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Tracking Anthropogenic Inputs Using Caffeine, Indicator Bacteria, and Nutrients in Rural Freshwater and Urban Marine Systems

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Our objective was to evaluate the hypothesis that measurements of caffeine, nutrients, and indicator bacteria can distinguish human versus non-human sources of surface water contamination in contrasting environments. A second objective was to determine if natural sources of caffeine were significant in unpopulated areas. Caffeine was measured in an isolated wetland, and a native plant source was identified. In two rural watersheds in southwest Georgia (U.S.), caffeine was detected in tributary creeks immediately below wastewater discharge sites and within towns. However, caffeine was not found in river main streams. Thus, although natural caffeine sources exist, background levels in stream drainage networks of these rural watersheds remained below detection. The presence of caffeine and elevated nitrate in streams was associated with anthropogenic inputs and population centers, whereas bacterial indicators did not correlate to these chemical indicators and appeared to have non-human sources. In contrast, caffeine in an urban coastal lagoon was generally linked to fecal coliform abundance. We observed sporadic relationships between caffeine and other water quality indicators, possibly due to differential rates of degradation. Creeks and bayous flowing into the lagoon contained the greatest caffeine concentrations and highest amounts of bacteria, nitrate, and radon, which is an indicator of groundwater discharge.

Introduction

Surface and groundwater contamination from anthropogenic sources has become widespread in the southeastern U.S. because of rapid population growth and a lack of adequate municipal infrastructure to keep pace with development. Microbial indicator organisms have been widely used to trace the origin of wastewater and determine the likelihood that the water poses a significant threat to human health. Fecal coliforms and enterococci are two of the most commonly used indicator bacteria (*1*, *2*). Nutrient concentrations are often elevated in wastewater and can have ecological consequences.

There are certain shortcomings with relying solely on indicator bacteria to assess risks associated with pathogenic microorganisms in water. The currently used groups of bacterial indicators are present in many animals, making distinctions between human and other sources difficult (*2*, *3*, *4*). Another problem is that fecal coliform abundance is not always consistent with the abundance of pathogenic viruses and human health risks (*2*, *3*). In some instances, human pathogens are more persistent in environmental waters than are fecal coliforms. Furthermore, fecal coliforms can adapt and survive in sediments and can later be mobilized into the water column via disturbances (*5*, *6*, *7*).

Methods for microbial source tracking in aquatic environments have been developed to distinguish animal from human sources. Such methods include monitoring for host specific organisms (viruses) or host related nucleic acid sequences (*1*−*4*, *8*, *9*). Considerable progress has been made in this arena, however, methods such as bacterial source tracking may be of limited geographic use. Expense, reproducibility, and standardization have also been problems for these approaches (*4*).

Caffeine has recently been examined as a tool for assessing human impacts on aquatic systems (*10*−*20*). When consumed, caffeine is metabolized (*21*, *22*), but a small amount (0.5−10%) of ingested caffeine remains intact when excreted (*16*, *18*, *21*). Most work in the past decade has focused on heavily polluted systems and efficiency of caffeine removal in sewage treatment plants (*10*, *13*, *14*, *19*, *23*−*25*). However, with improvements in technique (*13*, *16*, *17*, *26*, *27*) and lowered detection limits the scope of application has broadened to include stream, wetland, estuarine, and groundwater systems (*11*−*20*). In many instances, there appears to be an association between elevated caffeine concentrations and high population densities (*10*−*12*, *18*). However, to definitively trace caffeine to humans, the potential for natural sources needs to be more carefully considered. While caffeine is present in more than 60 species of plants (*28*), few are native to the U.S. (*16*). One species (*Ilex vomitoria* or yaupon holly) found in the southeast U.S. is known to contain caffeine (*29*).

The application of caffeine and other anthropogenic markers show promise for understanding wastewater contamination of groundwater in coastal zones, yet only a few studies have examined caffeine in groundwater (*13*). The added use of the geochemical tracer radon would be beneficial for understanding the distribution and transport of contaminants such as caffeine in groundwater. Radon is a dissolved gas whose presence in surface waters is frequently attributed to groundwater discharge (*30*).

The objective of this work was to evaluate the hypothesis that measurements of caffeine, nutrients and indicator bacteria can distinguish human versus non-human sources of surface water contamination in contrasting environments. We further hypothesized that natural background concentrations of caffeine would be low and examined this hypothesis in rural areas. In Sarasota Bay, an urbanized lagoon, we hypothesized that the presence of caffeine would correlate to bacterial and nutrient contamination.

