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Detection of the human specific *Bacteroides* genetic marker provides evidence of widespread sewage contamination of stormwater in the urban environment

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ABSTRACT

Human sewage contamination of surface waters is a major human health concern. We found urban stormwater systems that collect and convey runoff from impervious surfaces act as a conduit for sewage originating from breeches in sanitary sewer infrastructure. A total of 828 samples at 45 stormwater outfalls were collected over a four-year period and assessed by culture based methods, PCR, and quantitative PCR (qPCR) to test for traditional and alternative indicators of fecal pollution. All outfalls had the HF183 (human) *Bacteroides* genetic marker detected in at least one sample, suggesting sewage contamination is nearly ubiquitous in the urban environment. However, most outfalls were intermittently positive, ranging from detection in 11%–100% of the samples. Positive results did not correlate with seasonality, rainfall amounts, or days since previous rainfall. Approximately two-thirds of the outfalls had high (>5000 copy number, i.e. CN, per 100 ml) or moderate levels (1000–5000 CN per 100 ml) of the human *Bacteroides* genetic marker. *Escherichia coli* (*E. coli*) and enterococci levels did not correlate to human *Bacteroides*. A total of 66% of all outfall samples had standard fecal indicator levels above 10,000 CFU per 100 ml. A tiered assessment using this benchmark to identify high priority sites would have failed to flag 35% of the samples that had evidence of sewage contamination. In addition, high fecal indicators would have flagged 33% of samples as priority that had low or no evidence of sewage. Enteric virus levels in one outfall with high levels of the human *Bacteroides* genetic marker were similar to untreated wastewater, which illustrates stormwater can serve as a pathway for pathogen contamination. The major source of fecal pollution at four of five river sites that receive stormwater discharge appeared to be from sewage sources rather than non-human sources based on the ratios of human *Bacteroides* to total *Bacteroides* spp. This study shows the feasibility and benefits of employing molecular methods to test for alternative indicators of fecal pollution to identify sewage sources and potential health risks and for prioritization of remediation efforts.

1. Introduction

Urban stormwater routinely contains high levels of fecal indicator bacteria and is a major contributor to degraded water quality at urban beaches, lakes and rivers (Marsalek and Rochfort, 2004; Noble et al., 2006; USEPA, 2009). The fecal indicator bacteria found in non-point source runoff, e.g. stormwater, are assumed to be derived from animal sources including domestic pets and wildlife. However, there is growing evidence that stormwater systems can be contaminated with

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sewage due to failing infrastructure and illicit cross connections between the stormwater and sewage systems (O'Shea and Field, 1992; Haile et al., 1999; Gaffield et al., 2003; Noble et al., 2003; Salmore et al., 2006; Rajal et al., 2007). High levels of human enteric viruses have also been detected in stormwater runoff from outfalls further confirming the presence of human sources of fecal pollution in stormwater (Haile et al., 1999; Noble et al., 2006; Rajal et al., 2007; Sercu et al., 2009). Because stormwater systems are designed to release collected runoff untreated to surface waters, any sewage contamination poses a risk to human health, especially when the outfalls are located on rivers or near beaches used for recreational purposes. The extent to which the urban environment is impacted by sewage contamination delivered through stormwater systems has not been widely explored (Sercu et al., 2009; Parker et al., 2010).

Traditionally, culture methods for *Escherichia coli* (*E. coli*) and enterococci have been used for water quality monitoring due to low cost and ease of use. However, these standard indicators are found in both animal and human sources and vary greatly in their potential to carry human pathogens, consequently measuring their levels contributes little to our knowledge of the source of contamination (Boehm et al., 2009). Alternative indicators of fecal pollution that can be detected by PCR and quantitative PCR (qPCR) have proven to be promising for identifying human specific fecal pollution (Ahmed et al., 2007; Stewart et al., 2008; Converse et al., 2009; Sercu et al., 2009; Parker et al., 2010). Testing for traditional fecal indicators augmented with more sensitive and specific approaches would improve our ability to identify and prioritize sources that have a high likelihood of contributing pathogens to surface waters. Such approaches may be useful to investigate unrecognized sewage sources in surface waters, which may be the result of breeches in the sanitary sewer infrastructure.

In many cities around the Great Lakes, urban stormwater runoff drains into tributaries leading to nearshore waters that are both a drinking water source and the location of heavily used public beaches. The metropolitan area of Milwaukee in Wisconsin is a typical Great Lakes urban center with three urban rivers that converge and discharge to Lake Michigan in close proximity to several popular beaches. Fecal indicator bacteria such as *E. coli* have been detected as high as 20,000 colony forming units (CFUs) per 100 ml in metropolitan Milwaukee rivers during wet weather events and have also exceeded water quality standards during dry weather events (Salmore et al., 2006; McLellan et al., 2007). Stormwater in Milwaukee has been identified as a large contributor of fecal pollution in this system and therefore plays a significant role in the number of exceedances of recreational standards and the degradation of water quality (McLellan et al., 2007; SEWRPC, 2008).

In this study, we assessed the extent of human fecal contamination in stormwater outfalls in a dense urban area in metropolitan Milwaukee during wet weather flows. We examined samples collected over a four-year period from 45 stormwater outfalls, with roughly half of these fitted with inline samplers that capture both the initial water discharge during the first 60 min (i.e. the first flush) and the subsequent discharge during the remainder of the storm event (i.e. second flush). Traditional culture methods, as well as PCR and qPCR for standard and alternative indicators were used to assess the human fecal contamination at the outfalls. We demonstrated that the human *Bacteroides* genetic marker was routinely detected across the study area, a strong indication that sewage contamination is a chronic source of fecal pollution in urban stormwater. This study illustrates that molecular approaches designed to test for alternative indicators of fecal pollution can be used to improve and prioritize remediation projects and provide a higher level of information toward decision making processes aimed at protecting human health.

2. Materials and methods

2.1. Study site and sampling methods

The study area included four watersheds within metropolitan Milwaukee with the most intensive sampling carried out in the highly urbanized Kinnickinnic and Menomonee River watersheds (Fig. 1). The Kinnickinnic River watershed encompasses 25 square miles with 53 outfalls along 31 river miles. The Menomonee River watershed and its tributaries encompass 136 square miles with 101 outfalls discharging directly to the river over a 144 mile stretch. This study focused on Milwaukee's separated sewer area, where stormwater outfall discharges should be urban runoff as opposed to Milwaukee's combined sewer area where the stormwater and sanitary sewer systems are combined.

