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Molecular Detection of Pathogenic Leptospira and Microbial Source Tracking of Fecal Pollution in San Juan, Puerto Rico

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Preface

This thesis has been written in journal format and conforms to the style appropriate to my discipline. This manuscript will be submitted for publication in the Journal of Applied Microbiology, a peer reviewed interdisciplinary scientific journal, and therefore reflects the required formatting for this publication. This thesis does not contain a list of tables or a list of figures since these are not included in the submission directions for contributors to this journal. Figures and tables follow the text of the manuscript as required by the Journal of Applied Microbiology and this thesis committee.

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Abstract

Leptospirosis, caused by pathogenic *Leptospira*, is endemic to tropical regions. *Leptospira* is released into the environment through the secretion of urine from animals, making it easily transmissible through water sources. The estuarian environment surrounding the area of San Juan, Puerto Rico and its high density of urban development creates ideal conditions for transmission of Leptospirosis. The goal of this study was to determine the presence of *Leptospira* in these surface waters and use Microbial Source Tracking (MST) to identify the possible source of pathogenic *Leptospira*. Eighty-seven water samples were collected during the dry (44) and wet (43) seasons. Phosphorus and nitrogen levels were determined using standard USEPA methods. The level of *Leptospira interrogans* was determined using quantitative polymerase chain reaction (qPCR) targeting the *Lipl32 gene*. Human (HF183), dog (BacCan-UCD), and horse (HoF597) MST assays were performed to determine the likely sources of fecal contamination at each site. Total phosphorus and total nitrogen exceeded USEPA safety guidelines in multiple locations. *Leptospira interrogans* was detected in 32% of samples collected in the dry season and was not detected in the wet season. There was a positive correlation (r =0.89) between the presence of *L. interrogans* and human fecal bacterial MST marker (HF183). The MST also indicated a positive correlation between horse fecal contamination and total phosphorus and total nitrogen. The correlation between *L. interrogans* gene copies and MST makers warrants further examination of the water quality in the estuaries of San Juan, Puerto Rico due to the possible public health implications.

Introduction

Puerto Rico

The San Juan Bay Estuary (SJBE) is a wetland located in the northern part of the Caribbean island of Puerto Rico. The SJBE is a productive estuarian environment full of various wildlife and plant life, which can be vital in keeping the coastline intact particularly mangrove forests (Fretwell 1996). The San Juan metropolitan area is home to close to three million people, which is in close proximity to these important environments (Figures 1- 4). This particular area of Puerto Rico receives an abundant amount of rainfall, approximately 1500-1700 mm per year (Fretwell 1996, Brandeis *et al*. 2014). Rainfall is typically higher in the wet season, with the most extreme in August and September (Sánchez Colón and Schaffner 2021). This can be linked to the strong effects of hurricane season on the island (Mendez-Lazaro *et al*. 2014).

Nutrients and pathogenic bacteria from human activity can enter surface waters during storm events which could create a public health concern for individuals relying on these resources. Nitrogen and phosphorus are key nutrients to these aquatic environments, but an excess can indicate eutrophication and fecal contamination from a number of non-point sources (Conley *et al*. 2009). Pathogenic bacteria, specifically pathogenic *Leptospira* that causes Leptospirosis, and Microbial source tracking (MST) can lead to a better description of the possible contamination. Leptospirosis is considered an endemic disease among tropical regions due to the warm, wet environment with poor sanitation (Bharti *et al*. 2003). Cases of Leptospirosis have been linked as a cause of hurricane-associated mortality, this was especially seen after Hurricane Maria struck in September 2017. The large number of estimated cases that go unreported and misdiagnosed is a current public health concern for the island of Puerto Rico (Briskin *et al*. 2019). It is estimated that there were 15-100 cases in 2010 cases of Leptospirosis,

with the assumption that many went unreported (CDC 2012). In 2014-2015, 114 cases were reported to the Puerto Rico Health Depart.

Factors contributing to the spread of Leptospirosis are weather and rodent population. Based on evidence from Hacker *et al*. 2020, we would expect more cases of Leptospirosis during times of heavy precipitation due to dissolution of biofilm, higher oxygen concentration, and better mobility. Another contributing factor, previous studies have linked the rodent population and infestations in communities in San Juan to be a direct threat to human infection rates (Briskin *et al.* 2019). Pathogenic *Leptospira* has also been detected in many rodent species that were found in cattle farming communities in San Juan (Benavidez *et al*. 2019).

Leptospira

Leptospira are free-living bacteria that can survive in soil, freshwater, marine habitats, or in their animal hosts (Holt *et al*. 1994). Brock and Madigan (1984) describe this bacterium as a spirochete that is approximately 0.1 μm in diameter and 6 to 24 μm in length. They are typically stained with aniline dyes to be seen through a microscope. The helical form coils clockwise and both ends of the cell have hooked ends with two flagella per cell, one on each end. They move in rotations along the long axis in liquid solutions. In more viscous locations, more serpentine movement occurs. It is an aerobic bacterium that uses fatty acids or alcohols that have 15 or more carbon atoms as their carbon and energy sources. They do not use carbohydrates or amino acids as a source of energy.

Since its original description, *Leptospira* has been divided into two saprophytes. The saprophytes being a nonpathogenic, *Leptospira biflexa*, and a pathogenic saprophyte, *Leptospira interrogans,* both of these saprophytes have a very broad range (Stimson 1907). It is now

understood that *Leptospira* can be divided into three saprophytes-based lineages that correlate with pathogenicity. The three saprophytes are saprophytic, intermediate, and pathogenic (Perolat *et al*. 1998). The intermediate species have a common ancestor with the pathogenic species, but they present less pathogenicity in humans and animals. All of the saprophytes are able to survive in moist soil and freshwater environments for multiple weeks (Andre-Fontaine *et al.* 2015, Casanovas-Massana *et al*. 2018 b).

Fouts *et al.* (2016) consider *Leptospira* to be one of the most complex genera of pathogenic bacteria present. Evolutionary changes have helped the bacteria to have very diverse properties to help it survive. One major difference that separates the pathogenic species from the nonpathogenic species is the *B12* biosynthetic pathway. The pathogenic species are the only ones that possess this pathway for B12 synthesis. This pathway may serve as a way for these pathogens to survive in specific environmental niches. Another difference between the pathogenic and nonpathogenic species is the catalase activity in the pathogenic *Leptospira*. The catalase enzyme*, KatA,* is linked to the phagocyte-produced oxidative burst-mediated killing of pathogens after phagocytosis inside the cell. This may be the reason for intracellular host killing. Both the pathogenic and nonpathogenic serotypes have similar genes when it comes to movement using their flagella. The specific changes in motility between species are most likely due to the specific environmental niche that it fills. Only pathogenic *Leptospira* contain CRISPR/Cas systems potentially leading to the modification of this bacteria.

Leptospira causes the globally widespread disease, Leptospirosis. It is a neglected and emerging zoonotic disease (Fouts *et al.* 2016). The symptoms of leptospirosis are commonly associated with those of influenza, dengue, and malaria (Haake and Levett 2015). Leptospirosis can be hard to clinically diagnosed because the symptoms vary greatly. It can start as simple flu

symptoms and progress into renal failure, pulmonary hemorrhage, or meningitis. A more severe form of Leptospirosis is called Weil's disease. The bacteria can be tested for in the blood and urine of individuals, most commonly through quantitative polymerase chain reaction (qPCR) and microscopic agglutination test (MAT). Cultured *Leptospira* does not benefit clinical diagnoses well because of the slow growth (Marinova-Petkova *et al*. 2019). Cultures are typically performed using Ellinghausen and McCullough modified by Johnson and Harris (EMJH) media and can take days to weeks to see visible culture (Phillip *et al.* 2018).

Leptospira can enter the organism through mucous membranes or skin abrasions. Then *Leptospira* travels through the body of the infected organism and locates in the kidneys or the liver, causing jaundice and nephritis. The *Leptospira* that has caused the infection will then pass through the body through the urine. Infection can be caused by coming in contact with urine containing *Leptospira* (Fouts *et al*. 2016). The infections can be treated with antibiotics like penicillin, streptomycin, and tetracyclines. There is a lack of previous knowledge of antibiotic resistance on pathogenic *Leptospira*. Liegeon *et al.* (2018), suggested that it was susceptible to penicillin, amoxicillin, clavulanate, cephalexin, ceftriaxone, doxycycline, tetracycline, streptomycin, enrofloxacin, and spectinomycin. There is potential resistance to polymyxin, along with enrofloxacin, streptomycin, and tetracycline in the case of high inculcation of *Leptospira*. *Leptospira* grows slowly, so it may cause the need for further treatment to be expelled from the kidneys (Fouts et al. 2016). Early identification is key to the successful treatment of the disease (Gorbea et al. 2018). In cases severe enough to require hospitalization, the mortality rate is between 5-15%. *Leptospira* causes disease worldwide affecting 1 million people and causing 60,000 deaths per year. Lack of timely diagnosis and lack of awareness cause Leptospirosis to go unreported (Costa *et al*. 2015, Gorbea *et al.* 2018).

