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RESEARCH

Tracking the sources of *Leptospira* and nutrient flows in two urban watersheds of Puerto Rico

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Abstract This study investigated the relationship between nutrient levels, source of fecal contamination, and pathogenic *Leptospira* in Puerto Rico's northern coast and San Juan Bay Estuary (SJBE) aquatic ecosystems. Microbial source tracking (MST) was also used to investigate the connections between sources of feces contamination and the presence of *Leptospira*. Eighty-seven water samples were collected during the June (n=44) and August (n=43) in 2020. To quantify phosphorus and nitrogen concentrations, standard USEPA protocols were utilized, specifically Methods 365.4 for total and dissolved phosphorus, 351.2 for total Kjeldahl nitrogen and ammonium, and 353.2 for nitrate. Lipl32 gene-specific quantitative polymerase chain reaction (qPCR) was used to detect the presence of *Leptospira*. Human (HF183), canine (BacCan-UCD), and equine (HoF597) MST assays were utilized to trace the origins of fecal contamination. Forty one percent of the locations exceeded Puerto Rico's authorized total phosphorus limit of 160 g L^{-1}, while 34% exceeded the total nitrogen limit of 1700 g L^{-1}. Nearly half of the streams examined are affected by eutrophication. The MST analysis identified human and canine feces as the most prevalent contaminants, affecting approximately 50% of the sites. In addition, *Leptospira* was detected in 32% of the June samples. There was a significant correlation ($r = 0.79$) between the incidence of pathogenic *Leptospira* and the human bacterial marker (HF183). This study illuminates the central role of anthropogenic inputs in nutrient enrichment and pathogen proliferation in Puerto Rico's aquatic ecosystems.

Keywords *Leptospira* · Microbial source tracking · Fecal pollution · Puerto Rico

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Introduction

The northern coastal zones of Puerto Rico, including the San Juan Bay and Rio Grande de Loiza (below dam) watersheds, are increasingly becoming focal points of environmental concern due to wastewater originating from human activity. This runoff, which consists of effluents from sewage treatment, septic tanks, and animal husbandry, enters coastal lagoons and estuaries, posing critical threats to these vital ecosystems. The San Juan Bay Estuary (SJBE), a significant ecological center located within these watersheds, is essential to the integrity of the coastline, especially its mangrove

forests (Fretwell et al., 1996). The estuary, which serves as the drainage for the densely populated San Juan-Caguas-Guaynabo metropolitan area, home to an estimated 2.7 million people, faces the difficulty of large volumes of refuse runoff. This situation is exacerbated during the wet season, when the region receives between 1500 and 1700 mm of precipitation annually (Brandeis et al., 2014; Fretwell et al., 1996), with the heaviest precipitation occurring between August and September during hurricane season (Colón & Schaffner, 2021).

Particularly during these rain events, San Juan has been identified as a significant source of fecal pollution and nutrient discharge, raising significant public health and environmental concerns (Holman et al., 2014; Sutter & Sosa Pascual, 2018; Walker et al., 2013).

Among these threats, eutrophication, predominantly caused by excessive nitrogen (N) and phosphorus (P) from urban and fecal sources, is the most damaging. The toxic algal outbreaks that result pose a threat to marine ecosystems, with the potential for significant ecological consequences (Conley et al., 2009; Sutter & Sosa Pascual, 2018; USEPA, 2012). Attributed to accelerated population growth and agricultural expansion within these watersheds, (Mesa et al., 2015; Ngatia et al., 2019).

The Puerto Rico Environmental Quality Board regulates the water quality in these watersheds and has established total P and total N nutrient criteria of 0.160 mg L^{-1} and 1.70 mg L^{-1}, respectively (USEPA, 2019). Ngatia et al. (2019) report that most island streams and rivers exhibit alarmingly high N and P concentrations, far exceeding the established thresholds. This unsettling circumstance highlights the need for stricter pollution management measures and extensive research to comprehend the full scope of these environmental challenges.

Environments influenced by fecal runoff, especially during heavy rainfall, are potential reservoirs of harmful pathogens, such as *Leptospira*, the causative agent of *leptospirosis*. This aerobic, gram-negative bacterium is arranged in a unique spiral morphology and is currently classified into 35 species within four subclades. Notably, pathogenic *Leptospira* species like *L. interrogans*, *L. borgpetersenii*, *L. kirschneri*, *L. noguchii*, and *L. weilii*, all belonging to the P1 subclade, pose significant health risks as they are often expelled into the global environment through the urine of infected hosts (Pappas et al., 2008; Vincent et al., 2019).

This disease accounts for approximately 60,000 yearly fatalities (Bierque et al., 2020). Humans and other animals become susceptible to infection upon exposure to contaminated water, often filled with bacterium-laden urine (Pappas et al., 2008; Rawlins et al., 2014). Within Puerto Rico, the estimated cases of *Leptospirosis* ranged from 15 to 100 in 2010, with 114 reported cases during 2014 to 2015 (Briskin et al., 2019). However, it is crucial to note that these figures likely underestimate the actual disease burden, given that many cases of *Leptospirosis* are asymptomatic and often go unreported (Briskin et al., 2019).

Parallel to fecal waste nutrient contamination, environmental levels of pathogens such as *Leptospira* tend to spike during flooding or heavy rainfall in tropical islands. These periods of increased environmental prevalence usually precede human infection outbreaks (Barragan et al., 2017; USEPA, 2012). Truitt et al. (2020) discovered pathogenic *Leptospira* in a number of rural rivers in Puerto Rico during the wet season but found no meaningful correlation between physicochemical parameters (such as pH, dissolved oxygen (DO), and temperature), concentrations of fecal indicator bacteria (*Escherichia. coli*), and the presence of pathogenic *Leptospira* at the sampling sites. This was attributed to the fact that *Leptospira* is transmitted through urine that co-occur with feces but may not always contain these pathogens (Pratt & Rajeev, 2018; Truitt et al., 2020). A limitation of this study was the need for more evaluation regarding the potential sources of these pathogenic bacteria in the environment (Truitt et al., 2020).

