

## Delineation of a chemical and biological signature for stormwater pollution in an urban river

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### ABSTRACT

Stormwater is a major source of *Escherichia coli* in urban waterways. This study assessed the chemical and bacterial contaminant load in stormwater entering an urban river using chemical analyses, *E. coli* enumeration and antibiotic resistance testing. Inline monitoring of five major stormwater outfalls revealed that *E. coli* concentrations can range from 100 to >240,000 CFU 100 ml<sup>-1</sup> in stormwater; 15% of the samples contained >240,000 CFU 100 ml<sup>-1</sup>. *E. coli* levels increased from <200 CFU 100 ml<sup>-1</sup> to between 1,500 and >20,000 CFU 100 ml<sup>-1</sup> in the receiving waters following storm events. Metals and nutrients were significantly elevated ( $p < 0.05$ ) in inline stormwater samples compared with baseflow river water. Chromium, zinc, lead and total phosphorus were co-ordinately elevated in stormwater, but *E. coli* levels did not correlate to this chemical signature. *E. coli* isolates from stormwater and river water samples ( $n = 2,478$ ) were tested for resistance to 10 antibiotics and compared with over 1,800 isolates from human and non-human sources. The antibiotic resistance frequencies of isolates from human sources were considerably higher than those found in non-human sources. Isolates from human sources were resistant to multiple antibiotics; however, no specific multiple resistance pattern was found. One of five stormwater outfalls tested contained *E. coli* resistant to antibiotics at frequencies that were not significantly different from those of *E. coli* isolates from wastewater treatment plant influent ( $p < 0.05$ ). The results from antibiotic resistance testing suggested that the elevated *E. coli* levels observed after storm events are a mixture of human and non-human sources. The results of this study demonstrate that there is a considerable *E. coli* load to receiving waters from stormwater and that sanitary sewage inputs may not be limited to recognized sewer overflows.

**Key words** | antibiotic resistance, combined sewer overflows, *E. coli*, fecal pollution, microbial source tracking, stormwater

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### INTRODUCTION

With significant regulation of point source pollutant discharges to waterways since the passage of the Clean Water Act in 1972, non-point source contaminants have emerged as the major impediment to achieving water quality goals in the United States (USEPA 2000a). High levels of pollutant transfer from land to water occur during storm events, including heavy metals, chemical and biological contaminants (Bannerman *et al.* 1993; Wyer *et al.* 1995; Bickford *et al.* 1999; Faulkner *et al.* 2000). Stormwater

runoff is collected and discharged directly to waterways with no treatment in areas with separated sewer systems. Combined sewer systems capture both sanitary sewage and stormwater and convey these flows to the wastewater treatment plant. The treatment of stormwater in this fashion may be beneficial; however, combined sewer systems are highly susceptible to overflows during periods with large amounts of precipitation. The degree and type of land use in a watershed may influence the amount of bacteria and other

doi: 10.2166/wh.2006.006

contaminants carried by stormwater (Bannerman *et al.* 1993; Young & Thackston 1999; Mallin *et al.* 2000). Human sources of fecal contamination can be introduced to waterways by sanitary and combined sewer overflows. Bacterial contaminants in urban stormwater, e.g. non-point source runoff, are expected to originate from multiple, non-human sources. Bacterial fecal indicators alone may not be predictive of health risk from storm sewer discharge, particularly in instances where mixed sanitary and stormwater inflow are suspected (O'Shea & Field 1992). Given the age and condition of sewer conveyance systems in some urban areas, unrecognized sanitary inputs into the stormwater system should be considered.

Surface waters in and downstream from urban centres are complex systems subjected to numerous pollution sources. Of the 548 waterborne disease outbreaks reported between 1948 and 1994, 24% were from surface water contamination and 40% were from an unknown water contamination source; most followed an extreme precipitation event (Curriero *et al.* 2001). Fecal pollution released into surface waters from human sources is considered a serious health risk since it is likely to carry human pathogens (Moe 2002; Scott *et al.* 2002). Non-human sources of fecal pollution can also carry human pathogens; for example, *Escherichia coli* 0157:H7, *Salmonella* spp., *Giardia* and *Cryptosporidium parvum* are found in zoonotic reservoirs (NRC 2004). The health risk associated with non-point runoff (e.g. urban stormwater) has not been characterized extensively; however, there is increasing evidence that urban stormwater may pose a serious human health risk (O'Shea & Field 1992; Pruss 1998; Gaffield *et al.* 2003). Human viruses have been found in stormwater discharges (Jiang *et al.* 2001) and there are reports of increased incidence of illness associated with swimming near stormwater outfalls (Haile *et al.* 1999).

The origin of fecal pollution is relevant since the human health risk may vary depending upon the source (NRC 2004). Microbial source tracking methods for biological contaminants have been explored including genetic and phenotypic techniques (reviewed in Scott *et al.* 2002 and Field *et al.* 2003a). Several approaches are aimed towards detecting markers that are associated with a particular host and include culture independent methods for F+ coliphages (Havelaar *et al.* 1990; Cole *et al.* 2003), human

enteroviruses or adenoviruses (Jiang *et al.* 2001; Noble *et al.* 2003), host specific species of *Bacteroides* (Bernhard & Field 2000), or virulence genes in *E. coli* (Chern *et al.* 2004). Other methods rely on the indicator organisms themselves and require a characterization of host sources of fecal pollution for comparisons; these methods include DNA fingerprinting (Dombek *et al.* 2000; Carson *et al.* 2001) and antibiotic resistance testing (Parveen *et al.* 1997; Wiggins *et al.* 1999; Harwood *et al.* 2000). The various methods for culture independent (Field *et al.* 2003b) and library dependent (Harwood *et al.* 2003; Myoda *et al.* 2003) approaches have been recently compared. Antibiotic resistance (AR) may be useful because it is founded on a well-supported hypothesis that animals (specifically humans or certain agricultural animals) that are exposed to antibiotics will harbour antibiotic resistant bacteria more frequently than hosts not exposed to antibiotics. Studies have demonstrated that there is an increased proportion of fecal indicator bacteria with antibiotic resistance traits associated with human sources of fecal pollution (Parveen *et al.* 1997; Hagedorn *et al.* 1999; Harwood *et al.* 2000; Guan *et al.* 2002).