Materials and Methods

Sampling Sites-Freshwater. Three sets of samples were collected during January and February 2004 from rural areas in south Georgia and north Florida (Figure 1). "Grab" samples were collected from the middle of the stream or wetland and all were obtained from below the surface to avoid the surface micro-layer. Separate water samples were also collected in

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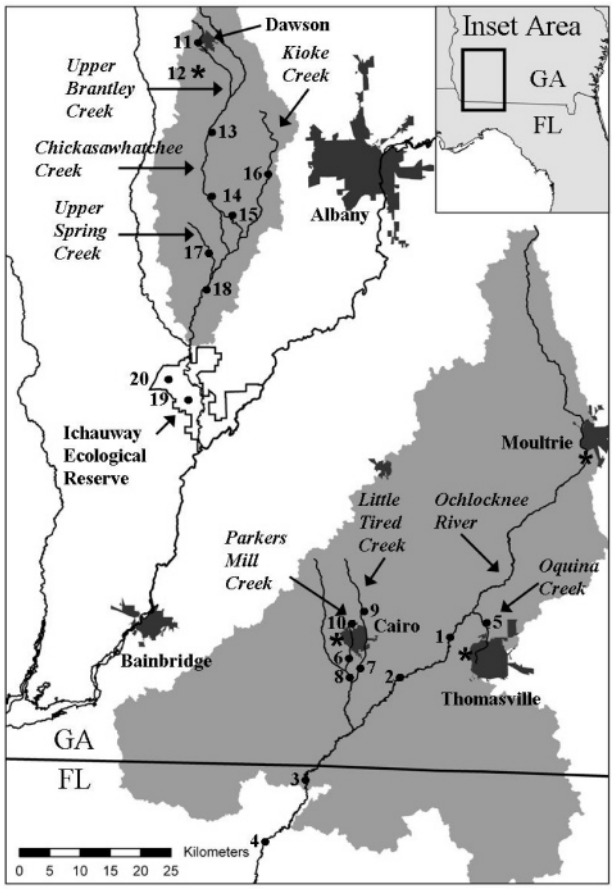

FIGURE 1. Locations of sampling sites in the Chickasawhatchee Creek and Ochlocknee River watersheds (shaded gray) and the 15 000 ha Ichauway Ecological Reserve (outlined). Most of the population resides in urbanized areas (shaded dark gray) with wastewater treatment plants (∗). The land cover of the surrounding rural area consists primarily of agriculture with some forested areas.

250 mL bottles for nutrient analysis and in 100 mL whirlpack bags containing sodium thiosulfate tablets for bacterial analysis. Nutrient and bacteria samples were iced immediately after collection and all samples were kept in the dark at 4 °C until processing.

The first sample set included four sites along the main stream of the Ochlocknee River (Figure 1) and four tributary creeks sampled when river streamflow was 159 m^3s^{-1}, well above the normal mean of 36 m^3s^{-1}. Three additional samples were collected from tributary creeks 10 days later after streamflow had declined to 41 m^3s^{-1}. Within the Ochlocknee River watershed, there are several small towns including Thomasville, GA (pop. ∼18 500) and Cairo, GA (pop. ∼9300) with multiple wastewater discharge sites. The population density in the three surrounding counties from which samples were collected is low (134−226 persons km^{-2}) indicating a predominantly rural landscape in this watershed.

The second set of freshwater samples was collected from the Chickasawhatchee Creek watershed (Figure 1). Upper Brantley Creek, a tributary, flows through Dawson, GA (pop. ∼5000). Streamflow at the Chickasawhatchee Creek during sampling (6.1 m^3s^{-1}) was only slightly above the long-term average for this day (4.5 m^3s^{-1}). The Dawson wastewater treatment plant discharges into Brantley Creek between the upper and lower sampling sites. The population density of Terrell County is only 13 persons km^{-2}, about half of which live in Dawson reflecting a very rural landscape dominated by agriculture and wetlands.

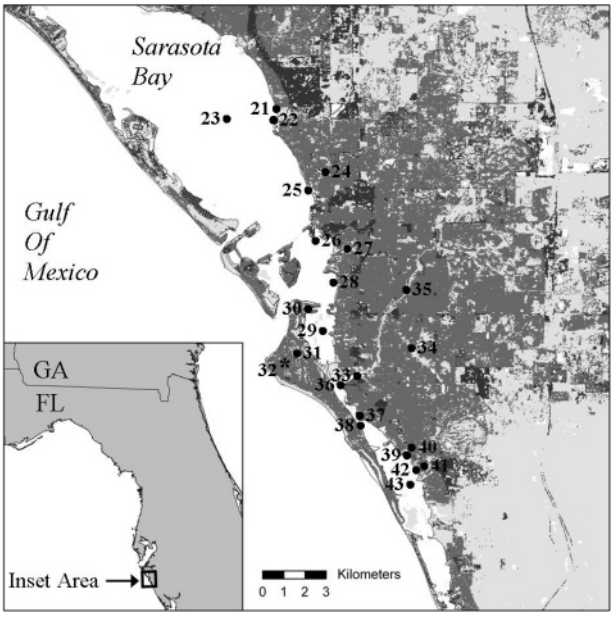

FIGURE 2. Sampling sites in the urbanized Sarasota Bay system (white indicates open water). The darkest gray reflects industrial/commercial land use, the medium gray indicates residential land use, light gray represents relatively low population areas, most of which are found outside the city limits.