A diverse array of animal hosts, including dogs, pigs, cattle, rodents, mongooses, bats, and frogs, can carry these bacteria and thus contribute significantly to disease transmission (Levett et al., 2001; Pratt & Rajeev, 2018). However, crucial information about the potential sources of pathogenic *Leptospira* and fecal nutrients still needs to be discovered, leaving a substantial gap in our understanding of these environmental health issues. The development of polymerase chain reaction (PCR) methodologies and microbial source tracking (MST) approaches have introduced a novel capability to detect *Leptospira* in environmental samples rapidly and to infer their potential sources (Bridgemohan et al., 2020; Rawlins et al., 2014; Riediger et al., 2016). The successful use of a hydrolysis probe assay targeting the *Lipl32* gene, which encodes a 32-kDa membrane protein unique to pathogenic

Leptospira species in the P1 subclade, represents a recent advancement in this context (Rawlins et al., 2014; Stoddard et al., 2009; Truitt et al., 2020).

Microbial source tracking methodologies can link the presence of pathogens or fecal contamination in aquatic systems to a particular source (Bridgemohan et al., 2020; González-Fernández et al., 2021). The central tenet of MST is that certain animals harbor species-specific fecal bacteria to its host, such as within the genus *Bacteroidales*, which can be detected using PCR (Bachoon et al., 2010; Bridgemohan et al., 2020). There have been reports of the successful use of MST to identify sources of fecal pollution and pathogenic bacteria in Puerto Rico's marine and freshwater systems (Bachoon et al., 2010; Wade et al., 2015).

Given the extant information about the San Juan Bay and Rio Grande de Loiza watersheds, we hypothesized that humans, horses, dogs, and rodents were the most likely sources of nutrient contamination and *Leptospira* (Benavidez et al., 2019). For this investigation, an MST assay for rodent fecal bacteria was unavailable. However, we propose the integration of MST, nutrient level assessments, and PCR detection of *Leptospira* to determine the source of surface water contamination. This study aims to determine the relationship between nutrient concentrations (N and P) with fecal pollution sources, the presence of pathogenic *Leptospira*, and the potential sources of these contaminants in the SJBE and Rio Grande de Loiza watersheds during June and August of 2020. Once the sources of contamination are identified, appropriate mitigation measures can be implemented to address these environmental challenges.

Materials and methods

Sampling sites

To represent urban and agricultural regions within the San Juan Bay Estuary (SBJE) and Rio Grande de Loiza watersheds, sampling stations were strategically chosen. The investigation included a minimum of two stations per subbasin in 2020 (see Appendix Table 3 for details). Following established protocols (Wilde et al., 1998), 87 water samples were collected using sterile plastic receptacles in June (which had weekly rainfall cumulative average of 17.53 mm to 24.38 mm) and August (dry period) with a weekly cumulative average of 4.06 mm to 2.03 mm.

Nutrient concentration evaluation

Chemical analyses included dissolved (DP) and total reactive (TP) P (EPA method 365.4), total Kjeldahl nitrogen (TKN), dissolved ammonium (NH_4^+) (EPA method 351.2), and nitrate (NO_3^-) (EPA method 353.2). Total nitrogen (TN) was obtained from the sum of (NO_3^-) and TKN. Samples for (NO_3^-), (NH_4^+), and DP were passed through a 0.45-µm pore size Gelman-Acrodisc filter (Waltham, MA, USA).

DNA extraction

Each sample (100 mL) was filtered through a nitrocellulose membrane filter (Type GS, Millipore, Billerica, MA, USA) with a 0.45-µm pore size (Millipore, Billerica, MA, USA) and then stored at −20 °C before being transported to Georgia College and State University. The DNA was extracted from the filters using the QIAGEN PowerSoil Kit (Qiagen, Hilden, Germany) in accordance with a modified version of the procedure described by Bachoon et al. (2010). The modification involved separating the bead solution from the beads and placing it in a 15-mL centrifuge tube containing the filter. Solutions S1 and IRS were added to the tube and vortexed vigorously for 15 min. The solution was removed from the centrifuge tube and placed in the bead tube. The manufacturer's protocol was followed from this point. After determining the DNA concentration of each sample using a Nanodrop ND-1000 Spectrophotometer (Wilmington, DE), the samples were stored at −20 °C.

Quantitative polymerase chain assays of *Leptospira interrogans*

Leptospira interrogans was quantified by qPCR using a 242-bp segment of the *Lipl32 gene*, as previously described (Rawlins et al., 2014; Truitt et al., 2020), by running samples in duplicate using QuantiTect Probe PCR kit (Qiagen) with the Bio-Rad CFX96 (Hercules, CA, USA), as described by Truitt et al. (2020). Each reaction had a final volume of 20 µl using ~10ng of extracted DNA, 500 nM of each primer (Table 1), and 200 nM of the probe. The thermal conditions were an initial 95°C for 15 min, followed by 40 cycles at 95°C

Table 1 Primers and probes for *Leptospira interrogans* detection and microbial source tracking