A total of 45 stormwater outfalls were sampled over a four-year period in 2006–2009. Outfalls were sampled a minimum of four times during the sampling seasons (April through November). Samples were collected from 23 outfalls using automated inline samplers installed at the last manhole access point before each outfall to ensure the first flush was captured. High flow (rise in pipe flow $>$0.51 cm) triggered the samplers to collect. Following the first 60 min of sample collection, the stormwater is diverted to a new sample bottle to collect flow from the remainder of the storm (the second flush). Grab samples were also collected from an additional 22 outfalls to increase outfall sampling coverage. Outfall sample locations are designated with the abbreviation for the watershed or subwatershed followed by a numerical assignment. Inline samples are given an "S" prefix. Watershed and subwatershed abbreviations are as follows: Menomonee River (MN), Honey Creek (HC), Underwood Creek (UC), Lincoln Creek (LC), and Lake Michigan (LM).

Quantification of the human *Bacteroides* genetic marker was conducted on a subset of inline and grab samples from 16 of the stormwater outfalls discharging to the Menomonee River watershed including two subwatersheds, Honey Creek and Underwood Creek. Two additional outfalls outside of this watershed with known sewage contamination were sampled for comparison. These outfalls are designated as SLC07 and SLM09 and discharge to Lincoln Creek and directly to Lake Michigan, respectively. Inline samplers were deployed at these two locations.

A total of ten wastewater treatment plant influent samples were analyzed for comparison with stormwater. Samples consisted of 24-h flow weighted samples collected at the two major wastewater treatment plants servicing metropolitan Milwaukee.

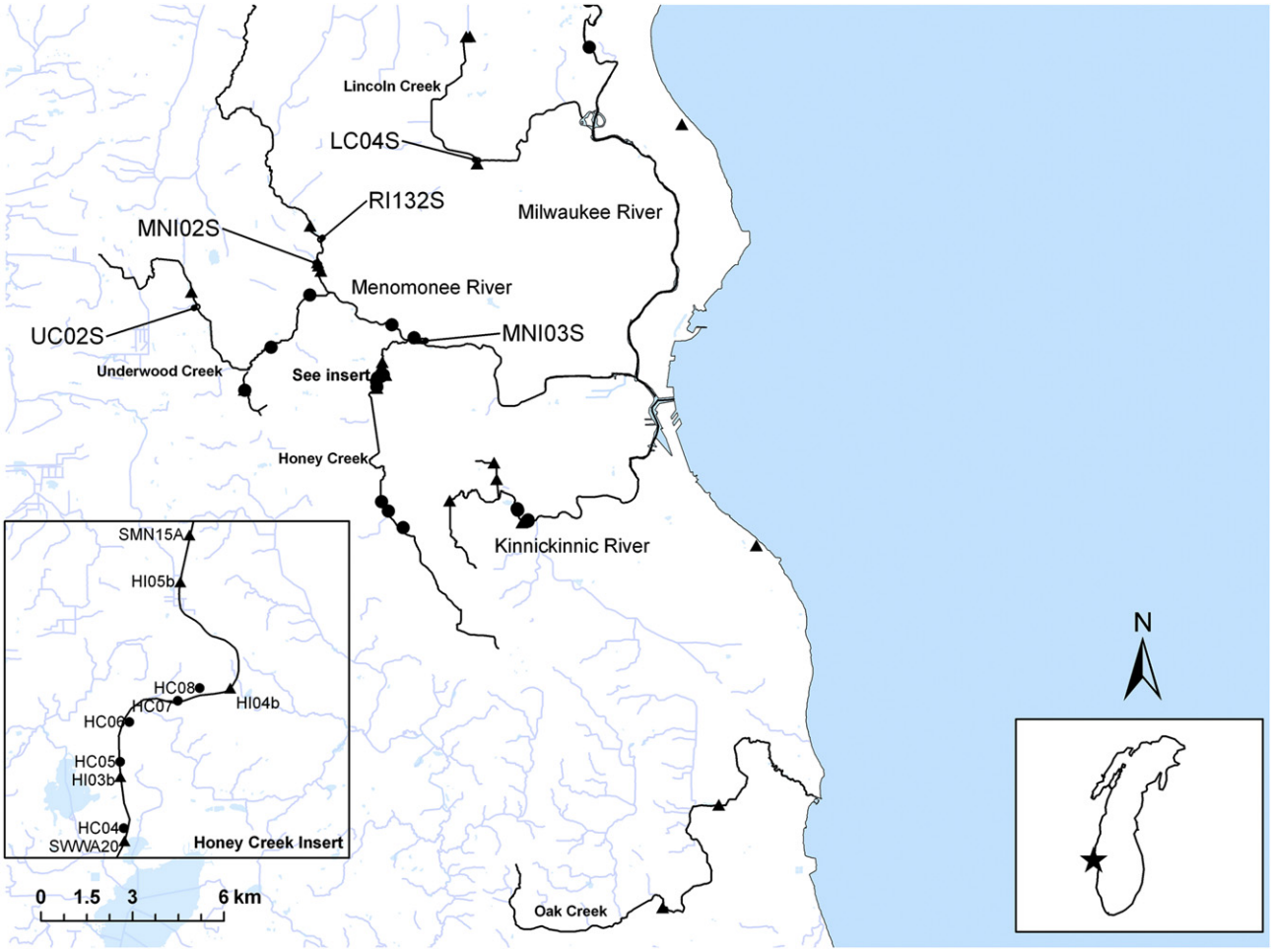

Fig. 1 – Sampling locations in the Milwaukee metropolitan area. Inline (triangle) and grab (circle) samples taken from outfalls and grab samples (arrow) taken from river locations.

Systematic sampling within the stormwater conveyance system was also conducted, with samples taken "up the pipe", e.g. at upstream manhole access points. The terminal outfall locations (last manhole access point) were designated with an "A", with upstream location or branch points assigned letters alphabetically from downstream to upstream (e.g. "B" to "E", depending on the number of points sampled).

