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Comparison of Microbial and Chemical Source Tracking Markers To Identify Fecal Contamination Sources in the Humber River (Toronto, Ontario, Canada) and Associated Storm Water Outfalls

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ABSTRACT

Storm water runoff is a major source of pollution, and understanding the components of storm water discharge is essential to remediation efforts and proper assessment of risks to human and ecosystem health. In this study, culturable *Escherichia coli* and ampicillin-resistant *E. coli* levels were quantified and microbial source tracking (MST) markers (including markers for general *Bacteroidales* spp., human, ruminant/cow, gull, and dog) were detected in storm water outfalls and sites along the Humber River in Toronto, Ontario, Canada, and enumerated via endpoint PCR and quantitative PCR (qPCR). Additionally, chemical source tracking (CST) markers specific for human wastewater (caffeine, carbamazepine, codeine, cotinine, acetaminophen, and acesulfame) were quantified. Human and gull fecal sources were detected at all sites, although concentrations of the human fecal marker were higher, particularly in outfalls (mean outfall concentrations of 4.22 $\log_{10}$ copies, expressed as copy numbers [CN]/100 milliliters for human and 0.46 $\log_{10}$ CN/100 milliliters for gull). Higher concentrations of caffeine, acetaminophen, acesulfame, *E. coli*, and the human fecal marker were indicative of greater raw sewage contamination at several sites (maximum concentrations of 34,800 ng/liter, 5,120 ng/liter, 9,720 ng/liter, 5.26 $\log_{10}$ CFU/100 ml, and 7.65 $\log_{10}$ CN/100 ml, respectively). These results indicate pervasive sewage contamination at storm water outfalls and throughout the Humber River, with multiple lines of evidence identifying Black Creek and two storm water outfalls with prominent sewage cross-connection problems requiring remediation. Limited data are available on specific sources of pollution in storm water, though our results indicate the value of using both MST and CST methodologies to more reliably assess sewage contamination in impacted watersheds.

IMPORTANCE

Storm water runoff is one of the most prominent non-point sources of biological and chemical contaminants which can potentially degrade water quality and pose risks to human and ecosystem health. Therefore, identifying fecal contamination in storm water runoff and outfalls is essential for remediation efforts to reduce risks to public health. This study employed multiple methods of identifying levels and sources of fecal contamination in both river and storm water outfall sites, evaluating the efficacy of using culture-based enumeration of *E. coli*, molecular methods of determining the source(s) of contamination, and CST markers as indicators of fecal contamination. The results identified pervasive human sewage contamination in storm water outfalls and throughout an urban watershed and highlight the utility of using both MST and CST to identify raw sewage contamination.

Storm water runoff has been identified as one of the most prominent non-point sources of both biological and chemical contaminants, degrading water quality and posing risks to public health in impacted recreational waters (1–3). The high level of fecal contamination present in storm water runoff has been noted as one the leading causes for beach closures and advisories in the United States (4) and has been directly linked to disease outbreaks (5, 6). The major sources of human fecal contamination in storm water runoff are failing sewage infrastructures and cross-connections between sewage and storm water networks (7, 8). However, in addition to sewage contamination, storm water runoff can also carry other forms of animal waste, as well as a variety of pesticides and other chemical contaminants (2, 9, 10). Understanding the composition of the resultant runoff is therefore essential to tracking storm water pollution and assessing overall risk to public health.

While surface waters are monitored using concentrations of fecal indicator bacteria (FIB), such as *Escherichia coli* and enterococci (11, 12), the FIB paradigm is imperfect. Among other problems, the presence of FIB does not always correlate with the occurrence of pathogens, particularly viral or protozoan pathogens (1, 13, 14). Further, elevated concentrations of FIB do not give any indication of the source of fecal contamination, which can hinder remediation efforts. Inability to accurately identify the source of contamination can also lead to inaccurate decisions relating to public health, particularly as different sources of contamination can pose different risks to human health, with human sewage contamination generally posing the greatest risks (15). Other methods to identify the source of contamination, particularly with regard

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TABLE 1 List of sampling site locations and percent detection for endpoint MST markers

Site name	Type	Location[a]	GPS coordinates	Sampling events (*n*)	% marker			
					Human	Ruminant	Gull	Dog
R1	River	Upper Humber (E)	43°47′51.13″N, 79°34′52.17″W	16	31	63	38	13
R2	River	Upper Humber (M)	43°47′27.64″N, 79°35′39.45″W	16	19	6	38	13
R3	River	Upper Humber (W)	43°43′9.34″N, 79°32′35.40″W	16	13	6	44	25
R4	River	Middle Humber	43°40′43.10″N, 79°30′26.24″W	16	31	25	81	19
T1	Creek	Black Creek	43°40′32.25″N, 79°29′48.38″W	16	88	31	81	13
R5[b]	River	Middle Humber	43°38′30.39″N, 79°29′25.57″W	7	29	29	71	14
R6	River	Lower Humber	43°39′6.80″N, 79°29′29.61″W	16	44	25	81	38
R7	River	Humber mouth	43°37′55.32″N, 79°28′15.30″W	15	47	13	87	27
T2	Outfall	Black Creek	43°40′30.85″N, 79°29′20.63″W	14	71	21	29	21
T3	Outfall	Black Creek	43°40′32.38″N, 79°29′13.65″W	14	57	0	21	7
S1	Outfall	Middle Humber	43°39′42.69″N, 79°30′13.69″W	13	69	46	31	15
S2	Outfall	Lower Humber	43°39′6.00″N 79°29′28.01″W	14	93	21	21	7
S3	Outfall	Middle Humber	43°39′25.63″N, 79°29′58.83″W	9	56	0	22	22
S4	Outfall	Lower Humber	43°38′17.49″N, 79°28′39.58″W	9	89	22	44	44
S5	Outfall	Lower Humber	43°38′3.18″N, 79°28′28.22″W	14	43	14	64	7

[a] E, east; M, middle; W, west.
[b] R5 had a smaller sample size than other sampling sites. Rain events and other seasonal influences were missed at site R5 that were sampled at other sites, precluding simple comparisons among other sites.

to human sewage, are often necessary to accurately guide remediation efforts.