For the past several summer seasons, there have been numerous closings of Lake Michigan beaches in and near Milwaukee, Wisconsin, due to persistent levels of *E. coli* above the USEPA recommended limits for recreational water quality. Theories to explain the elevated *E. coli* levels have included localized fecal pollution from waterfowl and shore birds, as well as regional contamination from sewer overflows or agricultural runoff delivered to the beach *via* rivers. The relative contribution of stormwater to overall degraded water quality in the rivers is unknown. The Menomonee River system may serve as a model for other cities that have been developed on the shores of the Great Lakes in close proximity to rivers that discharge to coastal waters. A combination of spatial surveys, chemical analyses, and antibiotic resistance traits of *E. coli* offers a possible strategy for identifying stormwater as a key contributor of pollutants entering urban waters. Chemical parameters such as nutrients, metals and solids and biological parameters were evaluated for a signature that could be used to assess urban stormwater loads and would differentiate bacterial contamination present in stormwater from contamination due to sanitary sewage.

## METHODS

### Site description

The Menomonee River watershed in Metropolitan Milwaukee consists of suburban and urban land use and covers 352 km<sup>2</sup>; the Menomonee River is 97 km long and joins the Milwaukee River approximately 1.5 river km before it enters Lake Michigan. Average yearly precipitation in the Menomonee River watershed is 84 cm and average temperature between May and September is 18°C. Stormwater outfalls enter the Menomonee throughout its length and include combined sewer outfalls in the lower reaches of the river course in downtown Milwaukee. The combined sewer area of Milwaukee extends approximately 45 km<sup>2</sup> around the most densely developed area of the city and during most storm events stormwater is captured and conveyed to the wastewater treatment plant. Following storm events with large amounts of precipitation, the combined sewer system will reach capacity and overflow to the Menomonee, Milwaukee or Kinnickinnic Rivers, resulting in a combined sewer overflow (CSO) that discharges both stormwater and sanitary sewage to the receiving waters. There have been an average of 2.5 CSO events per year in Milwaukee since 1995.

Ten sites were chosen along 24 river kilometres; three of the sites were within the combined sewer area of the Menomonee (sites 6–8) and two additional sites were below the confluence with the Milwaukee River (sites 9–10), also within the combined sewer system (Figure 1). The upper Menomonee River flows through a natural channel with intermittent stone reinforcement walls on one or both sides surrounded by suburban/urban residential land (sites 1–5). The river bed is channelized in cement for 1.4 km above site 6, while the lower reaches (sites 6–8) and confluence with the Milwaukee River (sites 9–10) flow through a dredged bed with iron piling or concrete sides, running through the city's urban and industrial core.

### Sampling

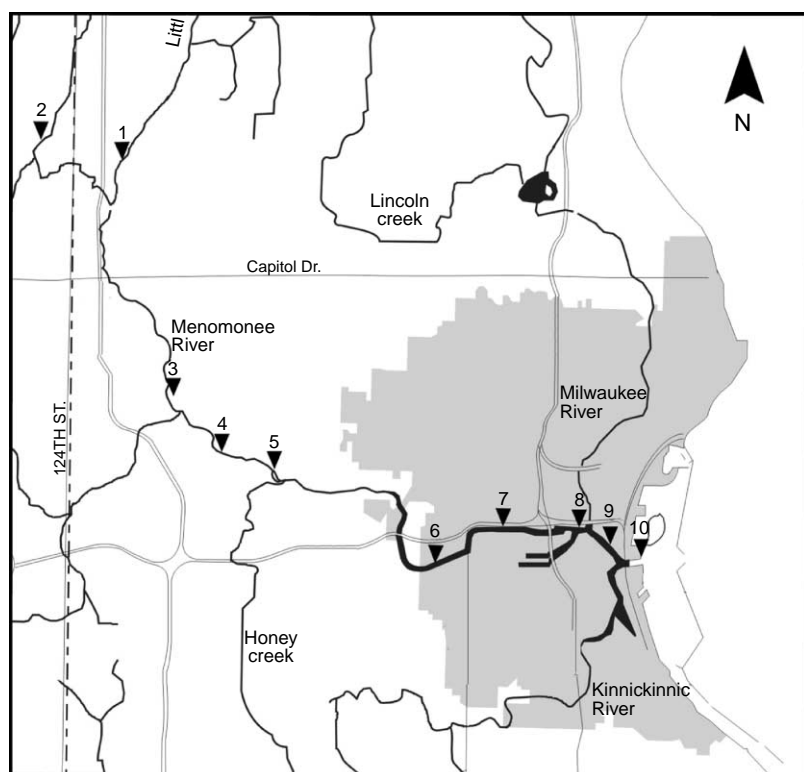
River surface water samples were collected at each of the ten sites as close to mid-channel as possible in triplicate using a 1-l grab sampler on days preceding a storm

(no rain in the previous 72 hours) and after a storm event (within 14 hours after rainfall began). Three storms were sampled during July–September 2002 where there was no CSO; rainfall was 30.5, 6.1 and 10.2 mm, respectively. One storm with 73.7 mm over a 48-hour period was followed by a CSO event, which delivered CSO contaminated water to sites 6–10 on two consecutive days. Two surveys were conducted during the CSO event: 18 hours after the start of the CSO, and 24 hours later during a second period of CSO discharge. All samples were stored on ice in the dark until processing. Measurements for pH, dissolved oxygen, temperature and conductivity were recorded in the field with a Multiprobe Hydrolab (Hydrolab, Hach Company, Loveland, Colorado).

Milwaukee Metropolitan Sewerage District (MMSD) automated inline sampling stations operated during storm events at the final collection point of underground storm sewers before the outfall that discharges to the Menomonee River. Samples for *E. coli* enumeration were obtained from five of these stations: three stations in the suburban stretches of the Menomonee and two sites in urban stretches near the confluence. Two of these sites (one suburban and two urban) were used to obtain *E. coli* isolates in order to determine the antibiotic resistance patterns found in stormwater isolates. Additional stormwater inline monitoring sites from urban areas of metro Milwaukee were also used to obtain *E. coli* isolates for comparisons. The inline samplers collected two flow-weighted composite samples for each station per storm event, one from 'first flush' stormflow (first 2 hours of the rain period) and one from 'second flush' stormflow (2–4 hours after the beginning of the rain period). The samples were removed from the automated samplers within 8 hours of the beginning of precipitation and placed on ice in the dark until analysis.

### *E. coli* identification and enumeration

River and inline stormwater samples were analysed according to the EPA original method for *E. coli* enumeration (USEPA 2000b). Presumptive identification of *E. coli* was made by observing yellow, yellow-brown or yellow-green colonies on m-TEC media. These results were confirmed by testing a subset of isolates for β-glucuronidase activity using



**Figure 1** | Menomonee River tributary of the Milwaukee River Basin, Wisconsin, USA. Sample sites along the Menomonee include primarily suburban/urban residential land (sites 1–5) and dense urban land within the combined sewer service area (sites 6–8); the Menomonee River and Milwaukee River confluence is in the downtown Milwaukee area (site 9), 1.5 km from where the rivers discharge to Milwaukee Harbor (site 10). The combined sewer area is shown in grey.

