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Sewage pollution in urban stormwater runoff as evident from the widespread presence of multiple microbial and chemical source tracking markers

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HIGHLIGHTS

- Presence of multiple MST and CST markers suggests ubiquitous sewage contamination.
- MST and CST markers suggest ubiquitous sewage contamination in urban environment.
- Good consensus (>80%) between the occurrence of MST and CST markers
- HF183 had high concurrence with human adenovirus and acesulfame.

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ABSTRACT

The concurrence of human sewage contamination in urban stormwater runoff ($n = 23$) from six urban catchments across Australia was assessed by using both microbial source tracking (MST) and chemical source tracking (CST) markers. Out of 23 stormwater samples human adenovirus (HAv), human polyomavirus (HPv) and the sewage-associated markers; *Methanobrevibacter smithii nifH* and *Bacteroides* HF183 were detected in 91%, 56%, 43% and 96% of samples, respectively. Similarly, CST markers paracetamol (87%), salicylic acid (78%) acesulfame (96%) and caffeine (91%) were frequently detected. Twenty one samples (91%) were positive for six to eight sewage related MST and CST markers and remaining two samples were positive for five and four markers, respectively. A very good consensus (>91%) observed between the concurrence of the HF183, HAv, acesulfame and caffeine suggests good predictability of the presence of HAv in samples positive for one of the three markers. High prevalence of HAv (91%) also suggests that other enteric viruses may also be present in the stormwater samples which may pose significant health risks. This study underscores the benefits of employing a set of MST and CST markers which could include monitoring for HF183, adenovirus, caffeine and paracetamol to accurately detect human sewage contamination along with credible information on the presence of human enteric viruses, which could be used for more reliable public health risk assessments. Based on the results obtained in this study, it is recommended that some degree of treatment of captured stormwater would be required if it were to be used for non-potable purposes.

1. Introduction

Urban stormwater can be used to augment non-potable and potable water supplies within cities and other urban areas (Sidhu et al., 2012). However, stormwater may also contain a variety of chemicals, metals and fecal material of human and animal origin. There are several impediments to reuse of stormwater for non-potable and potable purposes in urban residential areas. The most significant issue appears to be associated with the presence of pathogens in stormwater, potentially originating from human sewage contamination (Cizek et al., 2008; Noble et al., 2006; Rajal et al., 2007; Sauer et al., 2011; Sercu et al., 2009).

There is a growing evidence that stormwater conveyance networks can be contaminated with sewage due to failing sewer infrastructure and cross connections between stormwater and sewage networks (Noble et al., 2006; Rajal et al., 2007; Sercu et al., 2009).

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Exposure to stormwater runoff impacted waters has been linked to increased risk of gastrointestinal (GI) diseases (Curriero et al., 2001; Gaffield et al., 2003). Human health risk assessment and remediation strategies for microbial contamination from stormwater can be more effectively implemented if sources of contamination are known.

Traditional fecal indicator bacteria (FIB) such as *Escherichia coli* and *Enterococcus* spp. are routinely monitored to assess the microbiological quality of surface waters, however, the presence of FIB does not necessarily correlate with the presence of pathogens especially viral and protozoan pathogens (Horman et al., 2004; McQuaig et al., 2009; Selvakumar and Borst, 2006). Furthermore, monitoring for the FIB numbers in stormwater does not provide definitive information on the possible sources of contamination which is a major shortcoming of such evaluations. As identification of sources of pollution is difficult, microbial source tracking (MST) and chemical source tracking (CST) methods have been developed and used to discriminate between human and non-human sources of fecal contamination in environmental waters (Glassmeyer et al., 2005; Nakada et al., 2008; Parker et al., 2010; Sauer et al., 2011).

