along its banks. Furthermore, antibiotic resistant bacteria (ARB) has been found in raw sewage and treated sewage being released into the environment in the bayous. Ultimately, the ARB and antibiotic resistance genes (ARG) in the treatment plants affect wildlife, where the treated water is being released (Naquin et al., 2015). These water sources drain into the Gulf of Mexico, so monitoring antibiotic levels, ARB, and ARG, especially in seafood populations, are critical.

➢In Louisiana, one out of every seventy jobs are related to the seafood industry, and this region exports roughly 1 billion pounds of seafood each year at a value of 2.4 billion USD. Although the implications of an increasing presence of antibiotic resistance in the rural environment is alarming, there are no studies reported on this issue in Southeast Louisiana and the Gulf region.

Objectives:
1. Isolate and identify if antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARG) are present in samples collected from various seafood species.
2. Determine if bacteria isolated in pure culture can thrive in environments with various concentrations of antibiotics.
3. Analyze concentrations of antibiotics in our waterways.

Methods

1. Pure Cultures were isolated and identified from various seafood species

a. Colonies were isolated as pure cultures in Tryptic Soy Broth (TSB) from quadrant streak method on Tryptic Soy Agar (TSA) from Black Drum, Blue Crab, Shrimp, Oyster, Redfish, Speckled Trout, Cobia, Mahi-Mahi, and Amberjack.

b. BIOLOG identification technique was used to identify bacterial species.

2. Antibiotic resistance was observed using Kirby-Bauer Assay:

a. Pure Cultures were streaked as a bacterial lawn onto Mueller Hinton (MH) Agar and antibiotic discs were stamped onto the agar using an antibiotic disc dispenser. The following eight antibiotics were used: Tetracycline (TE 30), Streptomycin (S 10), Penicillin (P 10), Ampicillin (AM 10), Sulfamethoxazole-Trimethoprim (SXT), Vancomycin (VA 5), Bacitracin (B 10), and Clindamycin (CC 2).

b. Plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured. Bacteria were classified as Susceptible (S), Intermediate (I), or Resistant (R).

3. Antibiotic resistance genes were identified

a. Genome sequencing was performed on two isolates

b. BLAST was used for antibiotic resistance gene identification

4. Bacterial Growth Curve was performed

a. One bacteria resistant to tetracycline was chosen at random from a fish species to perform a growth curve at varying concentration of tetracycline (0 mg/L, 250 mg/L, 500 mg/L, 750 mg/L, and 1000 mg/L) in Basic Mineral Salt Media.

b. *Lactococcus garvieae* resistant to TE 30 was chosen from Redfish.

The bacterial growth was analyzed using HACH DR6000 spectrophotometer:

  – Ammonia: absorbance on Day 0 and Day 10 (initial and final)
  – Optical Density (OD): absorbance on Day 0 through Day 10

5. Antibiotic levels in local waterways were analyzed

- One site along Bayou Lafourche and one site along Bayou Terrebonne were sampled for antibiotic pollution.

Figure 1. Local Speckled Trout on ice. Samples were taken by swabbing the external extremities of the fish using a sterile cotton swab and sterile DI water.

Figure 2. Sites in Southeast Louisiana where water samples were analyzed. (1) Represents the Bayou Lafourche site, (2) represents the Bayou Terrebonne site, and (N) represents Nicholls State University.

Results

Kirby-Bauer Results:

Table 1. Kirby-Bauer Assay results of isolated colonies from various seafood species. (n) Denotes number of colonies. (S) stands for susceptible, (I) stands for intermediate, and (R) stands for resistant.

