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The Identification and Quantification of Sewage Contamination in the Milwaukee Estuary

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ABSTRACT

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Under the Supervision of Professor Sandra McLellan

Sewage contamination from failing infrastructure and sewer overflows is a major environmental and human health concern in waterways, especially in urban communities bordering the Great Lakes such as Milwaukee, Wisconsin. Culture-based fecal indicator bacteria, such as *Escherichia coli*, enterococci, and fecal coliforms are traditionally used to indicate the presence of a human health risk due to fecal contamination. These indicators, however, fail to distinguish between sources of fecal contamination (human vs. non-human). Two human-specific fecal indicators, human *Bacteroides* and human *Lachnospiraceae*, were used to identify and quantify sewage contamination in the Milwaukee estuary, which discharges to Lake Michigan, as well as the Milwaukee, Menomonee, and Kinnickinnic Rivers immediately upstream.

Chapter 1 provides an overview of the health and environmental impacts of fecal pollution in waterways and the use of alternative indicators to track sewage pollution. Chapter 2 describes the concentrations of human fecal indicators, used as a proxy for human sewage, in the three urban rivers upstream of the Milwaukee estuary and how this information can be used for the implementation stage of the current fecal coliforms Total Maximum Daily Load (TMDL)

process. Chapter 3 describes how human fecal indicators were used to characterize sewage contamination across the hydrograph. Intensive monitoring at sites in the rivers and the estuary was used to calculate event loads for storm and combined sewer overflow events and investigate relationships between loads and the degree of watershed urbanization and the amount of rainfall during an event. Chapter 4 discusses how the information generated in this research can be used in the TMDL implementation process and can be used to focus efforts of local agencies and municipalities to investigate and remediate unrecognized sources of sewage contamination. More specific information about sources of fecal pollution will be useful to create appropriate water quality goals to address the human health concerns of sewage contamination.

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1. Introduction

1.1 The Milwaukee Estuary Area of Concern

The Milwaukee River Basin is an over 2,000 square kilometer watershed in southeastern Wisconsin, which drains to Lake Michigan via the Milwaukee Estuary in the heart of downtown Milwaukee, Wisconsin. The basin is located in portions of seven counties and is divided into six sub-watersheds. The Milwaukee River North, Milwaukee River East-West, and Milwaukee River South watersheds make up the entire length of the Milwaukee River. The other three watersheds in the basin—the Cedar Creek, Menomonee River, and Kinnickinnic River watersheds—are named after the major rivers that they contain. The Milwaukee River is the most diverse in terms of land use. The Milwaukee River North and East-West watersheds have rural land use with the majority of land used for agriculture, whereas the Milwaukee River South watershed is a mix of rural and urban uses, with one-third of the land classified as urban and the remaining classified as agriculture, grasslands, forests, and wetlands. The Cedar Creek watershed is similar to the northern Milwaukee River watersheds in that it has primarily rural land use. The Menomonee River watershed has a majority of the land classified as urban land use, followed by agricultural uses. The Kinnickinnic River watershed is the smallest and most urban, with much of the streams in this watershed modified by straightening and concrete lining (Wisconsin Department of Natural Resources, 2001).

The Milwaukee Estuary has been classified as an Area of Concern (AOC) since 1987. The eleven beneficial use impairments (BUIs) listed for the estuary include: degradation of fish and wildlife populations, loss of fish and wildlife habitat, degradation of benthos, restrictions on dredging, restrictions on fish and wildlife consumption, suspected bird, animal, and fish deformities or reproduction problems, beach closings and recreational restrictions, degraded

phytoplankton and zooplankton populations, eutrophication or undesirable algae, and degradation of aesthetics. Although loading of toxic substances such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and heavy metals, was one of the main drivers behind naming the Milwaukee Estuary as an AOC, point source and runoff pollution, habitat fragmentation, and impacts of urbanization also play a big role in many of the BUIs (Wisconsin Department of Natural Resources, 2012).

Total Maximum Daily Load (TMDL) allocations are underway for the Milwaukee Estuary and the Milwaukee, Menomonee, and Kinnickinnic Rivers (Wisconsin Department of Natural Resources, 2012). TMDL calculations are the maximum amount of pollutant that a water body can receive and still meet water quality standards (U.S. Environmental Protection Agency, 2002a). This involves the allocation of loads to both point sources and non-point sources of a particular pollutant, which has proved to be a problem in the Milwaukee Estuary due to the lack of information regarding non-point sources of bacteria loading (Wisconsin Department of Natural Resources, 2012). TMDLs are being calculated to address the loading of fecal indicator bacteria in accordance with the EPA's ambient and recreational water quality criteria (U.S. Environmental Protection Agency, 2012, 1986); however, there is a need for source-specific information in order to more accurately characterize non-point sources as well as the risk to human health associated with these sources (Benham et al., 2006).

1.2 Tracking of sewage pollution in Milwaukee

The city of Milwaukee is known for one of the largest modern day waterborne disease outbreaks of the pathogen *Cryptosporidium* in the United States (Mac Kenzie et al., 1994), leading to increased awareness of waterborne illness associated with fecal contamination not only in

Milwaukee, but the entire United States. This outbreak was proceeded by record heavy spring rains that brought agricultural runoff to Lake Michigan from the upper part of the watershed and triggered sewage overflows. Sanitary sewer overflows (SSOs) and combined sewer overflows (CSOs) are of concern as point source fecal pollution. According to the Environmental Protection Agency's Report to Congress on the Impacts and Control of CSOs and SSOs, about 850 billion gallons of untreated sewage is discharged annually into the United States' waterways by CSOs and 10 billion gallons from SSOs (U.S. Environmental Protection Agency, 2004). The densest urban areas of downtown Milwaukee have combined sewers, allowing the runoff from impervious surfaces to be treated with the sanitary sewage. In past years, this meant that heavy rain could easily result in a CSO; however, with the installation of the "Deep Tunnel" stormwater storage system in 1993 by the Milwaukee Metropolitan Sewerage District (MMSD), CSOs have drastically decreased from fifty to sixty overflows per year to an average of 2.4 per year from 1994 to 2013 (Behm, 2013). However, even in the absence of CSOs, chronic human sewage pollution has been identified in the Milwaukee Estuary (Newton et al., 2013, 2011). Stormwater runoff may be a major source of sewage pollution in Milwaukee's urban rivers (Sauer et al., 2011) due to sewage leaking from sanitary sewage lines and being flushed out with urban runoff during rainfall (McLellan and Sauer, 2009). Despite persistent sewage pollution in the rivers and harbor, Razak and Christensen (2001) found a significant water quality benefit after the installation of the Deep Tunnel, especially in the Menomonee River (Razak and Christensen, 2001).

1.3 Sources of waterborne pathogens and human health impacts

A common concern with recreational water and beach use is the possibility of illness due to waterborne pathogens. Beach closures and recreational restrictions are frequently listed BUIs for the Great Lakes' AOCs. In the United States, bacteria, protozoa, and viruses are the pathogens of greatest concern to human health (Arnone and Walling, 2007). A major source of these pathogens in urban areas is stormwater runoff, which has been associated with risks to human health (Gaffield et al., 2003; Haile et al., 1999). A significant association has been found between rainfall events and pediatric emergency room visits due to acute gastrointestinal illness, which suggests that waterborne pathogens are a definite risk to gastrointestinal illness in recreational water users (Drayna et al., 2010). Additionally, CSOs and SSOs are a source of pathogens in urban waters (U.S. Environmental Protection Agency, 2004) and have the potential to effect both recreational and drinking water sources (Marsalek and Rochfort, 2004).

1.4 Standard and alternative indicators of fecal pollution

The U.S. Environmental Protection Agency (USEPA) recommends the regulation of recreational waters and beaches using fecal coliforms, *Escherichia coli* (*E. coli*), and enterococci (U.S. Environmental Protection Agency, 2012). Because these fecal indicator bacteria (FIB) are present in the gastrointestinal tract of humans and most warm-blooded animals and are easily grown and quantified in the laboratory, they have been used as indicators of risk to human health in recreational waters for decades (McLellan et al., 2013). However, because of these characteristics, they also fall short of identifying the source of fecal pollution (human versus non-human) (Scott et al., 2002) and often fail to identify the occurrence of pathogens (Borchardt et al., 2004; National Research Council, 2004). Human pathogens are more likely to be present

in human fecal pollution, therefore non-human specific FIB can be unsuccessful at bringing to attention threats to human health in recreational or drinking water (Field and Samadpour, 2007; McLellan et al., 2013). Recent advances in molecular techniques have allowed for the microbial source tracking of more host-specific alternative fecal indicators. Fecal anaerobes of the order *Bacteroidales* and the family *Lachnospiraceae* are of interest as indicators of fecal contamination. Because culture techniques for isolation of these bacteria are difficult to perform, molecular techniques using quantitative polymerase chain reaction (qPCR) have been developed to detect, amplify, and quantify human-specific sequences of 16S rRNA genes (Bernhard and Field, 2000; Fremaux et al., 2009; Kreader, 1995; Newton et al., 2011). Quantitative PCR assays developed for human-specific *Bacteroides* and *Lachnospiraceae* have shown a tight correlation with each other in sewage influent and Milwaukee estuary samples, which suggests that using them in tandem could provide a more accurate picture of human sewage contamination rather than using one on its own (Newton et al., 2011).

2. Quantification of human fecal indicators reveal urban watershed sources of sewage to Lake Michigan

2.1 Abstract

Sewage contamination of urban waterways from failing infrastructure and sewer overflows is a major environmental and public health concern. Fecal coliforms are commonly employed fecal indicator bacteria, but they fail to distinguish between sources of fecal contamination (human vs. non-human). Human *Bacteroides* and human *Lachnospiraceae*, two human fecal indicators, were used to identify sewage signals in two urban rivers and an estuary that drains to Lake Michigan. Grab samples were collected from the rivers throughout 2012 and 2013 and hourly samples were collected in the estuary across the hydrograph during summer 2013. Human *Bacteroides* and human *Lachnospiraceae* were highly correlated with each other in river samples (Pearson's r = 0.86), with average concentrations at most sites elevated during wet weather. However, no statistically significant differences were found between concentrations of human fecal indicators at baseflow and wet weather events at all but three sites, indicating that sewage contamination is chronic in these waterways, even at baseflow. Fecal coliforms are used for determining total maximum daily loads (TMDLs) in management plans, however this indicator alone often fails to recognize potential health risks. Fecal coliform concentrations did not have a strong correlation with human fecal indicator concentrations. Of 197 samples collected, a total of 84% of samples (n=64) with >1,000 CFU/100 ml fecal coliforms had sewage contamination; however, a similar number of samples (n=60) with moderate (200 to 1,000 CFU/100 ml) or low (<200 CFU/100 ml) fecal coliform levels also had evidence of human sewage. Analysis of human fecal indicator loading in the Milwaukee estuary revealed storm driven sewage loading varied greatly among events and was highest during an event with a short duration of intense rain. Further analysis of

sewage sources in Milwaukee's urban tributaries of the watershed is needed to determine relationships between land use, storm characteristics and other factors that drive sewage contamination in urban waterways.

2.2 Introduction

Fecal pollution is a growing concern in urban waterways, especially those that have recreational value. A common concern with recreational water, including beaches and rivers used for boating, is the possibility of illness due to waterborne pathogens following exposure to contaminated water. In the United States, bacteria, protozoa, and viruses are the pathogens of greatest concern to human health (Arnone and Walling, 2007). Stormwater which can be contaminated by sanitary sewage from leaking sewer lines or cross connections, has been shown to be a source of pathogens in urban areas (Sauer et al., 2011; Sercu et al., 2011, 2009) and has been associated with risks to human health (Gaffield et al., 2003; Haile et al., 1999). Additionally, combined sewer overflows (CSOs) and sanitary sewer overflows (SSOs) are a source of pathogens in urban waters (U.S. Environmental Protection Agency, 2004) and have the potential to effect recreational and drinking water sources (Marsalek and Rochfort, 2004). A significant association has been found between extreme rain events and gastrointestinal illness, which suggests precipitation increases waterborne pathogens in the environment (Curriero et al., 2001; Drayna et al., 2010).

Fecal coliforms, *Escherichia coli* (*E. coli*), and enterococci have historically been used to monitor rivers and recreational beaches for fecal pollution (U.S. Environmental Protection Agency, 2012, 1976). Because these fecal indicator bacteria (FIB) are present in the gastrointestinal tract of humans and most warm-blooded animals, and are easily grown and quantified in the laboratory, they have been used as indicators of risk to human health in

recreational waters for decades (Dufour and Schaub, 2007; McLellan et al., 2013). However, because of these same characteristics, they also fall short of identifying the source of fecal pollution as human or non-human (McLellan and Eren, 2014) and often fail to specifically indicate the occurrence of pathogens (Field and Samadpour, 2007; McLellan et al., 2013; National Research Council, 2004). Identifying sources that are most likely to carry pathogens is important to prioritize management strategies for mitigating fecal pollution. Several studies have had success employing molecular techniques using quantitative polymerase chain reaction (qPCR) to detect and quantify alternative indicators for human-specific fecal pollution (Ahmed et al., 2010; Converse et al., 2011; Newton et al., 2013; Nshimyimana et al., 2014). Specifically, qPCR assays developed for human-specific *Bacteroides* (HF183) and *Lachnospiraceae* (Lachno2) have exhibited a tight correlation in sewage influent and freshwater harbor samples, which suggests that using them in tandem could provide a more accurate picture of human sewage contamination rather than using one on its own (Newton et al., 2011).

Many coastal Great Lakes cities experience fecal pollution, which threatens recreational water quality in nearby rivers and beaches. The metropolitan area of Milwaukee, Wisconsin is located on the shores of Lake Michigan, where the Milwaukee, Menomonee, and Kinnickinnic Rivers drain into the lake via the Milwaukee estuary. The Milwaukee River has the largest drainage area and has mainly agricultural land uses, but becomes highly urbanized near the mouth of the river. The Menomonee River is a smaller drainage area with mainly urban land uses, but is influenced by some agricultural land uses and natural space. The Kinnickinnic River is the smallest and most urban river with more than half of its drainage area covered with impervious surfaces. The densest urban areas of downtown Milwaukee have combined sewers, allowing the runoff from impervious surfaces to be treated with the sanitary sewage. Even in the

absence of a CSO, chronic human sewage pollution has been identified in the Milwaukee estuary (Newton et al., 2013, 2011). Stormwater may be a major source of sewage pollution in Milwaukee's urban rivers (Sauer et al., 2011) due to sewage leaking from sanitary sewage lines and being flushed out with urban runoff during rainfall.

In this chapter, we evaluated the degree of human sewage contamination in the two most urbanized watersheds and the Milwaukee estuary by analyzing samples collected year-round and during a variety of weather conditions. This chapter seeks to characterize human fecal contamination at assessment points that are used in total maximum daily load (TMDL) assessments and demonstrate the value of using alternative fecal indicators to assess human health risks. To gain a watershed scale assessment of sewage contamination, automated sampling was incorporated to collect samples at regular intervals across the hydrograph in the Milwaukee estuary throughout a variety of weather conditions in 2013. These measurements allowed us to examine both quantitative loads entering Lake Michigan and human fecal indicator dynamics during storm events. This chapter demonstrates that human-specific indicators can be especially useful in the TMDL implementation process for identifying and prioritizing river reaches based on human health risks that may not be revealed by using traditional FIB alone. Additionally, continuous sampling provides estimates of sewage loadings from urban areas and is useful for determining storm-driven sewage patterns.

2.3 Materials and Methods

2.3.1 Study area and sampling methods

The Milwaukee estuary is the confluence of three major rivers, which drain to Lake Michigan. The Kinnickinnic (KK) River has the smallest, most urban watershed. The

Menomonee (MN) River has a larger, mainly urban watershed, with some agricultural land uses in its headwaters. The Milwaukee (MKE) River has the largest drainage area and is the most diverse in the terms of land use. The characteristics of these watersheds are provided in Table 1. Land use and impervious surface percentages were determined for each watershed using National Land Cover Database 2011 data (Jin et al., 2013; Xian et al., 2011). The primary focus of this study was the urbanized KK and MN Rivers. Four sampling sites were located on the KK River, five sites on the MN River (including one reference site upstream of urban land use), and one site was included below the confluence of the MN River and the MKE River, near the Milwaukee estuary. Grab samples were collected by the Milwaukee Metropolitan Sewerage District (MMSD) in 2012 and 2013 as a part of their long-term monitoring program. Samples were collected approximately once per month during a variety of weather conditions from June 2012 through August 2013. Samples were collected in one liter Nalgene bottles at the water surface in the center of the river channel and immediately placed on ice. All samples were filtered and incubated within six hours of collection for *E. coli*, enterococci, and total fecal coliforms using culture-based methods. Approximately 200 grab samples from the ten sites were analyzed for human fecal indicators by qPCR.

An automated sampler was used to collect hourly composite samples immediately downstream from the confluence of the MKE, MN, and KK Rivers in the Milwaukee estuary. Samples were collected with an automated sampler throughout event hydrographs for selected runoff events from May through September in 2013. A Teledyne ISCO 3700 full size portable sequential sampler was used for automated sampling at this site. The sampler was housed within the U.S. Geological Survey (USGS) monitoring station at Jones Island water reclamation facility in Milwaukee, Wisconsin. The sampler was programmed to collect 250 ml every 15 minutes for

up to 24 hours into one-liter polypropylene bottles, meaning each one-liter bottle represented a composite one-hour sample. During storm events, the sampler was generally activated prior to rainfall and collected samples until at least 24 hours following rainfall. Two sample bottles were combined into one, two-liter Nalgene bottle when the samples were collected, resulting in eight sub-samples. Samples were immediately placed on ice and taken back to the lab for analysis. One-liter sample bottles were sanitized with deionized water and replaced in the sampler after each sample was removed. A total of 118 samples from the Milwaukee estuary were analyzed for human fecal indicators by qPCR. Seven events, with sampling conducted over one to four days, were selected for analysis to represent a variety of precipitation events.

