DETECTION OF HUMAN- DERIVED FECAL CONTAMINATION IN PUERTO RICO USING CARBAMAZEPINE, HF183 *BACTERIODES*, AND FECAL INDICATOR BACTERIA

By

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Abstract

Carbamazepine (CBZ) and fecal indicator bacteria were used to detect fecal pollution and its human origins in 17 sites located mostly in coastal areas of Puerto Rico. CBZ was quantified using an enzyme-linked immunosorbent assay (ELISA) and *Escherichia coli* (*E.coli*) was enumerated using Colilert® enzyme substrate medium and Quanti-Tray®/2000. A quantitative polymerase chain reaction (qPCR) was used to detect the human-specific genetic marker, HF183 *Bacteriodes*. CBZ was detected in 16 sites, including Condado Lagoon, which is a popular recreational area. Average CBZ concentrations ranged from 0.005 μgL^{-1} to 0.482 μgL^{-1}. In general, CBZ concentrations were lower in less-densely populated areas, which support its use as a more specific wastewater indicator. Elevated *E.coli* levels (>410 CFU $100mL^{-1}$) were detected in 13 sites, and 7 sites were positive for HF183. *E.coli* and CBZ were significantly correlated ($R= 0.485$, P value = < 0.05). Higher CBZ concentrations were associated with the detection of HF183 (Mann-Whitney test;$U= 42.0$; $df=7$; 1-tailed P value = < 0.05). The widespread detection of CBZ indicates that rivers and streams near the coast in Puerto Rico may be a significant source of pollutants related with human-derived fecal contamination. This was the second study to determine surface water concentrations of CBZ in the Caribbean and the first in Puerto Rico.

Resumen

Carbamazepina (CBZ) y bacterias indicadoras de material fecal fueron utilizadas para detectar la contaminación fecal y y su origen humano en 17 lugares, mayormente cercanas a la costa de Puerto Rico. CBZ fue cuantificado utilizando ensayos de inmuno-adsorción enzimática (ELISA) y *Escherichiacoli* (*E.coli*) fue enumerado usando un el medio de substrato de enzimas Colilert ® en conjunto con Quanti-Tray®/2000. La detección del marcador genético especifico para material fecal humano, HF183 *Bacteroides*, fue detectado usando reacciones de polimerasa en cadena cuantitativa (qPCR). CBZ fue detectado en 16 de las 17 localidades, incluyendo la Laguna del Condado, la cual es un área popular para actividades recreativas. La concentración promedio de CBZ fluctuó entre 0.005µgL^{-1} a 0.482 µgL^{-1}. Niveles elevados de *E.coli* (>410 CFU 100mL^{-1}) fue detectado en 13 lugares, 7 dieron positivas para HF183. *E.coli* y CBZ se correlacionaron significativamente (R= 0.485, P value = < 0.05). Las concentraciones mayores de CBZ fueron asociadas con la detección de HF183 (Mann-Whitney test;U= 42.0; df=7; 1-tailed P value= 0.013). La amplia detección de CBZ indica que los rios y quebradas cercanas a la costa de Puerto Rico pueden ser una fuente significativa de contaminantes asociados a los desechos fecales humanos. Este es el segundo estudio que determina la concentración de CBZ en las aguas superficiales en el Caribe y el primero realizado en Puerto Rico.

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Introduction

Many water bodies in the US are contaminated with sewage and are considered impaired due to elevated bacterial levels (US EPA 2012a). Fecal indicator bacteria are used to monitor recreational waters for fecal contamination. High bacterial densities represent the elevated risk that disease-causing pathogens, such as *Salmonella* and *Cryptosporidium*, may be present in the water. Traditional microbial indicators cannot be used to identify the source of fecal contamination ((Seidler et al. 1981; Lopez-Torres et al. 1987; Oshiro and Fujioka 1995). The use of complementary, source-specific indicators would help prevent future contamination and protect public health.