												Antibiotic Tested												
	TE 30			SXT			AM 10			P 10			CC 2			B 10			VA 5			S 10		
Seafood Sample	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Amberjack (n = 9)	89%	11%	0%	100%	0%	0%	55%	0%	45%	11%	11%	78%	11%	11%	78%	0%	22%	78%	22%	0%	78%	0%	44%	56%
Black Drum (n = 2)	100%	0%	0%	100%	0%	0%	100%	0%	0%	50%	50%	0%	50%	50%	0%	0%	100%	0%	100%	0%	0%	0%	100%	0%
Blue Crab (n = 4)	0%	100%	0%	50%	0%	50%	100%	0%	0%	0%	0%	100%	0%	0%	100%	0%	0%	100%	0%	0%	100%	0%	0%	100%
Cobia (n = 2)	0%	0%	100%	0%	0%	100%	0%	0%	100%	0%	0%	100%	0%	0%	100%	0%	0%	100%	0%	0%	100%	0%	50%	50%
Mahi-mahi (n = 6)	33%	0%	67%	67%	0%	33%	67%	0%	33%	17%	17%	66%	17%	0%	83%	17%	17%	66%	33%	17%	50%	0%	33%	67%
Redfish (n = 4)	75%	25%	0%	100%	0%	0%	75%	25%	0%	0%	0%	100%	25%	0%	75%	0%	25%	75%	25%	0%	75%	0%	100%	0%
Shrimp (n = 7)	0%	14%	86%	72%	14%	14%	14%	29%	57%	0%	14%	86%	0%	14%	86%	0%	14%	86%	0%	14%	86%	0%	14%	86%
Speckled Trout (n = 7)	29%	0%	71%	71%	0%	29%	14%	0%	86%	0%	0%	100%	0%	0%	100%	14%	0%	86%	14%	0%	86%	0%	57%	43%

Figures 3 and 4. Kirby-Bauer Assay of *Pseudomonas aeruginosa* isolated from Speckled Trout (left) and *Bordetella trematum* isolated from Blue Crab (right)

Gene Display:

Table 2. Gene display results from BLAST identification of *Lactococcus garvieae* and *Serratia marcescens* searching for antibiotic resistance genes
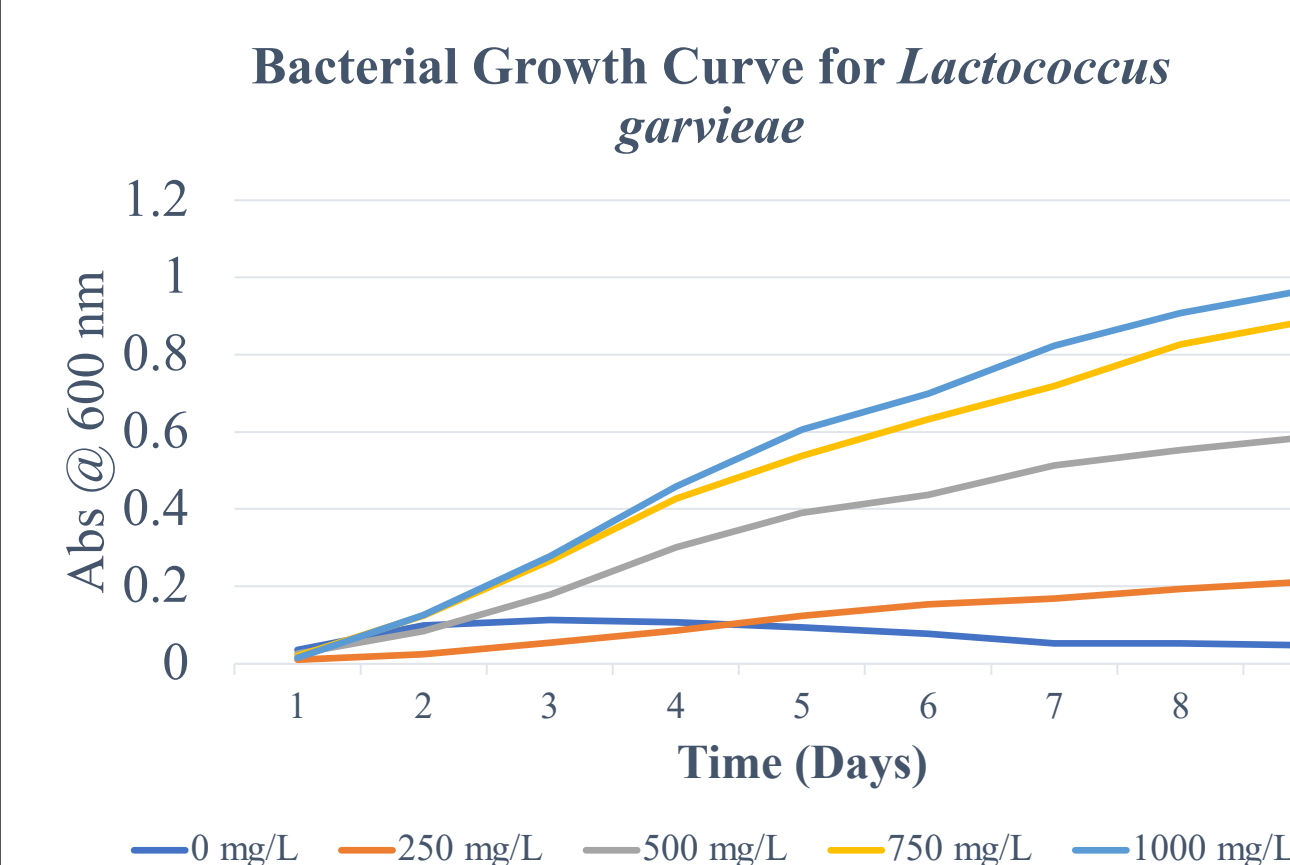
Molecular Data on Isolates