Table 1: Watershed characteristics of three major metropolitan Milwaukee, Wisconsin waterways which drain to Lake Michigan via the Milwaukee estuary. Base data from the National Land Cover Database (2011) Land Cover and Percent Developed Imperviousness datasets.

River	Drainage Area (mi^2)	Percent Impervious Surfaces	Percent Urban	Percent Agriculture	Percent Natural Areas[a]
Milwaukee	700	7	20	50	30
Menomonee	136	29	69	17	14
Kinnickinnic	25	52	98	0	2

[a] Forests, grasslands, wetlands, and open water are classified as natural areas

2.3.2 Culture-based analysis

All samples were analyzed within six hours for total fecal coliforms using standard methods (American Public Health Association et al., 2003). Each sample was filtered through a 0.45-µm-pore-size nitrocellulose filter (0.47-mm diameter; Millipore, Billerica, MA), placed on mFC

agar, and incubated for 24 hours at 44.5°C. After 24 hours, plates were removed from the incubator and counted for colony forming units (CFU).

2.3.3 DNA extraction and qPCR quantification of fecal indicators

Each sample was filtered and archived for future DNA extraction. A volume of 200 ml of each sample was filtered onto a 0.22-µm-pore-size mixed cellulose esters filter (47-mm diameter; Millipore, Billerica, MA). Filters were then folded and placed in 2-ml screw-cap tubes and immediately stored at -80°C until extraction. Frozen filters were broken into small pieces using a metal spatula. DNA was extracted from the fragmented filters using the MPBIO FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Anna, CA) and eluted using 150µL of DNase/Pyrogen-Free Water (DES).

Quantitative PCR was carried out using an Applied Biosystems StepOne Plus™ Real-Time PCR System Thermal Cycling Block (Applied Biosystems; Foster City, CA) with Taqman hydrolysis probe chemistry. Previously published primers and probe were used for the human *Bacteroides* (HF183) assay (Kildare et al., 2007) with the exception that the HF183F was used as the forward primer (Bernhard and Field, 2000). Analysis for human *Lachnospiraceae* (Lachno2) followed previously published methods for the Lachno2 assay (Newton et al., 2011). Standard curves were created during each run and consisted of a linearized plasmid containing the targeted gene sequence. Standard curves were run with DNA serially diluted from $1.5x10^6$ to $1.5x10^1$ copies per reaction. Standards were run in triplicate and each sample was run in duplicate in a final volume of 25µL with a final concentration of 1µM for each primer, 80nM for the probe, 5µL of sample DNA, and 12.5µL of 2X Taqman® Gene Expression Master Mix Kit (Applied Biosystems; Foster City, CA). Amplification consisted of the following cycles: one cycle at 50°C

for two minutes to activate the uracil-N-glycosylase (UNG), then one cycle at 95°C for ten minutes to inactivate the UNG and activate the Taq polymerase, and 40 cycles of 94°C for 15 seconds followed by one minute at 60°C.

Each qPCR reaction results in a raw number of copies (CN) per reaction. The number of copies is then converted to a concentration of CN/100 ml of original sample based on the proportion of the sample used in the reaction. For all river and estuary samples, 200 ml was filtered. We determined the limit of reliable quantification was 15 copies per reaction, or 225 CN/100 ml. Therefore, any samples which show positive amplification, but are below 15 copies are reported as below the limit of quantification (BLQ). For all statistical analyses and load calculations, BLQ samples were given a concentration of 225 CN/100 ml.

2.3.4 Determination of sewage benchmarks

We set thresholds for a positive sewage detection to be HF183 concentrations of 1,000 CN/100 ml or Lachno2 concentrations of 1,500 CN/100 ml based on the distribution of these genetic markers in stormwater samples collected from the Milwaukee urban area (Sauer et al., 2011) and concentrations of human viruses in comparison to alternative indicators (Newton et al., 2011). Sauer et al., 2011 found that human fecal indictors in stormwater outfall samples were nearly ubiquitous and outfalls with >1,000 CN/100 ml HF183 were consistently positive for human sewage (Sauer et al., 2011). These concentrations equate to approximately 0.003% of the average concentrations of HF183 and Lachno2 in sewage influent, which was determined by analysis of 94 influent samples collected at Jones Island and South Shore wastewater reclamation facilities in Milwaukee, WI from 2008-2013. Results from a subset of these samples were presented in Newton et al., 2011. In our analysis, samples with at least one human fecal indicator exceeding

the threshold were considered positive for human sewage. Five samples (2.5% of the dataset) were removed from the analysis because one human fecal indicator was absent while the other was above the sewage threshold. These inconsistent results suggest cross reactivity, differential survival, or other interfering factors.

2.3.5 Characterization of samples collected at baseflow

Each river grab sample was categorized as either collected at baseflow or during wet weather, meaning the majority of flow could be attributed either to baseflow or overland-runoff. Baseflow separation was carried out by the EcoHydRology package in the R suite of statistical packages (Fuka et al., 2015), which uses the recursive digital filter method for the separation of baseflow from quickflow (Nathan and McMahon, 1990). Grab samples were considered to be collected at baseflow when the ratio of daily baseflow to instantaneous discharge was greater than or equal to 0.80. Additionally, the time lag for runoff in the watershed was estimated by calculating the drainage area in square miles raised to the exponent 0.2 (Viessman et al., 1977). If a sample determined to be collected at baseflow was collected within this time lag of the hydrograph's peak, the sample was not considered to be collected at baseflow. Runoff time lags were calculated at 1.8 days in the KK River, 2.6 days in the MN River, and 3.7 days in the MKE River. This method for characterizing samples collected at baseflow was adapted from Corsi et al., 2013.

2.3.6 Determination of total event rainfall depths

Average rainfall accumulation for the Milwaukee estuary watershed, which includes the MKE, MN, and KK River watersheds, was determined using radar-indicated rainfall models, retrieved

from the National Weather Service North-Central River Forecast Center (National Weather Service, 2015). ESRI ArcGIS software was used to delineate the watershed for the Milwaukee estuary, using the location of the auto-sampler at Jones Island wastewater reclamation facility as the outlet point. The lower-Milwaukee River watershed was used to represent the urban influenced area of the watershed and average one-hour rainfall accumulation was computed for the defined area.

2.3.7 Statistical analysis

Results which were below the limit of quantification were assigned a value equal to the limit of quantification for statistical analysis. The Pearson's correlation (r) was used on log-transformed data to test the correlation between human fecal indicators. Correlations between human fecal indicators and other water quality indicators were tested using the Spearman's rank correlation (rho). The Wilcoxon rank sum test was used to determine whether significant differences exist between concentrations of human fecal indicators collected at baseflow compared to wet weather. Tests were considered significant at $p \leq 0.05$. The R suite of packages (R Core Team, 2015) were used for all statistical analyses.

2.3.8 Loads and maximum 24-hour calculations

Loads for individual samples collected continuously across the hydrograph in the Milwaukee estuary were calculated by multiplying river discharge by bacteria concentration (Porterfield, 1972). Velocity and water level data were retrieved from the USGS station 04087170 Milwaukee River at mouth at Milwaukee, WI. To characterize the bi-directional flow of the Milwaukee River estuary, velocities were measured using acoustic doppler current

profiling instrumentation (SonTek, San Diego, CA). Velocity was measured separately on both sides of the river channel (north and south). River discharge was calculated by multiplying the average of the north and south velocity measurements by the cross-sectional area of the channel at the time of measurement. The cross-sectional area was computed using a base area that was determined by the ADCP and adjusted for variations in water level. Maximum 24-hour mean concentrations and maximum 24-hour mean loads were computed for each event sampled in the Milwaukee estuary by taking a running 24-hour average of the instantaneous concentrations and loads for each event and selecting the largest value.

2.4 Results

2.4.1 Concentrations of human fecal indicators in river samples in metropolitan Milwaukee

Samples collected in the KK River and downstream MN River demonstrated chronic contamination from human fecal sources (Figure 1). At baseflow conditions, 70% of samples in the upstream and 80% of samples in the downstream KK River sites were considered positive for human sewage. Similarly, samples collected during wet weather were 73% positive for human sewage in both the upstream and downstream KK River sites. Of the downstream MN River sites, 59% of samples were positive for human sewage at baseflow, and this increased to 81% positive for samples collected during wet weather.

The upstream MN River sites and the MKE River site did not demonstrate chronic contamination. None of the samples collected from the upstream MN River sites and only 25% of MKE River samples were positive for sewage at baseflow. During wet weather, 33% of samples in both the upstream MN River sites and the MKE River site were positive for sewage. The low concentrations of human fecal indicators measured at the urbanized MKE River site,

below the confluence of the MKE and MN Rivers, is likely due to the large diluting flow delivered from the MKE River and backflow from Lake Michigan.

Overall there were statistically significant differences between baseflow and wet weather concentrations among all samples ($p<0.05$). Comparisons of concentrations among sites, however, demonstrated no statistically significant differences between baseflow and wet weather concentrations at most sites, with the exception of three of the sampling sites in the MN River (Figure 2). MN River sites MN RI-22, MN RI-09, and MN RI-20 were the only sites to demonstrate a statistically significant difference in concentrations of one or both human fecal indicators when comparing baseflow and wet weather conditions ($p<0.05$).

When samples collected in the upstream and downstream sites of the MN River were compared, concentrations in the downstream sites of the MN River were statistically greater than those in the upstream MN River sites during both rain and baseflow conditions ($p<0.05$). When KK River samples were compared in the same way, no statistically significant differences were found. KK River sites KK RI-33, 34, and 35 were found to have greater average concentrations at baseflow than during wet weather.

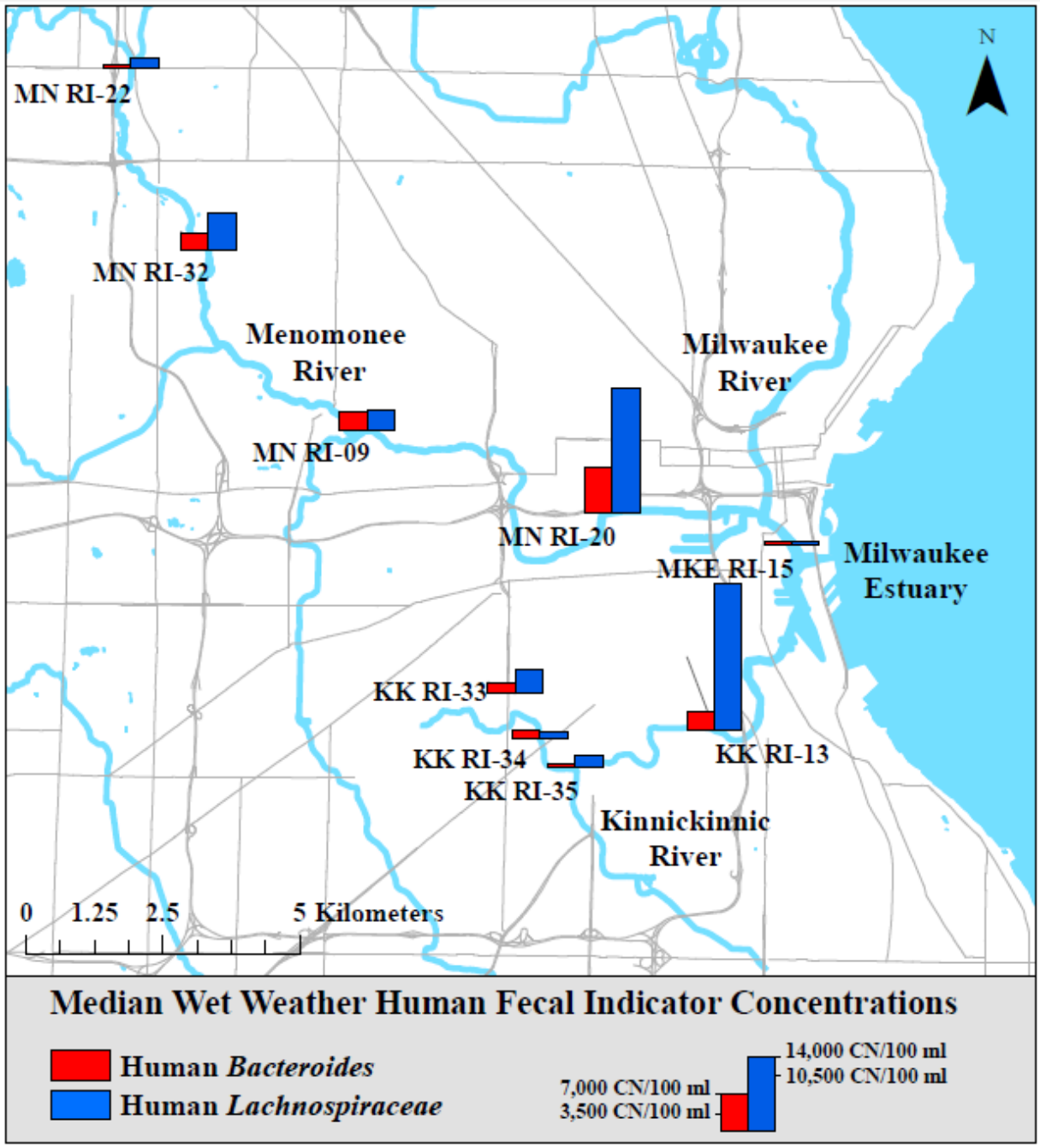

Figure 1: Median concentrations of Human *Bacteroides* and human *Lachnospiraceae* during wet weather on rivers in metropolitan Milwaukee, Wisconsin in 2012 and 2013. MN RI-36 is approximately 14 miles upstream of MN RI-22 and is not shown in this figure.

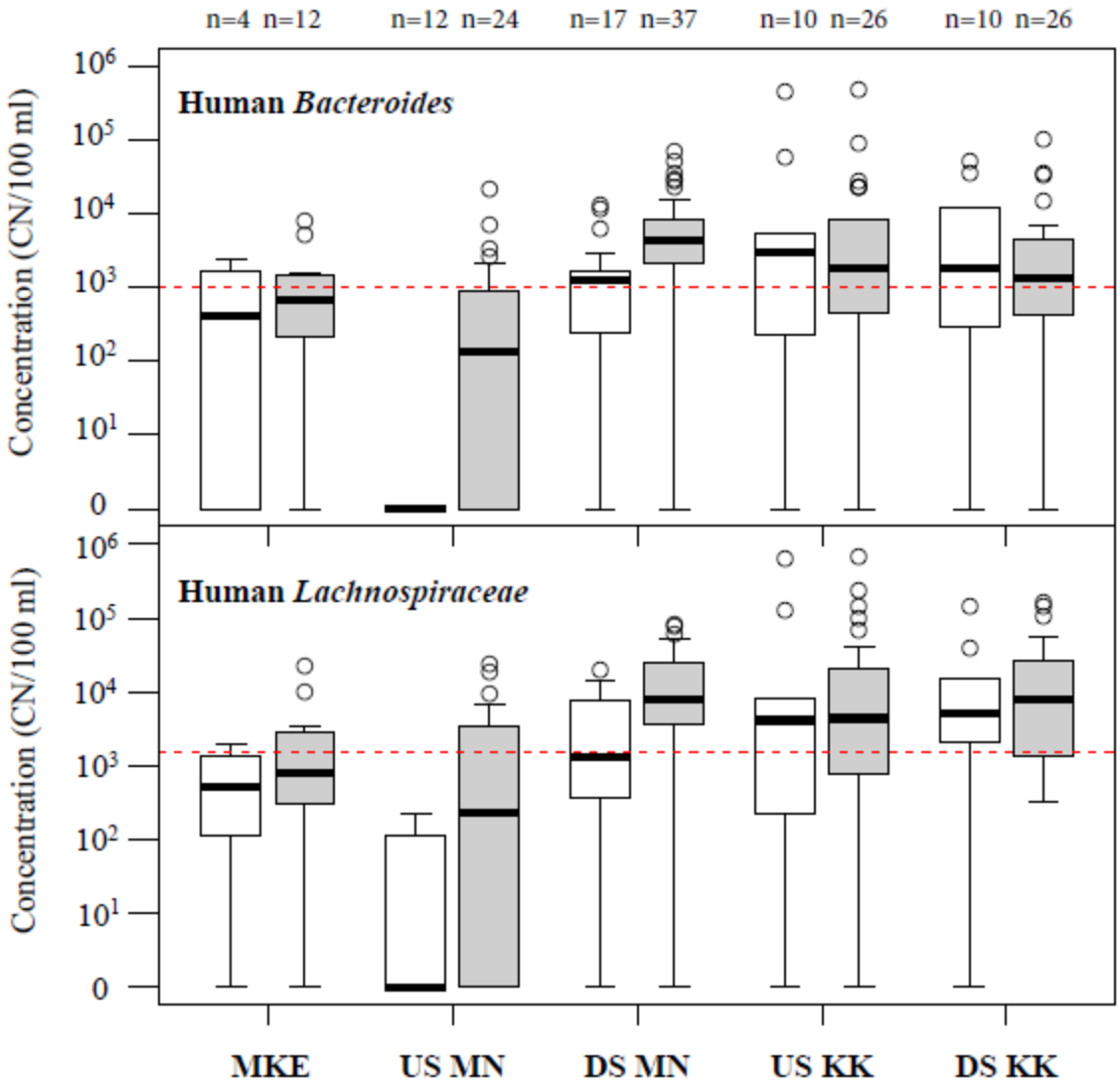

Figure 2: Human fecal indicator concentrations at baseflow (white plots) and wet weather (gray plots) in metropolitan Milwaukee, Wisconsin in 2012 and 2013. Dotted red lines represent sewage thresholds for human *Bacteroides* (1,000 CN/100 ml) and human *Lachnospiraceae* (1,500 CN/100 ml). The Milwaukee (MKE) River site MKE RI-15 is denoted as MKE. Upstream and downstream Menomonee (MN) River sites are denoted as US MN and DS MN, respectively. US MN sites include MN RI-36 and 22. DS MN sites include MN RI-32, 09, and 20. Upstream and downstream Kinnickinnic (KK) River sites are denoted as US KK and DS KK, respectively. US KK sites include KK RI-33 and 34. DS KK sites include KK RI-35 and 13.

