Science of the Total Environment 789 (2021) 147818

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Klebsiella spp. isolates from Houston bayous exhibit increased resistance to lead exposure and possess enhanced virulence potential

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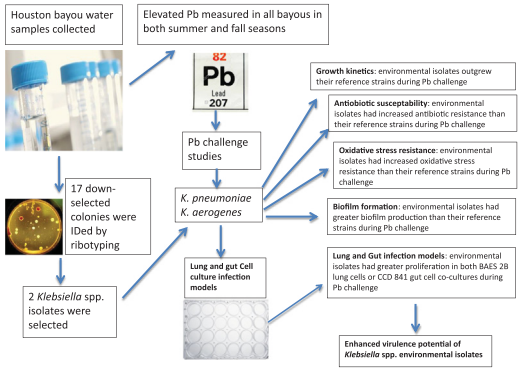
HIGHLIGHTS

- Mustang Bayou had elevated Pb levels as well as the greatest bacterial loads.
- *Klebsiella* isolated from Houston area bayous had increased antibiotic resistance.
- Pb levels measured in Houston bayous in 2018 were between 0.28 and 11.5 ppb.
- Pb-exposed *Klebsiella pneumoniae* produced more biofilm than its reference strain.
- Pb-exposed *Klebsiella* spp. proliferated better in lung and gut cell co-culture.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 26 March 2021
Received in revised form 6 May 2021
Accepted 12 May 2021
Available online 24 May 2021

Editor: Filip M.G. Tack

Keywords:
Environmental isolate
Eukaryotic co-culture
Lung infection model
Evolutionary adaptation

ABSTRACT

Houston watersheds are susceptible to microbial contamination as well as chemical contaminations from bordering industrial facilities. Bacterial loads in various Houston bayous were determined, and pathogenic Gram-negative bacteria were isolated for characterization. Isolates included *Klebsiella aerogenes* and *Klebsiella pneumoniae*. To determine whether environmental exposures to lead (Pb), measured in our Houston bayou samples, resulted in bacterial adaptations, we compared growth kinetics, biofilm production, oxidative stress resistance, and eukaryotic co-culture growth of environmentally isolated *K. aerogenes* and *K. pneumoniae* to their respective commercially acquired reference strains. Interestingly, the *K. aerogenes* environmental isolate displayed significantly better growth than the reference strain in the presence of 50 ppb of Pb. Unexpectedly, we did not observe any differences in biofilm production of the aforementioned strains when challenged with a range of Pb (0.5–50 ppb). However, when comparing our *K. pneumoniae* environmental isolate to its reference strain, there were significantly higher levels of biofilm produced by the environmental isolate when challenged with Pb concentrations of 10 and 50 ppb. When grown in eukaryotic cell co-culture with either BAES 2B lung cells or CCD 841 colon epithelial cells in the presence of 20 ppb Pb, the environmental isolates of *K. aerogenes* and *K. pneumoniae* had a significantly higher fold-increase over 6 h than their respective reference strains. Taken together, the environmentally isolated *Klebsiella* spp. appeared to be more Pb-tolerant than their respective reference strains, a possible environmental adaptation. Such enhanced tolerance can promote environmental persistence and increase the possibility of causing human disease.

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https://doi.org/10.1016/j.scitotenv.2021.147818
0048-9697/

1. Introduction

Houston is the fourth-largest city in the United States, with a population nearing 6 million. Surrounding the city are run-off watersheds and internal wastewater outflows, which could promote fecal and chemical contamination (Petersen et al., 2009). With regards to chemical contaminants, trace metals are of particular concern. More specifically, exposure to metals above certain thresholds can become deleterious to all living organisms, including microorganisms (Järup, 2003). Due to increased urbanization surrounding Houston, there is a concomitant increase in impervious surfaces, which intensifies the frequency of flooding while promoting increased runoff of environmental pollutants (Bhandari et al., 2017; Sridhar et al., 2020; Bukunmi-Omidiran and Maruthi Sridhar, 2021). Anthropogenic activities have significantly impacted Houston bayou water quality, which eventually flow into Galveston Bay (Jensen et al., 1999). The trace metal lead (Pb) is identified as one of the ten chemicals of major public health concern in the world (WHO, 2019). Worldwide, Pb exposure in 2017 accounted for 1.06 million deaths and left 24.4 million people with disability, of which 63.2% had intellectual disability, 15.9% had heart diseases, and 6.2% had stroke (WHO, 2019). Pb is a cumulative toxicant, and it affects multiple body systems such as brain, liver, kidney, and bones, particularly among young children (WHO, 2019). Children of preschool age and unborn fetuses are most vulnerable to Pb exposure, contamination, and toxicity (CDC, 1992). Increased flooding events in recent years has resulted in elevated metals in the Houston Bayou waters, among which Pb is found to be significantly higher (Sridhar et al., 2020; Bukunmi-Omidiran and Maruthi Sridhar, 2021). Additionally, Galveston Bay contains one of the largest concentrations of petroleum and chemical industries. Beyond chemical contaminants, alarmingly, nearly 50% of streams in the Houston and Galveston area are negatively impacted by elevated levels of indicator bacteria (TCEQ, 2014).

In that vein, the Houston Ship Channel is one of the most polluted water bodies in the United States (EPA, 1980). Despite that fact, surprisingly, many greater Houston-area bayous serve as a source of recreation for Houstonians. Therefore, the presence of pathogenic bacteria and/or environmental toxicants in the water pose significant human health threats (Stewart et al., 2008). To assess water safety, indicator bacteria (*Escherichia coli* and coliform bacteria) are often used to suggest the presence of enteric bacterial pathogens, like *Enterococcus* spp. (Franz et al., 1999; Ferguson and Signoretto, 2011; Suthar et al., 2009). Typically, indicator bacteria are used to determine water quality/safety for recreational activities, agriculture, industrial activities, and municipal water supplies. Previously, *E. coli* loads and dissolved oxygen levels were measured over an extended period in a Houston watershed to determine the influence of urban development on small watersheds (Quigg et al., 2009). However, urban flooding can significantly impact the local microbial landscape and increase the risk of waterborne infection in flooded areas. To evaluate that, *E. coli* and fecal coliform levels were measured in Houston bayou water samples over a 6-month period following Hurricane Harvey in 2017 and were compared to historical levels. Not only were *E. coli* levels elevated post-flood but also antibiotic resistance gene expression was upregulated, suggesting that the elevated bacterial load could be largely antibiotic resistance (Pingfeng et al., 2018).