EC growth medium containing 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG) (Remel, Lenexa, Kansas). MUG-positive isolates were cultured on agar plates and then confirmed for indole production using a colorimetric spot test of p-dimethylaminocinnamaldehyde (Remel, Lenexa, Kansas). The identification rate of *E. coli* using this combined protocol has been found to be 98% accurate when compared with PI20E biochemical panels (bioMérieux, Marcy L'Etoile, France). Additional data on *E. coli* levels from 14 inline sites, including the five in this study, were obtained for 2000–2002 for multiple storm events from MMSD (EPA method SW846 SM(20) 9221F). Fecal coliform levels in the inline stormwater samples and combined sewer outfalls were provided by MMSD (EPA method SM 9221E).

### Chemical analyses

Analyses of water samples for chemical parameters were provided by MMSD. Water samples were analysed by

MMSD according to the EPA standard methods as follows: alkalinity, EPA Method 310.2; chloride, EPA Method 325.2; turbidity, EPA Method 180.1; total suspended solids (TSS), SW846 SM (20) 2540D; ammonia nitrogen, EPA Method 350.1; total phosphorus (total P), EPA Method 365.1; nitrate and nitrite nitrogen, EPA Method 353.2; mercury, EPA Method 245.2; arsenic, cadmium, calcium, chromium, copper, lead, magnesium, nickel, selenium, silver and zinc (ICP method), SW846 6010A.

### Antibiotic resistance arrays

*E. coli* isolates were inoculated into LB media in 96-well microtitre trays, incubated overnight at 37°C, and then stored in the trays with LB supplemented with 25% glycerol at –80°C. The isolates were thawed and stamped with a 96-pin replicator (Nalge Nunc International, Naperville, Illinois) onto a series of LB plates containing the following antibiotic concentrations: ampicillin at 10 and 20  $\mu\text{g l}^{-1}$ ; streptomycin at 6.25, 12.5, and 25  $\mu\text{g l}^{-1}$ ;

chlorotetracycline, nalidixic acid, oxytetracycline, and tetracycline at 12.5, 25, and 50  $\mu\text{g l}^{-1}$ ; kanamycin and neomycin at 25, 50, and 100  $\mu\text{g l}^{-1}$ ; sulfathiazole at 500  $\mu\text{g l}^{-1}$  and 1 mg  $\text{l}^{-1}$ ; and penicillin at 45 and 90  $\mu\text{g l}^{-1}$ . Control plates containing no antibiotics were stamped for all isolates to document viability of isolates and establish normal colony growth morphology. Each isolate was scored as 0 (no growth), 1 (intermediate growth) or 2 (resistance); intermediate growth was considered as <50% of growth observed on control plates. Isolates were considered resistant only when growth was >50% of the growth observed on control plates containing no antibiotics; isolates with intermediate growth were regarded as sensitive. *E. coli* obtained directly from known sources of pollution were also tested. For non-human hosts, one isolate per fecal sample was obtained, and for sewage samples, 5–20 isolates per sample were collected. Sample locations, time periods and methods of isolation for these isolates have been described previously by our laboratory (McLellan 2004). Antibiotic resistance (AR) frequencies for each group of isolates were compiled using SAS (v.8 for Windows, Inc., Cary, North Carolina). Cluster analysis was performed using the UMPGA method based on Dice coefficients in Bio-numerics v. 3.0 (Applied Maths, Kortrijk, Belgium) to determine if common patterns of multiple antibiotic resistance were present and the relationships between multiple AR patterns and the host or environmental source of the isolates.

### Statistics

$\text{Log}_{10}$  transformations were applied to bacteria data. One-way non-parametric ANOVAs and post-hoc rank sum scores were used to assess differences in measured bacterial and chemical parameters, and step-wise multiple regression models were used to determine the relationship between *E. coli* levels and chemical parameters, and among chemical parameters (SAS v.8 for Windows). Differences in AR frequencies of *E. coli* isolates were evaluated using a two-sided test of binomial proportion using SAS software. All tests were assessed at the 95% confidence interval. For paired t-tests, replicates were considered as either individual samples from one storm event (15–150 isolates

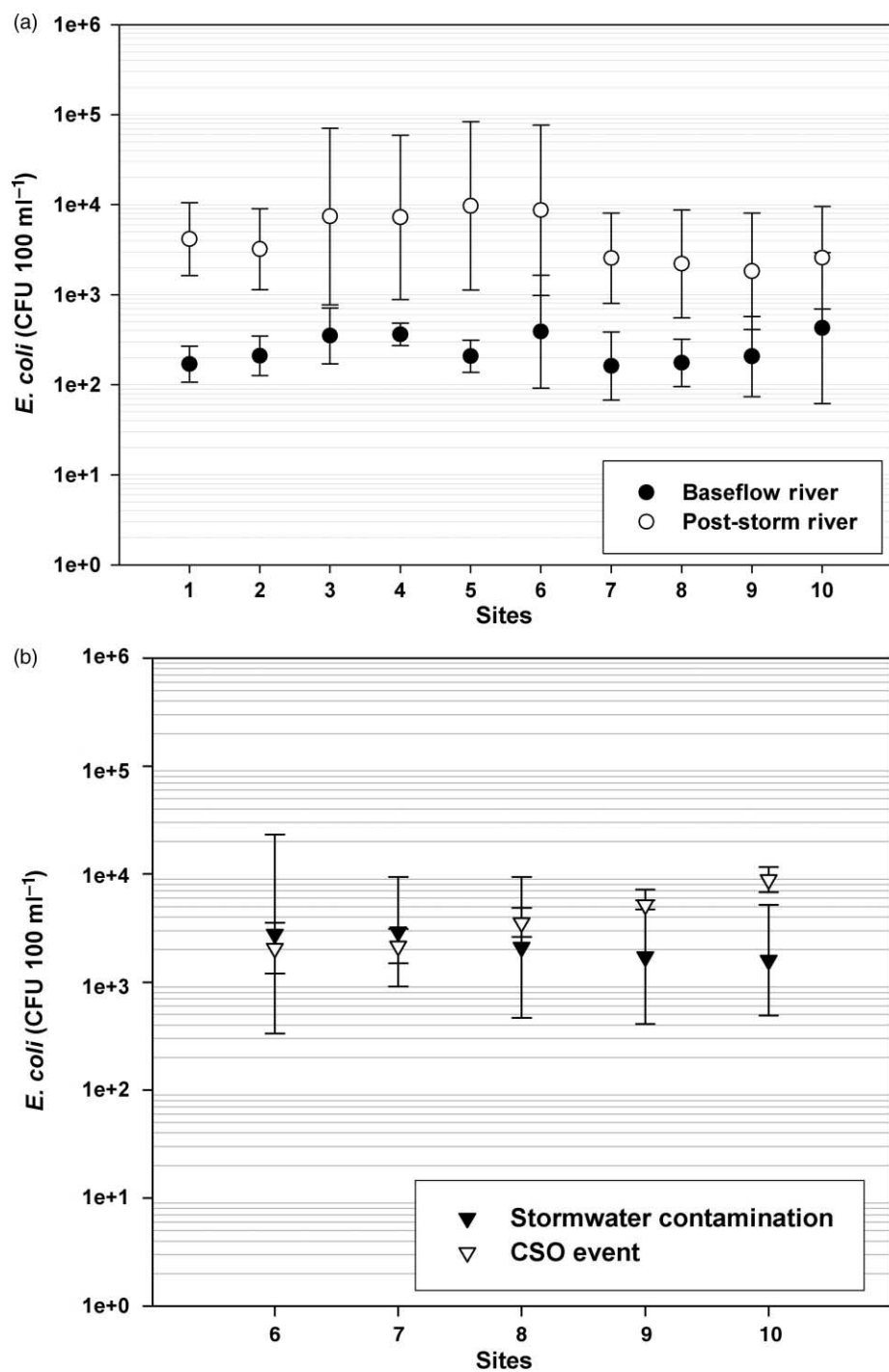
per sample), or composite datasets of isolates from a specific site from multiple storm events.