MST methods based on polymerase chain reaction (PCR) can be used to detect the presence of specific genes associated with certain groups of bacteria (Bernhard et al., 2003; Scott et al., 2005) or viruses (Fong et al., 2005; McQuaig et al., 2009) from human and animal hosts. PCR based methods have been successfully used for the detection of sewage-associated *Bacteroides* HF183 and *nifH* markers in surface waters (Ahmed et al., 2012b; Sercu et al., 2011; Seurinck et al., 2005; Ufnar et al., 2006). Human adenovirus (HAv) and human polyomavirus (HPv) are known to be highly prevalent (10^2 to 10^5/l) in sewage contaminated surface waters (Hamza et al., 2009; Muscillo et al., 2008; Sauer et al., 2011) and due to their stringent host specificity they are considered as most accurate MST markers (Ahmed et al., 2012b; Fong et al., 2005; Wong et al., 2012). In addition, viral MST assays provide more reliable information on potential health risks from water resources.

During the past decade, a number of studies have extensively surveyed the prevalence of pharmaceuticals and personal care products in sewage effluent and aquatic environments (Benotti and Brownawell, 2007; Clara et al., 2004; Duan et al., 2013; Glassmeyer et al., 2005; Nakada et al., 2008; Verlicchi et al., 2012). A number of CST markers including fecal sterols/stanols (Gregor et al., 2002), caffeine (Buerge et al., 2003; Heberer et al., 2002), and artificial sweeteners (Nakada et al., 2008; Scheurer et al., 2011) have also been suggested as specific sewage markers. Persistent markers such as acesulfame are reported to be useful for tracing the pathways of treated sewage, whereas, biodegradable compounds such as caffeine are indicators of untreated wastewater ingress into fresh water (Buerge et al., 2006).

Each of the MST and CST marker described in the literature to date has advantages and disadvantages (Hagedorn and Weisberg, 2009; Scott et al., 2002). These limitations include inadequate host specificity, lack of prevalence of the markers in host groups, lack of temporal and geographical stability and their environmental persistence. The consequence of inaccurate source tracking based on false positive results (if a non-specific marker is used) may lead to expensive infrastructure improvements that may not actually improve the water quality in question. MST analysis approach involving several markers is reported to improve the accuracy of identification of polluting sources (Ahmed et al., 2012a; Boehm et al., 2003; Mauffret et al., 2012; Noble et al., 2006). To date, most published studies on the characterizing of fecal contamination in stormwater are limited to MST markers (Noble et al., 2010; Sauer et al., 2011; Sidhu et al., 2012; Surbeck et al., 2006). Only a few studies have evaluated the advantages of using both MST and CST markers for the assessment of human sewage contamination in surface water (Blanch et al., 2006; Gourmelon et al., 2010; Litton et al., 2010; Peeler et al., 2006; Sauve et al., 2012). Application of a set of markers may provide additional information such as confidence in source identification, differentiation between recent and prior sewage contamination events and accurate health risk assessments which are vital from a regulatory point of view.

In our previous study (Sidhu et al., 2012), human-specific HF183 *Bacteroides* marker was detected in most of the stormwater samples collected from Brisbane, Australia suggesting ubiquitous sewage contamination in the urban environment. This study was carried out to determine if the contamination of stormwater runoff with sewage is limited to sub-tropical Brisbane or is a broader issue in urban catchments in other major cities across Australia. Stormwater samples were collected from six residential and commercial catchments in Brisbane, Sydney and Melbourne were assessed by using a set of sewage associated MST and CST markers. The MST markers investigated included both bacterial and viral markers. The human specific *Bacteroides* HF183 and *Methanobrevibacter smithii nifH* were tested to detect presence of human origin fecal pollution. The enteric viruses, HAv and HPv were also tested due to their specificity as MST markers and as index virus for the presence of other human enteric viruses. The chemical markers proven as useful indicators of anthropogenic sewage pollution (Glassmeyer et al., 2005) including readily biodegradable (caffeine, paracetamol, and salicylic acid) and recalcitrant marker acesulfame were also tested.

The specific aims were to determine; (i) the frequency of occurrence of sewage pollution in stormwater runoff in urban catchments across Australia; (ii) to assess the efficacy of using a set of MST and CST markers for differentiation between recent and prior contamination event; (iii) to determine the concurrence of HAv and HPv in stormwater runoff. This was done with an aim to improve understanding of the extent of potential health risks associated with reuse of stormwater for non-potable and potable purposes.

