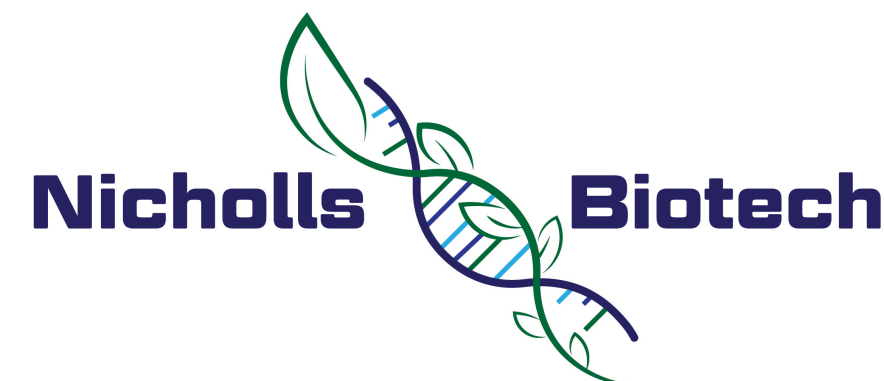




Preliminary analysis of antibiotic resistant bacteria, antibiotic resistant genes, and contaminants in sludge following Hurricane Ida.

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ABSTRACT

On August 29, 2021, Hurricane Ida made landfall as a catastrophic Category 4 hurricane in Southeast Louisiana and left extensive damage to homes and businesses, with almost total demolition of the power grid of lower Lafourche and Terrebonne Parishes. Due to the push of water into Barataria Bay, Terrebonne Bay, and Bayou Lafourche from storm surge, the Gulf Intracoastal Waterway overtopped and leaked sediments and sludge into a community in Larose, Louisiana. In this study, sludge samples were collected, and the occurrence of antibiotic-resistant bacteria was monitored. This survey of antibiotic-resistant bacteria and genes was accomplished using Kirby-Bauer Assay as well as polymerase chain reaction techniques for the presence of antibiotic resistance genes and for human polyomavirus BK, an anthropogenic marker for human fecal contamination. Sludge samples were also tested for levels of antibiotic contaminants, using HPLC, and heavy metal contaminants using ICP-OES. A community survey was used to survey the presence of health-related side-effects coinciding with the appearance of the sludge. The results of this study show the presence of human polyomavirus BK. The sludge also showed significant levels of antibiotics across the Larose neighborhood, along with the presence of *sul1* and *sul2*, sulfonamide resistance genes, and *tetA*, a gene for tetracycline resistance. The community survey showed a range of health-related side effects, with significant amounts of exposure of sludge to residents. ICP-OES analysis revealed an elevated presence of heavy metal contamination, exceeding recommended soil limits set by the World Health Organization.

INTRODUCTION

Antibiotic resistance has been previously recorded and studied by a laboratory in Southeast Louisiana; however, few studies have been performed in rural coastal areas regarding antibiotic resistance, with no studies on this issue involving the Gulf Intracoastal Waterway. Furthermore, there are no studies in the literature relating to a post-Hurricane environmental phenomenon of this magnitude and nature in Southeast Louisiana. Of particular interest to this study is the potential exposure of multi-drug resistant bacteria and contaminants to the local population in Larose, LA due to leaked sediments and sludge that resulted from the storm surge. Antibiotic resistance is a major global health problem as resistance in bacteria is increasing worldwide, and in contrast treatment options for infectious diseases are becoming rare. Wastewater treatment plants are considered as an ideal habitat for the evolution and spread of antibiotic resistance (Everage et al., 2014; Naquin et al., 2015). Once the bacteria become resistant to antibiotics, acquired antibiotic resistance genes are transferred through horizontal gene transfer between various species of bacteria (Salyers et al., 2004). Because of the flow of water and sediments of Hurricane Ida into North Larose, it is important to keep track of acute side-effects that could be incurred due to heavy metal contamination in the sludge that had settled in local waterways.