Also members of the gut bacteria family, the *Klebsiella* genus includes two notable human pathogens: *K. pneumoniae* and *K. aerogenes*. Both *Klebsiella* spp. produce polysaccharides capsules; however, only *K. aerogenes* is motile. Whereas *K. aerogenes* is primarily an opportunistic human pathogen capable of causing urinary tract infections, *K. pneumoniae* is a bona fide human pathogen capable of causing a wide-range of human disease ranging from pneumonia to bacteremia (Wang et al., 2020). Of growing concern are *K. pneumoniae* strains that are carbapenemase resistant (Ernst et al., 2020) or are hyper-virulent, capable at striking otherwise healthy adults in the community (Russo and Marr, 2019). In fact clinical *Klebsiella pneumoniae* carbapenease (KPC) resistant *K. aerogenes* (aka *Enterobacter aerogenes*) have also been isolated in Brazil (Tuon et al., 2015). Further, drug resistance concerns have prompted the monitoring of *K. pneumoniae* in wastewater outflows. In fact extended spectrum β-lactamase producing *K. pneumoniae* isolates from a municipal wastewater treatment plant in Brno, Czech Republic have been identified (Dolejska et al., 2011). Based on those concerns, this work sought to isolate and evaluate *Klebsiella* spp. isolated from metal-polluted Houston watersheds. Further, this study also sought to: 1. Determine metal contaminant levels in water samples of Houston watersheds, 2. Quantify bacterial loads in Houston watersheds (i.e., Horsepen, Mustang, Dickinson, and Cypress Creek Bayous), 3. Isolate Gram-negative enteric pathogens (including the pathogenic *Klebsiella* spp.), and 4. Evaluate the impact of Pb exposure on *Klebsiella* spp. isolates' growth kinetics, biofilm production, oxidative stress response, and proliferation in modeled lung and gut environments.

2. Material and methods

2.1. Study area and sample collection

A total of 63 water samples were collected (triplicate samples at each site) during summer and fall of 2018 in Cypress Creek, Horsepen, Mustang, and Dickinson Bayous (Fig. 1). The air temperatures at the sampling locations ranged from 27–32 °C and 11–13 °C, respectively. The longitude and latitude of each sampling location was recorded using a handheld Global Positioning System (GPS) receiver (Table 1). Samples were collected in 1000 mL sterile plastic bottles using a dipper. After the samples were collected, they were stored at 4 °C for downstream bacterial and chemical analysis.

2.2. Bacterial strains and eukaryotic cell lines and culture conditions

For all studies, the metal salt, lead (II) nitrate (Carolina Biological Burlington, NC USA), was used at concentrations of 0.5, 1, 5, 10, and 50 μg/mL in sterile dissolved water creating the corresponding concentration in parts per billion (ppb). Reference strains *K. pneumoniae* 155095A (Carolina Biological), and *K. aerogenes* 155030A (Carolina Biological) were purchased and were compared to two enteric environmental isolates *K. pneumonia* (MB 56 km, Mustang Bayou) and *K. aerogenes* (DKB 9.5 km Dickinson Bayou) in all experiments. For all experiments, Luria-Bertani (LB) medium (Sigma-Aldrich) was used to grow bacterial strains with agitation (250 rpm) at 37 °C. All absorbance readings were taken using a BioTek™ ELx800™ microplate reader at 600 nm. Altogether, from Dickinson Bayou, *Pseudomonas fluorescens*, *K. aerogenes* (as mentioned above) *Serratia marcescens*, *Pseudomonas lini*, and *Pseudomonas oleovorans* were isolated. From Mustang Bayou, *K. pneumoniae*, *Pseudomonas mendocina*, *Bacillus megaterium*, *Planomicrobium chinense*, and *Lysinibacillus* sp. strain 7 were isolated. From Cypress Creek Bayou, *Chryseobacterium jeonii*, *Pseudomonas stutzeri*, *Brevibacterium casei*, and *Bacillus indicus* were isolated. Finally, from Horsepen Bayou, *Pseudomonas nitroreducens*, *B. megaterium*, and *Enterobacter aerogenes* were isolated.

The eukaryotic cell lines used in this study, BEAS-2B cells (ATTCC CRL-9609) and CCD 841 (ATCC CRL 1790), were grown in DMEM medium (Thermo Fisher 11965092) supplemented with 10% FBS (Thermo Fisher 16140071) and 5% pen-strep (Thermo Fisher 15140122). Cells were cultured in tissue culture flasks and maintained at 37 °C in a humidified atmosphere with 5% CO_2. Flasks medium was changed every 3 days.

2.3. Antibiotic susceptibility plate assay

A previously published method (Bauer et al., 1966) was employed with some minor modifications. Ampicillin (10 μg), Gentamicin (10 μg), Streptomycin (10 μg), Tetracycline

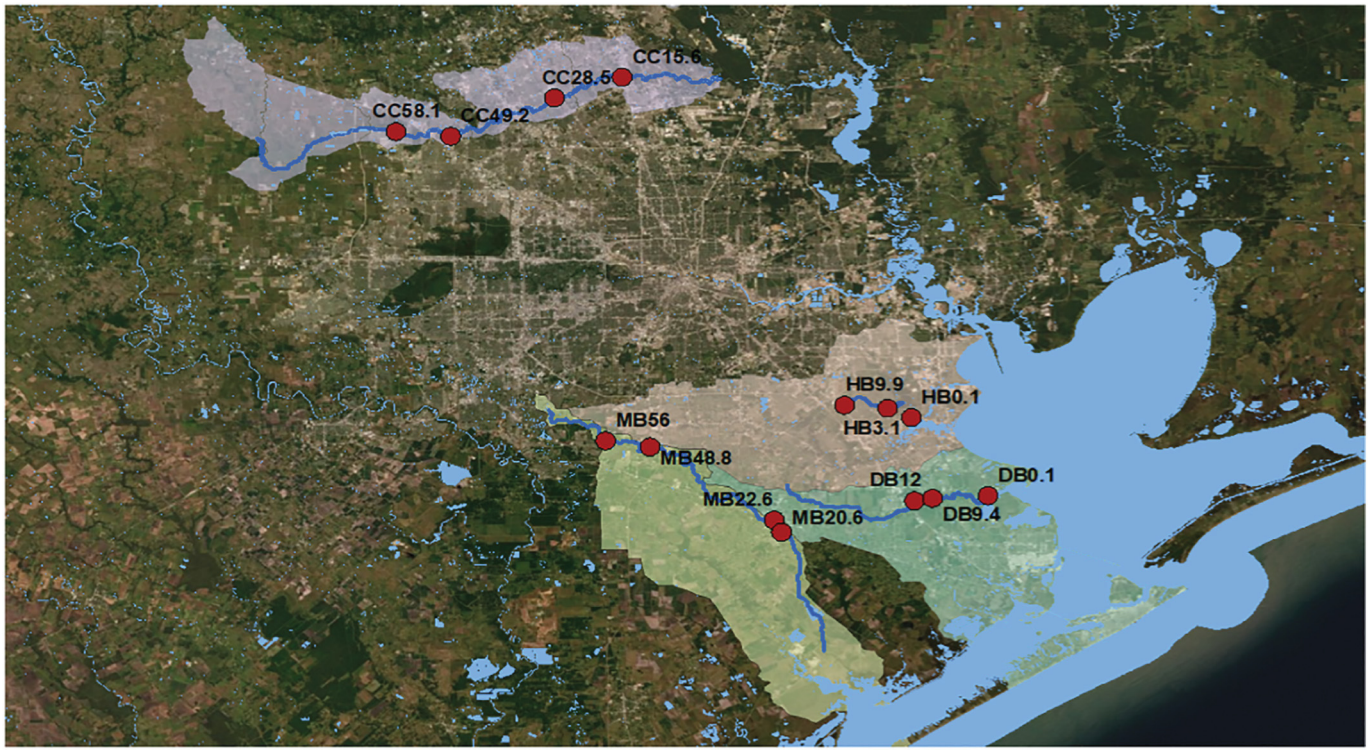

Fig. 1. GIST geographical map of water sample locations. Water samples were collected from the Cypress Creek, Dickinson, Horsepen, and Mustang Bayous during the summer and fall of 2018. Locations are indicated as distances from the bayou mouths. All samples were collected in triplicate.