Target	Primer	Sequence	Annealing temp. (°C)	Reference
Lipl32 gene	*Lipl32*-45F *Lipl32*-286R *Lipl32*-189P	AAGCATTACCG CTTGTGGTG GAACTCCCATTTCAGCGATT FAM-AAAGCCAGGACAAGCGCCG-BHQ1	64	Rawlins et al. (2014)
Human	HF-183-1 BtheR1 Probe	ATCATGAGTTCACATGTCCG CGTAGGAGTTTGGACCGTGT 6-FAM-CTGAGGAGAAGGTCCCCCACATTG GA-TAMRA	60	Green et al. (2014)
Dog	BacCan-545f1 BacUni-690r2 probe	GGAGCGCAGACGGGTTTT AATCGGAGTTCCTCGTGATATCTA 6-FAM-TGGTGTAGCGGTGAAA-TAMRA	61.4	Kildare et al. (2007)
Horse	HoF597 F Bac708 R	CCAGCCGTAAATAGTCGG CACATGTTCCTCCGCTCGTA	59	Dick et al. (2005)

for 10 s and 64°C for 30 s. *Leptospira interrogans* serovar Pomona was used as the positive control, *E. coli* strain K-12 as a negative control, and no-template controls were performed in each assay. The positive samples were then retested in triplicate. The number of genome copies was quantified using standard curves of *L. interrogans* serovar Pomona gene copies (Truitt et al., 2020). DNA extracted from *L. interrogans* serovar Pomona was used as the positive control; *E. coli* strain K-12 as a negative control; and tubes lacking DNA as no-template controls. Based on the genome size of *L. interrogans* (4.659 Mb), genome equivalents were calculated as described (Levett et al., 2005). The lower limit of the assay was set at 5 genome copies of *L. interrogans* determined using serial dilutions of positive control DNA. Deoxyribonucleic acid extracts were evaluated for the presence of PCR inhibitors by evaluating shifts in Cq-values between a sample and its 10-fold diluent (Dick et al., 2010; Truitt et al., 2020). Statistical analyses were performed with the R® program.

Microbial source tracking (MST)

Human and animal fecal bacteria were detected using quantitative polymerase chain reaction (qPCR) assays on the Bio-Rad CFX 9600 Real-Time Detection system (Hercules, California, USA). Hydrolysis probe-based assays utilizing the QuantiTect Probe PCR (Qiagen) reagents were utilized for human and canine samples, whereas the SsoFast Evagreen (Bio-Rad) dye-based assay was utilized for equine samples. Based on previously reported sensitivity and selectivity metrics, assay primers were selected and optimized to prevent non-specific cross-reactions with fecal DNA from local horse, cattle, dog, and human sources. This was done by testing each fecal host MST assay against the panel of host fecal DNA extracts (Table 1).

Polymerase chain reaction assays were conducted with 1 µL of sample DNA (approximately 10 ng μL^{-1}) and a ten-fold diluted DNA sample with annealing temperatures and primer/probe sequences for each source marker gene as listed in Table 1. Standard curves using host-specific fecal DNA for each qPCR (controls) were linear with coefficients of determination (r^2) of 0.99 and efficiencies of over 90%. Negative controls without DNA, which were run with each reaction, always exceeded the cycle threshold at a mean CT limit of the assay which was set at 5 genome copies of *L. interrogans* of 39.58. Each hydrolysis probe (human and dog) was labeled at the 5' end with the reporter dye 6-FAM (6-carboxy-fluorescein) and at the 3' end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine). For human (HF183-1), the qPCR assay used a modified protocol of Green et al. (2014), with *Bacteroides dorei* DSM 17855 (DSMZ) and human sewage used as a positive control and *E. coli* strain B from Sigma® D48890-1UN and horse and dog fecal DNA extracts as a negative control. The 20 µl assay contained 250 nM of each primer, 0.2 mg of bovine serum albumin (Sigma), and 80 nM 6-FAM™ labeled Hydrolysis® probe. Water samples were assessed for possible PCR inhibition by amending with positive control bacteria DNA as described by Bachoon et al. (2010). Changes of less than two CT value were observed, which indicates that the extracted DNA did not contain impurities that significantly inhibited the MST assay (Bachoon et al.,

2010). In addition, ten-fold dilutions of sample DNA extracts were compared to the undiluted sample DNA extract, and a change in CT shift of < 3 CT indicated no significant PCR inhibition (Dick et al., 2010). The samples were run at 95 °C for 10 min, 40 cycles at 95 °C for 10 s, and at the required annealing/extension temperature (Table 1). The qPCR detection limits were determined from the lowest concentration of target DNA that could be detected at 95% confidence and gene copies ranged from human 8, dog 10, and horse 25 gene copies. Data was statistically analyzed using InfoStat 2012 (Di Rienzo et al., 2012). Data from all the experiments were analyzed using a one-way ANOVA as a completely randomized design. Significant differences among treatment means were determined using Fisher's protected LSD at $p = 0.05$. Spearman correlation was also used to analyze data with significant correlation coefficients with a value greater than 0.7. Statistical analysis of the relationship between TP and TN with the marker for human fecal contamination was performed using an odds ratio.

Results

Nutrients concentration levels

Thirty-six of the eighty-seven water samples (41%) collected in 2020 had TP concentration that exceeded 160 µg L^{-1} which is the regulatory limit for Puerto Rico (USEPA, 2019) (Appendix Table 4). The highest levels of TP were observed after periods of rain during the June sampling events in the Quebrada Angela subbasin (3210 µg L^{-1}) and in the Josefina subbasin (1860 µg L^{-1}, 1,574 µg L^{-1}). These same sites exhibited elevated levels of DP ranging from ~1500 to 2800 µg L^{-1}, while the other sites ranged from ~2 to 955 µg L^{-1} (Appendix Table 4). Statistical analysis of the relationship between TP and the marker for human fecal contamination indicates that the probability of a water sample being positive for the human fecal bacterial marker when TP exceeds the regulatory limit of 160 µg L^{-1} is 16 times greater than when TP is less than 40 µg L^{-1} ($p < 0.000$).