## RESULTS

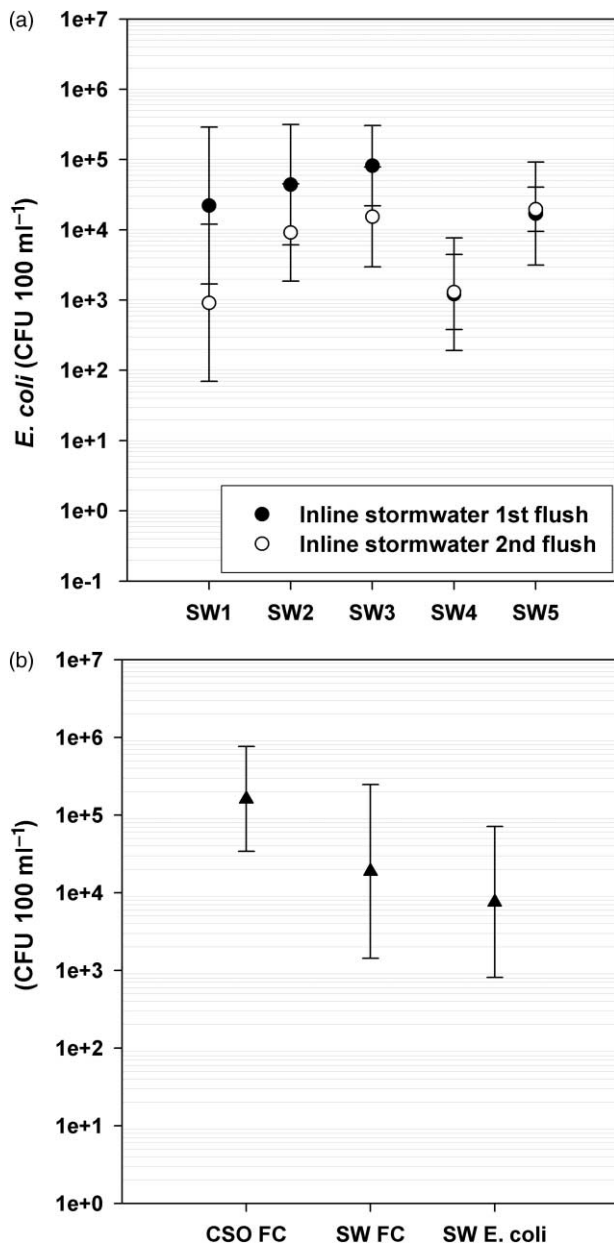
### Fecal indicator levels

*E. coli* levels in the Menomonee River increased by one to three orders of magnitude following storm events across all sites, from pre-storm means of 100–300 to post-storm means of 1,700–10,000 CFU 100 ml<sup>-1</sup> (Figure 2a). Sites 3–6 demonstrated greater, more variable differences between pre- and post-storm *E. coli* levels than did upstream or downstream sites. *E. coli* levels were lower in the combined sewer area (sites 6–10), where stormwater is captured and diverted to the wastewater treatment plant, than in upstream sites that receive stormwater (sites 1–5). The exception to this was during the one CSO event that occurred during the study period in 2002; however, even under these conditions, there was no significant difference in *E. coli* levels at river sites receiving stormwater and CSO (sites 6–10) compared with when the sites were receiving stormwater alone (Figure 2b). This suggests that stormwater contributes a major fecal bacteria load to this river system whether or not there is a CSO event.

Inline stormwater samples from the five sites used in this study (discharging directly to the Menomonee River) demonstrated high levels of *E. coli*, with means ranging from 1,000–100,000 CFU 100 ml<sup>-1</sup> across sites; these levels were one to three orders of magnitude higher than baseflow river samples (Figure 3a). At these same five sites, more than 15% of the samples exceeded 240,000 CFU 100 ml<sup>-1</sup> and more than 30% of the samples exceeded 100,000 CFU 100 ml<sup>-1</sup>. *E. coli* levels in the first flush of stormwater from three outfalls draining suburban areas were significantly higher than the second flush ( $p < 0.05$ ), while *E. coli* levels from two outfalls draining the urban centre were not significantly different between flushes. Data on fecal coliform levels in combined sewer overflow samples (prior to discharge to river) and inline stormwater samples for 14 sites monitored during the study year demonstrated that considerable bacterial loads are delivered to the rivers, and subsequently into Lake Michigan, from both sources (Figure 3b).



**Figure 2** | *E. coli* levels in river samples: (a) Comparison of pre-storm (baseflow) and post-storm levels across all ten sites when there was no CSO discharge; (b) comparison of levels at river sites within the combined sewer system when there was only stormwater present vs. during a CSO event.



**Figure 3** | Fecal indicator concentrations in stormwater and CSO discharge measured in inline samples prior to release to the river. (a) In-line *E. coli* levels in first flush collected in the first 2 hours of a storm event vs. second flush collected 2–4 hours after the beginning of the event. (b) Comparison of fecal coliform levels in CSO discharge (CSO FC) and stormwater (SW FC) and *E. coli* levels in stormwater (SW EC) across 14 inline monitoring sites within the urban area. These data were compiled as a part of this study and by the Milwaukee Metropolitan Sewage District during the same period (please refer to Methods).