Objectives:

1. To determine the presence of multi-drug resistant (MDR) bacteria and isolate and identify antibiotic resistance genes (ARG)
2. Identify a biomarker for human fecal contamination via genes of human polyomavirus BK as an anthropogenic marker
3. Identify antibiotic and heavy metal contaminants and determine any possible health-related side effects due to sludge exposure in the community

METHODS

1. Sludge samples were collected from four sites in Larose, LA as shown in figure 1.

a. A small sample of sludge from each site were inserted into sterile TSB to propagate bacterial cultures. The tubes were incubated for 48 hours at 37C. 1 mL of the culture in consortium was vortexed with 9 mL phosphate buffered saline and plated, using the quadrant streak method, onto (TSA). Plates were incubated at 37 C for 24 hours. Colonies were selected and isolated as pure cultures.

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. Antibiotics used were tetracycline, sulfamethoxazole-trimethoprim, streptomycin, penicillin, ampicillin, erythromycin, bacitracin, meropenem, clindamycin, and ampicillin.
- b. Plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured. A standard laboratory caliper was used to measure the zone of inhibition.

3. Isolation and identification of pure culture.

- a. The sample was taken from the zone of inhibition around the SMX and TE discs. Subculture in TSA was propagated.
- b. Quadrant streaking onto tryptic soy agar (TSA) was done to obtain pure culture for ID. BIOLOG was used for bacterial ID.

4. Detection of biomarker for human fecal contamination, sulfonamide, and tetracycline resistance genes using PCR.

- a. MoBio PowerSoil DNA Extraction Kit was used for molecular genetics analysis.
- b. Process of amplifying DNA: 25µL Promega GoTaq Green Master Mix, 2 µL forward primer, 2 µL reverse primer, 16 µL nuclease-free water, and 5µL sample DNA (template) were added to PCR tubes. A 2% agarose gel was made with 2 µL EtBr. The gel was run at 120 V for 45 minutes.

6. Antibiotic contaminant identification using HPLC.

- a. Sludge was sonicated with HPLC-grade water, centrifuged, and analyzed at a reference lab.

7. Heavy metal contaminant identification using ICP-OES.

- a. 0.5 g sludge was digested with 2 mL HNO₃ and 2 mL H₂O₂ for 48 hours, filtered, analyzed on inductively coupled plasma-optical emission spectrometer (ICP-OES). Pb, Cd, Cr, and Ni were analyzed.

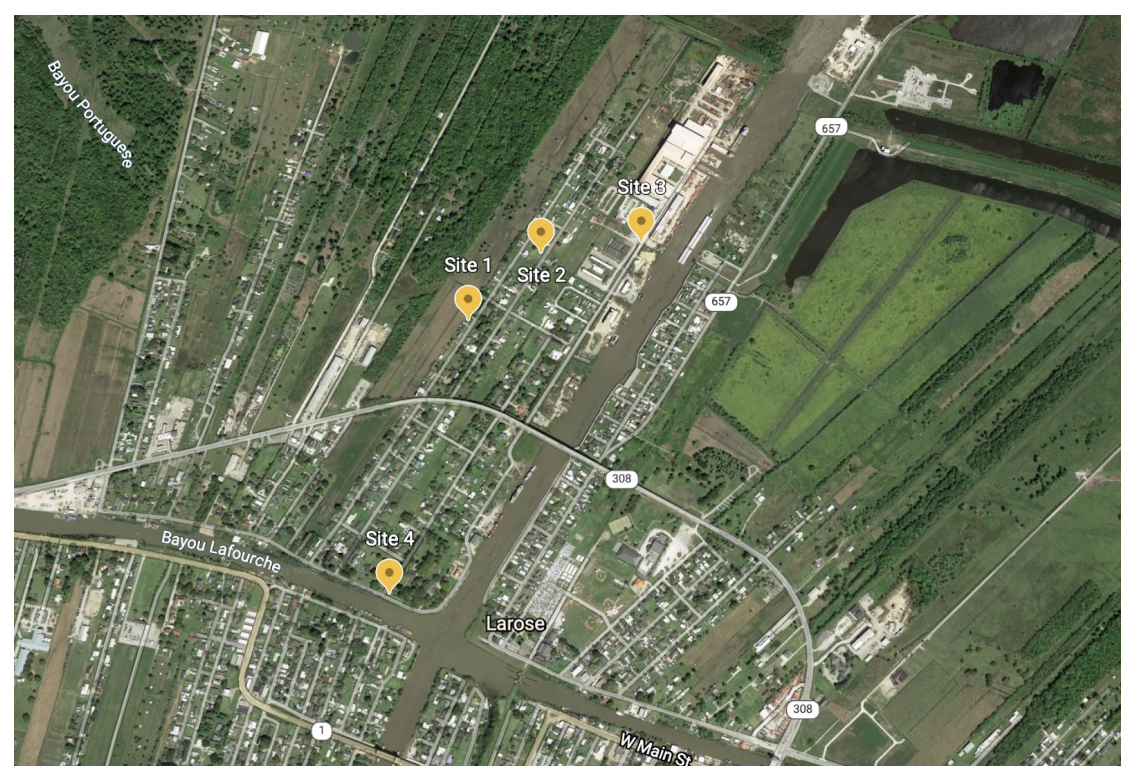


Figure 1. Sample sites in Larose, Louisiana, U.S.A. close view using Google Earth.