In addition to rodents and other mammals, dogs can be infected with *Leptospira* that causes similar infections as humans, but they are typically vaccinated for it. The most common serotypes for dog infection are *L. grippotyphosa*, *L. pomona*, *L. canicola, and L. icterohemorrhagiae* (Brown and Prescott 2008). These particular serotypes do not usually infect humans, particularly since most dogs are vaccinated. Along with vaccinations, rat elimination in cities would be an effective method towards the prevention of human infection (Brock and Madigan 1991). Not only does it have negative effects on humans and animals, but it also has a negative effect on the economy (Ellis 2014). For example, Leptospirosis in cattle can cause spontaneous abortions, stillbirth, infertility, and loss of milk (Rajeev *et al*. 2014).

Microbial Source Tracking

Microbial source tracking (MST) is a method used to detect fecal contamination using *Bacteroidales* that are present in the gut microbiome of specific animal hosts (Kongprajug *et al.* 2019). By using different host-specific *Bacteroidales,* the fecal contamination can be attributed to a specific animal which is more efficient than using ubiquitous fecal coliforms that are known to reproduce in the environment. These techniques have been proven useful to determine water quality and sources of fecal contamination in other tropical and subtropical settings (Bridgemohan *et al*. 2020, Gonzalez-Fernandez *et al*. 2021). Once the source of contamination is determined the appropriate mitigation techniques can be used. The potential pathogens and contaminants linked to fecal pollution can cause major issues for human health (Ahmed *et al.* 2018).

With high rainfalls, specifically due to hurricane season in the tropics, and developing urban environments, the area of the SJBE is subject to poor water quality and the presence of disease-causing pathogenic *Leptospira*. Poor water quality in these areas can be attributed to elevated nitrogen and phosphorus levels and fecal contamination. The objectives of this study were to determine the water quality of the SJBE in reference to nutrient presence, pathogenic *Leptospira,* and fecal contamination. The goal was to determine the presence of *L. interrogans* in the surface waters of the SJBE and use MST to identify the possible sources of the pathogenic *Leptospira*. Another aspect of this study was to determine the possible correlation between nutrient richness, pathogenic *Leptospira,* and MST.

Methods

Sampling Sites

Sites were chosen based on urban and agricultural locations in the SBJE. Multiple stations in specific subbasins were chosen to further examine the areas. Eighty-seven water samples were collected from these sites during one single sampling event in June (dry season) and another sampling event in August (wet season). Sites with the location and sample number are located in Table 1. Sample collection methods followed standard operating methods #024W (see appendix).

Table 1. 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
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

Nutrient Collection

Samples were analyzed for both the total and dissolved fractions. Total phosphorus (TP), dissolved phosphorus (DP), total Kjeldahl nitrogen (TKN), total nitrogen (TN), ammonium (NH_4^+), and nitrate (NO_3^-) were determined chemically using the USEPA Method 365.4 for TP and DP; Method 351.2 for TKN and NH_4^+; and Method 353.2 for (NO_3^-) (see appendix).

DNA extraction

One hundred milliliters of each sample were filtered through 0.22-µm-pore-size nitrocellulose membrane filters (Type GS, Millipore, Billerica, MA, USA), were frozen at -20 ºC, and shipped to Georgia College and State University. DNA was extracted from the filters using the QIAGEN PowerSoil Kit (Qiagen, Hilden, Germany) following the procedure of Truitt *et al.* 2020. Total genomic DNA from each sample was quantified using a Nanodrop ND-1000 Spectrophotometer (Wilmington, DE) and then frozen at -20 ºC.

Quantitative Polymerase Chain (qPCR) Assays of Leptospira interrogans

Leptospira interrogans was quantified using a 242 bp segment of the *Lipl32 gene*, as previously described (Rawlins *et al.* 2014), was targeted for this qPCR assay by running samples in duplicate using QuantiTect Probe PCR (Qiagen) with the Bio-Rad CFX96 (Hercules, California 94547, USA), as described in Rawlins et al. 2014. Each reaction had a final volume of 20 µl using 2 µl of extracted DNA, 500 nM of each primer (Table 2), and 200 nM of the probe. The thermal conditions were an initial 95°C for 15 minutes, followed by 40 cycles at 95°C for 10 s and 64°C for 30 s. *Leptospira interrogans* serovar Pomona was used as the positive control, *Escherichia coli* strain K-12 as a negative control, and no-template controls were performed in each assay.

Leptospira interrogans positive DNA extracted samples were then performed in triplicate under the same conditions with a 10-fold serial dilution as a positive standard curve to determine genome copy numbers. The genome size of *L. interrogans* (4.659 Mb) was used to calculate the amount per 100 ml (Rawlins *et al.* 2014, Truitt *et al*. 2020).

Quantitative Polymerase Chain (qPCR) Assays of Microbial Source Tracking (MST)

Human (HF-183) and Dog (BacCan)Assays. The QuantiTect Probe PCR (Qiagen) assays were both performed in duplicate with a total volume of 20 µl using 2 µl of extracted DNA, 500 nM of each primer (Table 2), and 200 nM of the probe The thermal conditions for the human MST assay were an initial 95°C for 15 minutes, followed by 40 cycles at 95°C for 10 s and 60°C for 40 s. Human-specific *Bacteriodales* were used as a positive control, *E. coli* strain K-12 as a negative control, and no-template controls were performed in each assay (Haugland *et al*. 2010). The thermal conditions for the dog MST assay was an initial 95°C for 15 minutes, followed by 40 cycles at 95°C for 10 s and 61.4°C for 30 s. Dog-specific *Bacteriodales* were used as a positive control, *E. coli* strain K-12 as a negative control, and no-template controls were performed in each assay (Kildare *et al*. 2007).

Horse. A SsoFast Evagreen (BIO-RAD) assay was performed in duplicate with a total volume of 20 µl using 2 µl of extracted DNA and 500 nM of each primer(Table 2). A melt curve was used to analyze positive samples instead of the use of Cq values. Horse-specific *Bacteriodales* were used as a positive control, *E. coli* strain K-12 as a negative control, and no-template controls were performed in each assay (Dick *et al*. 2005).

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 (Illowsky and Dean, 2021).

Table 2. Primers and probes for *Leptospira interrogans* detection and microbial source tracking.

Target	Primer	Sequence	Reference:
Lipl32 gene	Lipl32-45F Lipl32-286R Lipl32-189P	AAGCATTACCG CTTGTGGTG GAACTCCCATTTCAGCGATT FAM-AAAGCCAGGACAAGCGCCG-BHQ1	Rawlins *et al*. 2014
Human	HF-183-1 BtheR1 Probe	ATCATGAGTTCACATGTCCG CGTAGGAGTTTGGACCGTGT 6-FAM-CTGAGGAGAAGGTCCCCCACATTGGA-tamra	Haugland *et al*. 2010
Dog	BacCan-545f1 BacUni-690r2 probe	GGAGCGCAGACGGGTTTT AATCGGAGTTCCTCGTGATATCTA 6-FAM-TGGTGTAGCGGTGAAA-TAMRA-MGB	Kildare *et al*. 2007
Horse	HoF597 F Bac708 R	CCAGCCGTAAATAGTCGG CACATGTTCCTCCGCTCGTA	Dick *et al*. 2005

Results

Nutrients

The majority (84/87) of sampled sites had TP concentration < 1000 μg L^{-1}. The greatest levels of TP were observed in the June sampling event at two locations in Josephina (1,860 μg L^{-1}, 1,574 μg L^{-1}) and one in Quebrada Angela (3,210 μg L^{-1}). These same sites exhibited elevated levels of DP ranging from ~1,500 – 2,800 μg L^{-1}, while the other sites ranged from ~ 2 – 955 μg L^{-1} (Appendix Table 1). In addition to phosphorus, elevated levels of TN were found at Quebrada Angela (34,013 μg L^{-1}) during the June sampling event and two sites in Bocaforma (5,683 μg L^{-}

^{1}, 5,962 μg L^{-1}) subbasin during the August sampling month. The majority of sampled sites had TN concentrations <5,000 μg L^{-1}, with those sites raging from ~108 – 4,800 μg L^{-1}(Appendix Table 1). Quebrada Angela subbasin had extreme numbers exceeding both TP and TN at the June sampling event. Juan Méndez and San Antón had relatively high above 4,800 μg L^{-1} TN concentrations compared to the other sample sites in June.

Leptospira 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 $r^2 = 0.987$ (E = 88.2% and slope = 3.641). The results from the *Lipl32 gene* qPCR assay indicated that pathogenic *Leptospira* was present only in the dry season in June and was undetectable in the wet season in August ($p < 0.0001$) (Table 3). During the dry season sampling event, 14 of the 44 water samples were positive for *L. interrogans*, with an average gene copy of ~ 400 ± 199 leptospires per 100 mL sample, ranging from ~736-101 leptospires per sample.