2.4.2 Consistency of both human *Lachnospiraceae* and human *Bacteroides* detection in river and estuary samples

Detection of human sewage was supported by consistent detection of two independent human fecal indicators. The Lachno2 and HF183 indicators in river and estuary samples were highly correlated (Spearman's rho = 0.92 river; 0.91 estuary). Out of 197 river grab samples used in the analysis, both Lachno2 and HF183 were detected in 74% of samples and were absent in approximately 14% of samples (Table 2). The remaining 12% of samples had inconsistent detection of human fecal indicators, meaning there were detectable levels of one human fecal indicator but not the other. In 19 samples, Lachno2 was present while HF183 was absent; however, Lachno2 concentrations were generally low in these samples, ranging from below the limit of quantification (15 copies per reaction) to 83 copies per reaction, which is equivalent to a concentration of 1,200 CN/100 ml. In only 4 samples, HF183 was detected and Lachno2 was not. These samples also had consistently low concentrations of HF183, ranging from 16 copies per reaction (250 CN/100 ml) to 56 copies per reaction (840 CN/100 ml). A total of five samples (not included in Table 2) had one human fecal indicator absent and the other detected at a concentration greater than the benchmarks considered positive for sewage and were not used in the analyses.

Of the 118 estuary samples (each consisting of eight sub-samples) collected by the automated sampler, 74% were positive for both human fecal indicators and 19% negative for both. Similar to the grab samples, the remaining 7% of samples had inconsistent detection of human fecal indicators and concentrations of these samplers were near the limit of quantification (Table 2).

Of the samples with both human fecal indicators present, Lachno2 was on average 3 (± 3) times higher than HF183 in river samples and 2 (± 2) times higher than HF183 in samples collected in the Milwaukee estuary. The ratio of Lachno2 to HF183 in sewage influent is approximately 2 (± 3), based on 94 influent samples collected at Jones Island and South Shore wastewater reclamation facilities in Milwaukee, WI from 2008-2013.

Table 2: Number and percentage of total river and estuary samples collected in metropolitan Milwaukee, Wisconsin in 2012 and 2013, which were found to have both human fecal indicators (human *Bacteroides* (HF183) and human *Lachnospiraceae* (Lachno2)) present, both absent, and those with inconsistent human fecal indicator detection.

	Lachno2 and HF183 Present	**Lachno2 Present HF183 Absent[a]**	**Lachno2 Absent HF183 Present[a]**	**Lachno2 and HF183 Absent**	**Total**
Number of River Samples [% of Total]	147 [74%]	19 [10%]	4 [2%]	27 [14%]	197
Number of Estuary Samples [% of Total]	88 [74%]	7 [6%]	1 [1%]	22 [19%]	118

[a] Concentrations were generally near the limit of quantification

2.4.3 Correlations between human-specific indicators and a standard fecal indicator

Of 197 river samples collected at ten sites, both human fecal indicators were moderately correlated with fecal coliforms using a Spearman correlation (HF183 rho=0.41, Lachno2 rho=0.43, $p<0.05$). Of these samples, 39% had fecal coliform concentrations greater than 1,000 CFU/100 ml, which is the level set as a variance standard for surface water in Wisconsin. The water quality standard for fecal coliforms is 200 CFU/100 ml, and 37% of samples exceeded this limit but were below the 1,000 CFU/100 ml variance standard. Only 24% of the river samples actually met both water quality standards for fecal coliforms.

. Samples with concentrations exceeding the variance standard for fecal coliforms were ranked as “high”, those which met the water quality criteria for fecal coliforms were considered “low”, and samples with concentrations falling between the standards were considered “moderate”. Of the samples with high fecal coliforms levels, 84% were positive for sewage. Samples with moderate fecal coliforms levels were 52% positive for sewage, and those with low fecal coliforms levels were 48% positive for sewage (Figure 3). Some patterns exist as to how these samples are distributed among sampling locations. Specifically, samples with low fecal coliforms and evidence of sewage contamination occurred most often in the KK River, and samples with moderate fecal coliforms that were sewage positive were found more often at downstream sites. Samples with the highest human fecal indicator concentrations did not necessarily have the highest fecal coliform concentrations (Figure 4). Notably, among samples meeting the water quality standards for fecal coliforms, nearly half were positive for human sewage.

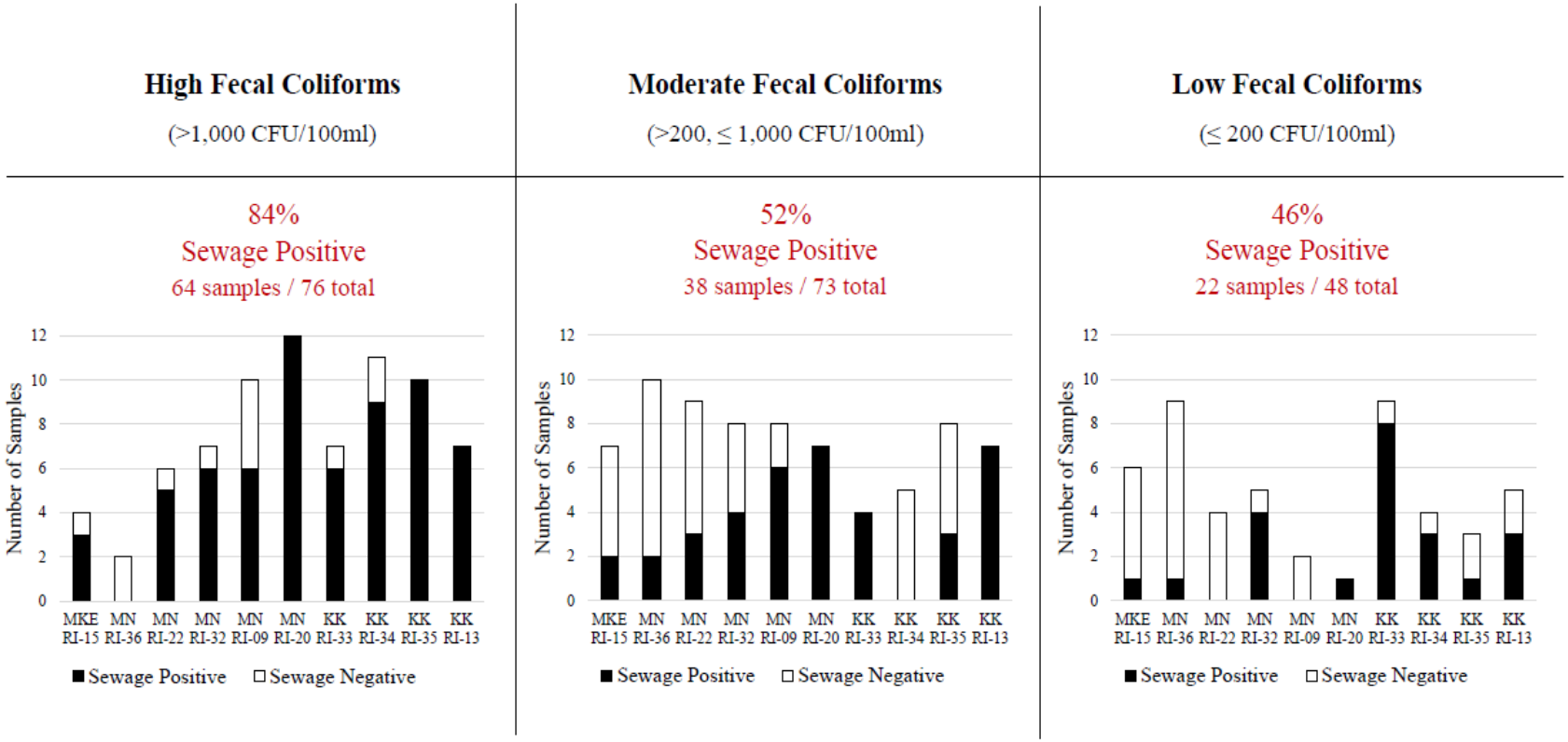

Figure 3: Distribution of sewage positive samples under three different fecal coliform concentration levels, across sampling locations in the Milwaukee (MKE), Menomonee (MN), and Kinnickinnic (KK) Rivers in metropolitan Milwaukee, Wisconsin in 2012 and 2013. Sites are listed from upstream to downstream in each river.

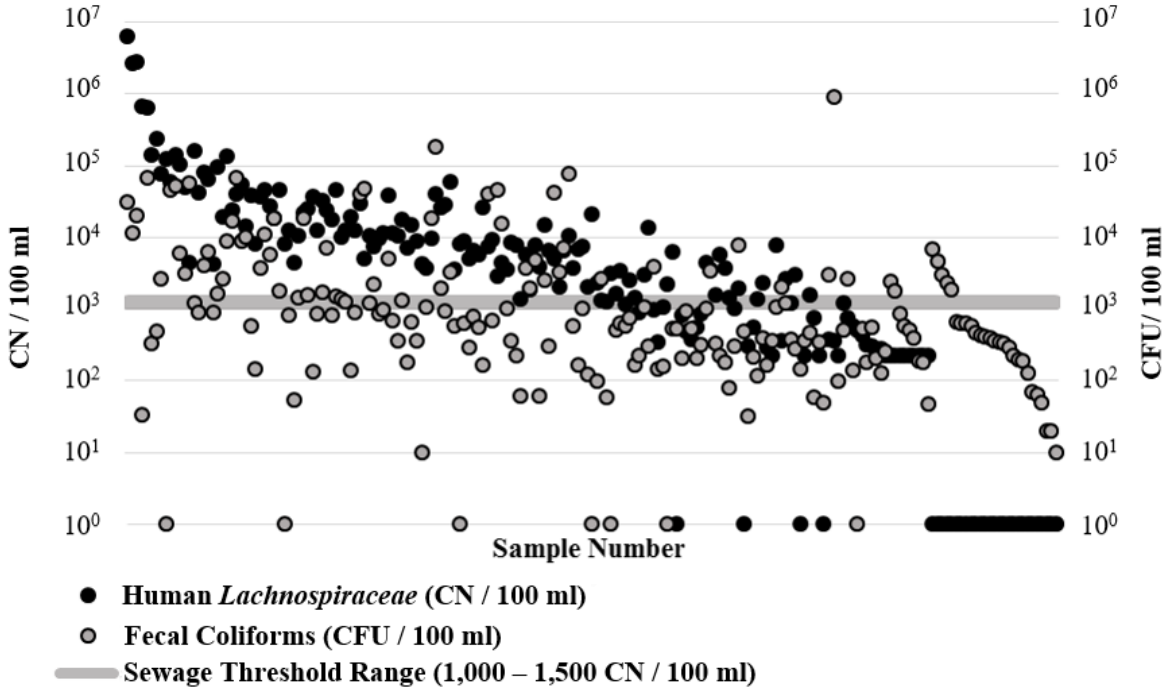

Figure 4: Human *Lachnospiraceae* concentrations from river samples collected in metropolitan Milwaukee, Wisconsin in 2012 and 2013, plotted in order of high to low human *Bacteroides* concentrations with corresponding fecal coliform concentrations.

2.4.4 Correlations between human-specific indicators and nutrient measures

Human fecal indicators were compared to a variety of physical and water quality measures. Turbidity, ammonia (NH_3), and chloride were specifically compared because they are measures commonly associated with the presence of sewage contamination. Samples were binned into three categories based on the weather conditions during or prior to sampling: baseflow, wet weather, and CSOs. Spearman's rank correlations were determined between human indicators and turbidity, NH_3, and chloride for each weather condition (Table 3).

NH_3, turbidity, and chloride did not have consistent correlations with human indicators and in only a few specific situations were correlations found to be significant. The strongest correlations were found between both human fecal indicators and NH_3 following a CSO.

Moderate, statistically significant correlations were found between turbidity and chloride during wet weather. During baseflow sampling, however, no water quality measures appeared to be good predictors for human fecal indicators. Samples were also categorized based on whether the sampling sites were located within metropolitan Milwaukee's combined sewer area or within the separated sewer area. In the combined sewer area during wet weather sampling, significant negative correlations were found between NH_3 and human fecal indicators (HF183 rho = -0.34, $p<0.05$; Lachno2 rho = -0.50, $p<0.05$) and significant positive correlations were found between chloride and human fecal indicators (HF183 rho = 0.37, $p<0.05$; Lachno2 rho = 0.50, $p<0.05$). Additionally, in the separated sewer area during wet weather sampling, significant positive correlations were found between all three water quality measures and both human fecal indicators (NH_3-HF183 rho = 0.28, $p<0.05$; NH_3-Lachno2 rho = 0.25, $p<0.05$; turbidity-HF183 rho = 0.37, $p<0.05$; turbidity-Lachno2 rho = 0.40, $p<0.05$; chloride-HF183 rho = 0.37, $p<0.05$; chloride-Lachno2 rho = 0.43, $p<0.05$).

Table 3: Spearman's rank correlations (rho) between human *Bacteroides* (HF183), human *Lachnospiraceae* (Lachno2), ammonia (NH^3), turbidity, and chloride (Cl^-) under various weather conditions in river samples collected in Milwaukee, Wisconsin in 2012 and 2013. Asterisks indicate tests that were significant at $p \leq 0.05$.

Spearman's Rank Correlations (rho)	**Baseflow** (n = 53)			**Wet Weather** (n = 125)			**CSO** (n = 19)		
	NH_3	Turbidity	Cl^-	NH_3	Turbidity	Cl^-	NH_3	Turbidity	Cl^-
HF183	0.11	-0.07	0.14	0.07	0.32*	0.35*	0.80*	0.40	-0.29
Lachno2	0.10	-0.11	0.10	-0.004	0.35*	0.43*	0.78*	0.33	-0.33

2.4.5 Event loading of human indicators in the Milwaukee estuary

Of the samples collected in the Milwaukee estuary, higher concentrations and loads were measured during events with greater rainfall amounts (Table 4). For the two baseflow events analyzed, peak 24-hour mean concentrations of HF183 and Lachno2 ranged from zero to < 30 CN/100 ml. In one of these events, a Lachno2 peak load of $< 5.0 \times 10^9$ CN was measured. This peak load is on the same order of magnitude as the loads measured for an event with light, scattered rainfall totaling only 4.1 mm. All concentrations measured during this rain event were near the limit of quantification, indicating that the low rainfall volumes did not have a considerable impact on sewage loading. Two events were sampled during which the majority of rainfall fell in a period of only a few hours (event numbers three and seven). For both of these events, peak human fecal indicator levels occurred during the 24 hours after rainfall ended. The largest rainfall amounts were sampled in May and June (event numbers one and two), with rainfall amounts of approximately 22.6 and 33.3 mm across the watershed. In both of these events, the majority of heavy rainfall occurred over a 24-hour period. In event number one, maximum human fecal indicator concentration levels occurred within the 24 hours following

peak discharge. These maximum concentrations, however, were observed at the end of the sampling event, which ideally would have been sampled longer in order to observe loading over a 48-hour period following rainfall. This event is represented in Figure 5. In event number two, sampling began after over an inch of rain had already fallen, so the beginning of the event was not captured. Maximum HF183 levels occurred in the 24 hours after about one inch of rain had fallen, while peak Lachno2 levels began approximately 18 hours following the peak HF183 load.

Table 4: Peak 24-hour mean event concentrations and peak loads of human fecal indicators, human *Bacteroides* (HF183) and human *Lachnospiraceae* (Lachno2), for seven wet and dry weather events sampled in the Milwaukee estuary at Jones Island wastewater reclamation facility in Milwaukee, Wisconsin in 2013.

