Greater Milwaukee Watersheds Pathogen Source Identification

Report: March 1, 2006 to July 28, 2009

MMSD Contract No. M03016P02

GREAT LAKES WATER INSTITUTE

UNIVERSITY of WISCONSIN UWMILWAUKEE

[redacted]

Greater Milwaukee Watersheds Pathogen Source Identification

Report: March 1, 2006 to July 28, 2009

MMSD Contract No. M03016P02

Authored by:
Sandra L. McLellan and Elizabeth P. Sauer
Great Lakes WATER Institute
University of Wisconsin-Milwaukee

Released: November 2, 2009

[redacted]

TABLE OF CONTENTS

List of Figures

List of Tables

EXECUTIVE SUMMARY

Background and Significance

The overall goal of this project was to determine sources of high fecal indicator bacteria in stormwater discharges within the Milwaukee Metropolitan Sewerage District (MMSD) service area. This research addresses a high priority area for the MMSD, whose mission is to protect public health and the environment and improve water quality. Prior analysis during the MMSD's *2020 Facilities Planning* effort, which also included work by the Southeastern Wisconsin Region Planning Commission (SEWRPC) to update the *Regional Water Quality Management Plan (RWQMP)*, and the McLellan Bacterial Genetics Laboratory (functioning as part of the University of Milwaukee's Great Lakes WATER Institute) had identified stormwater inputs as a major source of water quality impairment in the rivers of the Greater Milwaukee Watersheds (**Figure ES-1**) and subsequently Lake Michigan. High levels of fecal coliforms and *Escherichia coli* (*E. coli)* have been routinely detected in the absence of reported sanitary or combined sewage overflows; however the source of this bacterial contamination is currently unknown or unrecognized.

The water quality goals for the region include reducing the number of days surface waters exceed the State of Wisconsin's water quality standard for fecal coliforms. According to SEWRPC's 2009 *Regional Water Quality Management Plan*, Milwaukee Area rivers often exceed recreational standards. In 1998 to 2001, fecal coliform levels in both the Menomonee and Kinnickinnic Rivers often exceeded the water quality standard of 1,000 colony forming units (CFUs) per 100 ml of sample (water), which is a special variance standard for limited recreational use. For example, the Menomonee River station located between 25th Street and North 70th Street exceeded this standard in 38% of samples and the Kinnickinnic River station located between South 7th Street and South 27th Street exceeded the standard in 49% of samples (SEWRPC, 2008). Sites along the Milwaukee River that are

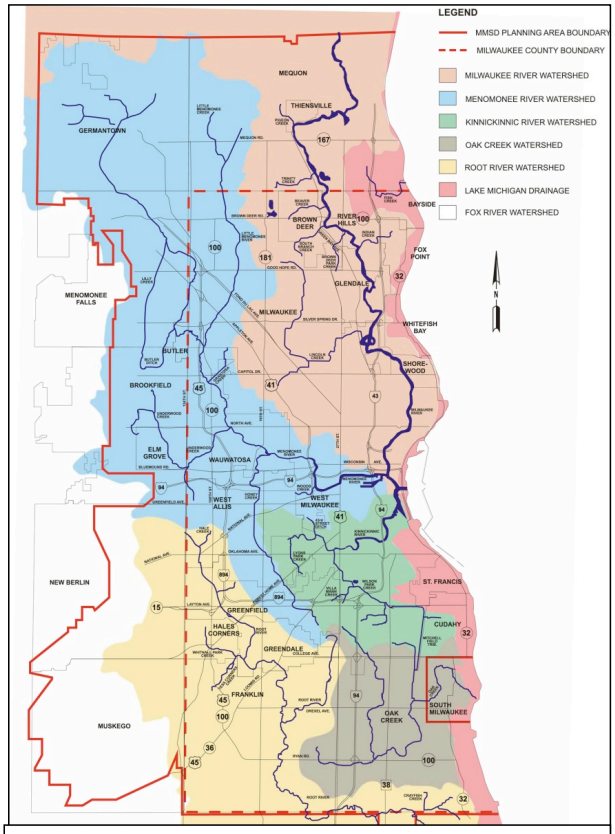


Figure ES-1. Greater Milwaukee Watersheds

designated as limited recreational use were usually in compliance (~75% of samples in compliance). However, Milwaukee River sites that are designated full recreational use are expected to meet a more stringent standard of 200 CFUs/100 ml; these sites often exceeded the limit (SEWRPC, 2008).

In order to meet water quality goals for swimmable, fishable waters, it is critical to determine what the major sources of pollution are so that remediation strategies can be formulated and implemented. While upstream rural sources account for a portion of the fecal indicator bacteria, a larger portion is derived from stormwater runoff in the urbanized areas (SEWRPC, 2008). It should be noted that water quality standards and monitoring are based upon an indicator bacteria that is only a general proxy for the presence of fecal pollution and disease-causing organisms (e.g. pathogens). Fecal pollution from different sources will carry different pathogens; however fecal pollution from sanitary sewage generally constitutes a more serious public health risk because multiple human pathogens including bacteria, viruses, and protozoan can be present in high concentrations. Additionally, agricultural/rural waste may also pose a public health risk due to pathogens such as *E. coli* O157:H7, *Giardia*, or *Cryptosporidium parvum*. The numbers and types of pathogens associated with stormwater (non-point) runoff (pet and wildlife waste) are not well known. Domestic pets and wildlife may carry some organisms that are pathogenic to humans such as *Salmonella* or *Campylobacter*, but overall, pathogen occurrence is expected to be much less than what is found in sanitary sewage. This means that even small inputs of sanitary sewage that results in only a modest increase in fecal indicator bacteria may be more significant in terms of health risk than large fecal indicator inputs from surface runoff that is free of sanitary sewage contamination.

Results and Discussion

The McLellan Bacterial Genetics Laboratory has developed DNA based methodology for detecting human sources of fecal pollution. This approach is based upon detecting a certain bacteria type that is specifically found in humans, a species of *Bacteroides*, which was first described by Field and co-workers (Bernhard & Field, 2000). This bacterium is found and is present in almost all humans, but not other animals. The McLellan Laboratory and others have found that the human *Bacteroides* genetic marker (e.g. the specific 16S rRNA gene sequence that identifies this organism) is a sensitive and specific indicator of sanitary sewage contamination (Stewart*, et al.*, 2003, Bower*, et al.*, 2005, Santoro & Boehm, 2007). Detection is based upon amplification of the 16S rRNA gene using polymerase chain reaction (PCR). If the human *Bacteroides* is present in a water sample, the genetic marker for this organism can be amplified and thereby be measured as to its relative strength.

The McLellan Bacterial Genetics Laboratory analyzed more than 1,000 stormwater samples (including inline stormwater and grab samples) from 62 municipal stormwater discharge locations over a three-year period for the presence of the human *Bacteroides* genetic marker (**Figure ES-2**). Three stormwater outfalls along Lake Michigan had positive results in more than 70% of the samples tested. Stormwater outfalls along the Menomonee River were positive in 73% of the samples tested (**Table ES-1**).

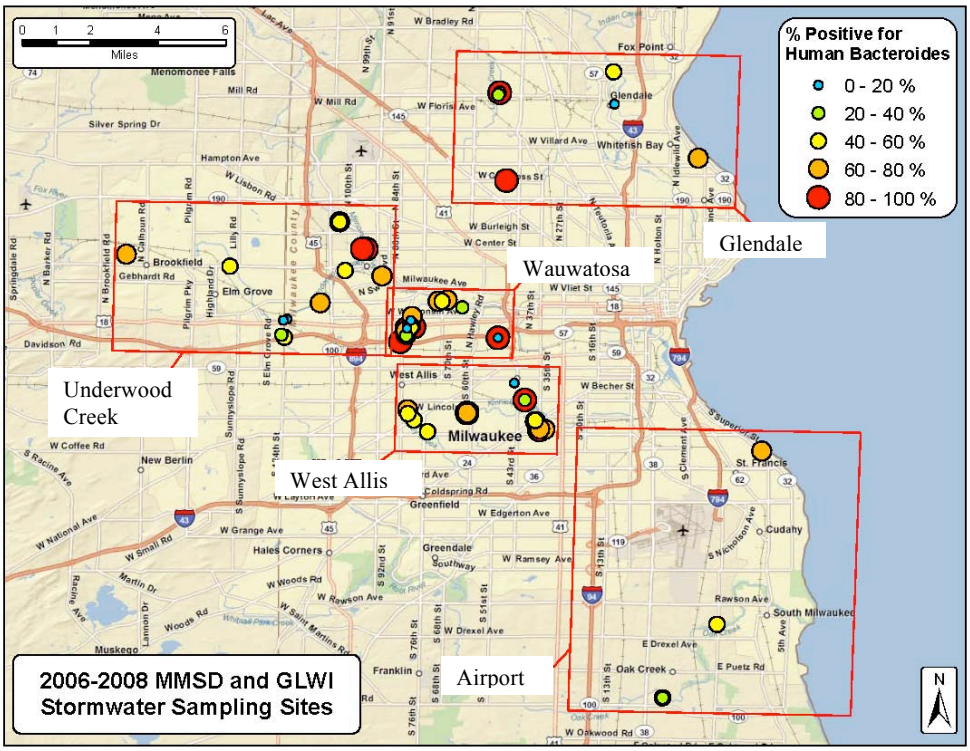


Figure ES-2: Percent of samples positive for the human *Bacteroides* genetic marker at MMSD and McLellan Laboratory stormwater site locations.

Similarly, stormwater outfalls along the Kinnickinnic River and Lincoln Creek were found to have a persistent signal of human *Bacteroides*, with 60% and 65% of the samples testing positive, respectively. The other four receiving waters; Honey Creek, Underwood Creek, Oak Creek, and the Milwaukee River, also demonstrated evidence of sanitary sewage contamination of stormwater. Overall, these results indicate that sanitary sewage contamination of stormwater is a serious concern throughout the Greater Milwaukee Watersheds, and locally owned stormwater collection & conveyance systems in specific areas should be given high priority for further investigation: those discharging directly to Lake Michigan, and those within the Menomonee, Lincoln Creek, and Kinnickinnic watersheds.

One stormwater outfall along Lincoln Creek was tested for the presence of human viruses since samples from this location demonstrated a persistent signal of human *Bacteroides* marker. Virus detection was performed by Dr. Mark Borchardt's laboratory at Marshfield Clinic (Marshfield, WI). High concentrations of three human viruses were found, including adenovirus, enterovirus, and G1 norovirus. These concentrations are similar to what is found in sewage influent and confirms the presence of human sewage contamination in this stormwater outfall. This level of human virus contamination in stormwater constitutes a potential public health risk.

Table ES-1: Summary of *E. coli*, Enterococcus and human *Bacteroides* genetic marker data compiled by receiving waters for stormwater samples collected by MMSD and GLWI during the 2006-2008 sampling seasons.

			E. coli		Enterococcus	
Receiving Waters	Number of Samples 2006-2008	% Positive for human *Bacteroides*	Average (CFUs/100ml)	Geometric Mean (CFUs/100ml)	Average (CFUs/100ml)	Geometric Mean (CFUs/100ml)
Milwaukee River	12	33%	6,550	4,040	6,020	4,250
Oak Creek	93	39%	358,000	9,020	153,000	11,000
Underwood Creek	74	42%	113,000	12,600	158,000	13,500
Honey Creek	203	51%	40,300	5,850	43,400	6,990
Kinnickinnic River	201	60%	329,000	19,700	175,000	21,600
Lincoln Creek	108	65%	51,100	14,600	60,500	9,770
Menomonee River*	209	73%	382,000	24,300	111,000	21,100
Lake Michigan	95	71%	364,000	36,400	211,000	29,400

*two representative samples from each SWWA13 pollutograph were included in this analysis. Special sampling at Miller Park, SWMI18 is not included in this analysis.
** Russell Ave outfall is not included because it is a submerged outfall; indicator levels may not accurately reflect fecal pollution levels.

The findings of this research showed no correlation between the presence of the human specific *Bacteroides* marker and culturable *E. coli* and enterococci. This demonstrates that human sources are only one contributor (of many) to fecal indicator bacteria. However, even a low amount of human sources may carry pathogens and therefore could pose a significant health risk, regardless of the contribution of fecal indicator bacteria. The relationship between rainfall amounts and the numbers of human marker positives were evaluated to explore possible connections between, for example, high rainfall amounts and subsequent ground saturation favoring infiltration/exfiltration of the sanitary system. No relationship between rainfall and positive results were found. Certain dates had higher numbers of stormwater outfalls positive across the five watersheds, suggesting that the dynamics of a storm event may contribute to the process by which sanitary sewage enters the stormwater collection & conveyance systems and different conditions can vary by community and soil types. Identifying the environmental and climate variables that are linked with the presence of sanitary sewage contamination in the stormwater collection & conveyance systems warrants further research.

A major challenge in advancing this research is to develop a quantitative assay for the human specific marker. The McLellan Bacterial Genetics Laboratory has developed a qPCR (quantitative PCR) assay for three fecal indicators (*E. coli*, enterococci, and total *Bacteroides* spp.) and the human *Bacteroides* genetic marker. This allows for the comparison of "total fecal pollution" to the "human specific" portion using the same methodology (e.g. by DNA based methods). A high ratio of human to total fecal pollution would indicate the major source is

sanitary sewage. Another major challenge in this work is to recover and amplify bacterial DNA from environmental samples that contain compounds that inhibits the PCR reaction. This is a problem when working with any environmental sample (soil, water, etc.), but stormwater appears to be particularly problematic in this regard due to high amounts of other confounding contaminates in the sample. To address this issue an inhibitor assay was developed in this study to determine if the PCR reaction is impaired, and to what degree. This assay was utilized in conjunction with the quantitative assay to accurately quantify the human *Bacteroides* genetic marker, and in traditional PCR to determine if there are potentially false negative results, e.g. PCR completely impaired due to inhibition. The quantitative assay requires validation before it can be employed for investigative studies; however it was used in this study to give a general sense of the level of sanitary sewage contamination.

Successes

The Human *Bacteroides* genetic marker has been used successfully in a number of spin-off investigations that originated from early results of this study. Working with MMSD monitoring crews, an outfall near Miller Park in Milwaukee was found to have positive results for the human *Bacteroides* genetic marker in nearly 100% of the samples. In March of 2007, dye testing was conducted to investigate the potential of illicit connections (**Figures ES-3 and ES-4**). It was found that some of the stadium's sanitary sewage lines (Luxury Box level) were mistakenly connected to a stormwater line. The problem was immediately remedied and the outfall has since tested negative for sanitary waste.

Figure ES-3. Dye testing of sanitary sewer lines near Miller Park. Testing for the human Bacteroides genetic marker was positive in a nearby stormwater outfall.

Figure ES-4. Fluorescein dye, which was introduced into sanitary facilities within Miller Park, was released through a stormwater outfall on the Menomonee River.