A variety of methods have been used to indicate human sewage contamination, such as the use of culturable antibiotic-resistant strains of bacteria, which tend to be more prevalent in wastewater (16–20). Additionally, many field studies have utilized microbial source tracking (MST) methods such as host-associated molecular markers to identify multiple sources of contamination (e.g., human, dog, gull, cow) (21–29). Further, in recent years, efforts to characterize the human microbiome have started to reveal the predominant bacteria within the human gut and associated with human skin. Several of these species, such as *Bacteroides thetaiotaomicron*, *B. dorei*, *Clostridium perfringens*, *Bifidobacterium adolescentis*, and *Faecalibacterium prausnitzii*, have previously been used as microbial source tracking markers (30–33). However, other prevalent gut-associated bacteria, such as *Eubacterium rectale* and *Ruminococcus bromii*, as well as skin-associated bacteria, such as *Staphylococcus epidermidis* and *Propionibacterium acnes*, may also be useful markers for human-specific contamination (34–36). Finally, a variety of chemical source tracking (CST) markers have been identified as potential indicators of sewage contamination, including caffeine (37, 38), carbamazepine and other pharmaceuticals (38–40), cotinine (41), and chemical sweeteners (42).

In this study, multiple sampling sites throughout the Humber River watershed (Toronto, Ontario, Canada), including associated tributaries and storm water outfalls, were sampled to identify hot spots of fecal contamination using concentrations of culturable *E. coli* and ampicillin-resistant (Amp^r) *E. coli*. To attempt to discriminate human and animal sources of the fecal contamination and potentially to identify sewage cross-connections in storm water outfalls, a suite of MST and CST markers were measured at all sampling sites. Additionally, the preliminary application of a quantitative PCR (qPCR) array with a variety of potential MST markers was explored to determine whether these potential markers would be useful in determining the source(s) of fecal contamination. Identification of the source(s) of elevated *E. coli* concentrations at Sunnyside Beach at the mouth of the Humber River is needed to guide remediation efforts to reduce beach postings and a beach Beneficial Use Impairment within the Toronto Area of Concern (AOC).

MATERIALS AND METHODS

Study area. This study was conducted within the Humber River watershed in the Toronto AOC. The Humber River is a relatively large river (with the main branch extending 126 km), draining an area of 911 square kilometers into Lake Ontario. Land use in the Humber watershed is 54% rural, 33% urban, 13% urbanizing, and 32% natural cover, and the population in the watershed area in 2014 was 856,200 inhabitants (43). The only sewage treatment plant discharge into the Humber River occurs from two small plants (serving about 7,000 people) that discharge in the central branch of the Humber River above our R2 sampling site. The Humber watershed has been characterized by poor water quality, with contamination in some upper branches of the river historically attributed predominantly to livestock and agricultural contamination (44), while contamination in the lower portion of the river has been attributed to storm water and combined sewer overflows containing raw sewage (45). Sampling sites were selected to represent the major tributaries in the Humber River and those larger storm water outfalls in the lower watershed in proximity to Sunnyside Beach.

Sample collection. Water samples were taken at both river sites (sites R1 to R7) and storm water outfall sites (S1 to S5) in the Humber River, as well as at the Black Creek tributary (site T1 and associated outfalls T2 and T3), from May to September 2014 (Table 1). Outfalls S1and S5 were not readily accessible and were sampled immediately below the outfall and were thus blended with river water. Two water samples were collected at the same time from each river and outfall site: a 500-ml sample collected in an autoclaved polypropylene bottle for *E. coli* enumeration and MST assays and an additional 100-ml sample collected in an amber glass bottle for chemical marker analysis (see below). All water samples were placed on ice and transported to the laboratory for processing within 6 h of collection. Additionally, 20 samples each of wastewater influent and effluent were collected from Toronto's four wastewater treatment plants (Ashbridges Bay, Highland Creek, Humber River, and North Toronto) on five separate days (8, 15, and 29 September and 6 and 20 October of 2014). These wastewater samples were used as a reference to compare the concentrations of MST and CST markers in ambient river and storm water

samples with concentrations found in wastewater untreated influent and treated effluent.

***E. coli* enumeration.** For enumeration of both culturable *E. coli* and Amp^r *E. coli*, water samples were filtered (using 0.45-μm-pore-size, 47-mm-diameter filters) over a range of dilutions according to standard membrane filtration methods (46). *E. coli* bacteria were enumerated on differential coliform (DC) media, supplemented with cefsulodin, while Amp^r *E. coli* bacteria were also enumerated on the same DC media additionally supplemented with ampicillin (32 μg/ml). Both culturable *E. coli* and Amp^r *E. coli* cultures were incubated at 44.5°C for 22 h. Results were reported as CFU counts per 100 milliliters. Filtration blanks were included in every batch of water samples.

DNA extraction and PCR. An additional 300 ml was filtered (using 0.45-μm-pore-size, 47-mm-diameter filters), as described above, for DNA extraction. Filters were frozen, for no more than 1 week, at −80°C until ready for DNA extraction. Filters were then folded and placed into Powerbead tubes, and the filter contents were extracted using Powersoil DNA isolation kits (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Extraction blanks were included in every batch of DNA extractions.

PCR and quantitative PCR (qPCR) assays were performed on all extracted DNA samples (including filtration and extraction blanks). PCR assays included general (Bac32), human (HF183), ruminant (CF128), and dog (DG37) *Bacteroidales* assays and gull *Catellicoccus* (Gull2) assays, performed with previously published primer sets (47–49). MST assays for qPCR included general (GenBactF3), human (HF183), cow (CowM2), and dog (DG37) *Bacteroidales* assays and gull *Catellicoccus* (qGull4) assays, performed with previously published primer and probe sets (49–53).