River water sampling (water quality monitoring) was conducted to assess the level of the human *Bacteroides* genetic marker following rain events when there was no sewage overflow. River water was collected in 2008 and 2009 at sites adjacent to stormwater outfalls at three locations on the Menomonee River, one location on Underwood Creek and one location on Lincoln Creek on a subset of the outfall sample collection days. River sites were collected with a 1 L grab sampler within 100 m of the corresponding outfall. These outfalls were greater than 36 inches in diameter and deliver large volumes of stormwater to the rivers.

2.2. *Culture-based microbial analysis*

All water samples were analyzed using the USEPA method for *E. coli* and enterococci enumeration (USEPA, 2002, 2006). The samples were filtered through a 0.45 μm pore size 47 mm nitrocellulose filter and placed on modified m-TEC and MEI agar. The volume of sample filtered was varied according to the expected level of contamination. The plates were incubated for 18 h and CFUs were counted and recorded.

2.3. *DNA extraction*

A volume of 200 ml from each water sample was filtered onto a 0.22 μm pore size 47 mm nitrocellulose filter and immediately stored at −80 °C, prior to extraction. A volume of 100 ml was filtered for sewage influent samples. The frozen filters were broken into small fragments using a sterile metal spatula. DNA was extracted using the MPBIO FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Anna, CA) according to the manufacturers instructions, with the exception of the lysis step in which a bead beater (Biospec, Bartlesville, OK) was used for 1 min. Extraction efficiencies were determined using enterococci BioBalls (bioMerieux, Marcy-l'Etoile, France). Briefly, 500, 5000 and 50,000 cells were added to 100 ml sterile water and extracted using the above procedure ($n = 10$ for each concentration). Recovery was 15.3 ± 2.7%. Crude cell extracts were also prepared by lysing cells on filters in 10 mM Tris 0.5 mM EDTA, pH 9.0 using 212–300 μm glass beads (Sigma, St. Louis, MO) with a bead beater. Recovery was on

average 55%, but high levels of inhibition were present and dilution of the samples to 1:10 was necessary to remove inhibition, therefore, we chose to perform DNA extractions on all samples to optimize recovery without inhibition.

2.4. PCR inhibition assays

Prior to all PCR and qPCR reactions, all sample DNA extractions were initially diluted to a DNA template concentration of 4 ng per ul (20 ng per reaction) based on pilot studies that demonstrated samples with higher DNA concentrations displayed inhibition of PCR. For PCR gel electrophoresis assays, we used *E. coli* as a control reaction since previous studies in our laboratory has demonstrated that *E. coli* and human *Bacteroides* are at similar levels. Samples were tested for *E. coli* using primers targeting the *uidA* gene, uidA298F and uidA884R (Table 1). PCR was carried out as described in Bower et al. (Bower et al., 2005). Samples containing ≥200 CFU per 100 ml *E. coli* were expected to be positive based on a previous study that examined the limit of detection by PCR of *E. coli* in sewage influent samples (Bower et al., 2005). In this study, all stormwater samples had *E. coli* levels above this threshold. If the *E. coli* control reaction demonstrated a negative result, samples were diluted 1:5, 1:10, 1:20, 1:50, depending upon cell counts, and individual dilutions were retested for *E. coli*. Samples that required dilution to detect *E. coli* were also tested at multiple dilutions for the human *Bacteroides* genetic marker as described below. Samples were considered negative for the human *Bacteroides* genetic marker when *E. coli* was detected in the original sample or two samples in the dilution series and the human *Bacteroides* reaction was negative.

For qPCR assays, additional inhibition studies were performed using a method described by Shanks et al (Shanks et al., 2009). In this case an internal amplification control (pIAC) plasmid was constructed (Integrated DNA Technologies, Skokie, Il) containing the human *Bacteroides*, *E. coli* and enterococci primer sites with a unique binding site for the IAC probe; UC1 (Zhang et al., 2003; Sivaganesan et al., 2008). In a subset of samples, qPCR was carried out in triplicate for each of these targets as described below with samples containing 50, 100, and 500 copies of the pIAC. The remainder of samples were only tested for inhibition using the human *Bacteroides* primers and 50 copies of the pIAC. In almost all cases, adjustment of the DNA template to 4 ng per ul (20 ng per reaction) was adequate to remove inhibition. Less than 1% of the samples required additional dilution to remove inhibition. In all subsequent qPCR assays, samples were diluted and final concentrations were calculated from this initial dilution.

2.5. PCR detection and qPCR quantification of fecal indicator genetic markers

After extraction and PCR inhibition determination, PCR was preformed for the human *Bacteroides* genetic marker using the HF183F and Bac708R primers (Table 1) (Bernhard and Field, 2000) according to previously published methods (Bower et al., 2005). PCR products were visualized under UV light on a 2% agarose gel after staining with ethidium bromide. Samples with weak bands were considered positive.

Samples positive for the human *Bacteroides* genetic marker using gel electrophoresis assays were further analyzed using qPCR ($n = 168$). qPCR assays were performed using previously published methods for the human *Bacteroides* genetic marker and total *Bacteroides* spp., (Bernhard and Field, 2000; Dick and Field, 2004; Kildare et al., 2007), *Enterococcus* (Behr et al., 2000) and *E. coli* (Li et al., 2006) (see Table 1 for details). The qPCR reactions were run with 25 μl reaction volumes and consisted of the 1X Taqman® Gene Expression Master Mix (Applied Biosystem; Foster City, CA) and primers and probes at a final concentration of 1.0 μM and 80 nM, respectively. DNA template was added at 20 ng per reaction. PCR cycling conditions were as follows: 2 min at 50 °C to activate the uracil-N-glycosylase (UNG), 10 min at 95 °C to inactivate the UNG and activate the Taq polymerase, 40 cycles of 95 °C for 15 s followed by 1 min at 60 °C. Reactions were carried out on a StepOne™ Real Time PCR System (Applied Biosystems, Foster City, CA). Results were reported as copy number (CN) per 100 ml.

Table 1 – Primers and probes used in PCR and qPCR assays.