Stormwater outfalls along Honey Creek north of I-94 were tested for the presence of the human *Bacteroides* marker because of consistently high bacteria levels reported by MMSD's Water Quality monitoring group for this section of Honey Creek. Based upon these results (consistent detection of the human *Bacteroides* genetic marker) a separate investigation was conducted by the City of Milwaukee, in the area near 72nd Street and Mt Vernon Avenue. Smoke and dye testing was conducted and in this case it was found that the sanitary sewer line was directly on top of the stormwater line, both which had multiple fractures/cracks. The lines were televised during dye testing and showed infiltration into the stormwater lines at several points. The sanitary sewer pipes have since been lined and the area is currently being retested.

Communication and Outreach

One of the McLellan Bacterial Genetics Laboratory's primary goals is to educate the public and integrate this information into the decision making process. As citizens understand the impacts of urban stormwater on rivers and Lake Michigan, they will be more willing to support investments in infrastructure and expect their communities to address serious pollution inputs such as illicit cross connections or failing sewer lines. The McLellan Bacterial Genetics Laboratory staff has made numerous presentations in the past three years conveying research findings and illustrating the impact of urban stormwater on water quality.

Figure ES-5. Green roof at the GLWI, which is used as a demonstration project to highlight strategies for mitigating stormwater runoff.

The McLellan Bacterial Genetics Laboratory has also worked with nonprofit groups to cooperate with citizen monitoring efforts and also continues to maintain a green roof (**Figure ES-5**) that was funded through MMSD in 2003 as a model for others to implement their own stormwater runoff reduction/retention strategies. The McLellan Bacterial Genetics Laboratory provides the public with tours of the green roof, which also serves as an outdoor classroom for GLWI and UWM. In addition Dr. McLellan has served on two key committees, SEWRPC Water Quality Modeling Subcommittee and the Southeastern Wisconsin Watersheds Trust Science Council (SWWT), and has highlighted research findings from this work during discussions.

Conclusions and Recommendations

Overall, this research demonstrates that unknown or unrecognized sanitary sewage inputs into the local municipal stormwater collection & conveyance system are a major source of fecal pollution in area waterways and Lake Michigan. This poses a potential, yet serious public health risk due to the likelihood of other pathogen occurrence. The following recommendations are made from this research:

(1) Investigate sanitary and storm sewers infrastructure integrity by community using traditional engineering approaches (dye testing, smoke testing, etc.) in areas of the stormwater collection & conveyance system where stormwater has shown evidence of chronic sanitary sewage discharges. This project has identified approximately 41 high priority stormwater outfalls of the 62 investigated. Stormwater outfalls that tested positive for the human *Bacteroides* genetic marker most frequently should be a high priority for follow up investigation by the locally responsible communities. Additional stormwater outfalls in these high priority areas should also be tested (see recommendation #2). A concentrated investigative effort in the stormwater system may provide valuable insight into the nature of infrastructure failures (sanitary sewers and storm sewers) in the urban environment.

(2) Broaden the investigation in areas of concern using quantitative methods to identify the stormwater outfalls and locations within the stormwater collection & conveyance system that have significant sanitary sewage contamination. There are specific areas of concern; Lake Michigan, Lincoln Creek, Menomonee and Kinnickinnic watersheds. This study has only investigated a very small fraction of the stormwater system within these areas. Stormwater outfalls should be prioritized using quantitative results for the human *Bacteroides* genetic marker by qPCR. A comprehensive investigative effort in the stormwater collection & conveyance systems that discharge to these receiving waters should be conducted to guide formulation of watershed management plans and improvement/restoration goals.

(3) Perform human virus testing in conjunction with human *Bacteroides* genetic marker sampling. The McLellan Bacterial Genetics Laboratory found that conducting virus testing on a small volume of stormwater is feasible; it was found that the same concentration of human derived viruses found in sewage was also present in one stormwater sample. Quantification of human viruses will provide the basis for assessment of public health risks associated with sanitary sewage discharges caused either by illicit connections or failing integrity of the associated infrastructure.

(4) Identify hydrological, physical, and meteorological parameters that correlate with high levels of sanitary sewage contamination in stormwater outfalls to better understand the conditions that favor contamination processes. Findings from this research demonstrate that the human *Bacteroides* signal is intermittent; suggesting that exfiltration of sewage from sanitary sewer systems and subsequent migration into stormwater collection & conveyance systems is the mechanism in which contamination occurs. Mapping infrastructure age using geographical information systems (GIS) in conjunction with sanitary sewage detection in stormwater collection & conveyance systems may provide insight into relationships between sanitary sewer system age and lack of integrity, which would help prioritize capital improvement investments.

(5) Quantify the overall human contribution to fecal pollution in Lincoln Creek, Menomonee River and Kinnickinnic River and correlate levels with hydrological and climate parameters. Determining the contribution of human sources will allow a direct comparison of stormwater inputs to combined sewer overflows in terms of health risk. The real time monitoring stations operated by MMSD and USGS and the sampling capabilities at these stations offer an investigative platform to accomplish this goal. A quantitative estimate of sanitary sewage

contamination in rivers can serve as a benchmark that can be used to evaluate the effectiveness of management strategies that are formulated and implemented through stakeholder groups like Watershed Action Teams (WATs) of the Southeastern Wisconsin Watersheds Trust (SWWT).

(6) Incorporate estimates of human sources to supplement fecal coliform levels in future modeling efforts. Considerable effort has been invested in defining fecal coliform loads in relation to specific assessment points contained within the Greater Milwaukee Watersheds.. Information as to the percentage of sanitary sewage contamination, in relation to total fecal pollution, can be incorporated into modeling efforts as a higher tier of information

(7) Assimilate water quality data (e.g. *E. coli*) and physical parameters (e.g. age of infrastructure and diameter of pipe) from the GLWI, MMSD, and Milwaukee Riverkeepers into a GIS database. The GIS database would be able to (1) perform initial analysis; (2) serve as a central repository for geographic data; and (3) produce visually intuitive map figures that supplement study findings.

(8) Continue public education and outreach. The McLellan Bacterial Genetics Laboratory works with the Milwaukee Riverkeepers, the Urban Ecology Center, and other grassroots organizations to help educate the public and collaborate on citizen monitoring programs.

These recommendations provide the framework to progress to the next level of achieving better water quality in the Greater Milwaukee Metropolitan area. Quantification of human sources of bacteria in stormwater discharges and surface waters will provide a benchmark from which the improvements due to investments in removing illicit connections and repairing/replacing sanitary or storm sewer lines (or privately owned laterals) may be measured. Such repairs are expensive, and the benefits need to be evaluated on an ongoing basis. The research contained herein may offer new tools for more comprehensive assessments that include fecal pollution source identification, to enable better preservation, planning, and management of our water resources.
.

1.0 INTRODUCTION

Pathogens in Urban stormwater

Urban stormwater runoff (non-point sources) are the largest contribution of fecal coliform bacteria to the Kinnickinnic, Milwaukee, and Menomonee Rivers (68.7%, 59%, and 83.7% of total fecal coliform loads, respectively) (SEWRPC, 2008). The high volumes of polluted stormwater entering the rivers cause the majority of river samples to exceed a variety of State water quality standards. Prior work by the Milwaukee Metropolitan Sewerage District (MMSD) 2020 Facilities planning effort recognized bacteria (fecal coliform and *E. coli*) as a priority pollutant in the Greater Milwaukee Watersheds (**Figure 1**).

Research in the McLellan Bacterial Genetics Laboratory has developed DNA based methods for identifying sources of fecal pollution. These methods have been applied to field based studies to identify stormwater as a source of high bacteria and the possible role that sanitary sewage contamination has in the overall water quality of Greater Milwaukee Watersheds, its rivers and ultimately Lake Michigan.

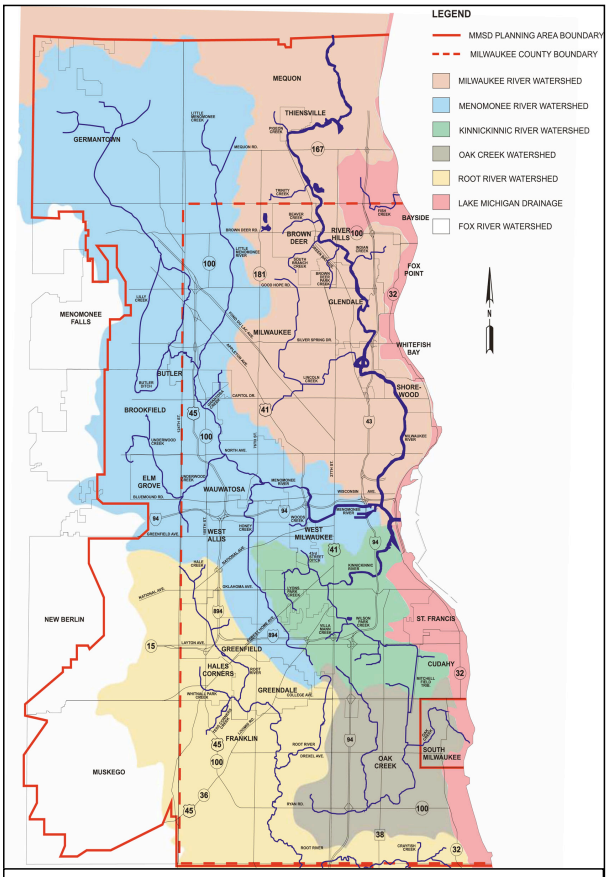


Figure1. Greater Milwaukee Watersheds

Goals and Objectives

- Assess the overall contribution of urban stormwater to *E. coli* levels in receiving waters.
- Determine which stormwater outfalls in the Greater Milwaukee Watersheds are discharging elevated levels of bacterial pollution.
- Identify the source of contamination, e.g. either human or non-human in these areas.
- Determine the proportion of non-point pollution vs. sewage contamination (e.g. stormwater runoff vs. human sewage contamination).
- Coordinate volunteer activities, disseminate research findings, and educate the public.

2.0 BACKGROUND

Urban stormwater remains one of the most difficult environmental and fiscal challenges in the United States and is a major source of pollution, including fecal bacteria pollution to the Nation's waterways. Urban areas are particularly vulnerable to degraded water quality and habitat loss. In many cases, the hydrology of these regions has been severely altered to accommodate development, which has resulted in an increase in the amount of impervious surfaces. During storm events, high levels of pollutants are washed off the land, transported and discharged directly into the waterways. Sewage contamination from failing infrastructure and septic systems, overflows or illicit connections can also contribute to the bacteria pollutant load.

Surface water is evaluated for fecal pollution by testing for fecal indicator bacteria, such as fecal coliforms, *E. coli*, or enterococci. Water quality standards are different for various bodies of water (**Table 1**). Standards for recreational water, e.g. deemed safe for "bathing" or full body contact, is recommended by the EPA, but each state will adopt its own criteria. Rivers and streams are regulated by Wisconsin State Administrative Code and are designated by either limited or full recreational use.

Table 1. Bacteria standards for recreational water in the Greater Milwaukee Metropolitan area.

Body of Water	Designated use	Indicator organisms	Criteria (CFU/100ml)[1]	Agency
Lake Michigan beaches	Bathing waters	*E. coli*	235	Clean Water Act
Rivers and Streams	Full recreational use	Fecal coliform	200[a]	WI Administrative Code; NR 102
Rivers and Streams	Limited recreational use	Fecal coliform	1,000 [a]	WI Administrative Code: NR 104

[1] CFU - colony forming units
[a] as a monthly geometric mean based on not less than 5 samples per month

Urban stormwater runoff adversely impacts the rivers, beaches, and subsequently Lake Michigan. Stormwater outfalls are extensively located along the three Milwaukee area urban rivers (**Figure 2**); Milwaukee River, Menomonee River, and Kinnickinnic River which converge and ultimately discharge into the Milwaukee Harbor. These three rivers, have been shown to have consistently high *E. Coli* levels and fecal coliform concentrations orders of magnitude higher than limited recreational (1,000CFUs/100ml (CFU= colony forming units) or full body contact recreational use (200 CFUs/100ml) during wet weather and fecal coliform levels in both

Figure 2: Example of Stormwater Outfalls.

the Menomonee and Kinnickinnic Rivers often exceeded 1,000 CFUs/100ml even during dry weather. Additionally, the majority of fecal coliform levels in the Milwaukee River exceeded 200 CFUs/100ml (SEWRPC, 2008). In previous research, the McLellan Bacterial Genetics Laboratory measured *E. coli* levels in the Menomonee River. The mean *E. coli* levels varied from 100-300 CFUs/100ml but after a storm event (with no sewer overflows) *E. coli* and fecal coliform levels can increased by three levels of magnitude; 10,000 – 100,000 CFUs/100ml have been observed (Salmore*, et al.*, 2006; MMSD, 2008).

Several Milwaukee area beaches which receive direct stormwater discharge on the beach and in some instances receives contaminated water from the outflow of Milwaukee's Outer harbor, have had numerous beach closings due to high *E. coli* levels. In 2008, Milwaukee County beaches were issued an advisory or closed for 39% of the monitoring days (WI Beach Health Website: http://www.wibeaches.us/traverse/f?p=BEACH:HOME:2184359401190081), with the majority of these advisories due to high *E. coli* levels. In numerous research studies of Lake Michigan beaches, the McLellan Lab has found that localized stormwater inputs greatly influence water quality as does the local gull population which uses the beach areas for food resources and roosting. *E. coli* levels following rain events increased between two and fifty times greater than pre-rain levels, which demonstrates that pollution inputs are highly variable in response to rainfall amounts and intensity (McLellan & Salmore, 2003).

Furthermore, the public health risk posed by very low levels of pathogens in Lake Michigan is difficult to estimate, since concentrations may not exceed the infectious dose needed to cause illness. However, Lake Michigan serves as the source for drinking water to the City of Milwaukee and other nearby communities; the *Cryptosporidium* outbreak in 1993 is a sound reminder that drinking water treatment should not be the only protective measure against pathogen exposure in Lake Michigan waters. Rather, contamination of source waters (e.g. CSOs, SSOs, urban and rural stormwater runoff, etc.) should be minimized as much as possible to protect public health. Moreover, evidence of human sources of contamination in stormwater strongly indicates that there is a need for a more detailed pathogen assessment of urban stormwater for disease causing organisms, including human viruses. Given the difficulty in direct pathogen assessments in Lake Michigan, stormwater outfalls dischargess should periodically be tested for the presences of human pathogens by the community in which they are located.

Human sources of fecal contamination are known to carry human pathogens, therefore, any contamination from sanitary sewage can be considered a potentially serious public health risk. Little is known about the types of pathogens and ultimately, the health risk that is associated with sources of fecal pollution in urban stormwater, however there is increasing evidence that urban stormwater may pose a serious human health risk (O'Shea & Field, 1992, Haile*, et al.*, 1999, Gaffield*, et al.*, 2003, Rajal*, et al.*, 2007). This may be due to the inadvertent introduction of sanitary sewage into the stormwater collection & conveyance system. During storm events, non-human sources of fecal indicator bacteria can rise to magnitudes well above water quality standards, masking the human sources of fecal indicator bacteria. However, using the technique of antibiotic resistance testing, the source of contamination (human vs. non-human) can be determined by testing the response of *E. coli* to an array of antibiotics that the human population is routinely administered. After a storm event or during baseflow conditions, low levels of fecal indicator bacteria were detected but antibiotic resistant *E. coli* was also detected. The antibiotic

resistant *E. coli* demonstrate a persistent source of sanitary sewage is present in the rivers, regardless of CSO or rain events.