Thirty of the eighty-seven water samples (34%) exceeded the regulatory limit of 1700 µg L^{-1} of TN (Appendix Table 4) (USEPA, 2019). It should be noted that TN in five water samples was > 4800 µg L^{-1}, and the sites that these samples were collected from include Juan Méndez, San Antón, Quebrada Angela, and Bocaforma. More specifically, the greatest concentrations of TN were measured at one site in Quebrada Angela during the June sampling event (34,013 µg L^{-1}) followed by two sites in the Bocaforma (5683 µg L^{-1}, 5,962 µg L^{-1}) subbasin in August. The lowest concentrations of TN were measured in Río Canóvanas during the June sampling event (116.56 µg L^{-1}, 108.43 µg L^{-1}) and the August sampling event (81.10 µg L^{-1}). Statistical analysis of the relationship between TN and the marker for human fecal contamination indicates that the probability of a water sample being positive for the human marker when TN exceeds the regulatory limit of 1700 µg L^{-1} is 16 times greater than when TN is less than 40 µg L^{-1} ($p = 0.001$). Nitrate in water samples during both sampling events ranged from 0.84 µg L^{-1} in Río Canovanillas to 1339.59 µg L^{-1} in Rio Piedras Sur. Fourteen of the water samples collected contained NO_3^- concentrations > 1000 µg L^{-1}, and most of these samples were collected during the dry period in August (Appendix Table 4).

Leptospira detection and MST

DNA amplified from water samples with a cycle threshold (Ct) < 40 were considered positive when evaluating qPCR assay results for both *L. interrogans* and MST markers. The qPCR assay for *L. interrogans* was considered successful with an efficiency of over 95% with an $r^2 = 0.987$ or greater, and the detection limit of the assay was set at 5 genome copies of *L. interrogans*. The results from the *Lipl32 gene* qPCR assay indicated that pathogenic *Leptospira* was present only in June samples after rainfall and was undetectable in the dryer month of August ($p < 0.0001$) (Table 2). During June, *L. interrogans* were detected in 14 of the 44 water samples, with an average gene copy of ~ 400 ± 199 *Leptospirosis* per 100 mL sample. Bocaforma had the highest level of *L. interrogans* with approximately 737 genome copies per 100ml of water. Most of the samples that contained *L. interrogans* were from the western regions, Rio Piedras, Juan Mendez, and Dona Ana, that experienced between 17.53 and 24.38 mm of rain over 7 days (Table 2).

Water samples were assayed for the presence of human, dog, and horse fecal bacteria using the MST markers listed in Table 1. The BacCan MST assay indicated that dogs (26/44) were the most common source of fecal pollution in June. The next most prevalent source of fecal contamination in June was by humans (20/44),

Table 2 Pathogenic *Leptospira* gene copy enumeration and microbial source tracking data. Gene copy number represents a positive qPCR result, and (−) represents a negative result. For the microbial source tracking results, (+) represents a positive qPCR result, and (−) represents a negative qPCR for fecal markers. Samples labeled "a" indicate a June 2020 sampling event. The August sampling event (b) was negative for the *Leptospira* gene

Sample site	*Leptospira* No. Avg. genome copy	Human (HF183)	Dog	Horse
1a	634.9±153.7	-	-	-
2a	284±6.6	-	+	-
3a	101.74±6.2	+	+	-
4a	450.4±256.8	+	+	+
5a	-	+	+	-
6a	-	+	+	-
7a	-	+	-	-
8a	326.4±177.7	+	+	-
9a	365.2±205.3	-	-	-
10a	178.6±81.5	+	+	-
11a	-	+	+	-
12a	386.9±276.9	+	+	+
13a	-	-	-	-
14a	-	-	-	-
15a	289.5±193.1	-	-	-
16a	-	-	-	-
17a	-	+	+	-
18a	-	-	-	-
19a	-	+	+	-
20a	491±97.5	+	+	-
21a	593.3±131.9	+	+	-
22a	-	+	+	-
23a	-	+	+	-
24a	128.1±121	-	-	-
25a	-	+	-	-
26a	-	-	+	+
27a	-	-	-	-
28a	-	-	+	-
29a	-	-	+	-
30a	-	-	-	-
31a	-	-	-	-
32a	640.8±204	-	+	-
33a	-	-	+	-
34a	736.6±186.9	-	-	+
35a	-	-	+	-
36a	-	-	-	-
37a	-	-	-	-
38a	-	-	+	-
39a	-	-	+	-
40a	-	-	-	-
41a	-	+	+	-
42a	-	+	+	-
43a	-	+	-	-
44a	-	+	+	-

and horse fecal pollution was present in only four sites. During the August sampling, human fecal pollution was detected in 22 sites, dog fecal waste was detected in 15 sites, and horse fecal bacteria were detected in four sites (Fig 1, Table 2). Spearmen's correlation indicated a significant positive relationship (r =0.89) between the sites with human fecal contamination and the sites with pathogenic *Leptospira*. These sites were mostly located on the western part of the SJBE (Fig. 1). There was a moderate correlation (r = 0.58) between dog fecal contamination and the sites with pathogenic *Leptospir*a which could be influenced by the cross-reaction of the BacCan assay (Schriewer et al., 2013), and horse fecal pollution did not have a significant co-relationship (r = 0.14) to the presence of *L. interrogans* in the water samples.