Primer	Sequence	Target	Method
HF183F	5′ATCATGAGTTCACATGTCCG3′	Human *Bacteroides*	PCR
Bac708R	5′CAATCGGAGTTCTTCGTG3′	Total *Bacteroides* spp.	PCR
*uidA*298F	5′AATAATCAGGAAGTGATGGAGCA3′	*E. coli*	PCR
*uidA*884R	5′CGACCAAAGCCAGTAAAGTAGAA3′	*E. coli*	PCR
HF183F	5′ATCATGAGTTCACATGTCCG3′	Human *Bacteroides*	qPCR
BacHum241R	5′CGTTACCCCGCCTACTATCTAATG3′	Human *Bacteroides*	qPCR
BacHum193 (probe)	5′6-FAM-TCCGGTAGACGATGGGGATGCGTT-MGB-NFQ3′	Human *Bacteroides*	qPCR
BacsppF	5′GCTCAGGATGAACGCTAGCT3′	Total *Bacteroides* spp.	qPCR
BacsppR	5′CCGTCATCCTTCACGCTACT3′	Total *Bacteroides* spp.	qPCR
Bacspp346 (probe)	5′6-FAM-CAATATTCCTCACTGCTGCCTCCCGTA- MGB-NFQ3′	Total *Bacteroides* spp.	qPCR
uidA1663F	5′GCGACCTCGCAAGGCATA3′	*E. coli*	qPCR
uidA1790R	5′GATTCATTGTTTGCCTCCCTGCTGCG 3′	*E. coli*	qPCR
uidA1729 (probe)	5′6-FAM-TGCAGCAGAAAAGCCGCCGACTTCGG-MGB-NFQ3′	*E. coli*	qPCR
Entero1F	5′AGAAATTCCAAACGAACTTG3′	Enterococci	qPCR
Entero2R	5′TCAGTGCTCTACCTCCATCATT3′	Enterococci	qPCR
Entero1 (probe)	5′6-FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-MGB-NFQ3′	Enterococci	qPCR

The qPCR limit of detection was 15 CN in a reaction, which is equivalent to 112 CN per 100 ml filtered sample based upon the final volume of the sample following DNA extraction and the volume of sample used in PCR. Gel-based assays were slightly less sensitive, with the limit of detection at 500 CN per 100 ml.

2.6. *Viral analysis of SLC07 stormwater discharge*

Four liters of stormwater (the largest volume we could obtain from the automated sampler) was collected directly from the outfall during a rain event and shipped overnight on ice for analysis. Analysis for human enteric viruses, including enteroviruses, rotavirus group A, hepatitis A (HAV), G1 noroviruses, GII noroviruses, and adenoviruses (serotypes 1, 2, 5, 6, 40 and 41) was carried out in the laboratory of Dr. Mark Borchardt according to previously published methods (Borchardt et al., 2004; Borchardt et al., 2007; Lambertini et al., 2008).

2.7. *Statistical analysis*

All statistical analyses were performed in SPSS v11.0. After statistical tests showed non-normally distributed data, all data were log10 transformed before statistical analysis. The relationship between rainfall, days since previous rainfall, and the human *Bacteroides* genetic marker was explored using logistic regression. The correlation between the human *Bacteroides* genetic marker and standard fecal indicators was tested using Pearson's correlation coefficient. All other data was analyzed using the *t*-test. All tests were considered significant at $p \leq 0.05$.

3. Results

3.1. *Detection of human* Bacteroides *genetic marker in stormwater outfalls in metropolitan Milwaukee*

The human *Bacteroides* genetic marker was detected in outfalls in all urban watersheds using a gel-based assay (Fig. 1). During the course of four years, 828 samples from 45 stormwater outfalls were collected during rain events. The stormwater outfalls were intermittently positive for the human *Bacteroides* genetic marker, which was detected in at least one sample from every outfall tested. Only one outfall was positive in 100% of samples. The frequency of detection for the human *Bacteroides* genetic marker was categorized as low (0–40%), medium (41–60%), high (61–80%), and very high (81–100%). There was very high or high detection frequency in 20 outfalls. Twenty five outfalls had either medium or low detection levels. Overall, 476 of the 828 samples (57%) contained the human *Bacteroides* genetic marker.

We examined the relationship between rainfall amounts, days since previous rainfall, and detection of human *Bacteroides* genetic marker using a logistic regression on data from 18 outfalls in the Menomonee River watershed, including Honey Creek and Underwood Creek subswatersheds. There was no significant relationship between the number of outfalls positive for the human *Bacteroides* genetic marker across the watershed and rainfall amounts for that day, the number of days since previous rainfall, or a combination of these two parameters. We also did not observe seasonal differences in the percent of outfalls testing positive for the human *Bacteroides* genetic marker (spring, 67%; summer, 60%; and fall, 70%).

We hypothesized that individual outfalls have sewage migrating into the stormwater flows under different conditions, accounting for the lack of a "global correlation" of rainfall parameters and human *Bacteroides* across the watershed. Therefore, we examined these relationships at two individual outfalls ($n = 19$ for outfall SUC02A and $n = 32$ for outfall SMN01A) that had been sampled intensively to determine if patterns could be determined at individual sites. In both these cases, there was no significant relationship between antecedent conditions or rainfall amounts and the detection of the human *Bacteroides* genetic marker. We could not assess the remaining outfalls for patterns because the human *Bacteroides* genetic marker was detected in nearly all of the samples (seven outfalls), or only five to six samples had been collected which reduced the power of our analysis (nine outfalls). Overall, these results suggest that complex dynamics are involved and individual sites may be influenced differently by rainfall, preceding conditions, or physical attributes of the infrastructure.

3.2. *Quantification of the human* Bacteroides *genetic marker and total* Bacteroides *spp. using qPCR*

For the 18 Menomonee River watershed outfalls and two comparison sites, all samples that were positive for the human *Bacteroides* genetic marker using the gel-based assay were tested by qPCR. High levels of the human *Bacteroides* genetic marker were found in outfalls discharging to the Menomonee River, Lincoln Creek, and Lake Michigan (Fig. 2a). The median levels of human *Bacteroides* genetic marker in these watersheds were found to be at least an order of magnitude higher than outfalls located on Underwood Creek and Honey Creek.

Total *Bacteroides* spp., which is derived from human and non-human sources and includes the human *Bacteroides*, was used as a measure of "total fecal pollution". Outfalls located in the Menomonee River, Lincoln Creek, and Lake Michigan watersheds had high total *Bacteroides* spp., but the proportion of human *Bacteroides* comprising this total was variable (Fig. 2b).