Currently, numerous bacterial source-tracking methods exist such as testing for antibiotic resistance of fecal indicator bacteria, detection of host specific molecular markers using Polymerase Chain Reaction (PCR), and assessment of man-made chemical tracers such as artificial sweetener and caffeine. These methods have recently been reviewed by Field and Samadpour (2007). Methods that utilize host specific markers are based on the premise that certain microorganisms are found in one host type, such as humans, and not found in other hosts. Marker based approaches do not rely upon isolating many different strains of fecal indicator bacteria from environmental samples and host sources (e.g. human, animals), and are therefore less cumbersome for widespread studies. Several microorganisms have been identified that can serve as host-specific indicators including certain members of the order *Bacteroidales, Methanobrevibacter smithii*, and more recently *Faecalibacterium* sp. (Bernhard & Field, 2000, Carson*, et al.*, 2005, Dick*, et al.*, 2005, Ufnar*, et al.*, 2006, Lamendella*, et al.*, 2007, Layton*, et al.*, 2009, Zheng*, et al.*, 2009). Although each method has its own merits, studies have shown it is more effective to use a combination of source detection methods/techniques rather than rely on one particular test to discriminate between bacterial host sources found in environmental water samples (Scott*, et al.*, 2002, Stewart*, et al.*, 2003).

The McLellan Bacterial Genetics Laboratory has employed the human *Bacteroides* genetic marker (Bernhard & Field, 2000) using Polymerase Chain Reaction (PCR) to isolate the gene sequence that identifies this organism. *Bacteroides* spp. may be one of the most sensitive fecal indicator genetic markers since *Bacteroides* are present in fecal pollution at a much higher abundance (1000X) than fecal coliforms. Certain species of *Bacteroides* have been found to commonly be harbored in humans, but not other sources of fecal pollution such as cows or gulls (Bernhard & Field, 2000, Dick*, et al.*, 2005, Lamendella*, et al.*, 2007). The human specific *Bacteroides* genetic marker was detected in sewage influent samples (n=15) from five different treatment plants in Wisconsin, including plants from Milwaukee, Manitowoc, and Door Counties. This marker was found to be positive when influent samples were diluted to 1:25,000. Further, fecal samples from gulls (n=240) and feed lot manure samples (n=48) were always negative for this genetic marker. These results demonstrate that the human *Bacteroides* is potentially a sensitive and specific marker for human sources of fecal pollution.

Bacteroides, which are obligate anaerobes, are not expected to survive for extended periods of time in the environment (Kreader, 1995); however, the relationship between standard measures of water quality (e.g. culturing *E. coli*) and the capability to detect these organisms in surface waters over time is unknown. Therefore, the distribution of *Bacteroides* spp., and other fecal indicator genetic markers, was determined in nearshore Lake Michigan following a sewage overflow, and the results were compared with the levels of culturable *E. coli* measured in the same samples. Results from this study demonstrate that the human *Bacteroides* genetic marker may be useful for detecting low levels of fecal pollution, particularly in a system such as Lake Michigan where dilution makes it difficult to track pollution inputs from watershed drainage. The simultaneous disappearance of the *E. coli* (detected by PCR) and human specific genetic markers in Lake Michigan suggests that *E. coli* and the human *Bacteroides* cells remain intact for

similar amounts of time. Molecular techniques, e.g. *Bacteroides* host specific PCR, offer promising results for human source identification in environmental water samples.

3.0 METHODOLOGY

3.1 Sample Collection

More than 1,000 stormwater samples, from 62 different locations along the Milwaukee, Menomonee, and Kinnickinnic Rivers (including Honey Creek, Underwood Creek, Oak Creek, and Lincoln Creek) were collected and analyzed during the 2006-2008 sampling seasons for this project (**Appendix A**). Overall, 236 stormwater samples were collected by the McLellan Bacterial Genetics Laboratory for this study. An additional 870 stormwater samples were collected by MMSD as part of their stormwater monitoring program, these samples include automatic inline stormwater samples collected from inside the storm sewer via manholes and instream water quality grab samples collected from the rivers downstream of stormwater outfall discharge points. A regional approach was taken when choosing stormwater outfall sites. Areas of concern where given high priority when choosing locations and a more concentrated sampling effort was conducted.

Samples collected by the McLellan Bacterial Genetics Laboratory were collected during rain events in 500 ml Nalgene bottles directly from the outfall. The samples were placed on ice until analysis. Samples collected by MMSD were obtained from automated inline samplers (ISSCO®) or in some cases were manual grab samples; all samples were stored on ice until delivered to UWM's Great Lakes WATER Institute (McLellan Bacterial Genetics Laboratory) for analysis.

3.2 *E. coli* and Enterococcus Enumeration

All water samples are analyzed within 12 hours using the USEPA method for *E. coli* enumeration (USEPA, 2002). The samples are filtered through a 0.45 µm pore size 47 mm nitrocellulose filter and placed on modified m-TEC and MEI agar. The volume of sample filtered is varied according to the expected contamination. The plates are incubated for 18 hours and colony forming units (CFUs) are counted and recorded.

3.3 Polymerase Chain Reaction (PCR)

All water samples were filtered within 12 hours for DNA extraction. A volume of 100 to 200 ml of sample was filtered onto a 0.45 µm pore size 47 mm nitrocellulose filter and stored at -80° C. The frozen filters were broken into small fragments using a metal spatula. DNA was extracted using the MPBIO FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Anna, CA). After extraction, PCR was performed using previously published methods (Bower*, et al.*, 2005). Human *Bacteroides* and *E. coli* primers are used in the PCR reaction, unless otherwise noted. In some cases, *Bacteroides* spp. (for total Bacteroidales) or cow specific *Bacteroides* was used.

The presence of various fecal bacteria DNA was confirmed by PCR analysis using primers that target specific genetic sequences in bacterial DNA. The presence of a product from the amplification process (e.g. PCR) confirms the presence of the specific bacteria sequence that was

targeted. The presence of *E. coli* was detected using the uidA1318F and uidA1698R (Bower*, et al.*, 2005) that target the *uid*A gene of *E. coli*. Human specific *Bacteroides* spp. was detected using HF183F and Bac708R (Bernhard & Field, 2000). All reactions were run using Taq PCR Master Mix Kit (QIAGEN Co., Valencia, CA) with 7.5 pmol forward and reverse primers and between 10 and 80 ng of DNA per 25 µl reaction. The thermocycler conditions for PCR were as follows: 1 cycle of 94° C for 4 minutes; 35 cycles of 94° C for 30 seconds; the annealing temperature (60° C for the uidA primers, 53° C for total *Bacteroidales* primers and 59° C for *Bacteroides* human-specific primers) for 30 seconds; 72° C for one minute; a final cycle at 72° C for six minutes and then a 10° C hold. PCR products were visualized on a 2% agarose gel stained with ethidium bromide and compared to a 100 bp DNA ladder molecular weight marker (**Figure 3**) (Fisher Scientific Co., Pittsburgh, PA).

PCR results are typically reported as positive (present) or negative (absent), however results can also be reported as weak, sample interference, or below the level of detection.

PCR and culture based methods are used concurrently because the culture based method allows the human *Bacteroides* PCR findings to be compared to culturable fecal indicators, which is the metric used for water quality standards. The *E. coli* is also detected by using PCR, which serves as an important control reaction. Previous work in the McLellan Laboratory has shown that if more than 500 *E. coli* cells/100 ml (or CFU/100 ml) are present in a sample of cells in sterile water, they can be easily detected by PCR. However, environmental water samples often contain many interfering substances; therefore if the culture based method confirms *E. coli* is at higher densities than 500 CFU/100 ml, but the control PCR reaction is negative, the sample is noted to have sample interference. This means that the environmental sample has substances that are inhibiting the PCR analysis or cross-reacting, so that the reactions do not occur may actually be interpreted as false negative results. These results may also be due to low DNA recovery from environmental samples, e.g. substances in the environmental sample reduce the efficiency of DNA extractions. When sample interference is suspected, the DNA is serially diluted and retested. This is done to dilute the interfering compounds to a level that will no longer inhibit the PCR reaction. When *E. coli* can be detected at only a very high dilution factor (with concurrent high cell counts), and the human *Bacteroides* genetic marker is negative, the negative *Bacteroides* result may be due to the fact that the *Bacteroides* target has been diluted to an undetectable level, therefore, the final result for these samples are designated as having possible sample interference. Results listed as below the level of detection (BLD) have *E. coli* cell counts less than 500 CFU/100 ml.

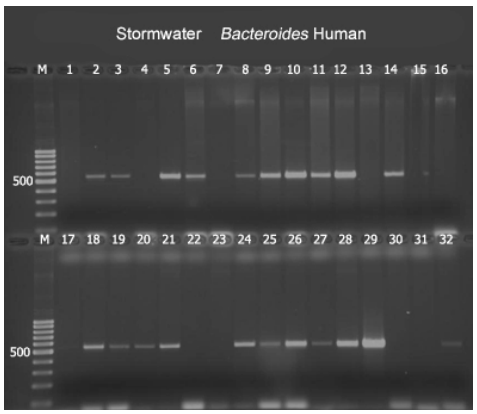


Figure 3. Visualization of 525 base pair PCR products on a 2% agarose gel stained with ethidium bromide. Lanes designated as "M" are the DNA size marker.

3.4 Data Management

All samples are tracked by the MMSD-assigned Laboratory Information Management System (LIMS) number or the GLWI-assigned Fate and Transport number. Data is logged into a laboratory notebook and then transferred to an MS Access® database and Excel® spreadsheets (2007 & 2008 human *Bacteroides* data contained in an Excel® spreadsheet in Appendix B of this report). Data entry is reviewed by project manager on a weekly basis. Monthly reports are generated to review the data on a regular basis and subsequent progress reports are generated. MMSD staff and McLellan Laboratory staff meet on a regular basis to discuss field and laboratory experimental progress and data management and reporting. Final reports are submitted to MMSD and Dr. McLellan gives presentations to disseminate the information.

4.0 RESULTS AND DISCUSSION

The goal of this study was to identify and characterize local stormwater inputs (discharges) within the Greater Milwaukee Watersheds (six in total) that are discharging elevated levels of bacterial contamination from the stormwater collection & conveyance system into the waterways, and subsequently determine if the origin of the pollution is from primarily human or non-human sources and at what level (concentration).

4.1 Identification of Stormwater Outfalls with Human Sources of Fecal Pollution.

High *E. coli* and enterococcus values and positive human *Bacteroides* genetic marker results were widely distributed throughout the Greater Milwaukee Watersheds. The sites located along Lake Michigan and the Menomonee River have the largest percentage of samples positive for the human *Bacteroides* genetic marker, with 71% and 73% of the samples positive, respectively (**Table 2**). Similarly, the Kinnickinnic River and Lincoln Creek were found to have a high number of samples positive for human *Bacteroides* marker, with 60% and 65% of the samples positive, respectively. The other four receiving waters, Honey Creek, Underwood Creek, Oak Creek, and the Milwaukee River, also demonstrated evidence of sanitary sewage contamination of stormwater. Results from individual stormwater samples (outfalls, inline stormsewers, instream) are shown in **Table 3**. These stormwater inputs create a public health risk at the rivers, the lake, and beaches due to the high bacteria levels and presences of the human *Bacteroides* marker.

Table 2: Summary of *E. coli*, Enterococcus and human *Bacteroides* genetic marker data compiled by receiving waters for stormwater samples collected by MMSD and GLWI during the 2006-2008 sampling seasons. (See **Appendix A** for site locations).

			E. coli		Enterococcus	
Receiving Waters	Number of Samples 2006-2008	% Positive for human *Bacteroides*	Average (CFUs/100ml)	Geometric Mean (CFUs/100ml)	Average (CFUs/100ml)	Geometric Mean (CFUs/100ml)
Milwaukee River	12	33%	6,550	4,040	6,020	4,250
Oak Creek	93	39%	358,000	9,020	153,000	11,000
Underwood Creek	74	42%	113,000	12,600	158,000	13,500
Honey Creek	203	51%	40,300	5,850	43,400	6,990
Kinnickinnic River	201	60%	329,000	19,700	175,000	21,600
Lincoln Creek	108	65%	51,100	14,600	60,500	9,770
Menomonee River*	209	73%	382,000	24,300	111,000	21,100
Lake Michigan	95	71%	364,000	36,400	211,000	29,400

*two representative samples from SWWA13 pollutographs were included this analysis. Special sampling at Miller Park, SWMI18 is not included in this analysis.
** Russell Ave outfall not included because it is a submerged outfall; indicator levels may not be accurate

Overall, these results confirm MMSD's 2020 Facilities Plan and SEWRPC's Regional Water Quality Management Plan findings that stormwater is a major contributor to bacterial loads in the Greater Milwaukee Watersheds. This study further indicates that sanitary sewage contamination of the local stormwater collection & conveyance system contributes to these bacterial loads. As many as 15% of stormwater samples in 2006-2008 had bacteria counts over 100,000 CFUs/100 ml for *E. coli* and 18% had counts over 100,000 CFUs/100 ml for enterococcus. These levels are **500 times higher** than the water quality standard of 200 CFU/100 ml of fecal coliforms and since *E. coli* is only one type of bacteria that make up fecal coliforms, the total fecal coliform levels in the stormwater samples are expected to be even higher. In addition, these findings suggest that human sewage is present in the local stormwater collection & conveyance systems at higher amounts throughout the Greater Milwaukee Metropolitan area than previously thought. Several stormwater outfalls were intensively sampled and tested positive for the human *Bacteroides* genetic marker 100% of the time; KI02S, KI05S, LC04S, SMN03A, and SWMI18 (**Table 3, street locations found in Appendix A**). In addition, 44 of the 62 regularly sampled stormwater outfalls (e.g. **nearly three quarters of the sites)** tested positive for the human *Bacteroides* genetic marker over 50% of the time.

Table 3: Summary of *E. coli*, Enterococcus and human *Bacteroides* genetic marker data analyzed by outfall for stormwater samples collected by MMSD and GLWI during the 2006-2008 sampling seasons. See **Appendix A** for site code locations and **Appendix B** for 2007 & 2008 human *Bacteroides* data (stormwater outfalls, inline stormsewers, & instream locations).