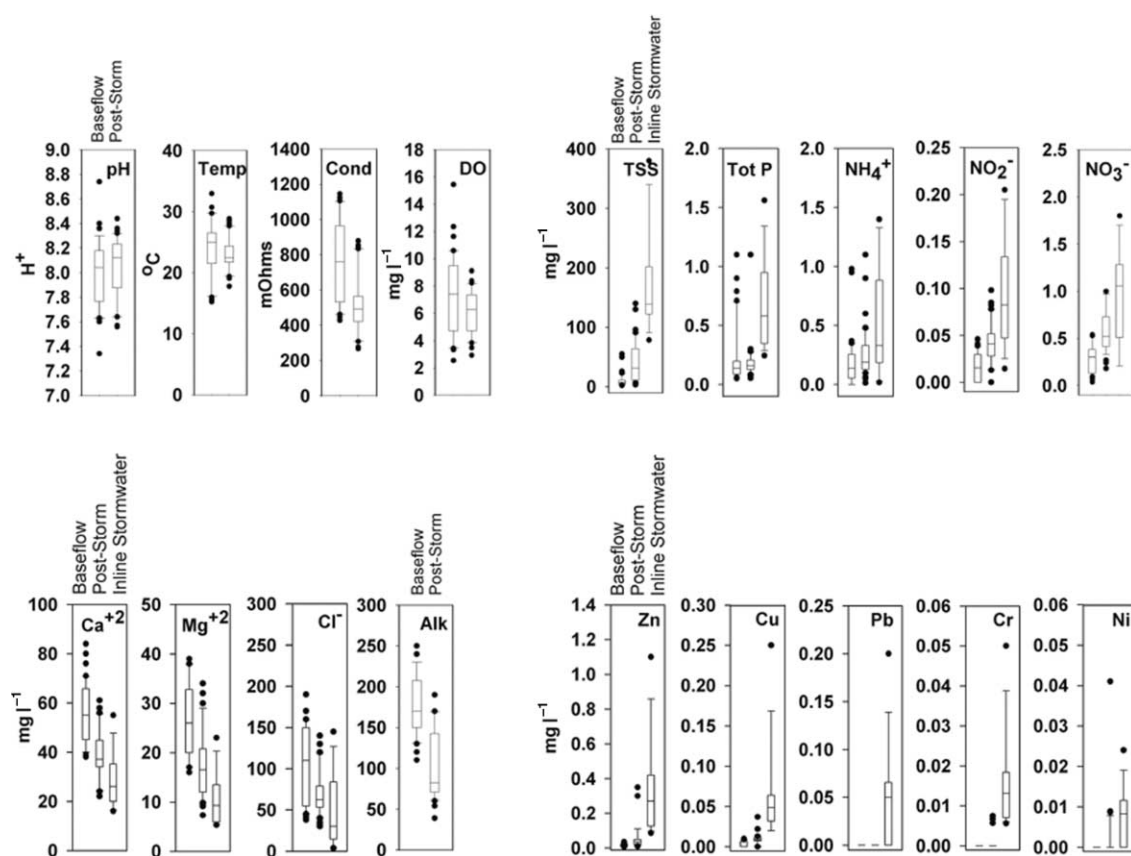
### Pre- and post-storm chemical analysis of river water

Chemical parameters averaged across all sites showed significant ( $p < 0.05$ ) differences between sample types:

for example, pre and post-storm river water and inline stormwater samples. Inline stormwater data collected over a 3-year period at 14 sites showed significantly higher levels of the metal contaminants chromium, copper, lead, nickel and zinc than pre- and post-storm river water samples ( $p < 0.05$ ); in contrast, magnesium and calcium were significantly lower,  $p < 0.05$  (Figure 4). Notably, chromium, lead and nickel were present at or below the level of detection in river samples. Inline stormwater levels of nutrients, including nitrite, ammonium nitrogen and total P were all significantly higher compared with pre-storm levels ( $p < 0.05$ ), while chloride and hardness demonstrated the opposite trend (Figure 4). Cadmium, arsenic, mercury, selenium and silver levels fell below the limit of detection in most samples and were not included in the statistical analyses.

Step-wise regression models were used to delineate a chemical signature that reflected non-point source loading of pollutants. Data provided by MMSD from 560 inline stormwater samples collected over a 2-year period, which included the study year, were used in the analyses. Overall, *E. coli* levels were significantly correlated with fecal coliform levels (as expected), nitrite and total P ( $p < 0.05$ ), but with no other parameter, indicating that urban runoff is highly variable in terms of the chemical and biological constituents, rather than just differences in the overall concentration of a uniform pollutant load. One possible explanation for this finding is that these analyses may be complicated by high *E. coli* levels that were not solely due to non-point runoff, but rather sanitary infiltration into the stormwater system. In order to identify parameters that may be co-ordinately elevated as a group regardless of *E. coli* levels, step-wise regression models were constructed using TSS as a dependent variable based on the assumption that TSS reflects the dilution of surface swept contaminants with rainwater. These analyses demonstrated that TSS was significantly correlated with chromium, total P, zinc and lead (all parameters,  $p < 0.0001$ ) and copper ( $p < 0.0194$ ).

The largest changes in nutrient and chemical parameters between pre- and post-storm samples were seen at sites 1–6, which correspond to the river sites that receive stormwater, in contrast to below site 6, where stormwater is



**Figure 4** | Comparison of Menomonee River water quality parameters in pre-storm samples under baseflow conditions (first box), post-storm event (second box), and inline stormwater (third box) samples. Temperature, pH, conductivity and dissolved oxygen (DO) were not measured in inline stormwater samples.

captured by the combined sewer system. Chemical and bacteria levels for inline stormwater from this study did not significantly differ from values recorded by MMSD for inline stormwater over a 3-year period across metropolitan Milwaukee (14 sites). This indicates that the 2002 dataset of the study site is consistent with stormwater pollutant loads in other parts of the metro area and may be representative of typical urban stormwater composition in areas of similar land use and development.

#### Antibiotic resistance patterns of *E. coli*

A total of 1,816 *E. coli* isolates from river water samples and 662 isolates from stormwater were tested for resistance to ten antibiotics and compared with the AR frequency found in isolates from known sources of fecal pollution (Table 1). *E. coli* isolates from sewage treatment plant influent (representing human sources) were found to

have the highest percentage of resistant strains for all ten antibiotics at all of the concentrations with rare exceptions. Chlorotetracycline was the only antibiotic of the ten tested that did not show significant differences between human and non-human sources ( $p < 0.05$ ). The proportion of strains resistant to multiple antibiotics was also highest for sewage isolates (Figure 5). In contrast, isolates from gulls (representing wildlife) had considerably fewer strains that were resistant to single or multiple antibiotics. Other host source isolates (dog, cow, pelican or raccoon) demonstrated the lowest numbers of strains with resistance to antibiotics, with no significant differences among host sources of this group ( $p < 0.05$ ); these isolates were categorized together as 'other host' for comparisons and statistical analysis. Only 39.8% of the sewage isolates were sensitive to all ten antibiotics, whereas 89.9% of the gull and 79.6% of other host isolates were sensitive (Figure 5).



