
Background

Impairment of rivers and beaches by fecal pollution is an ongoing problem for water resource managers and threatens ecosystems and human health worldwide (Garbossa et al., 2017; Reeves et al., 2004; Reischer et al., 2013; Stewart et al., 2008; USEPA, 2009). Urban coastal regions are especially stressed by fecal pollution from watershed sources that can include upstream agricultural runoff and densely populated urban areas near estuaries (Paul and Meyer, 2001; Rothenberger et al., 2009; Sidhu et al., 2013). In the US, more than 50% of the population lives in counties within coastal watersheds, highlighting the disproportionate anthropogenic stress on these watersheds (NOAA, 2013a). This trend is mirrored worldwide with more than 1 billion people living within 50 km of a coast (Kummu et al., 2016). The freshwater coasts of the Great Lakes are particularly sensitive to fecal pollution impacts as these bodies of water serve as drinking water sources to more than 40 million people in the US and Canada (NOAA, 2013b).

Aging sewer infrastructure increasingly threatens water quality as the gap between investments and deterioration widens. An estimated amount of almost one trillion dollars is needed for capital investments to assure the integrity of wastewater infrastructure in the US (USEPA, 2007). The American Society of Civil Engineers ranks the nation’s wastewater conveyance and treatment systems as a D+ (ASCE, 2017). Sewage has been detected in urban rivers and coastal waters in the absence of recognized sewage overflows (Sercu et al., 2009; Wiegner et
al., 2017). Sewage can enter surface waters in a number of different ways. It can leak from deteriorating pipes into the surrounding ground and subsequently infiltrate stormwater systems or groundwater, which can then transport contamination to rivers and beaches. Sewage overflows continue to be a major problem in older cities in the Midwest, Northeast, and Pacific Northwest, where nearly 860 communities have combined sewer systems (USEPA, 2004), where both stormwater and sanitary sewage are captured in the same system of pipes. These systems become inundated with stormwater and overflow to rivers, resulting in the release of 850 billion gallons of untreated sewage mixed with stormwater each year (USEPA, 2004). A total of 184 of these systems discharge to the Great Lakes drinking water sources (USEPA, 2016). With the number of extreme storms expected to increase in areas of the US that have a high density of combined sewer systems, combined sewer overflows (CSOs) may rise, threatening drinking water sources (Patz et al., 2008; Trtanj et al., 2016).

To track sewage pollution, water quality indicators more specific than those used traditionally are needed (McLellan and Eren, 2014). For more than 100 years, rivers and streams have been monitored for waterborne pathogen risk using fecal organisms that are easy to culture, including fecal coliforms, E. coli and enterococci. However, human sources cannot be distinguished from domestic pet, wildlife or agricultural animal waste, because all of these hosts carry E. coli in their guts. Using new indicators that target fecal anaerobic bacteria (Fiksdal et al., 1985; Eren et al., 2015) specifically associated with humans can provide evidence of sewage contamination in waterways. Identifying and remediating sources of human fecal pollution is important, because they have a higher probability of carrying human pathogens, especially human viruses, compared to many animal sources (Schoen et al., 2011). Agricultural runoff can also present a high health risk from certain bacterial and protozoan pathogens such as Salmonella, E. coli O157:H7, and Cryptosporidium (Ferguson et al., 1996; Medema et al., 1997; Chekabab et al., 2013).

In this study, our goal was to couple intensive sampling before, during, and after storm events with measurements of both traditional fecal indicators and host-associated indicators to quantify sources of fecal pollution. Our work sheds light on the causes of chronic fecal contamination observed in an estuary, and attempts to benchmark quantitatively the amount of sewage being released from a highly urbanized watershed following rainfall. Concurrent pathogen measurements in sewage allowed us to relate human-associated indicator levels to relative risk of illness from pathogen exposure. Quantifying sources of agricultural and sewage fecal pollution across the hydrograph illustrates the dynamic and complex contamination inputs from watershed sources.

**Methods**

**Area of study and sampling.** The Milwaukee River Basin encompasses 2280 km² of mixed land use with three main rivers that converge in the Milwaukee estuary within 0.5 km of the Milwaukee harbor, which is located along the western shore of Lake Michigan. The study site is shown in Figure 1. This basin is typical of coastal watersheds, with the largest river, the Milwaukee (MKE) River, receiving drainage (1813 km²) from upstream agricultural land that includes dairy farms, suburban areas, and highly developed areas of several small communities and the northern parts of metropolitan Milwaukee. The Menomonee (MN) River receives drainage (352 km²) from primarily suburban and urban areas. The Kinnickinnic (KK) River drains the smallest watershed of 65 km² and is highly urbanized. There are approximately 190 combined sewage outfalls in the lower reaches of these rivers that discharge during extreme rain events. Two wastewater treatment plants (WWTPs) service this area: treated effluent is discharged from Jones Island WWTP to the Milwaukee harbor, adjacent to the estuary and below our sampling site; and South Shore WWTP discharges to Lake Michigan approximately 15 km south of the Milwaukee harbor.