			E. coli		Enterococcus	
Site Code	Number of Samples 2006-2008	% Positive for Human *Bacteroides*	Average (CFUs/100ml)	Geometric Mean (CFUs/100ml)	Average (CFUs/100ml)	Geometric Mean (CFUs/100ml)
Honey Creek						
HC01	14	50%	9,540	5,180	29,800	10,900
HC01S	12	70%	3,560	1,520	4,750	592
HC02	15	53%	12,700	3,370	22,100	8,050
HC02S	11	89%	10,000	5,150	38,200	6,660
HC03	14	50%	15,100	4,560	18,500	5,760
HC03S	11	78%	13,800	4,300	5,130	1,880
HC04	14	64%	28,100	6,850	25,700	12,100
HC05	14	64%	40,600	7,270	57,000	26,200
HC06	13	15%	45,400	2,280	43,900	3,920
HC07	12	50%	11,900	1,250	24,100	4,090
HC08	14	54%	37,500	16,300	44,600	27,100
HI03b	11	89%	7,790	6,460	10,200	6,680
HI04b	11	89%	223,000	28,100	113,000	13,900
HI05b	11	11%	19,500	7,090	7,300	4,600
SWWA20	26	38%	101,000	29,600	135,000	7,790

Table 3: continued

Site Code	Number of Samples 2006-2008	% Positive for Human *Bacteroides*	*E. coli* Average (CFUs/100ml)	*E. coli* Geometric Mean (CFUs/100ml)	Enterococcus Average (CFUs/100ml)	Enterococcus Geometric Mean (CFUs/100ml)
Kinnickinnic River						
KI02S	6	100%	9,680	8,620	185,000	34,500
KI05S	6	100%	5,850	3,190	12,000	5,200
KK01	15	67%	49,400	9,600	58,700	19,400
KK02	9	67%	12,200	8,760	15,200	3,010
KK03	10	78%	121,000	25,100	233,000	58,000
KK04	16	56%	30,800	10,100	113,000	17,300
RI33S	7	86%	9,020	6,170	66,300	32,100
S4301A	47	34%	108,400	24,600	192,000	39,900
S4302A	17	18%	10,300	3,600	14,400	2,580
SKK01A	19	95%	742,000	29,500	172,000	33,600
SLP01A	50	76%	913,000	75,100	324,000	41,700
Lake Michigan						
SWMI04	23	65%	103,000	23,400	122,000	29,400
SWWB09	71	75%	286,000	38,700	158,000	62,900
Russell Ave*	30	74%	5,800	640	6,500	420
Menomonee River						
MN01	6	33%	102,000	16,700	59,500	17,700
MN04	13	69%	90,900	18,500	77,700	22,800
MN06	9	56%	56,200	30,600	116,000	49,900
MN07	6	67%	20,400	14,700	63,700	28,900
MNI01S	6	67%	8,160	6,150	38,300	12,700
MNI02S	6	83%	9,970	7,880	35,100	28,400
MNI03S	6	67%	10,600	9,150	23,300	19,600
SMN01A	31	48%	793,000	34,800	82,000	22,700
SMN02A	25	72%	237,000	33,900	184,000	45,300
SMN03A	13	100%	6,400	3,870	12,700	7,310
SMN04A	14	93%	6,670	3,310	78,300	3,480
RI32S	7	71%	6,590	4,680	14,000	9,730
SWMI18 (before)	15	93%	238,000	2,810	176,000	1,260
SWMI18** (after)	60	7%	14,700	135	17,240	51
SWWA13	107	89%	145,000	10,600	111,000	17,800

Table 3: continued

Site Code	Number of Samples 2006-2008	% Positive for Human *Bacteroides*	*E. coli* Average (CFUs/100ml)	*E. coli* Geometric Mean (CFUs/100ml)	Enterococcus Average (CFUs/100ml)	Enterococcus Geometric Mean (CFUs/100ml)
Milwaukee River						
MWO1	5	60%	7,440	4,850	7,520	7,120
MWR1	7	14%	5,930	3,550	4,960	2,940
Oak Creek						
OC03S	7	57%	5,130	4,140	28,500	8,950
OC04S	7	43%	6,980	4,000	15,600	8,000
SOC01A	39	44%	800,000	22,000	203,000	23,500
SOC02A	40	33%	42,600	5,620	146,000	9,130
Lincoln Creek						
SLC01A	18	28%	48,600	16,900	96,600	34,600
LC04S	7	100%	11,300	8,240	22,900	15,600
LC02S	7	86%	8,530	4,330	18,600	7,570
SLC02A	25	32%	67,200	4,450	99,900	18,300
SWMI07	51	88%	55,500	13,600	39,500	10,700
Underwood Creek						
SUC01A	18	22%	189,000	23,100	83,400	15,200
SUC02A	19	53%	144,000	36,200	321,000	31,800
UC02S	7	43%	16,300	2,990	23,000	11,100
UND01	5	60%	9,580	8,090	8,630	7,050
UND02	4	0%	219,000	4,590	299,000	7,850
UND03	8	63%	132,000	12,300	188,000	33,700
UND04	10	60%	7,670	4,760	14,060	11,140
UNDR1	3	0%	4,300	3,960	10,300	8,410

*Russell Ave outfall is submerged so indicator levels may be diluted

**SWMI18 was tested before and after a cross-connection problem was identified and solved at Miller Park.

4.2 Relationship between Inline Stormwater Samples and River Water Quality Samples.

It was evident throughout this study that instream water quality monitoring locations were influenced by surrounding stormwater discharge points. The McLellan Laboratory analyzed instream river water quality samples provide by MMSD and found some of these instream water quality monitoring sites have tested positive for the human *Bacteroides* marker in all of the

samples collected (100% of the time); these results appear to carry further downstream affecting other instream water quality sampling locations as well.

Instream water quality monitoring sites on the Kinnickinnic River, KI02S (35th Street & Manitoba Street) and KI05S (60th Street & Kinnickinnic River Parkway) tested positive for human specific *Bacteroides* marker in 100% of their samples (n=6 samples per site). KI02S and KI05S have corresponding inline storm sewer water monitoring sites in close proximity (SKK01A and SLP01A respectively) which also tested positive for the human specific *Bacteroides* marker on a regular basis. SKK01A (35th Street & Manitoba Street) tested positive in 95% of all samples taken (n=19) and SLP01A (61St Street & Harrison Avenue) tested positive in 76% of all samples taken (n= 50).

In the Menomonee River, instream water quality monitoring location at West Center Street and the Menomonee River Parkway (MNI02S) tested positive for human specific *Bacteroides* marker in 83% of the samples collected (n=6) while samples collected from the corresponding upstream inline storm sewer sites located at W. Center Street and 97th Street (SMN03A) tested positive for human *Bacteroides* marker 100% of the time (n=13) and site SMN04A (W. Center Street and 96th Street) tested positive for human specific *Bacteroides* marker 93% of the time (n= 14) (**Figure 4**).

Figure 4. Instream Water Quality

There are more "high priority" sites indicated in this area of the Menomonee River; inline storm sewer and outfall SMN04A and SWWA13 (near Ridge & Harding Boulevard) and instream water quality monitoring location MNI01S (Ridge & Harding Boulevard) are of importance. SMN04A tested positive for human specific *Bacteroides* marker in 93% of the 14 samples taken and SWWA13 tested positive for human specific *Bacteroides* marker in 89% of

the samples taken (n=107). The stormwater monitoring locations, SMN03A, SMN04A and SWWA13 have affected the water quality of the succeeding downstream water quality instream site, MNI01S. This site tested positive for human specific *Bacteroides* marker in 67% of the samples taken (n=6). The high frequencies of positive results from the upstream inline storm sewer sites paired with the high frequency of positive results for successive downstream water quality instream sites indicates that these inline storm sewers and their associated stormwater outfall discharges are having a negative effect on the instream water quality of the Menomonee River.

Another instream water quality monitoring site of interest is in Lincoln Creek - LC04S, located at 47th Street & Congress Street. Here 100% of the samples collected tested positive for human specific *Bacteroides* marker (n=7). This instream site is within 90 feet of the realm of influence of the corresponding downstream inline storm sewer site at N. 47th Street (SWMI07) which tested positive for human specific *Bacteroides* marker in 88% of the 51 samples collected and tested positive for human derived viruses (see Sction 4.6 for discussion).

4.3 Relationship of Fecal Indicator Bacteria, Precipitation, and Seasonality to the Human *Bacteroides* Genetic Marker.

The relationship between microbiological data for *E. coli* and enterococcus and molecular data for the human *Bacteroides* genetic marker was examined. There was found to be no statistically significant correlation between culturable *E. coli* or enterococci and the human *Bacteroides* genetic marker. Samples positive for the human *Bacteroides* genetic marker were found over a wide range of *E. coli* and enterococcus values (**Figure 5**). Samples containing *E. coli* and enterococci as low as 100 CFU/100 ml were found to have as many as 30% of these samples positive for the human specific *Bacteroides* marker. Samples with *E. coli* and enterococci in the range of l01 to 500 CFU/100 ml had 50% and 60% of the samples test positive for human specific *Bacteroides* marker, respectively. *E. coli* and enterococci levels over 1,000 CFUs/100 ml had the highest average of over 60% of samples positive for the human *Bacteroides* genetic marker. These findings suggest that sanitary sewage inputs into the local stormwater collection & conveyance system may be present but may not always be a major contributor to overall fecal indicator levels. However, any sanitary sewage contamination can pose a serious health risk, due to the possible presences of pathogens, regardless of fecal indicator levels.

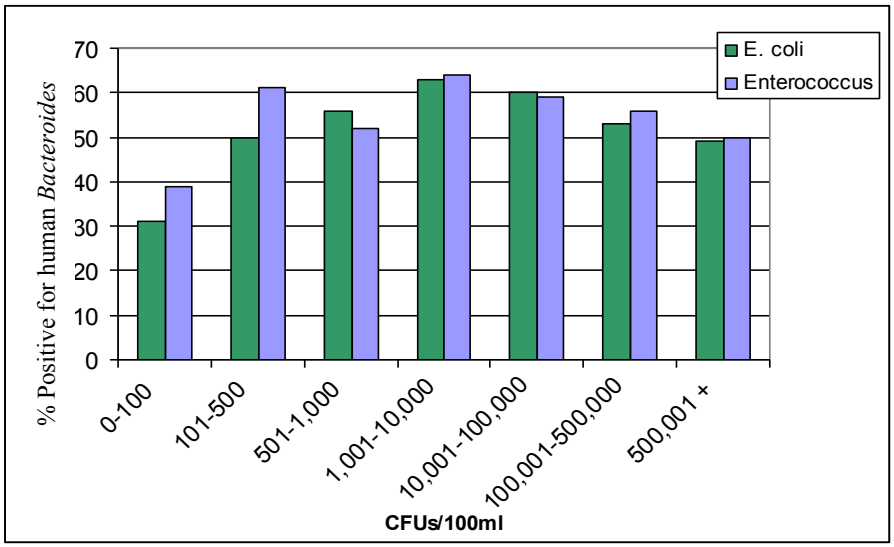


Figure 5: General trends found in the relationship between *E. coli* and enterococcus values when compared to percent positive for the human *Bacteroides* genetic marker, data for 2006-2008.

Human *Bacteroides* genetic marker results were also compared to rainfall amounts. The sampling events in this study spanned a wide range of precipitation, from trace amounts up to 6 inches within 48 hours. The relationship between rainfall and positive results at individual stormwater outfalls, as well as for all stormwater outfalls as a group was analyzed. None of the stormwater outfalls included in this study were observed to have discharge under baseflow conditions. At increasing amounts of rainfall, the number of samples positive for the human *Bacteroides* genetic marker did not significantly change. The lack of relationship with rainfall suggests that the mechanism for sanitary sewage contamination into the stormwater collection and & conveyance system is complicated and appears to be a chronic problem that can occur not only with heavy precipitation but also with only minimal precipitation. While some of the positive results may be due to direct (illicit) connections, exfiltration from deteriorating sanitary sewer lines and migration into the stormwater collection & conveyance system most likely accounts for a high number of these results. The findings of intermittently positive human *Bacteroides* marker with no clear association with rainfall, suggests that a number of factors in combination result in conditions that favor this exfiltration/migration phenomenon. The relationships between sanitary sewage contamination and other factors: soil type and moisture, days since last rainfall, age and location of the sanitary and storm sewer infrastructure, etc., needs to be considered in combination with meteorological conditions. It's anticipated that these relationships might be localized (neighborhood scale), or even community (site) specific, and would require more intensive sampling efforts to define.

Seasonal trends among samples positive for human *Bacteroides* genetic marker were also examined. Samples in early spring (April) and autumn (November) have the highest percentage of samples positive for human *Bacteroides* genetic marker in 2008 (**Figure 6**). However, in general, *E. coli* levels were lower in early spring samples, possible due to high salt condition (road salt) and cold water temperatures. The 2006 samples also showed the highest number of positive samples for the human specific *Bacteroides* marker in autumn. Samples from 2007 were not included in this analysis since some of these are being reanalyzed for the possibility of inhibitors (see Section 4.7 for discussion). Summer months generally had lower numbers of positives for the human specific *Bacteroides* marker, but none of these differences were statistically significant.

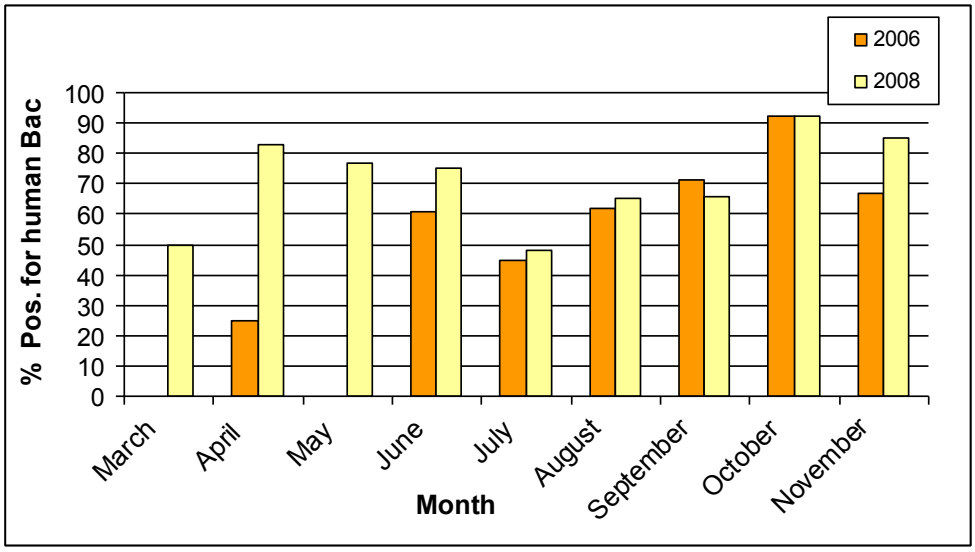


Figure 6: Seasonal variation in percent of samples positive for human *Bacteroides* genetic marker over the 2006 and 2008 sampling seasons. April and October have the largest percentage of samples positive for human *Bacteroides* genetic marker.

4.4 Results of Pollutographs

The McLellan Laboratory analyzed two sites using pollutographs (5 or more consecutive samplings at timed intervals during a specific precipitation event) at two separate locations in 2007. The first pollutograph sampling site was located at Miller Park (SWMI18). Two pollutographs collected after remediation of an illicit connection found at Miller Park demonstrated low to moderate levels of *E. coli* (<100 to 4,200 CFU/100 ml) throughout the sampling of 10 flushes. All samples were negative for the human *Bacteroides* genetic marker.