A third set of samples was collected from two (<5 ha) isolated limesink wetlands in southwest Georgia that are part of the Ichauway Ecological Reserve (Figure 1). Predest Pond is classified as a cypress/gum swamp and Rhexia Pond is classified as a marsh/savannah. These wetlands are relatively pristine and have distinct watersheds which are hydrologically isolated from known pathways of human effluent.

Sampling Sites-Marine. Sarasota Bay is a 400 km^2 barrier island enclosed lagoon located on the southwest coast of Florida surrounded by the city of Sarasota (1385 persons $km^{-2,}$ Figure 2). Freshwater enters Sarasota Bay via several small tidal bayous and creeks which receive some of their input though stormwater runoff and submarine groundwater discharge. The major tributaries to the Bay are Whitaker Bayou, Hudson Bayou, and Phillippi Creek. The Phillippi Creek watershed contains approximately 32 000 septic tanks (*31*). Whitaker Bayou receives advanced treated wastewater effluent from the city of Sarasota municipal treatment plant.

Samples were collected four times (January, March, May, and July 2004) from five types of locations: keys, open bay, nearshore, canals, and bayous/creeks. The Keys group included samples collected from a drainage feature bordered by houses that was cut into Siesta Key, a barrier island. Open bay samples were collected in the centers of water bodies. Nearshore samples were collected within 50 meters of the mainland shoreline. Canals are dredged man-made waters without freshwater input. Bayous and creeks are natural drainage features with significant freshwater input.

Groundwater samples were collected at two sites on two dates (May and July) from seepage meters. Seepage meters are chambers placed over the bay floor with a vent tube to which a plastic sample bag is attached to collect groundwater seeping from the bay floor (*30*). Deployment sites were selected because they could be accessed from shore. Seepage meters were placed at 1 meter water depth. In July, the tide was much lower, consequently, the meters were placed further from the shoreline. Samples for caffeine and nutrient chemistry from Sarasota Bay were collected and processed as described above.

Caffeine Extraction and GC/MS Analysis. The procedure for caffeine analysis was an adaptation of the method

presented in ref *18*. Briefly, filtered samples were spiked with ^{13}C-labeled caffeine as an internal recovery standard. Reversed phase solid-phase extraction (SPE) with LC-18 columns was used to extract caffeine from water samples. Procedural blanks were run periodically to check for caffeine contamination during extraction. Analysis was conducted on an Agilent 6890 GC equipped with a J&W HP-5MS column, and an Agilent 5973 MSD. The MSD was run in SIM mode and the peaks produced by the 194 and 197 ions were used to quantify ^{12}C-caffeine (natural) and ^{13}C-caffeine (internal standard), respectively. The instrument detection limit for caffeine standards was 50 pg/injection. The ^{13}C-caffeine internal recovery standard accounts for any losses during extraction (e.g., breakthrough during SPE) and GC/MS analysis assuming that ^{12}C-caffeine and ^{13}C-caffeine are processed and analyzed with the same efficiency. The extraction efficiency of caffeine was also examined using duplicate 1 L samples of DI water with caffeine at concentrations of 20, 40, and 100 ng/L. The percent recovery of caffeine in DI samples ranged from 81 to 97%. The mean extraction efficiency of ^{13}C-caffeine from natural waters was somewhat lower than in DI water (84%) and showed a larger range (34−113%).

Analysis of Caffeine in Plant Tissues. Twigs, leaves, and berries from three species of the herbaceous holly shrubs, yaupon holly (*Ilex vomitoria*), inkberry (*Ilex glabra*), and myrtle-leaved holly (*Ilex myrtifolia*) were analyzed. Holly is common in coastal wetlands of the southeast U.S., and the species in this study were collected from Rhexia Pond (Figure 1). Samples were dried at 45 C and ground in a Wiley Mill through a 500 μm mesh. Ten milliliters of methylene chloride were added to tubes containing 0.5−1.0 g of material. The samples were soaked for 3 days with periodic shaking. Following this period, the methylene chloride was removed from each tube and reduced. The extract was transferred to ethyl acetate/methanol (95:5) and run on the GC/MS as described above. These extractions were performed as a spot check to determine whether any of the plant species had very high concentrations of caffeine and whether it appeared to be localized in a particular tissue. We did not study the extraction efficiency of caffeine from plant tissues, and values reported should be considered minimums.