**Table 1** | Antibiotic resistance frequency by host or environmental sample type

Antibiotic	Conc ( $\mu\text{g ml}^{-1}$ )	% of isolates resistant			% of isolates resistant			
		Sewage (n = 1,042)	Gull (n = 475)	Other <sup>1</sup> (n = 285)	Pre-storm river (n = 638)	Post-storm river (n = 1,009)	Post-storm river w/CSO (n = 169)	Inline stormwater (n = 662)
Ampicillin <sup>2</sup>	10	45	9	1	36	22	21	27
	20	28	7	0	12	13	12	17
Chlorotetracycline	12.5	24	16	4	24	9	14	41
	25	16	13	2	23	9	4	17
	50	6	5	1	4	4	4	10
Kanamycin	25	6	<0.5	0	<1	<1	1	<1
	50	5	<0.5	0	<1	<0.5	1	<1
	100	4	0	0	<0.5	<0.5	1	<1
Nalidixic Acid	12.5	9	<0.5	2	<0.5	<1	0	3
	25	7	<1	1	<0.5	<1	0	4
	50	6	<0.5	0	<1	<0.5	0	3
Neomycin	25	5	<0.5	0	<1	0.5	0	<1
	50	4	<0.5	1	<1	<0.5	0	<1
	100	3	0	0	<0.5	<0.5	0	<0.5
Oxytetracycline	12.5	23	8	1	5	6	14	22
	25	22	8	1	4	6	13	18
	50	21	7	1	4	5	13	17
Penicillin <sup>2</sup>	45	15	6	4	14	13	24	8
	90	16	6	0	9	10	21	5
Streptomycin	6.25	18	3	2	9	5	13	8
	12.5	16	2	0	3	4	11	7
	25	10	3	1	3	4	9	4
Sulfathiazole <sup>2</sup>	500	12	2	3	2	4	8	3
	1,000	8	1	<0.5	1	2	6	4
Tetracycline	12.5	19	6	2	5	7	20	19

Table 1 | Continued

Antibiotic	Conc (ug ml <sup>-1</sup> )	% of isolates resistant			% of isolates resistant			
		Sewage (n = 1,042)	Gull (n = 475)	Other <sup>1</sup> (n = 285)	Pre-storm river (n = 638)	Post-storm river (n = 1,009)	Post-storm river w/CSO (n = 169)	Inline stormwater (n = 662)
	25	19	5	2	4	6	14	18
	50	16	6	<0.3	4	5	13	14

<sup>1</sup>Other' host category includes dog, cow, pelican and raccoon.

<sup>2</sup>Low concentrations of antibiotics resulted in low intermediate growth across all isolates, indicating breakthrough growth with no resolution for resistant strains; therefore a third concentration was not used.

Shaded areas indicate that antibiotic resistance frequencies were not significantly different from sewage isolate antibiotic resistance frequencies (p < 0.05) using a two-sided binomial test of proportions.

The environmental isolates were divided into stormwater (from inline samples), river pre-storm, river post-storm, or river with known combined sewage overflow (CSO) groups for comparisons with host sources (Table 1). Stormwater isolates gave the highest percentages of antibiotic resistance among the environmental sample types, where 63.8% of stormwater isolates were resistant to at least one of the antibiotics compared with 47% of the isolates from CSO contaminated samples, which suggests possible contamination of the stormwater with sanitary sewage.

Multiple antibiotic resistance was found more frequently in stormwater isolates than the other environmental sample types, and was similar to the frequencies found in sewage isolates (Figure 5).

Stormwater isolates were assessed according to inline sampling site and date of collection. Isolates from one inline sampling site (n = 241) could account for elevated AR frequencies for eight of ten antibiotics; where 58%, 29%, 10%, 31%, 11%, 15%, 9%, 35% of isolates were resistant to the intermediate concentrations of ampicillin,

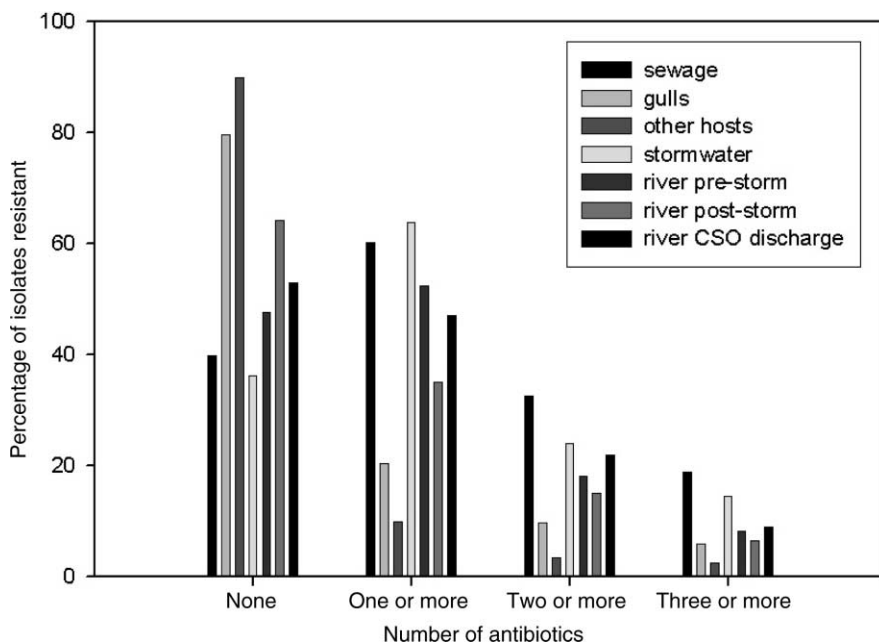


Figure 5 | Antibiotic resistance in host and environmental sample groups. The bars indicating percentage resistance for host and environmental isolates, from left to right, correspond to the figure legend designations from top to bottom.

chlorotetracycline, nalidixic acid, oxytetracycline, penicillin, streptomycin, sulfathiazole and tetracycline, respectively. The trends in *E. coli* levels from the inline samples did not coincide with AR frequencies of *E. coli* isolates; the stormwater outfall with elevated resistance traits ranged from 150,000 CFU to 250,000 CFU for four storms; however, the remaining outfalls ranged from 5,400 CFU 100 ml<sup>-1</sup> to equally elevated *E. coli* levels of 240,000 for the same four storms. These data suggest that bacterial counts alone would not be a good indicator for the presence of sanitary sewage; however, AR frequencies might be useful to investigate sanitary sewage contamination in stormwater systems.