A portable automated sequential sampler (3700 full size, Teledyne ISCO, Lincoln, NE) was used to collect hourly composite samples (15-min time-weighted subsamples in a single 1-L bottle) downstream from the confluence of the three rivers in the Milwaukee estuary. This site is approximately 190 combined sewage outfalls in the lower reaches of these rivers that discharge during extreme rain events. Two wastewater treatment plants (WWTPs) service this area: treated effluent is discharged from Jones Island WWTP to the Milwaukee harbor, adjacent to the estuary and below our sampling site; and South Shore WWTP discharges to Lake Michigan approximately 15 km south of the Milwaukee harbor.

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of the MKE, MN, and KK rivers in the Milwaukee estuary. Samples were collected before, during, and after rain events from April to October during 2009, 2010, and 2011. The sampler was packed with ice at the start of sampling and replenished with ice each day. Samples were analyzed for *E. coli* and enterococci by culture as described below. Human-associated indicators were analyzed by quantitative polymerase chain reaction (qPCR) in the 1-h samples, or composites of these samples representing 2-h or 4-h time frames. For this analysis, sample volumes of 200 mL were filtered onto a 0.22 µm mixed cellulose ester filter (47-mm diameter; Millipore, Billerica, MA) and filters were stored at −80°C until processed for DNA extraction. Analysis of the traditional culturable indicators, *E. coli* and enterococci, and of qPCR genetic markers in the 1-h samples demonstrated that 2-h and 4-h composites captured the changes in concentrations similar to what was measured in the individual 1-h samples. Table S1 shows the event types, dates and number of samples analyzed by culturing and qPCR.

**WWTP influent samples.** Untreated sewage influent samples were collected as 24-h flow-weighted samples from a single day, or were combined as seven-day composites. Samples were collected by the Milwaukee Metropolitan Sewerage District as part of their daily monitoring program two to three times per month over a two-year period (n = 98). Seven-day composites were held at 4°C prior to processing within 48 h of the end of collection. Single-day samples were processed the same day as collection. A volume of 50 mL was filtered onto a 0.22 µm mixed cellulose ester filter (47-mm diameter; Millipore, Billerica, MA), and the filters were stored at −80°C until processed for DNA extraction. Composite and single-day samples were analyzed for human *Bacteroides* (HB) and human *Lachnospiraceae* (Lachno2), as well as the traditional indicators, *E. coli* and enterococci, by qPCR as described below. Culturing of traditional indicators was not performed on these samples. Data were log-transformed, and geometric concentrations and standard deviations of the geometric mean were calculated.

Norovirus concentrations were determined in these same samples, using 2 L of sample, as part of another study. Text S1 details the methods for GI and GII noroviruses, which were used for risk assessment calculations. Data for norovirus concentrations are deposited in the U.S. Geological Survey (USGS) National Water Information System Web Interface and can be retrieved by USGS parameter codes, parameter names, and microbiological category. For GI norovirus, parameter code is 31765, parameter name is “Norovirus genogroup I, qPCR”, and microbiology category is “Human Virus”. For GII norovirus, parameter code is 31766, parameter name is “Norovirus genogroup II, qPCR”, and microbiology category is “Human Virus”.

**Traditional and alternative indicator analysis.** Estuarine samples and selected WWTP influent samples were analyzed for culturable *E. coli* and enterococci using USEPA methods by filtering the appropriate volume to obtain a countable density of colonies, 0 to 220 colonies per plate (USEPA, 2002, 2006). Samples were also analyzed for *E. coli*, enterococci, and host-associated markers by qPCR as described previously (Sauer et al., 2011; Templar et al., 2016). Briefly, DNA was extracted from stored filters using MPBIO FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Anna, CA). Samples were generally analyzed within six to nine months after collection, with the exception of samples from 2009 and 2010 which were analyzed for Lachno2 in 2011 as the assay was developed. These latter samples were reanalyzed for HB, and no significant difference was detected between HB values determined after <9 months of storage and those determined within 12–30 months of storage (p < 0.05).

