Comparative microbial source tracking methods for identification of fecal contamination sources at Sunnyside Beach in the Toronto region area of concern

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ABSTRACT

Several beaches within the Toronto region area of concern have persistent issues with fecal contamination, causing a beach beneficial use impairment (BUI). In this study, *Escherichia coli*, including ampicillin-resistant strains, were enumerated via culturable and quantitative polymerase chain reaction (qPCR) methods. Microbial source tracking (MST) markers (for general *Bacteroidales*, human, ruminant/cow, gull, and dog) were detected and enumerated via PCR and qPCR to identify sources of fecal contamination at Sunnyside Beach and in the Humber River. Human, cow, and dog markers had good host-specificity, while gull markers sometimes amplified a few other bird species. The ruminant endpoint PCR marker amplified a variety of other animal species rendering it less useful. Both human and gull fecal contamination were prevalent in the Humber River, while Sunnyside Beach was predominantly impacted by gull fecal contamination. Human sewage impacts were more prevalent in the lower Humber River, particularly in Black Creek. However, to reduce Sunnyside beach postings, reducing bird fecal contamination in the river and at the beach would be necessary. When there are high levels of *E. coli* throughout a beachshed, an MST toolbox approach can add value to discriminate source(s) of *E. coli* contamination and guide decisions relating to public health risk and remediation strategies.

Key words | antibiotics, *E. coli*, environmental, microbial source tracking, qPCR, recreational water

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INTRODUCTION

As a consequence of historically poor water quality, certain regions around the Great Lakes have been designated as areas of concern (AOC). Within these AOCs, many beaches have had consistent problems with fecal contamination, and beach beneficial use impairments (BUIs) have been the subject of efforts to remediate sources of fecal contamination (City of Toronto 2009; Environment Canada & Ontario Ministry of the Environment 2011). In general, BUIs are chemical, physical or biological changes in a Great Lakes system sufficient to cause specified use impairments, including beach closures. Fecal contamination at many Great Lakes beaches is an on-going problem, resulting in beach postings and closures, loss of recreational and economic opportunities and creating potential risks to human and ecosystem health (The Brookings Institution 2007; Nevers *et al.* 2014).

Presently, regulatory standards focus on concentrations of fecal indicator bacteria (FIB), such as *Escherichia coli* (Health Canada 2012; US Environmental Protection Agency 2012). Although widely used, the existing FIB paradigm is imperfect. The ideal FIB should only be present and persist for as long as the pathogens they are used to predict; however, a number of studies have shown that FIB are capable of extended persistence and possibly growth, particularly in foreshore beach sands (Whitman *et al.* 2014). Additionally, while elevated concentrations of FIB may be indicative of fecal contamination, they provide no information regarding

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the source(s) of contamination. The lack of information regarding the source(s) of fecal contamination can be problematic, as it can hinder remediation efforts. Additionally, different sources of contamination can pose different risks to human health, with human sewage contamination generally posing the greatest risks (Craun *et al.* 2005; Soller *et al.* 2010).

One early method which has been employed to identify sewage contamination is the use of antibiotic resistant bacteria, which can be indicative of human wastewater (Parveen *et al.* 1997; Reinthaler *et al.* 2003). However, more recent studies have utilized microbial source tracking (MST) methods which use host-specific molecular markers to identify multiple sources of contamination in addition to human sewage (e.g., dog, gull, cow) (US Environmental Protection Agency 2005; Edge *et al.* 2010; Hagedorn *et al.* 2011).

In this study, the Sunnyside beachshed (Sunnyside Beach and the adjacent Humber River) in the Toronto region AOC was sampled to indicate both the occurrence and source(s) of fecal contamination throughout the watershed and along the beach. To identify source(s) of fecal contamination, MST assays were performed to identify human, ruminant/cow, gull, and dog fecal contamination. Additionally, enumeration of ampicillin-resistant (ampicillin^R) *E. coli* was tested as a method to detect potential sewage contamination. Further, analyses were conducted to identify correlations and patterns among *E. coli*, ampicillin^R *E. coli*, and MST DNA markers.

MATERIALS AND METHODS

Site description

This study was conducted throughout the Humber River (upper tributary sites designated PG (east branch), CL (main branch), AL (west branch); middle reaches SC, OM, Hum Boat; and the river mouth H0; Toronto, ON, Canada), and its major middle reach tributary Black Creek (site JA; Toronto, ON, Canada). Three transects were studied along Sunnyside Beach (sites SS1–SS3; Toronto, ON, Canada) progressing away from the river mouth (see Table 1 for a list of GPS coordinates for all sites and their relative location within the watershed and Figure 1 for a map of the sampling area). The Humber River extends 100 km, and its watershed includes both rural (55%) and urban (45%) land use (Toronto & Region Conservation 2015). Sunnyside Beach, at the mouth of the Humber River, is an urban beach protected from Lake Ontario by a breakwall.