SAMPLE	#FILE	SEQUENCE	START	END	GENE	COVERAGE	%COVERAGE	%IDENTITY	DATABASE	ACCESSION
L. garvieae	contigs.fasta	NODE_14_length_16326_cov_117.352676	12795	14735	tet(S)_	1-1941/1941	100	99.9	resfinder	X92946
Serratia sp. (marcescans)	contigs.fasta	NODE_2_length_354217_cov_51.111576	226620	227060	aac(6')-Ic_1	1-441/441	100	92.52	resfinder	M94066
Serratia sp. (marcescans)	contigs.fasta	NODE_2_length_528804_cov_50.540965	522689	525809	oqxB_1	1-3121/3153	98.76	82.45	resfinder	EU370913
Serratia sp. (marcescans)	contigs.fasta	NODE_1_length_1471378_cov_49.649016	131499	132168	QnrB56_1	12-679/681	97.94	75.26	resfinder	JX259317
Serratia sp. (marcescans)	contigs.fasta	NODE_1_length_1471378_cov_49.649016	581077	582251	tet(41)_1	1-1175/1182	99.41	93.28	resfinder	AY264780

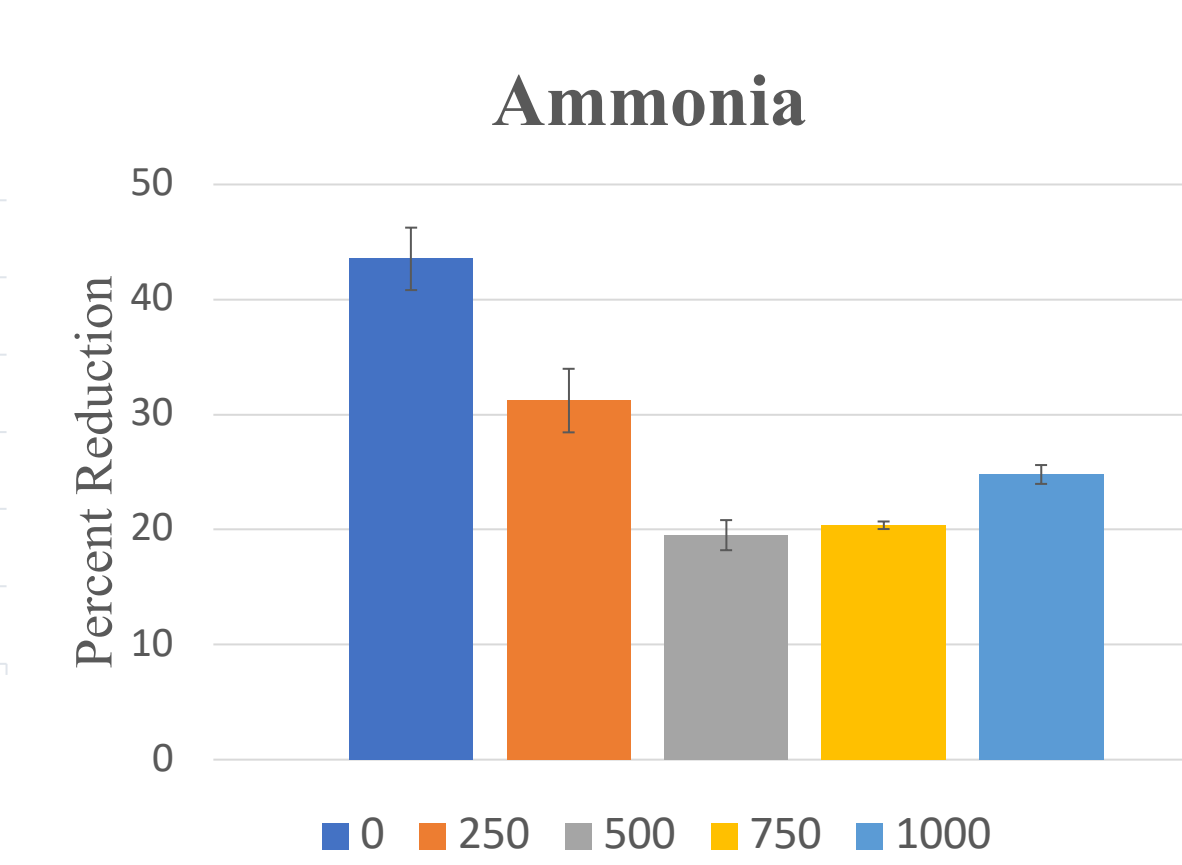**_Lactococcus garvieae_ from Redfish w/Tetracycline:**

Figure 5. Kirby-Bauer Assay of *Lactococcus garvieae* isolated from Redfish. This isolate, being resistant to tetracycline, will be used in the bacterial growth curve study.

Bacterial Growth Curve:

Bacterial Growth Curve for *Lactococcus garvieae*

— 0 mg/L — 250 mg/L — 500 mg/L — 750 mg/L — 1000 mg/L

Ammonia:

Ammonia

■ 0 ■ 250 ■ 500 ■ 750 ■ 1000

Figures 6 and 7. Bacterial Growth Curve (Abs @ 600nm) and percent reduction of Ammonia.

Figure 8. Red/orange metabolite production from growth curve experiment.

Results (cont.)

Water Sample Results:

October 2019 Antibiotic Concentration in Waterways

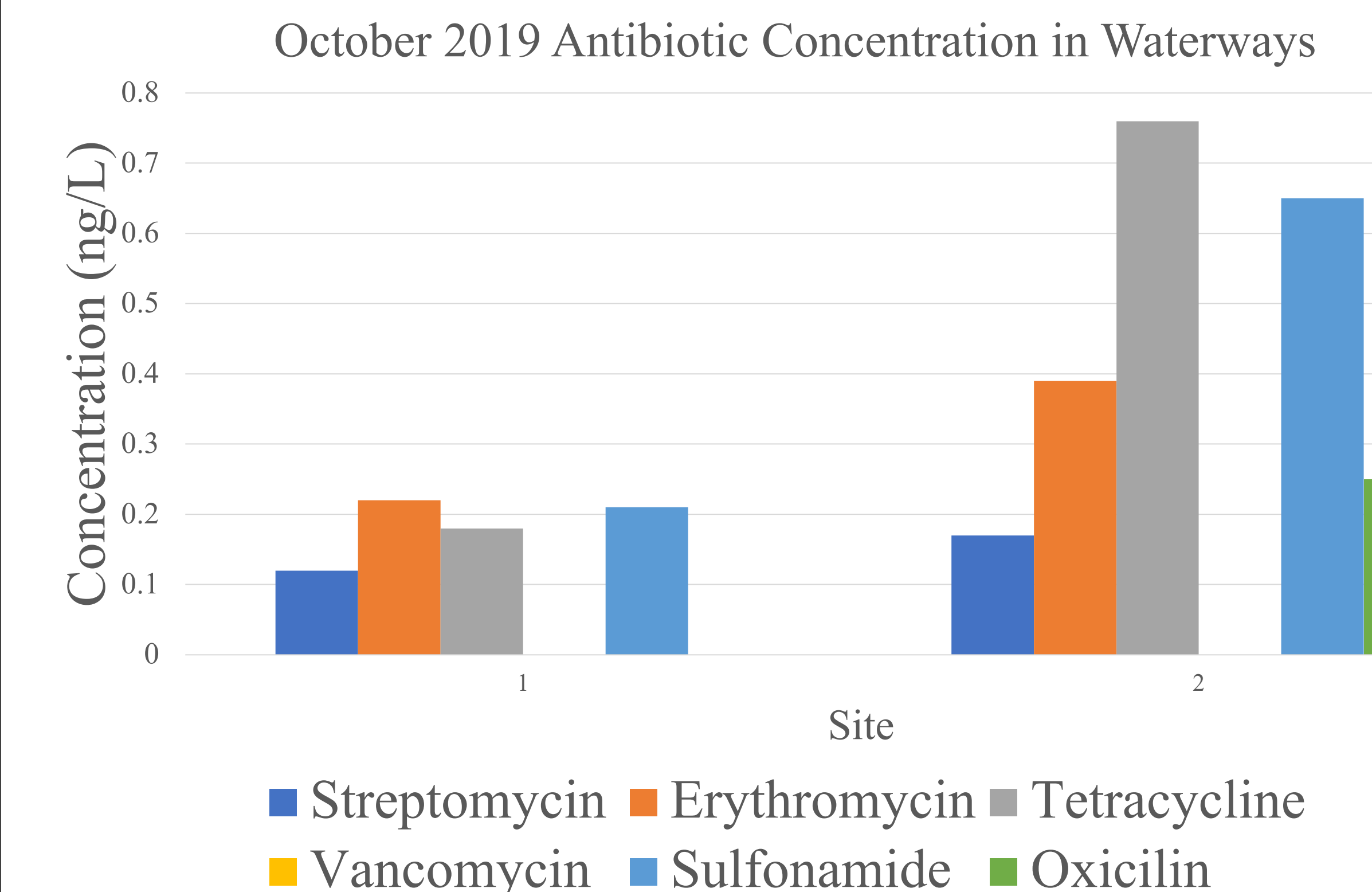■ Streptomycin ■ Erythromycin ■ Tetracycline ■ Vancomycin ■ Sulfonamide ■ Oxicilin

Figure 9. October 2019 antibiotic levels at Site 1 along Bayou Lafourche (29° 47' 48.48" N, 90° 49' 29.28" W) and Site 2 along Bayou Terrebonne (29° 35' 56.652" N, 90° 42' 48.96" W).

Discussion/Conclusions

It has been identified that there is antibiotic resistance in all seafood samples with resistance to all eight antibiotics tested in the Kirby-Bauer assay as well as the presence of antibiotic resistance genes in seafood samples tested. *Pseudomonas aeruginosa*, one of the multi-drug resistant pathogens isolated from seafood in this study, is on the list of bacteria in which new antibiotics are urgently needed (Lawe-Davies and Bennett, 2017). Moreover, the presence of antibiotics in our waterways will exert selection pressure on bacteria to develop antibiotic resistance. In the Growth Curve conducted on *Lactococcus garvieae* isolated from redfish, it was observed that it grew in all concentrations of tetracycline and had a higher growth at the highest concentration tested.