Water Chemistry and Bacterial Assays. Nutrient samples were filtered through glass fiber filters (size 0.7 μm) and kept at 4 °C. Samples were analyzed for dissolved organic carbon (DOC), nitrate, phosphate, and ammonium within 2 weeks. Sodium phenolate was added as a preservative to ammonium samples before refrigeration. DOC measurements were made according to the method of Benner and Strom (*32*) using a Shimadzu TOC-V series total carbon analyzer. Other nutrient analyses were conducted using a Latchet auto-analyzer according to manufacturer's guidelines. Phosphate determinations represent soluble reactive phosphorus. Bacteria samples were packed on ice and measured at a state certified lab using U.S. Environmental Protection Agency (USEPA) method 9222D for fecal coliforms and EPA method 1600 for enterococci. Radon was determined on 3 liter samples collected in airtight bottles with a paristatiltic pump and sparged with He upon return to the laboratory. Rn was trapped on a cold trap and transferred to an alpha scintillation cell. Following a 3-hour in-growth period, alpha emissions were counted (*30*).

Data Analysis. After samples were analyzed, caffeine concentrations were compared to biological and chemical data using Model II regressions that do not assume dependence of either variable. Correlation coefficients (r) from regressions and degrees of freedom ($n - 2$) were used to calculate p-values. P-values less than 0.05, corresponding to a 95% confidence interval, were interpreted as significant correlations. We report 1-tailed p-values because we had clear expectations about the sign of the co-variation of the analyzed parameters. Samples which were below detection were treated as 0 values.

Results and Discussion

Rural Freshwater, Ochlocknee Watershed. Caffeine was not detected in the main stream of the Ochlocknee River but it was present in tributary creeks (Table 1). Each of these creeks receives discharge from a wastewater treatment plant (WWTP). However, WWTP effluent does not always contain high levels of caffeine (Table 1, 2, and ref *33*). Wastewater treatment plants are co-incident with greater population density and likely other anthropogenic wastewater inputs (nearby septic tanks, stormwater runoff, or treatment plant overflow) that must be the source of the caffeine if typical WWTP effluent is not (*33*). When detected, caffeine concentrations ranged from 34 to 196 ng/L. As the water from the tributary creeks flowed into the Ochlocknee River, caffeine appeared to be diluted to levels below the detection limit. Dilution, rather than degradation is the most likely explanation for low concentrations in the Ochlocknee River main stream because flow was relatively high (159 m^3s^{-1}) compared to the long-term average for this time (36 m^3s^{-1}), and substantial degradation is unlikely given that transit time between stations was short (<1 day) (*34*). Dilution is also consistent with the reduced nutrient concentrations observed in the main stream relative to the tributaries (Table 1).

The abundance of bacterial indicators ranged from 132 to 946 cfu/100 mL and from 280 to 3010 cfu/100 mL for fecal coliform and enterococci, respectively (Table 1). USEPA water quality standards for swimming are 126/100 mL and 33/100 mL CFU for fecal coliforms and enterococci respectively over a 30 day period (*35*). The relationship between enterococci and fecal coliform concentrations was highly significant ($r = 0.89$, $n = 11$, $p < 0.0001$). However, neither fecal coliform nor enterococci were correlated with caffeine ($r = 0.379$, $n = 11$, $p = 0.12$, and $r = 0.448$, $n = 11$, $p = 0.084$ respectively).

High nutrient concentrations in most of the tributary creek samples indicate an impact of wastewater on water quality in this system (Table 1). Nitrate had a significant positive relationships with caffeine ($r = 0.88$, $n = 11$, $p = 0.0015$) although phosphate did not. The sample with the greatest ammonium concentration had the greatest caffeine concentration. DOC showed a significant negative relationship with caffeine ($r = 0.57$, $n = 11$, $p = 0.035$), with DOC being lower in tributary creeks that we sampled than in the river main stream most of the time. Nitrate and ammonium did not correlate to either fecal coliform or enterococci abundance ($p > 0.05$). It appears as though the chemical tracers (nitrate and caffeine) track each other well and become diluted downstream. The abundance of indicator bacteria was variable. Bacteria were often measured at high concentrations well downstream and did not follow a pattern of dilution, suggesting natural sources.