Sample collection

Water samples were collected weekly from May–September 2014 from all river and beach sites. At each transect along Sunnyside Beach, surface water samples were collected at increasing distances from the shoreline at ankle depth (about 10 cm) and chest-depth (about 1.2 meters) within

Table 1 | List of sampling sites and GPS coordinates

Site name	Type	Location	GPS coordinates	Sampling events (*n*)
PG	River	Upper Humber (E)	43 °47′51.13″N, 79 °34′52.17″W	16
CL	River	Upper Humber (M)	43 °47′27.64″N, 79 °35′39.45″W	16
AL	River	Upper Humber (W)	43 °43′9.34″N, 79 °32′35.40″W	16
SC	River	Middle Humber	43 °40′43.10″N, 79 °30′26.24″W	16
JA	Creek	Black Creek	43 °40′32.25″N, 79 °29′48.38″W	16
Hum Boat	River	Middle Humber	43 °38′30.39″N, 79 °29′25.57″W	7
OM	River	Middle Humber	43 °39′6.80″N, 79 °29′29.61″W	16
H0	River	Humber Mouth	43 °37′55.32″N, 79 °28′15.30″W	15
SS1	Beach	Sunnyside	43 °37′57.99″N, 79 °28′9.73″W	16
SS2	Beach	Sunnyside	43 °38′8.97″N, 79 °27′45.36″W	16
SS3	Beach	Sunnyside	43 °38′13.84″N, 79 °27′27.18″W	16

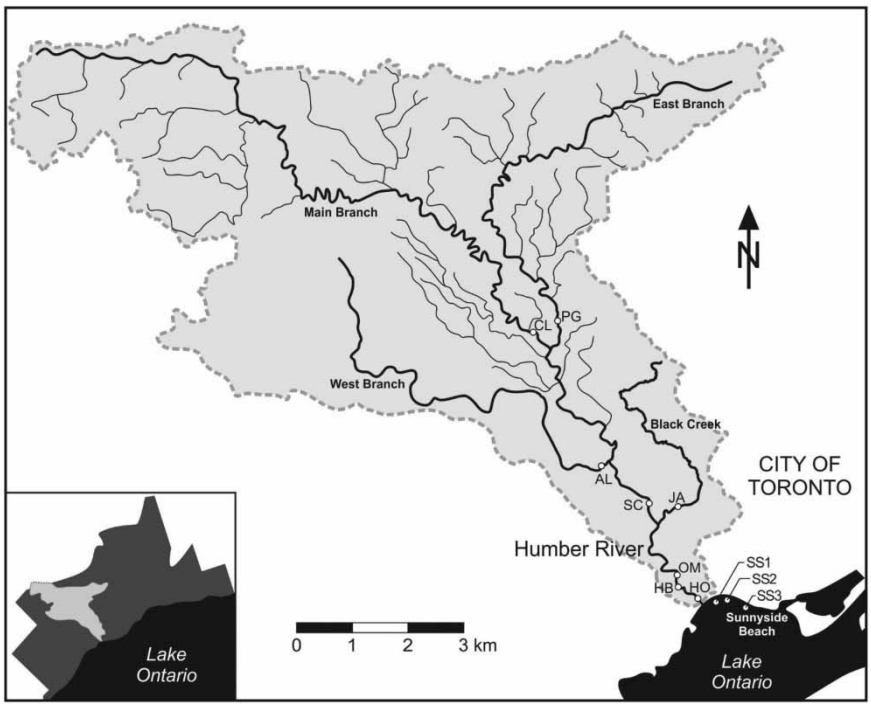

Figure 1 | Map of the Humber River watershed with marked sampling sites.

the lake, along with a sand pore water sample. To collect the sand pore water sample, a hole was dug down to the water table in the foreshore sand about 1 meter inland from the lake and a 250 ml polypropylene bottle was inserted into the hole to collect the water that accumulated (while minimizing sand collection). All other water samples were grab samples collected in 500 ml autoclaved polypropylene bottles. All water samples were placed on ice and transported to the laboratory for processing within 6 hours of collection.

E. coli enumeration

Water samples were processed for the enumeration of both *E. coli* and ampicillin resistant (ampicillin^{R}) *E. coli*. For enumeration of both, water samples were filtered (0.45 μm pore size, 47 nm diameter nitrocellulose membranes) over a range of dilutions (0.1, 1, 10, and 100 ml) according to standard membrane filtration methods (American Public Health Association 1999). *E. coli* were enumerated on differential coliform (DC) agar, supplemented with cefsulodin, and incubated at 44.5 °C for 22 hours. Ampicillin^{R} *E. coli* were enumerated on DC agar, as described above, additionally supplemented with ampicillin (32 μg ml^{-1}) and incubated at 44.5 °C for 22 hours. The concentration of 32 μg ml^{-1} was selected as it has been previously shown to represent a high level of resistance to ampicillin (Briñas *et al.* 2016) and it is the clinical breakpoint for the antibiotic. Results were reported as CFU 100 ml^{-1}. Laboratory filtration blanks were included for every sampling day and no inadvertent contamination was noted. *E. coli* were also quantified via quantitative polymerase chain reaction (qPCR) (targeting the *uidA* gene) using a previously published primer set (Lee *et al.* 2005) and qPCR reaction and thermocycler conditions listed below.