(30 μg), and Erythromycin (15 μg) infused disks were placed equidistantly, and zones of inhibition were measured in millimeters.

2.4. Heavy metal contaminant measurements

Concentrations of heavy metal elements in water samples were estimated by using inductively coupled plasma mass spectrometry (ICP-MS). Following treatment with 10 mL nitric acid (HNO_3), samples were placed into Mars 6 microwave vessels and digested using the EPA 3015a method for water (Dirk et al., 1999). Digested water samples were then transferred to 50 mL centrifuge tubes for an additional 24 h to allow for further digestion and settling of solids. Then, 0.2 μL of supernatant was diluted in water and analyzed by ICP-MS following calibrations with appropriate standards.

2.5. Bacterial enumeration and colony isolation

The broad-based Luria Bertani (LB) agar (BD Difco™) medium was used to culture total bacteria while the differential and selective MacConkey agar (Difco®) medium was used to cultivate Gram-negative enteric bacteria. Bacterial enumeration was carried out, in triplicate, via 10-fold serial dilutions followed by plate counts. 17 colonies were down-selected (based upon phenotypic characteristics) from each of the 4 bayous evaluated to generate single colony-isolates for downstream characterization and identification.

Table 1
Houston bayou sampling locations are indicated by longitude and latitude as recorded by a handheld Global Positioning System (GPS) receiver. Total and enteric bacterial loads (cfu/mL) are included for summer and fall collections. Lead (Pb) levels were measured by ICP-MS analysis and are indicated in parts per billion (ppb).

Bayous	Location from mouth	Latitude	Longitude	Total CFU/mL		Enteric CFU/m	
				Summer	Fall	Summer	Fall
	HB9.9 km	29.5843	−95.1549	3400	1220	1200	330
Horsepen	HB3.1 km	29.5799	−95.1011	7500	1100	1800	300
	HB0.1 km	29.5673	−95.071	11,400	1300	2000	700
	DKB12 km	29.4533	−95.071	10,000	2600	2040	990
Dickinson	DKB9.4 km	29.4602	−95.0446	14,000	2700	2500	900
	DKB0.1 km	29.4602	−94.9750	19,000	3100	3480	1670
	MB56 km	29.5355	−95.4551	24,800	38,000	2500	1500
Mustang	MB48.8 km	29.5269	−95.3996	32,000	19,500	3000	2000
	MB20.6 km	29.4102	−95.2342	16,900	15,000	3300	2500
	CC58.1 km	29.9594	−95.7182	2100	700	940	100
Cypress Creek	CC49.2 km	29.9529	−95.6495	1500	270	980	55
	CC28.5 km	30.0057	−95.5195	2800	1000	980	120

Criteria	Fecal coliform
EPA	200 cfu/100 mL
TCEQ	104 cfu/100 mL

2.6. 16S rDNA PCR amplification

Colony PCR reactions were set up as previously described (Rosenzweig and Jejelowo, 2011). In short, 25 μL reaction volumes were prepared using a 2× concentrated Taq master mix (New England Biolabs cat #: M0270L). A single isolated colony of the cultured bacteria was used as template. Universal 27F forward (AGAGTTTGATCCTGGC TCAG) and 1387R reverse primers (GGGCGGGTGTACAAGGC) (Ferris et al., 2007; Rosenzweig and Jejelowo, 2011), were employed to amplify a conserved DNA region encoding part of the 16S ribosome. Reactions were carried out on the Bio-Rad T100 thermal cycler for 35 cycles. PCR amplicons of 1360 nucleotides were confirmed on a 0.7% agarose gel. Sequences were then checked against bacterial libraries using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) to generate a hit list of related organisms.

2.7. Pb influence on bacterial growth

Growth curves were generated for each strain using a 96-well plate platform and the Synergy HTX plate reader (BioTek). Pb was identified as a primary environmental toxicant in Dickinson, Horsepen, and Mustang Bayous water samples above the Texas Human Health (THH) protection criteria of 1.15 ppb (Table 2). As a result, *K. pneumoniae*, and *K. aerogenes* environmental and reference strains were challenged with various Pb concentrations [0.5, 1, 5, 10 (~environmentally-relevant measured concentration), or 50 ppb]. Saturated cultures were diluted in LB broth to an optical density of 0.2_{600nm} in 96-well plates in 200 μL/well final volumes. Growth rates were monitored for 12 h and conducted in triplicate.

2.8. Oxidative stress resistance assay

For plate-based H_2O_2 assays, a modified version of our previously published protocol was employed (Henry et al., 2012). In short, 10-fold serial dilutions of saturated cultures of *K. pneumoniae* or *K. aerogenes* (environmental or references strains) were grown in either 0, 0.5, 5 or 10 ppb of Pb. Cultures were then spotted, in duplicate, (using a pronger) in ~2 μL volumes on 2 LB agar (Difco) plates containing either 0 or 0.4 mM H_2O_2.

Table 2
Trace metal concentrations in the water samples collected from the different locations along the Horsepen (HB), Dickinson (DKB), Mustang (MB), and Cypress Creek (CC) Bayou (in $\mu g\ L^{-1}$). Given are mean values ($n = 3$) of three replicates. Also given are the proposed critical limits for human health protection in water for specific metals (TCEQ, 2014; USEPA, 2018). THH = Texas Human Health protection criteria. The underlined values exceed the proposed critical limits.