				Maximum 24-hour Mean Concentration (CN/100ml)		Maximum 24-hour Mean Load (CN)	
Event Number	Dates	Duration (hours)	Total Rainfall Depth (mm)	HF183	Lachno2	HF183	Lachno2
1	5/21-5/23/2013	37	22.6	2,100	2,700	$5.5x10^{10}$	$7.7x10^{10}$
2	5/28 – 6/2/2013	122	33.3	15,000	38,000	$8.7x10^{11}$	$1.4x10^{12}$
3	6/12 – 6/14/2013	34	32.0	105,000	131,000	$5.1x10^{12}$	$6.1x10^{12}$
4	7/19 – 7/20/2013	24	0.0	0	0	0	0
5	8/8 – 8/9/2013	24	0.0	0	< 30	0	$< 7.1x10^{8}$
6	8/11 – 8/12/2013	24	4.1	100	95	$1.0x10^{9}$	$1.3x10^{9}$
7	8/22 – 8/23/2013	29	13.5	4,200	4,100	$6.9x10^{10}$	$6.8x10^{10}$

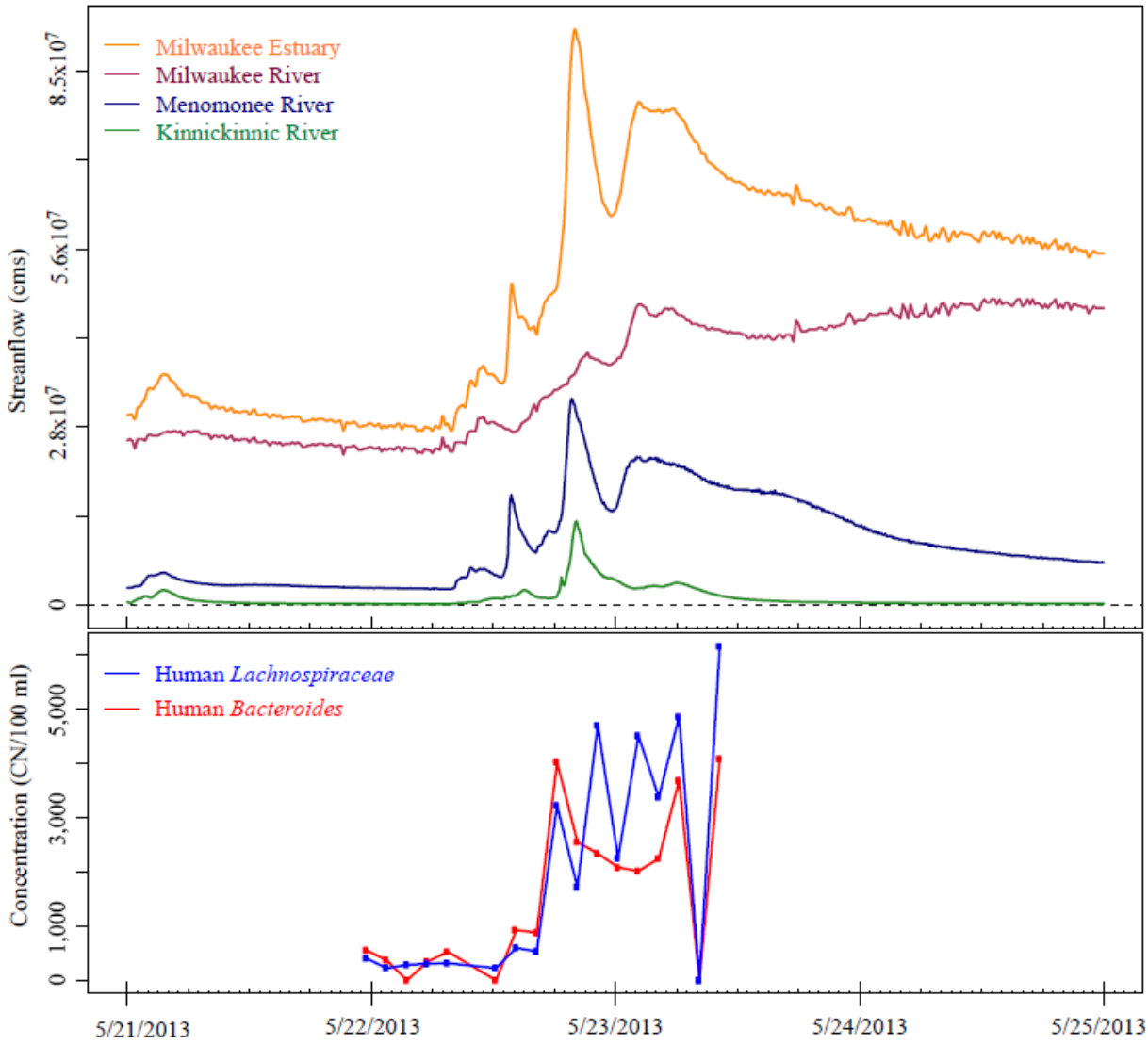

Figure 5: Streamflow in three rivers and the Milwaukee estuary (upper panel) and corresponding concentrations of human fecal indicators (lower panel) for a single rain event sampled across the hydrograph in the Milwaukee estuary at Jones Island wastewater reclamation facility in Milwaukee, Wisconsin in 2013.

2.5 Discussion

The goal of implementing bacterial TMDLs is to reduce fecal pollution sources so that waterways are fishable and swimmable. General fecal indicator bacteria, including fecal coliforms, serve as a metric to assess human health risks associated with exposure to pathogens

carried in fecal material. Fecal sources commonly considered by TMDLs in urban areas include sewage contamination from SSOs, CSOs, leaky sewer lines, illicit cross-connections, domestic pet waste, urban wildlife, and urban stormwater (Benham et al., 2006). Because fecal contamination from human sewage is likely to carry human pathogens (Sedmak et al., 2005, 2003), it is important to distinguish sewage contamination from other sources of fecal pollution in order to effectively assess risk. Although bacterial TMDLs generally use standard FIBs such as fecal coliforms, *E. coli*, or enterococci, microbial source tracking is beginning to be used in TMDL development and implementation to identify sources of fecal contamination (Tetra Tech Inc. and Herrera Environmental Consultants, 2011). As indicated by Benham et al., 2006, improved characterization of bacterial sources is needed in the TMDL process (Benham et al., 2006).

The use of fecal coliforms alone is not sufficient to identify risk, as they do not always coincide with the presence of human sewage and pathogens. Previously published studies found poor correlations between standard fecal indicators and human fecal indicators, especially in urban areas, which our data agrees with (Converse et al., 2011; Nshimyimana et al., 2014; Sauer et al., 2011). Of our river samples that were at or below the variance standard for fecal coliforms (1,000 CFU/100 ml), approximately 50% were identified as positive for human sewage. Conversely, we found that river sites that frequently have high levels of fecal coliforms were not always the sites with sewage contamination a high percentage of the time. Thus prioritizing sites for TMDL management and risk assessment using fecal coliforms alone can give a very different picture than using human fecal indicators to prioritize. Additionally, there is increased potential for health risks being incorrectly evaluated. For example, one study found that the risk of

gastrointestinal illness is greater when exposed to recreational water contaminated by human and cattle feces, compared to gull, chicken, or pig feces (Soller et al., 2010).

In urban rivers, one often unrecognized source of sewage pollution is stormwater discharges. Stormwater outfalls have been found to contribute sewage to urban waterways in both dry and wet weather (Converse et al., 2011; Parker et al., 2010; Sauer et al., 2011; Sercu et al., 2011, 2009), acting as a conduit for sewage contamination from leaking sewer infrastructure and illicit connections. Thus, receiving waters have the potential to cause adverse health effects for recreational-users (Gaffield et al., 2003; Haile et al., 1999). Using two human fecal indicators, we found widespread sewage contamination in Milwaukee waterways, even in the absence of known sewage sources such as CSOs and SSOs.

Human fecal indicators were highly correlated with each other among river and estuary samples (Spearman's rho = 0.92 river; 0.91 estuary) when both indicators were present, which suggests that there is a high likelihood that the fecal contamination originated from human sources. However, there is the potential for a sample to have fecal contamination by a non-human source. When this non-human fecal contamination is present at a high concentration, one or the other human fecal indicator may cross-react (Fisher et al., 2015). It is unlikely that a cross-reacting fecal source would create a false positive in two human fecal indicators; therefore, using both indicators on each sample can reduce the likelihood of false positives. We found that the samples in which only one indicator was present and the other was absent generally had low concentrations of the indicator that was present. This discrepancy can be attributed to measuring a value near the limit of reliable quantification for the assay. Samples in which one human fecal indicator was present at very high levels and the other was absent, were removed from the analysis, as it is likely that a cross-reacting fecal source is present at a high concentration. In the

majority of both river and harbor samples with inconsistent human indicator detection, Lachno2 was present while HF183 was absent. Additionally, Lachno2 is higher on average than HF183 in river and estuary samples, which could be attributed to Lachno2 existing at a greater concentration in raw sewage or Lachno2 persisting longer in the environment.

Generally, concentrations of human fecal indicators at most river sites were elevated during wet weather compared to baseflow. Increases in concentration during wet weather demonstrate that incoming waters have more concentrated contamination levels than receiving waters. When upstream and downstream sites were compared in the MN River, downstream sites were significantly higher than upstream sites for both baseflow and wet weather samples, demonstrating that the downstream sites exhibit chronic fecal contamination. None of the sites on the KK River had statistically significant differences between human fecal indicator concentrations when comparing baseflow to wet weather samples. In fact, KK River sites KK RI-33, 34, and 35 had on average greater human fecal indicator concentrations at baseflow, demonstrating that the entire KK River is chronically contaminated, even with no inputs from stormwater contaminated with sanitary sewage.

Deteriorating wastewater conveyance infrastructure can be a substantial contributor to human fecal pollution in urbanized rivers. While the results from samples collected at rural sites in the upstream MN River indicate that sewage contamination increased during runoff periods, the results from samples collected at urbanized sites in the KK River and downstream MN River indicate chronic sewage pollution, meaning concentrations are elevated regardless of the weather conditions.

Of samples collected across the hydrograph in the Milwaukee estuary, an increase in loading was seen during rain events, suggesting rainfall was a driver of human sewage pollution

even in the absence of a sewer overflow. Total depth, duration, and intensity of rainfall events also appear to have an impact on sewage loading. Intense rainfall events with large volumes of precipitation tended to produce the greatest sewage loadings compared to events with scattered rainfall spread across a longer period of time. From these events, it was evident that sampling could be more representative by beginning at least two hours prior to the beginning of rainfall and ending at least 48 hours following precipitation in order to fully characterize sewage loading across the hydrograph and define the effect of storm characteristics on loads. Additionally, elevated concentrations in the rivers compared to the estuary indicate that diluted flow from the MKE River, as well as backflow from Lake Michigan, is likely causing dilution of human fecal indicator concentrations measured in the estuary. Continuous monitoring in the MKE, MN, and KK Rivers would allow a better estimation of sewage loads discharging to the harbor, and provide a better indication of which river(s) contribute most to sewage pollution in the estuary.

Continuous integrated sampling allowed for fine-scale hydrologic characterization of human fecal contamination in waterways. We showed that this method of sampling gave a clear picture of fecal contamination from human sewage sources. The average ratio of the two human fecal indicators (Lachno2: HF183) in integrated samples was 2 (± 2), which was very similar to the average ratio found in raw human sewage samples (2 (± 3)). Continuous integrated sampling across the hydrograph has the potential to bring microbial source tracking to a watershed scale.

2.6 Conclusions

This chapter demonstrated the utility of using two human fecal indicators to quantify human sewage contamination. Human fecal indicators were detected in samples collected both at baseflow and during wet weather in the KK River, MN River, and MKE River near the

Milwaukee estuary. Samples collected in the highly urbanized KK River and downstream MN River sites showed no significant differences between concentrations collected at baseflow and wet weather, demonstrating that human fecal contamination is chronic in the urbanized rivers of metropolitan Milwaukee.

The use of fecal coliforms and other culture-based fecal indicator bacteria alone fail to identify the risk of human sewage contamination in recreational waters, as demonstrated by the inconsistent detection of human fecal indicators among samples that had both high and low fecal coliform concentrations. TMDLs must take into account the sources of fecal contamination before informed decisions can be made for implementation and river reach prioritization.

An assessment of sewage loading can be useful for determining the relationships between sewage contamination and other environmental factors such as rainfall volume, rain event duration, and antecedent rainfall. In order to determine the major contributors to sewage contamination in the Milwaukee estuary, quantification of loads in each of the three rivers is needed.

3. Intensive monitoring of three urban rivers and an estuary reveals storm-driven patterns of sewage loading to a Lake Michigan estuary

3.1 Abstract

Fecal contamination in urban waterways is a major public and environmental health threat. Sources of fecal contamination and pathogens to urban waterways include major inputs from sanitary and combined sewer overflows as well as inputs from stormwater and failing sewer infrastructure. Samples were collected every 15 min over several days during storm events to quantify loads of sewage based on two human-specific fecal indicator bacteria (human *Bacteroides* and human *Lachnospiraceae*). Samples were collected at an estuary that discharges to Lake Michigan and at three rivers sites immediately upstream. These samples were analyzed using quantitative polymerase chain reaction (qPCR) assays and loads were calculated from streamflow data collected at each location. Human fecal indicators were found during periods of low flow and loads increased significantly (one to two orders of magnitude) during rain events. Combined sewer overflow (CSO) events generally contributed the highest loads of human indicator bacteria to these urban waterways, which were up to several orders of magnitude higher than rainfall events with no CSO. Sampling in the rivers upstream of the estuary indicated sewage contamination is related to the degree of urbanization in the watershed. When yields were calculated considering only the urban land use (load per urban km^2), all three watersheds showed similar values. This information will be useful for directing the efforts of local agencies and municipalities to investigate failing sewer infrastructure, as well as helping agencies at the state and federal levels to create appropriate goals to address the human health concerns posed by sewage contamination in urban waterways.

3.2 Introduction

In many urban waterways, sewer overflows are a major source of point-source sewage contamination, resulting in considerable impacts to microbiological water quality in receiving waters following the overflow event (McLellan et al., 2007; Passerat et al., 2011). The U.S. Environmental Protection Agency estimates 850 billion gallons of untreated sewage is discharged annually into United States waterways by combined sewer overflows (CSO) and up to 10 billion gallons from separated sewer overflows (SSOs) (U.S. Environmental Protection Agency, 2004). In fact, pathogen contamination, which is measured by fecal indicator bacteria (FIB), is a frequent water quality impairment. Non-point sources of sewage contamination, on the other hand, are more elusive because they are often the result of leaking infrastructure, stormwater runoff, and illicit cross-connections. It is possible for leaking sanitary sewer lines to discharge sewage into the groundwater, which may ultimately infiltrate into leaking storm sewers and wash into a receiving waterway, even during dry weather (Sercu et al., 2011).

Fecal contamination is usually measured by fecal indicator bacteria (FIB) which are used as indicators of human health risk in recreational water. *Escherichia coli (E. coli*), enterococci, and fecal coliforms are all common FIB, as they are easily grown in a laboratory and present in the gastrointestinal tract of humans and most warm-blooded animals (McLellan et al., 2013). These FIB, however, frequently fail to detect the occurrence of human sewage and pathogens, as they are not specific to the source of fecal contamination. Alternative indicators are used in microbial source tracking to determine the source of fecal contamination and more accurately anticipate risk to human health. Human fecal indicators such as human *Bacteroides* (HF183) and human *Lachnospiraceae* (Lachno2) are used as proxies for human sewage. These indicators are highly correlated in sewage, thus using them in tandem is beneficial while tracking sewage in an

urban environment where many fecal sources are present. HF183 has been found to amplify fecal sources other than human sewage, therefore using two human fecal indicators reduces the risk of finding a false positive.

Milwaukee, Wisconsin is a city located on the shores of Lake Michigan and similar to most highly urbanized areas, has ongoing sewage contamination issues in urban waterways. The oldest parts of the city have a combined sewer system, which collects runoff from impervious surfaces and conveys both sanitary sewage and stormwater runoff to the water reclamation facility. In past years, this meant that heavy rain could easily result in a CSO; however, since the installation of the "Deep Tunnel" stormwater storage system in 1993 by the Milwaukee Metropolitan Sewerage District (MMSD), CSOs have drastically decreased from fifty to sixty overflows per year to an average of 2.4 per year from 1994 to 2013 (Behm, 2013). Despite the improvements made by the Deep Tunnel, chronic human sewage pollution continues to be present in the Milwaukee estuary, which is the confluence of three major rivers (the Milwaukee, Menomonee, and Kinnickinnic Rivers) which drain to Lake Michigan via the Milwaukee estuary (Newton et al., 2013, 2011).

As described in this chapter, samples were collected across the hydrograph at locations in each of the three rivers and the estuary during low flow periods, rain events, and CSOs to characterize sewage pollution in these urban waterways. This chapter seeks to (1) quantify event loads of sewage discharged into Lake Michigan by each of three rivers and the estuary during low flow periods and rain events; (2) compare the loads produced during rain events to CSO events; (3) investigate relationships between the degree of urbanization in multiple watersheds and the fluxes of sewage they are contributing; and (4) determine the relative sewage contributions of the watersheds in the Milwaukee estuary.

3.3 Materials and methods

3.3.1 Study sites and sampling methods

This research was conducted in metropolitan Milwaukee, Wisconsin which is located on the shores of Lake Michigan with the Milwaukee estuary at the heart of the city. The Milwaukee estuary is the confluence of three major rivers – the Milwaukee (MKE) River from the north, the Menomonee (MN) River from the northwest, and the Kinnickinnic (KK) River from the south. Table 5 represents drainage area, land use, and impervious surface percentages for each river. Land use and impervious surface percentages for each watershed were determined by using the ESRI ArcGIS® software package and data from the 2011 National Land Cover Database (Jin et al., 2013; Xian et al., 2011). Urban land use was defined as any land cover classified as developed land, including low, medium, and high intensity developed land, and developed open space. This includes all low to moderately developed areas, including urban parks, golf courses, and residential areas, as well as highly developed commercial and industrial areas. The MKE River drains the largest area and is the most diverse in terms of land use, with mainly rural and agricultural land uses in the headwaters and dense urban area near the mouth. The MN River drains a much smaller area with mainly urban and residential land uses and some agriculture and natural areas in the headwaters. The KK River drains by far the smallest area, with nearly all urban and industrial land uses and over half of the watershed covered by impervious surfaces.