DNA extraction and MST PCR

In addition to filtration for *E. coli* enumeration, an additional 300 ml (100 ml for pore water samples) was filtered (0.45 μm pore size, 47 mm diameter nitrocellulose membranes) for DNA extraction. Filters were frozen at −80 °C until analysis. Filters were then allowed to thaw, folded and placed into Powerbead tubes and extracted using Powersoil™ DNA Isolation Kits (MO BIO Laboratories, Inc., Carlsbad, CA,

USA) according to manufacturer's instructions. Extraction and filtration blanks were included in every batch of DNA extractions and no inadvertent contamination was noted.

PCR and qPCR assays were performed on all extracted DNA samples (including filtration and extraction blanks). MST assays for both PCR and qPCR consisted of a general *Bacteroidales* assay, as well as host-specific assays for human, ruminant (PCR) or cow (qPCR), gull, and dog (Table 2). Primer and probe sets for all assays have been previously published (Bernhard & Field 2000; Lu *et al.* 2008; Shanks *et al.* 2008; Shanks *et al.* 2011; Ryu *et al.* 2012; Green *et al.* 2014a, 2014b).

Endpoint MST PCR reactions

Each PCR reaction consisted of 2.5 μl 10x IDTE Buffer (Integrated DNA Technologies, Coralville, IA, USA), 0.2 μl 100 mM dNTP mixture, 0.16 μl 10% BSA, 0.5 μl each of forward and reverse primers, 0.25 μl Hotmaster Taq DNA polyermase (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 the respective host-specific fecal source. Cycler conditions were consistent with previously published assays (Bernhard & Field 2000; Lu *et al.* 2008; Green *et al.* 2014b).

Table 2 | List of PCR and qPCR assays including name, source-specific target and reference

Name	Target	Reference
Endpoint PCR		
Bac32	General *Bacteroidales*	Bernhard & Field (2000)
HF183	Human	Bernhard & Field (2000)
CF128	Ruminant	Bernhard & Field (2000)
Gull2	Gull	Lu *et al.* (2008)
DG37	Dog	Green *et al.* (2014b)
qPCR		
GenBac	General *Bacteroidales*	Shanks *et al.* (2011)
HF183	Human	Green *et al.* (2014a)
CowM2	Ruminant	Shanks *et al.* (2008)
qGull4	Gull	Ryu *et al.* (2012)
DG37	Dog	Green *et al.* (2014b)

MST and *E. coli* qPCR reactions

Each qPCR reaction consisted of 2 μl of an internal amplification control (IAC), 2.5 μl 2 mg ml^{-1} 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, and 2 μl of extracted DNA. Reactions were carried out in 96-well plates using a Bio-Rad CFX96 cycler (Hercules, CA, USA). All reactions were carried out in duplicate, including no-template controls, negative controls consisting of 2 μl salmon testes DNA, and positive controls consisting of 2 μl of DNA extracted from a known fecal source. 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) 100 ml^{-1}.

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 per 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 for each assay and included in every reaction to verify that there was no inhibition. The limit of detection for all MST assays was ~10 CN $reaction^{-1}$ and ~200 CN $reaction^{-1}$ for the *E. coli* qPCR. All qPCR runs had an efficiency between 90 and 110% with an R^2 of > 0.95 and results were normalized to reaction efficiency.

PCR/qPCR fecal validation

To assess the sensitivity and specificity of the PCR and qPCR methods, each assay was run against a number of fecal and sewage samples from known sources from the Toronto region and eastern Canada (Table 3). Sewage samples

Table 3 | Percent detection of endpoint PCR and qPCR MST markers in fecal samples and sewage influent and effluent

		Endpoint PCR (%)					qPCR (%)				
Source	**Sample size (*n*)**	**Bac32**	**HF183**	**CF128**	**Gull2**	**DG37**	**GenBac**	**HF183**	**CowM2**	**qGull4**	**DG37**
Canada Goose	10	80	0	0	30	0	100	0	0	20	0
Cat	13	85	0	15	0	15	100	0	0	0	0
Chicken	8	100	0	63	0	0	100	0	0	0	0
Cormorant	17	18	6	0	12	0	59	0	0	18	0
Cow	5	100	0	100	0	0	100	0	100	0	0
Dog	15	93	0	7	0	67	100	0	0	0	67
Goose	1	100	0	0	100	0	100	0	0	100	0
Gull	14	57	0	0	86	0	50	0	0	93	0
Horse	12	100	0	67	0	0	100	0	0	0	0
Rabbit	3	67	33	0	0	0	100	67	0	0	0
Raccoon	6	50	0	0	0	0	67	0	0	17	0
Rat	1	100	0	0	0	0	100	0	0	0	0
Seal	15	87	0	0	0	0	93	0	0	0	0
Sheep	1	100	0	100	0	0	100	0	0	0	0
Tern	13	38	0	0	46	0	69	0	0	54	0
Influent	20	100	100	60	20	75	100	100	0	5	65
Effluent	20	100	95	0	20	20	100	100	0	10	0

Percent detection is based on an average of the sample size.