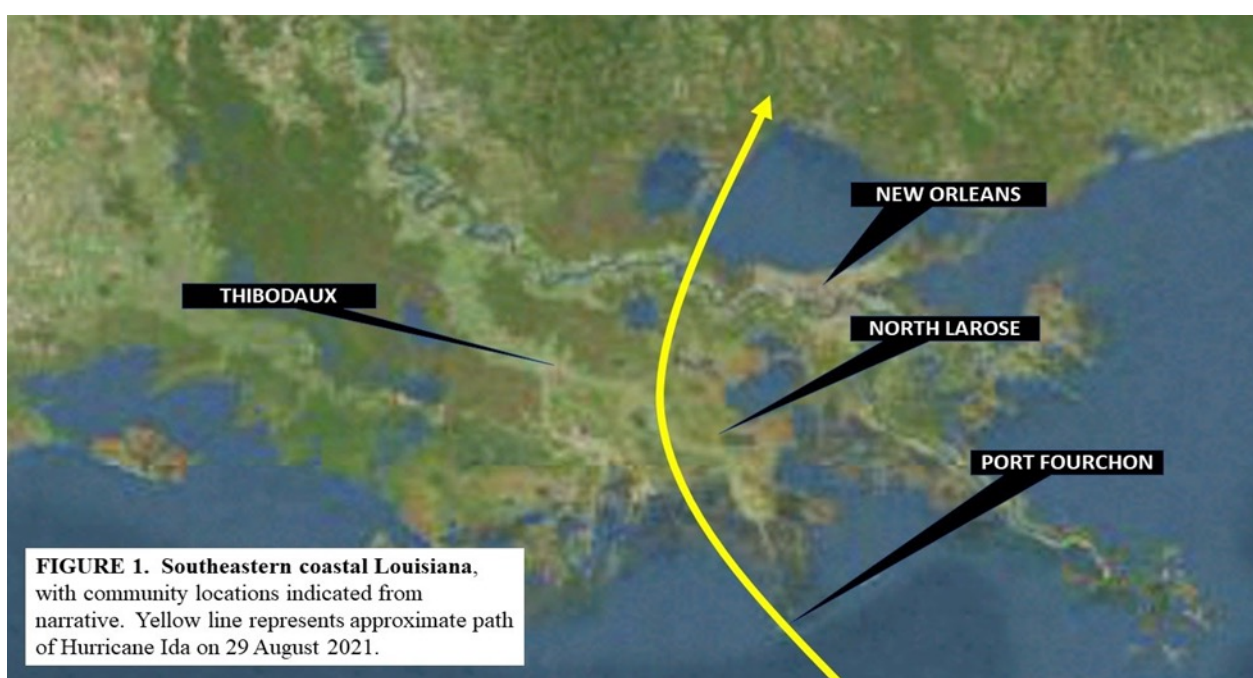
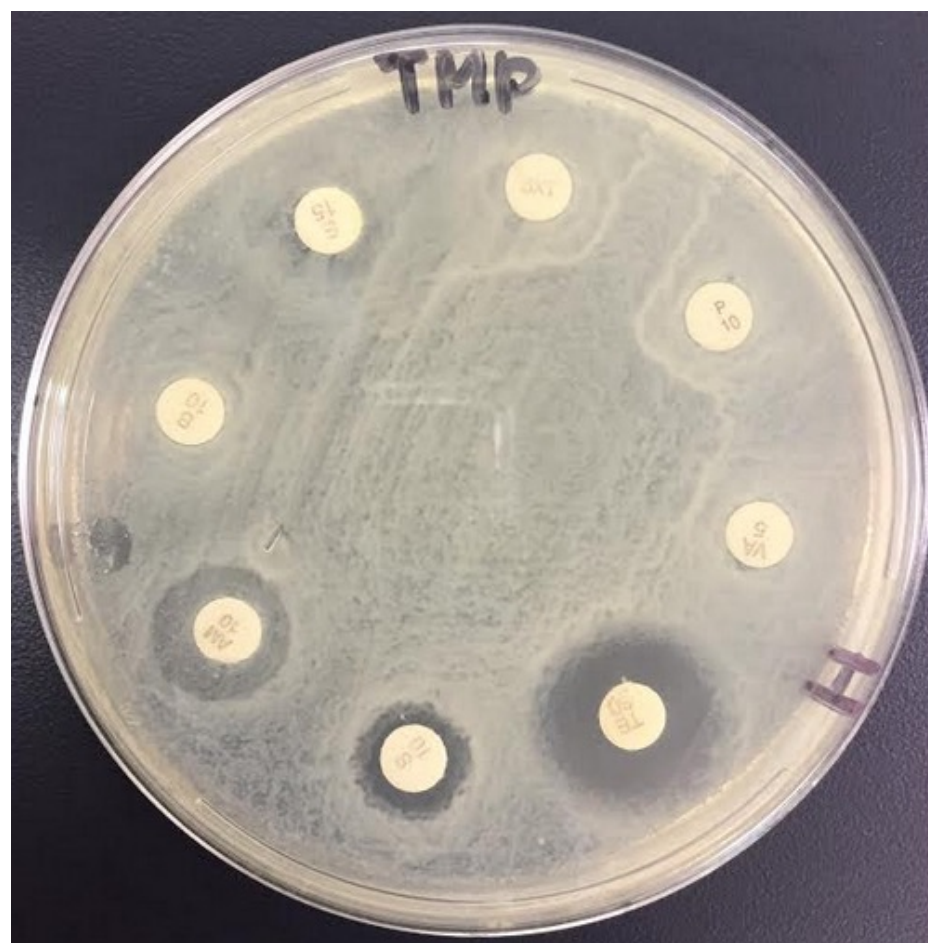