Discussion

Nutrient concentration in SJBE and MST

The primary objective of this study was to assess nutrient concentrations in the waters flowing into the San Juan Bay Estuary (SJBE) and to employ microbial source tracking (MST) to identify potential fecal sources contributing to elevated nitrogen (N) and phosphorus (P) concentrations. Prior research conducted in Puerto Rico by Larsen and Webb (2009) demonstrates a startling ten-fold increase in N and P concentrations compared to presettlement values, which is primarily attributable to human landscape modification.

The SJBE in Puerto Rico, which is characterized by extensive urban and residential development, is susceptible to increased nutrient loading from a variety of sources, such as sewage discharges, animal waste, fertilizers, fossil fuels, and industrial wastewater (Conley et al., 2009; Galloway et al.,

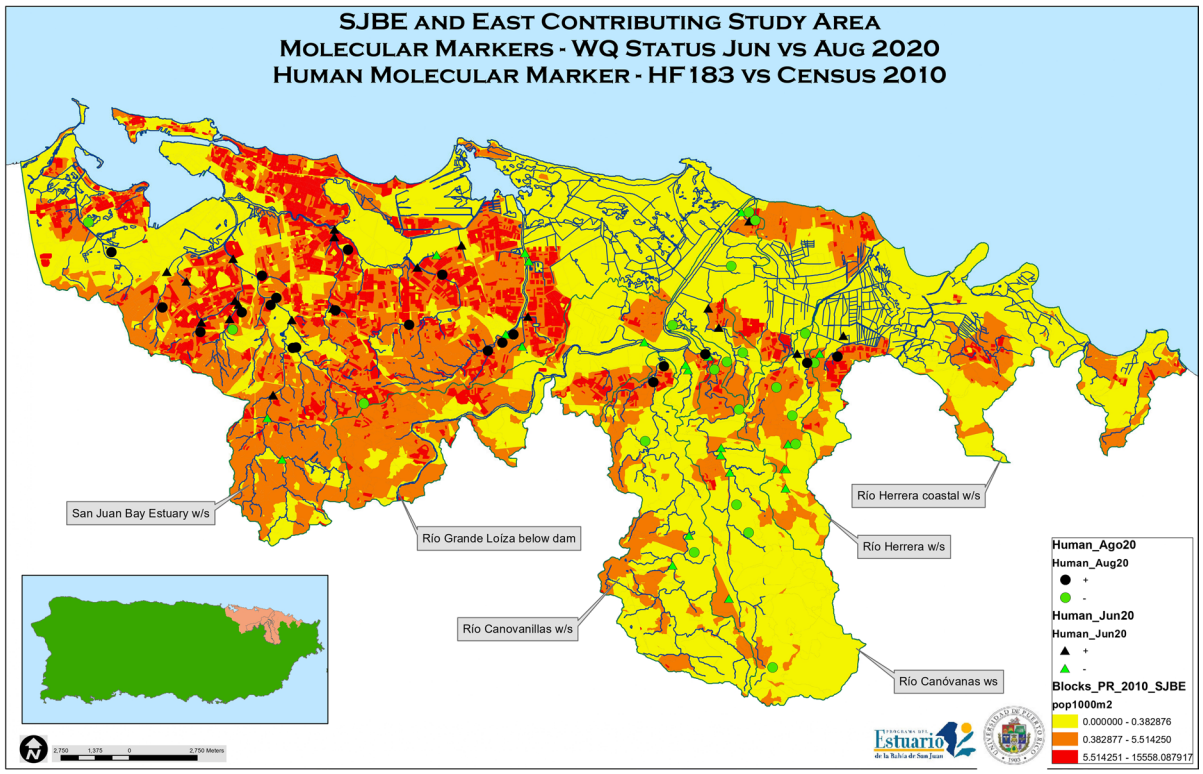

Fig. 1 Sampling sites with detectable levels of human fecal pollution in the wet and dry seasons

2008). This nutrient enrichment, particularly of N and P, is well documented to have detrimental effects on the integrity of estuarine ecosystems, thereby compromising their healthy functioning (Correll, 1999; Ngatia et al., 2019). To contribute to a broader understanding of human-induced nutrient loading and its implications for environmental health and resilience, the purpose of this study is to conduct a comprehensive evaluation of these crucial aspects.

Thirty-six out of the eighty-seven samples exceeded 160 µg L^{-1} of TP indicating that nearly half of the water sampled was in hypereutrophic conditions (Carlson, 1977). The areas that had the greatest levels of TP, Josephina (1860 µg L^{-1}, 1574 µg L^{1}) and Quebrada Angela (3210 µg L^{-1}), were also impacted by human (Fig. 1) and dog fecal pollution (Table 2, Appendix Table 3). In addition, the greatest concentration of TN measured in this study (34,013 µg L^{-1}) came from water collected at the Quebrada Angela location after periods of rainfall in June. Molecular markers for dog and human fecal pollution were also detected in these waters during the wet period in June (Table 2), while only the human fecal pollution was detected during the dry sampling event (data not shown). These results are not surprising since Quebrada Angela is located near urban areas with a high human population density. It should be noted that the concentration of NO_3^- in all water samples was below the USEPA regulatory limit of 10,000 µg L^{-1} (Appendix Table 4).

Dogs have been previously identified as significant sources of fecal pollution in coastal areas (Martin & Gruber, 2005; Wright et al., 2009), and the BacCan MST assay in our study indicates that dogs were the most common source of fecal pollution in June (26 sites) and the second most common source in August (15 sites). However, this assay is very sensitive but is reported to sometime exhibit cross-reactions with non-canine species (Boehm et al., 2013; Schriewer et al., 2013). However, the population of dogs in an area should be considered when developing management practices to limit the input of N and P in surface waters.