The AR frequencies of river water isolates from pre- and post-storm samples across ten sites generally fell between the values for the sewage and gull host groups, which may be a product of a mixture of human and wildlife sources. Likewise, the occurrence of multiple resistance traits for pre- and post-storm samples and notably CSO contaminated samples fell between the sewage and gull groups (Figure 5). No differences in single and multiple AR were found between upstream and downstream sites, with the exception of the river sites during a CSO event (Tables 1 and 2). A higher percentage of antibiotic resistant strains were found across all sites in pre-storm samples compared with post-storm samples (Table 2).

Cluster analysis of multiple antibiotic resistance patterns was performed to determine if there was an

antibiotic resistance signature/s associated with sewage isolates that could serve as a marker for sewage contamination more specific than observing individual AR frequencies. Of the 3,800 isolates that were analysed, 782 demonstrated resistance to more than one antibiotic (distribution of these isolates across host and environmental source groups is shown in Figure 5). A total of 133 patterns (different combinations of resistance to two or more antibiotics) were observed. The major resistance pattern was ampicillin–penicillin and accounted for 18.5% of the isolates. The other common resistance patterns were ampicillin–chlorotetracycline–oxytetracycline–tetracycline (7.8% of isolates), chlorotetracycline–oxytetracycline–tetracycline (6.1% of isolates), ampicillin–chlorotetracycline (6.1% of isolates), and ampicillin–chlorotetracycline–penicillin (5.5% of isolates). The remaining patterns of multiple resistance occurred in less than 5% of the isolates and occurred infrequently, e.g. found in only one or two isolates. Isolates from the sewage group were intermixed with other groups for the majority of the patterns, and the number of host type isolates with a certain multiple AR pattern was proportional to the percentage of isolates from that host type that demonstrated resistance to multiple antibiotics. This suggests that the frequency of multiple resistance occurring in isolates may be indicative of sewage contamination, but distinctive patterns are not specifically associated with sewage. Using one or more of

**Table 2** | Antibiotic resistance frequencies of *E. coli* isolates in river samples in pre-storm samples compared with post-storm samples

Site	% of isolates sensitive*		% of isolates resistant to					
	Pre-storm	Post-storm	1 antibiotic*		2 antibiotics		3 or more antibiotics*	
	Pre-storm	Post-storm	Pre-storm	Post-storm	Pre-storm	Post-storm	Pre-storm	Post-storm
1–3	44.0	60.0	34.2	26.2	11.1	9.2	8.1	4.5
4	39.8	62.5	35.7	14.0	11.4	14.0	8.8	9.6
5	46.9	77.8	39.8	10.2	6.2	5.1	8.9	6.3
6	54.0	61.9	38.3	23.3	10.2	7.4	8.6	7.4
All sites	47.6	64.2	34.2	20.1	10.0	8.6	8.1	6.4

\*Values are significantly different between pre- and post-storm ( $p < 0.05$ ) by paired t-test across sites.

these patterns as a sewage indicator may not be useful since their overall occurrence was  $<0.01\%$  in sewage influent, and in most cases, not detected at all in water samples with known sewage contamination.

## DISCUSSION

Stormwater is a major threat to water quality in most US cities (USEPA 2000a). Contaminants are introduced into stormwater as rainwater washes across surfaces and accumulates metals, chemicals and bacteria. The pollutant loads entering the stormwater system are generally dependent on the sanitation in the area, such as street sweeping. In some cities, stormwater is highly managed and conveyed to a detention system for temporary or permanent holding. In other cases, stormwater might be conveyed through stormwater sewer systems and discharged directly to rivers or other receiving waters. The contamination of stormwater with sanitary sewage is also of concern; illicit cross connections or ageing infrastructure in older cities may result in unrecognized sanitary inputs (O'Shea & Field 1992). The Menomonee river system may be a good model for studying the impacts of stormwater on water quality since its features are common to many Great Lakes coastal urban areas, including mixed land use, ageing infrastructure, and both separate and combined sewer systems.

The bacteria levels observed in the Menomonee River during this study support the trend seen in other river systems, where the level of fecal coliforms and fecal streptococci observed during storm events reached orders of magnitude above the acceptable bacterial indicator levels for recreational waters (Bannerman *et al.* 1993; USEPA 2000a; Schiff & Kinney 2001). In considering the effects of the physical characteristics of the riverbed on bacterial loading and transport, it was expected that more bacteria would travel further in the lined, impervious concrete channels and that an accumulation would be observed as a gradient from upstream to downstream sites. We observed the opposite effect, where post-storm bacterial levels were lower in sites 7–10 than upstream sites, which is consistent with other studies in this riverine system (Razak & Christenson 2001). The downstream segment of the river corresponds to the beginning of the combined sewer system

(site 6), which captures stormwater so that it is not discharged to the river, and the confluence of the Milwaukee River (site 9), which carries large flow volumes. In addition, bacterial settling along the river may account for the general reduction in levels, as river sediments have been noted as reservoirs for *E. coli* and fecal coliforms (Solo-Gabriele *et al.* 2000; Desmarais *et al.* 2002; Byappanahalli *et al.* 2003). Similar studies have noted bacterial densities to be as high at the head of a watershed and the mouth of the channel, without appreciable contributions from sub-watersheds, or differences in residential, commercial or industrial land uses (Schiff & Kinney 2001). While reductions in *E. coli* levels were observed at sites within the combined sewer area for most sampling dates, this was not the case when there was a CSO event. However, during a CSO event, *E. coli* levels in the CSO area did not exceed levels found at upstream sites that were impacted only by stormwater.

Several chemical and nutrient parameters were found to be elevated in stormwater, and river water after storm events, compared with river water during baseflow conditions. No specific group of chemical and nutrient parameters was found to be co-ordinately elevated with *E. coli*, with the exception of nitrite and total P, which were significantly correlated ( $p < 0.05$ ) to *E. coli* in step-wise regression models. It is perhaps noteworthy that ammonia was also identified as a parameter that meets the significance level for entry into the model, which would suggest sanitary inputs (Baker *et al.* 2003), but did not demonstrate a strong correlation to the other parameters ( $F$  value = 3.75,  $p = 0.0534$ ). Interestingly, step-wise multiple regression models that did not consider *E. coli* levels as a dependent variable, showed that TSS was correlated with chromium, total P, zinc, lead and copper, but not ammonia. *E. coli* was correlated with these six parameters, but not above the 95% confidence interval ( $p = 0.10$ ). These data would indicate that two separate signatures might be present: stormwater with elevated *E. coli* resulting solely from non-point runoff, and stormwater that also contains contamination from sanitary sewage. That is, *E. coli* levels may be increased in some samples owing to sanitary inputs (e.g. cross connections or leaking infrastructure), accounting for high *E. coli* levels in the absence of specific chemicals. These findings suggest

that chemical parameters have a consistent signature for urban stormwater, but correlations between chemicals and *E. coli* levels may be inconsistent, particularly in cases where large *E. coli* loads are introduced into stormwater independent of chemical contaminants (e.g. sanitary inputs rather than impervious surface runoff).