The second pollutograph sampling site was located at Ridge and Harding streets (City of Wauwatosa) on the Menomonee River (SWWA13). Seven pollutographs were conducted over the 2007 sampling season (**Figure 7**). The two pollutographs collected in spring (4-25-07 and 5-1-07) had low levels of *E. coli*, which was most likely due colder water temperatures and residual salt from roadways. In the other five pollutographs, *E. coli* levels were two to four orders of magnitude lower after the 5th flush sample was collected (approximately 4-5 hours after the start of sampling). On one sample day, 6-4-07, flushes 7 and 16 had increased levels of *E. coli*, but these were not significant. The pollutograph samples were consistently positive for the human *Bacteroides* genetic marker, in all flushes; up to 15 flushes. There were sporadic negative results, which accounted for approximately 10% of the samples. These negative results correlated to very high *E. coli* and enterococcus levels and were most likely due to sample interference. These findings show a persistent input of sanitary sewage that is not washed out of the storm sewer pipe after the initial first flush.

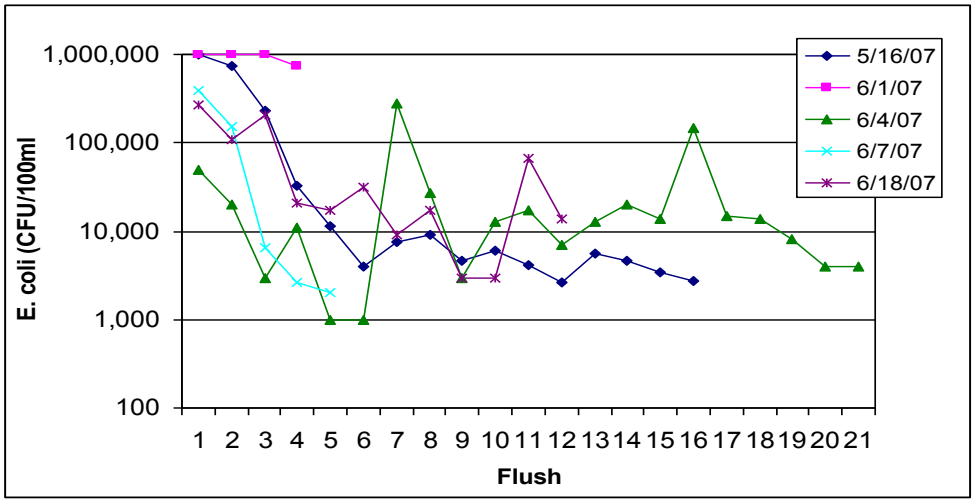


Figure 7. Pollutograph samples from five sampling dates. *E. coli* levels were two to four orders of magnitude lower after the 4th and 5th flush samples. Nearly 90% of the samples from all dates were positive for the human *Bacteroides* genetic marker. Negative samples corresponded to very high *E. coli levels*, and may be negative due to sample interference (see Section 4.7 for explanation of conditions that cause interferences).

4.5 Assessment of Beach Stormwater Outfalls.

As part of a project funded by the University of Wisconsin Sea Grant Program, stormwater outfalls that discharge adjacent to beach areas were sampled. Results are shown in **Table 4**. The percentage of samples positive for the human specific *Bacteroides* marker was similar (and in some cases higher) to what was found in stormwater outfalls discharging to rivers. These findings demonstrate that stormwater outfalls discharging directly to Lake Michigan have infrastructure concerns that are comparable to what was found in other areas of the Greater

Milwaukee metropolitan area. Aging/deteriorating sanitary sewer infrastructure or illicit connections can contribute sanitary sewage into the stormwater collection & conveyance system. Any sanitary sewage contamination released near recreational use waters, such as beaches, is of high concern due to the potential presence of pathogens. Maps and tables with locations and summary data for each beach related stomwater outfall are found in **Appendix C**.

Table 4: Summary of the human *Bacteroides* genetic marker detected in beach stormwater outfalls.

Beach	Number of Outfalls	Number of Samples	% Positive for Human *Bacteroides*
Bradford Beach	14	136	21%
Klode Park	3	42	31%
North Beach Dr.	8	30	50%
Atwater Beach	1	8	63%
Doctors Park	1	10	70%
Bay View	1	17	71%
South Shore	1	26	73%
Big Bay Beach	1	16	75%

4.6 Human Virus Detection

The McLellan Laboratory investigated the occurrence of human derived viruses in one stormwater outfall site located along Lincoln Creek (47th Street & Congress Street) that chronically tested positive for the human *Bacteroides* genetic marker. On 7/8/08, MMSD collected 4 L of water from stomwater outfall SWMI07 after a rain event. The sample was analyzed by Marshfield Clinic for enteroviruses, rotavirus group A, hepatitis A (HAV), G1 noroviruses, GII noroviruses, and adenoviruses. The sample was positive for three different viruses: adenovirus at 1.3 x 10E3 genomic equivalents/L (ge/L), enterovirus at 1.9 x 10E4 ge/L and G1 norovirus at 1.5 x 10E3 ge/L. These concentrations of viruses are similar to what is found in sewage influent and confirms the presence of human sewage contamination in this stormwater outfall. This level of human virus contamination in stormwater constitutes a potential public health risk.

The stormwater monitoring site SWMI07 was chosen for virus sampling because it was ranked as a high priority stormwater outfall based upon the number of samples positive for the human *Bacteroides* genetic marker. For this stormwater outfall, the mean and geometric mean *E. coli* levels were 55,500 and 13,600 CFU/100 ml respectively, and the mean and geometric mean for enterococci levels were 39,500 and 10,700 CFU/100 mL respectively (**Table 3**). Interestingly this stormwater outfall has mean and geometric mean *E. coli* and enterococci levels similar to other sites that are ranked as low priority because of the low percentage of times the human *Bacteroides* genetic marker was detected. In addition, the sample in which the human virus sampling was performed had relatively low *E. coli* levels on that day compared to other sample days at this site. These findings further corroborate other evidence that sanitary sewage inputs

into the local stormwater collection & conveyance system may be present but may not always be a major contributor to overall fecal indicator levels (see Section 4.3 for discussion) and human virus presence is not necessarily correlated with high levels of *E. coli*.

4.7 Inhibition Assay Development

4.7.1 Inhibitor Assays

Environmental samples contain many organic and inorganic compounds such as phenolic compounds, humic acid, clays, heavy metals, and large amounts of non-target DNA that may interfere with PCR reaction. In all of the PCR assays, a control is included to assure that the reaction worked properly and that there were no inhibitors present that would lead to a false negative result. Prior work in the McLellan Bacterial Genetics Laboratory has demonstrated that in sewage, *E. coli* and the human *Bacteroides* genetic marker are at similar levels (Bower et al. 2005), therefore, *E. coli* is used as a PCR target for the control reaction. Further, *E. coli* is present based on microbiological results, so all samples should have a positive reaction if culturable *E. coli* is above 500 CFU/100 ml (the limit of detection determined in the laboratory, see methods section). If there is a mixture of human and non-human sources contributing to fecal pollution, the non-human sources are expected to contribute *E. coli* but not the human *Bacteroides* genetic marker to the sample. In these instances, it may be possible to have a positive *E. coli* control reaction, but inhibition may prevent detection of the human *Bacteroides* genetic marker. Further, samples may produce a positive result, but the magnitude of the positive result may be diminished due to inhibition, which needs to be determined in order to accurately quantify the human *Bacteroides* genetic marker (see Section 4.7.2 for discussion). Therefore, it was necessary to develop a quantitative inhibition assay that could distinguish a true negative from a sample that produces a negative result due to inhibition.

This inhibition assay is based upon amplification of an internal control in the same reaction or under the same conditions as the PCR assay for human *Bacteroides* genetic marker. This assay has been under development in the McLellan Bacterial Genetics Laboratory since 2008. Briefly, the McLellan Bacterial Genetics Laboratory has adopted an approach described by Sivaganesan et. al. (2008); which entailed spiking in a known concentration of internal control target DNA and performing quantitative PCR (qPCR). The target is essentially a synthesized gene that is an internal amplification control (IAC). The IAC contains a known (nonbacterial) control genetic sequence that is flanked by human *Bacteroides*, *E. coli*, enterococcus, and total *Bacteroides* genetic sequences complementary to the primers used in PCR. This gene is carried on a plasmid that can be introduced into the reaction at a known concentration. The IAC should amplify with human *Bacteroides* primers, but can be distinguished from the human *Bacteroides* genetic marker based upon the intervening control sequence. When added to a stormwater sample, the strength of the signal is compared to the same amount of target IAC with no stormwater sample present to determine if it is diminished by inhibition, and to what extent (e.g. 2 fold, 4 fold, complete inhibition).

Approximately 2-4% of the samples in 2006 and 2008 were suspected to have inhibition based upon negative *E. coli* control reactions. The 2007 samples had a slightly higher rate, with 10% of the samples inhibited. A subset of archived 2007 samples was tested with the inhibitor assay to confirm inhibition and determine to what extent. These results indicated that only two of

twenty samples had significant inhibition. Further work is necessary to determine the sensitivity of the inhibitor assay. This assay can be used in conjunction with development of quantitative assays (qPCR) for the human *Bacteroides* genetic marker (see Section 4.7.2 for discussion).

4.7.2 Quantification of the Human *Bacteroides* Genetic Marker

The high number of stormwater samples testing positive for the human *Bacteroides* genetic marker highlights the need for prioritizing sampling sites for further investigation using dye or smoke testing. The human *Bacteroides* genetic marker is a very sensitive measure of sanitary sewage contamination. For example, the McLellan Bacterial Genetics Laboratory has detected this marker in Lake Michigan following a sewage overflow, when *E. coli* levels were found to be 0 CFU/100 ml, therefore knowing how human *Bacteroides* genetic marker relates to *E. coli* levels would be useful in understanding contamination level and risk.

Quantitative PCR (qPCR) is based upon the exponential phase of the amplification process. During PCR, every cycle will produce a doubling of the DNA template. When the reaction reaches a certain point, the doubling produces a dramatic increase in amplification product (e.g. the exponential phase). In qPCR, the reactions can be tracked during each amplification cycle, so this exponential phase can be detected using fluorescently labeled probes. The amount of starting template corresponds to the cycle in which the exponential phase is detected. This is illustrated in **Figure 8**, which shows a typical standard curve where each sample represents increasing concentrations of DNA template. Test samples are assigned a concentration based upon the standard curve.

Two different assays are being developed to determine the concentration of the human marker. Both assays are based upon the human *Bacteroides* genetic marker that is currently used for the standard PCR that provides a positive/negative result, but each has a different conserved region, which is targeted further down the gene sequence. These are the two assays that are being developed by the EPA for source tracking at a national level. Dr. McLellan is currently collaborating with the USEPA to validate the qPCR assays for the human *Bacteroides*. This collaboration offers a unique opportunity to interface with the most current approaches for microbial source tracking. Other real time PCR targets for total *Bacteroides*, *E. coli* and enterococci are currently used in the McLellan Bacterial Genetics Laboratory. Through this work, the proportion of the human *Bacteroides* genetic marker in relation to standard water quality fecal indicators (e.g. fecal coliform and *E. coli*) can be determined.

A. B.

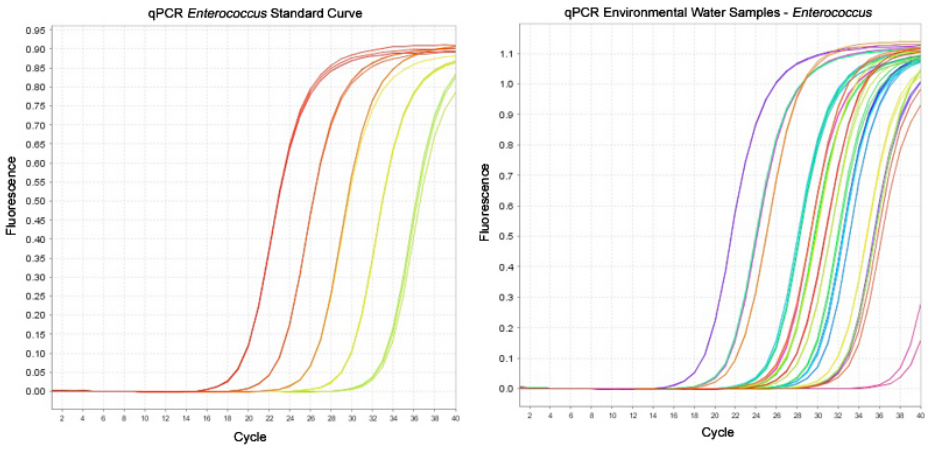


Figure 8. Panel A illustrates a typical standard curve with each sample representing increasing amounts of DNA template. Panel B illustrates qPCR results for 20 samples testing for enterococcus. The cycle in which exponential increases in DNA amplification product are detected are compared to the standard curve in order to assign a *Bacteroides* concentration value.

The human *Bacteroides* genetic marker was quantified in a select number of samples from 2007-2008 (**Table 5**). Stormwater samples are notated to have significant human sewage contamination if 10,000 or more copy numbers of human *Bacteroides* per 100 ml was detected. Samples found to have fewer than 10,000 copy numbers (e.g. equivalent to 2,000 cells per 100 ml) of human *Bacteroides* are not ranked as a priority. The priority ranking is based on the range of copy numbers found in each organism of the human *Bacteroides*; the McLellan Bacterial Genetics Laboratory uses an average of 5 copy numbers per organism. Highlighted (in color) stormwater samples in Table 5 are ranked as a high priority site (samples with >10,000 copy numbers (CN)/100mL).

Table 5: qPCR was conducted on 96 Stormwater samples from 2007-2008. The human *Bacteroides* genetic marker is reported as copy number per 100mL. (See **Appendix A** for site maps).

Date	Site Code	Location	Human *Bacteroides* (Copy Number/100mL)
Honey Creek Watershed			
7/2/08	HC01	74th St. & Bennett	112,000
8/4/08	HC05	84th St. & Hill St.	9,000
Kinnickinnic River Watershed			
9/5/08	KI02S	KK River @ 35th & Manitoba St	54,800
9/4/08	KI05S	KK River @ 60th & KK River Pkwy	11,800
9/5/08	KI05S	KK River @ 60th & KK River Pkwy	1,470,000

Table 5: continued.