We examined the ratio of the human *Bacteroides* to total *Bacteroides* spp. found in untreated sewage (e.g. human sources). The average human *Bacteroides* genetic marker and total *Bacteroides* spp. levels were 4.8×10^7 and 9.8×10^8 CN per 100 ml, respectively, which corresponded to 5.1% (±2.93) of the total *Bacteroides* spp. being accounted for as human *Bacteroides*. The average percent human *Bacteroides* genetic marker detected in individual outfalls was highly variable (Table 2) across the study area. The outfall discharging to Lincoln Creek had the highest percentage of human *Bacteroides* (1.45%), which suggests human sources are a predominate source of fecal pollution. The Menomonee River, Honey Creek, and Lake Michigan outfalls collectively also had a high percentage of human *Bacteroides*, with averages of 0.82, 0.79, 0.51%, respectively for outfalls discharging to each of these receiving waters. Outfalls discharging to Underwood Creek had a much

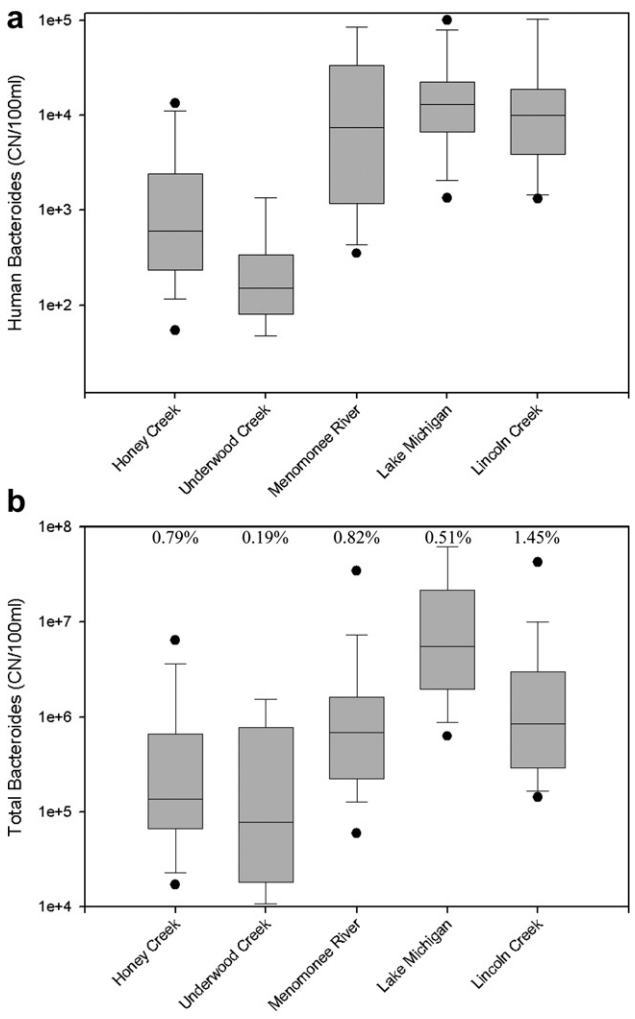

Fig. 2 – The contribution of Human *Bacteroides* and total *Bacteroides* spp. to receiving waters in the Milwaukee Metropolitan area. Box and whisker plots A: Human *Bacteroides*, B: Total *Bacteroides* ($n = 168$). The percentage of human *Bacteroides* to total *Bacteroides* spp. is listed.

lower percentage of human *Bacteroides*, with an average of only 0.19%.

3.3. *Individual outfall human* Bacteroides *patterns and correlations with infiltration and inflow*

Levels of the human *Bacteroides* genetic marker at individual outfalls ranged from an average of 300 CN per 100 ml to >400,000 CN per 100 ml (Table 2). Levels were highly variable, with standard deviations nearing 30–50% of the average for several locations. These results are likely highly influenced by dilution from different amounts of rainwater. Inline samples capture the first flush of the storm, whereas grab samples are collected later in the storm. Inline samples collected at the beginning of the storm had significantly higher levels of total *Bacteroides* spp. than grab samples. However, overall, inline samples did not have significantly higher levels of the human *Bacteroides* genetic marker compared to the grab samples.

We examined the correlation of infiltration and inflow (I&I) in the sanitary sewer system to the *Bacteroides* genetic marker results. Rainwater can enter the sanitary system through direct connections such as downspouts (inflow) or through cracks and leaks (infiltration). I&I scores in part reflect the integrity of the sanitary sewer lines and are classified as exceeds or acceptable and range from low to high for each category. Levels of the human *Bacteroides* genetic marker were significantly higher ($p \leq 0.05$) in areas with I&I scores of exceeds (all levels) or acceptable (high) compared with areas with I/I scores of acceptable (medium or low).

3.4. *Correlations between the human* Bacteroides *genetic marker and standard fecal indictors detected by qPCR and culture methods*

We examined the relationship between qPCR for the human *Bacteroides* genetic marker and total *Bacteroides* spp., and two standard indicators measured by qPCR and culture. The human *Bacteroides* genetic marker did not correlate with enterococci or *E. coli* culture results, which are the most commonly used water quality measures (Table 3). Human *Bacteroides* genetic marker also did not correlate with enterococci qPCR, but did have a very weak correlation to *E. coli* qPCR. We examined how stormwater outfall samples would be ranked differently based on culture results for *E. coli* and/or enterococci and human *Bacteroides* results. Table 4 shows the number of samples distributed among different strata: high, moderate and low human *Bacteroides*, and high, moderate and low *E. coli* and/or enterococci. Only 44 samples were found to have high human *Bacteroides* with high *E. coli*. A total of 37 samples with a moderate amount of human *Bacteroides* (100–1000 CN per 100 ml) had *E. coli* levels that were high. However, 49 samples had low to moderate *E. coli* but moderate to high levels of the human *Bacteroides* genetic marker so these samples would not have been flagged as a priority.

3.5. *Human virus detection*

A stormwater outfall that chronically tested positive for the human *Bacteroides* genetic marker, located along Lincoln Creek (SLC07), was investigated for the occurrence of human derived viruses. This outfall also demonstrated high levels of the human *Bacteroides* genetic marker using qPCR. One sample collected during a rain event was analyzed for enteroviruses, rotavirus group A, hepatitis A (HAV), G1 noroviruses, GII noroviruses, and adenoviruses. The sample was positive for three different viruses: adenovirus at 1.3×10^3 genomic equivalents per L (ge/L), enterovirus at 1.9×10^4 ge/L and G1 norovirus at 1.5×10^3 ge/L. These concentrations of viruses are similar to what is found in sewage influent (M. Borchardt, personal communication) and confirm the presence of human sewage contamination in this stormwater outfall.