(influent and effluent from Toronto's Ashbridge Bay Wastewater Treatment Plant) were filtered and DNA extractions were performed as described above. For raw fecal samples, ~0.3 g was weighed and placed in a MoBio Powerbead tube and extracted as described above. All endpoint PCR and qPCR MST assays were performed on the extracted DNA from all samples as described above.

Statistical analysis

Comparison between the Humber River and Sunnyside Beach

All measures of culturable *E. coli* (culturable and ampicillin^R; CFU 100 ml^{-1}) and qPCR quantification of *E. coli* and MST markers (CN 100 ml^{-1}) were log transformed prior to analysis. T-tests were used to assess differences in *E. coli* and qPCR-enumerated MST marker concentrations between river and beach sites. Bayes Theorem was applied to assess the probability of correctly detecting host-specific endpoint or qPCR markers (Kildare *et al.* 2007). Pearson correlation coefficients were used to assess relationships among qPCR markers and measures of *E. coli*.

Analysis of site-specific differences among Humber River or Sunnyside Beach sites

To determine the main effect of sampling site among river sites or among beach transects (transects 1–3) and sub-locations (ankle-, chest-depth, and pore water samples), multiple analysis of variance (MANOVA) was used, where response variables were *E. coli* concentrations (culturable, ampicillin^R and qPCR-enumerated). The main effect of sampling site was similarly assessed using qPCR-enumerated MST 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. All analyses were performed in Statistica v.12.

RESULTS

E. coli enumeration

T-tests revealed that there were no significant differences in culturable *E. coli* and ampicillin^R *E. coli* concentrations between river and beach sites. MANOVA detected significant differences in *E. coli* and ampicillin^R *E. coli* concentrations among river sites ($F_{28,301} = 2.70$, $P < 0.001$; Figure 2). Tukey's *post hoc* test determined that site JA had significantly higher *E. coli* concentrations (enumerated via culture and qPCR) than site CL ($P = 0.012$ and 0.007, respectively; Figure 2(a) and 2(b)) and significantly higher ampicillin^R *E. coli* concentrations than at all other sites except Hum Boat ($P \leq 0.024$ for all sites; Figure 2(c)).

Among beach sites, MANOVA revealed a significant effect of transect on *E. coli* concentrations ($F_{8,218} = 2.95$, $P = 0.004$). *Post hoc* tests determined that transect 2 had significantly higher concentrations of qPCR-enumerated and ampicillin^R *E. coli* than transect 3 ($P = 0.009$ and 0.016; Figure 2(b) and 2(c)). A significant difference was observed depending on the shore-relative sub-location (foreshore pore, ankle-, or chest-depth; $F_{6,270} = 16.4$, $P < 0.001$). Tukey's *post hoc* test showed that culturable *E. coli* and ampicillin^R *E. coli* concentrations, as well as *E. coli* concentrations enumerated via qPCR, were significantly higher in foreshore pore water than ankle- or chest-depth water samples ($P < 0.001$ for all analyses; Figure 2). Additionally, for all measures of *E. coli* (culturable, qPCR and ampicillin^R), significantly higher concentrations were detected in ankle- than chest-depth water samples ($P < 0.001$, $= 0.009$ and $= 0.030$, respectively; Figure 2).

Validation of MST PCR and qPCR markers

Fecal validation of the MST PCR assays revealed that none of the host-specific markers exhibited 100% specificity, though human, cow and dog markers showed good specificity. The least specific marker was the ruminant marker (CF128), which was detected occasionally in cat and dog fecal samples, as well as in the majority of horse and chicken fecal samples. The qPCR MST assays tended to be more host-specific, with the cow and dog markers (CowM2 and DG37, respectively), being detected exclusively in target fecal samples, although in the case of the dog marker, also in sewage influent samples. Whenever cross-reactivity was detected by qPCR assays, the level of cross-reactivity in non-target host fecal samples was always orders of magnitude lower than in target host species fecal samples.

Bayesian analysis revealed differences in the probability that each endpoint and qPCR marker was detecting a true positive. Among the endpoint markers, the probability of correctly detecting a true positive was calculated to be 95.48% for HF183, 43.89% for CF128, 96.65% for Gull2, and 85.11% for DG37. Among the qPCR markers (when assessed based on presence/absence of marker detection), the probability of correctly detecting a true positive was 98.68% for HF183 and 91.19% for qGull4. Calculations were not conducted on the CowM2 or DG37 qPCR markers as these were never detected in water samples.