Date	Site Code	Location	Human *Bacteroides* (Copy Number/100mL)
6/4/07	KK01	KK River Pkwy. & 33rd St.	51,500
8/14/07	KK01	KK River Pkwy. & 33rd St.	1,400
9/10/07	KK01	KK River Pkwy. & 33rd St.	1,100
6/4/07	KK02	KK River Pkwy. & 35th St.	10,600
8/14/07	KK02	KK River Pkwy. & 35th St.	14,400
9/4/08	KK03	KK River Pkwy. & Forest Home	31,600
6/4/07	KK04	KK River Pkwy. & Forest Home	37,100
8/14/07	KK04	KK River Pkwy. & Forest Home	4,400
9/10/07	KK04	KK River Pkwy. & Forest Home	1,600
9/4/08	SKK01A	35th & Manitoba	28,900
3/19/08	SLP01A	61st and Harrison Ave.	14,200
11/7/08	SLP01A	61st and Harrison Ave.	133,000
		Lake Michigan Watershed	
7/8/08	SWWB09	4939 N. Newhall	195,000
9/5/08	SWWB09	4939 N. Newhall	138,000
9/5/08	SWWB09	4939 N. Newhall	826,000
11/7/08	SWWB09	4939 N. Newhall	51,300
11/7/08	SWWB09	4939 N. Newhall	126,000
		Menomonee River Watershed	
8/15/07	MN01	Honey Creek Pkwy. & S. 68th St.	2,590,000
8/20/07	MN01	Honey Creek Pkwy. & S. 68th St.	3,160,000
8/27/07	MN01	Honey Creek Pkwy. & S. 68th St.	60,800
9/10/07	MN01	Honey Creek Pkwy. & S. 68th St.	200
8/15/07	MN04	Men River Pkwy. & 90th St.	58,200
8/20/07	MN04	Men River Pkwy. & 90th St.	14,800
8/27/07	MN04	Men River Pkwy. & 90th St.	9,400
9/10/07	MN04	Men River Pkwy. & 90th St.	60,900
8/4/08	MN04	Men River Pkwy. & 90th St.	3,040,000
8/20/07	MN06	Hart Park	18,300
8/27/07	MN06	Hart Park	19,500
9/10/07	MN06	Hart Park	23,800
6/9/08	MN06	Hart Park	130,000
10/15/08	MN06	Hart Park	115,000
8/20/07	MN07	N. 68th & River Pkwy.	2,500
8/27/07	MN07	N. 68th & River Pkwy.	700
7/2/08	MN07	N. 68th & River Pkwy.	66,000
9/4/08	MN07	N. 68th & River Pkwy.	15,500
9/11/07	MN101S	Men Rvr @ Ridge & Harding Blvd.	5,300
6/27/07	MNI01S	Men Rvr @ Ridge & Harding Blvd.	17,000
9/26/07	MNI01S	Men Rvr @ Ridge & Harding Blvd.	2,400

Table 5: continued.

Date	Site Code	Location	Human *Bacteroides* (Copy Number/100mL)
10/9/07	MNI01S	Men Rvr @ Ridge & Harding Blvd.	900
5/30/08	MNI01S	Men Rvr @ Ridge & Harding Blvd.	4,000
6/5/08	MNI01S	Men Rvr @ Ridge & Harding Blvd.	906,000
9/5/08	MNI01S	Men Rvr @ Ridge & Harding Blvd.	48,900
6/5/08	MNI02S	Men Rvr @ West Center St. & Men River Pkwy	20,300
6/13/08	MNI02S	Men Rvr @ West Center St. & Men River Pkwy	3,900
9/4/08	MNI02S	Men Rvr @ West Center St. & Men River Pkwy	177,000
5/30/08	MNI03S	Men Rvr @ N 69th St. & Men Rvr Pkwy	34,200
6/5/08	MNI03S	Men Rvr @ N 69th St. & Men Rvr Pkwy	19,600
6/13/08	MNI03S	Men Rvr @ N 69th St. & Men Rvr Pkwy	4,400
7/8/08	MNI03S	Men Rvr @ N 69th St. & Men Rvr Pkwy	33,500
5/12/08	SMN01A	10435 Concordia Ave.	38,400
6/5/08	SMN01A	10435 Concordia Ave.	4,900
6/6/08	SMN01A	10435 Concordia Ave.	800
8/4/08	SMN01A	10435 Concordia Ave.	1,100
9/4/08	SMN01A	10435 Concordia Ave.	17,000
9/5/08	SMN01A	10435 Concordia Ave.	900
5/12/08	SMN02A	69th Ext'd and Hart Park Lane Ext'd	5,700
6/5/08	SMN02A	69th Ext'd and Hart Park Lane Ext'd	67,800
7/3/08	SMN02A	69th Ext'd and Hart Park Lane Ext'd	6,500
9/5/08	SMN02A	69th Ext'd and Hart Park Lane Ext'd	2,600
9/5/08	SMN02A	69th Ext'd and Hart Park Lane Ext'd	19,800
4/25/08	SMN03A	Center St. and 97th St.	46,000
7/3/08	SMN03A	Center St. and 97th St.	842,000
7/8/08	SMN03A	Center St. and 97th St.	56,200
9/5/08	SMN03A	Center St. and 97th St.	1,210,00
10/6/08	SMN03A	Center St. and 97th St.	5,250,000
6/13/08	SMN04A	96th and Center St.	235,000
6/23/08	SMN04A	96th and Center St.	57,800
8/4/08	SMN04A	96th and Center St.	45,000
9/4/08	SMN04A	96th and Center St.	50,000
4/25/08	RI32S	Men Rvr @ Burleigh Avenue	26,700

Table 5: Continued.

Date	Site Code	Location	Human *Bacteroides* (Copy Number/100mL)
6/13/08	RI32S	Ridge and Harding Blvd. Men Rvr @ Burleigh Avenue	870
5/30/08	SWWA13	Ridge and Harding Blvd.	10,800
5/30/08	SWWA13	Ridge and Harding Blvd.	142,000
6/5/08	SWWA13	Ridge and Harding Blvd.	47,500
6/23/08	SWWA13	Ridge and Harding Blvd.	3,400
7/8/08	SWWA13	Ridge and Harding Blvd.	400
9/4/08	SWWA13	Ridge and Harding Blvd.	2,600
9/5/08	SWWA13	Ridge and Harding Blvd.	12,400
9/5/08	SWWA13	Ridge and Harding Blvd.	20,400
		Lincoln Creek Watershed	
7/8/08	SWMI07	4345 N. 47th Street	90,900
7/8/08	SWMI07	4345 N. 47th Street	99,000
9/5/08	SWMI07	4345 N. 47th Street	120,000
9/5/08	SWMI07	4345 N. 47th Street	95,900
6/19/07	LC04S	Lincoln Ck @ N. 47th & Congress St.	7,600
6/27/07	LC04S	Lincoln Ck @ N. 47th & Congress St.	3,100
10/1/07	LC04S	Lincoln Ck @ N. 47th & Congress St.	115,000
9/5/08	LC04S	Lincoln Ck @ N. 47th & Congress St.	32,400
		Underwood Creek Watershed	
9/26/07	SUC01A	Fairview Ext'd. and Curtis Rd.	6,700

4.7.3 Differences in Commercial DNA Isolation Kits

Several commercial kits are available for DNA isolation. DNA is extracted from water samples using a DNA Isolation kit and PCR is conducted on the eluted DNA. There are no documented differences between kits sold by different companies. However, in 2007 there was a significant lower amount of stormwater samples positive for the human *Bacteroides* genetic marker. In 2007, a different DNA isolation kit (MO BIO PowerSoil DNA Isolation kit) was used than in previous years due to availability. To determine if the DNA isolation kit was a factor in the lower percentage of human *Bacteroides* positive (due to lower extraction efficiency) the McLellan Laboratory isolated the replicate filters for 34 stormwater samples from 2007 with the extraction kit that was used in previous years (MPBIO FastDNA Spin Kit for Soil). The human *Bacteroides* genetic marker was not detected in any of these samples using the MO BIO PowerSoil kit but when isolated using the MPBIO Spin kit 32% of the samples were positive for human *Bacteroides*. The MO BIO PowerSoil kit was found to have lower extraction efficiency which caused difficulty in detecting the human *Bacteroides* genetic marker due to a low number of target cells. However, the additional positives results did not change the priority level for any of the stormwater monitoring sites. The majority of the samples that tested positive for the human *Bacteroides* genetic marker with the different kit were already categorized as high priority sites so it increased the amount of positive samples results for the human *Bacteroides*

genetic marker but it did not cause any low priority sites to be categorized differently (i.e. samples did not change from <50% positive to >50% positive).

4.8 Investigation of Assay Specificity

4.8.1 Cross Reactivity of the Human *Bacteroide*s Genetic Marker

Urban wildlife, such gulls, geese, deer and raccoons, can all be sources of *E. coli* in stormwater. The McLellan Bacterial Genetics Laboratory explored in this study the possibility that bacteria found in raccoon feces could be cross-reacting with the human *Bacteroides* genetic marker, causing false positive results for the marker. Raccoons were selected as target species for this analysis because they live in high densities in the storm sewer along the Menomonee River Parkway and their waste is clearly visible in storm sewer catch basins and pipes. Through this additional work it was found that the *Bacteroides* spp. contained in raccoon feces is similar, but not identical to the 16S rRNA gene sequences found in sewage samples (**Figure 9).**

The region of the 16S rRNA gene that is used as the human genetic marker was not found in raccoons; however the amino acid base-pairs sequences that were found in raccoons were also detected in sewage, what this means is that if these "raccoon" base-pairs sequences are detected in stormwater they could be from either raccoons or sewage or both.

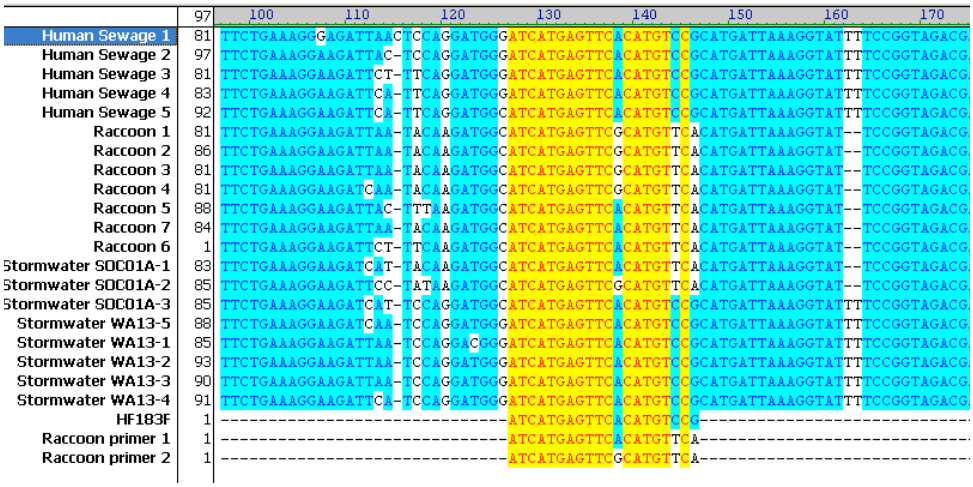


Figure 9. Sequence alignment of *Bacteroides* sequences from sewage, raccoons, and stormwater. The human specific primer that is used to detect the human *Bacteroides* genetic marker is designated as HF183. Raccoons have similar but not identical base-pair sequences as the human specific *Bacteroides*. Sequences identical to the human *Bacteroides* found in sewage were found in two stormwater outfalls, demonstrating the presence of sewage at these sites.

The McLellan Bacterial Genetics Laboratory sequenced *Bacteroides* spp. from stormwater samples at SOC01A and SWWA13 to compare to the sequences obtained from sewage and raccoons. The human *Bacteroides* genetic marker present in stormwater matched the human maker in sewage. Other similar (but not identical) *Bacteroides* spp. were found to be carried by either raccoon or humans but it can be concluded from these results that while raccoon fecal waste may be present in stormwater, it does not account or cross- react with the positive human specific *Bacteroides* genetic marker also found in stormwater and therefore is not being misidentified as a human derived bacteria source.

4.8.2 Milorganite Analysis

Milorganite®, dry granular organic fertilizer manufactured from wastewater biosolids, can be applied to lawns, gardens, and parks. During heavy rainfall, stormwater runoff could wash Milorganite® into the stormwater system. Because Milorganite® is made from microbes used to breakdown human waste during the wastewater treatment process it was necessary to consider whether the application of Milorganite® and potential for some subsequent surface run-off could produce false positives for the human *Bacteroides* genetic marker in stormwater samples. To investigate this possibility a sample of Milorganite® received directly from MMSD was analyzed using PCR for *E. coli*, *Bacteroides* spp., human specific *Bacteroides*, and total bacteria. All of these genetic targets could be detected in the Milorganite® sample; however, 10 grams of undiluted Milorganite® was necessary for the analysis. This means, in a typical stormwater sample volume of 200 milliliters, at least 10 g of Milorganite® would need to be present to obtain a positive human specific *Bacteroides* result. A concentration of Milorganite® this high would not typically be found in the environment and therefore it is concluded that the standard application of Milorganite® could not cause the positive results for human *Bacteroides* genetic marker being found in stormwater samples throughout the Great Milwaukee Watersheds.

5.0 SUCCESSES – Highlighted Case Studies

The research conducted by the McLellan Bacterial Genetics Laboratory in conjunction with MMSD has resulted in several real-world applications throughout the Milwaukee metropolitan area.

- Miller Park. In 2006, a stormwater outfall that was sampled as part of MMSD's stormwater monitoring program was suspected to have sanitary sewage contamination based upon human *Bacteroides* genetic marker testing performed by the McLellan Laboratory. A total of 14 of the 15 samples taken were positive for the human *Bacteroides* genetic marker. Dye testing was conducted in March of 2007 and an illicit connection was identified that connected a portion of the stadium (luxury box sanitary facilities) to a stormwater line that discharged to the Menomonee River. After the cross-connection issue was remediated, the McLellan Laboratory analyzed 60 additional samples to confirm the cross-connection was remediated properly. The culture counts dropped significantly and the human *Bacteroides* genetic marker was only found in 7% of samples. It can be concluded that this site has been remediated. These low levels are considered background levels in the environment.

- State Fair. The McLellan Bacterial Genetics Laboratory analyzed samples collected by MMSD from the Wisconsin State Fairgrounds in 2007 and 2008. Samples were taken from storm sewers and Honey Creek pre, during, and post-State Fair, during wet and dry weather and analyzed were for microbiological and molecular data, including quantification on a subset of samples from 2008 (See memos McLellan and Sauer, Bacterial Investigation of State Fair Park for data and further information). Since the implementation of the sampling program, Wisconsin State Fair has implemented several best management practices to reduce contaminated runoff (e.g. street sweeping, covering manholes, containment of barnyard animals, and monitoring portable bathrooms and RV waste hook-ups) which have proven to reduce bacterial loads. However, high bacterial loads and the presences of the human *Bacteroides* genetic marker were detected in samples from stomwater sewers coming from surrounding communities(Milwaukee and West Allis) into State Fair Park. These areas should be further investigated for the source of contamination.

- Honey Creek – N 72nd Street and Mt Vernon Ave. In a separate investigation prompted by early results of this study the City of Milwaukee began an investigation of the area near 72nd Street and Mt Vernon Avenue using smoke and dye testing because of consistent detection of the human *Bacteroides* genetic marker. In this case, the sanitary sewer line was directly on top of the stormwater line and both had multiple cracks. The lines were televised during dye testing and showed exfiltration from the sanitary line with migration into the stormwater lines at several points. The sanitary sewer pipes have since been lined and the area is currently being retested.