3.6. *Up the pipe investigations*

Five outfalls discharging to the Menomonee River (Table 5) had high levels of fecal indicator bacteria and human *Bacteroides* and were thus chosen for up the pipe investigations. Inline samplers were placed at different branches of the

Table 2 – Summary of outfalls sampled in each subwatershed. Average values for culturable *E. coli* and enterococci are shown. qPCR data is shown as average human *Bacteroides* genetic marker and the percent of the human *Bacteroides* genetic marker to total *Bacteroides* spp. Infiltration and Inflow (I&I) scores are categorized as exceeds or acceptable and range from high to low for each category.

Receiving Body of Water	Number of Samples	Outfall	Type of Sample	Average *E. coli* (CFU/100 ml)	Average Enterococci (CFU/100 ml)	Average Human *Bacteroides* (CN/100 ml)	Average Percentage Human/Total	I&I	Ranking
Honey Creek	5	HC06	Grab	21,400	28,800	326	0.41	Acceptable	Medium
	6	HC03	Grab	21,200	26,000	749	0.35	Exceeds	Medium
	6	HC01	Grab	4900	32,600	3640	0.52	Exceeds	Low
	6	HC02	Grab	11,700	16,500	1330	2.22	Exceeds	Low
	6	HC05	Grab	4900	19,900	4100	2.03	Acceptable	Medium
	5	HC08	Grab	43,500	47,600	1760	0.78	Acceptable	Medium
	5	HC07	Grab	1120	3380	804	0.03	Acceptable	Medium
	6	HC04	Grab	5800	10,500	4790	4.42	Acceptable	Medium
Menomonee River	5	SMN15A	Inline	3,410,000	609,000	18,700	1.81	Acceptable	High
	32	SMN01A	Inline	787,000	82,000	993	0.33	Acceptable	Medium
	6	MN06	Grab	40,100	47,200	360,000	2.73	Acceptable	High
	25	SMN02A	Inline	237,000	151,000	1400	0.44	Acceptable	High
	14	SMN04A	Inline	222,000	213,000	408,000	3.76	Exceeds	Medium
	4	MN07	Grab	17,500	41,000	5460	1.13	Acceptable	High
	13	SMN03A	Inline	428,000	105,000	153,000	3.91	Exceeds	Medium
Underwood Creek	19	SUC02A	Inline	144,000	268,000	298	0.43	Acceptable	Medium
Lincoln Creek	24	SLC07	Inline	28,200	22,700	32,500	1.44	Exceeds	Low
Lake Michigan	27	SLM09	Inline	252,000	169,000	22,900	0.77	Exceeds	Low

stormwater system upstream of the original outfall site to pinpoint the area of contamination. In four of five outfalls, at least one upstream location had approximately two fold higher levels of the human *Bacteroides* genetic marker suggesting that the source of contamination originated upstream. In one location, the human *Bacteroides* genetic marker was not detected using gel-based assays in any of the samples, which suggests the point of contamination lies between the outfall and the upstream locations. Further sampling between these segments is necessary to isolate the breech in the sanitary system.

3.7. *Impact on water quality*

We sampled five rivers that serve as receiving waters for stormwater discharges from outfalls in this study. There was no significant difference in levels of *E. coli* and enterococci among sites (Fig. 3). On average, the levels of the human *Bacteroides* genetic maker showed large differences among sites, however, these differences were not statistically significant because within site variation was great. The three Menomonee River sites and the Lincoln Creek site had ratios of human *Bacteroides* to total *Bacteroides* spp. in the range of what was found with untreated sewage. Lincoln Creek has five- to ten-fold higher levels of the human *Bacteroides* genetic marker than Underwood Creek although they have similar levels of fecal indicator bacteria. Across all sites, the levels of the human *Bacteroides* genetic marker and total *Bacteroides* spp. in river water were similar or higher than what was found at adjacent outfalls. Among the Menomonee River sites, the middle river site and the adjacent outfall had high levels of the human *Bacteroides* genetic marker (20,000 and 153,000 CN per 100 ml, respectively) and a high ratio of the human *Bacteroides* genetic marker to total *Bacteroides* spp. (>4.0% for both the river and outfall) indicating that the outfall may be a direct source of human contamination. All the river sites, with the exception of Underwood Creek, were found to be heavily impacted by fecal pollution and appear to have sewage as the major source.

Table 3 – Correlations between culturable fecal indicators and qPCR targets (n = 168). Significant correlations are flagged.*

	Human *Bacteroides*	Total *Bacteroides* spp.
Human *Bacteroides*	1	0.552*
Total *Bacteroides* spp.	0.552*	1
E. coli	0.158*	0.464*
Enterococci	0.057	0.473*
E. coli culturable	0.027	0.336*
Enterococci culturable	−0.105	0.328*

4. Discussion

Human sewage contamination of surface waters is a widespread problem in the urban environment (Lipp et al., 2001; Marsalek and Rochfort, 2004; Salmore et al., 2006; Arnone and Walling, 2007), however, identifying the primary mechanisms that introduce sewage into waterways is elusive. Sewage can enter stormwater systems as a result of breeches in sanitary sewage infrastructure, cross-connections, and abandoned sewer bypass locations, which can be exacerbated by wet weather flows (O'Shea and Field, 1992; Marsalek and Rochfort, 2004). This study attempted to quantify the extent

Table 4 – Stormwater outfall samples stratified by levels of fecal indicator bacteria (*E. coli* and enterococci) and the human *Bacteroides* genetic marker detection ($n = 214$).

	High Fecal Indicator Bacteria (>10,000 CFU/100 ml)	Moderate Fecal Indicator Bacteria (1000–10,000 CFU/100 ml)	Low Fecal Indicator Bacteria (<1000 CFU/100 ml)
High Human *Bacteroides* (>5000 CN/100 ml)	44	33	4
Moderate Human *Bacteroides* (1000–5000 CN/100 ml)	28	11	2
Low Human *Bacteroides* (<1000 CN/100 ml)	32	12	2
Negative Human *Bacteroides* (gel based)	39	6	1

in which stormwater systems act as a conduit for sewage from failing sanitary sewer infrastructure. We sampled nearly one quarter of the major stormwater outfalls in two urbanized watersheds in addition to less dense sampling in other areas of metropolitan Milwaukee. Sewage contamination was intermittently detected at every site examined, attesting to the extent of occurrence for unrecognized sewage fecal pollution sources. Further, there was on average high to moderate levels of human *Bacteroides* (e.g. >1000 CN per 100 ml) at two-thirds of the outfalls tested.