Rural Freshwater, Chickasawhatchee Watershed. The only sample in the Chickasawhatchee Creek watershed that contained caffeine above our detection limit was Upper Brantley creek within the city limits of Dawson, GA (Table 1; Figure 1). The presence of caffeine in this portion of Brantley Creek must come from surface water runoff or from septic tank seepage. The wastewater treatment effluent that discharges into Brantley Creek did not contain measurable levels of caffeine although inorganic nutrient concentrations were elevated. None of the main stream samples from Chickasawhatchee Creek or those from the tributaries contained caffeine. This portion of the watershed is predominantly rural and there are no municipal wastewater treatment facilities. An absence of caffeine throughout most of this rural stream system supports the possibility that background caffeine levels from natural sources are below the analytical detection

TABLE 1. Station Information and Water Quality Data for the Ochlocknee River Basin, the Chickasawhatchee Creek Basin, and Two Isolated Wetlands[a]

station #	site name	caffeine (ng/Lu)	1/2 RANGE	F. coliform (# colonies/ 100 mL)	enterococci (# colonies/ 100 mL)	DOC (mg/L)	NO_3 (μM)	NH_4 (μM)	PO_4 (μM)
				Ochlocknee River Watershed					
	main stream (16 Feb 04)								
1	Ochlockonee River	BD		946	3,010	14.0	3.1	2.1	0.29
2	Ochlockonee River	BD		420	1,800	12.2	2.6	3.0	0.25
3	Ochlockonee River	BD		520	1,190	12.2	3.1	2.9	0.25
4	Ochlockonee River	BD		132	280	11.8	3.0	2.7	0.22
	tributaries (16 Feb 04)								
5	Oquina Creek*	68.9	14.4	240	1,052	6.4	27.7	9.0	0.96
6	Parker's Mill Creek	33.9	5.1	120	740	7.9	20.6	16.4	4.02
7	Little Tired Creek[b]	195.8	9.6	160	350	6.1	53.9	108.2	0.71
8	Tired Creek	BD		460	1,250	11.1	6.5	4.3	0.37
	tributaries (26 Feb 04)								
9	Little Tired Creek[b]	BD		260	1,100	19.8	0.1	3.4	0.08
10	Parker's Mill Creek[b]	43.7	1.9	480	1,600	7.3	5.0	11.7	0.08
6	Parker's Mill Creek	BD		340	1,800	7.5	25.4	16.6	4.40
				Chickawawhatchee Creek Watershed (7 Jan 04)					
11	Upper Brantley	62.3	22.7	93	ND	1.9	4.9	2.4	0.07
12	Dawson WWTP	BD		17	ND	4.1	18.7	4.8	1.74
13	Lower Brantley[b]	BD		152	ND	3.1	4.9	3.2	0.60
14	Chickasawhatchee Creek	BD		240	ND	3.7	2.6	1.6	0.20
15	Chickasawhatchee Creek	BD		960	ND	4.2	1.4	1.6	0.05
16	Kioke Creek	BD		139	ND	0.0	0.0	0.6	0.02
17	Upper Spring Creek	BD		169	ND	0.6	24.1	1.5	0.02
18	Chickasawhatchee Creek	BD		70	ND	4.6	2.4	0.7	0.03
				Isolated Wetlands on the Ichauway Ecological Reserve (7 Jan 04)					
19	Predest	BD		1,620	ND	17.3	0.0	2.7	0.03
20	Rhexia	14.6		16	ND	14.0	0.0	3.2	0.03

[a] Caffeine concentrations represent averages from replicate extraction and analysis. One-half the range is used as an estimate of error. The detection limit was 5 ng/L. [b] First sampling station below wastewater effluent discharge sites. BD, below detection; ND, not determined.

limits. There were two sites along the Chickasawhatchee Creek that had fecal coliform bacteria at levels above 200 cfu/100 mL. However, based on low nutrient concentrations and a lack of caffeine, these samples had no other indication of being contaminated by wastewater effluent suggesting a non-anthropogenic source of the elevated fecal coliforms.

Rural Freshwater, Isolated Wetlands. Two samples were collected from isolated wetlands that do not exchange water with other surface water or groundwater sources and there is no anthropogenic source of contamination. Predest Pond did not contain measurable levels of caffeine but fecal coliform counts were 1600 cfu/100 mL (Table 1). Such high fecal coliform abundance in this system must come from animal sources that include native species of turtles, birds, alligators, etc. Rhexia Pond had a caffeine concentration of 15 ng/L, but fecal coliform abundance was low (16 cfu/100 mL). Of the three species of holly present in the system, caffeine was only detected in yaupon holly and concentrations were very high. Leaves contained the highest amount of caffeine (0.5 mg/g dry weight). Twigs contained 0.1 mg/g dry weight, and the berries contained 2 μg/g dry weight.