In the US, poor water quality affects public health, tourism, and significantly impacts the economy. In 2012, 54% of the more than a million miles of assessed rivers and streams in the US were impaired (US EPA 2012a). In Puerto Rico close to all (96%) of the more than 5,000 miles of assessed rivers and streams were impaired (US EPA 2012a). Elevated bacteria levels are the most common cause of impairment and cause thousands of beach closures each year. The economic impact associated with beach closures is hard to estimate, but Rabinovici et al. (2004), concluded that a typical beach closure on Lake Michigan could result in net economic losses of up to $37,000/day. Given et al. (2006) estimated that fecal contamination resulted in 627,800-1,479,200 gastrointestinal illnesses annually in two southern California beaches and carried a public health cost of 21-51 million dollars. It is important to rapidly detect fecal contamination to reduce the risk to public health and prevent unnecessary economic losses.

Fecal indicators are used to represent the elevated risk that disease-causing pathogens may be present. An ideal fecal indicator should be easily detected using simple laboratory methods, should not be present in unpolluted waters or multiply outside the intestinal tract, appear in concentrations correlated with the level of contamination, and have a die-off rate that is not faster than the rate of the pathogens (Thomann and Mueller 1987; Sloat and Ziel 1992; Hurst etal. 2002). The United States Environmental Protection Agency (US EPA) currently recommends using *Escherichia coli* (*E.coli)* to detect fecal contamination in recreational fresh water (US EPA 2012b). Results from epidemiological studies have shown that *E.coli* correlates well with swimming-associated gastroenteritis and should be used instead of fecal coliforms to provide improved public health protection (US EPA 1986; US EPA 2012b). *E.coli* is a more specific fecal indicator than fecal coliforms, but several limitations still make it an unsuitable wastewater marker. *E.coli* has been detected in pristine tropical environments (Hazen 1988; Bermúdez and Hazen 1988), can multiply in sediments (Lopez-Torres et al. 1987; Solo-Gabriele et al. 2000; Desmarais et al. 2002), and is present in the intestinal tract of all warm-blooded animals and thus is not source-specific.

Traditional microbial indicators cannot be used to discriminate between human and other animal sources (Seidler et al. 1981; Lopez-Torres et al. 1987; Oshiro and Fujioka 1995). The use of complementary, source-specific indicators would help prevent future contamination and protect public health. Recent technological advances in polymerase chain reaction (PCR) analyses have made it possible to rapidly detect bacteria that can identify the source (Green et al. 2014). HF183 *Bacteriodes* is specific to sewage and can reliably detect human fecal pollution (Ahmed et al. 2008). Despite technological advances, widespread applicability of PCR is still limited because of the high cost of instrumentation and expertise needed to run the tests. A

source-specific, cost- and time-effective method would be convenient to quickly detect the potential for fecal contamination and efficiently allocate resources for confirmation and remediation of the problem.

Chemical detection methods may offer several advantages over microbial methods. Ideal chemical indicators generally require a shorter analysis, are not found naturally in the environment, and are source-specific. Fecal sterols, such as cholesterol and coprostanol, have been studied to assess their suitability as indicators. Cholesterol is found in the gut of mammals, but is not an ideal indicator because it can also be found in milk, lard, and eggs. Coprostanol has a relatively short half-life (<10 days) and could be used to indicate fresh fecal contamination (Isobe et al. 2002). However, Coprostanol is hydrophobic and relatively resistant to biodegradation and can potentially persist in the sediments for over a year (Isobe et al. 2002). Pharmaceuticals and personal care products (PPCP) have also been evaluated as potential alternative fecal indicators.

Much research has been done assessing the use of caffeine as a fecal indicator. Caffeine is a source-specific, non-conservative tracer (Seiler et al. 1999; Chen et al. 2002) that can be biodegraded in the environment (Clara et al. 2004; Bradley et al. 2007), and has been proposed as a useful indicator of recent fecal contamination (Daneshvar et al. 2012). In contrast, Carbamazepine (CBZ) is relatively resistant to biodegradation (Clara et al. 2004; Joss et al. 2006) and has been proposed as a cumulative wastewater discharge indicator due to its long half-life (Madoux-Humery et al. 2013).