The hypothesis that antibiotic resistant fecal indicator bacteria, such as *E. coli*, will be isolated more frequently from hosts that are subjected to antibiotics is supported by multiple studies (Parveen *et al.* 1997; Wiggins *et al.* 1999; Harwood *et al.* 2000) and can be accounted for by the mechanism of selective pressure on commensal intestinal bacteria. We compared 3,800 isolates to determine statistical parameters that would allow us to discern human sources of contamination from wildlife in an urban watershed. Notably, this study did not focus on agricultural animals, which were not considered as a possible source in the study watershed; however, agricultural animals may contribute antibiotic resistant fecal indicator bacteria in watersheds with farm land use (Hagedorn *et al.* 1999; Wiggins *et al.* 1999). There are reports of antibiotic resistance bacteria isolated from wildlife, but the AR frequencies were lower than what has been observed from human or sewage sources (Guan *et al.* 2002). These procedures may be limited by the degree of uncertainty in the classification of isolates in large watersheds that have multiple sources of contamination (Harwood *et al.* 2000; Field *et al.* 2003a).

In this study, we did not rely on discriminant or similarity analysis (e.g. library based comparisons) to differentiate specific sources, but rather statistical probability. Discriminant analysis is useful when there are adequate 'discriminating' characteristics, and this approach appears to be fairly reliable for classifying isolates using library-based comparisons that are representative of watershed sources (Wiggins *et al.* 2003). Within our isolate group, almost 30% of the sewage isolates and 80–90% of the non-human isolates (wildlife) were sensitive to all antibiotics. Because a high number of isolates could not be compared since they did not have an AR trait, we utilized a statistical probability approach similar to Parveen *et al.* (1997). This approach is based on the underlying hypothesis that higher frequencies of AR would be found in sewage compared with wildlife hosts, which is supported by our host data. In this

study, the percentages of sewage isolates resistant to ampicillin, streptomycin, neomycin, kanamycin, tetracycline and sulfathiazole, were similar to what has been reported previously (Parveen *et al.* 1997). Results from other studies differed for certain antibiotics: Reinthaler *et al.* (2003) report resistant frequencies for sewage isolates as ampicillin 18%, nalidixic acid 15% and tetracycline 57%. These differences may be accounted for by differences in methods; the disc diffusion method was employed rather than the agar dilution plate method that was used in this study. These frequencies, as well as the patterns of multiple antibiotic resistance, will most likely need to take into account the geographic region, the antibiotic usage in the human population, or suspected sources in the watershed (Harwood *et al.* 2000). Recent comparison studies of different microbial source tracking methods identify a need to standardize methods (Stewart *et al.* 2003).

Overall, the AR frequency of stormwater isolates was not as high as that found in sewage, but elevated AR frequencies for some of the antibiotics were suggestive of sewage contamination. River water isolates that were collected following a CSO event serve as a benchmark of the expected frequencies when stormwater is contaminated with human and non-human sources (Table 1). The AR frequencies of *E. coli* may be a composite of two or more sources of contamination and these relationships might be observed in the trends rather than statistical significance. This was clearly illustrated in further breaking down the stormwater data; one outfall was found to have antibiotic resistance bacteria at levels equal to that found in sewage. *E. coli* levels alone would not have identified this outfall as a potential source of sanitary sewage.

Samples collected prior to storm events, e.g. under baseflow conditions, demonstrate low, but not absent *E. coli* levels, which rose significantly ( $p < 0.05$ ) after storm events. In post-storm river samples, the proportion of antibiotic resistant bacteria decreased. This suggests that residual, persistent contamination in rivers contains human sources, whereas following storm events there is a disproportionate increase in contamination from non-human sources. Other studies in urban watersheds have noted that when fecal coliform levels were low, or during baseflow conditions, the primary source was human (Whitlock *et al.* 2002; Burnes 2003). However, given the overall magnitude

of the increase in *E. coli* levels following storm events, which rose up to three orders of magnitude at some sites, large amounts of bacterial loading from non-human sources may mask the contamination from human sources (Whitlock *et al.* 2002). While there is evidence of sanitary sewage contamination, non-point sources of fecal indicator bacteria appear to be major contributors to the *E. coli* levels observed in this system. Antibiotic resistance testing may prove useful for investigating discharge points to waterways prior to mixing with river water, where inputs from multiple sources may make differences less distinct. In addition, this approach may be attractive as it ties microbial source tracking to the same indicator of pollution.

## CONCLUSIONS

This study demonstrates how stormwater delivers heavy metals, chemicals and fecal indicator bacteria into receiving waters and can be a major contributor to degraded water quality in urban rivers. Further, these findings illustrate that stormwater impacts can equal those of combined sewer overflows in terms of fecal bacteria loading.

Importantly, these data provide evidence for human sources of fecal contamination in stormwater in the absence of recognized combined sewer or sanitary sewer overflows. High *E. coli* levels alone did not suggest sanitary inputs; however, elevated *E. coli* levels with frequent antibiotic resistance among isolates appeared to serve as a signature for potential sanitary inputs. Several chemical parameters were co-ordinately elevated in stormwater, suggesting a chemical signature, but the relationship of this signature to elevated *E. coli* was difficult to discern in stormwater samples with suspected sanitary contamination. While the health risk due to non-point source urban runoff, i.e. stormwater, remains under-characterized, sanitary sewage contamination of stormwater systems has been cited as a serious health risk. Discerning sources of fecal pollution will require a multifaceted approach, as no single chemical or biological measure is expected to differentiate human vs. non-human fecal pollution. Overall, the antibiotic resistance data suggest that there is sanitary sewage contamination in stormwater systems, and this method may be useful for screening outfalls prior to discharge to rivers.

Since the stormwater outfall sites were chosen for location, rather than for suspected sanitary infiltration, it is unlikely that this occurrence is an isolated incident and, therefore, it would be of interest to evaluate stormwater discharges for sanitary sewage contamination that is unrecognized and therefore unreported.

## ACKNOWLEDGEMENTS

We thank M. Tulod, J. Harris, M. Luebke and E. Lewis for field and laboratory assistance, and H. Bootsma and anonymous reviewers for their comments during the preparation of this manuscript. This project was made possible through logistical support and in kind services for chemical analyses from the Milwaukee Metropolitan Sewerage District and through a grant from the Charles A. and Anne Morrow Lindbergh Foundation.

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