Urban Sarasota Bay. Caffeine was measured in a majority of surface water samples, demonstrating that caffeine is released to and persists within the Sarasota Bay system (Table 2). Caffeine concentration ranged from below detection to 166 ng/L in surface water and was higher in canals, bayous, and Philippe Creek. The relationships between caffeine and other water quality measurements during each of the four sampling periods are presented in Table 3. Positive correlations between caffeine and fecal coliform abundance were significant for three of four sampling periods (Table 3). Significant correlations were observed when coliform abundance was greater than 200 cfu/100 mL which is indicative of more heavily impacted water. Enterococci abundance did not correlate with caffeine or with fecal coliform abundance.

Significant correlations between caffeine and other water chemistry parameters were evident but occurred irregularly. In March, the relationship between DOC and caffeine was positive demonstrating higher caffeine concentrations in DOC-rich waters that were derived from inland sources. Nitrate was correlated with caffeine in January and July, whereas ammonium correlated with caffeine only in July. Radon concentrations correlated with caffeine on only one occasion, but this relationship was among the strongest observed in the study ($r = 0.9$).

During the first two sampling periods, caffeine showed significant negative relationships with salinity suggesting caffeine contamination from non-saline sources. The latter two sampling periods exhibited nearly significant negative relationships ($P = 0.07, 0.055$) between caffeine and salinity. The regression equation for each sampling date was used to extrapolate to zero salinity in order to estimate the caffeine concentration of the local freshwater endmember. During January, a zero salinity sample was estimated to contain 61 ng/L of caffeine, whereas a much higher zero salinity sample of about 190 ng/L was estimated for March. These concentrations are in the range of those observed for freshwater streams in Georgia that were impacted from known wastewater sources (Table 1). The use of freshwater endmembers derived in this manner may be advantageous because they represent an integrated estimate of combined surface water and groundwater inputs to the bay system that would be otherwise difficult to obtain.

Groundwater from seepage meters was analyzed for caffeine at two sites. In May 2004, the Anchorage site had a seepage rate of 3.9 cm/day and caffeine concentrations were high (145 ng/L, Table 2). Viable fecal coliform bacteria were absent in the seepage water but enterococci bacteria were present at 130 cfu/100 mL. The New College site had a seepage rate of 25.0 cm/day, and the caffeine concentration in the

TABLE 2. Caffeine Concentrations in Sarasota Bay Surface Waters in 2004. Two Groundwater Samples Collected from Seepage Meters Are Also Included[a]

		caffeine						
station ID	site name (group no.)	Jan 23 ng/L	±	May 18 ng/L	±	May 20 ng/L	±	Jul 27 ng/L
	surface water							
21	New College, Library (3)			BD		37.1	(0.5)	22.4
22	New College, Caples (3)					BD		22.6
23	Middle of Sarasota Bay (2)			BD				44.9
24	Whitaker Bayou (5)	51.7	(5.2)	71.5		5.0	(1.0)	62.9
25	Whitaker Bayou, Mouth (3)			16.0	(4.6)	21.2	(1.4)	56.3
26	Anchorage (3)			15.0	(2.9)[b]	BD	-	31.4
27	Hudson Bayou (5)	20.9	(2.4)[b]	87.0	-	34.9	(1.8)	90.0
28	Radon 24 h House (3)	7.9	-					
29	Middle of Roberts Bay (2)			BD				
30	Canal through Keys (1)			7.7				
31	Siesta Key (1)	8.5				35.6	(1.6)	11.0
32	Treatment Plant (1)			BD				
33	Philippi Creek, Bridge (5)	18.6	(1.6)					
34	Philippi Creek, Large Branch (5)	18.4	-	116.0	(10.7)[b]	33.9	(1.1)	41.6
35	Philippi Creek, Small Branch (5)			165.8	(69.4)			
36	Philippi Creek, Mouth (3)	8.7	-			8.1	(1.1)	
37	Stickney Point, Canal (4)	14.9	(3.6)					
38	Siesta Bridge, South (2)							BD
39	Coral Cove (3)					17.0	(2.1)	36.4
40	Coral Cove, Canal (4)	BD						
41	Hidden Harbor Marina Canal (4)	8.0	(0.1)			37.9	(2.1)	27.5
42	Hidden Harbor, Mouth (3)	9.9	(0.4)	BD	-	23.7	(4.8)	42.2
43	Middle of Little Sarasota Bay (2)			BD				BD
	ground water							
22	New College, Caples					204.0	-	BD
26	Anchorage					144.8	-	
	locations sampled:	11		13		14		15

[a] Numbers in parenthesis beside site names refer to groupings based on site location (see text). BD = below detection (5 ng/L). When duplicate samples were collected, half the range was used as an estimate of variability. [b] Standard deviation is reported as an estimate of variability (n = 4).