MST

A chi-squared test revealed significant differences in detection of endpoint markers between river and beach sites. The human (χ^2=52.3, $P < 0.001$), ruminant (χ^2=33.1, $P < 0.001$) and dog (χ^2=11.5, $P = 0.001$) endpoint markers were detected significantly more often in river than beach sites (Table 4). Conversely, the gull endpoint marker was detected significantly more frequently in beach than river sites (χ^2=19.6, $P < 0.001$; Table 4). MANOVA also showed a significant difference in qPCR marker concentrations between river and beach sites ($F_{3,57} = 27.4$, $P < 0.001$; Figure 3). Univariate analysis determined that there were significantly greater concentrations of human-specific *Bacteroidales* at river sites ($F_{1,59} = 52.2$, $P < 0.001$; Figure 3), whereas concentrations of the qPCR gull marker were significantly greater at beach sites ($F_{1,59} = 7.75$, $P = 0.007$; Figure 3). The cow and dog qPCR markers were not detected in any water samples.

Among river sites, chi-squared tests found significant differences in detection of the human, ruminant and gull markers. The human marker was detected significantly more often at site JA than other river sites (χ^2=25.5, $P = 0.001$; Table 4), and the ruminant marker was detected significantly more often at site PG than other river sites (χ^2=20.0, $P = 0.006$; Table 4). MANOVA also revealed significant differences in qPCR marker concentrations among

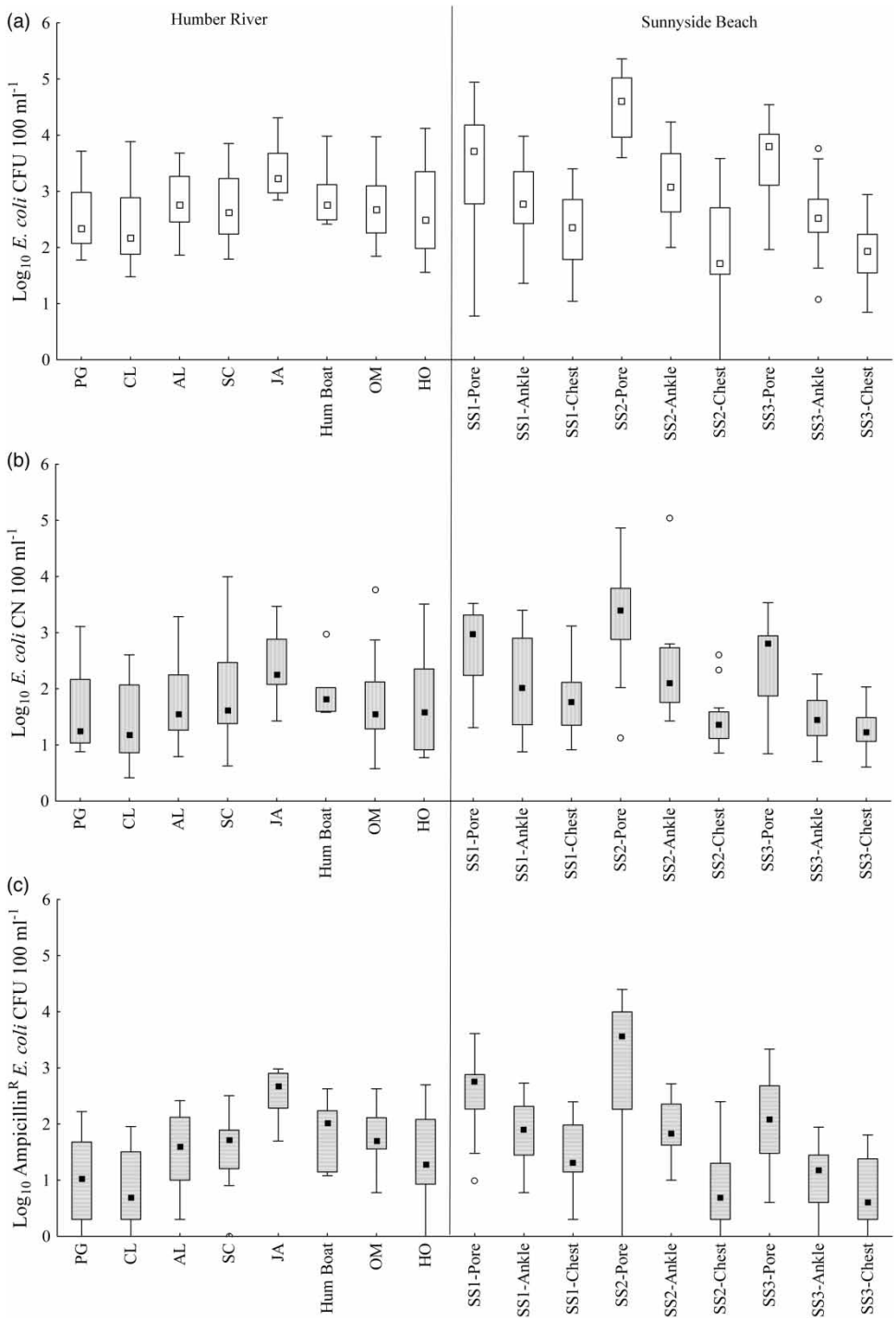

Figure 2 | Box plots of (a) culturable *E. coli*, (b) qPCR-enumerated *E. coli* and (c) ampicillin^R *E. coli* at each sampling site. Box plots show the median *E. coli* concentration between the 25th and 75th data quartiles; whiskers extend to the outermost data point within ±1.5 this interquartile range. Open circles depict outlier values.

river sites ($F_{21,311} = 3.62$, $P < 0.001$; Figure 3). *Post hoc* analyses determined that site JA had significantly greater concentrations of human-specific *Bacteroidales* than all river sites except Hum Boat and OM ($P \leq 0.009$; Figure 3). No significant differences were found among river sites for concentrations of the qPCR gull marker.