CBZ, an anti-epileptic, is one of the most ubiquitous and persistent pharmaceuticals in the environment (Lin and Reinhard 2005; Zhang et al. 2008; Calisto et al. 2011). An estimated

1,014 tons of CBZ is consumed worldwide (Zhang et al. 2008). In 2000, over 40 tons of CBZ was consumed in the US alone (Thacker 2005). CBZ is extensively metabolized in the liver by cytochrome P450 enzymes (Kerr et al. 1995; Valentine et al. 1996). Metabolites and unchanged forms of the parent compound are excreted in human waste and transported to sewage treatment plants. At the treatment plant the concentration of CBZ can increase during common wastewater treatment processes (Zuehlke et al. 2004; Vieno et al. 2007). Zuehlke et al. (2004) found concentrations of CBZ in the effluent to be 2x as high as in the influent, which could be explained by the cleavage of the glucuronic moiety from carbamazepine-N-glucuronide and release of parent compound during secondary treatment (Vieno et al. 2007). CBZ is not significantly removed during sludge processes (Clara et al. 2005). The limited removal of CBZ during sewage treatment can be explained by its resistance to biodegradation and low distribution coefficient between water and sludge (K_d). Joss et al. (2006) reported CBZ had a very low kinetic degradation constant and was not biodegraded or removed. CBZ is not removed by attachment to sludge either. A distribution coefficient (K_d) of 500 L kg_{ss}^{-1} is required for significant sorption to the sludge, and CBZ has a (K_d) of 1.2 L kg_{ss}^{-1} (Ternes et al. 2004).

Photodegradation is an important removal processes in surface waters for compounds like CBZ that are refractory to biodegradation. Many studies have been conducted to estimate the photodegradation rate of CBZ, but differences in methodologies result in a wide range of photochemical half-lives being reported. Andreozzi et al. (2002) used distilled water in simulated solar radiation experiments and reported a half-life of 121.6 hours. Matamoros et al. (2009) used natural aqueous matrices and a solar light simulator and estimated the half-life of CBZ to be 8 to 39 hours. Predictive models suggest that photodegradation is highly variable with CBZ half -life ranging from weeks to several months (Laurentis et al. 2012). Environmental conditions may

play an important role in the rate of photodegradation. Organic matter can inhibit CBZ direct photolysis by acting as a UV quencher and decreasing degradation rates (Andreozzi et al. 2002, 2003; Chiron et al. 2006; Laurentis et al. 2012). Calisto et al. (2011) reported that CBZ could persist in the environment from 4.5 to 25 days. Thus, CBZ is not significantly degraded or removed during wastewater treatment and enters the environment where it is relatively resistant to photodegradation and persistent.

Because of its persistence to degradation, CBZ has been detected in various environmental matrices from surface water to drinking water wells (Ternes 1998; Herberer et al. 2002; Kolpin et al.2002; Clara et al. 2004; Fenz et al, 2005; Hua et al. 2006; Bahlmann et al. 2009). CBZ surface water concentrations range from 0.0023 to 1.075 μgL^{-1} (Herberer et al. 2002; Kolpin et al. 2002; Metcalfe et al. 2003; Calisto et al. 2011; Bahlmann et al. 2012). CBZ is not easily degraded or adsorbed and can reach aquifers (Clara et al. 2004). In Germany, Osenbrück et al. (2007) detected concentrations up to 0.083 μgL^{-1} in groundwater and found that river water infiltration was the main factor leading to CBZ presence in groundwater. Rabiet et al. (2006) detected CBZ concentrations of 0.0432 and 0.0139 ugL^{-1} in 2 out of 7 drinking water wells investigated in the Mediterranean region. The presence and persistence of CBZ in a variety of environments and its sole use as a pharmaceutical make CBZ a suitable human-specific fecal indicator (Clara et al. 2004, 2005; Benotti and Brownawell 2007; Doummar et al. 2014; Tran et al. 2014).