Figure 2. This picture shows the southeastern coast of Louisiana with community locations indicated in the narrative. The yellow line represents the approximate path of Hurricane Ida on August 29, 2021.

RESULTS

Antibiotic Resistance Assay in one of the isolates:



Figures 3 and 4. Kirby-Bauer Antibiotic Disc plate and isolation of pure culture

Kirby-Bauer and BIOLOG Results:

Table 1. Kirby-Bauer and BIOLOG result from multi-drug resistant bacterium from Sites 1, 2, 3, and 4.

Sample Site: 1		
Bacterial Identification: <i>Bacillus cereus</i>		
Antibiotic	Zone of Inhibition (mm)	Interpretation
CC2	16.98	I
SXT	0	R
E15	18.62	I
TE30	16	I
S10	16.59	S
P10	0	R
MEM10	16.28	S
AMC10	8.76	R
AM10	0	R
OX1	0	R

Sample Site: 2		
Bacterial Identification: <i>Proteus mirabilis</i>		
Antibiotic	Zone of Inhibition (mm)	Interpretation
CC2	0	R
SXT	13.74	I
E15	0	R
TE30	0	R
S10	0	R
P10	0	R
MEM10	23.87	S
AMC10	0	R
AM10	12.24	R
OX1	0	R

Sample Site: 3		
Bacterial Identification: <i>Bordetella avium</i>		
Antibiotic	Zone of Inhibition (mm)	Interpretation
CC2	0	R
SXT	0	R
E15	20.54	I
TE30	7.58	R
S10	0	R
P10	0	R
MEM10	25.2	S
AMC10	24.4	S
AM10	21.62	S
OX1	0	R

Sample Site: 4		
Bacterial Identification: <i>Pseudomonas aeruginosa</i>		
Antibiotic	Zone of Inhibition (mm)	Interpretation
CC2	0	R
SXT	0	R
E15	0	R
TE30	0	R
S10	6.85	R
P10	0	R
MEM10	28.62	S
AMC10	0	R
AM10	0	R
OX1	0	R

Human Polyomavirus Results:

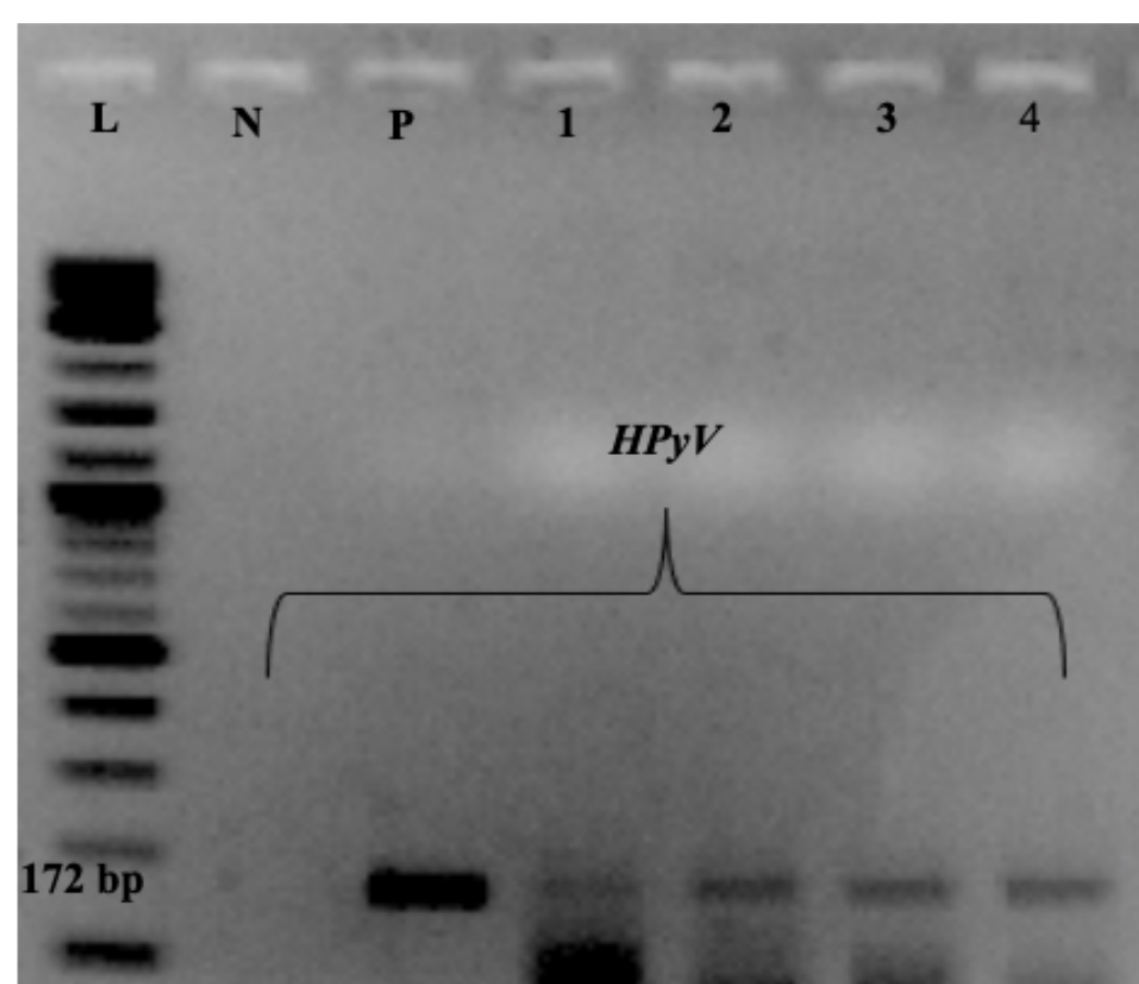


Figure 5. Figure 5. Gel showing presence of human polyomavirus in sludge sample sites. L = DNA ladder, N = negative control, P = positive control, 1-4 = sludge sites, respectively.

ARG Results:

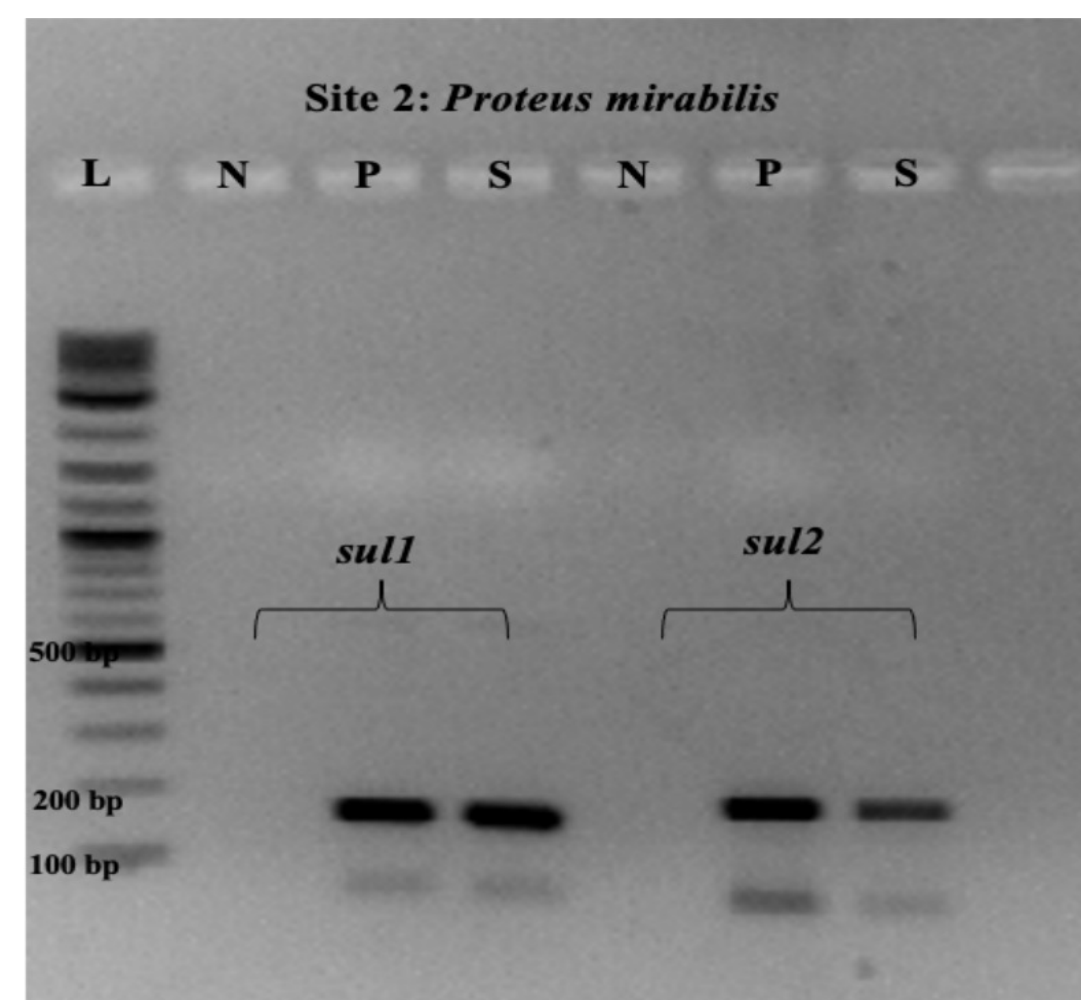