Horses are common in the sampled watersheds due to recreational and personal use. The molecular marker of horse fecal bacteria indicating horse fecal contamination was only present in four sites in each month; however, molecular markers for human and/or dog fecal contamination were also present in six of the eight horse-positive samples (Table 2). This was not surprising because humans, dogs, and horses are usually present in the same areas of the island. Horses typically produce a larger volume of waste per animal unit compared to humans and dogs, and their activity makes soil more susceptible to nutrient loss. For example, horse activity has been shown to decrease soil aggregate stability (Beever & Herrick, 2006) and increase bare ground in riparian buffers (Boyd et al., 2017) both of which increase the chance of surface runoff and subsequent nutrient loading. Furthermore, elevated nutrient levels have been previously linked to horse fecal pollution from uncontrolled horse manure runoff (Parvage et al., 2015; Skelly, 2015). Even though horse activity has been shown to increase nutrient loading in other watersheds, our MST results indicate that concentrations of N and P in surface waters of Puerto Rico are not significantly impacted by the presence of horses alone. Instead, our MST in Figure 1 suggests that fecal pollution in the affected watersheds is due to human population densities. Therefore, nutrient pollution in these watersheds is the result of high human population density, and dogs and horses can be considered complementary sources that are derived from human settlement.

Leptospira detection in wet and dry season

The concurrent occurrence of fecal waste and urine contamination in environmental samples implies that surface water containing high nitrogen and phosphorous from fecal runoff may also contain higher concentrations of *Leptospira* that is carried in the urine of infected host (Bierque et al., 2020; Fouts et al., 2016). Another aim of this study was to determine the relationship among nutrient levels, MST of human, horse and dog fecal contamination, and qPCR detection of pathogenic *Leptospira* in SJBE. Water samples were screened using the *Lipl32* gene qPCR assay that was optimized for environmental samples (Riediger et al., 2016; Truitt et al., 2020). During June sampling event, *L. interrogans* were detected in approximately 32% of the sites with an average gene copy number of 400 *leptospires* per 100 ml. (Table 2). This low concentration of *L. interrogans* may still pose a public health risk since reports suggest that a low dose of *L. interrogans* is infectious to humans and these bacteria can remain infectious for up to 20 months in water (Adesiyun et al., 2011; Hochedez et al., 2013). In addition, the DNA extraction protocol used recovers approximately 42% of bacterial DNA which implies that the actual concentration of *leptospires* in the water could be greater than the detected value (Morrison et al., 2008). These trends in the detection of *L. interrogans* are comparable to those in another Caribbean Island (St. Kitts) and rural Puerto Rico (Rawlins et al., 2014; Truitt et al., 2020). It was not surprising that *L. interrogans* were detected following rainfall in June because most studies have reported an increase in the detection of *L. interrogans* in freshwater environments following rain events (Keenum et al., 2021; Riediger et al., 2016; Truitt et al., 2020).

The molecular source tracking of humans, horses, and dogs was used to link the presence of *L. interrogans* in sampling sites to a possible source of fecal pollution in watersheds that flow into the SJBE. Unfortunately, an MST assay for rodents, which are a major reservoir of *Leptospira*, was not available during this study (Casanovas-Massana et al., 2018). Urban development in the San Juan region will attract rodents, which are the most common animal to shed *L. interrogans* in their urine (Casanovas-Massana et al., 2018). Molecular source tracking for human fecal contamination using the HF183-1 marker (Green et al., 2014) indicated a significant correlation between human fecal contamination and pathogenic *L. interrogans* ($r = 0.79$) during the June sampling event. There was also a positive correlation ($r = 0.70$) between dog fecal contamination and *L. interrogans* in the June samples which is likely due to the large population of stray dogs in Puerto Rico (The Sato Project, 2021) that could be potential host for *L. interrogans* and easily transmit it to waterways. In contrast, there was no meaningful relationship between horse fecal pollution and *L. interrogans* in the sampled watersheds ($r = 0.02$).

Conclusions

- The SJBE and Rio Grande de Loiza watersheds are impacted by elevated levels of TN and TP due to human activity.

- *Leptospira* was only present in the June samples and was linked to human and dog fecal pollution.
- The combined use of nutrient measurements, MST, and the qPCR assay for the *Lipl32 gene* and the source of the pathogenic *Leptospira* and nutrient contamination in environmental samples can be determined.
- Further studies need to be conducted to examine other seasonal changes in the area regarding *L. interrogans* presence.
- There is a need for mitigation efforts towards public health concerns and environmental impacts regarding nutrient concentrations and fecal pollution in SJBE.

Appendix

Table 3 Sample sites indicated by their subbasin, station, latitude, and longitude. Samples labeled "a" indicate a June 2020 sampling event and samples labeled "b" indicate an August 2020 sampling event