DNA extraction efficiency was tested in a subset of river water samples (n = 20) using salmon testes DNA (Sigma, catalog# D1626) according to USEPA Method 1611 (USEPA, 2012a). This method involved adding 0.2 µg of salmon testes DNA to 1 mL DNA extraction buffer and extracting a blank filter (n = 5), followed by qPCR analysis of the extracted samples. The mean (± standard deviation) extraction efficiency was 19.8% (±6%), which was similar to the efficiency we determined previously (15.3 ± 2.7%; Sauer et al., 2011). When the salmon testes DNA was added to river water sample filters (n = 20), extraction efficiencies increased to an average of 46.5% (±3%), suggesting that sample DNA acts as a carrier for the low amounts of spiked DNA. Inhibition of the PCR was tested independently of extraction in a subset (n = 20) of river water samples by adding salmon testes DNA directly to extracted river water samples to a final concentration of 0.2 ng µL⁻¹ in each sample. DNA concentrations, quantified as described in Method 1611 (USEPA, 2012a), ranged from 84 to 105% of the expected concentrations, with an average of 95% (±7%), indicating no inhibition. As these results are consistent with other studies of stormwater samples, where no inhibition was noted using internal amplification controls consisting of synthesized plasmids with an unrelated target sequence (Sauer et al., 2011), we did not test for inhibition in individual samples.

Assays for *E. coli* (Sauer et al., 2011), enterococci (USEPA, 2012a), HB (Templar et al., 2016), Lachno2 (Newton et al., 2011; Templar et al., 2016), and ruminant markers (Reischer et al., 2006) have been described previously. An Applied Biosystems StepOne Plus™ system with Taqman chemistry (Applied Biosystems; Foster City, CA) was used for qPCR. Reactions were carried out in volumes of 25 µL, with 5 µL of sample added as template, using Taqman® Gene Expression Mastermix kit according to manufactures instructions (Applied Biosystems, Foster City, CA). Amplification products cloned into TA vector 2.1 (Invitrogen, Carlsbad, CA) were used as standards. Standard curves were determined using a range of 1.5 × 10⁰ to 1.5 copy number (CN) per reaction. The lower limit of quantification was determined to be 15 CN per reaction, which is equivalent to 225 CN per 100-mL sample considering a volume of 200 mL was filtered for each sample and extracted DNA was eluted in a volume of 150 µL. Signals below 35 cycles (and not within the quantifiable range) were considered detectable but not quantifiable. All qPCR runs included two previously analyzed environmental samples as controls. All no-DNA template controls were negative. All assays were performed in duplicate and compared to values in standard curves. Assay primers and standard curves are reported in Table S2.
Quantitative microbial risk assessment (QMRA). We predicted the risk of enteric illness associated with recreational exposure to human markers from sewage in the river water when the concentrations were 1, 10, 10^2, 10^3, 10^4, and 10^5 CN per 100 mL in river water. To do so, we modeled the distributions of HB, Lachno2, and norovirus measured in untreated sewage using the 98 WWTP influent samples described above. We combined the data from the two WWTPs and modeled them as log$_{10}$ normal with mean and standard deviation.

To calculate the risk, we followed the methods described in detail by Boehm et al. (2015). In brief, we started with the assumed concentration of human marker in river water (1, 10, 10^2, 10^3, or 10^5 CN per 100 mL in river water). We then used a Monte Carlo approach to randomly draw from the distribution describing the human marker concentration in sewage, and used this value to calculate the fraction of sewage present in a volume of river water. Subsequently, we drew a concentration of norovirus in sewage from its distribution to calculate the concentration of norovirus present in the river water. The volume of water ingested during swimming was assumed to follow the In-normal distribution reported by Dufour et al. (2006). A number was drawn from this distribution and, along with the concentration of norovirus present in river water, used to calculate the dose a swimmer consumes of norovirus. The norovirus dose-response curve was used to determine the probability of infection given that dose, and the probability of illness given infection was assumed to be 0.6 (Teunis et al., 2008; Boehm et al., 2015). This procedure was repeated 10,000 times for each human marker concentration in river water, and the process was completed for each of the two human markers (HB and Lachno2). The end result is a distribution of predicted illnesses per 100 swimmers for each concentration of human marker. The QMRA approach used herein differs from that described by Boehm et al. (2015), because norovirus is the only pathogen considered in the QMRA. We took this approach because norovirus contributes most of the risk in recreational water QMRAs that have modeled norovirus is the only pathogen considered in the QMRA.

Concentrations of human viruses in sewage and risk relationship to indicators. Human viruses were measured in 70 of the 98 untreated WWTP samples as part of another study (Lenaker et al., 2017). Detection of viruses was intermittent and levels considerably lower than the human-associated bacterial markers. Norovirus GI was detected in 30 of the 70 samples at concentrations of 10^4 to 10^5 GC L$^{-1}$, with a geomean concentration of 3.18×10^4 GC L$^{-1}$ for the positive samples. Norovirus was detected in every month except February. Genotype GI was not detected in any of the samples. In general, the frequency of detection was higher from spring to early fall, but norovirus concentrations were highest in January. The full dataset for human viruses is available in the USGS National Water Information System Web Interface (as referenced in Methods).