Twenty-seven out of the eighty-seven the water samples were positive for two markers, including the MST markers and *L. interrogans.* Five sites were positive for three markers and two sites were positive for all four markers tested. These two sites were in the June sample event at the Rio Pedras Notre and Blasina subbasins. Spearmen's correlation indicated a significant positive relationship (r =0.89) between the sites positive for human fecal contamination and the sites positive for pathogenic *Leptospira*. These sites were mostly located on the western part of the SJBE (Figures 1 and 2). There was a moderate correlation (r = 0.58) between dog fecal

contamination and the sites positive for pathogenic *Leptospira*. *Leptospira interrogans* prevalence did not correlate with horse fecal contamination ($r = 0.14$).

Leptospira interrogans had no significant correlation with TP, DP, TN, NH_4^+, or NO_3^- ($r \leq 0.50$). Furthermore, there was no correlation between nutrients and MST markers for dog and human fecal contamination ($r \leq 0.50$). However, data from MST assays with positive horse fecal pollution yielded significant correlations with TP ($r = 0.90$), TN ($r = 0.92$), and NH_4^+ ($r = 0.96$).

Site maps (Figures 1, 2, 3, and 4) were created to show the difference between the dry and wet sampling events and the positive and negative markers. The maps can also be used as a comparison to look at the differences between the MST and the *L. interrogans* data at the same locations. The correlation can be visibly seen in locations between the human and *L. interrogans* positive locations on the site maps.

Table 3. 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 and samples labeled (#b) indicate an August sampling event.

Sample Site	*Leptospira*	Human (HF183)	Dog	Horse
	No. Avg. Genome Copy			
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	-	+	+	-
1b	-	+	+	-
2b	-	+	-	-
3b	-	+	+	-
4b	-	+	-	-
5b	-	+	-	-
6b	-	+	-	-
7b	-	-	+	-
8b	-	+	+	-
9b	-	+	+	-
10b	-	+	+	-
11b	-	+	+	-
12b	-	+	+	-
13b	-	+	+	+
14b	-	+	-	-
15b	-	+	-	+
16b	-	-	-	-

17b	-	+	+	-
18b	-	+	+	-
19b	-	-	-	-
20b	-	+	+	-
21b	-	-	-	-
22b	-	-	-	-
23b	-	-	-	-
24b	-	-	-	-
25b	-	-	-	-
26b	-	-	-	-
27b	-	+	+	-
28b	-	+	+	-
29b	-	-	-	+
30b	-	-	-	-
31b	-	-	+	-
32b	-	-	-	-
33b	-	-	-	-
34b	-	-	-	-
35b	-	+	-	-
36b	-	-	-	-
37b	-	-	-	-
38b	-	+	-	-
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41b	-	-	-	-
42b	-	-	-	-
43b	-	-	-	+

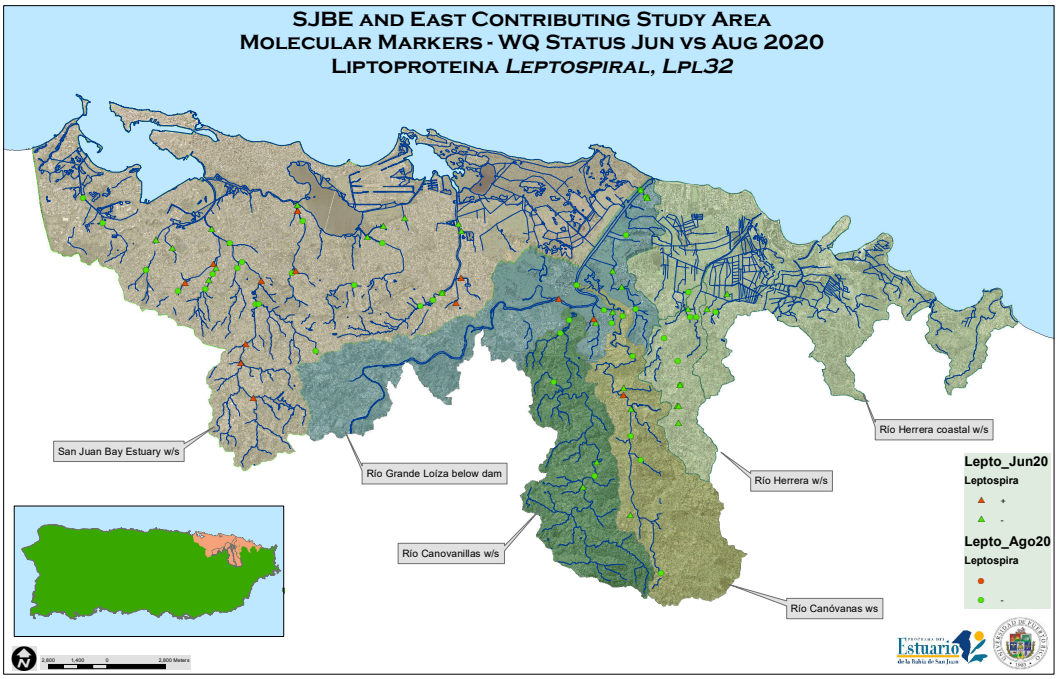

Figure 1. San Juan Bay Estuarian (SBJE) study area with indicated positive (red) and negative (green) data points for pathogenic *Leptospira* in both June (Jun20, triangles) and August (Ago20, circles) sampling events.

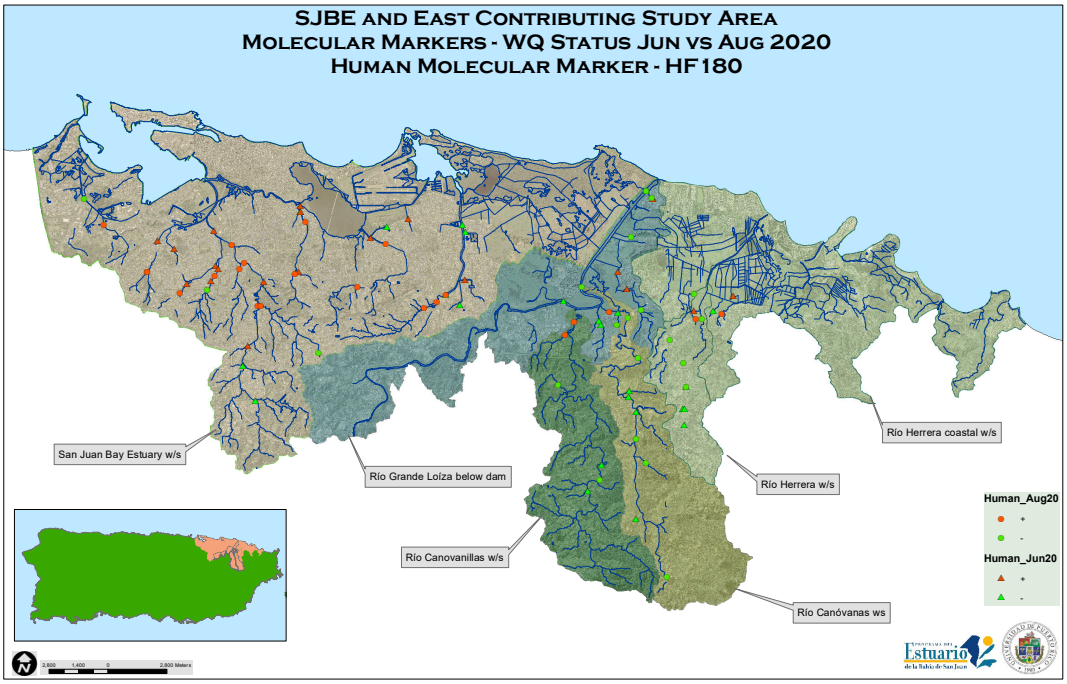

Figure 2. San Juan Bay Estuarian (SBJE) study area with indicated positive (red) and negative (green) data points for Human fecal contamination in both June (Jun20, triangles) and August (Aug20, circles)sampling events.

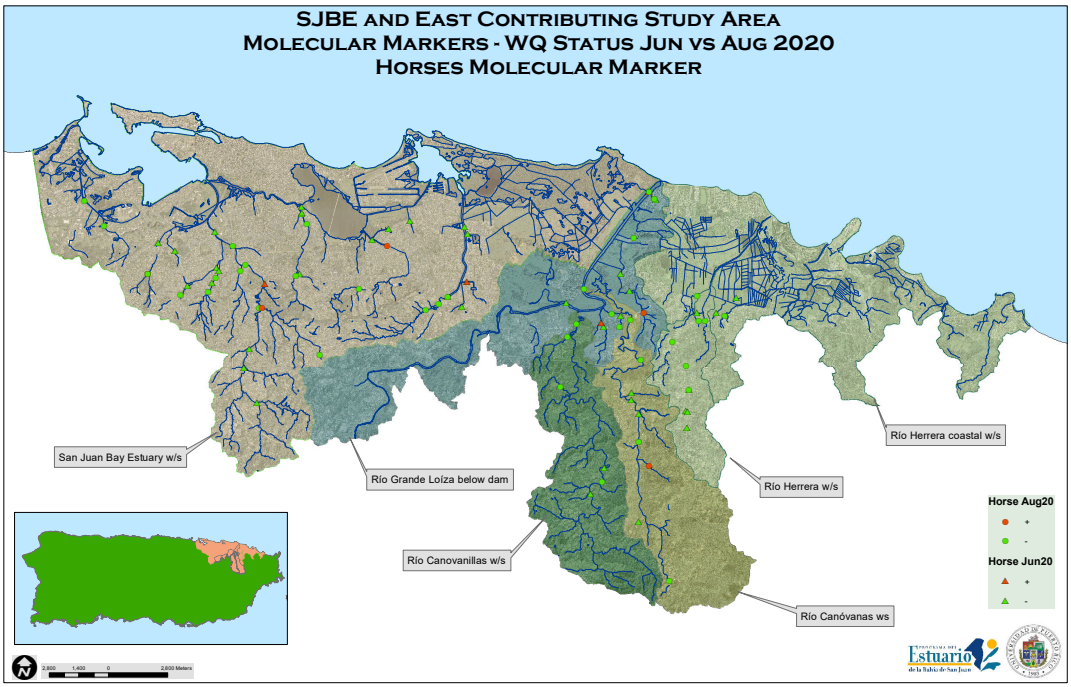

Figure 3. San Juan Bay Estuarian (SBJE) study area with indicated positive (red) and negative (green) data points for Horse fecal contamination in both June (Jun20, triangles) and August (Aug20, circles) sampling events.