TABLE 3. Relationships between Caffeine and Water Quality Measurements for Sites in Sarasota Bay[a]

	Jan 21		March 18		May 20		July 27	
	R	P	R	P	R	P	R	P
salinity	**0.54**	***0.043***	**0.59**	***0.016***	0.45	*0.072*	0.43	*0.055*
f. coliform	**0.74**	***0.005***	**0.72**	***0.003***	0.22	*0.250*	**0.72**	***0.001***
entero	ND	*ND*	0.35	*0.123*	0.35	*0.136*	0.33	*0.115*
DOC	0.51	*0.054*	**0.80**	***0.0005***	0.45	*0.071*	0.31	*0.132*
nitrate	**0.77**	***0.003***	0.19	*0.255*	0.17	*0.295*	**0.60**	***0.009***
ammonium	0.41	*0.105*	0.11	*0.360*	0.04	*0.890*	**0.56**	***0.015***
phosphate	0.28	*0.202*	0.13	*0.355*	0.42	*0.086*	0.28	*0.160*
radon	0.35	*0.153*	**0.90**	***0.0005***	0.10	*0.378*	0.03	*0.458*

[a] For January 21 for all sites, n = 11; March 18, n = 13, except Rn, n = 12; May 20, n = 14, except Rn, n = 9; July 27, n = 15, except Rn, n = 12. R is the correlation coefficient of the linear regression of the listed parameter with caffeine. P (in italics) is the probability that the observed relationship occurred by pure chance and that in the population from which the sample was drawn, no such relationship exists. Bold values indicate statistically significant results, $P < 0.05$.

seepage water was the highest measured in the study (204 ng/L). Enterococci bacteria were also abundant in this sample (1,550 cfu/mL) but fecal coliform bacteria were not detected.

During July 2004 the seepage rate at the New College site was only 5.9 cm/day. Caffeine in the seepage meter water was below the detection limit. Neither fecal coliform nor enterococci bacteria were present in the seepage meter water at this time. During the July sampling date the tide was much lower than on the May date so the seepage meters were much further from shore (50 meters in May and 150 meters in July). Freshwater discharge is focused close to shore while further from shore recirculation of seawater may be more important (*36*). This difference could explain the difference in caffeine results between the two sampling trips.

The presence of high caffeine concentrations and enterococci bacteria was consistent with wastewater contamination of groundwater as observed in other work in the Sarasota Bay system. Lipp et al. (*37*) conducted a study of microbial indicators of fecal pollution in the Sarasota Bay area and presented compelling evidence for subsurface transport of contamination to surface waters, with effluent from shallow septic systems suspected as being the source of contamination. Pierce and Brown (*38*) measured the distribution of the fecal sterol coprostanol in Sarasota Bay sediments. While the main source of coprostanol at the time was the sewage outfall in Whitaker Bayou, other sources were observed, and postulated to be associated with marinas, septic tank leaching and periodic overflow of sewer lines in the area.

Data from Sarasota Bay were divided into five groups as described earlier. Because of a non-normal distribution, statistical analysis was conducted by the nonparametric Wilcoxon rank-sum test and the Kruskal Wallis comparison of medians (Table 4). Significantly greater concentrations of caffeine, enterococci, fecal coliforms, nitrate, and radon were found in the bayou/creek grouping relative to the other areas. Based on this combination of water quality measurements, the bayous/creeks show the most obvious signs of impairment. Elevated radon concentrations suggest a large groundwater contribution to these areas again linking groundwater with surface water contamination.

To summarize, in rural areas, elevated caffeine and nitrate were correlated and linked to population centers and their associated wastewater treatment plants. Indicator bacteria in these systems were not correlated with these chemical

TABLE 4. Summary of Collected Data Divided Into Groups by Location[a]

	min	max	median	count	group
Caffeine ng/L					
(1) keys	<0.5	35.6	7.7	5	A
(2) open bay	<0.5	44.9	7.4	6	A
(3) near shore	<0.5	56.3	16.5	20	A
(4) canals	<0.5	37.9	14.9	5	A
(5)bayous/creek	5.0	165.8	46.7	14	B
Salinity psu					
(1) keys	10.0	30.0	27.7	5	B
(2) open bay	27.8	34.6	29.0	6	A
(3) near shore	24.6	35.0	32.2	20	A
(4) canals	25.3	32.0	30.0	5	A
(5) bayous/creek	1.4	33.7	17.5	14	C
Fecal Coliform CFU/100 mL					
(1) keys	0	92	10	5	A
(2) open bay	0	92	10	6	A
(3) near shore	0	700	6	20	A
(4) canals	36	1400	130	5	B
(5) bayous/creek	40	3400	255	14	B
Enterococci CFU					
(1) keys	62	840	296	4	A
(2) open bay	10	820	57	6	A
(3) near shore	0	530	190	17	A
(4) canals	20	130	75	2	A
(5) bayous/creek	250	8100	745	10	B
Nitrate μM					
(1) keys	0.00	2.66	0.50	5	B
(2) open bay	0.00	0.35	0.07	6	A
(3) near shore	0.00	3.30	0.28	20	B
(4) canals	0.27	1.80	0.54	5	B
(5) bayous/creek	0.23	11.16	1.66	14	C