Sampling was conducted at four sites – one in each of the three rivers and one in the estuary. In April through September of the years 2014 and 2015, samples were taken across the hydrograph using an automated Teledyne ISCO 3700 full size portable sequential sampler at each site. The samplers were housed within U.S. Geological Survey (USGS) and Milwaukee Metropolitan Sewerage District (MMSD) monitoring stations. The Milwaukee estuary site was

located at the USGS station at Jones Island water reclamation facility in Milwaukee, Wisconsin (USGS 04087170). The MKE River sampler was housed within an MMSD real-time water quality station beneath the Cherry Street Bridge in Milwaukee, WI. The MN River sampler was housed within the USGS monitoring station on 16th Street in Milwaukee, WI (USGS 04087142). The KK River sampler was housed within the USGS monitoring station on 11th and Harrison Streets in Milwaukee, WI (USGS 04087159). Figure 6 shows the locations of each automated sampler. During sampling periods, a 250 ml sample was collected every 15 minutes into one of 24 one-liter polypropylene bottles contained within each sampler. Each bottle represented a composited one-hour sample with four subsamples. Samples were collected during each day of sampling, placed on ice, returned to the laboratory, and processed within six hours of collection. Each sample was filtered and archived for DNA extraction and analyzed for standard FIB via culture-based methods. For rain event sampling, the samplers were ideally activated at least two hours prior to expected rainfall and samples were collected continuously for at least 24 hours following the rainfall event. Two sample bottles were composited in the field, resulting in two-hour samples with eight subsamples. For dry weather sampling, the samplers were activated after at least 48 hours of dry weather. Four sample bottles were composited in the field, resulting in four-hour samples with 16 subsamples. The goal was to collect samples during storm events with a variety of different characteristics, and to collect samples during periods of dry weather approximately once per month. Over 2,000 samples were collected during eleven rain events, four dry weather events, and two CSO events.

Table 5: Watershed characteristics of sampling sites in Milwaukee, Wisconsin. Base data from the National Land Cover Database (2011) Land Cover and Percent Developed Imperviousness datasets (Jin et al., 2013; Xian et al., 2011).

Monitoring Location	USGS Site ID	Drainage Area (km^2)	Percent Impervious Surfaces	Percent Urban	Percent Agriculture	Percent Natural Areas[a]
Kinnickinnic River at 11th Street	04087159	51	52	99	0	1
Menomonee River at 16th Street	04087142	349	28	69	17	14
Milwaukee River at Cherry Street	n/a	1,781	7	19.5	50.5	30
Milwaukee River at Mouth	04087170	2,215	12	30	43	27

[a] Forests, grasslands, wetlands, and open water are classified as natural areas

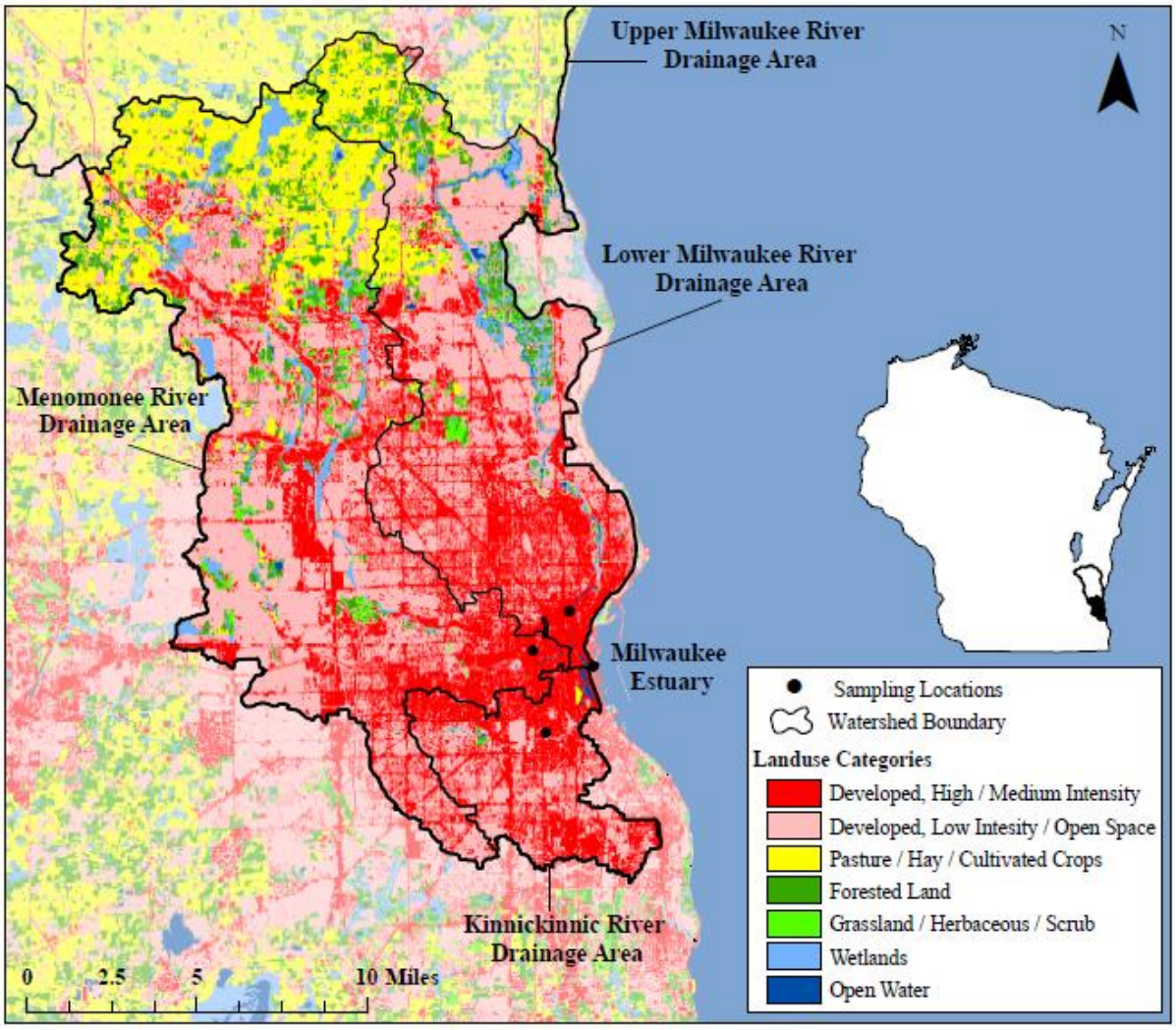

Figure 6: Sampling site locations, drainage areas, and land use in Milwaukee, Wisconsin. Base data from the National Land Cover Database (2011) Land Cover dataset (Jin et al., 2013).

3.3.2 Culture-based methods

Within six hours of collection, all samples collected by the automated samplers were analyzed for *E. coli*, enterococci, and total fecal coliforms using standard methods. A volume of sample ranging from 1-ml to 100 ml, based on expected bacteria levels, was filtered through a 0.45-µm-pore-size nitrocellulose filter (0.47-mm diameter; Millipore, Billerica, MA) and aseptically placed onto selective media and incubated for approximately 24 hours before being removed and

counted for colony forming units (CFUs). For *E. coli* enumeration, filters were placed on modified mTEC agar, and incubated for 24 hours at 41°C (U.S. Environmental Protection Agency, 2009). For enterococci enumeration, filters were placed on MEI agar and incubated at 35°C for two hours and the remaining 22 hours at 44.5°C to revive stressed or injured bacteria (U.S. Environmental Protection Agency, 2002b). For fecal coliforms enumeration, filters were placed on mFC agar and incubated for 24 hours at 44.5°C (American Public Health Association et al., 2003).

3.3.3 DNA extraction and quantitative polymerase chain reaction assays

Within six hours of collection, a volume of 200 ml or 400 ml of each sample was filtered onto a 0.22-µm-pore-size mixed cellulose esters filter (47-mm diameter; Millipore, Billerica, MA). Filters were then folded and placed in 2-ml screw-cap tubes and immediately stored at -80°C until DNA extraction. The frozen filters were broken into small fragments using a metal spatula and DNA was extracted from the crushed filters using the MPBIO FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Anna, CA) and eluted using 150 µL of DNase/Pyrogen-Free Water (DES). Quantitative PCR was conducted using an Applied Biosystems StepOne Plus™ Real-Time PCR System Thermal Cycling Block (Applied Biosystems; Foster City, CA) with Taqman hydrolysis probe chemistry. Previously published primers and probe were used for the human *Bacteroides* (HF183) assay (Kildare et al., 2007) with the exception that the HF183F was used as the forward primer (Bernhard and Field, 2000). Previously published methods for the Lachno2 assay were used for human *Lachnospiraceae* (Lachno2) analysis (Newton et al., 2011). The MKE estuary and MKE River sites were analyzed for a ruminant-specific fecal indicator because of the agricultural land uses in the headwaters of these watersheds. A previously published

ruminant qPCR assay was used for this analysis (Reischer et al., 2006). Standard curves were created during each run, consisting of a linearized plasmid containing the targeted gene sequence. Standard curves were run with DNA serially diluted from $1.5x10^6$ to $1.5x10^1$ copies per reaction and standards were run in triplicate. Each sample was run in duplicate in a final volume of 25 µL with a final concentration of 1-µM for each primer, 80 nM for the probe, 5-µL of sample DNA, and 12.5 µL of 2X Taqman® Gene Expression Master Mix Kit (Applied Biosystems; Foster City, CA). The following amplification cycles were used: one cycle at 50°C for two minutes to activate the uracil-N-glycosylase (UNG), then one cycle at 95°C for ten minutes to inactivate the UNG and activate the Taq polymerase, and 40 cycles of 94°C for 15 seconds followed by one minute at 60°C.

Each qPCR reaction results in a raw number of copies (CN) per reaction, which is then converted to a concentration of copies per 100 ml of original sample based on the proportion of the sample used in the reaction. For all samples collected in 2014, 200 ml was filtered for all samples. For samples collected in 2015, 200 ml was filtered for all storm events and 400 ml was filtered for low flow samples. The limit of reliable quantification was determined to be 15 copies per reaction, or 225 CN/100 ml. Therefore, any samples with positive amplification, but were below 15 copies were reported as below the limit of quantification (BLQ). For all statistical analyses and load calculations, BLQ samples were given a concentration of 225 CN/100 ml.

3.3.4 Benchmarks for positive sewage detection

Thresholds for positive sewage detection in individual samples was set to HF183 concentrations of 1,000 CN/100 ml or Lachno2 concentrations of 1,500 CN/100 ml. Samples were considered to have a positive sewage signal if one or the other indicator was above its respective threshold.

These thresholds correspond to the level at which there is a high risk of the presence of pathogens (specifically adenovirus) and what would be considered an unacceptable risk of infection (Newton et al., 2011). Sauer et al., 2011 also demonstrated that stormwater outfalls in the Milwaukee urban area with >1,000 CN/100 ml HF183 were consistently positive for human sewage (Sauer et al., 2011). These concentrations of HF183 and Lachno2 are equivalent to approximately 0.003% of the mean concentrations of HF183 and Lachno2 in sewage influent, which was determined by analysis of 94 influent samples collected at Jones Island and South Shore water reclamation facilities in Milwaukee, WI from 2008-2013. Results from a subset of these samples were presented in Newton et al., 2011.

3.3.5 Statistical analysis

For statistical analysis, results which had detectable concentrations below the limit of quantification were assigned a value equal to the limit of quantification. The Spearman's rank correlation (rho) was used to determine correlations between quantities of human fecal indicators and other event characteristics. The Wilcoxon signed rank test was used to determine whether significant differences existed between event loads that were computed in the Milwaukee estuary and the sum of the event loads in the KK, MN, and MKE Rivers for each event. A two-tailed t-test was used on log-transformed data to determine whether a significant difference existed between human fecal indicators in sewage influent during low flow compared to high flow periods in the water reclamation facility. All tests were considered significant at $p \leq 0.05$. The R suite of packages (R Core Team, 2015) were used for all statistical analyses.

3.3.6 Defining hydrologic events

Events were defined by visually inspecting the MKE, MN, and KK River hydrographs to identify the urban runoff portion of each event. The beginning of each event was visually defined as the beginning of the rising limb of the hydrograph. The end of each event was defined as the approximate inflection point of the falling limb of the hydrograph, which was defined as the point where the falling limb begins to change concavity, indicating that the majority of flow can be attributed to baseflow rather than runoff.

3.3.7 Calculating maximum 24-hour mean concentrations, loads, and fluxes

Maximum 24-hour mean concentrations were computed for each event sampled in the Milwaukee estuary. A running 24-hour average of the instantaneous concentrations for each event was taken and the largest value was selected.

River streamflow was retrieved from U.S. Geological Survey continuous monitoring stations on the KK River at 11th Street (USGS 04087159), the MN River at Wauwatosa, WI (USGS 04087120), and the MKE River at Milwaukee, WI (USGS 04087000). Drainage area ratio corrections were used on the MN and MKE River streamflow data to correct for the difference in watershed area between the continuous monitoring stations and the locations at which samples were collected. Streamflow at the Milwaukee estuary was calculated by summing the instantaneous streamflow values of the MKE, MN, and KK Rivers and multiplying the streamflow of the MN and KK Rivers by the drainage area ratio. Instantaneous loads of HF183 and Lachno2 were determined by multiplying streamflow by concentration and the integration method was used to determine the event loads of each human fecal indicator (Porterfield, 1972). Concentrations at the beginning of storm events were estimated by using samples collected

immediately prior to rainfall. Concentrations at the end of storm events were estimated by using samples collected immediately after the end of the events.

Hourly fluxes were calculated by dividing event loads by the number of hours that were sampled. Daily fluxes were then calculated by multiplying each events' hourly flux by 24 hours. This allowed for an equal comparison between fluxes measured during low flow periods and storm events.

3.3.8 Estimating dilution of concentrations measured in the Milwaukee estuary

To characterize bi-directional flow at the Milwaukee estuary, velocity and water level data were retrieved from the USGS continuous monitoring station at the MKE River at the mouth in Milwaukee, WI (USGS 04087170). Velocities on the north and south sides of the river channel were measured using acoustic doppler current profiling instrumentation (ADCP) (SonTek, San Diego, CA). Streamflow was calculated by multiplying the average of the north and south velocity measurements by the cross-sectional area of the channel at the time of measurement. For each rain or CSO event, the absolute value of the sum of the negative streamflow values was divided by the absolute value of the sum of the negative and positive streamflow values to determine the percentage of total event flow that was negative flow, or "backflow" from Lake Michigan. Concentrations in backflow were assumed to be zero, providing an estimate of the highest possible dilution effect.

3.3.9 Determination of rainfall event characteristics

The watersheds of the Milwaukee estuary and the MKE, MN, and KK Rivers were delineated using ESRI ArcGIS® software, using the locations of the automated samplers at each site as the

outlet points. The lower-Milwaukee River watershed was used to represent the urban influenced area of the Milwaukee estuary and MKE River watersheds. Average rainfall accumulation for each watershed was determined using radar-indicated rainfall models, retrieved from the National Weather Service North-Central River Forecast Center (National Weather Service, 2015). Average one-hour rainfall accumulation was computed for each watershed-defined area. For events during which rainfall amounts from the National Weather Service were missing, the Thiessen polygon method was used in ESRI ArcGIS® to determine weights that were placed on MMSD rain gauges located in each watershed. Rainfall depths from each rain gauge within each watershed were multiplied by their respective weights and these values were summed to compute average hourly rainfall depths across each watershed.

3.3.10 Calculation of untreated sewage equivalents

Concentrations of HF183 and Lachno2 found in sewage influent were used to convert loads of each human fecal indicator to a quantity of "untreated sewage equivalents". A total of 54 samples from Jones Island water reclamation facility were used to determine mean concentrations of HF183 and Lachno2 in influent sewage. Concentrations were log-transformed and the 25% of samples with the highest influent flow rates and 25% of samples with the lowest influent flow rates were compared using a two-tailed t-test to determine if dilution from infiltrated rainwater occurred during high flow days. No statistically significant difference ($p<0.05$) was found between high and low flow concentrations of HF183 ($p=0.37$) and Lachno2 ($p=0.14$). Mean influent flow was 63 million gallons/day for the lowest 25% of samples. The mean HF183 concentration for the lowest 25% of samples was $3.1x10^7$ CN/100 ml and Lachno2 was $4.3x10^7$ CN/100 ml. Mean flow was 148 million gallons/day for the highest 25% of samples.

The mean HF183 concentration for the highest 25% of samples was $2.9x10^7$ CN/100 ml and Lachno2 was $3.6x10^7$ CN/100 ml. A combination of samples collected during high and low flow were used to calculate mean concentrations of the two human fecal indicators. The mean concentration of HF183 and Lachno2 in raw sewage collected from Jones Island water reclamation facility were found to be $1.38x10^9$ ($\pm$ $1.46x10^9$) and $2.0x10^9$ ($\pm$ $2.0x10^9$) CN/gallon, respectively. Event loads (CN) were divided by mean concentrations of the Lachno2 indicator in raw sewage (CN/gallon) to get estimates of untreated sewage equivalents (gallons). The Lachno2 indicator was used to calculate estimates of untreated sewage equivalents because it had more consistent concentrations in sewage influent samples.