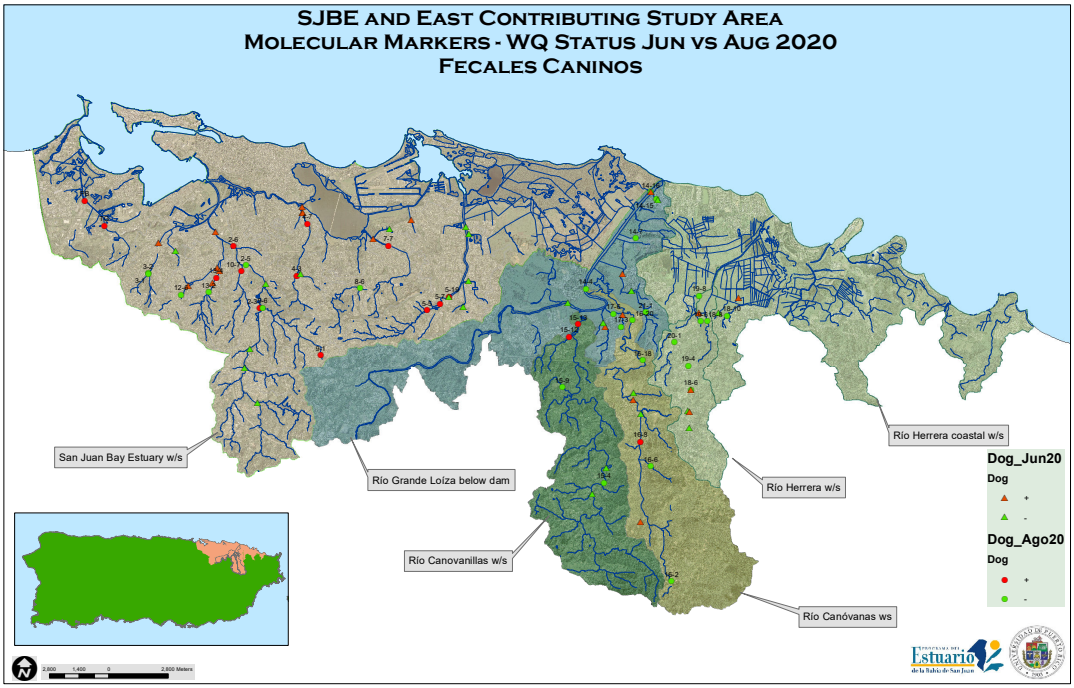

Figure 4. San Juan Bay Estuarian (SBJE) study area with indicated positive (red) and negative (green) data points for Dog fecal (fecales caninos) contamination in both June (Jun20, triangles) and August (Ago20, circles) sampling events.

Discussion

The main objective of this study was to determine the presence of *L. interrogans* in the surface waters of the SJBE and use MST to identify the possible sources of the pathogenic *Leptospira*. Another aspect of this study was to determine the possible correlation between nutrient richness, pathogenic *Leptospira,* and MST markers. During this study, *L. interrogans* were only detected in the dry season (June) samples ($p < 0.0001$). There was a significant correlation between human fecal contamination and pathogenic *L. interrogans* (r = 0.89). Additionally, there were significant correlations between horse fecal contamination and TP (r = 0.90), TN (r = 0.92), and NH_4^+ (r = 0.96).

USEPA standards in Puerto Rico for estuarian environments are 1000 μg L^{-1} for TP and 5000 μg L^{-1} for TN (USEPA 2019). The eutrophication indicator for TP in water is indicated when a site has > 100 μg L^{-1} of TP (Sánchez Colón and Schaffner 2021). Forty-nine out of the eighty-seven sampled sites exceeded this limit, indicating that approximately half of the water sampled was in eutrophic conditions. Eutrophication can be extremely detrimental to a marine estuarian environment, such as the SJBE community. Eutrophication can lead to anoxia, hypoxia, poor water quality, habitat and diversity loss, and harmful algal blooms (Ngatia *et al.* 2019). Phosphorus has been indicated as the limiting nutrient for some surface waters similar to the ones sampled in the SJBE (Correll 1999). There are multiple key species found in the SJBE that could be affected by this, including the mangrove forests which are important to the integrity of the coast.

The SJBE is a highly developed urban and residential area with the potential for elevated N and P coming from fertilizers, fossil fuels, and municipal and industrial wastewater (Galloway *et al.* 2008, Conley *et al.* 2009). The areas that had exceedingly high TP levels, Josephina (1860 μg L^{-1}, 1574 μg L^{-1}) and one in Quebrada Angela (3210 μg L^{-1}), were also positive for human

and dog MST markers (Table 3, Appendix Table 1). Quebrada Angela is located near urban areas making it a probable source for industrial pollution and fertilizers to enter surface water. These high levels in the specific area could be a potential health hazard if the subbasins are used recreationally or for drinking water. These levels need to be consistently monitored to gain a better understanding of public health concerns.

The infectious dose of pathogenic *Leptospira,* causing Leptospirosis, is unknown. There have been studies using an ELISA method to quantify *Leptospira* from infected human blood. These results indicated a detection limit of 50 gene copies per 10 µl and a critical threshold of 10^4 leptospires per milliliter of blood (Truccolo *et al.* 2001). The gene copy enumeration for this study was the number of Leptospira gene copies per 100 ml. Bacteria are known to have about one gene copy (O'Donnell *et al.* 2013). With that being said, the average gene copy number for our sites was ~ 400 leptospires per 100 ml. Although it is below the critical threshold, blood and water are two different solutions so the possibility for human infection is still possible and likely

During the June sampling event, roughly 32% of the samples collected were positive for *L. interrogans* (Table 3). These results are comparable to those in another Caribbean island (St. Kitts) and rural Puerto Rico (Rawlins *et al.* 2014, Truitt *et al.* 2020). The island of St. Kitts is significantly smaller than Puerto Rico but has a similar land size to the SJBE. Researchers surveyed various water sources, and ~20% of the sampled waters returned a positive result for pathogenic *Leptospira*. In addition, the authors also found a higher prevalence in the rainy season compared to the dry season (Rawlins *et al.* 2014). Additionally, Truitt et al. (2020) found that rural areas in Puerto Rico had higher positives detection of *L. interrogans* in the wet season than that of the dry season, which directly contradicts the finding of our study. Hurricanes and expected rainfall have been known to increase disease prevalence. This was specifically seen

before and after Hurricane Maria (Briskin *et al.* 2014). After Hurricane Maria, *L. interrogans* was detected in surface water, including ones that were used for drinking water in Puerto Rico (Keenum *et al.* 2021). In a study by Casanovas-Massana *et al.* (2018a), the presence of *Leptospira* during the rainy seasons yielded more positive results. The rainier seasons may have been more favorable for the *Leptospira* because of better mobility, dissolution of biofilms, or higher levels of oxygen. They are the first to claim this data supporting the higher positivity in rainy seasons, which is the opposite of what was seen in the results of our study.

There were no positive *L. interrogans* locations during the August sampling event (Table 3). August is known to be the rainier month in the SJBE, and the additional rainfall could have diluted *L. interrogans* below our detection limit (Mendez-Lazaro *et al.* 2014, Sánchez Colón and Schaffner 2021). In our study, the dryer season could have generated a higher concentration of *L. interrogans* in the area due to reduced water volume and flow.

The most significant finding of our study was the positive correlation between human fecal contamination and *L. interrogans* ($r = 0.89$). Human environments with trash and sewage often attract rats, which are the most common animal known to shed *L. interrogans* in their urine (Brickin *et al*. 2014, Casanovas-Massana *et al*. 2018 a). With Leptospirosis infecting around 1 million people globally each year, this is a significant public health concern. It was not surprising that there was a moderate correlation ($r = 0.58$) between dog fecal contamination and *L. interrogans* due to the large population of stray dogs in Puerto Rico that could contract *L. interrogans* and easily spread it to waterways (The Sato Project). Although it is unlikely for strays, dogs can be vaccinated but it is not the given with the most common vaccinations. The sites that contained all four markers (*Lipl32 gene*, horse, dog, human) were located in urban

residential areas, creating a multitude of pathways that fecal contamination could enter the water source.