Table 4 | Percent detection for each PCR and qPCR markers at each sampling site

			Endpoint marker (%)				qPCR marker (%)			
Site name	**Type**	**Sampling events (*n*)**	**General *Bacteroidales***	**Human**	**Ruminant**	**Gull**	**Dog**	**General *Bacteroidales***	**Human**	**Gull**
PG	River	16	100	31	63	38	13	100	100	14
CL	River	16	100	19	6	38	13	100	71	14
AL	River	16	100	13	6	44	25	100	63	31
SC	River	16	100	31	25	81	19	100	100	38
JA	River	16	100	88	31	81	13	100	80	53
Hum Boat*	River	7*	100	29	29	71	14	100	56	0
OM	River	16	100	44	25	81	38	100	86	43
H0	River	15	93	47	13	87	27	100	93	7
Average	River	–	99	38	25	65	20	100	81	25
SS1-P	Beach	16	88	0	0	56	0	100	13	31
SS1-A	Beach	16	88	25	6	100	6	100	56	88
SS1-C	Beach	16	81	13	6	94	13	100	56	63
SS2-P	Beach	16	81	6	0	88	13	100	6	75
SS2-A	Beach	16	81	0	0	100	6	100	50	100
SS2-C	Beach	16	88	6	0	88	6	100	25	75
SS3-P	Beach	16	81	0	0	75	13	100	6	31
SS3-A	Beach	16	63	6	0	100	0	100	31	94
SS3-C	Beach	16	81	6	0	88	0	100	25	69
Average	Beach	–	81	7	1	88	6	100	30	69

*Hum Boat had a smaller sample size than other sampling sites. Rain events and other seasonal influences were missed at this site that were sampled at other sites, precluding simple comparisons among sites and river-beach averages.

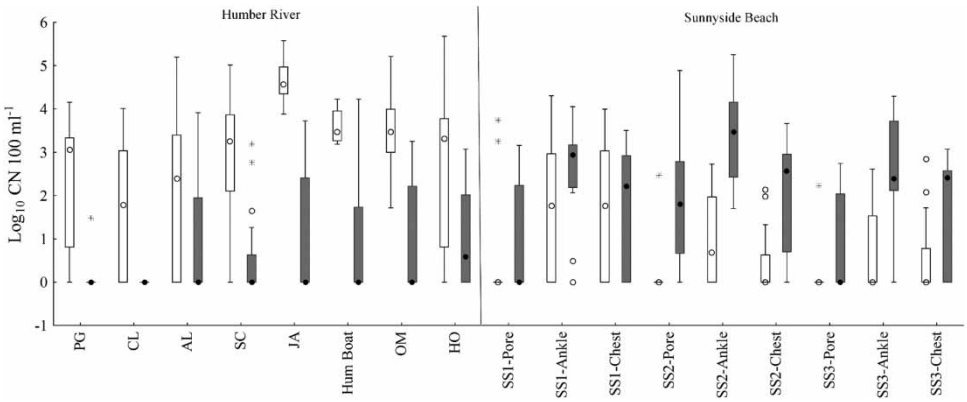

Figure 3 | Box plots of human and gull qPCR marker concentrations for each site. Light bars represent human qPCR marker concentrations, whereas dark bars represent gull qPCR marker concentrations. Box plots show the median qPCR marker concentration between the 25th and 75th data quartiles; whiskers extend to the outermost data point within ± 1.5 this interquartile range. Open circles depict outlier values and asterisks depict extreme values.

Among beach sites, no significant differences in endpoint marker detection were observed among the different transects; however, MANOVA revealed significant differences in qPCR marker concentrations (Table 2 and Figure 3). *Post hoc* analysis determined that transect 1 had significantly greater concentrations of human-specific

Bacteroidales than transects 2 or 3 ($P = 0.009$ and 0.002, respectively; Figure 3), while transect 2 had significantly greater concentrations of the qPCR gull marker than transects 1 or 3 ($P = 0.016$ and 0.027, respectively; Figure 3). This was consistent with informal weekly field observations of more gulls and other birds and their fecal droppings around transect 2.