Sample #	Subbasin	Station	Latitude	Longitude
1a	Rio Piedras Sur	9	18.3435	−66.0598
2a	Rio Piedras Sur	7	18.35853	−66.0656
3a	Rio Piedras Norte	1	18.36687	−66.0633
4a	Rio Piedras Norte	4	18.39435	−66.056
5a	Rio Piedras Norte	7	18.41659	−66.0785
6a	Margarita	9	18.41197	−66.1039
7a	Margarita	11	18.40846	−66.0963
8a	Juan Méndez	4	18.39855	−66.0405
9a	Juan Méndez	10	18.42451	−66.0397
10a	Juan Méndez	11	18.42454	−66.0395
11a	Juan Méndez	12	18.42725	−66.0395
12a	Blasina	8	18.39529	−65.9655
13a	Blasina	9	18.41554	−65.9652
14a	Blasina	10	18.41848	−65.9665
15a	Blasina	17	18.38433	−65.9677
16a	Blasina	19	18.38887	−65.9741
17a	San Antón	8	18.41328	−66.0078
18a	San Antón	11	18.41798	−66.0006
19a	San Antón	12	18.42146	−65.991
20a	Dona Ana	5	18.39349	−66.0906
21a	Dona Ana	11	18.40133	−66.0778
22a	Josefina	3	18.3947	−66.0798
23a	Josefina	5	18.39996	−66.0766
24a	Río Grande de Loíza	2	18.3859	−65.9209
25a	Río Grande de Loíza	14	18.42989	−65.8806
26a	Río Grande de Loíza	16	18.43329	−65.8837
27a	Río Canovanillas	2	18.30439	−65.9103
28a	Río Canovanillas	5	18.3153	−65.9041
29a	Río Canóvanas	3	18.29217	−65.8889
30a	Río Canóvanas	15	18.33826	−65.8884
31a	Río Canóvanas	16	18.34728	−65.8917
32a	Río Canóvanas	17	18.34455	−65.8919
33a	Bocaforma	1	18.37559	−65.9042
34a	Bocaforma	2	18.3775	−65.9053
35a	Bocaforma	5	18.38054	−65.8966
36a	Río Herrera	2	18.33242	−65.867
37a	Río Herrera	3	18.33947	−65.8675
38a	Río Herrera	4	18.3394	−65.8668

Table 3 (continued)

Sample #	Subbasin	Station	Latitude	Longitude
39a	Río Herrera	6	18.34865	−65.8661
40a	Río Herrera	11	18.3815	−65.8538
41a	Quebrada Angela	7	18.38796	−65.8446
42a	Quebrada Cambalache	4	18.3815	−65.8623
43a	Canal San Isidro	2	18.39106	−65.892453
44a	Canal San Isidro	4	18.39817	−65.896519
1b	Rio Piedras Norte	3	18.38402	−66.0587
2b	Rio Piedras Norte	5	18.40246	−66.0649
3b	Rio Piedras Norte	6	18.41052	−66.0704
4b	Margarita	1	18.39897	−66.1086
5b	Margarita	2	18.39891	−66.1086
6b	Catano	Toro Greek	18.41919	−66.1281
7b	Catano	Puente Blanco	18.43014	−66.1369
8b	Juan Méndez	3	18.39778	−66.0422
9b	Juan Méndez	7	18.41992	−66.0374
10b	Blasina	5	18.38292	−65.9839
11b	Blasina	7	18.38577	−65.9782
12b	Blasina	19	18.38887	−65.9741
13b	San Antón	7	18.41064	−66.0012
14b	Sabana Llana	6	18.39249	−66.014
15b	Guaracanal	6	18.38432	−66.0574
16b	Guaracanal	1	18.36378	−66.0314
17b	Buena Vista	7	18.39982	−66.0671
18b	Dona Ana	6	18.38989	−66.094
19b	Josefina	2	18.3908	−66.0816
20b	Josefina	4	18.3972	−66.0781
21b	Río Grande de Loíza	4	18.39203	−65.913
22b	Río Grande de Loíza	7	18.41363	−65.8905
23b	Río Grande de Loíza	15	18.43035	−65.8813
24b	Río Grande de Loíza	16	18.43329	−65.8837
25b	Río Canovanillas	4	18.30906	−65.9051
26b	Río Canovanillas	9	18.34989	−65.9236
27b	Río Canovanillas	12	18.37135	−65.9205
28b	Río Canovanillas	13	18.37699	−65.9164
29b	Río Canóvanas	2	18.26704	−65.8751
30b	Río Canóvanas	6	18.31625	−65.8842
31b	Río Canóvanas	8	18.32654	−65.8888
32b	Río Canóvanas	18	18.36137	−65.8877
33b	Río Canóvanas	20	18.37866	−65.8922
34b	Bocaforma	3	18.37585	−65.8972
35b	Bocaforma	6	18.38137	−65.9006
36b	Río Herrera	6	18.34865	−65.8661
37b	Río Herrera	8	18.37807	−65.8588
38b	Río Herrera	10	18.38031	−65.85
39b	Quebrada Angela	4	18.35905	−65.8672
40b	Quebrada Angela	6	18.37815	−65.8616
41b	Quebrada Angela	8	18.38893	−65.8623
42b	Quebrada Cambalache	1	18.36911	−65.8733
43b	Canal San Isidro	1	18.3819	−65.886270

Table 4 Nitrogen and phosphorous concentration in the watershed's sites during June (a) and August (b), 2020. All measurements are in micrograms per liter