A QMRA was used to estimate the risk expected from exposure to raw sewage in river water. We used observed concentrations of HB, Lachno2, and norovirus GI in untreated sewage to construct log$_{10}$-normal distributions (Table S3). These distributions were used as inputs to the QMRA. Distributions of risk obtained from the 10,000 simulations for each indicator concentration are shown as box and whisker plots in Figures S1 and S2. The median values for risk of illness for each concentration of human-associated indicators that was generated in the simulation are plotted in Figure 2.
Quantitative measurements of human Bacteroides and Lachnospiraceae signals during known sewage contamination events in the environment. We used two known CSOs to evaluate how well our human-associated genetic markers measured sewage contamination quantitatively. We assessed the human fecal pollution signals in the estuary before, during, and after two storm events with CSOs of different magnitudes. The rain event of 19 June 2009 was accompanied by a large CSO approximately five times the magnitude in volume and duration of a smaller CSO that occurred during the 20 June 2011 rain event (Table 2). The magnitude difference in volume of combined sewage was mirrored in the differences in mean concentrations during the event, as well as the overall load of human indicators into the lake (Table 2). The mean concentration of the HB marker was 5.7-fold higher, and the HB load 6.2-fold higher, during the large CSO compared to the smaller event. In addition to reported CSO volumes, upstream communities also reported sanitary sewage overflows (SSOs) for both events, which would be expected.
to increase the amount of untreated sewage in the estuary during the same time frame. Lachno2 in sewage was highly correlated to HB ($r = 0.96$) across both CSO events.

Using the geomean of HB concentrations in untreated sewage, we estimated that the discharge from the estuary to the lake was comprised of 0.81% and 0.14% untreated sewage for the large and the small events, respectively. From the loads measured in the estuary (assuming that all sewage was from the CSO) and the total volume of CSO release reported, we estimated that the larger CSO was comprised of 2.76% untreated sewage mixed with stormwater in the actual CSO release. Estimates from the smaller event suggested that the CSO discharge was slightly more concentrated, with 3.14% untreated sewage mixed with stormwater.

In the 24-h period following the larger CSO, mean concentrations of the HB marker dropped an order of magnitude to $2.71 \times 10^4$. The smaller event followed a similar pattern, but for a sharp increase in HB concentrations 14 h after the end of the CSO. This second peak in HB concentrations declined to $<10^4$ within the next 16 h, and may have corresponded to SSO discharges from communities upstream that extended past the period of CSO discharge. A third CSO event was partially sampled, with samples taken 18 h after the start of the event and again one day after the event (Table 3). This storm event resulted in greater than 20 cm of rain in the watershed in a 24-h period and was accompanied by widespread flooding. Due to lack of access to the sampling site, the peak concentrations were likely missed. These results indicate

### Table 2: Comparison of two combined sewer overflow (CSO) events of different magnitudes. DOI: https://doi.org/10.1525/elementa.301.t2

<table>
<thead>
<tr>
<th>Event</th>
<th>Duration (h)</th>
<th>Volume CSO (MG)$^a$</th>
<th>Mean HB$^b$ (CN 100 ml$^{-1}$)</th>
<th>Mean sewage (%)$^c$</th>
<th>Load$^d$ (HB CN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 June 2009</td>
<td>38.5</td>
<td>935.7</td>
<td>2.26 $\times 10^0$</td>
<td>0.81</td>
<td>1.70 $\times 10^6$</td>
</tr>
<tr>
<td>20 June 2011</td>
<td>14.0</td>
<td>170.5</td>
<td>3.97 $\times 10^4$</td>
<td>0.14</td>
<td>2.74 $\times 10^3$</td>
</tr>
</tbody>
</table>

$^a$ Million gallons.

$^b$ Mean HB (human Bacteroides) concentration at estuary station during the CSO event.

$^c$ Copy number.

$^d$ Computed using a geomean of $2.77 \times 10^7$ HB 100 ml$^{-1}$ in untreated sewage.

### Table 3: Peak 24-h mean concentrations and event loads under various hydrological conditions. DOI: https://doi.org/10.1525/elementa.301.t3