Methods

The primary objective of this study was to quantify CBZ in tropical surface waters throughout Puerto Rico in order to assess the incidence of human-derived fecal contamination. The secondary objective was to examine the potential relationship between CBZ concentrations and fecal indicator bacteria. Surface water samples were taken in replicates from 17 stations throughout Puerto Rico (Figure 1) during the wet season in November and December 2014. Rivers, creeks, and lagoons were sampled and the majority of locations (ca. 70%) were in urban or semi-urban areas. Rural locations were also sampled to assess the relationship between CBZ concentrations and population density. One stream, located on a mountain summit near Cayey, was the only non-coastal site sampled, and served as a negative control. The Río Guayanilla site was downstream from the Guayanilla Wastewater Treatment Plant sewage outfall. Whirl-pak® bags attached to a line sampler were used to collect the samples. Care was taken to avoid disturbing the bottom sediment. The Whirl-pak® bags were sealed, placed into gallon size re-sealable bags, preserved on ice, and transported to the laboratory. Microbiological analysis was carried out within 6 hours of the sampling. Colilert® media and Quanti-Tray®/2000 were used to determine most probable numbers (MPNs) of *E.coli* (USEPA, 2007). IDEXX MPN Generator Software Version 3.2 was used to estimate final densities as CFU/100mL.