Horse fecal contamination strongly correlated with the TP (r = 0.90), TN (r = 0.92), and NH_4^+ (r = 0.96) levels in the water sampled. High nutrient levels linked to horse fecal pollution have been noted in previous research due to uncontrolled manure run-off (Parvage *et al.* 2015, Skelly 2015). Not only can this affect the surface waters of the SJBE, but also the groundwater if it is used for drinking water (Skelly 2015). In one study, N and P levels were measured through leaching loss, which indicated the loss of water-soluble nutrients. They found that N and P were found in topsoil in horse paddocks, specifically the feeding and excreting areas. Paddocks in sandy soils also had a higher rate of N leaching. They determined that the excess leaching of P and N from horses could cause a potential threat to water quality (Parvage *et al.* 2015). Horses are common in the SJBE community due to recreational use and personal use and should be considered in the future when developing nutrient management practices.

In conclusion, *L. interrogans* abundance was only present in the dry season and was strongly correlated with human fecal pollution. Leptospirosis is an underreported and dangerous threat to tropical and subtropical regions. With the combined use of MST and the qPCR assay for the *Lipl32 gene*, the source of the *L. interrogans* abundance can be determined. This can be used in mitigation efforts to bring down the prevalence of disease in the SJBE. Further studies need to be conducted to examine other seasonal changes in the area regarding *L. interrogans* presence. Elevated levels of TN and TP indicated fecal contamination and possible eutrophication, these sites also correlated with horse fecal pollution. In addition, our study indicates other mitigation efforts are needed towards public health concerns and environmental impacts regarding nutrient concentrations and fecal pollution.

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Appendix

AGRICULTURAL EXPERIMENTAL STATION
SOIL AND WATER QUALITY LABORATORY

APPENDIX 5. STANDARD OPERATING PROCEDURES FOR MICROBIOLOGICAL ANALYSIS OF SURFACE WATERS (ENTEROCOCCI ENUMERATION *AND BACTEROIDALES* HUMAN SPECIFIC MARKER HF183– SOP #024W

1. Enterococci enumeration

1.1. Cleaning of bottles to be used for sampling

1. Wash each sample bottle and cap with a brush and phosphate-free detergent.
2. Rinse three times with distilled water.
3. Immerse bottles for 10 min in 10% HCL.
4. Rinse bottles three times with deionized water.
5. Leave bottles to dry and cap after drying.
6. Seal bottles across the cap and the bottle with sterile indicator tape.
7. Autoclave bottles (American Sterilizer Corp) at 121°C, 15 psi for 15 min. Do not remove "sterile" indicator tape after autoclave.
8. After autoclave, place bottles in plastic box or container for storage.

1.2. Grab sampling[1]

1.2.1 Laboratory

1. Identify bottles to be used for sampling during the incursion.
2. Adhere identification labels on bottles.
3. Fill out labels with all of the required information.

1.2.2. Field

1. Put on disposable, powder-less gloves.
2. Select bottle to be used for sampling at the particular site and fill out any other missing information required in the label.
3. Remove cap and immerse the bottle in the stream to a depth of 6 to 8 inches; fill the sample bottle about one-quarter full, cap bottle, shake gently.
4. Discard rinse water by swirling the solution out of the bottle.
5. Repeat the procedure (steps 3 and 4)
6. Remove cap and immerse the bottle in the stream to a depth of 6 to 8 inches, fill the sample bottle to the top and cap.
7. Place bottles in a cooler with ice, shielding from direct sunlight.
8. Transfer samples to UPRM-BNF laboratory
9. Process samples for fecal indicator bacteria within the allowed time limit. 6 hours is allowed for samples to reach the lab with an additional 2 hours is allowed for the analysis. The analysis must be completed within 8 hours of collection.

[1] See Appendix 2 (APPENDIX 2. STREAM WATER SAMPLING FOR NUTRIENTS AND MICROBIAL INDICATORS IN WATER, SOP #019W), Section 3.0. for details.

1.3. Storm sampling

1. Put on disposable, powder-less gloves.
2. Remove each bottle from the sampling rack and cap
3. Identify each bottle with all of the necessary information on the label.
4. Transfer samples to UPRM-BNF laboratory

1.4. Analysis of fecal enterococci[2]

1. Permit water sample in each sample bottle to reach room temperature
2. Transfer a 100 mL water-sample aliquot to sterile 100-mL manufacturer supplied polystyrene bottle; this will be the undiluted sample
3. Transfer 10 mL of water sample to a 90 mL sterile dilution tube (this will be the diluted samples and must be labeled as 10^{-1} D); if further dilution is needed the 10-1 D sample must be used and the procedure repeated.
4. Transfer the 10-1 D sample to 120-mL polystyrene bottles
5. Mix each sample with manufacturer-supplied growth medium until dissolved.
6. Pour the contents of each bottle into sterile Quanti-Tray® panel containing 97 wells and heat-seal.
7. Incubate Quanti-Tray® panels for fecal enterococci enumeration at 41 ± 0.5°C for 24 to 28 hours after sealing.
8. Determine the presence of fecal enterococci wells by detection of fluorescence with UV light at 365 nm.
9. Use a manufacturer-supplied table to convert the number of positive wells to most probable number (MPN) values. As needed use the proper dilution used in each subsample to quantify final MPN values.

2. *Bacteroidales* human specific marker HF183

2.1. Cleaning of bottles to be used for sampling – Follow procedures as in section 1.1 of this appendix.

2.2. Grab sampling – Follow procedures as in section 1.2 of this appendix.

2.3. Storm sampling – Follow procedures as in section 1.3 of this appendix.

2.4. Sample Processing

1. Each sample will be filtered twice and labeled as A and B.
2. Label tubes (MoBio DNA extraction).
3. Set up vacuum filtration unit. Unit consists of side-arm vacuum flask (500 – 1000 mL) fitted with a filter holder (for 25 mm filters) with capacity for > 100 mL water sample. Filter units must be washed, scrubbed, and well-rinsed prior to use.
4. Rinse filtration unit thoroughly (without filter) with 70% methanol and vacuum dry.
5. Place filter membrane (nitrocellulose, 0.22-µm-pore-size GSWP, Millipore, Cat. # GSWP04700) on filter unit using flame-sterilized forceps. Do not touch filter with your hands/fingers. Preferably wear gloves.

[2] Enumeration of fecal enterococci with the Enterolert™ system (IDEXX Laboratories, Westbrook, ME).

6. Filter 100 mL of water sample. You may use less water if the sample is cloudy/high in suspended solids and refuses to go thru the filter, but you need to note how much water sample is filtered.
7. Remove filter membrane from unit using flame-sterilized forceps. Using a second set of flame-sterilized forceps, roll filter loosely and place it in labeled tube. The procedure is show in the following video: http://www.mobio.com/water-dna-isolation/powerwater-dna-isolation-kit.html . Try not to place lid of tube down on bench while doing this as you want it to remain sterile.
8. If you are going to reuse filter units, you need to wash, scrub, and rinse them before reusing them.
9. Run a blank extraction using ~100 ml of the purest water available (de-ionized/ distilled water or better quality). The blank should show the absence of human specific marker HF183 - be sure you use a clean/methanol-rinsed filtration unit and sterile forceps!
10. Freeze tubes at -20°C once filtration is finished.
11. Place tubes in a cooler with ice packs and send by overnight courier to GSU.

Notes:

- Aseptic techniques must be followed at all times.
- Manage all samples, glassware and materials in accordance with Good Laboratory Practices.
- Dispose and wash all materials in accord with laboratory SOPs and Good Laboratory Practices.

AGRICULTURAL EXPERIMENTAL STATION
SOIL AND WATER QUALITY LABORATORY

STANDARD OPERATING PROCEDURES FOR TKN ANALYSIS IN WATER – SOP #-013W

PROCEDURE FOR DETERMINATION OF TOTAL KJELDAHL NITROGEN ANALYSIS BY BRAN + LUEBBE ION AUTO ANALYZER 3 (Based on EPA method No 351.2)

1. Introduction

Nitrogen is found in several different forms in terrestrial and aquatic ecosystems. These forms include ammonia (NH3), nitrates (NO3), and nitrites (NO2). Nitrates are essential plant nutrients, but in excess amounts they can cause significant water quality problems. Together with phosphorus, nitrates in excess amounts can accelerate eutrophication, causing dramatic increases in aquatic plant growth and changes in the types of plants and animals that live in the stream. This, in turn, affects dissolved oxygen, temperature, and other indicators. Excess nitrates can cause hypoxia (low levels of dissolved oxygen) and can become toxic to warm-blooded animals at higher concentrations (10 mg/L) or higher) under certain conditions. The natural level of ammonia or nitrate in surface water is typically low (less than 1 mg/L); in the effluent of wastewater treatment plants, it can range up to 30 mg/L.

Ammonia (NH3) is a colorless gas with a strong pungent odor. It is easily liquefied and solidified and is very soluble in water. One volume of water will dissolve 1,300 volumes of NH3. Ammonia will react with water to form a weak base.