Sampling locations	As		Cd		Pb	
	Summer	Fall	Summer	Fall	Summer	Fall
HB9.9	11.23	1.53	0.50	0.20	5.00	6.87
HB3.1	14.37	3.63	0.47	0.40	5.33	5.30
HB0.1	8.20	1.49	0.77	0.25	4.07	6.13
DKB12	8.50	5.55	0.60	0.40	10.07	6.93
DKB9.4	8.10	7.30	0.37	0.20	4.27	4.43
DKB0.1	8.47	5.00	0.80	0.50	4.13	8.40
MB56	10.40	2.70	0.53	0.10	17.30	13.13
MB48.8	14.27	3.40	0.13	0.13	6.03	8.93
MB20.6	20.55	3.13	0.27	0.10	7.23	3.77
CC58.1	9.30	0.90	0.40	0.30	1.90	6.83
CC49.2	8.27	3.53	0.83	0.20	4.70	4.77
CC28.5	5.50	4.50	0.30	0.35	5.30	7.17
Criteria						
THH	10		5		1.15	
EPA	10		5		10	

2.9. Biofilm assay

Previously described methods (Suraju et al., 2015; Bado et al., 2017) were employed with some modifications. Briefly, saturated cultures of *K. pneumoniae* and *K. aerogenes* reference and environmental strains were grown in LB broth, were diluted to an OD_{600nm} of 0.2 in a 96-well plate (final volume, 200 μL/well), and were incubated for 24 h with agitation (~100 rpm) at 37 °C. Wells were washed with sterile distilled water and incubated with 0.1% (vol/vol) crystal violet (total volume, 250 μL/well) for 1 h. Unbound crystal violet was removed by again washing with water, and wells were dried overnight. Biofilm-bound crystal violet was dissolved in 250 μL of 30% acetic acid, and ODs of solubilized crystal violet were measured at 580 nm. Biofilm produced was normalized based on relative biomass (optical densities of planktonic cells) to account for any differences in the growth rates of the various bacterial strains used. All experiments were carried out in triplicate or quadruplicate.

2.10. BAES2B and CCD 841 cell culture infection assay

Our previously published method (Bado et al., 2018) was employed with some modifications. In short, BEAS-2B or CCD 841, human lung and colon epithelial cells respectively, were seeded into 24-well plates at densities of ~1.0×10^5 cells/well, 24 h prior to bacterial infection. At this seeding density, monolayers were sub-confluent (~60–80% confluency) at the time of the experiment. Bacteria were grown to saturation in LB broth at 37 °C with agitation (~250 rpm), washed with 1× PBS, and diluted to optical densities 1.0_{600nm} in DMEM + 10% FBS. Diluted cultures of *K. pneumoniae* and *K. aerogenes*, were further diluted to achieve multiplicities of infection of ~1. Following a 30-minute attachment period, each well was washed with PBS, and DMEM containing 20 μg/mL (20 ppb) of heavy metal Pb was added to each well. Viable colony plate counts were enumerated for both the 0- and 6-h end points (in triplicate), and fold-increases over that time period were calculated.

2.11. Statistical analysis

All experiments were carried out in triplicate and averaged. Standard errors of the mean are represented by error bars. Using the Student's *t*-test (two-tailed and unequal variance), *p*-values less than or equal to 0.01 (represented by two asterisks) and p-values less than or equal to 0.05 (represented by one asterisk) were considered significant.

3. Results

In seeking to isolate enteric pathogens from Houston watersheds and assess bacterial loads, various bayous surrounding the Houston area were sampled (Fig. 1). The Cypress Creek Bayou lies northwest of the city center while the Horsepen, Dickinson, and Mustang Bayous lie southeast, due south, and slightly southwest of the city center, respectively (Fig. 1). To determine temporal differences in bacterial loads, the aforementioned bayous were sampled both during the warmer summer months (07/30/2018–08/03/2018) and again during the cooler fall months (11/07/2018–12/05/2018). Using broad, non-selective medium, total summer bacterial loads ranged from the lowest of 1.5×10^3 cfu/mL at Cypress Creek Bayou to the highest at 3.2×10^4 cfu/mL at Mustang Bayou (Table 1). Interestingly, Mustang Bayou also had the highest total load during the fall period as well at 3.8×10^4 cfu/mL while Cypress Creek Bayou again had the lowest bacterial load of 2.7×10^2 cfu/mL (Table 1). Similarly, with the exception of one Dickinson Bayou sample (3.5×10^3 cfu/mL), we observed the Mustang Bayou to again have the highest enteric loads in both the summer (3.3×10^3 cfu/mL) and fall samples (2.5×10^3 cfu/mL) when using selective medium enriching for Gram-negative enteric growth (Table 1). Again, Cypress Creek Bayou had the lowest enteric bacterial loads of both summer (9.4×10^2 cfu/mL) and fall (5.5×10) (Table 1). The reason

Table 3
Bacterial environmental isolates from various Houston bayous were identified using16S ribotyping and characterized by biochemical tests and Gram staining.

Bayou	Location	Ribotyping	Gram stain	Biochemical tests	
				Catalase	Oxidase
Dickinson	DKB0.1 km	*Pseudomonas fluorescens strain DH-27*	Negative	+	+
	DKB9.4 km	*Klebsiella aerogenes strain KA32282a*	Negative	+	−
		Serratia marcescens subsp. *sakuensis strain RY21*	Negative	+	−
	DKB12 km	*Pseudomonas lini strain FRT6*	Negative	+	−
		Pseudomonas oleovorans strain JZY3-48	Negative	+	−
Mustang	MB56 km	*Pseudomonas mendocina ymp*	Negative	+	−
		Klebsiella pneumoniae	Negative	+	−
	MB48.8 km	*Bacillus megaterium strain S15 16S*	Positive	+	+
	MB20.6 km	*Planomicrobium chinense strain MN-JXJ:3*	Positive	+	+
		Lysinibacillus sp. *strain 7*	Positive	+	+
Cypress Creek	CC49.2 km	*Chryseobacterium jeonii* strain NCTC13459	Negative	−	−
	CC28.5 km	*Pseudomonas stutzeri strain KGS-2*	Negative	+	−
		Brevibacterium casei	Positive	+	−
	CC58.1 km	*Bacillus indicus strain ZW3*	Positive	+	+
		Pseudomonas nitroreducens	Negative	+	−
Horsepen	HB9.9 km	*Bacillus megaterium NBRC 15308 = ATCC 14581*	Positive	+	+
	HB3.1 km	*Klebsiella aerogenes strain SPUKJM2*	Negative	+	+

for why Mustang Bayou had the highest bacterial loads, relative to neighboring bayous, remains unclear; however, fertilizer run-off from neighboring agricultural fields could be a potential contributor.

The heavy metal concentrations in the Bayou water samples collected in both the seasons are provided in Table 2. Our results indicate that the Pb concentrations in water samples were higher and remained above their THH criteria of 1.15 ppb in all the bayous during both summer and fall seasons (Table 2). In sharp contrast, only samples from Horsepen and Mustang Bayou collected during the summer had arsenic (As) concentrations exceeding Texas Human Health (THH) criteria of 10 ppm, while cadmium (Cd) concentrations from all bayou sampling locations were well below the THH criteria of 5 ppm (Table 2).

Following bacterial load determinations, 17 colonies were down-selected from all 4 sampled bayous that had been grown on both broad- and selective-media for identification and downstream analysis. All 17 down-selected colony isolates (including 6 Gram-positive isolates) were identified (using ribotyping and BLAST analysis) and included 3 human enteric pathogens: *K. pneumoniae*, *K. aerogenes*, and *Serratia marcescens* (Table 3). While *K. pneumoniae* was isolated from Mustang Bayou (Table 3), the bayou with the highest enteric loads (Table 1), *K. aerogenes* was isolated from both the Horsepen and Dickinson Bayous (Table 2). For all downstream applications, we characterized the Dickinson Bayou *K. aerogenes* isolate.