Each PCR consisted of 2.5 μl 10× IDTE buffer (Integrated DNA Technologies, Coralville, IA, USA), 0.2 μl 100 mM deoxynucleoside triphosphate (dNTP) mixture, 0.16 μl 10% bovine serum albumin (BSA), 0.5 μl each of forward and reverse primers (78 pM), 0.25 μl HotMaster *Taq* DNA polymerase (5Prime GmbH, Hilden, Germany), 19.89 μl nuclease-free water, and 1 μl of extracted DNA. Reactions were carried out in 96-well plates using an Eppendorf Mastercycler (Hamburg, Germany). Each 96-well plate included a negative control consisting of nuclease-free water and a positive control of DNA extracted from a known fecal source. For all plates, the negative control produced no band on the subsequent gel, while the positive control produced a band of the correct molecular weight for the corresponding target. Cycler conditions were consistent with previously published assays (47–49).

Each of the qPCRs consisted of 2 μl of an internal amplification control (IAC), 2.5 μl 2 mg/ml BSA, 3 μl nuclease-free water, 12.5 μl TaqMan universal master mix 2.0 (Applied Biosystems, Carlsbad, CA, USA), 3 μl of a primer/probe mixture (100 μM for both primers and probe), and 2 μl of extracted DNA. Reactions were carried out in 96-well plates using a CFX96 cycler (Bio-Rad, Hercules, CA, USA). All reactions were carried out in duplicate, including no-template controls (NTC), negative controls consisting of 2 μl salmon testes DNA, and positive controls consisting of 2 μl of DNA extracted from a known fecal source. Standard curves were run on every qPCR plate (13 total standard curves were run for each target). For all qPCR runs, NTC and negative-control samples never showed amplification, while amplification was observed in all positive controls. Samples that amplified at cycle 30 ± 3 cycles in the IAC were considered uninhibited. No samples were deemed inhibited. Thermocycler settings were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min for all targets except gull. Thermocycler settings for the gull qPCR assay were 95°C for 5 min and 45 cycles of 95°C for 15 s and 60°C for 30 s. All qPCR results were reported as copy numbers (CN) per 100 milliliters.

Standard curves for all qPCR assays were constructed using synthesized plasmid DNA (pIDTSMART with ampicillin resistance; Integrated DNA Technologies, Coralville, IA, USA). DNA used for the standard curve was serially diluted using AE buffer (Qiagen, Valencia, CA, USA) to concentrations ranging from 10^2 to 10^5 gene copies/reaction. DNA used for the IAC was similarly constructed using synthesized plasmid DNA (pIDTSMART with ampicillin resistance; Integrated DNA Technologies, Coralville, IA, USA) with complementary primer sites included in each assay and every reaction to verify that there was no inhibition from the ambient water matrices. All qPCR runs had an efficiency level of between 90% and 110%, with an R^2 of >0.95, and results were normalized to reaction efficiency.

qPCR arrays. One wastewater influent sample and 36 water samples were selected for exploratory testing using Qiagen microbial DNA qPCR arrays (Qiagen, Valencia, CA). The water samples were collected on one rain event sampling day in late July and one dry weather sampling day in early August. The qPCR array design was customized in a 96-well format to include DNA markers for *B. thetaiotaomicron*, *B. dorei*, *Bif. adolescentis*, *Catellicoccus marimammalium*, *Clostridium perfringens*, *Eu. rectale*, *F. prausnitzii*, *P. acnes*, *R. bromii*, *S. epidermidis*, and *Turicibacter sanguinis*. The qPCR arrays were run according to the manufacturer's instructions. As these arrays are not quantitative without standard curves, the results were reported in a semiquantitative form using an inverse cycle threshold: this value was obtained by subtracting the cycle at which a sample amplified from the maximum number of 40 cycles. Therefore, the greater the inverse cycle, the more copies of a given target detected within that sample.

CST marker analysis. CST marker analysis for all samples was performed by Environment Canada's National Laboratory for Environmental Testing (Burlington, Ontario, Canada). The full methods are presented in "Additional methods" in the supplemental material. For comparison, chemical analysis was performed on wastewater samples (influent and effluent collected from Toronto's four wastewater treatment plants; n = 20 for both influent and effluent samples). Concentrations were measured for the compounds caffeine, carbamazepine, codeine, cotinine, and acetaminophen and the artificial sweetener acesulfame. For quantitative analyses, if a sampling site had detectable levels of a chemical in greater than 50% of water samples, water samples with values below the detection limit ("nondetects") were adjusted to values of one-half the detection limit for that chemical. For sites where a chemical was detected less than 50% of the time, nondetects were assigned a value of 0. The results of the statistical analyses were robust regardless of whether data corresponding to one-half the detection limit or untransformed data were used.

Statistical analysis. Data from both measures of *E. coli* (culturable and Amp^r; CFU counts per 100 milliliters) and all MST markers (CN per 100 milliliters) were log transformed prior to analysis. *t* tests were used to assess differences in *E. coli* and MST marker concentrations between river sites and outfalls. Multivariate analysis of variance (MANOVA) was used to determine the main effect of sampling site among river sites or among outfalls, where response variables were *E. coli* concentrations. The main effect of sampling site (including wastewater treatment plant influent and effluent samples) was similarly assessed via MANOVA where response variables were chemical marker concentrations. Tukey's *post hoc* test was performed if a significant effect was detected. Chi square tests were used to determine differences in endpoint MST marker detection. Spearman correlations were used to assess relationships among *E. coli*, qPCR, and MST and CST marker concentrations. All analyses were performed in Statistica v.12, and results were considered significant at the α level of 0.05.