The combination of stormwater outfall and river water assessments for human *Bacteroides* demonstrates that sanitary sewage sources of fecal pollution are major contributors to poor water quality within our study area. Nearly half of the 18 outfalls in which qPCR was conducted had ratios of human *Bacteroides* to total *Bacteroides* spp. consistent with 25% or more sanitary sewage composition based upon our analysis of untreated sewage. River water within all of the subwatersheds had very high levels of human *Bacteroides* (Fig. 3) and reflected the water quality of the outfalls within the vicinity. These findings support the concept that outfall discharges directly influence receiving waters. It is difficult to estimate actual sewage loading contributions from individual outfalls because there are many variables that influence the concentration of indicator organisms and the volume of water discharge to the river (drainage area, amount of rain runoff, timing of sampling, etc.), but such a calculation would be critical for assigning sources to fecal pollution loads in this system.

River water samples consistently exceeded recreational standards for *E. coli* and enterococci. *E. coli*, one fecal coliform, exceeded a variance standard criteria of 1000 CFU per 100 ml fecal coliforms in 97% of samples for the Menomonee River and 100% of samples for Lincoln Creek and Underwood Creek. However, there were greater differences in human *Bacteroides* levels among river sites, an indicator that likely serves as a better benchmark of potential human health risk. Quantitative risk assessment studies have estimated that levels of $>8.6 \times 10^3$ copies per L (860 copies per 100 ml) of human *Bacteroides* genetic marker may pose a health risk in recreation waters (Soller et al., 2010); Based on previous studies of total culturable viruses in Milwaukee sewage using EPA method for total culturable viruses, (Sedmak et al., 2005), concentrations averaged 2×10^4 total culturable viruses per L. Therefore, concentrations of 860 copies per 100 L of human *Bacteroides* corresponds to ~0.1–3 total culturable viruses per L during summer months. More recent estimates using qPCR detected adenovirus concentrations ranging from 5×10^2 to 1×10^6, depending upon the time of year (M. Borchardt, personal communication); this would correspond to 0.01–20 genomic copies of adenovirues per L for Milwaukee sewage. In other

Table 5 – Human *Bacteroides* and total *Bacteroides* spp. for investigations moving upstream from the original discharge point.

Original Site with high frequency of Human *Bacteroides*	Upstream Site	% Positive for Human *Bacteroides*	Human *Bacteroides* (CN/100 ml)	Total *Bacteroides* spp. (CN/100 ml)
SMN01A	SMN01C	0	n/a[a]	n/a[a]
	SMN01E	0	n/a[a]	n/a[a]
SMN02A	SMN02B	40	12,350	1,920,000
	SMN02C	0	n/a[a]	n/a[a]
SMN03A	SMN03B	40	44,700	5,050,000
SMN04A	SMN04B	93	51,500	1,150,000
SMN06A	SMN06B	n/a[b]	101,000	213,000,000
	SMN06C	n/a[b]	31,000	6,080,000
	SMN06D	n/a[b]	543,000	4,800,000
	SMN06E	n/a[b]	175,000	14,800,000

a Not applicable, qPCR is not performed when gel-based assays are negative.
b Not applicable, gel-based assays were not preformed on these samples.

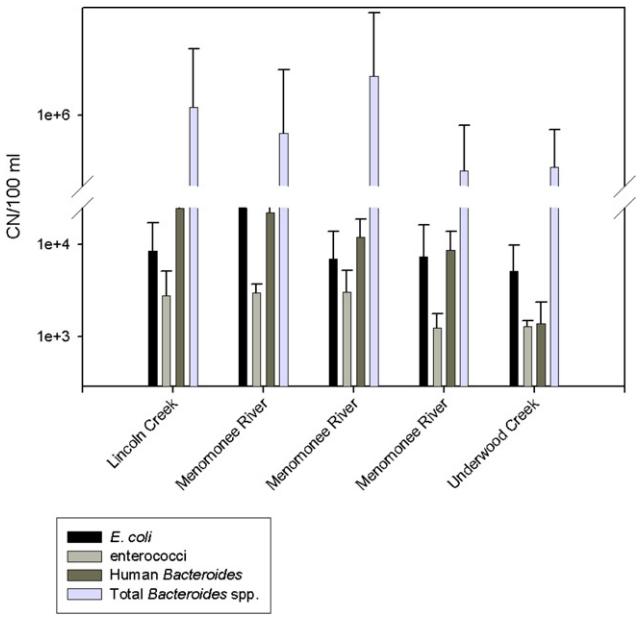

Fig. 3 – *E. coli*, enterococci, the human *Bacteroides* genetic marker, and total *Bacteroides* spp. levels found in river samples for metropolitan Milwaukee subwatersheds (Lincoln Creek $n = 6$, Menomonee River $n = 4$, Menomonee River $n = 3$, Menomonee River $n = 4$, and Underwood Creek $n = 2$).

areas, adenovirus has been detected at average concentrations of 3×10^4 genomic copies per L, with a similar large seasonal variation (Bofill-Mas et al., 2006). All of our river sites exceeded 860 copies per 100 ml, and some sites were 10–25 times greater than this target.

Alarmingly, these rivers discharge near Lake Michigan beaches and are used for numerous recreational activities including canoeing and fishing. Other urban areas on marine and freshwater coasts send stormwater discharges directly to public swimming beaches (Haile et al., 1999; Marsalek and Rochfort, 2004; Converse et al., 2009). Ironically, public lands are often the location of stormwater outfall discharge points and simultaneously, the location of recreational beaches. These two uses are in direct conflict with one another. Studies have documented enteroviruses in stormwater (Rajal et al., 2007; Converse et al., 2009) and increased illness associated with swimming near stormwater outfalls. Collectively, these studies highlight the widespread nature of failing urban infrastructure and the potential health risk this problem poses.