Earlier findings suggested that environmentally isolated *E. coli* from Houston watersheds was potentially more resistant to antibiotics based upon upregulated antibiotic resistance gene expression (Pingfeng et al., 2018). As a result, the environmentally isolated *Klebsiella* spp. were evaluated for increased resistance to antibiotic chemotherapeutics: ampicillin, gentamicin, streptomycin, penicillin, tetracycline, and erythromycin. Interestingly, the *K. aerogenes* environmental isolate was less sensitive to gentamycin, streptomycin, and tetracycline than the reference strain as evidenced by smaller zones of inhibition [12 mm vs. 16 mm, 20 mm vs. 22 mm, and 13 mm vs. 15 mm, respectively] (Table 4). In fact, the zones of inhibition observed indicated that the *K. aerogenes* environmental isolate was resistant to ampicillin, gentamicin, tetracycline, and erythromycin based on Clinical Laboratory and Standards Institute thresholds (Table 4). The *K. pneumoniae* environmental isolate was similarly less sensitive than its reference strain to gentamycin, streptomycin, and tetracycline than its reference strain as evidenced by smaller zones of inhibition [13 mm vs. 16 mm, 18 mm vs. 20 mm, and 7 mm vs. 10 mm, respectively] (Table 4); however, based on CLSI standards, the *K. pneumoniae* environmental isolate was only resistant to ampicillin (Table 4).

To determine whether exposure to metal contaminants found in Houston watersheds promoted adaptations in the environmental isolates, both the environmental and reference strains were exposed to various Pb concentrations. Pb levels were elevated in bayou water samples (Table 2) exceeding THH criteria of 1.15 ppb and approaching the actionable levels of 15 parts per billion (ppb) limits on drinking water (https://www.epa.gov/dwreginfo/lead-and-copper-rule#rule-summary) prompting the investigation. Initially, a range of Pb concentrations including: 1 ppb, 10 ppb, and 50 ppb were tested. Only the 50 ppb Pb-challenge significantly slowed bacterial growth for all 4 strains evaluated while the *K. pneumoniae* environmental isolate appeared less sensitive to Pb-exposure than its reference strain (Fig. S1). When compared directly following 50 ppb Pb-challenge, both the *K. pneumoniae* and *K. aerogenes* environmental isolates had significantly greater biomass than their corresponding reference strains (Fig. 2), suggesting potentially beneficial environmental adaptations. Interestingly, the environmentally isolated *K. aerogenes* strain had significantly higher

Table 4
Klebsiella environmental isolates and their respective reference strains were evaluated for their antibiotic resistance. Zones of inhibition were measured (mm). The underlined values indicate antibiotic resistance based on CLSI standards (https://clsi.org/standards/products/microbiology/).

Antibiotic	*K. pneumoniae* (reference) zone of inhibition (mm)	*K. pneumoniae* (environmental) zone of inhibition (mm)	*K. aerogenes* (reference) zone of inhibition (mm)	*K. aerogenes* (environmental) zone of inhibition (mm)	Resistance for enteric bacteria (mm)
Ampicillin 10 μg	0	0	0	0	≤13
Gentamicin 10 μg	16	13	16	12	≤12
Streptomycin 10 μg	20	18	22	20	≤11
Tetracycline 30 μg	17	17	15	13	≤14
Erythromycin 15 μg	10	7	0	0	≤3

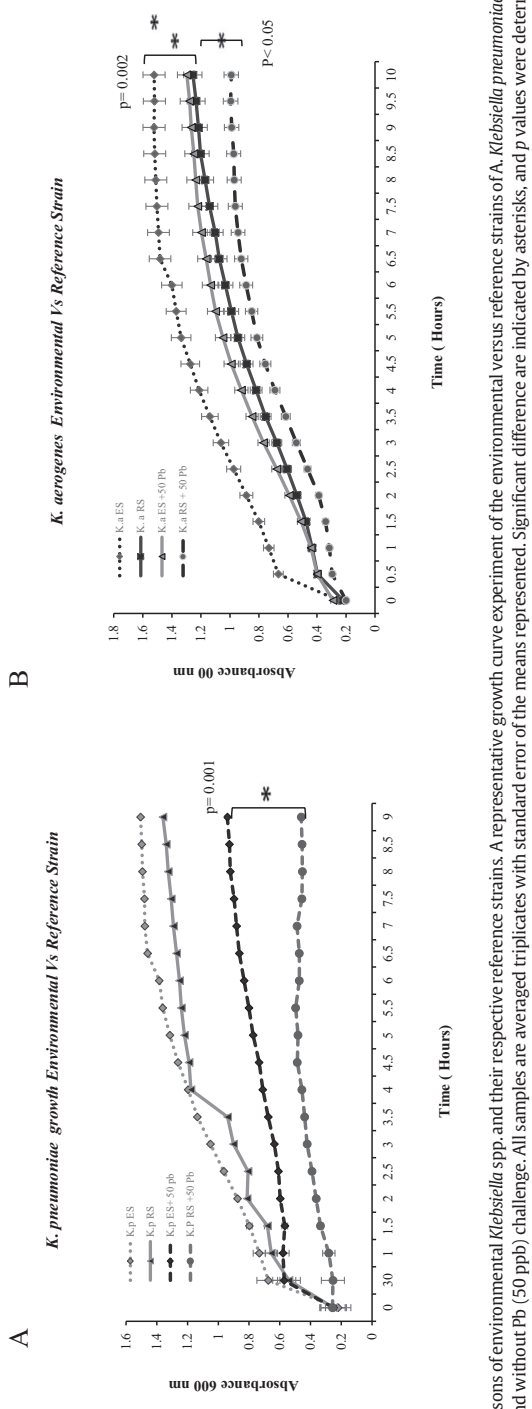

Fig. 2. Growth curve comparisons of environmental *Klebsiella* spp. and their respective reference strains. A representative growth curve experiment of the environmental versus reference strains of A. *Klebsiella pneumoniae* and B. *Klebsiella aerogenes* is graphically displayed with and without Pb (50 ppb) challenge. All samples are averaged triplicates with standard error of the means represented. Significant difference are indicated by asterisks, and *p* values were determined by the Student's *t*-test.

biomass than its reference strain at all-time points evaluated for reasons that remain unclear (panel B).