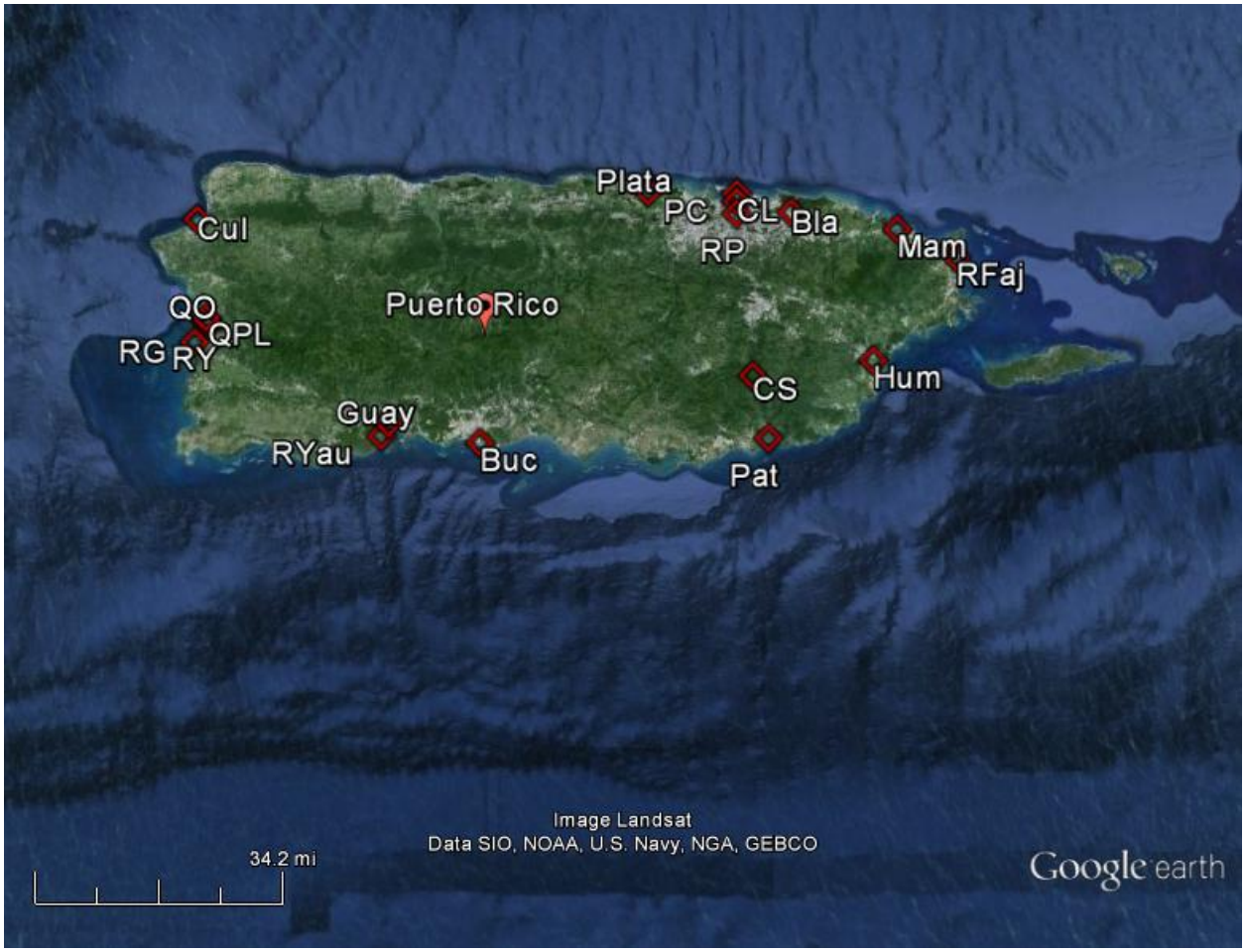

Figure 1: *Sampling Locations: CS (Cayey Stream), Mam (Mameyes), Cul (Culebrinas), Hum (Río Humacao), Pat (Río Patillas), CL (Condado Lagoon), RY (Río Yaguez), Buc (Río Bucaná), RP (Río Piedras), Bla (Blasina), PC (Parque Central), RFaj (Río Fajardo), RG (Río Guanajibo), QO (Quebrada del Oro), Guay (Río Guayanilla), QPL(Quebrada Parque Litoral), Plata (La Plata). Latitude and londitude for stations are provided in Table 1.*

A quantitative PCR-based (qPCR) analysis was used to detect HF183. 100mL aliquots were filtered through 0.2µm 47mm o.d. polycarbonate filters (Osmonics). Particles on filters were frozen (-80°C) in plastic tubes and shipped on dry ice to Georgica College and State University, Georgia for further analysis. A MoBio UltracleanTM Soil DNA Kit was used to process the filters, and a modified version of the manufacturer's instructions was followed

(Holman et al. 2014). Extracted DNA was quantified using a Nanodrop ND-1000 spectrophotometer. qPCR assays were conducted on a CFX 9600 (Bio RAD). All primers used in this study were optimized to avoid non-specific cross-reaction and increase specificity. The qPCR assay used a modified protocol of Haugland et al. (2010), with *Bacteroidesdorei*DSM 17855 (DSMZ) as a positive control and *Escherichia coli* strain B from Sigma® D48890-1UN as a negative control. The assay contained 1µM of each primer, 0.2 mg of bovine serum albumin (Sigma), 80 nMFam™ labeled Taqman® probe, 9 µl of deionized water, and 1 µl of sample DNA. The samples were run at: 95°C for 15 min; 40 cycles at 95°C for 10s and 66.3°C for 40s (Haugland et al. 2010). Standard curves and negative and positive controls were included during each run. The qPCR detection limit was 25 gene copies $100mL^{-1}$.

CBZ concentration was determined using the remaining portion of sample that was not used during the microbiological analysis. Solid phase extraction was used to concentrate CBZ and clean up the samples. Pre-concentration is an important step when targeting compounds that may be significantly diluted in the aquatic environment and close to the limit of detection. The protocol was based off of previously published methods for the solid phase extraction of pharmaceuticals (Vieno et al. 2006). Suspended particles in the water were removed using a 0.2 µm filter (GF 6, Schleiher & Schuell). The pH of the water samples was adjusted to 10.0 using 0.5M NaOH to reduce potential cross-reactivity with Cetrizine (Bahlmann et al. 2011). 200mL samples were passed through Oasis HLB (3 cc, 60 mg) cartridges using PTFE tubes. Cartridges were pre-conditioned with 6mL of MeOH, and equilibrated with 5mL of distilled water. After all the sample had passed through, the cartridges were washed with 5mL of distilled water and dried by allowing the vacuum pump run for 3 minutes. Elution of CBZ was done by passing 8mL of MeOH through the cartridges, which were collected in 20mL glass vessels. The

methanol extracts were then evaporated and stored at -18^{0}C until analysis. Prior to analysis extracts were reconstituted with 4mL of distilled water resulting in a 50x concentrated sample. This 50x concentrated sample was then diluted 5x. The average difference (n=13) between the mean concentrations of the 50x and 10x concentrated samples was very close to 5. It was determined after the first test that 50x was close to the method detection limit, so then samples were only concentrated 10x.