RESULTS

***E. coli* enumeration.** Culturable *E. coli* concentrations were significantly correlated ($P < 0.05$) with Amp^r *E. coli* concentrations ($r_s = 0.87$) for the pooled data set. *t* tests revealed significantly greater concentrations of *E. coli* by both measures (culturable and Amp^r) in outfall sites than in river sites ($P < 0.001$ for both measures). Among the river sites, sampling site had a significant effect on both measures of *E. coli* ($F_{21,259} = 3.06$, $P < 0.001$; Fig. 1). *Post hoc* analyses revealed that site T1 had significantly greater concentrations of culturable *E. coli* than sites R2 ($P = 0.003$ for both measures) and R1 ($P = 0.021$ and 0.025, respectively). Amp^r *E. coli*

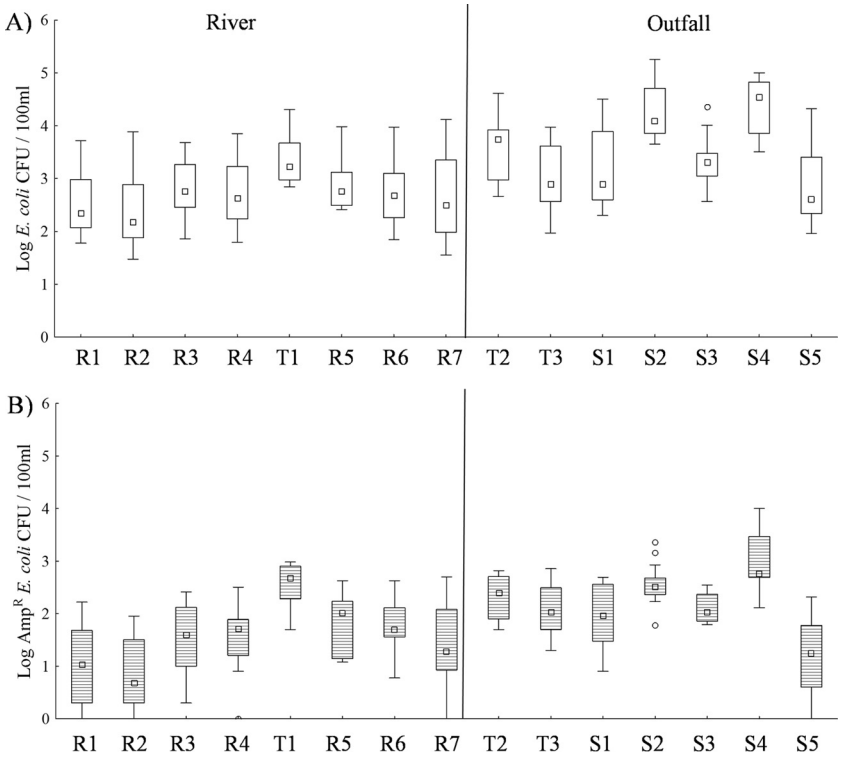

FIG 1 Box plots of (A) culturable *E. coli* and (B) Amp^r *E. coli* at each sampling site. Box plots show the median *E. coli* concentration between the 25th and 75th data quartiles; whiskers extend to the outermost data point within ±1.5 data points of this interquartile range. Open circles depict outlier values.

concentrations were also significantly greater in site T1 than in sites R1 to R4 and R7 ($P \leq 0.010$). Among outfall sites, sampling site also had a significant effect on both measures of *E. coli* ($F_{18,190} = 5.26$, $P < 0.001$; Fig. 1). *Post hoc* analysis revealed that outfall S2 had significantly greater culturable *E. coli* concentrations than all other outfalls, except S4 ($P \leq 0.009$), and that S4 had significantly greater culturable *E. coli* concentrations than outfalls T3, S5, and S1 ($P \leq 0.002$).

Microbial source tracking. The general *Bacteroidales* marker (Bac32) was detected in all but one sample and was therefore not included in further analyses. A chi square test revealed significant differences in the levels of detection of host-associated endpoint MST markers between river sites and outfalls (Table 1); the human marker was detected significantly more frequently in outfalls than in river sites ($\chi^2 = 16.32$, $P < 0.001$), while the gull marker was detected significantly more frequently in river sites ($\chi^2 = 19.35$, $P < 0.001$). As the qPCR cow marker was never detected in any river or outfall samples, and the qPCR dog marker was detected only rarely in outfalls, they were excluded from further analyses. *t* tests revealed that outfalls had significantly higher concentrations of the human *Bacteroidales* qPCR marker than river sites ($P < 0.001$; Fig. 2).

Among river sites, sampling location had a significant effect on endpoint MST marker detection (Table 1). The human *Bacteroidales* marker was detected significantly more frequently at site T1 than at other river sites ($\chi^2 = 25.5$, $P = 0.001$), the ruminant *Bacteroidales* marker was detected significantly more often at site R1 than at other river sites ($\chi^2 = 20.0$, $P = 0.006$), and the gull marker was detected significantly less often at sites R1 and R2 than at other river sites ($\chi^2 = 22.4$, $P = 0.002$). Sampling location also had a significant effect on qPCR marker concentrations among river sites ($F_{21,311} = 3.62$, $P < 0.001$; Fig. 2). *Post hoc* analyses determined that site T1 had significantly greater concentrations of human-specific *Bacteroidales* than all river sites except R5 and R6 ($P \leq 0.009$; Fig. 2).

Among outfall sites, no significant differences were observed for detection of any endpoint marker among sites. However, MANOVA detected a significant effect of outfall location on qPCR marker concentrations ($F_{18,221} = 4.75$, $P < 0.001$; Fig. 2). *Post hoc* analyses determined that outfall S2 had significantly greater concentrations of the human *Bacteroidales* marker than outfalls T2, T3, and S5 ($P = 0.007$, 0.037, and < 0.001, respectively) and that outfall S4 had significantly greater concentrations than outfalls T2, T3, and S5 ($P = 0.004$, 0.020, and < 0.001, respectively).