To determine whether the possible environmental adaptations extend beyond sustained growth during Pb-exposure, the impact of Pb-exposure on oxidative stress resistance was evaluated as well. On solid medium, no apparent differences were observed when the *K. pneumoniae* strains were challenged with a range of Pb concentrations (0.5, 5, or 10 ppb) (Fig. 3A), in agreement with what was observed in liquid culture (Fig. 2). However, differences became apparent when in the presence of 0.4 mM of H_2O_2. More specifically, even without Pb-challenge, the reference strain appeared more sensitive to 0.4 mM H_2O_2 than did the environmental strain, and Pb-exposure at all concentrations did not affect the environmentally isolated strain's growth (panel B). With regards to *K. aerogenes*, mirroring what was observed for *K. pneumoniae*, no apparent differences were observed when the *K. aerogenes* strains were challenged with a range of Pb concentrations (0.5, 5, or 10 ppb) (Fig. 4A). However, when challenged with either 5 or 10 ppb Pb, the environmental *K. aerogenes* outgrew the reference strain when in the presence of 0.4 mM of H_2O_2 (Fig. 4B). Growth on higher concentration H_2O_2 plates of 1 and 10 mM of H_2O_2 was not observed regardless of Pb exposure (data not shown).

Since it appeared that both *Klebsiella* environmental isolates were better able to tolerate Pb exposure as it related to their growth and oxidative stress resistance than their corresponding reference strains, their biofilm production was also examined to determine whether it was similarly enhanced. Interestingly, significantly enhanced biofilm formation was observed in the environmental *K. pneumoniae* strain relative to the reference strain when challenged with 10, 20, and 50 ppb of Pb (Fig. 5A). Similarly, the environmental *K. aerogenes* isolate exhibited significantly enhanced biofilm production at 10, 20, and 50 ppb of Pb (Fig. 5B). However, no significant differences in biofilm formation were observed for either set of *Klebsiella* species when exposed to lower concentrations of 0.5 and 1 ppb of Pb (Fig. 5).

Finally, determinations whether the influence of Pb exposure on *Klebsiella* spp. proliferation in modeled lung and gut environments (using BAES 2B human lung cells or CCD 841 human gut colon epithelial cells) were made. In a lung model system and in the absence of Pb-challenge, both the environmental and reference *K. pneumoniae* strains exhibited similar fold increases over a 6 h infection period, 15.5 and 13.5-fold increases respectively; however, the environmental isolate exhibited a significantly greater 4.75-fold increase relative to the reference strain when challenged with 20 ppb Pb (Fig. 6). More specifically, in the presence of Pb, the environmental isolate was able to proliferate 12.75-fold while the reference strain proliferated only 2.8-fold (Fig. 6A). Mirroring what was observed in the lung infection model, in the absence of Pb-challenge, both the environmental and reference *K. pneumoniae* strains exhibited similar fold increases in a gut infection model over a 6-h infection period, 1.80 and 1.46-fold increases respectively; however, the environmental isolate exhibited a significantly greater 5.92-fold increase relative to the reference strain when challenged with 20 ppb Pb (Fig. 6B). More specifically, in the presence of Pb, the environmental isolate was able to proliferate 1.37-fold while the reference strain actually decreased 0.23-fold (Fig. 6B). Unexpectedly, *K. pneumoniae* was better suited to proliferate in the lung model than the gut the model system.

In sharp contrast, both *K. aerogenes* strains proliferated much more modestly than did the *K. pneumoniae* strains in BAES 2B co-culture. In the absence of Pb-treatment, there was no significant difference between the proliferations of the environmental or reference strains, with proliferations of 1.88- and 1.39-fold, respectively (Fig. 7A). However, as was seen for *K. pneumoniae* (Fig. 6A), when challenged with 20 ppb Pb, the environmental *K. aerogenes* exhibited a significantly greater 3.47-fold increase relative to the reference strain over the 6-h infection (Fig. 7A). More specifically, the environmental strain expanded 1.77-fold relative to the 0.51-fold decline of the reference strain when challenged with 20 ppb Pb (Fig. 7A). Mirroring its performance in

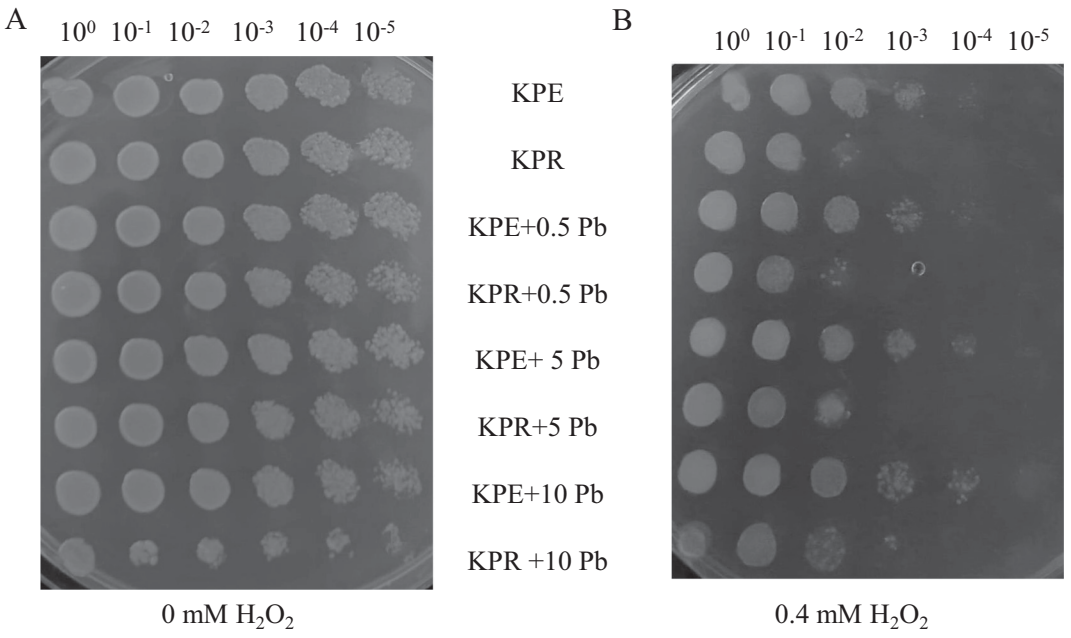

Fig. 3. Oxidative stress resistance of *Klebsiella pneumoniae* following Pb challenge. *K. pneumoniae* environmental and reference strains were grown in the presence of various Pb concentrations and subsequently spotted on either 0 mM (A) or 0.4 mM (B) H_2O_2. Following 16 h incubation at 30 °C plates were scanned. All strains were spotted in duplicate for internal dilution and spotting controls, and the figure is a representative experiment.

the lung model infection in the absence of Pb-challenge, there was no significant difference in fold-increases of either the environmental (1.88-fold) or reference strain (1.57-fold) following a 6-h infection in a gut model system (Fig. 7B). However, when challenged with 20 ppb Pb, the environmental strain exhibited a significantly higher 1.55-fold increased proliferation relative to its reference strain (Fig. 7B). More specifically, the environmental *K. aerogenes* strain expanded 1.58-fold in the gut model relative to the marginal 1.03-fold expansion of the reference strain (Fig. 7B). Unlike what was observed with *K. pneumoniae* (Fig. 6), *K. aerogenes* did not proliferate better in the lung infection model relative to the gut model system, for reasons that remain unclear (Fig. 7).