3.4 Results

3.4.1 Sewage concentrations and loads after storm events in the Milwaukee estuary

Concentrations and event loads of human fecal indicators in the Milwaukee estuary display seasonal patterns, as well as relationships with rainfall and river streamflow to the estuary. In 2014 and 2015, events were sampled from early spring to late summer, with total rainfall amounts ranging from 7.3 mm in August 2014 to 58.3 mm in April 2014 and mean event streamflow ranging from $1.0x10^7$ cubic meters per second (cms) in September 2014 to $1.7x10^8$ cms in April 2014. Events sampled in the spring of each year generally had greater total rainfall depths and mean event streamflow, with higher human fecal indicator concentrations measured during this time frame (Table 6). Maximum 24-hour mean concentrations of HF183 were up to 15 times during CSOs compared to the largest rain event and Lachno2 was up to six times higher during CSOs.

The lowest concentrations of human fecal indicators were found during low flow periods. Concentrations of human fecal indicators showed a consistent pattern of increased concentrations with increased flow across the hydrograph (Figure 7). This result is surprising because concentrations were expected to be diluted by increased streamflow. Of all samples collected in the Milwaukee estuary during a variety of weather conditions in 2014 and 2015 (n=188), concentrations of both human indicators were significantly correlated to streamflow volume (HF183 rho=0.56, Lachno2 rho=0.62, $p<0.05$) and maximum river streamflow (HF183 rho=0.75, Lachno2 rho=0.78, $p<0.05$) measured during sample collection. A ruminant-specific genetic marker was also analyzed in Milwaukee estuary samples because of the rural and agricultural land uses in the headwaters of the MKE River. The ruminant signal is generally either absent or present at low levels throughout the majority of a rain event, but then can be detected at higher levels several days following rainfall, once water from the headwaters of the MKE River make it to the MKE estuary (Figure 7).

Although HF183 and Lachno2 concentrations are highly correlated among all Milwaukee estuary samples (rho=0.97; $p<0.05$; n=188), Lachno2 concentrations were on average 1.9 times higher than HF183 concentrations. The percent difference between HF183 and Lachno2 maximum concentrations were generally greater in samples collected in the spring than during the summer. Concentrations of Lachno2 in samples collected in the spring range from two to five times higher than HF183 concentrations, whereas samples collected in the summer and during low flow generally have ratios of Lachno2 to HF183 that are closer to one.

Daily flux of HF183 and Lachno2 were also calculated for storm events and low flow periods (Figure 8). Storm event fluxes and total rainfall depth are significantly correlated for both loads of HF183 (rho=0.90, $p<0.05$) and Lachno2 (rho=0.82, $p<0.05$). Storm event fluxes

ranged from $7.4x10^{10}$ CN of HF183 and $8.0x10^{10}$ CN of Lachno2 during an event in September 2014 (Event 7, represented in Table 6 and Figure 8), to $7.2x10^{13}$ CN of HF183 and $3.4x10^{14}$ CN during a storm event in April 2014 (Event 1, represented in Table 6 and Figure 8).

Table 6: Peak instantaneous concentrations and maximum 24-hour mean concentrations of human *Bacteroides* (HF183) and human *Lachnospiraceae* (Lachno2), total rainfall, and mean streamflow of storm events sampled in the Milwaukee estuary at Jones Island water reclamation facility in Milwaukee, Wisconsin in 2014 and 2015.

Event Number	Dates	Total Rainfall Depth (mm)	Mean Event Streamflow (cms)	Peak Instantaneous Concentration (CN/100 ml)		Maximum 24-hour Mean Concentration (CN/100 ml)	
				HF183	Lachno2	HF183	Lachno2
1	4/13/2014 – 4/14/2014	58.3	$1.7x10^8$	56,000	270,000	39,000	190,000
2	4/27/2014 – 5/1/2014	32.9	$5.3x10^7$	11,000	79,000	7,100	30,000
3	5/11/2014 – 5/14/2014	56.7	$1.0x10^8$	19,000	50,000	11,000	31,000
4	6/10/2014 – 6/12/2014	22.6	$3.3x10^7$	4,700	4,800	2,800	3,300
5	8/18/2014 – 8/20/2014	28.9	$3.4x10^7$	4,500	4,200	2,700	2,500
6	8/21/2014 – 8/23/2014	7.4	$2.6x10^7$	2,200	2,000	1,400	1,200
7	9/10/2014 – 9/11/2014	9.2	$1.0x10^7$	1,800	1,900	1,100	1,100
8	6/11/15 – 6/13/15	33.2	$3.7x10^7$	9,900	16,000	4,700	7,800

9	6/14/15 – 6/15/15	20.4	$4.5x10^7$	2,000	3,100	1,100	1,800
10	7/6/2015 – 7/9/2015	32.0	$1.7x10^7$	4,400	4,300	3,200	2,900
11	9/8/15 – 9/10/15	32.8	$3.7x10^7$	4,800	4,200	2,500	2,600
	2014 CSO	86.9	$1.2x10^8$	850,000	2,000,000	370,000	860,000
	2015 CSO	69.6	$2.6x10^8$	1,000,000	2,000,000	570,000	1,100,000
	Low Flow[a]	0.00	$1.2x10^7$	1,000	1,700	340	520

[a]Peak instantaneous concentrations of low flow represents the peak concentration of all samples collected during low flow periods. Maximum 24-hour mean concentrations of low flow periods represent the mean concentrations of all samples collected during low flow periods

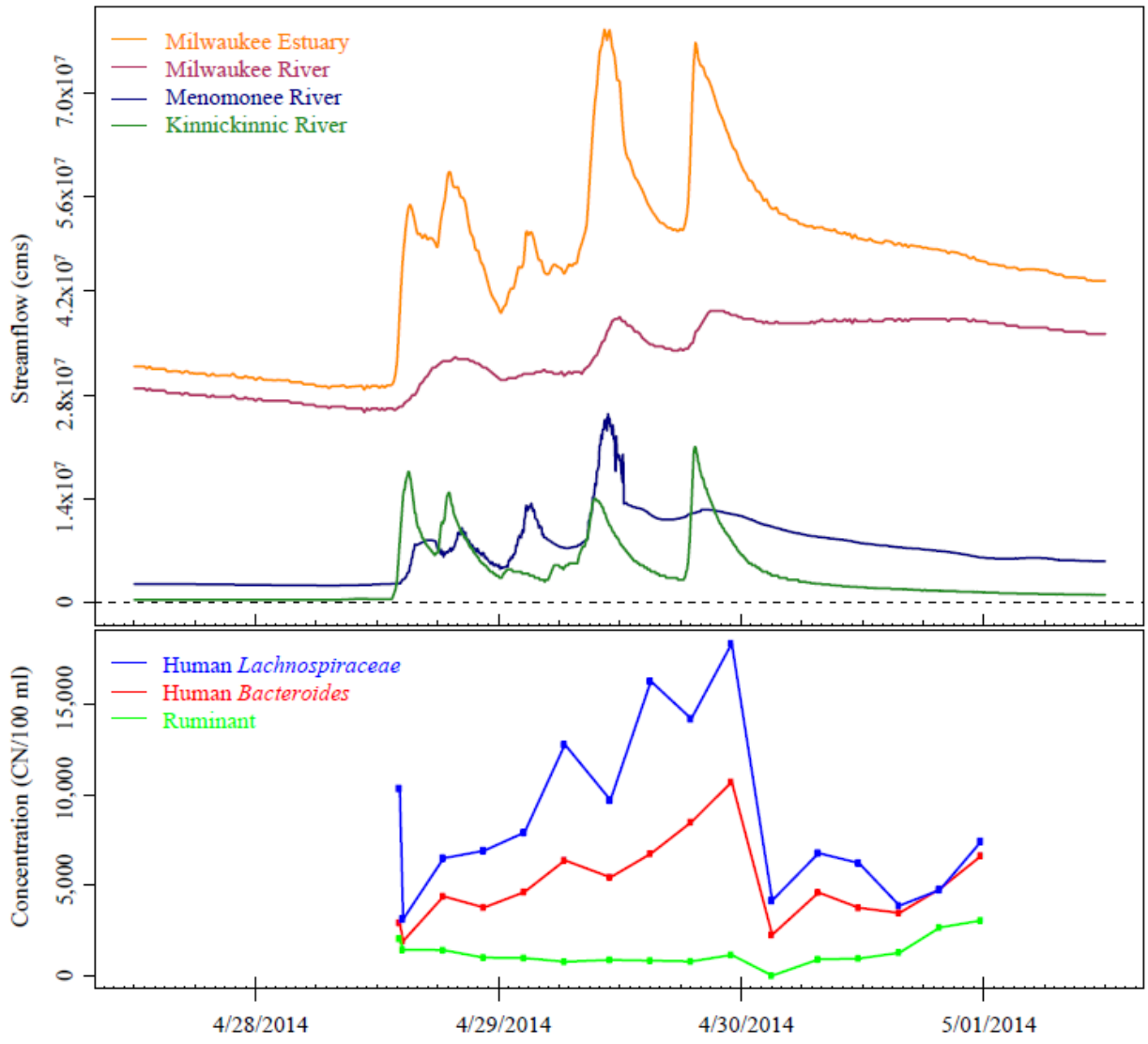

Figure 7: Streamflow (upper panel) and corresponding human *Bacteroides*, human *Lachnospiraceae*, and ruminant indicator concentrations (lower panel) measured during a single storm event in the Milwaukee estuary, collected at Jones Island water reclamation facility in Milwaukee, Wisconsin in 2014.

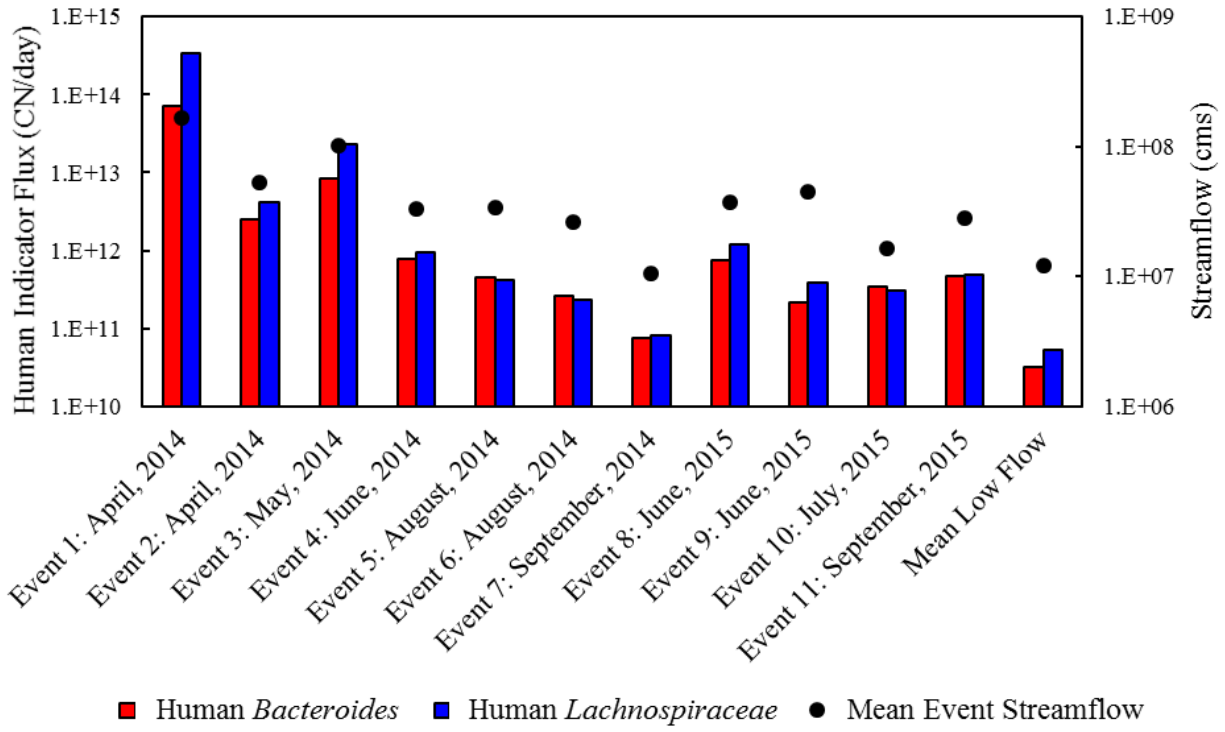

Figure 8: Daily fluxes of human *Bacteroides* and human *Lachnospiraceae* during 11 storm events, mean daily fluxes of these human fecal indicators collected during low flow, and mean streamflow of each event and low flow sampling period in the Milwaukee estuary in Milwaukee, Wisconsin in 2014 and 2015.

3.4.2 Upstream contributions to sewage loading in the Milwaukee estuary

The MKE, MN, and KK Rivers contribute varying fluxes (loads per day) of sewage to the Milwaukee estuary, and ultimately, Lake Michigan. On average, the MKE River, which has the highest flow, had the highest human fecal indicator event loads and daily fluxes during rain events (Figure 9). Among rain events sampled in 2014 and 2015, MKE River streamflow was on average two times greater than MN River streamflow and about five times greater than KK River streamflow and mean daily fluxes of both human fecal indicators in the MKE River were, in fact, about two times greater than those in the MN River and five times greater than those in the KK River. During low flow periods, mean daily fluxes of HF183 were greatest in the KK River,

which is the smallest watershed drainage area, with fluxes close to was found in the MKE River. The Lachno2 marker followed a similar pattern across the three rivers, except the fluxes of Lachno2 in the MKE River were slightly higher (3%) than fluxes in the KK River.

Because the three rivers that were studied have very different drainage areas, as well as different degrees of urbanization and imperviousness, fluxes were normalized by drainage area to calculate yield per day for total watershed area and for urban land cover area. Mean daily yields of both human fecal indicators in the KK River were nearly three times greater than those in the MN River and nearly seven times greater than those in the MKE River during rain events. Even greater differences were found between mean daily yields of human fecal indicators during low flow periods. Mean daily yields of HF183 in the KK River were nearly 30 times higher than the MN River and about 40 times higher than the MKE River. Similarly, mean daily yields of Lachno2 in the KK River were approximately 40 times higher than the MN River and 30 times higher than the MKE River.

Fluxes in each river were also normalized by urban area, meaning daily fluxes were divided by the area within each watershed classified as urban land cover. During rain events, mean daily flux per urban area of both human fecal indicators were more similar across the three watersheds, with the mean daily flux of human fecal indicators per km^2 of urban area from the KK River on average only 1.5 times higher than the MN and MKE Rivers (Figure 9). However, during low flow periods we observed that the KK River has a much larger flux per km^2 of urban area. Mean daily flux of HF183 per urban area in the KK River was approximately 20 times higher than the MN River and nine times higher than the MKE River. Similarly, Lachno2 in the KK River was nearly 30 times higher than the MN River and seven times higher than the MKE River (Figure 9).

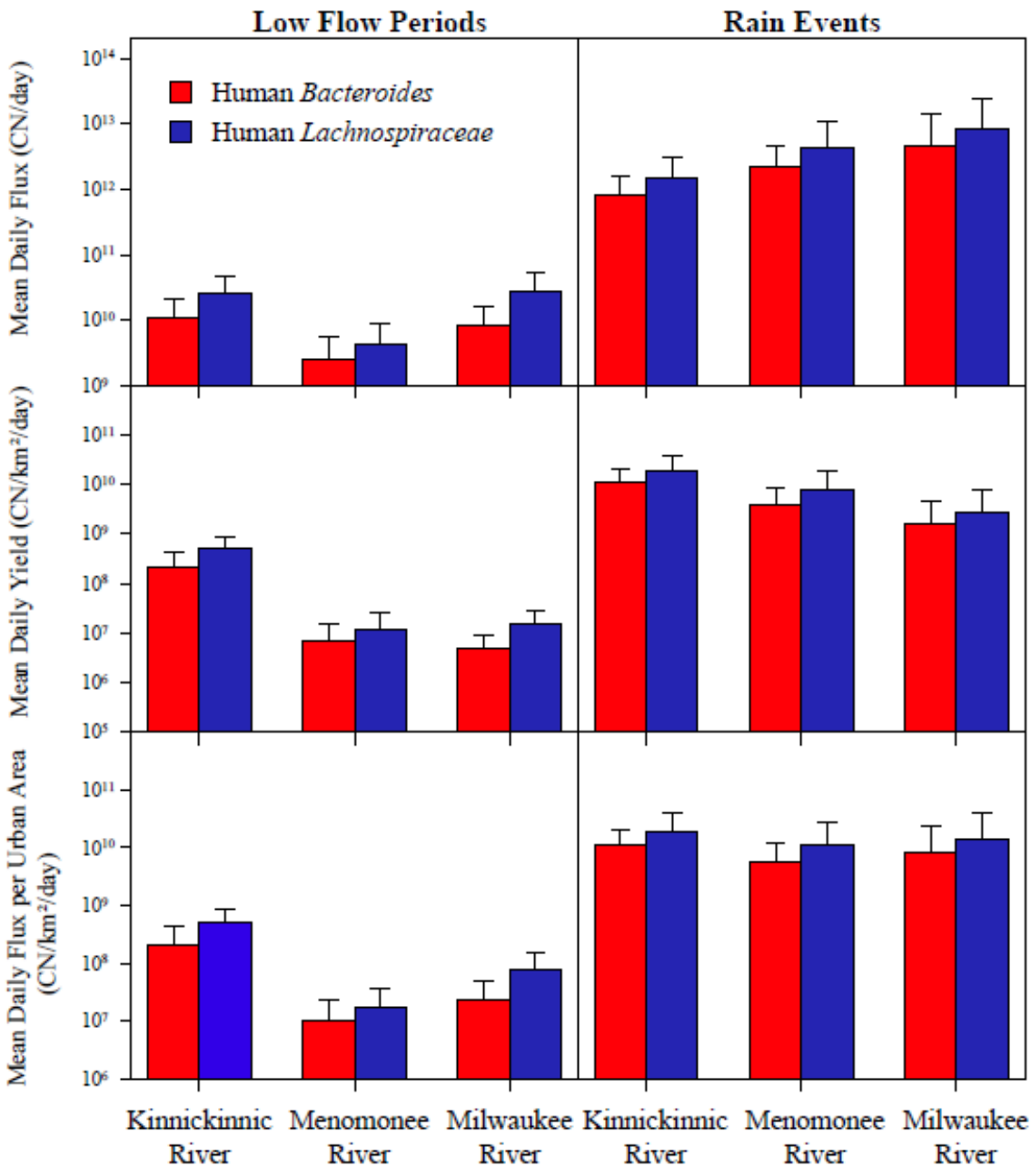

Figure 9: Mean daily fluxes, mean daily yields, and mean daily fluxes per urban area of human *Bacteroides* and human *Lachnospiraceae* in the Kinnickinnic (KK) River, Menomonee (MN) River, and Milwaukee (MKE) River for eleven rain events and four low flow periods collected in Milwaukee, Wisconsin in 2014 and 2015.