- Atwater Beach. As part of the McLellan Laboratory's funding through the Wisconsin Sea Grant program, a stormwater outfall located just north of Atwater Beach in the Village of Shorewood (**Figure 10**) was regularly tested. The stormwater outfall is consistently positive for the human *Bacteroides* genetic marker, suggesting that there is a direct sanitary sewage input. This stormwater outfall discharges to the public swimming beach, therefore posing a high risk to human health if the beach remains open. The McLellan Bacterial Genetics Laboratory has confirmed that the problems exists and continues to locate the source through additional water quality testing, flourescein dye testing and smoke testing.

Figure 10: Atwater Beach

6.0 COMMUNICATIONS AND WORKING RELATIONSHIPS

Since water quality issues, particularly non-point source pollution, are not confined by political boundaries it is imperative that there is a constant exchange of current water resource management data between agencies. Coordinating water quality data among stakeholder agencies is both environmentally and economically beneficial. It broadens our overall knowledge of water quality challenges within the region, and reduces the chance of duplicating efforts, efficient monitoring data and management efforts allow municipalities to allot additional financial resources towards water quality improvement projects rather than water quality studies.

We provide technical expertise, community education, and/or work directly with:

- Milwaukee Metropolitan Sewerage District
- Wisconsin Department of Natural Resources
- City of Milwaukee
- Milwaukee County
- City of Racine
- Village of Shorewood
- City of Wauwatosa
- Milwaukee Riverkeepers
- Urban Ecology Center
- Wisconsin State Fair
- Discovery World
- Wisconsin Initiative for Climate Change Impacts
- Southeastern Wisconsin Watersheds Trust
- Institute for Service Learning, UWM
- MillerCoors Brewing company

SELECTED PUBLICATIONS RELATING TO THIS WORK AND BEACHES

McLellan, S.L., S.M. Huse, S.R. Mueller-Spitz, E.N. Andreishcheva, and M.L. Sogin. Diversity and population structure of sewage derived microorganisms in wastewater treatment plant influent. (submitted)

Mueller-Spitz S.R., L.B. Stewart, and S.L. McLellan. Genetic diversity of Clostridium perfringens isolates from m-CP method. (submitted).

Kinzelman, JL, SL McLellan. Success of Science-based Best Management Practices in Reducing Swimming Bans - A Case Study from Racine, Wisconsin, USA. Aquatic Ecosystem Health and Management (in press)

Jeter, SN, CM McDermott, PA Bower, JL Kinzelman, MJ Bootsma, GW Goetz, and SL McLellan. 2009. Bacteroidales diversity in Ring-billed gulls (Laurus delawarensis) residing at Lake Michigan beaches. Appl. Environ. Microbiol. 75: 1525-1533.

Patz, J.A., S.J. Vavrus C.K. Uejio, S.L. McLellan. 2008. Climate Change and Waterborne Disease Risk in the Great Lakes Region of the U.S. Am J Prev Med. 35(5):451-8.

Kinzelman, J., S.L. McLellan, A. Amick, J. Preedit, C.O. Scopel, O. Olapade, S. Gradus, A. Singh, G. Sedmak. 2008. Identification of Human Enteric Pathogens in Gull Feces at Southwestern Lake Michigan Bathing Beaches. J. Can Microbiol. 54: 1006-1015.

McLellan SL, ET Hollis, MM Depas, M Van Dyke, J Harris, and CO Scopel. 2007. Distribution and fate of *Escherichia coli* in Lake Michigan following contamination with urban stormwater and combined sewer overflows. *J Great Lakes Res.* 33:566-580.

Beversdorf L, S Bornstein-Forst, SL McLellan. 2007. The potential for beach sand to serve as a reservoir for *E. coli* and the physical influences on cell die-off. J. *Appl. Microbiol.* 102:1372-1381.

Scopel, CO, J Harris, and SL McLellan. 2006. Sources and nearshore transport of *Escherichia coli* at Lake Michigan beaches. *J Great Lakes Res* 32:543–552.

Olapade, O, ET Jensen, MM Depas, and SL McLellan. 2006. Microbial communities on *Cladophora* mats along the coastline of Lake Michigan. *Appl. Environ. Microbiol*. 72:1932-38.

Salmore, AK, ET Jensen, SL McLellan. 2006. Delineation of a chemical and biological signature for stormwater pollution in urban rivers. *Journal of Water & Health* 4: 247-262.

Bower PA, CO Scopel, ET Jensen, MM Depas, and SL McLellan. 2005. Detection of genetic markers of fecal indicator bacteria in Lake Michigan and the relationship to *Escherichia coli* densities using standard microbiological methods. *Appl. Environ. Microbiol*. 71: 8305-8313.

Jensen, ET and SL McLellan. 2005. Beach Closings: Scientific Studies and Public Perception. ActionBioscience.org

7.0 CONCLUSIONS & RECOMMENDATIONS

In most urban areas stormwater collection & conveyance systems are separated from sanitary sewage systems and are designed to capture storm runoff from rooftops, parking lots, and other impervious surfaces and discharge directly to nearby waterways virtually untreated (rather recently new regulations require stormwater best management practices to improve water quality). Sanitary sewage should not be present in separate stormwater collection & conveyance systems. However, this study demonstrates that human sewage contamination is in the local stormwater collection & conveyance systems and this is widespread throughout the Greater Milwaukee Metropolitan area. Nearly two thirds of the stormwater outfalls the McLellan Bacterial Genetics Laboratory investigated are categorized as high priority for further follow up (**Table 6**).

These results indicate a persistence source of bacteria contamination to Lincoln Creek, the Kinnickinnic and Menomonee River watersheds from a sanitary sewage origin. Direct private lateral connections to the storm sewer could be one possibility for the high bacteria counts to these and other locations. A more likely scenario is failing sanitary sewers or private laterals which are allowing seepage of sanitary contamination to reach the storm sewer since there was no visible physical evidence of sanitary waste at any of the storm sewer outfalls examined during this investigation. Older residential areas could have exfiltration occurring from leaking laterals, sanitary sewer pipes or poor connections/seals due to subsidence or deterioration. Migration of

sanitary waste into the storm sewers can occur though leaking joints, poor connections, or from foundation drains and sump pumps connected to the storm sewer. This transfer of contaminated seepage can be accelerated during wet weather or even when lawns are watered. Conversely, wet weather has been shown regionally to be a source of significant clear water infiltration entering the sanitary sewer system thereby potentially contributing to sanitary sewer overflows. Another possibility for contamination is that former local sanitary sewer bypass locations; those that have been sealed and properly retired (e.g. no longer being used by the local municipality) years or even decades ago but could have deteriorated over time and may be seeping or leaking into close by storm sewers. All of these factors could be contributing to the findings of this study; therefore additional testing will help determine exact causes for the sanitary contamination in stormwater and water bodies.

Human sewage contamination poses a public health risk due to the likelihood of pathogen occurrence. It is imperative that the causes are discovered and documented in order to plan and design solutions to correct the problems. Specific conclusions and recommendations are as follows:

(1) Investigate sanitary and storm sewers infrastructure integrity by community using traditional engineering approaches (dye testing, smoke testing, etc.) in areas of the stormwater collection & conveyance system where stormwater has shown evidence of chronic sanitary sewage discharges. This project has identified approximately 41 high priority stormwater outfalls of the 62 investigated. Stormwater outfalls that tested positive for the human *Bacteroides* genetic marker most frequently should be a high priority for follow up investigation by the locally responsible communities. Additional stormwater outfalls in these high priority areas should also be tested (see recommendation #2). A concentrated investigative effort in the stormwater system may provide valuable insight into the nature of infrastructure failures (sanitary sewers and storm sewers) in the urban environment.

(2) Broaden the investigation in areas of concern using quantitative methods to identify the stormwater outfalls and locations within the stormwater collection & conveyance system that have significant sanitary sewage contamination. There are specific areas of concern; Lake Michigan, Lincoln Creek, Menomonee and Kinnickinnic watersheds. This study has only investigated a very small fraction of the stormwater system within these areas. Stormwater outfalls should be prioritized using quantitative results for the human *Bacteroides* genetic marker by qPCR. A comprehensive investigative effort in the stormwater collection & conveyance systems that discharge to these receiving waters should be conducted to guide formulation of watershed management plans and improvement/restoration goals.

(3) Perform human virus testing in conjunction with human *Bacteroides* genetic marker sampling. The McLellan Bacterial Genetics Laboratory found that conducting virus testing on a small volume of stormwater is feasible; it was found that the same concentration of human derived viruses found in sewage was also present in one stormwater sample. Quantification of human viruses will provide the basis for assessment of public health risks associated with sanitary sewage discharges caused either by illicit connections or failing integrity of the associated infrastructure.

(4) Identify hydrological, physical, and meteorological parameters that correlate with high levels of sanitary sewage contamination in stormwater outfalls to better understand the conditions that favor contamination processes. Findings from this research demonstrate that the human *Bacteroides* signal is intermittent; suggesting that exfiltration of sewage from sanitary sewer systems and subsequent migration into stormwater collection & conveyance systems is the mechanism in which contamination occurs. Mapping infrastructure age using geographical information systems (GIS) in conjunction with sanitary sewage detection in stormwater collection & conveyance systems may provide insight into relationships between sanitary sewer system age and lack of integrity, which would help prioritize capital improvement investments.

(5) Quantify the overall human contribution to fecal pollution in Lincoln Creek, Menomonee River and Kinnickinnic River and correlate levels with hydrological and climate parameters. Determining the contribution of human sources will allow a direct comparison of stormwater inputs to combined sewer overflows in terms of health risk. The real time monitoring stations operated by MMSD and USGS and the sampling capabilities at these stations offer an investigative platform to accomplish this goal. These sampling stations provide real-time water quality data available to the public (http://v3.mmsd.com/H2OInfo/index.html). A quantitative estimate of sanitary sewage contamination in rivers can serve as a benchmark that can be used to evaluate the effectiveness of management strategies that are formulated and implemented through stakeholder groups like Watershed Action Teams (WATs) of the Southeastern Wisconsin Watersheds Trust (SWWT).

(6) Incorporate estimates of human sources to supplement fecal coliform levels in future modeling efforts. Considerable effort has been invested in defining fecal coliform loads in relation to specific assessment points contained within the Greater Milwaukee Watersheds. Information as to the percentage of sanitary sewage contamination, in relation to total fecal pollution, can be incorporated into modeling efforts as a higher tier of information

(7) Assimilate water quality data (e.g. *E. coli*) and physical parameters (e.g. age of infrastructure and diameter of pipe) from the GLWI, MMSD, and Milwaukee Riverkeepers into a GIS database. The GIS database would be able to (1) perform initial analysis; (2) serve as a central repository for geographic data; and (3) produce visually intuitive map figures that supplement study findings.

(8) Continue public education and outreach. The McLellan Bacterial Genetics Laboratory works with the Milwaukee Riverkeepers, the Urban Ecology Center, and other grassroots organizations to help educate the public and collaborate on citizen monitoring programs. The McLellan Bacterial Genetics Laboratory also continues to maintain a green roof that was funded through MMSD in 2003 as a demonstration project for others to implement their own stormwater runoff reduction/retention strategies. The McLellan Bacterial Genetics Laboratory also provides the public with tours of the green roof, which serves as an outdoor classroom for GLWI and UWM.

Table 6: Conclusions and recommendations for stormwater sites. A high priority site is a stormwater site that the human *Bacteroides* genetic marker has been detected in more than 50% of samples. A low priority site is a stormwater site that the human *Bacteroides* genetic marker has been detected in less than 50% of samples. (See **Appendix A** for site maps).

Site Code	% Positive for Human *Bacteroides*	Location	Narrative
Honey Creek			
HC01	50%	74th Street and W. Bennett Ave.	Sampled 2006-2008 by GLWI. High Priority site.
HC01S	70%	Honey Creek @ McCarty Park	2006 MMSD Honey Creek Bacteria Investigation Survey. High priority site.
HC02	53%	Honey Creek Pkwy. & Beloit Rd.	Sampled 2006-2008 by GLWI. High priority site.
HC02S	89%	Honey Creek @ 84th Street. & O'Conner Street	2006 MMSD Honey Creek Bacteria Investigation Survey. High priority site.
HC03	50%	82nd St. & Arthur Ave.	Sampled 2006-2008 by GLWI. High priority site.
HC03S	78%	Honey Creek @ Honey CK Pkwy & Bluemound Rd.	2006 MMSD Honey Creek Bacteria Investigation Survey. High priority site.
HC04	64%	S. 84th St. & WI Lutheran H.S.	Sampled 2006-2008 by GLWI. High priority site.
HC05	64%	84th Street & Hill Street	Sampled 2006-2008 by GLWI. High priority site.
HC06	15%	84th Street & Hill Street	Sampled 2006-2008 by GLWI. Low priority site.
HC07	50%	N. Honey Creek Pkwy. & Honey Creek	Sampled 2006-2008 by GLWI. High priority site.
HC08	54%	N. Honey Creek Pkwy. & Honey Creek	Sampled 2006-2008 by GLWI. High priority site.

Table 6: continued.

Site Code	% Positive for Human *Bacteroides*	Location	Narrative
HI03b	89%	Honey Ck Pkwy @ SW outfall near 80th & Stevenson St	2006 MMSD Honey Creek Bacteria Investigation Survey. High priority site.
HI04b	89%	Honey Ck Pkwy @ 79th St. & Mt. Vernon Ct. from SW corrugated metal pipe	2006 MMSD Honey Creek Bacteria Investigation Survey. In 2008, conducted further testing of this outfall with the City of MKE. Human *Bacteroides* was detected during this testing. High priority site.
HI05b	11%	Honey Creek Parkway & Mary Ellen Pl. from SW corrugated metal pipe	2006 MMSD Honey Creek Bacteria Investigation Survey. Low priority site.
SWWA20	38%	Dana Ct. and 83rd St. Ext'd	Sampled in 2006. Low priority site.
Kinnickinnic River			
KI02S	100%	KK River @ 35th & Manitoba St	Sampled by MMSD in 2008. High Priority Site, continue sampling along with corresponding inline site (SKK01A).
KI05S	100%	KK River @ 60th & KK River Pkwy	Sampled by MMSD in 2008. High Priority Site, continue sampling along with corresponding inline site (SLP01A).
KK01	67%	KK River Pkwy. & 33rd St.	Sampled 2006-2008 by GLWI. High priority site.
KK02	67%	KK River Pkwy. & 35th St.	Sampled 2006-2008 by GLWI. High priority site.
KK03	78%	KK River Pkwy. & Forest Home Ave.	Sampled 2006-2008 by GLWI. High priority site.
KK04	56%	KK River Pkwy. & Forest Home Ave.	Sampled 2006-2008 by GLWI. High priority site.
RI33S	86%	43rd St Ditch @ Lincoln Ave	Sampled by MMSD in 2008. High priority site.
S4301A	34%	400 W. Lincoln Ave.	Sampled by MMSD in 2007and 2008. Low priority site.

Table 6: continued.