4. Discussion

In this work, bacterial responses to Pb exposure were investigated on account of Pb concentrations in all measured bayous (during both the seasons in this study) being at least 4 to 12 times higher than the THH criteria limit (Table 2). Furthermore, human exposure to Pb concentration in Bayou waters may occur through recreational activities,

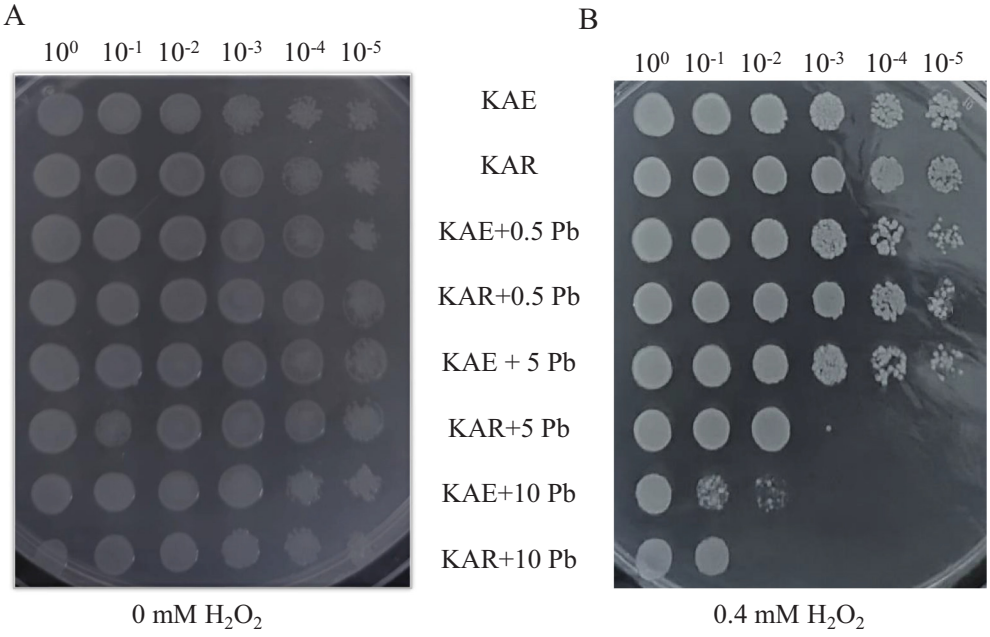

Fig. 4. Oxidative stress resistance of *Klebsiella aerogenes* following Pb challenge. *K. aerogenes* environmental and reference strains were grown in the presence of various Pb concentrations and subsequently spotted on either 0 mM (A) or 0.4 mM (B) H_2O_2. Following 16 h incubation at 30 °C, plates were scanned. All strains were spotted in duplicate for internal dilution and spotting controls, and the figure is a representative experiment.

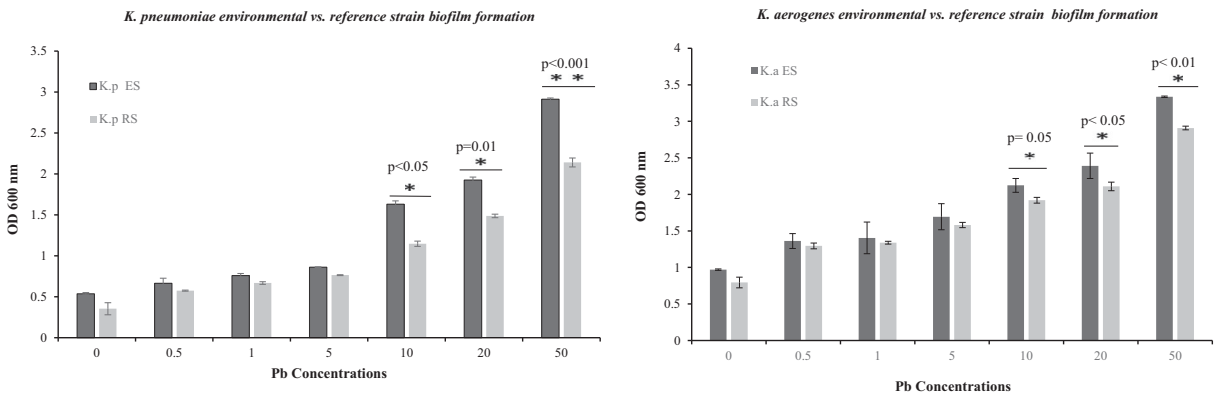

Fig. 5. Biofilm formation of environmental and reference *Klebsiella* spp. In this representative experiment, *K. pneumoniae* (A) and *K. aerogenes* (B) environmental and reference strains were exposed to 0.5, 1, 5, 10, 20, or 50 ppb Pb and measured for biofilm production. In this representative experiment, all samples are averaged triplicates with standard error of the means represented. Significant difference are indicated by asterisks, and *p* values were determined by the Student's *t*-test.

floodwater inundation, and ground water contamination. EPA has set the maximum contaminant level goal for Pb in drinking water at zero since Pb negatively affects human health at even low exposure levels (USEPA, 2020).

In this study, 3 Gram-negative enteric pathogens were isolated from the Houston watershed, and two of them, *K. pneumoniae* and *K. aerogenes*, were characterized. Since *K. pneumoniae* can be KPC resistant (Ernst et al., 2020) or hyper-virulent (Russo and Marr, 2019), has been recovered from European wastewater outflows (Dolejska et al., 2011), and poses human health threats, the *K. pneumoniae* isolate, as well as the *K. aerogenes* isolate, were challenged with a battery of antibiotics including: ampicillin, gentamicin, streptomycin, penicillin, tetracycline, and erythromycin. These interrogations were further motivated by an earlier report suggesting that Houston watershed-isolated *E. coli* potentially had increased resistance to antibiotics based on elevated antibiotic resistance gene expression (Pingfeng et al., 2018). As a result, the environmentally isolated *Klebsiella* spp. were evaluated for increased resistance to antibiotic chemotherapeutics. As was expected, the environmental *K. aerogenes* isolate was less sensitive to most antibiotics tested than its reference strain and resistant to gentamycin, ampicillin, and tetracycline. Interestingly, however, the *K. pneumoniae* isolate was not resistant to any antibiotic tested, with the exception of ampicillin, despite being less sensitive to the antibiotics tested than its reference strain. Furthermore, increased resistance to antimicrobial activities could prove problematic for recreationalists who are at increased risk for environmental *Klebsiella* exposures. It is possible that by adapting to elevated Pb levels in Houston waterways *Klebsiella* spp. become increasingly resistant to various chemical insults, including antimicrobials; however, those possibilities were not explored in this work.