3.4.3 Mass balance of human fecal indicators at four continuous monitoring stations

Because the three rivers feed into Lake Michigan via the Milwaukee estuary, theoretically, the sum of the event loads at each of the MKE, MN, and KK Rivers would be equal to the event load computed in the Milwaukee estuary for each event. However, due to the close proximity to Lake Michigan, backflow often results in diluted concentrations in the Milwaukee estuary and an underestimation of loads because the samples captured are river water diluted by Lake Michigan water. Negative flows in the estuary ranged from 10 to 52 percent of the total flow measured among eleven total rain events. Of these rain events, the event loads from the sum of the three rivers were greater than the event loads computed in the Milwaukee estuary for nine of the events. The total loads of HF183 from the three rivers ranged from approximately one to nine times greater than the loads computed in the Milwaukee estuary and loads of Lachno2 in the rivers ranged from approximately two to eleven times higher than the Milwaukee estuary, but overall there was less than an order of magnitude difference between the two values for each event. There were only two events (Events 1 and 3) which had greater loads in the Milwaukee estuary than the total loads from the three rivers. These two events had the greatest total rainfall depth and greatest streamflow of all of the events sampled. Additionally, during Event 3, blending occurred at the Jones Island water reclamation facility, which could potentially explain the elevated loads due to the additional input of sewage at the Milwaukee harbor and backflow into the Milwaukee estuary site, effectively reducing the dilution effects. Total loads of the three rivers and loads in the Milwaukee estuary were significantly correlated for both HF183 ($rho=0.89$, $p<0.05$) and Lachno2 ($rho=0.83$, $p<0.05$). Additionally, a comparison using the Wilcoxon signed rank test showed no significant differences between the total loads from the rivers and the loads in the Milwaukee estuary for both HF183 ($p=0.21$) and Lachno2 ($p=0.28$).

3.4.4 Combined sewer overflow events

Two CSO events were sampled on June 18th - 19th, 2014 and April 9th - 10th, 2015. Based on volumes reported by MMSD, approximately 341.2 million gallons (MG) of sewage was released during the 2014 CSO, and 681.1 MG was released during the 2015 CSO. The majority of sewage released during the CSOs occurred at discharge points from the combined sewer system along the KK, MN, and MKE rivers, and one location in each of these rivers was sampled every 15 minutes throughout the duration of each CSO event. The proportional loads of human indicators in each river were compared to the proportional volume released at CSO outfalls upstream of sampling locations and good correspondence was found (Table 7). CSO outfalls upstream of sampling locations in the MN River contributed the greatest portion of gallons of raw sewage released during the CSOs in 2014 and 2015, followed by the MKE and KK Rivers. The loads measured in each river followed a similar pattern, demonstrating that the large portion of CSO outfalls that discharged into the MN River caused sewage to be most concentrated there.

During CSOs, large volumes of stormwater is mixed with raw sewage and discharged into urban waterways, making it difficult to estimate what portion of the discharged water is raw sewage and what portion is stormwater. We used concentrations of human fecal indicators in raw sewage influent samples at the Jones Island water reclamation facility to estimate how many gallons of untreated sewage were discharged into the rivers and the estuary. Loads of the Lachno2 indicator were converted to gallons of "untreated sewage equivalents" in waterways throughout the duration of an event (Table 8). The mean concentration of Lachno2 in sewage influent samples collected from 2008-2015 (n=54) was 2.0×10^9 ($\pm 2.0 \times 10^9$) CN/gallon. Based on the volumes reported to be released from CSO outfalls upstream of the automated samplers and the sum of the loads measured from each automated sampler in the rivers, untreated sewage was

calculated to be only 0.4% of the total volume released in the 2014 CSO (equivalent to 1.2 MG of sewage equivalents) and only 0.3% of the volume released in the 2015 CSO (equivalent to 1.4 MG of sewage equivalents).

Human fecal indicator loads (expressed as sewage equivalents) from the CSO events were on average 35 (HF183) and 40 (Lachno2) times higher than the largest rain event with no CSO when considering contributions from all three rivers. The greatest difference between human fecal indicator loads during the largest rain event and CSOs was found in the MN River, which had over 100 times greater loads during the CSOs, likely because the majority of CSO volume was released in this river. Loads in the MKE River during the CSO were all close to an order of magnitude higher than the largest rain events and loads in KK River during the largest rain event were all nearly equal to loads during the CSO. Total rainfall amounts during the CSOs were only about 1.5 times higher than the largest rainfall event, which is small in comparison to the large differences in human indicator loads between CSOs and rain events.

The three rivers summed to a load two times larger than that measured in the Milwaukee estuary during the 2014 CSO and nearly five times larger during the 2015 CSO. Several factors impact the load convergence of the three rivers in the harbor, complicating a direct comparison between the sum of the three rivers and the Milwaukee estuary. These factors seem to affect the loading in the estuary not only during low flow periods and rain events, but also periods of intense rain and elevated river streamflow such as those occurring during a CSO.

Table 7: Volumes and percentages of total volumes of sewage released from combined sewer overflow (CSO) outfalls upstream of automated sampling locations in the Kinnickinnic (KK), Menomonee (MN), and Milwaukee (MKE) Rivers during CSO events in 2014 and 2015, as reported by the Milwaukee Metropolitan Sewerage District. Loads and percentages of total loads of human *Bacteroides* (HF183) and human *Lachnospiraceae* (Lachno2) measured at automated sampling locations in each river during these CSO events are also listed.

		Reported CSO volume upstream of sampling locations (MG)[a]	Percent of total CSO volume upstream of sampling locations	HF183 load [Lachno2 load]	Percent of total load of HF183 [Lachno2]
2014 CSO	KK River	0.2	0.1	$1.5x10^{12}$ [$1.7x10^{12}$]	0.1 [0.1]
	MN River	194.3	67.7	$8.3x10^{14}$ [$1.8x10^{15}$]	79.2 [79.9]
	MKE River	92.5	32.2	$2.2x10^{14}$ [$4.6x10^{14}$]	20.7 [20]
	Total	287		$1.1x10^{15}$ [$2.3x10^{15}$]	
2015 CSO	KK River	0	0	$1.9x10^{12}$ [$4.0x10^{12}$]	0.1 [0.2]
	MN River	323.4	74	$9.4x10^{14}$ [$1.9x10^{15}$]	68.6 [69.1]
	MKE River	116.4	26	$4.3x10^{14}$ [$8.2x10^{14}$]	31.3 [30.7]
	Total	439.8		$1.4x10^{15}$ [$2.7x10^{15}$]	

[a] Some CSO outfalls are located downstream of automated sampler locations, so these values only represent the volumes released upstream of sampling locations rather than the total volume from every CSO outfall

Table 8: Sewage equivalents and standard deviations calculated for two high-intensity rain events and two combined sewer overflow (CSO) events, as well as volumes of sewage released during each CSO in the Kinnickinnic (KK), Menomonee (MN), and Milwaukee (MKE) Rivers in Milwaukee, Wisconsin in 2014 and 2015.

				Untreated Sewage Equivalents (gallons)		
Event Type	Rainfall amount (mm)[a]	Rainfall intensity (mm/hr)[b]	Dates	KK River	MN River	MKE River
Mean Low Flow per day	0.0	0.0	varies	**13** ± 13	**2** ± 2	**14** ± 13
Rain Event	58.3	0.96	4/13/2014 – 4/15/2014	**1,400** ± 1,400	**8,800** ± 8,800	**$2.1x10^4$** ± $2.1x10^4$
Rain Event	56.7	1.16	5/12/2014 – 5/14/2014	**450** ± 440	**3,300** ± 3,300	**7,800** ± 7,700
CSO	86.9	2.4	6/17/2014 – 6/20/2014	**870** ± 860	**$9.3x10^5$** ± $9.2x10^5$	**$2.3x10^5$** ± $2.3x10^5$
CSO	69.6	2.4	4/9/2015 – 4/11/2015	**2,000** ± 2,000	**$9.3x10^5$** ± $9.3x10^5$	**$4.2x10^5$** ± $4.1x10^5$

[a] Rainfall amounts are reported for the Milwaukee estuary watershed as a whole
[b] Rainfall intensities are reported for the Milwaukee estuary watershed as a whole

3.4.5 Other sources of fecal contamination

Samples collected in the KK, MN, and MKE River and the Milwaukee estuary were binned based on whether they were collected within 48 hours of rainfall or dry weather for at least 48 hours prior to sampling. All samples were categorized as either meeting all the water quality standards for *E. coli* (126 CFU/100 ml), enterococci (35 CFU/100 ml), and total fecal coliforms (200 CFU/100 ml), or exceeding at least one of the standards. All samples were also categorized based on whether they were considered to be "positive" or "negative" for sewage. Samples were considered positive when the concentrations of HF183 or Lachno2 exceeded the thresholds considered to be positive for human sewage. The KK River consistently exceeded water quality standards and was also consistently positive for human sewage, even during dry weather (Figure 10). In the KK River, only one sample collected during a rain event met all water quality standards, and this sample was considered positive for sewage. Only one of the rain event samples was considered to be negative for sewage, though it did exceed all water quality standards. Among samples collected from the KK River during dry weather, all exceeded at least one water quality standard and only one sample was considered to be negative for sewage. In the MN and MKE Rivers, the majority of samples collected during rain events exceeded water quality standards and a majority of these also were positive for human sewage. A very small percentage of samples (1-3%) collected during rain events met all water quality standards; however, 50% and 75% of these samples were in fact found to be positive for human sewage in the MN River and MKE River, respectively. In both the MN and MKE Rivers, the majority of samples collected during dry weather exceeded at least one water quality standard, but at each site only one of these water quality exceedances was found to be positive for sewage, meaning 74% and 63% of dry weather samples were negative for sewage in the MN and MKE Rivers,

respectively. Many of these exceedances were due to elevated fecal coliforms or enterococci concentrations, which were not likely from human sources. One-third of dry weather samples in the MN and MKE Rivers which met all water quality standards were found to be positive for sewage. Similar to the MN and MKE Rivers, a majority of samples collected in the Milwaukee estuary during rain events exceeded at least one water quality standard and a majority of these samples were also positive for human sewage. Of the samples which met water quality standards during rain events, 47% of these samples were also positive for sewage. In contrast to the MN and MKE Rivers, a majority of dry weather samples collected in the Milwaukee estuary met all water quality standards and only two of these samples were positive for sewage.

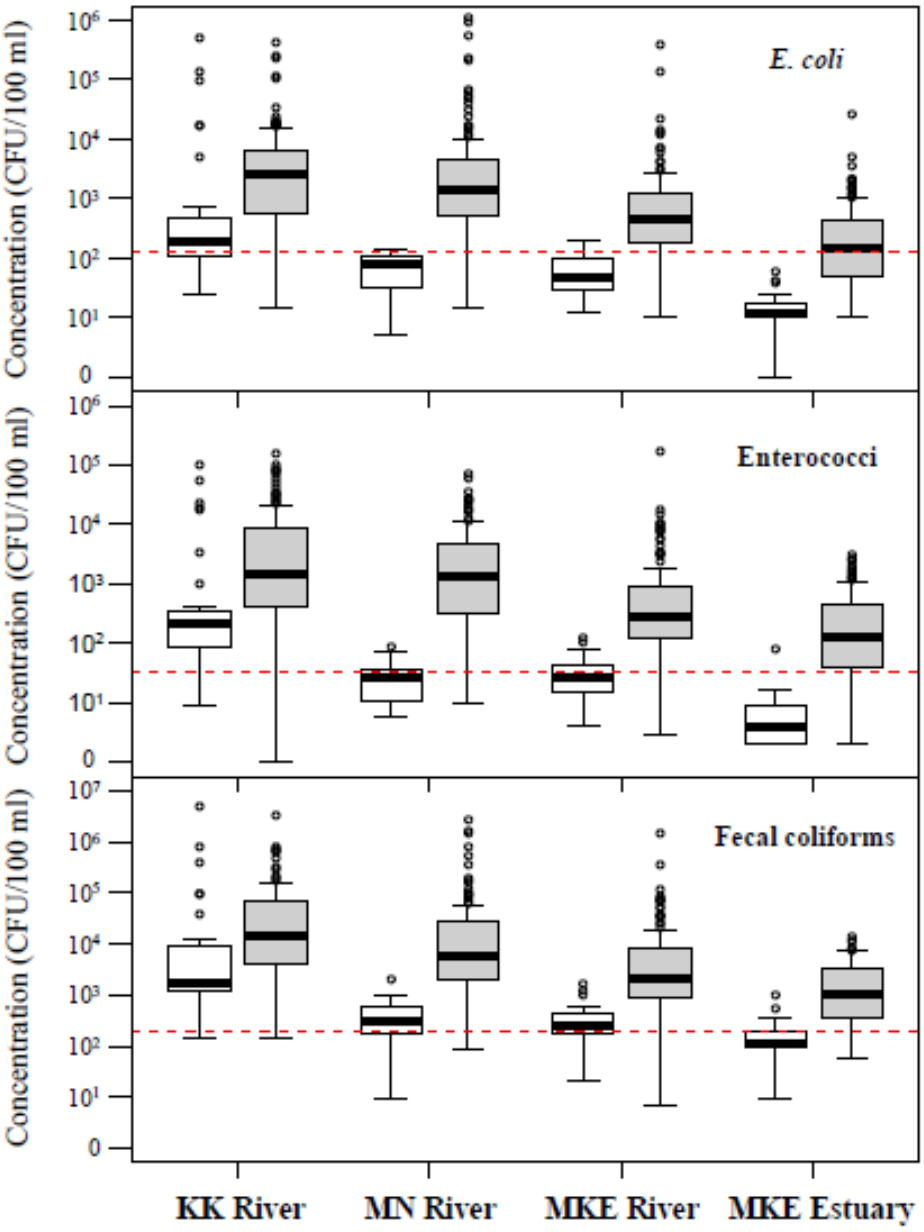

Figure 10: Concentrations of standard fecal indicator bacteria, *Escherichia coli (E. coli)*, enterococci, and fecal coliforms, measured in the Kinnickinnic (KK), Menomonee (MN), and Milwaukee (MKE) Rivers, as well as the MKE estuary during dry weather (white plots) and rain events (gray plots) in Milwaukee, Wisconsin in 2014 and 2015. Red dotted lines represent the ambient water quality standards for *E. coli* (126 CFU/100 ml), enterococci (35 CFU/100 ml), and fecal coliforms (200 CFU/100 ml).

3.5 Discussion

3.5.1 Rainfall as a driver of fecal pollution in Milwaukee waterways

In Milwaukee, Wisconsin, three waterways with varying drainage areas and degrees of urbanization discharge into Lake Michigan via the Milwaukee estuary. Fecal contamination due to sewage pollution has been measured in several studies in the rivers, estuary, and nearshore Lake Michigan and this pollution has been found to increase following rainfall (Newton et al., 2013). In urban Milwaukee, deteriorating, leaky infrastructure is likely a major source of sewage pollution, which seems to be driven by rainfall. We demonstrated this pattern by measuring concentrations of two human fecal indicators across the hydrograph throughout the duration of storm events, and consistently observing a pattern of increased concentrations with increased flow following rainfall. These results are consistent with other studies which have also found that concentrations of FIB increase and decrease with similar changes in streamflow (Rowny and Stewart, 2012; Stumpf et al., 2010). This is a widespread problem, because this trend was observed at sampling sites located in each of the three rivers, as well as in the Milwaukee estuary.