An enzyme-linked immunoassay (ELISA) was used to determine CBZ concentrations (CBZ ELISA: Abraxis LLC). Manufacturer instructions were followed, although the concentrated samples were used instead of raw samples to increase the detection limit. The detection limit of the test is reported to be 0.021ugL^{-1}, and saturated at 2.5µgL^{-1}. The assay was shown to correlate very well (R^2= 0.978) with traditional LC/MS methods (Bahlmann et al. 2012). The microtiter plate was placed on a phototransilluminator, a picture was taken of the plate with a Cannon PowerShot®, and Corel PaintShop Pro Version X6 was used to detect color differences. CBZ concentrations were determined using a standard curve with the standards treated as samples.

The limit of detection (LoD) was 0.027 µgL^{-1}, which closely matches the LoD reported by the manufacturer for the ELISA. LoD was determined by first calculating the limit of the blank (LoB), where LoB = [average concentration of the blank (n=4) + (3*standard deviation of blank)] and LoD = [LoB + (3*standard deviation of low concentration standard, 0.025µgL^{-1})] (Armbruster and Pry 2008).

To assess the effect of different initial sample concentrations on solid phase extraction and thus analyte recovery, the stream sample was separated into three 200mL aliquots and each

aliquot was spiked to a different concentration. The aliquots were spiked with the CBZ analytical standard, CAS no. 298-46-4, (Sigma-Aldrich, Milwaukee) to 0.04, 0.025, and 0.01µgL^{-1}. The equation, [(spiked sample concentration- unspiked concentration)/known spiked concentration]*100, was used to calculate relative percent recovery, and the average percent recovery was 77 ± 8% (mean±SD, n=3). From this test it was determined that initial sample concentration did not significantly affect analyte recovery.

Statistics were run using StatsXLVer1.8. A linear regression on log transformed data was used to determine the correlation between *E.coli* and CBZ, assuming a one-tailed distribution. A Mann-Whitney test was used to assess the relationship between CBZ and HF183.

Results and Discussion

Average CBZ concentrations and bacterial densities are given in Table 1. CBZ was detected in 16 out of 17 sites sampled, and concentrations ranged from 0.005ug$L^{-1}$to 0.482ugL^{-1}. CBZ was present below the detection limit (0.027 ugL^{-1}) in the Cayey Stream, a rural, pristine location presumably not impacted by sewage. Low concentrations of CBZ were detected in Mameyes, Culebrinas, Río Humacao, and Río Patillas. All of these locations are rural and the low concentrations detected are likely attributable to the lack of dense urban development. Higher CBZ concentrations were detected in urban and semi-urban areas. Río Guayanilla had the third highest concentration, which is probably related to the wastewater treatment plant outfall located just upstream from the sampling site. In general, lower CBZ concentrations were found in less densely populated areas.

Table 1: *Average carbamazepine concentrations (ugL^{-1}) and average bacterial densities (CFU 100mL^{-1}). n/a= not applicable, + = detected, - = not detected. Boxes around numbers indicate bacterial densities below the recommended statistical threshold value for E.coli (410 CFU100 mL^{-1}). ND= concentrations below detection limit. NQ= Detected, but not quantified, concentrations above assay detection limit.*

Sampling Site	Lat and Lon	CBZ	HF183	*E.coli*	Classification
Cayey stream	18.1038, -66.0463	0.0023 ND	n/a	159.7	Rural
Mameyes	18.3857, -65.7511	0.0046	-	296.9	Rural
Culebrinas	18.4056, -67.1763	0.0050	-	694.5	Rural
Río Humacao	18.1332, -65.8016	0.0073	-	25334.8	Rural
Río Patillas	17.9837, -66.0143	0.0121	-	392	Rural
Condado Lagoon	18.4550, -66.0770	0.0146	n/a	15634.5	Urban
Río Yagüez	18.2080, -67.1528	0.0187	+	48391.7	Urban
Río Bucaná	17.9747, -66.5985	0.0251	-	723.7	Urban
Río Piedras	18.4162, -66.0783	0.0384	+	48391.7	Urban
Blasina	18.4185, -65.9667	0.0439	+	>48392	Urban
Parque Central	18.4390 , -66.0770	0.0448	n/a	>48392	Urban
Río Fajardo	18.3311, -65.6308	0.0486	-	474.7	Urban
Río Guanajibo	18.1677, -67.1800	0.0495	-	2170.6	Semi-Urban
Quebrada del Oro	18.2144, -67.1568	0.0505	+	>48392	Urban
Río Guayanilla	18.0120, -66.7840	0.0591	+	241.2	Semi-Urban
Quebrada Parque Litoral	18.2016, -67.1523	0.1388	+	2888.2	Urban
La Plata	18.4584, -66.2581	>.25 NQ	+	21837.2	Urban