Total Kjeldahl nitrogen is defined as the sum of free-ammonia and organic nitrogen compounds which are converted to ammonium sulfate (NH4)2SO4.

2. Summary of Method

This method covers the determination of total Kjeldahl nitrogen in drinking and surface waters, domestic and industrial wastes.

The procedure converts nitrogen components of biological origin such as amino acids, proteins and peptides to ammonia, but may not convert the nitrogenous compounds of some industrial wastes such as amines, nitro compounds, hydrazones, oximes, semicarbazones and some refractory tertiary amines. The applicable range of this method is 0.1 to 20 mg/L TKN. The range may be extended with sample dilution. The AES-Laboratory method detection limit is 0.12 mg TKN/L. The AES-Laboratory reporting limits are 0.15 – 20.0 mg TKN/L (MDL limit study included following the SOP).

The sample is heated in the presence of sulfuric acid and K2SO4 and for 3.5 hours. The residue is diluted to 25 ml, this is followed by an automated colorimetric procedure in which an emerald-green color is formed by the reaction of ammonia, sodium salicylate, sodium nitroprusside and hypochlorite in a buffered alkaline medium. The ammonium salicylate complex is read at 660 nm and placed on the Auto Analyzer for ammonia determination. The digested sample may also be used for phosphorus determination

3. Quality Control Definitions:

3.1. System Blank (SB): A Volume of Matrix, same as the matrix used for the calibration standards but without the analytes.

3.2. System Standard (SS): A known standard concentration which has been used for the standard calibration curve, using the same matrix and from the same cup. The SS is used to check the system performance.

3.3. Method Blank (MB): An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents and reagents. The MB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents or the apparatus.

3.4. Check Point (CP): A known standard concentration that is prepared separately from the one used for the calibration curve and treated exactly as all other samples including exposure to all glassware, equipment, solvents and reagents. The CP is used to determine if the concentrations of the standards used for the calibration curve are correct.

3.5. Spike (SPK): A known concentration is added to replicate of a sample and treated exactly as all other samples including exposure to all glassware, equipment, solvents and reagents. The SPK is used to determine the % of recovery of analytes.

3.6. Precision (PRE): A sample is tested three times and treated exactly as all other samples including exposure to all glassware, equipment, solvents and reagents. The PRE is used to determine the Relative Standard Deviation (RSD).

3.7. Organic Standard: A known Organic Standard concentration that is treated exactly as all other samples including exposure to all glassware, equipment, solvents and reagents.

4. Interferences

4.1. High nitrate concentration (10X or more than the TKN level) results in low TKN values. If interference is suspected, samples should be diluted and reanalyzed.

4.2. Method interferences may be caused by contaminants in the reagent water, reagents, glassware, and other sample processing apparatus.

5. Safety

5.1. The toxicity or carcinogenicity of each reagent used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure should be low as reasonably achievable. Cautions are included for known extremely hazardous material or procedures.

5.2. Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of Material Safety Data Sheets (MSDS) should be made available to all personnel involved in chemical analysis. The preparation of a formal safety plan is also advisable

5.3. The following chemicals have the potential to be highly toxic or hazardous, consult MSDS.

5.3.1. Sulfuric Acid

5.3.2. Sodium nitroprusside

6. Conditions of sampling

6.1. General Preparation of Sampling Containers. This method should be used when preparing all sample containers and glassware for monitoring nitrates and phosphorus.

6.2. Wash each sample bottle or piece of glassware with a brush and phosphate-free detergent.

6.3. Rinse three times with cold tap water.

6.4. Rinse with 10 percent hydrochloric acid.

6.5. Rinse three times with deionized water.

Note: Sample collection: Sampling is performed following procedures delineated by USGS (Wilde et al., 1998) and others (Haygarth and Edwards, 2000).

7. Sample Preservation

7.1. Samples are preserved at pH <2.0, by the addition of concentrated Sulfuric Acid and stored at 4oC. Even when preserved in this manner, conversion of organic Nitrogen to Ammonia may occur therefore, samples should be analyzed as soon as possible.

8. Materials and equipment

8.1. Reagents Make-up (DI water refers to high quality Deionized water) Unless otherwise specified all chemicals should be of ACS grade or Equivalent.

8.2. Digestion Solution: (Sulfuric acid / potassium sulfate solution): Dissolve 133 g of K2SO4 in 700 ml of deionized water and 200 ml of conc. H2SO4. Dilute to 1 liter of DI water and mix thoroughly.

8.3. Sulfuric Acid Solution 4%- (Sampler Wash Receptacle solution): Add 80 ml of conc. sulfuric acid to 1600 ml of DI water, cool and 135g of Sodium Hydroxide dilute to two liters with DI water, Solution pH range should be 12.8 to 13.1.

8.4. Stock Sodium Hydroxide solution (20%): Dissolve 200 g of Sodium Hydroxide in about 700ml of DI water. Cool to room temperature and dilute to one liter with DI water. Store in a plastic bottle. Keep closed to prevent CO2 absorption. Can be used for as long as the solution remains clear.

8.5. Stock Sodium Potassium Tartrate Solution (20%): Dissolve 200 g sodium potassium tartrate in about 800 ml of DI water and dilute to one liter with DI water and mix thoroughly. Keep Closed and replace after one year. This reagent is a common source of contamination, as can be seen from a high reagent absorbance and baseline noise, so be sure to obtain high purity material.

8.6. Stock Buffer Solution 0.5M: Dissolve 70g of sodium phosphate, dibasic Anhydrous (Na2HPO4) in about 800 ml of ammonia free DI water. Add 20 g of NaOH and dilute to one liter with DI water, Keep Closed.

8.7. Working Buffer Solution 0.5M: Combine the reagents in the stated order; add 200ml of stock sodium potassium tartrate solution, 20% (4) to 160 ml of stock buffer solution, 0.5M (5) with swirling. Slowly, with swirling, add 110 ml of Sodium Hydroxide solution 20% (3) dilute to one liter with DI water. Add 2.0 ml of Brij-35 30% and mix thoroughly. NOTE: the pH in the reaction mixture should be pH 12.8 – 13.1

8.8. Sodium Salicylate / Sodium Nitroprusside: Dissolve 194 g of Sodium Salicylate and 0.4 g of Sodium Nitroprusside in about 600 ml of DI water. Dilute to one liter of DI water. Add 0.5 ml of Brij-35 30% and mix thoroughly. Store in a light resistant container. Make up fresh monthly.

8.9. Sodium Hypochlorite Solution: Dilute 5.0 ml sodium hypochlorite solution (Clorox) to 100 ml with ammonia free DI water. Add 0.1ml of Brij-35 30% (two drops) and mix thoroughly, prepare fresh daily. Use any commercial bleach solution containing 5.25% of available chlorine.

Note. The chemistry is sensitive to free chlorine concentration. The chlorine content of hypochlorite solution is not constant, and varies every time the bottle is opened, and on storage. Is the specified sensitivity and linearity are not met, vary the hypochlorite concentration until the optimum sensitivity and linearity are achieved. If a large variation was necessary, check the reaction pH, because hypochlorite solution is strongly alkaline.

9. Standards

9.1. Stock Standard A 100 mg / L N

9.1.1. In a 100 ml volumetric flask, weigh 10 g of certified Ammonium Standard, 1000 ppm and dilute to a volume of 100 ml by weight with DI water and mix thoroughly.

9.2. Standard Curve:

9.2.1. In a 100 ml volumetric flask, weigh as follows for the curve Standards:

mg Stock A	mg /L N
0.10	0.10
0.25	0.25
0.50	0.50
1.00	1.00
2.50	2.50
5.00	5.00

9.2.2. Dilute all standards to a volume of 100 ml by weight with DI water and mix thoroughly. Note: Include a System Blank (SB) as specified 3.2.1. All Curve standards including System Blank (SB) should processed and treated exactly as all other samples including exposure to all glassware, equipment, solvents and reagents.

9.2.3. Stock Standard B 100 mg /L N

9.2.3.1. In a 100 ml volumetric flask, weigh 10 g of certified Ammonium Standard, 1000 ppm and dilute to a volume of 100 ml by weight with DI water and mix thoroughly. Stock Standard B is prepared separately from the Stock Standard A used for the calibration curve and treated exactly as all other samples including exposure to all glassware, equipment, solvents and reagents with the purpose to determine if the concentrations of the standards used for the calibration curve are correct. With the use of Quality Control Samples: (CP) 3.4, (SPK) 3.5, and (PRE) 3.6.

9.2.4. Organic Nitrogen Standards

9.2.4.1. Stock Glycine p-Toluenesulfonate: 25 mg/L N

9.2.4.1.1. Weigh 0.4416g of Glycine p-Toluenesulfonate and dissolve in 800mL of DI water. Dilute to 1000mL with DI water and mix thoroughly.

9.2.4.2. Glycine 0.64 mg/L NH4 Standard

9.2.4.2.1. Dilute 2 mL of Stock Glycine p-Toluenesulfonate to 100 mL with DI water and mix thoroughly.

9.2.4.3. Glycine 1.29 mg/L NH4 Standard

9.2.4.3.1. Dilute 4 mL of Stock Glycine p-Toluenesulfonate to 100 mL with DI water and mix thoroughly.

9.2.5. Stock L-Analine 14 mg/L N

9.2.5.1. Weigh 0.089 g of L-Analine and dissolve in about 600 mL of DI water. Dilute to 1000 mL with DI water and mix thoroughly.