The inverse cycle thresholds obtained for select qPCR array markers are displayed in Fig. 3. Generally, *C. perfringens* was more prevalent in outfalls than in river sites (data not shown). *B. thetaiotaomicron* and *B. dorei* were present in all sites, with the highest levels in S2. *Bif. adolescentis* was present at all sites except for R1, with the highest levels in S2. *F. prausnitzii* and *Eu. rectale* were ubiquitous, and concentrations in S2 approached the levels found in wastewater influent. *R. bromii* was commonly found, with higher concentrations in the outfall and middle-to-lower Humber River sites (data not shown). *P. acnes* was found at higher concentrations in river than in storm water outfalls, while *S. epidermidis* was detected at only one storm water outfall (S2) and in wastewater influent (data not shown). *C. marimammalium* was present at the majority of sites but less often in the upper watershed away from Lake Ontario. *T. sanguinis* was not detected in the

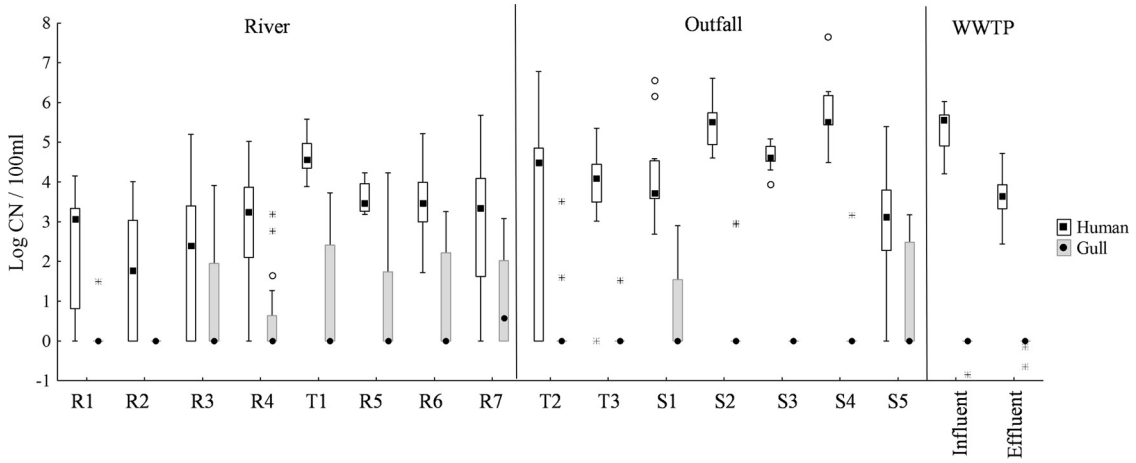

FIG 2 Box plots of human and gull qPCR marker concentrations for each site. Box plots show the median *E. coli* concentration between the 25th and 75th data quartiles; whiskers extend to the outermost data point within ±1.5 data points of this interquartile range. Open circles depict outlier values. WWTP, wastewater treatment plant.

river and was detected only in outfalls from the lower Humber River.

CST markers. MANOVA revealed a significant effect of sample type (river, outfall, influent, or effluent) on the concentrations of CST markers ($F_{18,620} = 172.17$, $P < 0.001$; select CST markers are shown in Fig. 4). *Post hoc* analysis revealed that wastewater influent had significantly higher concentrations of caffeine, cotinine, and acetaminophen than any other site type ($P < 0.001$ for all analyses). While there was no significant difference in carbamazepine concentrations between wastewater influent and effluent, both wastewater sample types had significantly higher concentrations of the chemical than samples from river or outfall sites ($P < 0.001$ for all analyses). Codeine and acesulfame concentrations were significantly higher in wastewater influent than in any other site type ($P < 0.001$ for all analyses), and effluent had significantly greater concentrations of the chemicals than samples from river or outfall sites ($P < 0.001$ for all analyses). Excluding wastewater samples from the analysis, *t* tests revealed that outfall sites had

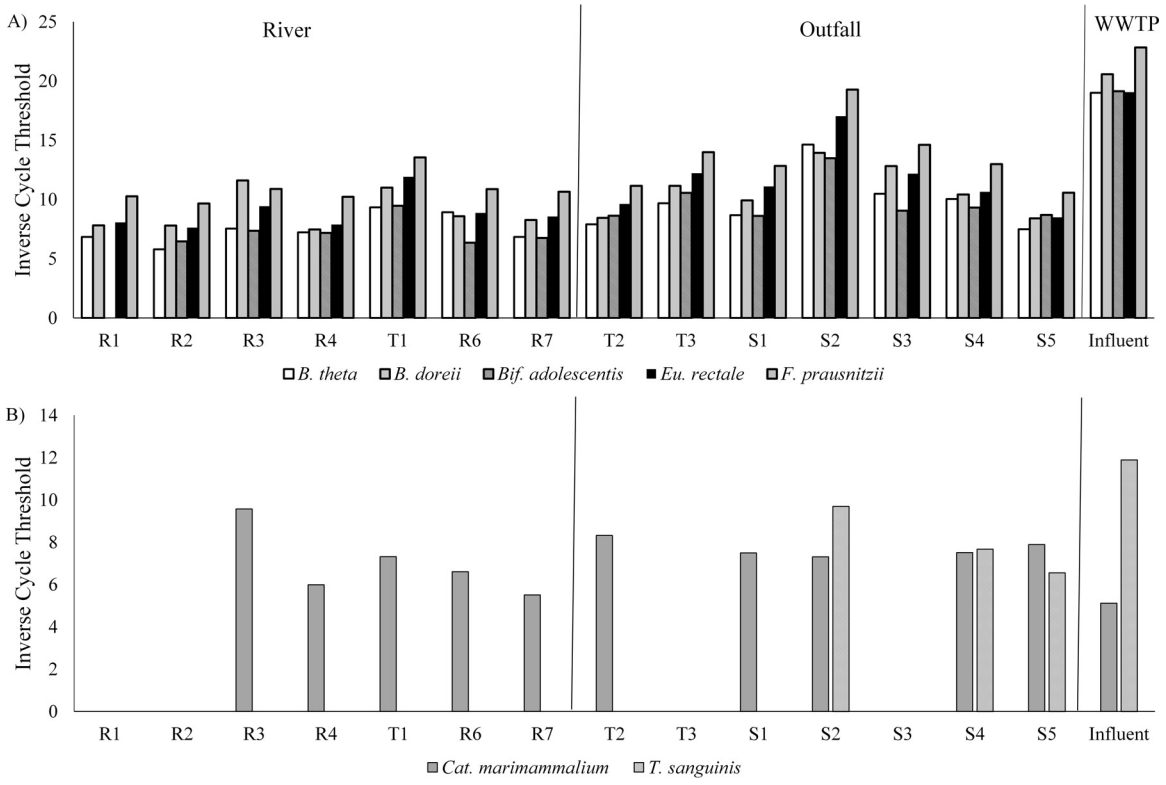

FIG 3 Mean inverse cycle thresholds obtained using qPCR arrays with targets for (A) human and (B) gull and Canada goose.