Densities of *E.coli* ranged from 159 to >48,392 CFU 100mL^{-1}, and 76% of sampling sites were impaired with *E.coli* levels well above the recommended 410 CFU 100mL^{-1} standard (USEPA 2012b). The extremely high *E.coli* level (>48,392 CFU 100mL^{-1}) in Parque Central was similar to previous reported levels above 60,000 CFU 100mL^{-1} for the same location (Bachoon et al 2010). Out of all the locations sampled, 7 sites had *E.coli* densities >15,000 CFU 100mL^{-1}. Condado Lagoon, a very popular recreational area in San Juan, had bacterial densities 38 times higher than acceptable recreational freshwater values.

The Cayey stream, Mameyes, Río Patillas, and Río Guayanilla had acceptable levels of fecal contamination based on *E.coli* abundance (<410 CFU $100mL^{-1}$). The lack of urban development around Cayey stream, Mameyes, and Río Patillas is probably a reason for the low bacterial densities. *E.coli* densities were expected to be higher in Río Guayanilla, because it was downstream from the nearby town and sewage outfall. However, CFU's were lower than the standard, either indicating the effectiveness of treatment or unculturable state of microbes after treatment (George et al. 2002). The widespread detection of *E.coli* was expected. Results from the 2012 US EPA water quality assessment showed that over 4,000 miles of rivers and streams in Puerto Rico were impaired due to elevated bacteria levels (US EPA 2012a). Bachoon et al. (2010) also reported widespread contamination, with approximately 50% of sites exceeding recommended *E.coli* standards. *E.coli* and CBZ were significantly correlated (R= 0.485, P value= 0.0285) after correcting for outliers (Figure 2).

HF183 was detected in one semi-urban and six urban sites, and was not detected in any of the rural locations. The absence of HF183 in rural areas supports its use as a more specific wastewater indicator. The co-occurrence of higher concentrations of CBZ and the detection of HF183 was examined by dividing the dataset in two groups based on HF183 detection. The median CBZ concentration for the group of sites where HF183 was found was $0.051\mu gL^{-1}$ and $0.012\mu gL^{-1}$ for those where HF183 was not detected. These test results support the assumption that higher CBZ concentrations are associated with the detection of the human-associated *Bacteroides* (Mann-Whitney test; U= 42.0; df=7; 1-tailed P value= 0.013) (Appendix 2). These results suggest that sites containing CBZ concentrations $>0.1\mu gL^{-1}$ may represent areas with significant human fecal contamination, corroborable using qPCR approaches.

Water samples were taken during the wet season and due to time constraints no attempt was made to determine temporal patterns. Therefore, the concentration of CBZ, *E.coli* densities, and detection of HF183 are indicative of the limited hydrologic conditions at the time of sampling and do not take into account seasonal variability. Urban and potentially contaminated sites were targeted and may in part account for the frequent detection of CBZ and high densities of *E.coli*.