Historically, tetracycline has been used extensively in the medical field because of its broad spectrum of use as well as its low toxicity. Tetracycline is furthermore used in meat production and in cattle and chicken farms (Speer et al., 1992).
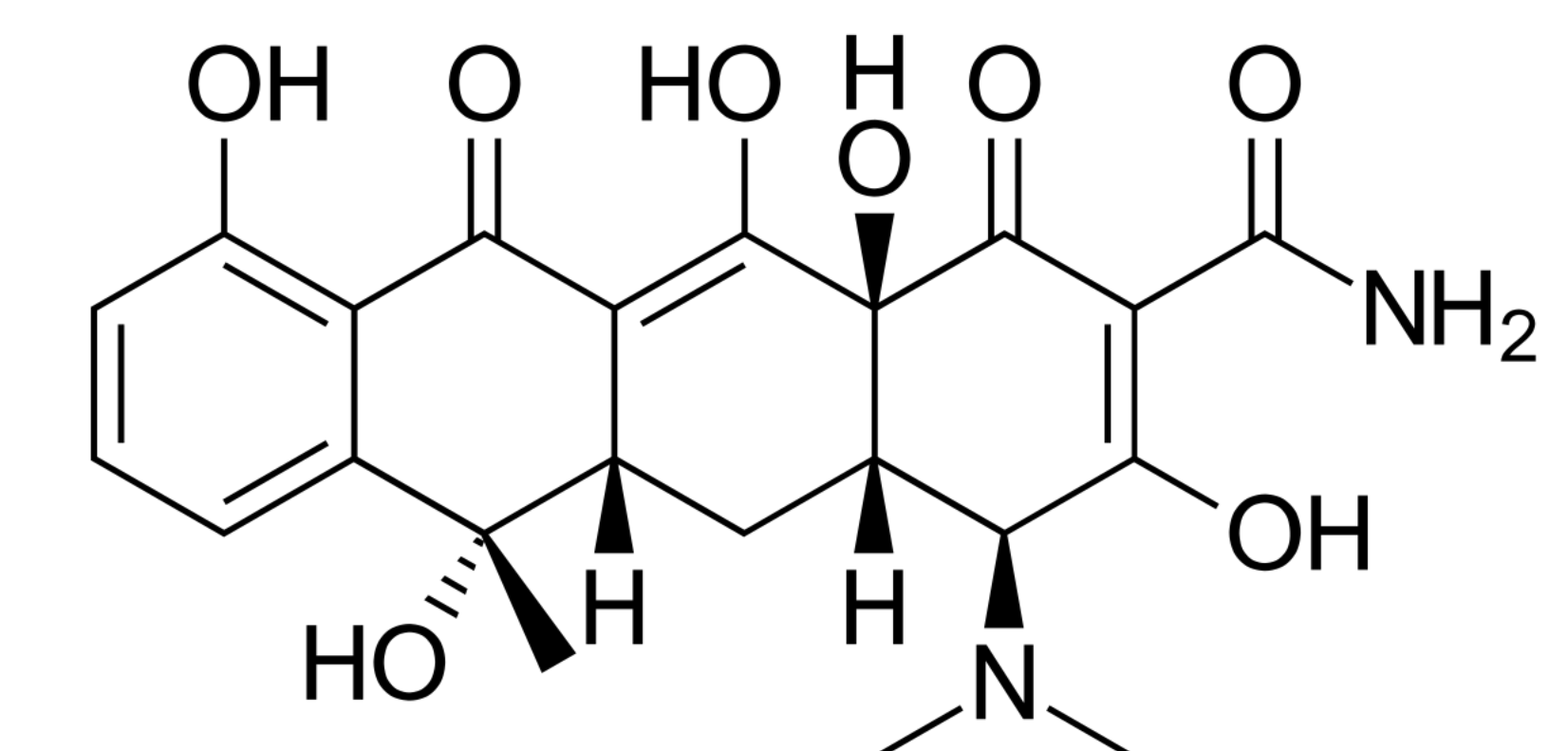

Figure 12: Tetracycline structure

It is highly recommended that all individuals who come into contact with our local seafood practice precautionary measures to avoid direct contact with seafood, especially if the individual has cuts or open wounds. It is recommended that extra sanitary procedures is to be exercised in the seafood industry, especially during the sale of seafood to the general public. Additionally, it is recommended that immunocompromised individuals exercise strict aseptic technique when handling local seafood. Further work will continue to isolate bacterial species.

References

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Acknowledgements