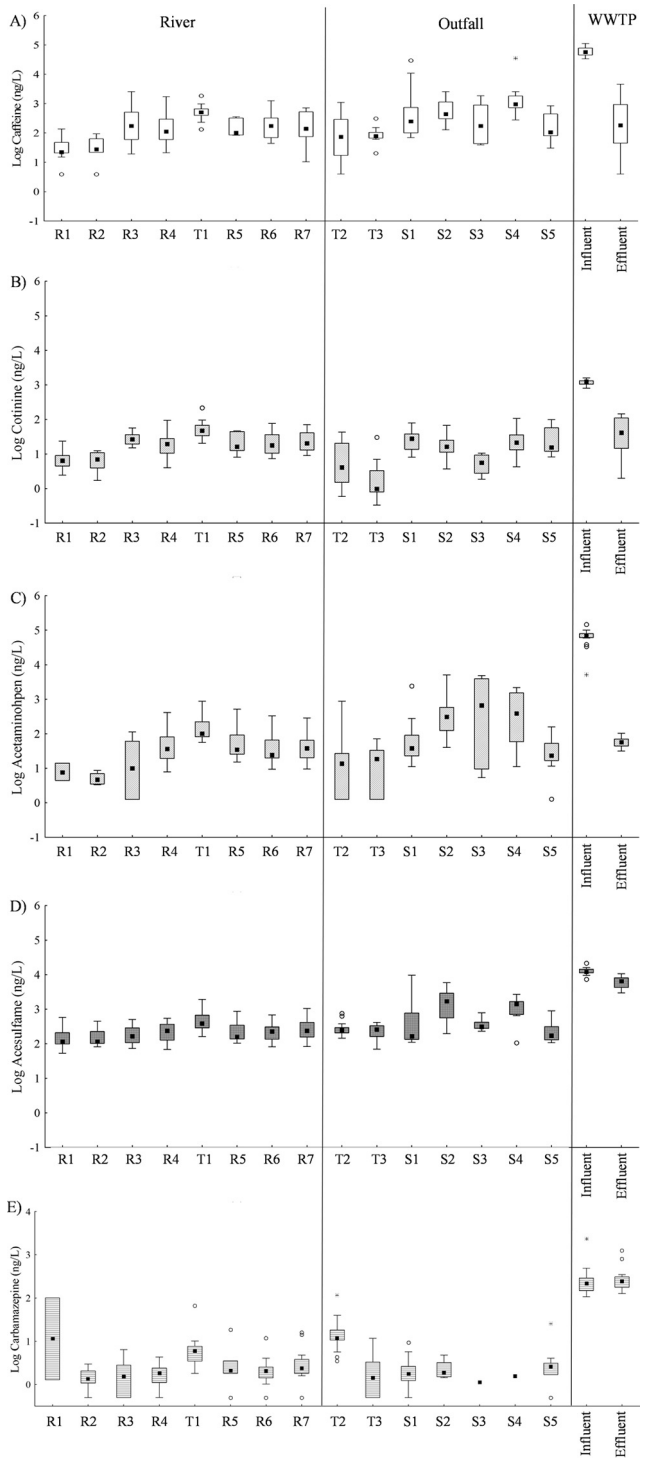

FIG 4 Box plots of (A) caffeine, (B) cotinine, (C) acetaminophen, (D) acesulfame, and (E) carbamazepine for each site. Box plots show the median *E. coli* concentration between the 25th and 75th data quartiles; whiskers extend to the outermost data point within ±1.5 data points of this interquartile range. Open circles depict outlier values, and asterisks depict extreme values.

significantly greater concentrations of caffeine, acetaminophen, and acesulfame than river sites ($P = 0.030$, <0.001, and <0.001, respectively) and that river sites had significantly greater concentrations of codeine than outfalls ($P = 0.009$).

Among river sites, MANOVA detected a significant effect of sampling location on chemical marker concentrations ($F_{42,454} = 2.90$, $P < 0.001$), with site T1 having significantly higher concentrations of the CST markers than many river sites (Fig. 4). *Post hoc* analyses revealed that site T1 had significantly greater concentrations of caffeine than site R2 ($P = 0.003$), significantly greater concentrations of carbamazepine than sites R1 to R4 ($P = 0.026$, 0.003, 0.033, and 0.017, respectively), and significantly greater concentrations of cotinine than all other river sites ($P \leq 0.033$).

Among outfalls, MANOVA detected a significant effect of outfall on CST marker concentrations ($F_{36,301} = 3.87$, $P < 0.001$; Fig. 4), although *post hoc* analyses revealed no significant differences among outfalls for caffeine or acetaminophen concentrations. Outfall T2 had significantly greater concentrations of carbamazepine than all other outfalls ($P \leq 0.006$). Outfall S2 had significantly greater concentrations of codeine than all other outfalls ($P \leq 0.013$) and significantly greater concentrations of acesulfame than outfalls T2, T3, and S5 ($P = 0.031$, 0.024, and 0.028, respectively).

Correlations between *E. coli*, qPCR, and CST markers. Spearman correlations between *E. coli* and qPCR marker concentrations are presented in Table 2 for river sites and Table 3 for outfall sites and wastewater samples. Among river sites, both measures of *E. coli* were significantly associated with human and gull qPCR markers, although the correlations were considerably higher with the human marker (Table 2). Among outfall sites, significant positive correlations were observed between concentrations of the human qPCR markers and both measures of *E. coli*, although no significant correlations were observed between either measure of *E. coli* and the qPCR gull marker (Table 3).

Analysis of river sites for CST markers showed significant positive correlations between culturable and Amp^r *E. coli* concentrations and caffeine ($r_s = 0.69$ and 0.72, respectively), codeine ($r_s = 0.36$ and 0.32, respectively), cotinine ($r_s = 0.67$ and 0.71, respectively), and acetaminophen ($r_s = 0.41$ and 0.53, respectively). Analysis of outfalls showed significant positive correlations between culturable and Amp^r *E. coli* concentrations and caffeine ($r_s = 0.63$ and 0.42, respectively), codeine ($r_s = 0.47$ and 0.36, respectively), and acetaminophen ($r_s = 0.61$ and 0.40, respectively). Significant positive correlations were also observed in outfall samples between concentrations of culturable *E. coli* and cotinine ($r_s = 0.40$) and acesulfame ($r_s = 0.27$).