Sample site	Total phosphorus ($\mu g\ L^{-1}$)	Dissolved phosphorus ($\mu g\ L^{-1}$)	Total Kjeldahl nitrogen ($\mu g\ L^{-1}$)	Total nitrogen ($\mu g\ L^{-1}$)	Ammonium (NH_4^+) ($\mu g\ L^{-1}$)	Nitrate (NO_3^-) ($\mu g\ L^{-1}$)
1a	84	73	0.00	705.03	0.00	705.03
2a	118	104	161.00	1123.33	0.00	962.33
3a	112	95	479.11	1412.76	0.00	933.64
4a	137	117	244.22	1214.69	0.00	970.47
5a	187	158	550.67	1325.96	219.75	775.29
6a	78	85	553.00	1606.15	76.87	1053.15
7a	59	44	419.22	987.14	0.00	567.91
8a	420	392	4489.35	4822.32	5525.57	332.98
9a	347	307	2208.12	2329.20	1257.93	121.08
10a	227	195	994.78	1629.11	373.50	634.33
11a	336	294	1575.78	1900.63	940.34	324.84
12a	373	350	2217.45	2236.43	1085.55	18.98
13a	381	252	2079.01	2101.82	215.09	22.82
14a	221	188	1448.23	1545.82	567.62	97.59
15a	265	245	581.00	1602.07	0.00	1021.07
16a	127	107	530.45	1292.86	0.00	762.41
17a	31	12	265.22	879.67	54.36	614.45
18a	352	300	1533.78	1584.16	554.42	50.38
19a	725	469	4778.68	4888.47	3738.85	109.79
20a	65	47	149.33	1115.73	0.00	966.40
21a	55	33	537.45	1373.95	0.00	836.51
22a	1860	1842	231.78	1124.31	46.59	892.53
23a	1574	1528	1186.11	2146.42	246.93	960.30
24a	14	0	735.78	749.33	0.00	13.55
25a	410	311	1783.45	1807.85	10.09	24.40
26a	36	2	529.67	530.12	0.00	0.45
27a	27	14	290.89	373.80	0.00	82.91
28a	131	130	794.11	1896.73	180.92	1102.62
29a	18	10	362.45	436.77	0.00	74.32
30a	17	9	0.00	116.56	0.00	116.56
31a	24	18	0.00	108.43	0.00	108.43
32a	24	12	134.56	283.88	0.00	149.32
33a	692	669	2150.56	2254.48	1371.30	103.91
34a	599	588	1393.00	1705.65	931.80	312.65
35a	263	247	462.78	624.98	201.89	162.20
36a	25	17	335.22	500.81	0.00	165.58
37a	28	23	0.00	174.17	0.00	174.17
38a	45	44	361.67	410.01	0.00	48.34
39a	31	16	136.11	420.07	0.00	283.96
40a	344	268	656.45	948.54	0.00	292.09
41a	3210	2840	33996.76	34013.71	25624.50	16.94
42a	190	162	105.00	573.07	0.00	468.06
43a	199	184	211.56	212.23	205.77	0.68

Table 4 (continued)

Sample site	Total phosphorus (μg L^{-1})	Dissolved phosphorus (μg L^{-1})	Total Kjeldahl nitrogen (μg L^{-1})	Total nitrogen (μg L^{-1})	Ammonium (NH_4^+) (μg L^{-1})	Nitrate (NO_3^-) (μg L^{-1})
44a	179	169	1057.00	1066.49	720.59	9.49
1b	250	211	2483.45	2782.99	1608.91	299.54
2b	168	150	843.89	2183.48	252.36	1339.59
3b	200	180	1198.56	2502.00	562.96	1303.44
4b	105	93	1363.44	1955.75	771.84	592.31
5b	42	33	608.22	1141.57	188.69	533.35
6b	14	3	295.56	393.14	48.14	97.59
7b	157	109	1415.56	1470.68	535.79	55.12
8b	177	155	1638.78	2887.55	733.02	1248.78
9b	346	285	3881.89	5163.19	1832.54	1281.30
10b	93	76	760.67	1867.58	133.56	1106.91
11b	99	85	671.22	1837.32	160.74	1166.10
12b	91	84	500.11	1566.36	63.67	1066.25
13b	490	380	2053.33	3081.18	217.42	1027.85
14b	502	390	2058.78	3099.27	94.73	1040.50
15b	52	127	339.11	1539.09	21.74	1199.98
16b	127	43	527.33	1096.38	243.04	569.04
17b	354	234	3146.11	3651.68	1938.92	505.56
18b	136	118	1341.67	2246.40	796.69	904.73
19b	53	49	275.33	1194.07	38.83	918.74
20b	161	140	496.22	1273.32	44.26	777.10
21b	29	3	795.67	1269.38	37.27	473.71
22b	291	219	1898.56	1912.11	111.82	13.55
23b	317	236	1250.67	1265.35	119.58	14.68
24b	75	22	1268.56	1503.04	111.82	234.48
25b	28	21	258.22	437.14	31.06	178.91
26b	77	71	412.22	1161.53	49.70	749.31
27b	144	132	689.11	1289.55	166.17	600.44
28b	93	73	775.44	1036.36	115.70	260.91
29b	58	53	214.67	319.94	40.38	105.27
30b	25	21	132.22	296.45	32.61	164.23
31b	6	2	94.11	181.08	39.60	86.97
32b	17	10	0.00	81.10	42.71	81.10
33b	12	2	230.22	294.83	67.56	64.61
34b	365	359	5063.33	5683.20	78.43	619.87
35b	990	955	5950.00	5962.43	5820.64	12.42
36b	22	19	104.22	296.01	92.40	191.79
37b	38	31	280.00	471.79	64.45	191.79
38b	156	142	465.89	538.40	95.51	72.51
39b	136	134	405.22	815.00	48.92	409.78
40b	240	220	700.00	950.75	157.63	250.75
41b	315	223	3300.89	3307.67	1399.25	6.78
42b	52	47	397.44	841.11	75.32	443.67
43b	73	100	464.33	474.72	72.21	10.39

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Author contribution Everyone contributed to the final version of this manuscript. D.S.B. and T.C. analyzed the data, conducted the PCR analysis, and wrote the main manuscript text. G.A.M. collected the samples and prepared the maps and figures and assisted with the manuscript editing and statistics. C.D.B. assisted with the proof reading, statistics, nutrient data analysis, and discussion. W.C.D. assisted with the sample preparation, PCR analysis, and tables.

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Data availability The data set used is included in this manuscript and is available from the corresponding author on reasonable request.

Declarations

Ethics approval All authors have read, understood, and have complied as applicable with the statement on "Ethical responsibilities of Authors" as found in the Instructions for Authors and are aware that with minor exceptions, no changes can be made to authorship once the paper is submitted. This is an observational study. The Research Ethics Committee has confirmed that no ethical approval is required.

Competing interests The authors declare no competing interests.

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