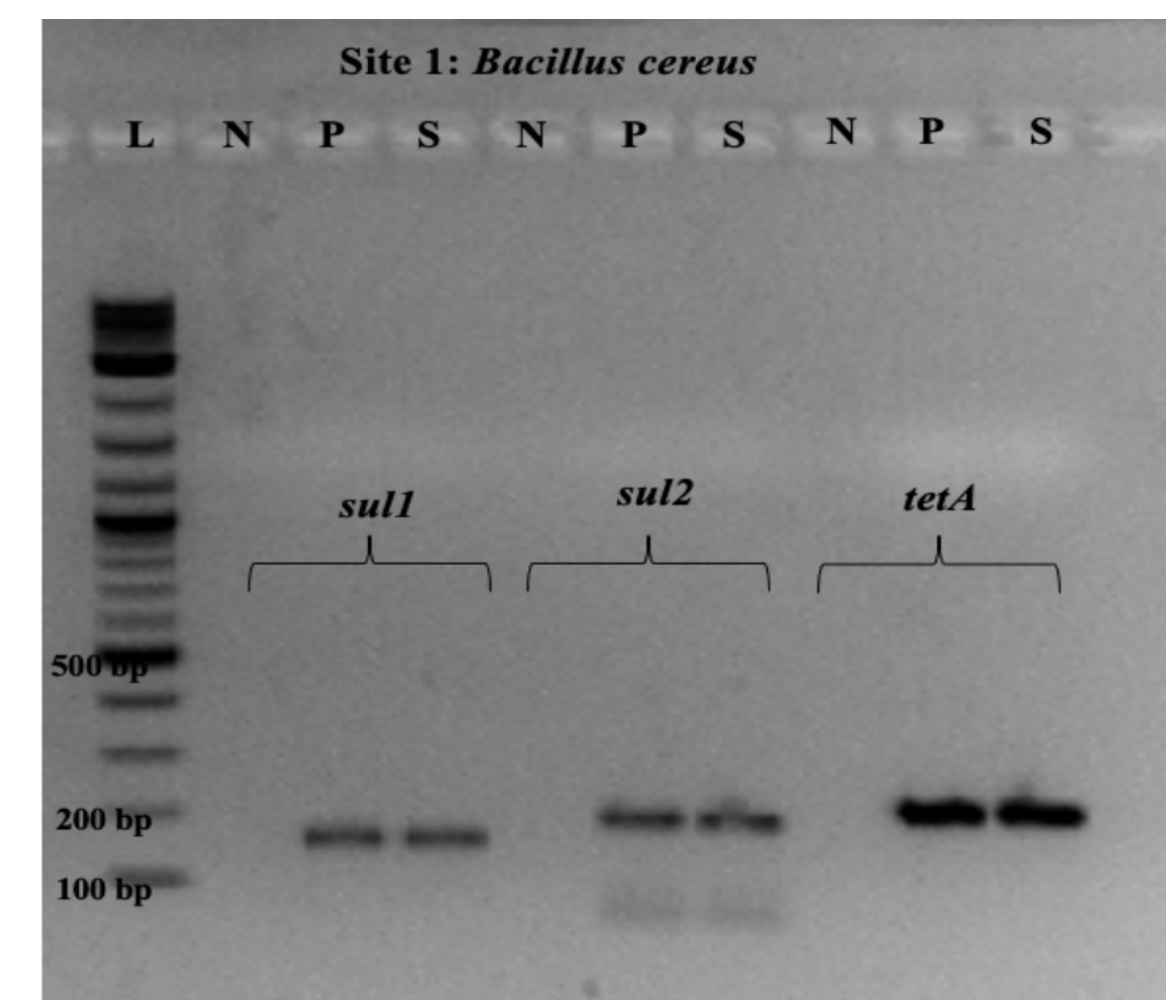