<table>
<thead>
<tr>
<th>Start date$^e$</th>
<th>Event type</th>
<th>Precipitation (cm prior 24 h)</th>
<th>Peak mean 24-h concentration of HB (CN 100 ml$^{-1}$)$^b$</th>
<th>Peak mean 24-h concentration of Lachno2 (CN 100 ml$^{-1}$)$^b$</th>
<th>Load HB (CN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 June 2009</td>
<td>CSO</td>
<td>11.9</td>
<td>2.98 $\times 10^3$</td>
<td>3.35 $\times 10^3$</td>
<td>1.78 $\times 10^6$</td>
</tr>
<tr>
<td>7 July 2010</td>
<td>CSO</td>
<td>21.6$^e$</td>
<td>2.68 $\times 10^4$</td>
<td>7.69 $\times 10^4$</td>
<td>ND</td>
</tr>
<tr>
<td>20 June 2011</td>
<td>CSO</td>
<td>10.2</td>
<td>2.36 $\times 10^{46}$</td>
<td>1.01 $\times 10^3$</td>
<td>3.71 $\times 10^{15}$</td>
</tr>
<tr>
<td>8 June 2009</td>
<td>Rain</td>
<td>3.8$^e$</td>
<td>6.88 $\times 10^3$</td>
<td>8.01 $\times 10^3$</td>
<td>3.45 $\times 10^4$</td>
</tr>
<tr>
<td>22 October 2009</td>
<td>Rain</td>
<td>5.3</td>
<td>9.72 $\times 10^4$</td>
<td>8.12 $\times 10^4$</td>
<td>6.49 $\times 10^4$</td>
</tr>
<tr>
<td>5 April 2010</td>
<td>Rain</td>
<td>7.1</td>
<td>8.18 $\times 10^4$</td>
<td>ND$^e$</td>
<td>1.48 $\times 10^1$</td>
</tr>
<tr>
<td>23 April 2010</td>
<td>Rain</td>
<td>4.4</td>
<td>1.17 $\times 10^4$</td>
<td>ND$^e$</td>
<td>ND</td>
</tr>
<tr>
<td>10 May 2010</td>
<td>Rain</td>
<td>5.1</td>
<td>4.79 $\times 10^3$</td>
<td>1.14 $\times 10^4$</td>
<td>ND</td>
</tr>
<tr>
<td>27 July 2011</td>
<td>Rain</td>
<td>3.1$^e$</td>
<td>1.89 $\times 10^3$</td>
<td>ND$^e$</td>
<td>ND</td>
</tr>
<tr>
<td>12 October 2011</td>
<td>Rain</td>
<td>1.4</td>
<td>1.59 $\times 10^3$</td>
<td>ND$^e$</td>
<td>ND</td>
</tr>
<tr>
<td>16 June 2009</td>
<td>Baseflow</td>
<td>0.4</td>
<td>7.20 $\times 10^2$</td>
<td>6.84 $\times 10^2$</td>
<td>ND</td>
</tr>
<tr>
<td>18 May 2010</td>
<td>Baseflow</td>
<td>0.0</td>
<td>2.62 $\times 10^2$</td>
<td>2.71 $\times 10^2$</td>
<td>ND</td>
</tr>
<tr>
<td>25 July 2011</td>
<td>Baseflow</td>
<td>0.0</td>
<td>8.27 $\times 10^2$</td>
<td>ND$^e$</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ Hydrographs with culture and qPCR data for indicators appear in Figures S3–S13 and S14–24, respectively.

$^b$ Copy numbers (CN) per liter.

$^c$ includes rainfall totals from 24 hours prior, for 23 July 2010 the main storm event was not sampled due to flooding.

$^d$ peak 12-h average; combined sewer overflow (CSO) event spanned 12 h.

$^e$ Not determined.
that human-associated indicators coupled with hydrological measurements and high frequency sampling can be used to estimate the quantity of untreated sewage entering water bodies.

**Sources of fecal pollution during baseflow and rain events.** There was evidence of sewage contamination during baseflow conditions; i.e., when there had been no rain within 24 h. Mean 24-h concentrations were generally low compared to means during rain events (Table 3). During rain events with no reported sewage overflows, the HB genetic marker was detected consistently but at significantly lower concentrations compared to the large CSO event (Figure 3). The full set of hydrographs for events in Table 3 with human-associated indicators and culture data for traditional indicators are presented in Figures S3–S13, and hydrographs with human-associated indicators with qPCR data for traditional indicators are presented in Figures S14–S24. The 24-h peak means of HB (and Lachno2) concentrations varied for different storms, ranging from $1.59 \times 10^4$ to $9.72 \times 10^4$ CN 100 mL$^{-1}$. All indicator concentrations rose as the MKE, MN, and KK rivers increased in flow. After the peak of the hydrograph in the urban rivers, the human bacterial signal decreased, but the traditional indicators remained elevated. In selected events, we measured the ruminant marker and found that concentrations increased at the estuary approximately one day following the start of the rain event and increased as the human indicators decreased. The ruminant marker was detected in all sampled rain events when the MKE River was a large contributor of flow to the estuary, indicating that agricultural inputs from the upper reaches of the watershed likely impacted estuary and nearshore waters.