3.5.2 The usefulness of two genetic markers to track sewage pollution in freshwater systems

This research shows the utility of using two human genetic markers, HF183 and Lachno2, in tandem to reliably track sewage contamination. The two indicators are highly correlated in river and estuary samples, indicating there is a high probably that the fecal pollution is from a human sewage source (Newton et al., 2011). Although greater differences between concentrations of the two indicators were found in grab samples collected in river samples upstream in the watersheds (Chapter 2), this research demonstrates that among composite samples collected across the

hydrograph, the ratio of the two indicators shows greater consistency than individual grab samples. In untreated sewage from Jones Island water reclamation facility, the ratio of Lachno2 to HF183 is on average 1.5 (± 0.6), and we found similar ratios among rain events, low flow periods, and CSO samples collected in the estuary and the rivers in 2014 and 2015. Lachno2 concentrations in the Milwaukee estuary, MN, and MKE Rivers were on average 2 (± 1) times higher than HF183 concentrations and in the KK River they were on average 3 (± 3) times higher, suggesting Lachno2 may persist for a longer time than HF183 in the environment. Since two different types of bacteria are being used as indicators, differences in their ecologies may contribute to the disparity in the concentrations we measured and these differences may give a clue to how long the sewage pollution has been in the environment. For example, in the Milwaukee estuary, the difference between HF183 and Lachno2 concentrations tended to be higher in samples collected in the spring compared to samples collected in the summer and during low flow periods. One explanation may be that in spring, contamination is "older", for example, sewage leaking from infrastructure may have built up over the winter months and then was flushed into the environment during heavy spring rainfall. We have hypothesized that members of the family *Lachnospiraceae* may last longer in the environment because it is comprised of gram positive organisms, compared to the genus *Bacteroides*, which is comprised of gram negative organisms. During the summer and low flow periods, the ratio of Lachno2 to HF183 may be closer to one because the pollution source is more recent, i.e. more frequent summer rains have not allowed pollution to build up as much. High levels of the indicators during low flow periods are likely caused by illicit cross-connections in sewer lines causing fresh sewage to flow directly into the waterway.

3.5.3 Potential targets for remediation

The KK River is the smallest and most urbanized river which leads directly to Lake Michigan via the Milwaukee estuary and it was also the greatest contributor per drainage area and per urban area to sewage contamination during low flow periods. Numerous stormwater outfalls line the concrete channel of the KK River, which likely serve as a conduit for leaking sanitary sewers and sewage from illicit cross-connections to reach the river. As a small watershed, the KK River is a potential target for remediation. An increase in concentrations and loads, was found following rainfall in all three of the rivers and the estuary. When comparing total loads during rainfall events, the MKE River had the highest loads, followed by the MN and KK Rivers. When rain event loads were normalized by drainage area, the KK River had the greatest yield, followed by the MN River and the MKE River, indicating that the difference in loads among the rivers is largely due to the differences in drainage areas and river streamflow between each site. When rain event loads were normalized by area of urban land use in each watershed, loads per km^2 of urban-classified land use were more similar among the three river sites, suggesting sewage contamination was related to the degree of urbanization in the watershed. Since the KK River watershed has nearly all urban land use, the entire watershed is a potential target for remediation. In addition, the highly urbanized regions of the MN and MKE Rivers may also be considered, in order to target areas that have the most impact on sewage loading to Lake Michigan.

3.5.4 Possible mechanisms driving sewage pollution

Non-point source loading of many environmental pollutants can be described by the “first flush” phenomenon, which is theoretically the first portion of runoff which contains a large fraction of contaminants conveyed to receiving waters. In this study, the MKE and MN River watersheds

are relatively large watersheds that were not expected to display the first flush phenomenon, as smaller watersheds or sewersheds would. Additionally, several studies have found that microbial contaminants such as FIB do not always follow this phenomenon (Rowny and Stewart, 2012; Stumpf et al., 2010; Surbeck et al., 2006), but environmental reservoirs of bacteria may contribute to fecal contamination in surface waters in a "mud puddle" effect (Rowny and Stewart, 2012; Surbeck et al., 2006), meaning FIB are ubiquitous in the environment and provide a continuous source of fecal pollution that is not diluted by high flow. A similar phenomenon was observed with human fecal indicators in samples collected at the three rivers and in the estuary site for this study, as concentrations were positively correlated with river flow and loads were positively correlated with total rainfall amount and rainfall duration. Because human fecal indicators are specific to human sewage, it is not likely that sewage is ubiquitous in the environment, but there are a few possible sources which could be contributing to a continuous source of sewage contamination. One source is leaking sewer lines and illicit cross connections. In many areas of the city of Milwaukee, sewer infrastructure is deteriorating, causing cracks in sanitary sewer lines, private laterals, and storm sewers. These leaks can cause sewage to infiltrate the groundwater and travel to storm sewers, which act as a direct route for sewage to reach a receiving waterway during rain events. Sercu et al., 2011 provided evidence that even during dry weather, leaking sanitary sewer lines can directly contaminate storm sewers (Sercu et al., 2011). Leaking sewer lines, along with illicit cross connections which divert sewage directly into storm sewers, could potentially provide a consistent source of human fecal contamination. Additionally, very large and intense rain events may not always cause a CSO, but many smaller communities such as those upstream in the MKE River may have SSOs that possibly go unreported and are difficult to quantify. Finally, river sediment could be a reservoir for human

fecal indicators, which could potentially re-suspend during rain events and contribute to human indicator loads measured in the rivers and the estuary.

3.5.5 Combined sewer overflow events

CSOs are a major impact to water quality, discharging millions of gallons of untreated sewage into urban waterways. Since the discharge from CSOs are actually a mixture of raw sewage and large volumes of stormwater, it is difficult to determine what portion is raw sewage. Using human fecal indicators as a proxy for raw sewage it was estimated that only a fraction of a percent of the total volume of CSO discharge was raw human sewage. Although sewage concentrations were not continuously monitored throughout the duration of an entire year, the human fecal indicator loads computed from each of 11 rain events, along with the total rainfall depth over the course of these events, was used to determine how the amount of sewage discharged during a CSO compares to the amount that is discharged over the course of a year with average rainfall. The average yearly rainfall in Milwaukee, Wisconsin is 884.2 mm. On average, loads of human fecal indicators during CSOs were still six to seven times greater than the total load over the course of a year from rain events alone. The load of sewage per year discharged to Lake Michigan via the MKE, MN, and KK Rivers during rain events was translated to approximately 175,00 (± 174,000) gallons of untreated sewage equivalents. Although CSOs pose an obvious health risk over a few days per year, sewage that is present during rain events and low flow periods result in some level of health risk 365 days a year.

3.5.6 Non-human sources of fecal contamination

Human sewage is a common cause of fecal contamination in urban waterways; however, there are many other sources of fecal contamination that may contribute to frequent water quality exceedances. When standard FIB concentrations were compared to human fecal indicator concentrations, it was evident that a majority of the samples collected in the KK River exceeded at least one water quality standard and had a positive human sewage detection. In both wet weather and dry weather samples, nearly all samples had both water quality exceedances and positive sewage detection, suggesting that human sewage contamination is a major cause of water quality exceedances in the KK River. The MN River, MKE River, and Milwaukee estuary watersheds are larger, mixed-use watersheds and are more likely to have sources of fecal contamination other than from human sewage. While a majority of rain event samples had elevated concentrations of both FIB and human fecal indicators, suggesting human sources for most of these samples, a large proportion of dry weather samples in the MN and MKE Rivers had high FIB concentrations but were negative for sewage. This suggests a portion of the water quality exceedances in these rivers may be the due to sources other than sewage, especially during dry weather. Possible sources include pet waste and urban wildlife in urban areas, and animal manure in rural and agricultural areas. A clear signal from the ruminant genetic marker was found in samples collected in the MKE River and the MKE estuary, indicating that agricultural runoff is a source of fecal pollution in the MKE River watershed. Though uncommon, several of the samples collected from the rivers and the estuary met all water quality standards, yet were positive for sewage. Because the assays used to analyze human fecal indicators detect both live and dead cells, whereas the culture-based methods used to analyze standard FIB detect only live cells, these results could be due to a longer persistence of human

fecal indicators in the environment. These results also suggest that standard FIB are not always a reliable method to identify health risks of exposure to the harmful pathogens that may be present in human sewage.

3.5.7 Complications to load and mass balance computations in a freshwater estuary

Although samples were collected in the KK, MN, and MKE Rivers and the Milwaukee estuary during nearly the same time periods, due to the complexity of the Milwaukee estuary, determining a mass balance between the sewage loads measured in the rivers and the loads measured in the estuary is more complicated than simply adding up the loads from the three rivers and expecting it to be equal to loads measured in the estuary. Because of the close proximity of the Milwaukee estuary to Lake Michigan, there is a significant amount of backflow which dilutes the concentrations measured in the samples collected in this study. The sampling location in the Milwaukee estuary is upstream of where the effluent from the Jones Island water reclamation facility is discharged, yet backflow could cause some of this effluent to flow upstream and potentially contribute to the concentrations measured in the estuary. Another issue is the distance between each sampling locations on the rivers and the sampling location in the estuary. Each of the rivers has a different velocity, effecting the travel time between the river monitoring sites and the estuary monitoring site. The bacteria we are measuring in the rivers may degrade or settle into the sediment before reaching the estuary, and likewise, bacteria can be re-suspended from the sediment into the water column. Settling could be significant due to the slowing of velocity in the estuary where the channels cross sections are much larger, and water surface slopes decrease due to influence from Lake Michigan. Additionally, samples are collected in the estuary from a relatively shallow intake of approximately two meters below the

surface, meaning there is a potential for bacteria to be settled further down in the water column before reaching our sampling point. Finally, additional contributions from the urban areas located between the sampling locations on the rivers and in the estuary were not accounted for; however, for most events the sum of the loads from the three rivers were greater than the load in the estuary, suggesting settling and dilution outweighs these additional contributions.

3.6 Conclusions

In summary, sampling two human fecal indicators continuously across the hydrograph allowed us to reliably identify and quantify loads of sewage contamination in urban waterways. During low flow periods, the smallest but most urbanized watershed (KK River) consistently delivered the largest amount of sewage contamination per unit drainage area. Fluxes of sewage in the KK, MN, and MKE Rivers, and the Milwaukee estuary increased several orders of magnitude between low flow periods and rainfall events, suggesting rainfall was a driver of sewage pollution. Additionally, significant correlations were found between loads of human fecal indicators, total rainfall amount, and rainfall duration. During extreme rain events, combined sewer systems exceed capacity and overflow, increasing sewage loads on average up to several orders of magnitude above heavy rainfall alone. Sewage contamination in these rivers related to the degree of urbanization in the watershed. When yields of human fecal indicators were calculated considering only the urban land use (load per urban km^2), all three watersheds showed similar values. This indicates that there are consistent sources of sewage throughout the urban areas of each watershed.

4. Concluding statements

This research demonstrated the usefulness of using two human-specific fecal indicators to identify and quantify sewage contamination in the Milwaukee estuary and three upstream urban rivers. This research showed that intensive monitoring of human fecal indicators across the hydrograph is useful to fully characterize sewage contamination that occurs both from point sources such as CSOs and non-point sources during average rainfall events. At all sampling locations in the rivers and the Milwaukee estuary, a consistent pattern was found between concentrations of human fecal indicators and streamflow. Concentrations were positively correlated with streamflow, suggesting a constant source sewage contamination that was driven by rainfall. This result was surprising, because intuitively, concentrations would be diluted out by increasing streamflow. Several hypotheses exist as to what is causing the constant source of sewage in these waterways. One hypothesis relates to leaking sewer infrastructure and sewer misconnections. During rainfall, groundwater levels rise and cause inflow and infiltration into sewer pipes. As the sewer pipes fill up, sewage could also potentially be pushed into the groundwater and carried to leaking storm sewers. Sewage would then infiltrate through the cracks in the storm sewer pipes and wash into a receiving waterbody. Additionally, it is possible for sewage to infiltrate into the groundwater during dry weather periods and ultimately get flushed out of a storm sewer during rain events. Another potential continuous source of sewage contamination is from sediment resuspension. There is potential for human fecal indicators to accumulate in sediment and some portion of this could be re-suspended during heavy rainfall. Additional research is needed to determine the exact source of sewage causing the phenomenon described in this report.

The knowledge generated as part of this work will be useful to create appropriate goals to address the human health concerns of sewage contamination, such as in the TMDL implementation process. Although ambient water bodies in the Unites States are regulated on standard fecal indicators for bacterial water quality, alternative indicators can be used to prioritize river reaches and target sources for remediation that are responsible for the most serious health risks. In addition, sampling for human fecal indicator bacteria could help identify health risks due to human sewage that would otherwise go undetected if fecal coliforms alone were used in monitoring. Overall, this research demonstrates the utility of alternative indicators for the assessment of fecal pollution sources and this data could be used to help direct the efforts of local agencies and municipalities to investigate and remediate unrecognized sources of sewage contamination, which pose a serious health concern.

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6. Appendix: Human fecal indicator loads at all sampling locations

Event Number	Dates	HF183 Event Loads (CN)					
		KK River	MN River	MKE River	Sum of 3 rivers	Milwaukee estuary	Fold difference
1	4/13/2014 – 4/14/2014	$1.4x10^{12}$	$6.8x10^{12}$	$2.6x10^{13}$	$3.4x10^{13}$	$9.8x10^{13}$	3
2	4/27/2014 – 5/1/2014	$2.3x10^{12}$	$3.7x10^{12}$	$5.6x10^{12}$	$1.2x10^{13}$	$5.9x10^{12}$	2
3	5/11/2014 – 5/14/2014	$3.5x10^{11}$	$3.0x10^{12}$	$5.9x10^{12}$	$9.3x10^{12}$	$1.2x10^{13}$	1
4	6/10/2014 – 6/12/2014	$5.5x10^{11}$	$6.0x10^{11}$	$1.7x10^{12}$	$2.9x10^{12}$	$9.8x10^{11}$	3
5	8/18/2014 – 8/20/2014	$2.4x10^{11}$	$6.3x10^{11}$	$1.5x10^{12}$	$2.4x10^{12}$	$6.0x10^{11}$	4
6	8/21/2014 – 8/23/2014	$3.9x10^{10}$	$1.3x10^{11}$	$1.5x10^{11}$	$3.2x10^{11}$	$2.6x10^{11}$	1
7	9/10/2014 – 9/11/2014	$1.0x10^{11}$	$5.6x10^{10}$	$8.0x10^{10}$	$2.4x10^{11}$	$9.5x10^{10}$	2
8	6/11/15 – 6/13/15	$3.4x10^{11}$	$2.5x10^{12}$	$9.3x10^{11}$	$3.8x10^{12}$	$1.1x10^{12}$	3
9	6/14/15 – 6/15/15	$9.9x10^{10}$	$4.3x10^{11}$	$6.0x10^{11}$	$1.1x10^{12}$	$3.8x10^{11}$	3
10	7/6/2015 – 7/9/2015	$2.1x10^{11}$	$1.1x10^{12}$	$1.7x10^{12}$	$3.0x10^{12}$	$6.6x10^{11}$	4
11	9/8/15 – 9/10/15	$2.7x10^{11}$	$2.8x10^{12}$	$1.6x10^{12}$	$4.7x10^{12}$	$5.2x10^{11}$	9
	Mean Low Flow	$1.7x10^{9}$	$5.4x10^{8}$	$3.2x10^{10}$	$3.4x10^{10}$	$9.4x10^{9}$	4

Event Number	Dates	Lachno2 Event Loads (CN)					
		KK River	MN River	MKE River	Sum of 3 rivers	Milwaukee estuary	Fold difference
1	4/13/2014 – 4/14/2014	$2.7x10^{12}$	$1.8x10^{13}$	$4.1x10^{13}$	$6.2x10^{13}$	$4.6x10^{14}$	7
2	4/27/2014 – 5/1/2014	$4.5x10^{12}$	$7.4x10^{12}$	$8.6x10^{12}$	$2.1x10^{13}$	$1.0x10^{13}$	2
3	5/11/2014 – 5/14/2014	$8.8x10^{11}$	$6.5x10^{12}$	$1.5x10^{13}$	$2.3x10^{13}$	$3.3x10^{13}$	1
4	6/10/2014 – 6/12/2014	$5.4x10^{11}$	$7.3x10^{11}$	$2.7x10^{12}$	$4.0x10^{12}$	$1.2x10^{12}$	3
5	8/18/2014 – 8/20/2014	$3.6x10^{11}$	$9.0x10^{11}$	$2.1x10^{12}$	$3.3x10^{12}$	$5.6x10^{11}$	6
6	8/21/2014 – 8/23/2014	$9.1x10^{10}$	$1.6x10^{11}$	$2.7x10^{11}$	$5.1x10^{11}$	$2.3x10^{11}$	2
7	9/10/2014 – 9/11/2014	$1.7x10^{11}$	$2.0x10^{11}$	$1.6x10^{11}$	$5.4x10^{11}$	$1.0x10^{11}$	5
8	6/11/15 – 6/13/15	$5.8x10^{11}$	$3.2x10^{12}$	$1.5x10^{12}$	$5.2x10^{12}$	$1.8x10^{12}$	3
9	6/14/15 – 6/15/15	$1.5x10^{11}$	$7.8x10^{11}$	$9.5x10^{11}$	$1.9x10^{12}$	$6.9x10^{11}$	3
10	7/6/2015 – 7/9/2015	$3.2x10^{11}$	$2.0x10^{12}$	$2.6x10^{12}$	$5.0x10^{12}$	$5.9x10^{11}$	8
11	9/8/15 – 9/10/15	$3.2x10^{11}$	$3.8x10^{12}$	$1.9x10^{12}$	$6.0x10^{12}$	$5.6x10^{11}$	11
	Mean Low Flow	$6.1x10^{9}$	$8.9x10^{8}$	$5.7x10^{10}$	$6.4x10^{10}$	$1.3x10^{10}$	5