Figure 6. Gel depicting ARG bands from Sites 1, 2, 3, and 4. L = DNA ladder, N = negative control, P = positive control, S = sample. Brackets indicate gene product.

RESULTS (Cont.)

Antibiotic Concentration in the Sediment Samples:

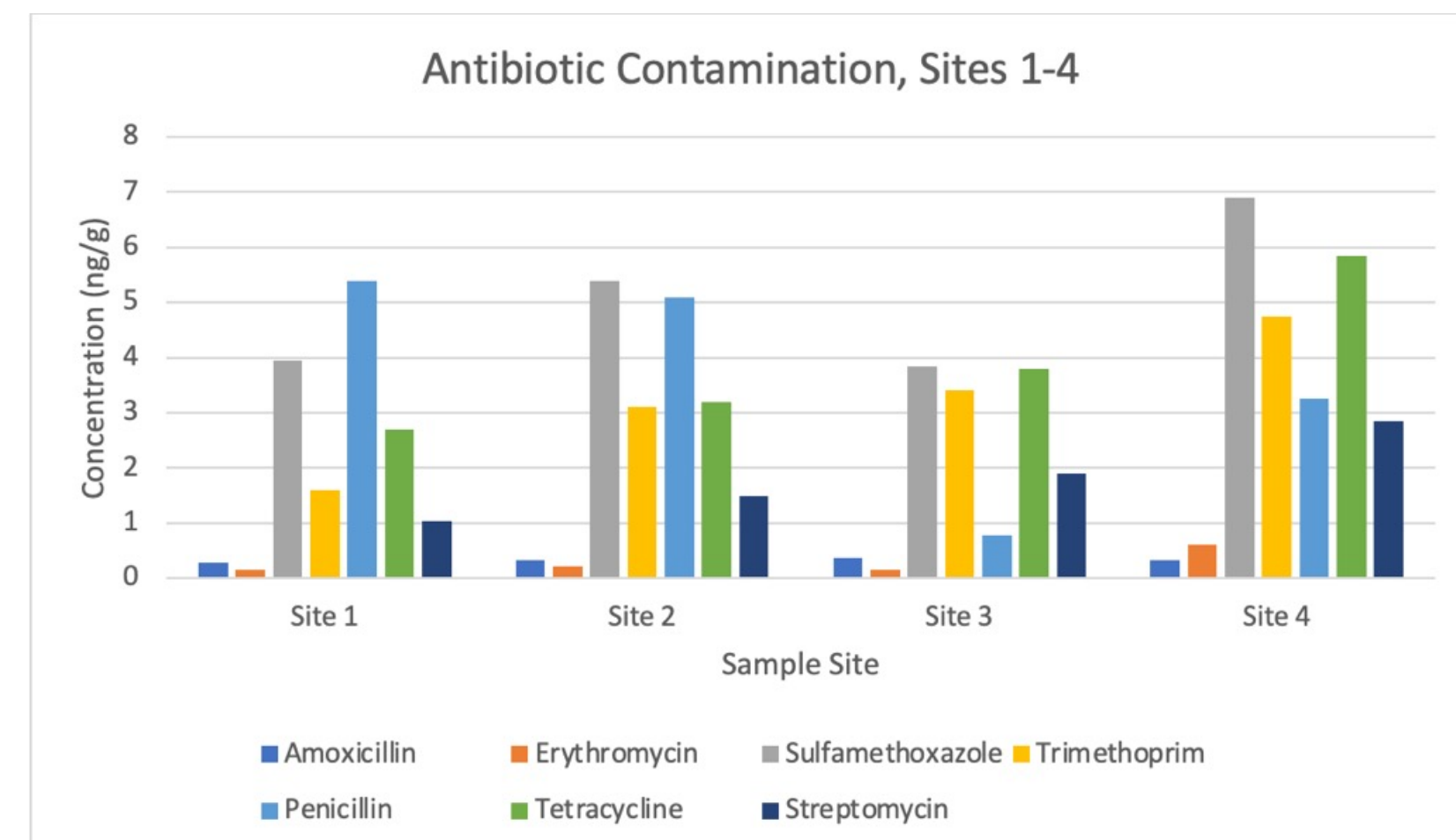


Figure 7. Antibiotic contamination per sample site in ng/kg.

Heavy Metal Concentration in the Sediment Samples:

Table 2. Heavy metal contaminants in sludge samples (ppm).

Sample	Pb (ppm)	Cd (ppm)	Cr (ppm)	Ni (ppm)
1	1.954	0.00346	0.99504	1.20942
2	1.89591	0.07113	0.72637	1.06054
3	1.69745	0.04383	0.66761	1.00423
4	1.94173	0.06288	0.55744	0.88394

DISCUSSION

Bacteria resistant to a wide array of antibiotics were observed across the four sample sites in the Larose community. Four multi-drug resistant bacteria were identified, and the three targeted antibiotic genes were identified. The *sul1* and *sul2* genes encode for enzymatic modifications against sulfonamide-derived antibiotics. The *tetA* gene encodes for an efflux pump protein against tetracycline-derived antibiotics. All bacteria isolated at least displayed both *sul1* and *sul2*. This shows support for a significant contribution of horizontal gene transfer between bacteria in acquiring these genes. All organisms expressing both *sul1* and *sul2* are expressing more than one resistance gene for the same class of antibiotics. This result is very uncommon.

Additionally, gene display revealed the presence of human polyomavirus BK in all four sample sites, confirming the presence of human fecal contamination in the sludge. This source could have come from sources ranging from the Gulf Intracoastal Waterway, Bayou Lafourche, or the failure of the Larose sewage system or home septic tanks that could have been destroyed and leaked human fecal contaminants into the sludge during the flooding event (Martinez et al. 2019). The presence of elevated levels of heavy metals in the sediments exceeded recommended targets set by the World Health Organization (Kinuthia et al. 2020).

CONCLUSION

- Four multi-drug resistant bacteria were identified.
- Three targeted antibiotic resistance genes were identified.
- Human fecal contamination was confirmed to be present.
- Antibiotic contamination present and heavy metal contaminations present.
 - Heavy metal levels above World Health Organization suggested limits
- Suggested to limit exposure to sludge
- Public should be warned about wearing protective gears during clean-up
- Future studies needed
 - Monitor ARB, ARG, and contaminate levels
 - Monitor growth curve in MDR

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