**Relationships between *E. coli*, enterococci, and human-associated indicators in untreated sewage and contaminated waters.** We compared human-associated indicators with traditional indicators measured by qPCR. During the large CSO event, the HB concentration was 14-fold higher than *E. coli* and 3-fold higher than enterococci. For the event with a lower CSO release volume, the HB concentration was only 5 times higher than *E. coli* and half the concentration of enterococci. These results demonstrate that there is an increasing human signal compared to traditional indicators with known amounts of increasing sewage. However, during rain events with no reported sewage overflow, as well as during baseflow conditions, ratios of HB to *E. coli* and to enterococci were significantly lower than under CSO conditions ($p < 0.05$ and highly variable across the hydrograph (Figure 4), indicating that a mixture of human and nonhuman sources were present, with human sources dominating for only a short time at the peak of the hydrograph. While the overall trends in these ratios were significant, individual samples had high variability (Figure 4). However, when considering the ratios of HB CN and cultured traditional indicators, we observed a different pattern. Rainfall and baseflow had higher HB CN per cultured cell of *E. coli* or enterococci than CSOs. Further, there was a larger disparity between HB CN and cultured traditional indicators overall compared with the differences between HB and traditional indicators measured by qPCR.

**Discussion**

Frequent detection of sewage in estuaries and on beaches (Ferguson et al., 1996; Bower et al., 2005; Korajkic et al., 2011; Johnston et al., 2013; Templar et al., 2016) illustrates the ongoing challenges of maintaining adequate sanitation infrastructure in dense urban areas. Sewer infrastructure networks can comprise more than 10,000 miles of sanitary sewer pipes in a large city. One study reported that up to 4% of sanitary sewer pipes may be at risk for failing (Baah et al., 2015). There are multiple pathways for sewage to escape these systems, including illicit connections or leaking pipes (O’Shea and Field, 1992; Marsalek and Rochfort, 2004). In the latter case, contamination may be mobilized from surrounding soils after rain events and infiltrate ground water (Yau et al., 2014), drinking water (Hunt et al., 2010) and adjacent stormwater systems (Sauer et al., 2011). In cases of heavy rain, systems can become inundated with rainwater and overflow causing CSOs or SSOs. In this study, we have demonstrated that there is a quantifiable pulse of sewage released from an urban area each time it rains, resulting in contamination levels that create a potential public health risk.

**Quantifying sewage inputs from urban areas**

We found that HB and Lachno2 were at predictable concentrations in untreated sewage, which offered the opportunity to benchmark environmental contamination against the equivalent volume of untreated sewage. Previous work in our laboratory demonstrated that levels of human fecal bacteria in untreated sewage from 71 different cities across the US were relatively consistent (Newton et al., 2015), and that levels at the Milwaukee study site were stable over a three-year period (McLellan et al., 2013). Human *Bacteroides* as defined by the HF183 genetic marker (Bernhard and Field, 2000) or the HB marker (Sauer et al., 2011) and Lachno2 have been detected widely in field studies (Ahmed et al., 2008; Newton et al., 2011; Jarde et al., 2018). The HF183, HB, and Lachno2 are highly abundant in humans but are not strictly specific, as these markers have been found sporadically in other hosts such as dogs and deer (Boehm et al., 2013; Fisher et al., 2015). Using the two genetic markers in combination is expected to improve reliability, as cross-reacting hosts are unlikely to have both indicators (Fisher et al., 2015; Wang et al., 2010). The consistent occurrence of human-associated fecal indicators across the US suggests that urban areas can be compared to each other, and quantitative water quality criteria could be developed based on new indicators of sewage contamination. In other regions of the world, differences in the human gut microbiome (influenced by diet and other factors) may determine which indicators are most useful (Muegge et al., 2011; Walker et al., 2011; Reischer et al., 2013; Koskey et al., 2014).

**Sewage signals following storm events**

High frequency sampling allowed us to quantify mean concentrations over long periods of time (i.e., 24 h or more), and hydrological measurements allowed us to quantify loads. As flow increased in the urban rivers, the concentrations of HB and Lachno2 human markers increased, sug-
Figure 3: Source-specific indicators (human) and general indicators (E. coli and enterococci) measured in the estuary. Samples were collected across the hydrograph during the CSO on 20 June 2009 (A) and the rain event on 15 May 2010 (B). Human fecal indicators, human Bacteroides and Lachnospiraceae (Lachno2), were detected at high concentrations following the peak of the discharge for the two urban rivers, the Menomonee and Kininnickinnic rivers. Hydrographs from all events are shown for culture and qPCR in Figures S3–13 and Figures S14–24, respectively. Lowess indicates locally weighted regression using the lowess function in R (R core team, 2017). Dates are day/month/year. DOI: https://doi.org/10.1525/elementa.301.f3
gesting rainfall intensities were a driver for transport of sewage into rivers. In rainfall events of greater than 5 cm in 24 or 48 h, there was a disproportionate increase in both peak mean 24-h concentrations and load. In addition, 12 h after the end of a CSO on 11 June 2011, there was an unexpected spike in human-associated indicators, suggesting that during this time of heavy rain unrecognized overflows in the sanitary sewage systems upstream might have contributed additional sewage. These results suggest that there may be a critical threshold for conveyance systems within a city. Establishing the sensitivity of a city to rainfall events could enable warning systems to advise the public to avoid risk of exposure, as well as help guide investments to improve capacity, particularly under changing climate conditions where storm events of increased intensity are predicted to increase in the northeast and Great Lakes regions of the US (USEPA, 2008; Trtanj et al., 2016).