When assessed by depth (i.e., pore, ankle-, or chest-depth water), chi-squared tests showed a significant difference in detection of the endpoint gull marker, with ankle-depth samples having significantly greater detection than chest-depth or pore water samples and chest-depth samples having significantly greater detection than pore water samples. MANOVA also revealed significant differences in qPCR marker concentrations as result of sampling depth ($F_{6,278} = 10.6$, $P < 0.001$; Figure 3). *Post hoc* analysis determined that pore water samples had significantly greater concentrations of general *Bacteroidales* than chest-depth water samples ($P = 0.001$), although significantly lower concentrations of the human-specific *Bacteroidales* marker than either ankle- or chest-depth samples ($P = 0.001$ and 0.027, respectively). Similar to endpoint results, concentrations of the qPCR gull marker were significantly greater in ankle-depth water samples than chest-depth or pore water samples ($P < 0.001$ for both) and significantly greater in chest-depth than pore water samples ($P = 0.048$).

Relationship between *E. coli* and MST markers

Among river sites, all measures of *E. coli* had significant correlations with concentrations of both the human and gull qPCR markers (Table 5), with correlations being strongest with concentrations of the human rather than the gull qPCR marker. Further, ampicillin^R *E. coli* concentrations had the strongest correlation with concentrations of the human qPCR marker (Table 5). Among all beach samples (including pore water samples) no significant correlations were observed between any measure of *E. coli* and either human or gull qPCR marker concentrations. However, when pore samples were excluded from the analysis, ampicillin^R and qPCR-enumerated *E. coli* concentrations were significantly correlated with concentrations of the human qPCR marker and all measures of *E. coli* were correlated with concentrations of the gull qPCR marker (Table 5). The strongest beach water correlations were observed between ampicillin^R *E. coli* and concentrations of the human qPCR marker and between concentrations of culturable *E. coli* and the gull qPCR marker (Table 5).

Table 5 | Pearson's *r*–values for correlations between qPCR MST assays and *E. coli* by enumeration method and water body type

		***E. coli* enumeration method**		
Samples	**qPCR target**	**Culturable**	**Ampicillin^R**	**qPCR**
River	Human	0.67*	0.76*	0.72*
	Gull	0.40*	0.44*	0.45*
Beach with pore	Human	−0.08	0.01	0.00
	Gull	0.02	−0.11	0.08
Beach without pore	Human	0.17	0.28*	0.26*
	Gull	0.41*	0.27*	0.45*

*Denotes a significant correlation ($P < 0.05$).

DISCUSSION

This study found that, while culturable *E. coli* and ampicillin^R *E. coli* concentrations were similar, the source(s) of fecal contamination were notably different between the Humber River and Sunnyside Beach. Detection of all endpoint PCR markers (except gull) was more frequent, and there were significantly greater concentrations of general and human *Bacteroidales* qPCR markers in river than beach sites, while the opposite was found with regard to the gull markers. These MST results are consistent with the results of a preliminary study where a different human *Bacteroidales* marker was detected significantly more often at several sites in the lower Humber River, whereas library-dependent antibiotic resistance and REP-PCR DNA fingerprinting of *E. coli* isolates identified greater levels of bird fecal contamination on Sunnyside Beach (Edge *et al.* 2010).

Within the Humber River, the highest *E. coli* concentrations (by all measures) and the greatest concentrations of the human-specific marker occurred at site JA in the Black Creek tributary. Black Creek is a receiving stream for 300 storm drains and combined sewer outfalls (Toronto & Region Conservation 2015) and preliminary research in this watershed identified frequent detection of a human-specific marker (Edge *et al.* 2010). It was notable that our

data show that all sites in the Humber River, from the upper watershed rural sites to the urban site at the mouth, were contaminated by human sewage at times. This widespread contamination could stem from two small sewage treatment plants (Kleinberg and Nobleton) discharging upstream of site PG, sewage cross-connects within stormwater systems throughout the watershed, or from failing septic systems in rural communities (Verhougstraete *et al.* 2015). While Black Creek had the highest concentrations of the qPCR human marker within the Humber River watershed, this human marker was detected at site PG on 100% of sampling days, indicating a consistent source of human fecal contamination, albeit at low levels, in the upper watershed.

Detection of the CF128 ruminant marker throughout the watershed is difficult to interpret based upon its relatively poor host specificity. While significantly greater detection of the ruminant endpoint marker at site PG is consistent with a history of fecal contamination from livestock and milkhouse wash water (Mar 1991), the detection of this marker downstream at sites associated with more urban surrounding land use (like site JA) is difficult to understand. Host specificity testing detected the CF128 ruminant marker in the majority of wastewater influent samples, and fecal samples from horses and chickens resulting in poor fecal source discriminatory power. Further, the cow-specific qPCR marker was never detected at site PG or elsewhere, which suggests that if there was cattle fecal contamination at site PG, it was only at low levels. Detection of the CF128 marker at other sites throughout the Humber River watershed could be indicative of fecal contamination from cattle, horse feces, poultry litter, or another unrecognized fecal pollution source in the upper watershed. Common detection of CF128 in our sewage influent validation samples, could also suggest its presence represents untreated sewage contamination sources in the upper (i.e. above site PG) and lower (above site JA) Humber River watershed. Additionally, CF128 could also be indicative of sewage contamination in stormwater runoff or sources like horse fecal contamination from horse-mounted police patrols common in some urban areas within the watershed.