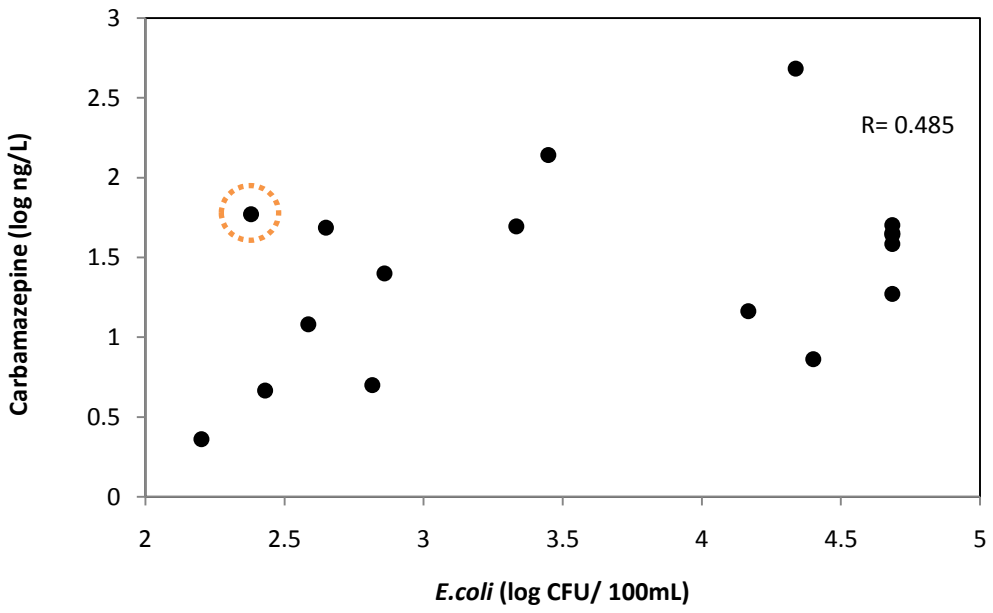

Figure 2: *Correlation between log transformed average carbamazepine concentration and E.coli abundance. The correlation was significant (P=0.0285). The marked point, Rio Guayanilla, was considered an outlier and not included in the correlation, because it was located downstream from a treatment plant and had low E.coli levels, and high CBZ.*

Conclusion

Results from this study and previously published work indicate widespread fecal contamination of rivers and streams in Puerto Rico (Bachoon et al. 2010; US EPA 2012a). The presence of CBZ and HF183 indicate that surface waters in Puerto Rico are significantly impacted by human fecal contamination. These findings highlight the importance of identifying the sources of pollution and taking appropriate remedial actions to minimize public health risks. Furthermore, these results indicate the presence of significant inputs of pollutants, biological and chemical, from human origins into coastal ecosystems, some of which may be presently threatened or protected (Bonkosky et al. 2008). Combining the use of source-specific chemical indicators, such as CBZ, with microbial methods would provide a more comprehensive water quality assessment. Epidemiological studies are needed to correlate CBZ concentrations with the incidence of gastrointestinal illnesses. This study was conducted to provide baseline data for CBZ, but future studies should include more sites to clearly assess the relationship of human population density in urban to rural watersheds with CBZ and other "novel" pollutants. These studies should preferably span at least a year to account for potential differences between wet and dry seasons (i.e. low and high flow). Future research should focus on the development of standardized methods for the combined use of CBZ and indicator bacteria to detect fecal contamination.

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Appendix Table 1

Sampling Site	Abbreviations
Cayey stream	CS
Mameyes	Mam
Culebrinas	Cul
Río Humacao	Hum
Río Patillas	Pat
Condado Lagoon	CL
Río Yagüez	RY
Río Bucaná	Buc
Río Piedras	RP
Blasina	Bla
Parque Central	PC
Río Fajardo	RFaj
Río Guanajibo	RG
Quebrada del Oro	QO
Río Guayanilla	Guay
Quebrada Parque Litoral	QPL
La Plata	Plata

Appendix Table 1: *Table of sampling locations and abbreviations*.

Appendix Table 2

Descriptive Statistics

	Value		Rank	
	detected	undetected	detected	undetected
	0.019	0.005	5.0	1.0
	0.038	0.005	7.0	2.0
	0.044	0.007	8.0	3.0
	0.051	0.012	11.0	4.0
	0.059	0.025	12.0	6.0
	0.139	0.049	13.0	9.0
	0.250	0.050	14.0	10.0
Median	0.051	0.012	11.0	4.0
Sum	0.599	0.152	70.0	35.0
N	7	7	7	7

1-tailed Test (detected > undetected)

U	DF 1	DF 2	P
42.000	7	7	0.013

Appendix Table 2: *Results from Mann-Whitney.*