Based on the aforementioned finding of Pb being elevated in Houston bayou water samples, *Klebsiella* spp. isolates were challenged with a range of various Pb concentrations. Their subsequent performances in pure culture and eukaryotic cell co-culture proliferation, oxidative stress resistance, and biofilm production assays were compared. Not surprisingly, the *K. pneumoniae* and *K. aerogenes* environmental isolates indeed appeared to be better adapted to Pb-exposure, likely on account of consistently high Pb levels in the waters from which they were derived. More specifically, both environmental isolates outgrew their reference strain counterparts in pure-culture and eukaryotic co-culture growth in the presence of Pb as well as exhibited both increased biofilm production as well as increased resistance to oxidative stress. With regards to eukaryotic co-culture experiments, it is worth noting that while Pb exposure to BAES 2B cells alone resulted in loss of viability; Pb exposures of 1, 5, and 10 ppb resulted in only 40% cytotoxicity after 6 h exposure (data not shown). Therefore, during the course of our 6 h co-culture infections, the majority of BAES 2B lung cells were viable during the modeled lung infection.

Interestingly, the two environmentally isolated *Klebsiella* spp. displayed different Pb-tolerance profiles. This was perhaps due, in part, to the fact that they were not isolated from the same Houston area bayous and adapted to slightly different water borne toxicants and stressors. Whereas *K. pneumoniae* was isolated form Mustang Bayou (measured levels of 3.2 and 7.3 ppb Pb in the summer and fall

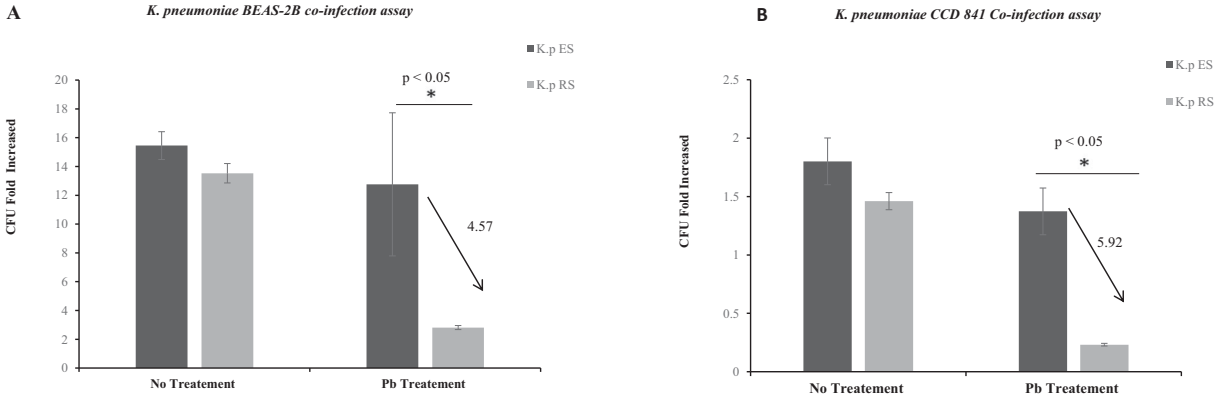

Fig. 6. Lung model infection with *Klebsiella* spp. in the presence of Pb. *K. pneumoniae* (A) and *K. aerogenes* (B) environmental and reference strains were co-cultured with BEAS-2B cells at a multiplicity of infection of ~1, modeling the lung environment. Bacterial proliferation was measured following a 6 h infection in the presence or absence of 20 ppb Pb 6-h infection period. In this representative experiment, fold increases are displayed, and all samples are averaged triplicates with standard error of the means represented. Significant difference are indicated by asterisks, and p values were determined by the Student's *t*-test.

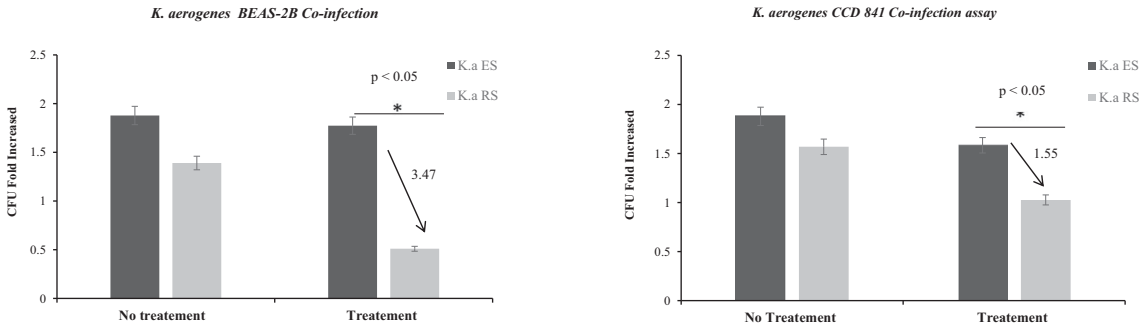

Fig. 7. Gut model infection with *Klebsiella* spp. in the presence of Pb. *K. pneumoniae* (A) and *K. aerogenes* (B) environmental and reference strains were co-cultured with CCD 841 cells at a multiplicity of infection of ~1, modeling the lung environment. Bacterial proliferation was measured following a 6 h infection in the presence or absence of 20 ppb Pb 6-h infection period. In this representative experiment, fold increases are displayed, and all samples are averaged triplicates with standard error of the means represented. Significant difference are indicated by asterisks, and p values were determined by the Student's *t*-test.

respectively), *K. aerogenes* was isolated from Dickinson Bayou (0.5 and 2.8 ppb Pb in the summer and fall respectively). It is possible that consistently higher levels of Pb in the water are what caused the *K. pneumoniae* isolate to better tolerate Pb exposures than the *K. aerogenes* isolate. However, there are potentially other unobserved contaminants in both bayous that may have also contributed to the Pb-tolerance threshold differences.

Taken together, these data demonstrate that environmental Gram-negative enteric pathogens have the potential to promote their virulence potential through exposures to various toxicants, including trace metals like Pb. Through increased biofilm formation, resistance to antibiotics, resistance to oxidative stress, and enhanced proliferation in both lung and gut environments, Gram-negative pathogens, like the *Klebsiella* spp., could pose additional threats to human health. As a result, people using the bayous for recreational purposes need to be aware of these potential threats.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147818.

CRediT authorship contribution statement

H Ali performed all experiments and contributed to the writing of the manuscript. BBM Sridhar contributed to sample collection, ICPMS measurement design, GIST map plotting, and contributed to the writing of the manuscript. JA Rosenzweig was responsible for all microbiological experimental planning and design as well as was responsible for the bulk of the manuscript's writing.

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.

Acknowledgment

This work was supported by the National Science Foundation (NSF) grants for Texas Southern University (TSU) under the award numbers HRD-1622993 and BCS-1831205. We acknowledge Djene Keita for the chemical analysis of water samples and Titilope Bukunmi-Omidiran, Alexander Cruz SanMugiel, and Folasade Adedoyin for help in water and soil sampling. We would like to thank Shishir Shishodia and Daniel Vrinceanu for their valuable insights and comments, and discussions.

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