DISCUSSION

This study found that all Humber river sites were usually in excess of the limit specified in Ontario provincial guidelines for recreational water of 100 *E. coli* CFU/100 ml (54) and had some level of human sewage contamination. The river hot spot for *E. coli* was site T1. It had the highest concentrations of *E. coli* and the human qPCR marker, which is consistent with a previous study which found high levels of an alternate HF183 marker in Black Creek (21). Our results identify the importance of reducing sewage contamination in Black Creek for reducing *E. coli* levels and the potential for health risks downstream. The source of the HF183 human marker in the upper branches of the Humber River could be two small wastewater treatment plants upstream of site R2, sewage cross-connects within storm water systems in smaller rural communities, or leaking septic systems. An MST study using the HF183 marker in Michigan in the United States recently detected widespread septic system impacts on surface water quality in rural

TABLE 2 Spearman correlation coefficients between *E. coli* concentrations, qPCR, and chemical assays for river sites[a]

Sampling site	qPCR assay	Spearman correlation coefficient							
		Culturable *E. coli*	Amp^r *E. coli*	Caffeine	Carbamazepine	Codeine	Cotinine	Acetaminophen	Acesulfame
All river samples	Human	0.75*	0.83*	0.72*	0.23*	0.51*	0.65*	0.60*	0.23*
	Gull	0.28*	0.44*	0.38*	0.08	0.13	0.38*	0.37*	0.18
R1	Human	0.62*	0.79*	0.43			0.55*	0.50	−0.16
	Gull	−0.25	0.24	−0.31			0.12		0.31
R2	Human	0.52*	0.68*	0.48	0	−0.52	0.41	0.80	0.01
	Gull								
R3	Human	0.54*	0.74*	0.57*	0.04	0.26	0.67*	0.60*	0.23
	Gull	0.50*	0.70*	0.57*	0.23	0.22	0.47	0.60*	0.18
R4	Human	0.78*	0.90*	0.78*	0.33	0.11	0.72*	0.32	−0.05
	Gull	0.35	0.08	0.57*	0.38	0.42	0.57*	0.77*	0.40
T1	Human	0.49	0.70*	0.46	−0.18	0.31	0.22	0.5	−0.14
	Gull	0.64*	0.46	0.27	−0.65*	−0.36	0.43	0.04	−0.55*
R5[b]	Human	0.43	0.59	0.14	−0.54	0.09	−0.09	0.67	0.12
	Gull	−0.45	−0.18	−0.68	−0.68	0.27	−0.03	−0.67	−0.26
R6	Human	0.77*	0.78*	0.76*	−0.31	0.57*	0.60*	0.64*	−0.01
	Gull	0.39	0.45	0.43	0.19	0.42	0.29	0.55*	0.06
R7	Human	0.70*	0.62*	0.65*	−0.39	0.64*	0.56*	0.64*	−0.27
	Gull	−0.09	0.46	−0.35	−0.24	−0.33	−0.25	−0.12	0.15

[a] Blank cells represent situations where nondetects limited the ability to obtain a correlation. *, significant correlation ($P < 0.05$).
[b] R5 had a smaller sample size than other sampling sites. Rain events and other seasonal influences were missed at site R5 that were sampled at other sites, precluding simple comparisons among other sites.

areas (55). The increases in both *E. coli* and human marker concentrations in the middle and lower Humber River are likely due to increased impacts from combined sewer overflows and storm water systems with sewage cross-connections.

River sites were also frequently impacted by gull fecal contamination, although at relatively low concentrations, which is consistent with a previous study that found widespread gull contamination along coastal and riverine systems in southern Ontario (56). Within the upper watershed, particularly at site R1, there also appeared to be high levels of ruminant contamination, as detected by endpoint PCR. While this is consistent with past research showing this area of the Humber River watershed to have a history of livestock operations (44), the qPCR CowM2 marker was never detected at this (or any other) site. It is possible that ruminant contamination was present at this site, albeit at low levels. Additionally, the concentrations of the CowM2 marker have been previously reported to vary based upon the age and diet of cows, which may have affected detection of this marker (57). However, CF128 marker detection may have been the result of false positives, as previous host specificity testing of this CF128 marker (58) revealed that it had only a 43.89% probability of correctly detecting a true positive. Detection of the CF128 marker could, therefore, have been indicative of another fecal pollution source upstream of site R1.

This study also found that all storm water outfalls had some level of human sewage contamination. Among storm water outfalls, S2 and S4 had the highest levels of *E. coli* as well as the highest frequency of the human PCR marker and the highest concentrations of the human qPCR marker. Consequently, these outfalls are likely impacted by sewage cross-connections and represent important targets for remediation. In contrast, outfalls S1 and S5 had higher levels of gull contamination than other outfalls. However, outfall S1 was sampled in the river slightly downstream of the outfall due to dangerous conditions encountered in accessing the outfall directly and results from that site therefore likely reflect the combined impacts from upstream river water quality and the outfall. Similarly, outfall S5 likely reflects the combined impacts from upstream river quality and this submerged outfall.

Aside from analysis of *E. coli* concentrations and employment of conventional PCR/qPCR techniques, this study also examined the utility of a variety of alternate markers of human sewage contamination, including the use of ampicillin-resistant *E. coli*, which has previously been shown to be present in high concentrations in treated and untreated wastewater (19). At river sites in this study, Amp^r *E. coli* was found to be more highly correlated with concentrations of the human qPCR marker than culturable *E. coli*, suggesting that Amp^r *E. coli* might be a more useful indicator of human sewage contamination in the Humber River. However, this did not extend to storm water outfalls, where a significant correlation between Amp^r *E. coli* and the human qPCR marker was observed at only one outfall site. Caution should therefore be used in using Amp^r *E. coli* as an indicator of sewage contamination, as these organisms can be ubiquitous in a given watershed.