**Comparison of traditional and human-associated indicators**

We examined patterns in the ratios of HB to traditional indicators over multiple hydrological conditions. Most notably, during rain events the ratios of HB to *E. coli* in river samples measured by qPCR were lower than in WWTP influent, which suggests that other fecal sources contributed *E. coli*, but not human markers, to the contaminated river water. These differences were illustrated in our high resolution sampling over the hydrograph, where ruminant sources dominated during the second half of the storm without a parallel drop in general indicators (Figures 3 and S3–S13).

The differences in indicators measured by qPCR (i.e., detection of intact cells) and culture (i.e., detection of viable cells) might provide clues as to the age of pollution. Microcosm studies have suggested that qPCR markers from different organisms are lost at a similar rate (Mattioli et al., 2017). However, decay of cultured indicators are expected to be more rapid than loss of qPCR signal. Differential decay can be influenced by temperature and sunlight (Korajkic et al., 2014; Maraccini et al., 2016), and sunlight has been shown to influence decay of viable cells more than molecular targets (Boehm et al., 2009; Green et al., 2011). Our results are consistent with this concept, as ratios of HB to cultured *E. coli* were greater than ratios of HB to qPCR-based *E. coli*. We also noted that recent pollution (i.e., from a CSO) had a smaller ratio of HB CN to

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**Figure 4: Ratios of HB human marker to traditional indicators measured by qPCR and culture methods.** Top panel truncates values above 50 to show detail of lower values. Lower panel shows full range of ratios, which has samples with HB to cultured *E. coli* or enterococci > 50. Sample date 4 June 2010 was excluded from analysis due to extremely high HB levels that were similar to sanitary sewer overflow conditions, but could not be confirmed. The enterococci copy number was divided by four, assuming four copies per cell, before calculating ratios of HB to enterococci by qPCR for visual comparisons with ratios of HB to enterococci by culture. *E. coli* qPCR targets the single-copy *uidA* gene. WWTP indicates wastewater treatment plant; CSO, combined sewer overflow. The width of the outlines for each data series is proportional to the relative density of points. DOI: https://doi.org/10.1525/elementa.301.f4
cultured _E. coli_ or enterococci than when pollution may have been in the environment longer (i.e., rain or baseflow samples). Multiple factors can influence the ratios of a host-associated indicator to traditional indicators in a single sample, including inputs from multiple sources, timing of human and nonhuman inputs, and differential decay of signals over time. As a result, the relationship between human-associated and traditional indicators cannot be interpreted in single samples; however, ratios may reflect a general trend in the system.

**Human-associated indicators of pathogens and waterborne illness risk**

Human markers were three to four orders of magnitude higher than virus concentrations in untreated sewage (Table 1), suggesting that human-associated indicators might be useful for estimating risk in surface waters with dilute amounts of sewage contamination. Pathogens are technically difficult and expensive to measure in the environment, and are often present at low levels (Gentry-Shields et al., 2012; Corsi et al., 2014); in cases where pathogen test results are negative, the pathogens could be present but below detection limit. In work conducted concurrently with the present study, viruses were sampled at the estuary during one of the CSO events (Lenaker et al., 2017); however, no viruses were recovered, despite known sewage contamination. Because virus occurrence in the human population is seasonal (Sedmak et al., 2005), the exposure pathway can be characterized by using genetic markers that are relatively stable in sewage (the primary reservoir for human waterborne pathogens), without depending on capturing virus occurrence at the sampling time.

The QMRA model indicates that we can expect greater than a 10% illness risk for swimmers exposed to river water after heavy rain in the absence of sewage overflows, based on the concentrations of HB and Lachno2 that we measured. Risk from CSO-contaminated water exceeded the upper bound of 10% in this analysis. The model results could be used to derive risk-based thresholds for these two indicators, which could be defined as the concentration at which the median simulated risk is 0.03, similar to the USEPA benchmark risk used for establishing recreational water quality criteria (USEPA, 2012b). Our estimates were very similar to what was reported in Boehm et al. (2015) for HF183 (4,200 CN 100 mL⁻¹), despite the two studies using different datasets for the human _Bacteroides_ marker in untreated sewage. In this study, norovirus was measured in the same samples as the HB marker, whereas Boehm et al. (2015) used previously published concentrations for norovirus that were independent of the samples in which the HF183 marker was measured. The human _Bacteroides_ HB and HF183 assays target the same organism, and both employ the HF183 primer, but utilize slightly different reverse primers and probes (Templar et al., 2016).