9.2.5.2. L-Analine 0.54 mg/L NH4 Standard

9.2.5.2.1. Dilute 3 mL of Stock L-Analine to 100 mL and mix thoroughly.

10. Equipment

10.1. Balance: Analytical, capable of accurately weighing to the nearest 0.0001g.

10.2. Glassware: Class A volumetric flask and pipetting devises as required.

10.3. Seal Analytical BD50s Digesting System.

10.4. Digestion tubes: 1"x 10.5" heavy-walled 100ML tubes.

10.5. Hengar, 136C Plain Granule Part # 901800Analyzed in, Seal Analytical AA3.

11. Laboratory Digestion Procedure

11.1. Analytical Procedure

11.1.1. Add (4-8) Hengar Boiling Plain Granules to a digester tube. CAUTION: too many boiling Granules or none will cause the sample to over boil. Add 25 ml of sample and 5 ml of digestion solution (8.2) and mix with a vortex shaker.

11.1.2. Set the Block Digester system low temperature at 160°C for 2 hours and high temperature to 380°C for 30 min. Preheat unit to stable 160°C. Place tubes in digester block and activate the digesting schedule run.

11.1.3. Remove samples rack from the block digester and cool to room temperature, then dilute to 25 ml with ammonia free DI water.

11.1.4. Analyze samples in Seal Analytical Autoanalyzer System AA3.

12. Calibration and Standardization

12.1. Prepare a series of at least three standards for the Standard Curve covering the desired range, and a blank by diluting suitable volumes of standard solution with DI water.

12.2. Prepare standards curve and blank as described in (section 9.2) procedure.

12.3. Set manifold and flow system for NH4-N in Seal Analytical AA3 as shown in Figure 1

12.4. Place appropriate standards curve in order of decreasing concentration, MB, CP, SPK, PRE, organic std and samples in sampler tray and perform analysis using (procedure 13.0) and Seal Analytical AA3 instrument procedure.

12.5. After the calibration curve has been established, it must be reviewed and verified by the analysis of a suitable Quality Control Check Point (CP) sample. If measurements exceed ± 10% of the established (CP) value, the analysis should be terminated and the instrument recalibrated. The new calibration must be verified before continuing analysis. Periodic reanalysis of the (CP) is recommended as a continuing calibration check.

13. Colorimetric Analysis

13.1. Avoid instrument unattendance and check periodically for appropriate level of all reagents containers to ensure an adequate supply.

13.2. Only when necessary flush all lines with 5N Sulfuric Acid for 30min with Proportioning pump in fast. Rinse for 30 minutes with DI water.

13.3. Place all reagent lines in their respective containers and start the proportioning pump.
13.4. Allow the system to equilibrate. If a precipitation occurs, the pH is too low, immediately stop the proportioning pump and flush the coils with water using a syringe. Before restarting the system, check the concentration of the wash solution and the working buffer solution.
13.5. When the stable baseline has been obtained, start analyzes run.

14. Quality Assurance and Quality Control Checks

14.1. Certified Reference Materials (CRMs) of the analyte of interest will be used to assess accuracy of a given analysis. At least one CRM fortified (spike) sample will be analyzed with each batch of 20 or fewer samples. Laboratory accuracy will be expressed in terms of percent recovery of the spiked sample. An accuracy goal of 70 – 130% must be met at all times. A CRM of the analyte of interest will also be used as an internal standard. The latter, which constitutes an analytical check point will be run 1 out of every ten samples. A deviation of more than 10% from the actual concentration would require establishment of a new calibration curve. All samples read between the before to last – and the last check point should be reanalyzed. Compliance with performing criteria (accuracy) will be checked prior to proceeding with the analysis. For every 20 samples analyzed triplicate analyses of an unknown sample will be performed to determine analytical precision. The acceptance criteria will be ≤ 20%RSD. If the laboratory fails to comply with either the precision or accuracy criteria the data for the entire batch will be considered suspect. Calculations and instrument will be checked, the CRM will be reanalyzed to confirm the results. If values are still outside the control limits in the repeat analysis, the laboratory is required to determine and correct the source of the problem and repeat the analysis until control limits are met. A field blank (trip blank) and a laboratory blank will be analyzed with every batch of 20 samples or less. The laboratory reagent blank (i.e. method blank) will be used to assess laboratory contamination during all stages of sample preparation and analysis. The reagent blank will be processed in a manner identical to the samples. A reagent blank concentration between the MDL and 3 times the MDL will require corrective action to identify and eliminate the source(s) of contamination before proceeding with sample analysis.

15. Calculations

15.1. Prepare standard curve by plotting peak heights of processed standards against concentration values. Compute concentrations by comparing sample peak heights with standard curve.

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AGRICULTURAL EXPERIMENTAL STATION
SOIL AND WATER QUALITY LABORATORY

STANDARD OPERATING PROCEDURES FOR TP ANALYSIS IN WATER – SOP #011w
(Based in EPA method No 365.2)

4.1. Introduction

Dissolved orthophosphate is the form of P most readily available to aquatic plants, but numerous studies have shown that other forms of P can be hydrolyzed to the orthophosphate form in wastewater-treatment facilities and in natural waters. Therefore, when assessing the long-term potential for accelerated eutrophication of surface water due to P loading, many researchers and watershed managers want to know the total P concentration (regardless of P form) in water samples.

Polyphosphates and phosphates bound to organic substances do not react with the molybdate reagent used for colorimetric P analysis. Therefore, analysis for total P content of water samples requires that all condensed and organic P compounds, including particulate P, first be converted (hydrolyzed) to orthophosphate so they can be determined colorimetrically. This is accomplished by digesting the sample in strong acid at high temperature to oxidize the organic matter and release P as orthophosphate.

Total phosphorus is first converted to ortho-phosphate by off-line Kjeldahl digestion with sulfuric acid. The determination of ortho-phosphate is then based on the colorimetric method in which a blue color is formed by the reaction of phosphate, molybdate and antimony followed by reduction with ascorbic acid at an acidic pH. The phosphor-molybdenum complex is read at 660nm. Chloride prevents the precipitation of mercury from the digestion catalyst. To determine total P (dissolved + particulate), an unfiltered sample is shaken (to suspend the particulate matter) just before submitting a subsample for digestion.

4.2. Summary of Method

This method covers the determination of specified forms of phosphorus in drinking, surface and saline waters, domestic and industrial wastes. The method is usable in the 0.01 to 0.5 mg P/L range. The AES-Laboratory method detection limit is 0.006 mg P/L. The AES-Laboratory reporting limits are 0.009 – 0.5 mg P/L (MDL limit study included following the SOP).
The sample (50mL) is heated in the presence of sulfuric acid and potassium persulfate to reduce the sample to a volume of 10 mL of the initial volume, the sample is allowed to cool down and is brought back the original volume (50mL) with distilled-deionized water. An aliquot is then submitted for color developing.

4.3. Conditions of sampling

4.3.1. General Preparation of Sampling Containers. This procedure should be used when preparing all sample containers and glassware for monitoring nitrates and phosphorus.

1) Wash each sample bottle or piece of glassware with a brush and phosphate-free detergent.

2) Rinse three times with cold tap water.

3) Rinse with 10 percent hydrochloric acid.

4) Rinse three times with distilled, deionized water.

4.3.2. Collect the sample.

1) Label the bottle with the site number, date, and time.

2) Wading. Follow AES SOP#019w. In general, try to disturb as little bottom sediment as possible. In any case, be careful not to collect water that has sediment from bottom disturbance. Stand facing upstream. Collect the water sample on your upstream side, in front of you. You may also tape your bottle to an extension pole to sample from deeper water. Collect a water sample 8 to 12 inches beneath the surface or mid-way between the surface and the bottom if the stream reach is shallow.

3) Boat. Follow. Use Van Dorn bottle as described in AES SOP#022w.

4) Leave a 1-inch air space (Except for DO and BOD samples). Do not fill the bottle completely (so that the sample can be shaken just before analysis). Recap the bottle carefully, remembering not to touch the inside.

5) Fill in the bottle number and/or site number on the appropriate field data sheet. This is important because it tells the lab coordinator which bottle goes with which site.

6) If the samples are to be analyzed in the lab, place them in the cooler for transport to the lab.

7) If analysis is not completed within 24 hours of sample collection, samples should be preserved at pH <2.0, by the addition of concentrated Sulfuric Acid and stored at 4oC.

4.4. Materials and equipment

4.4.1. Reagents

(1) Sulfuric Acid Solution (H_2SO_4 - 11N). Transfer approximately 600 mL of distilled water to a 1000 mL volumetric flask. Slowly (and carefully) add 310 ml of concentrated H_2SO_4. After the solution has cooled, dilute to 1000 mL with distilled deionized water and mix.

(2) Potassium Persulfate, $K_2S_2O_8$ solid.