The data obtained from the qPCR array were relatively consistent with the standard qPCR results. The human gut-associated bacteria *B. thetaiotaomicron*, *B. dorei*, *Bif. adolescentis*, *F. praus-*

TABLE 3 Spearman correlation coefficients between *E. coli* concentrations, qPCR, and chemical assays for outfall and wastewater (influent and effluent) samples[a]

Sampling site	qPCR assay	Spearman correlation coefficient							
		Culturable *E. coli*	Amp^r *E. coli*	Caffeine	Carbamazepine	Codeine	Cotinine	Acetaminophen	Acesulfame
All outfall samples	Human	0.77*	0.66*	0.66*	−0.06	0.52*	0.31*	0.61*	0.44*
	Gull	0.16	0.06	0.19	−0.04	−0.07	0.42*	0.10	−0.28*
T2	Human	0.86*	0.71*	0.90*	0.44	0.49	0.87*	0.82*	−0.31
	Gull	0.50	0.40	0.62*	−0.20	−0.04	0.52	0.64*	−0.29
T3	Human	0.40	0.31	0.35	−0.32	0.03	0.45	0.39	0.10
	Gull	0.31	0.31	0.23	0.48	0.46	0.31	−0.08	−0.46
S1	Human	0.60*	0.22	0.92*	−0.40	0.61*	0.62*	0.72*	0.45
	Gull	0.69*	0.74*	0.19	0.08	−0.20	0.06	0.14	−0.30
S2	Human	−0.04	−0.13	−0.09	−0.40	0.45	−0.15	0.37	0.23
	Gull	−0.01	0.05	−0.05	−0.26	−0.63*	0.40	−0.45	−0.56*
S3	Human	0.10	0.25	−0.18		−0.37	0.21	−0.39	−0.57
	Gull								
S4	Human	0.62	0.64	−0.07		0.37	0.20	0.15	−0.42
	Gull			0.55		0.55	0.55	0.27	0.14
S5	Human	0.61*	0.50	0.67*	−0.01	0.10	0.70*	0.41	−0.01
	Gull	0.25	0.48	0.19	−0.17	−0.27	0.41	0.11	0.04
Wastewater	Human	NS[b]	NS	0.78*	−0.22	0.76*	0.68*	0.70*	0.68*
	Gull	NS	NS	0.13	0.07	0.11	0.19	0	0.27

[a] Blank cells represent situations where nondetects limited the ability to obtain a correlation. *, significant correlation ($P < 0.05$).
[b] NS, the parameter was not sampled.

nitzii, *Eu. rectale*, and *R. bromii* appeared more abundant in outfalls than in river sites. There were higher concentrations of these markers in outfall S2 and at river site T1, consistent with our HF183 qPCR results, suggesting that these markers could be good indicators of human sewage contamination. In contrast, the skin-associated bacterium *P. acnes* showed an inverse pattern and was detected more frequently in river sites than in outfalls. As this bacterial species is skin associated, this may reflect sources such as gray water rather than sewage discharge into the river. The qPCR array markers for *Catellicoccus marimammalium* (gull) and *T. sanguinis* (possibly Canada geese) were also consistent with our gull qPCR results and were found where we might expect to find impacts from Canada geese closer to the lake. However, it should be noted that, as the qPCR arrays are not rigorously quantitative, and as only two sampling events were assessed by this method, these conclusions are only preliminary. Further study utilizing these arrays is necessary to evaluate the efficacy of these markers.

Differences were also detected among the CST markers regarding their efficacy in assessing sewage contamination sources. Comparison of wastewater influent and effluent samples revealed that CST markers had different levels of persistence. Caffeine, cotinine, and acetaminophen had significantly higher concentrations in sewage influent than in the associated effluent (Fig. 4), which suggests that these CST markers are more likely to degrade quickly and, therefore, might be more indicative of recent raw sewage contamination. Caffeine, cotinine, and acetaminophen were the CST markers most often correlated with the human qPCR marker (Tables 2 and 3), supporting the idea of their utility as predictors of raw sewage contamination. Other studies have also found that the presence of caffeine tends to be indicative of more-recent sewage contamination (59). Carbamazepine has previously been observed to be a useful indicator of sewage contamination, although perhaps from less-recent contamination events, or treated sewage, due to its greater potential to resist degradation (60, 61).

However, caution should be used when interpreting CST markers. While the most contaminated river site (based on *E. coli* concentrations, human PCR marker detection, and human qPCR marker concentrations), T1, tended to have higher concentrations of most CST markers, in particular, of cotinine, codeine, acetaminophen, and acesulfame, than most of the other river sites, this was not true of the most contaminated outfalls. Only outfalls S2 and S4 had concentrations of codeine and acetaminophen that were significantly greater than those at other outfalls. Further, none of the most sewage-contaminated sites (T1, S2, and S4) had significant correlations between any chemical marker and the human qPCR marker. The lack of significant correlations with the human qPCR marker at these sites may indicate a site-specific utility for CST markers. Storm water outfalls can exhibit unique "upstream" storm watershed conditions which may render the relationships between chemical and human qPCR markers unreliable depending upon aspects such as different human consumption patterns, the scale and mechanism of sewage input into a storm water system, and different attenuation mechanisms of markers.

MST methods were able to identify human sewage as the likely

source of fecal contamination at key Humber watershed *E. coli* hot spots, with Amp^r *E. coli* concentrations showing some potential for an incremental benefit in detecting human sewage contamination in Humber River sites. Some CST markers were also consistent in identifying human sewage contamination at key *E. coli* hot spots. The CST markers caffeine, cotinine, and acetaminophen showed promise as indicators of recent raw sewage contamination. However, these CST markers present potential challenges for source determination, as significant correlations with human qPCR markers were not observed at most sites. Consequently, the results of this study indicate that while CST markers can be helpful in identifying raw sewage contamination, the additional use of MST methodologies can provide more-reliable identification of the source(s) of fecal contamination and alleviate potential confounding factors related to use of CST methods alone.

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