Recent research estimates that nearly 90 million incidents of waterborne disease occur annually in the US due to recreational water exposure (DeFlorio-Barker et al., 2018). Moving forward, the risk-based thresholds we have developed could be particularly valuable for understanding human health risks associated with recreational contact, as they offer a stronger scientific basis for inferring risk than general indicators. Estuaries and harbors are generally not used for swimming; however, kayaking, rowing, and other recreational activities are becoming more popular. Direct exposure would most likely occur from mishaps, when individuals become submerged in the water. Of higher concern may be urban beaches near harbors and river outlets (Wiegner et al., 2017). In these cases, hydrodynamic models or other predictive tools would be useful to further estimate the amount of river water delivered to a beach site. Future work should examine whether aging of the contamination affects the estimation of threshold levels, as they were derived in this study for the specific scenario where the sewage contamination is very recent. The differential survival and transport of viruses and bacteria may further affect risk assessment relationships that employ bacterial indicators. Directly measuring pathogens, or surrogate viruses common to humans, in high volumes of the river or beach water under known contamination conditions could be the next step towards developing site-specific criteria. While norovirus was detected throughout the year in this study, concentrations were higher in winter than summer; therefore, next steps towards evaluating risk could incorporate a seasonal component into risk modeling.

**Evaluating and managing urban waters**

In the US, the current method for responding to impaired waters is to implement total maximum daily load (TMDL) regulations. In this framework, fecal coliforms are targeted to reduce risk from pathogens. However, different sources of fecal coliforms do not carry the same risk (Soller et al., 2010b). Stormwater-impacted rivers have shown evidence of sewage contamination with only a modest correlation to fecal coliforms (Sauer et al., 2011; Templar et al., 2016), suggesting that both human and nonhuman sources were present. Many watersheds have upstream agricultural land use, which can contribute fecal pollution from agricultural animal sources, as well as from leaking septic systems (Verhougstraete et al., 2015). The presence of traditional indicators in the absence of a sewage or agricultural signal demonstrates that stormwater carries non-point pollution from urban wildlife and domestic pets, contributing fecal coliforms to waterways that are not considered as serious a health risk as sewage (Soller et al., 2010b). To effectively address pathogens, TMDL regulations need to be developed with an understanding of the source of contamination (He et al., 2007).

Urban waters are economic drivers in cities and are used increasingly for recreation. Higher resolution methods for identifying sewage sources could offer information on areas where pathogens are more likely to occur, and could provide evidence of potential risk. Here we quantified on a watershed scale the amount of sewage released from an urban area, creating risk to humans and the ecosystem. The ability to track this contamination using newly developed host-associated indicators advances our ability to assess and manage freshwater resources.
McLellan et al: Sewage contamination of urban coasts

Data Accessibility Statement
Data have been archived using Dash (University of California Curation Center) in the DataOne Project under the title of this manuscript; https://doi.org/10.15146/R3S962.

Supplemental Files
The supplemental files for this article can be found as follows:

- **Text S1.** Detection of norovirus. DOI: https://doi.org/10.1525/elementa.301.s1
- **Table S1.** Urban estuary samples collected under low-flow, rainfall, and rainfall with combined sewer overflows (CSOs). DOI: https://doi.org/10.1525/elementa.301.s1
- **Table S2.** Traditional host-associated qPCR assay primers, standard curves, and references. DOI: https://doi.org/10.1525/elementa.301.s1
- **Table S3.** Log_{10} mean and standard deviation (in log space) of HB, Lachno2, and norovirus. DOI: https://doi.org/10.1525/elementa.301.s1
- **Figure S1.** Box and whisker plots of risk of illness from norovirus given a concentration of HB in river water. DOI: https://doi.org/10.1525/elementa.301.s1
- **Figure S2.** Box and whisker plots of risk of illness from norovirus given a concentration of Lachno2 in river water. DOI: https://doi.org/10.1525/elementa.301.s2
- **Figures S3–S13.** Full set of hydrographs for events in Table 3 with human-associated indicators and culture data for traditional indicators. DOI: https://doi.org/10.1525/elementa.301.s2
- **Figures S14–S24.** Hydrographs with human-associated indicators with qPCR data for traditional indicators. DOI: https://doi.org/10.1525/elementa.301.s3

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