(3) Sodium Chloride Wash Solution. Using a 1000 ml volumetric flask, dissolve 3.501 g of NaCl in approximately 800 mL of distilled deionized water. Add 40 mL sulfuric acid concentrated (H_2SO_4). Dilute to 1000 mL with distilled deionized water.

(4) Sodium Dodecyl Sulfate Diluent Solution. Using a 1000 ml volumetric flask, dissolve 2.0 g of $CH_3(CH_2)_{11}OSO_3Na$ in approximately 900 mL of distilled water, and dilute to volume.

(5) Ascorbic Acid Solution. Dissolve 8.0 g of L-ascorbic acid in 1000 mL of distilled deionized water.

(6) Antimony Potassium Tartrate Stock Solution. Using a 100 ml volumetric flask, dissolve 2.3 g of $C_8H_4K_2O_{12}Sb_2.3H_2O$ in approximately 60 mL of distilled deionized water, and dilute to volume. Store in an opaque plastic bottle at 4oC.

(7) Ammonium Molybdate Solution. Using a 1000 ml volumetric flask, dissolve 6 g of $(NH_4)_6Mo_7O_{24}.4H_2O$ in approximately 500 ml of distilled deionized water. Add 64 mL sulfuric acid concentrated (H_2SO_4 conc.) and 22 mL Antimony Potassium Tartrate Stock Solution. Dilute to 1000 mL

4.4.2. Equipment

1) Hot plate with adequate heating surface.

2) Balance: Analytical, capable of accurately weighing to the nearest 0.0001g.

3) Acid-washed glassware and plastic bottles: graduated cylinders (5 ml to 100 ml measurements), volumetric flasks (100 ml, 500 ml, and 1000 ml), storage bottles (opaque plastic).

4) Analyzer in Autoanalizer 3 Bran & Luebbe. Absorbance is measured at 660nm.

4.5. Procedure in the laboratory

4.5.1. Analytical Procedure

1) Thoroughly mix the sample, and measure a suitable portion (50 ml is recommended) into a 125 ml Erlenmeyer flask.

2) Add 1 ml of H2SO4 solution (1).

3) Add 0.4 g of solid Potassium Persulfate (K2S2O8) and mix.

4) Boil the sample solution gently on the preheated hot plate until the volume is reduced to approximately 10 mL.

5) Cool the sample and dilute to 50 mL with distilled deionized water.

4.5.2. Calibration and Standardization

4.5.3. Prepare a series of at least three standards for the Standard Curve covering the desired range, and a blank by diluting suitable volumes of the standard stock solution (1,000 µg P/L) with distilled, deionized water.

4.5.4. Process Standards Curve and blank as described in (Section 4.5.1.) procedure.

4.5.5. Set up Manifold and flow system as shown in Figure 1.

4.5.6. Place appropriate Standards Curve in sampler in order of decreasing concentration and perform analysis using (Section 4.6) procedure.

4.5.7. After the calibration has been established, it must be verified by the analysis of a suitable Quality Control Check Point (CP) sample. If measurements exceed ± 10% of the established (CP) value, the analysis should be terminated and the instrument recalibrated. The new calibration must be verified before continuing analysis. Periodic reanalysis of the (CP) is recommended as a continuing calibration check.

4.6 Instrument Calibration

The equipment will be calibrated prior to the analysis of each sample batch, after each major equipment disruption, and whenever on-going calibration checks do not meet recommended control limit criteria. All calibration standards will be traceable to a recognized organization for the preparation and certification of QA/QC materials. Calibration curves will be established for each element and batch analysis from a calibration (analytical) blank and a minimum of three analytical standards of increasing concentration, covering the range of expected sample concentrations. The calibration curve will be well-characterized and will be established prior to the analysis of the samples. Only data which results from quantification within the demonstrated working calibration range may be reported by the laboratory. Samples outside the calibration range will be diluted or concentrated as appropriate and reanalyzed.

4.7 Quality Assurance and Quality Control Checks

Certified Reference Materials (CRMs) of the analyte of interest will be used to assess accuracy of a given analysis. At least one CRM fortified (spike) sample will be analyzed with each batch of 20 or fewer samples. Laboratory accuracy will be expressed in terms of percent recovery of

the spiked sample. An accuracy goal of 70 – 130% must be met at all times. A CRM of the analyte of interest will also be used as an internal standard. The latter, which constitutes an analytical check point will be run 1 out of every ten samples. A deviation of more than 10% from the actual concentration would require establishment of a new calibration curve. All samples read between the before to last – and the last check point should be reanalyzed. Compliance with performing criteria (accuracy) will be checked prior to proceeding with the analysis. For every 20 samples analyzed triplicate analyses of an unknown sample will be performed to determine analytical precision. The acceptance criteria will be ≤ 20%RSD. If the laboratory fails to comply with either the precision or accuracy criteria the data for the entire batch will be considered suspect. Calculations and instrument will be checked, the CRM will be reanalyzed to confirm the results. If values are still outside the control limits in the repeat analysis, the laboratory is required to determine and correct the source of the problem and repeat the analysis until control limits are met. A field blank (trip blank) and a laboratory blank will be analyzed with every batch of 20 samples or less. The laboratory reagent blank (i.e. method blank) will be used to assess laboratory contamination during all stages of sample preparation and analysis. The reagent blank will be processed in a manner identical to the samples. A reagent blank concentration between the MDL and 3 times the MDL will require corrective action to identify and eliminate the source(s) of contamination before proceeding with sample analysis.

4.8. Colorimetric Analysis

- (1) Check the level of all reagents containers to ensure an adequate supply.
- (2) Flush all lines with 5N Sulfuric Acid for 30 minutes with proportioning pump in fast. Rinse for 30 minutes with distilled, deionized water.
- (3) Place all reagent lines in their respective containers, and start the proportioning pump.
- (4) When reagents have been pumping for at least five minutes, and after a stable baseline has been obtained start the sampler.

4.9. Calculations

Prepare standard curve by plotting peak heights of processed standards against concentration values. Compute concentrations by comparing sample peak heights with standard curve. Report results as P, mg/L.

4.6. Interpretation of results

Criteria: The following criteria for total phosphorus were recommended by US EPA (1986):

- No more than 0.1 mg/L for streams which do not empty into reservoirs,
- No more than 0.05 mg/L for streams discharging into reservoirs, and
- No more than 0.025 mg/L for reservoirs.

References

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

41a	3210	2840	33996.76	25624.50	16.94	34013.71
42a	190	162	105.00	0.00	468.06	573.07
43a	199	184	211.56	205.77	0.68	212.23
44a	179	169	1057.00	720.59	9.49	1066.49
1b	250	211	2483.45	1608.91	299.54	2782.99
2b	168	150	843.89	252.36	1339.59	2183.48
3b	200	180	1198.56	562.96	1303.44	2502.00
4b	105	93	1363.44	771.84	592.31	1955.75
5b	42	33	608.22	188.69	533.35	1141.57
6b	14	3	295.56	48.14	97.59	393.14
7b	157	109	1415.56	535.79	55.12	1470.68
8b	177	155	1638.78	733.02	1248.78	2887.55
9b	346	285	3881.89	1832.54	1281.30	5163.19
10b	93	76	760.67	133.56	1106.91	1867.58
11b	99	85	671.22	160.74	1166.10	1837.32
12b	91	84	500.11	63.67	1066.25	1566.36
13b	490	380	2053.33	217.42	1027.85	3081.18
14b	502	390	2058.78	94.73	1040.50	3099.27
15b	52	127	339.11	21.74	1199.98	1539.09
16b	127	43	527.33	243.04	569.04	1096.38
17b	354	234	3146.11	1938.92	505.56	3651.68
18b	136	118	1341.67	796.69	904.73	2246.40
19b	53	49	275.33	38.83	918.74	1194.07
20b	161	140	496.22	44.26	777.10	1273.32
21b	29	3	795.67	37.27	473.71	1269.38
22b	291	219	1898.56	111.82	13.55	1912.11
23b	317	236	1250.67	119.58	14.68	1265.35
24b	75	22	1268.56	111.82	234.48	1503.04
25b	28	21	258.22	31.06	178.91	437.14
26b	77	71	412.22	49.70	749.31	1161.53
27b	144	132	689.11	166.17	600.44	1289.55
28b	93	73	775.44	115.70	260.91	1036.36
29b	58	53	214.67	40.38	105.27	319.94
30b	25	21	132.22	32.61	164.23	296.45
31b	6	2	94.11	39.60	86.97	181.08
32b	17	10	0.00	42.71	81.10	81.10
33b	12	2	230.22	67.56	64.61	294.83
34b	365	359	5063.33	78.43	619.87	5683.20
35b	990	955	5950.00	5820.64	12.42	5962.43
36b	22	19	104.22	92.40	191.79	296.01
37b	38	31	280.00	64.45	191.79	471.79
38b	156	142	465.89	95.51	72.51	538.40
39b	136	134	405.22	48.92	409.78	815.00
40b	240	220	700.00	157.63	250.75	950.75
41b	315	223	3300.89	1399.25	6.78	3307.67
42b	52	47	397.44	75.32	443.67	841.11

43b	73	100	464.33	72.21	10.39	474.72