Numerous studies have been conducted on outfalls with high fecal indicator bacteria with no obvious source of contamination (e.g. direct misconnections or sewage overflows) highlighting the diffuse and problematic nature of fecal pollution in stormwater discharge (Schiff and Kinney, 2001; Sercu et al., 2009; Parker et al., 2010). No correlation has been found between rainfall, magnitude of storm, or the progression of the storm to *E. coli* and enterococci levels (Parker et al., 2010); however, these parameters have not been previously explored in respect to relationships with alternative, host specific markers that are indicative of a source such as sanitary sewage. In our study, there was no correlation between rainfall amounts or days since previous rainfall, and detection of the human *Bacteroides* genetic marker. Seasonality also did not appear to influence the frequency in which sewage was detected in stormwater. Lack of correlation to rainfall and intermittent positive results for the human *Bacteroides* genetic marker indicate that site specific characteristics may play a large role in stormwater discharge. Other studies have also found significant variability of the human *Bacteroides* marker within a single outfall (Sercu et al., 2009). Further, studies have suggested specific parameters and the source of contamination (sanitary exfiltration vs. surface runoff) can affect the levels of fecal indicator bacteria in the first flush of stormwater discharge (McCarthy, 2009). We found I&I scores in the sanitary sewer system adjacent to an outfall corresponded to elevated human *Bacteroides* in the stormwater released from that outfall (Table 2). I&I scores, age of development or other infrastructure characteristics may be useful parameters to consider when attempting to delineate patterns of sewage contamination across a large area, such as a major metropolitan city.

The high number of outfalls contaminated with human sewage along with the overall river water quality is a testament to the importance of developing an effective approach to identifying and monitoring these areas of contamination. Others (Noble et al., 2006; Converse et al., 2009; Sercu et al., 2009) have suggested a tiered approach to identifying areas of contamination. Samples are tested through a variety of methods, starting with culture based methods for traditional fecal indicators. Further analyses such as PCR, qPCR, or virus analysis are directed toward samples exceeding water quality standards or those with the highest fecal indicator levels. This may be beneficial and cost effective for areas with a known contamination source (e.g., septic tanks, agricultural runoff). However, in urban areas with significant aging infrastructure and numerous non-point sources of pollution, qPCR may be the best approach as the first tier assessment. Culturable indicators did not correlate with qPCR for the human *Bacteroides* genetic marker. For example, only moderate levels of *E. coli* or enterococci (1000 to 10,000 CFU per 100 ml) were found in nearly half the samples with high levels of human *Bacteroides* genetic marker, and a number of outfalls had high *E. coli* or enterococci with low or no human *Bacteroides* (Table 4). This suggests that other sources of *E. coli* and enterococci, besides human inputs, are in the stormwater system, which is consistent with other reports identifying urban wildlife and pets as fecal sources (Ram et al., 2007). If traditional indicators were used as a metric for identifying and prioritizing outfalls in our study system, then two-thirds of the outfalls with clear evidence of sewage contamination would not have been given a high priority. These findings illustrate the extent in which *E. coli* and enterococci levels may be uncoupled to evidence of sewage contamination in the urban environment.

Water resource managers and regulators will ultimately need to define priorities that target either human sources that likely carry pathogens (regardless of the level) or non-human sources in runoff that contribute high fecal indicators but are not a likely source of human pathogens. Total maximum daily load (TMDL) targets are meant to reduce water quality impairments; however, they are based on standard fecal indicators

and not the actual cause of the impairment, pathogens. Targeting sources for mitigation is a straightforward endeavor in cases where high fecal indicator bacteria correspond to evidence of sewage sources (Sercu et al., 2009). However, our study clearly demonstrates that there is an important decision point on a second tier of assessment; whether to target high fecal indicators, which may include non-human sources, or to prioritize sewage sources. It is important to note that few studies have rigorously tested common non-human sources in urban areas for zoonotic pathogens, with a few exceptions (Schueler and Holland, 1999; Kullas et al., 2002). In contrast, sewage sources have high levels of human pathogens (Sedmak et al., 2003). If the ultimate goal is to reduce pathogens, TMDLs need to consider more precise indicators.

Molecular methods offer the opportunity for a much wider variety of organisms to be used as alternative indicators (Field and Samadpour, 2007; Stewart et al., 2008; Boehm et al., 2009). The human genetic marker used in this study has been reported to be highly specific ranging from 83% to 95% (Shanks et al., 2010; Seurinck et al., 2005). Cross reactivity has been reported with 1 of 10 dogs tested in one study (Shanks et al., 2010) and 2 of 8 dogs in another study (Kildare et al., 2007). It is possible that dog waste could account for some of the positive results, but highly unlikely that this source was a major contributor to fecal pollution given the very high *E. coli* and enterococci levels found in many of the outfalls. Alternative indicators that are specific for fecal waste from animals commonly found in urban areas will be important for confirming the causes of high *E. coli* or enterococci levels.

As these methods become standardized and put into general use, it will be necessary to relate historical measurements to newer approaches. In our study, there is a correlation between culturable enterococci and qPCR enterococci targets (Byappanahalli et al., 2010; Whitman et al., 2010). As host specific qPCR markers are employed for water quality monitoring due to it's sensitivity and potential specificity toward new targets (Byappanahalli et al., 2010; Lavender and Kinzelman, 2009), research is needed to determine the prevalence of pathogens and viruses in correlation to alternative indicators and direct linkages to human health risks.

5. Conclusions

Urbanized coastal areas are among our oldest cities and are often challenged with maintaining aging infrastructure. Recognizing and mitigating sources of sewage contamination to surface waters is a high priority. Using PCR and qPCR methods, the sources of contamination can be systematically tracked through the stormwater system and ultimately monitored after remediation. However, a watershed approach with a long-term monitoring program may be the best approach to protect human health. With over 200 outfalls in the Milwaukee metropolitan area, tracking each outfall is time and cost prohibitive. High I&I scores indicate a high probability of sewer system leaks and these areas should therefore be the primary targets of monitoring for sewage entering stormwater systems. Determining "hotspots" of contamination through a watershed approach and then testing suspicious outfalls with traditional engineering approaches such as dye or smoke testing could be the most effective approach to protecting human health and assessing infrastructure. This approach may be particularly useful in urban areas where numerous non-human sources of fecal pollution cause standard fecal indicators to be of little use for prioritizing remediation efforts.

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