	min	max	median	count	group
Ammonium μM					
(1) keys	1.70	3.63	3.17	5	B
(2) open bay	0.37	1.76	1.04	6	A
(3) near shore	0.34	5.30	1.48	20	A
(4) canals	0.52	53.80	7.47	5	B
(5) bayous/creek	0.0	11.74	6.83	14	B
Phosphate μM					
(1) keys	0.39	4.93	0.80	5	B
(2) open bay	0.18	0.54	0.28	6	A
(3) near shore	0.20	0.83	0.34	20	A
(4) canals	0.37	1.15	0.51	5	B
(5) bayous/creek	0.44	2.54	1.06	14	B
DOC mg/L					
(1) keys	2.0	8.9	5.3	5	A
(2) open bay	3.5	6.4	5.6	6	A
(3) near shore	1.0	6.8	3.4	20	B
(4) canals	1.8	7.8	2.9	5	B
(5) bayous/creek	2.7	13.0	7.2	14	A
Radon dpm/L					
(1) keys	8.1	39.4	23.8	2	A
(2) open bay	6.5	47.9	12.2	6	A
(3) near shore	7.3	33.1	18.5	18	A
(4) canals	23.2	74.1	47.9	4	B
(5) bayous/creek	16.4	147.5	97.4	8	C

[a] The maximum, minimum, and median value for each sample group is shown as well as the number of samples collected at each location (count). The group column shows which locations are different from each other based on *p*-values. *P*-values less than 0.05 were interpreted to be different. Groups were standardized by always setting open bay as group A.

parameters and were not associated with human populations. Indeed, the greatest bacterial abundance was found in an isolated wetland free from human influence but heavily populated by wildlife. In contrast, caffeine in surface water in the urban Sarasota Bay system was more likely to be positively correlated with fecal coliform abundance and with nitrate. The results are consistent with our hypothesis and indicate that measurements of caffeine, nutrients, particularly nitrate, and indicator bacteria can aid in distinguishing human versus non-human sources of surface water contamination. To more accurately assess human impacts on water quality, multiple parameters should be measured and the mechanisms controlling inputs and turnover of different indicator parameters considered for each system.

For instance, Buerge et al. (*18*) showed that caffeine is susceptible to photochemical degradation, which can be more significant than microbial degradation under high light. This contrasts with the removal of nutrients, which is primarily a biological process. The persistence of bacterial indicators is determined in part by yet other biological factors including grazing and physiological stress. The relationships among the indicators measured in this study must be dependent on the extent to which these different removal mechanisms influence the persistence of the each indicator in a given environment. For example, caffeine may be particularly robust in the absence of sunlight and may prove to be a better indicator of effluent in groundwater systems that lack sunlight and have longer water residence times.

The relationship with caffeine and DOC also varied between the two systems, being negatively correlated in rural Georgia and northern Florida, and more likely to have a positive (albeit not always statistically significant) relationship in Sarasota Bay. In Sarasota Bay, Bayous/Creeks had the highest DOC levels, and displayed the greatest concentrations of caffeine. In the Southeastern U.S., high DOC is associated with dark tannic waters, often derived from swampy wetlands. In rural areas, these areas appear to be more free from human influence, while in our marine sampling area, the conduits delivering these waters to the lagoon bisect urban zones and become contaminated.

Questions about natural sources and background levels of caffeine in aquatic systems have not been well addressed. Caffeine concentrations were below the detection limit in the Chickasawhatchee Creek watershed (with the exception of the single occurrence within the Dawson city limits). This is a watershed with relatively low human impact on water quality. Low natural background levels are also indicated from the Ochlocknee River system where the main stream of the river did not contain measurable caffeine, but smaller tributary creeks with known point sources of wastewater releases often had elevated concentrations. The presence of measurable caffeine in an isolated wetland demonstrates that natural sources of caffeine, such as the yaupon holly, can impact aquatic systems. However, the general lack of caffeine in rural creeks and rivers suggests that this occurrence is an exception and that larger drainage networks have low background levels.

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