Sunnyside Beach transect 2 was characterized by observations of the greatest number of water fowl (similar to transect B in Edge *et al.* 2010), significantly greater concentrations of all *E. coli* measures in beach sand, as well as significantly greater concentrations of the qPCR gull marker than other transects. The greater concentrations of ampicillin^{R} *E. coli* at this transect could be the result of gull fecal contamination, as gulls have been found to carry a number of antibiotic resistant bacteria (Simões *et al.* 2010). Transect 1, nearest the mouth of the Humber River, had significantly greater concentrations of the human specific qPCR marker than other transects, likely due to impacts from the plume of the Humber River, as suggested in a previous study (Edge *et al.* 2010).

The shore-normal location of sampling (pore water, ankle- or chest-depth) was also found to significantly influence *E. coli* concentrations and MST marker detection. The greatest *E. coli* concentrations (by all measures) were found in the interstitial pore water, with concentrations decreasing with increasing lake depth, similar to results reported at other beaches (Whitman & Nevers 2003; Edge & Hill 2007). However, MST markers tended to show a different profile, with the greatest concentrations present in ankle-, then chest-depth water, and with the lowest concentrations in the interstitial pore water. Greater detection of gull markers in ankle-depth compared to pore water samples could be due to the higher prevalence of bird fecal deposition on the wave-swept foreshore sand and in the shallow water at the sand–water interface. This contamination would likely be diluted farther out towards chest-depth waters. It is also possible that, unlike *E. coli*, the gull marker does not persist well in the pore water of foreshore sands. Pore water samples had significantly lower concentrations of the human *Bacteroidales* markers than ankle- and chest-depth samples, similar to results reported at another fine sand beach (Staley *et al.* 2015). Further study is needed to understand the apparently different relationship between *E. coli* and the MST DNA markers in sand compared to beach waters.

The efficacy of using ampicillin^{R} *E. coli* concentrations as a determinant of human sewage contamination varied between river and beach sites. Within the Humber River, all measures of *E. coli* were significantly correlated with concentrations of both the human and gull qPCR MST markers, suggesting that *E. coli* in the Humber River are likely from both human sewage and gull fecal contamination. However, correlations were considerably greater with concentrations of the human MST marker, suggesting human sewage as the

more prevalent source of *E. coli* contamination within the river. Ampicillin^R *E. coli* concentrations, in particular, had the highest correlation with the human marker ($r = 0.76$), suggesting that spikes in ampicillin^R *E. coli* might be a good predictor of sewage contamination in river sites.

While all *E. coli* measures were highly associated with the human MST marker in the Humber River, culturable and qPCR-enumerated *E. coli* concentrations were more significantly correlated with the gull MST marker at Sunnyside Beach. This is consistent with *E. coli* at the beach more likely coming from gull fecal contamination. While ampicillin^R *E. coli* concentration was significantly correlated with the human qPCR marker at the beach, it is not likely as useful an indicator of human sewage contamination at Sunnyside Beach as it is in the Humber River. It should be noted that this relationship was only evident when pore samples were excluded from analyses. Pore water samples represent a much different matrix than ankle- and chest-depth water samples, and the relationship between *E. coli* and MST DNA markers may be less strong in sand than beach water, as previously discussed.

CONCLUSIONS

The remediation of beach BUIs is a persistent challenge in many AOCs around the Great Lakes. However, determining beach remediation strategies based only on *E. coli* data might lead to inappropriate actions to reduce beach postings. Although the Humber River in the Toronto region AOC has been recognized to be impacted by CSO discharges and sewage contamination in the lower reaches (City of Toronto 2009; Edge *et al.* 2010), this study detected human sewage contamination at all sampling sites within the watershed. However, the highest levels of the human qPCR marker were found in Black Creek (site JA) which represents a legacy sewage contamination problem, and a priority for remediation.

Importantly, this study also found gull fecal contamination to be very common in the lower reaches of the Humber River. While gull fecal contamination was the most prominent source of fecal contamination impacting Sunnyside Beach, this study identified a more complicated picture of gull fecal contamination likely originating from gull droppings on the beach, as well as from the plume of the Humber River containing urban runoff with gull fecal contamination. In addition to reducing sewage contamination sources in the Humber River watershed, remediation efforts to reduce Sunnyside Beach postings will need to address continuous loadings of fecal contamination from gulls at the beach, as well as in the Humber River stemming from sources like urban runoff. These results highlight the need for a toolbox approach to water quality assessment, including both *E. coli* enumeration and MST techniques, as the major source(s) of fecal contamination and associated remediation strategies can change throughout a beachshed. Multiple MST methodologies can add significant value when interpreting *E. coli* data to more comprehensively assess fecal contamination source(s) and risks to public health, as well as guiding cost-effective remediation strategies.

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