Site Code	% Positive for Human *Bacteroides*	Location	Narrative
S4302A	18%	44th St. & Burnham	Sampled by MMSD in 2007. Low priority site.
SKK01A	95%	35th St. & Manitoba	Sampled by MMSD in 2007and 2008. High Priority Site
SLP01A	76%	61st St. and Harrison Ave.	Sampled by MMSD in 2007and 2008. High priority site. 2007 replicate samples were isolated using different kit, found additional positives, 76% of samples positive for human *Bacteroides* is an underestimate
Lake Michigan			
SWMI04	65%	3500 S. Lake Dr. @ Bay View Park	Sampled by MMSD in 2007. High priority site. 2007 replicate samples were isolated using different kit, found additional positives, 65% of samples positive for human *Bacteroides* in an underestimate.
SWWB09	75%	4939 N. Newhall	
Menomonee River			
MN01	33%	Honey Creek Pkwy. & S. 68th St.	Sampled 2007 and 2008 by GLWI. Low priority site.
MN04	69%	Men River Pkwy. & 90th St.	Sampled 2006-2008 by GLWI. High priority site.
MN06	56%	Hart Park	Sampled 2007 and 2008 by GLWI. High priority site.
MN07	67%	N. 68th & Men River Pkwy.	Sampled 2007 and 2008 by GLWI. High priority site.
MNI01S	67%	Men Rvr @ Ridge & Harding Blvd.	Sampled by MMSD in 2008. High priority site.
MNI02S	83%	Men Rvr @ West Center St. & Men River Pkwy	Sampled by MMSD in 2008. High priority site.
MNI03S	67%	Men Rvr @ N 69th St. & Men Rvr Pkwy	Sampled by MMSD in 2008. High priority site.

Table 6: continued.

Site Code	% Positive for Human *Bacteroides*	Location	Narrative
SMN01A	48%	10435 Concordia Ave.	Sampled by MMSD in 2008. Low priority site.
SMN02A	72%	69th St. Ext'd and Hart Park Lane Ext'd	Sampled by MMSD in 2008. High priority site.
SMN03A	100%	Center St. & 97th St.	Sampled by MMSD in 2008. High priority site.
SMN04A	93%	96th & Center St.	Sampled by MMSD in 2008. High priority site.
RI32S	71%	North Ave. & Mt. Kisco Dr.	Sampled by MMSD in 2008. High priority site.
SWMI18	93% (Before) 7% (After)	Miller Park East Parking Lot at the Sausage House	Sampled by MMSD in 2006 and 2007. Found a cross-connection problem at Miller Park. The problem was fixed in 2007. Low priority site, low cell counts coupled with low percentage of positive Human *Bacteroides* results suggest that the problem has been solved and does not need further investigation.
SWWA13	89%	Ridge Blvd. & Harding Blvd.	Sampled by MMSD in 2007 (pollutographs) and 2008. It was suspected that raccoons could be causing a false positive at this outfall. After sequencing, it was determined that raccoons do not cross-react with Human *Bacteroides*. High priority site.
Milwaukee River			
MW01	60%	Green Tree Rd. & River Rd.	Sampled by MMSD in 2007 and 2008 by GLWI. High priority site.
MWR1	14%	Bender Rd. & N. Sunny Point Rd.	Sampled by MMSD in 2007 and 2008 by GLWI. Only found a positive at this site during CSO in June 2008. Low priority site. This site does not need further investigation, except during a CSO/SSO event.

Table 6: continued.

Site Code	% Positive for Human *Bacteroides*	Location	Narrative
Oak Creek			
OC03S	57%	Oak Ck. @ Forest Hill Road	Sampled by MMSD in 2008. High priority site.
OC04S	43%	Oak Ck @ Pennsylvania Ave.	Sampled by MMSD in 2008. Low priority site.
SOC01A	44%	2345 E. Montana Ave.	Sampled by MMSD in 2007 and 2008. In 2008, it was suspected that raccoons could be causing a false positive at this outfall. After sequencing, it was determined that raccoons do not cross-react with Human *Bacteroides*. Low priority site.
SOC02A	33%	S. Shedpard Ave. Ext'd & HWY 100 Ext'd.	Sampled by MMSD in 2007 and 2008. Low priority site.
Lincoln Creek			
SLC01A	28%	Mill Rd. & 51st St.	Sampled by MMSD in 2007 and 2008. Low priority site.
SLC02A	32%	49tt St. & Mill Rd.	Sampled by MMSD in 2008. Low priority site.
LC02S	86%	Lincoln Ck @ Mill Rd near 51st & Woolworth Av.	Sampled by MMSD in 2008. High priority site.
LC04S	100%	Linclon Ck @ 47th St and & Congress St.	Sampled by MMSD in 2008. High priority site, continue sampling along with corresponding inline site (SWMI07).
SWMI07	88%	4345 N. 47th St.	Sampled by MMSD in 2007 and 2008. In 2008, viruses were detected at the same concentration as human sewage. High priority site.
Underwood Creek			
SUC01A	22%	Fairview Ext'd & Curtis Rd.	Sampled by MMSD in 2007. Low priority site.

Table 6: continued.

Site Code	% Positive for Human *Bacteroides*	Location	Narrative
SUC02A	53%	S. Shedpard Ave. Ext & HWY 100 Ext'd	Sampled by MMSD in 2008. High priority site.
UC02S	43%		Sampled by MMSD in 2008. Low priority site.
UND01	60%	I-94 East Bank	Sampled in 2006 and 2007 by GLWI. High priority site.
UND02	0%	Bluemound Ave. & 124th St.	Sampled in 2006 and 2007 by GLWI. Low priority site, further investigation not recommended.
UND03	63%	Underwood Ck Pkwy. & 115th St.	Sampled in 2006 and 2007 by GLWI. High priority site.
UND04	60%	Fisher Pkwy. 103rd St.	Sampled in 2006 and 2007 by GLWI. High priority site.
UNDR1	0%	Pedestrian Bridge - North Branch of Watertown Plank Rd.	Sampled in 2007 by GLWI. Low priority site, further investigation not recommended.

8.0 Acknowledgements
Great Lakes WATER Institute:

Patricia Bower – Research Technician
Melinda Bootsma – Research Technician
Lisa Stewart - Research Technician
Sabrina Mueller-Spitz – Post doctoral fellow
Jeff Ecklund – Undergraduate Research Assistant

This work was funded by a Research Project Contract with through the Milwaukee Metropolitan Sewerage District (MMSD).

Special thanks to MMSD's staff for their assistance and review:
Christopher Magruder – Project Manager (Community Environmental Liaison)
Eric Waldmer – Water Quality Supervisor
Mary Singer - Stormwater Quality Supervisor
Bre McDonald – Water Resource & Sustainability Specialist
and the MMSD's stormwater field monitoring crews.

9.0 Work Cited

Bernhard AE & Field KG (2000) Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. *Appl Environ Microbiol* **66**: 1587-1594.

Bernhard AE & Field KG (2000) A PCR assay To discriminate human and ruminant feces on the basis of host differences in *Bacteroides*-Prevotella genes encoding 16S rRNA. *Appl Environ Microbiol* **66**: 4571-4574.

Bower PA, Scopel CO, Jensen ET, Depas MM & McLellan SL (2005) Detection of genetic markers of fecal indicator bacteria in Lake Michigan and determination of their relationship to Escherichia coli densities using standard microbiological methods. *Appl Environ Microbiol* **71**: 8305-8313.

Carson CA, Christiansen JM, Yampara-Iquise H*, et al.* (2005) Specificity of a *Bacteroides* thetaiotaomicron marker for human feces. *Appl Environ Microbiol* **71**: 4945-4949.

Dick LK, Bernhard AE, Brodeur TJ, Santo Domingo JW, Simpson JM, Walters SP & Field KG (2005) Host distributions of uncultivated fecal Bacteroidales bacteria reveal genetic markers for fecal source identification. *Appl Environ Microbiol* **71**: 3184-3191.

Field KG & Samadpour M (2007) Fecal source tracking, the indicator paradigm, and managing water quality. *Water Res* **41**: 3517-3538.

Gaffield SJ, Goo RL, Richards LA & Jackson RJ (2003) Public health effects of inadequately managed stormwater runoff. *Am J Public Health* **93**: 1527-1533.

Haile RW, Witte JS, Gold M*, et al.* (1999) The health effects of swimming in ocean water contaminated by storm drain runoff. *Epidemiology* **10**: 355-363.

Kreader CA (1995) Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. *Appl Environ Microbiol* **61**: 1171-1179.

Lamendella R, Domingo JW, Oerther DB, Vogel JR & Stoeckel DM (2007) Assessment of fecal pollution sources in a small northern-plains watershed using PCR and phylogenetic analyses of Bacteroidetes 16S rRNA gene. *FEMS Microbiol Ecol* **59**: 651-660.

Layton BA, Walters SP & Boehm AB (2009) Distribution and diversity of the enterococcal surface protein (esp) gene in animal hosts and the Pacific coast environment. *J Appl Microbiol*.

McLellan SL & Salmore AK (2003) Evidence for localized bacterial loading as the cause of chronic beach closings in a freshwater marina. *Water Res* **37**: 2700-2708.

O'Shea ML & Field R (1992) Detection and disinfection of pathogens in storm-generated flows. *Can J Microbiol* **38**: 267-276.

Rajal VB, McSwain BS, Thompson DE, Leutenegger CM & Wuertz S (2007) Molecular quantitative analysis of human viruses in California stormwater. *Water Res* **41**: 4287-4298.

Salmore AK, Jensen ET & McLellan SL (2006) Delineation of a chemical and biological signature for stormwater pollution in urban river. *Journal of Water and Health* **4**: 247-262.

Santoro AE & Boehm AB (2007) Frequent occurrence of the human-specific *Bacteroides* fecal marker at an open coast marine beach: relationship to waves, tides and traditional indicators. *Environ Microbiol* **9**: 2038-2049.

Scott TM, Rose JB, Jenkins TM, Farrah SR & Lukasik J (2002) Microbial source tracking: current methodology and future directions. *Appl Environ Microbiol* **68**: 5796-5803.

SEWRPC (2008) Regional Water Quality Plan Update, Technical Report 39. ed.^eds.), p.^pp.

Stewart JR, Ellender RD, Gooch JA, Jiang S, Myoda SP & Weisberg SB (2003) Recommendations for microbial source tracking: lessons from a methods comparison study. *J Water Health* **1**: 225-231.

Ufnar JA, Wang SY, Christiansen JM, Yampara-Iquise H, Carson CA & Ellender RD (2006) Detection of the nifH gene of *Methanobrevibacter smithii*: a potential tool to identify sewage pollution in recreational waters. *J Appl Microbiol* **101**: 44-52.

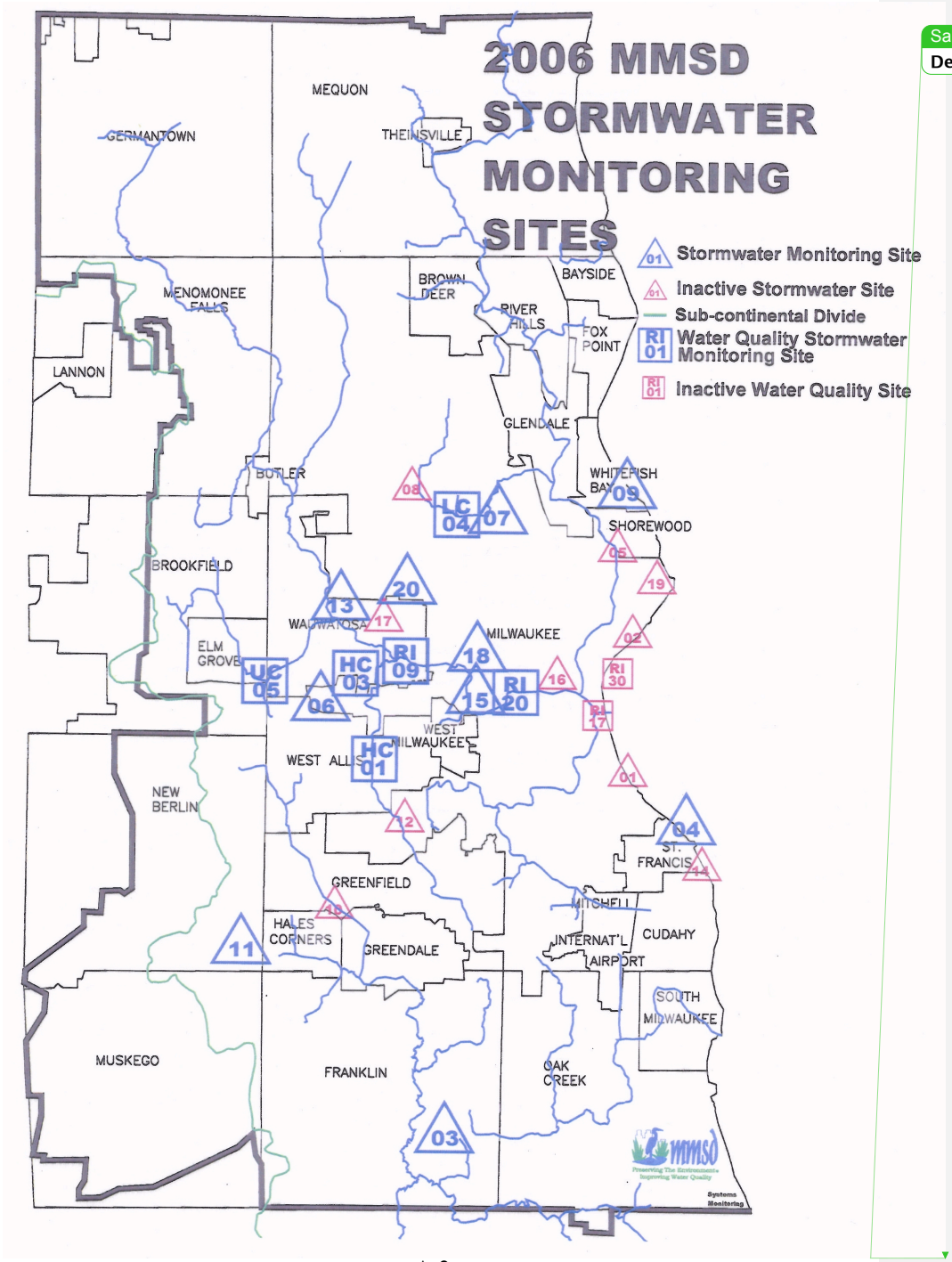
USEPA (2002) Method 1603: *Escherichia coli* (*E. coli*) in water by membrane filtration using modified membrane-thermotolerant Escherichia coli agar (modified mTEC). ed.^eds.), p.^pp. US Environmental Protection Agency Office of Water, Washington, D.C.

Zheng G, Yampara-Iquise H, Jones JE & Andrew Carson C (2009) Development of Faecalibacterium 16S rRNA gene marker for identification of human faeces. *J Appl Microbiol* **106**: 634-641.

10.0 Appendices

10.1 Appendix A: Stormwater Sites and Map Locations
10.2 Appendix B: Human *Bacteroides* Data (stormwater outfalls, Inline stormsewers, & instream Locations) (2007 & 2008)
10.3 Appendix C: Beach Outfalls Sites and Locations

10.1 Appendix A
Stormwater Sites and Locations
(2006-2008)



Sandra McLellan 7/23/09 12:42 PM
Deleted: ..