2. Materials and methods

2.1. Stormwater sampling sites

The studied catchments differ with respect to the size of their drainage area, impervious area and land use. A brief site description and potential sources of contamination in the six catchments is presented in Table 1. Three catchments, Fitzgibbon (north of Brisbane), Banyan Creek (south of Melbourne) and Ku-Ring-Gai (north of Sydney), represented medium density residential catchments covering total area of 290 ha, 235 ha and 8.9 ha, respectively. The impervious surface coefficient was estimated by using an image classification and cadastral filtering of high-resolution visible aerial photography method and was determined to be 30–39%. The remaining three sites, Makerston Street (Brisbane), Hornsby (Sydney), and Smith Street (Melbourne) are located in high density commercial areas. The Makerston Street catchment covers a total area of 30 ha, Hornsby 1.1 ha and Smith Street 23 ha. Impervious area in these catchments was determined to be ≈90%. Site specific rainfall data was collected from the Australian Bureau of Meteorology (BOM) website which varied across catchments from 5.8 to 82 mm depending upon storm intensity (Table 3).

2.2. Stormwater sampling strategy

Multiple stormwater samples were collected from each of the six sampling sites after the storm events on multiple occasions. On each sampling occasion, volume proportional composite samples were taken using automated sampling infrastructure (ISCO 6700 or equivalent) triggered by automated flow measurement (either using a Doppler flowmeter or a weir, depending on site characteristics). The automatic samplers were programmed to the site specific requirements, overall allowing to reliably determined event mean concentrations via composite samples. These samplers were programmed to fill up to 20 l high density polyethylene containers (HDPE) (Food and Drug approved grade) during a storm event which were then mixed to obtain a composite

Table 1
Stormwater sites and brief description of land use and potential sources of contamination.

Sites	GPS coordinates	Land use	Total area (ha)	Impervious area (%)	Potential source of fecal contamination
Fitzgibbon Drain, Brisbane	27°20′08″S; 153°01′14″E	Residential, large blocks	290	30	Sewage pipe network, pets, water fowls, bird, rodents, small numbers of horses, sheep and cattle
Makerston Street, Brisbane	27°28′2.4″S; 153°1′4.5″E	City, commercial	30	>90	Sewage pipe network, birds and rodents
Hornsby, Sydney	33°42′6.6″S; 151°5′50.1″E	City roads/commercial	1.1	87	Sewage pipe network, birds and rodents
Banyan Reserve, Melbourne	38°5′44.4″S; 145°10′58.3″E	Residential with a small percentage of commercial precincts (<5%)	235	35	Sewage pipe network, pets, birds, possums and rodents
Smith Street, Melbourne	37°47′47.9″S 144°59′4.6″E	Commercial with around 20% high density residential developments	23	≈90	Sewage pipe network, birds and rodents
Ku-Ring-Gai, Sydney	33°44′53.9″S; 151°6′58.9″E	Residential, site close to oval	8.8	39	Sewage pipe network, pets, birds and rodents

sample. To avoid cross-contamination, stormwater collection containers, were cleaned using sodium hypochlorite solution (1%) and then rinsed with ultra-pure water (MilliQ system, Millipore) in the laboratory before replacing the used containers at the field sites.

Stormwater samples ($n = 23$) were collected after multiple storm events from six sites around Australia: Fitzgibbon (FG) Drain ($n = 5$) and Makerston Street (MA) ($n = 5$) in Brisbane, Hornsby Site (HN) ($n = 5$) and Ku-Ring-Gai (KG) ($n = 2$) in Sydney and Banyan Creek (BA) ($n = 4$) and Smith Street (SM) ($n = 2$) in Melbourne. Most of the stormwater samples were collected from the stormwater drains which flow during the wet period. Background samples that were collected from the FG and MA sites and tested for the presence of HF 183 marker were found to be negative for the presence of this sewage contamination marker (data not shown). Approximately, 20 l of composite sample was used for microbiological analysis from each site, whereas, 1 l sample was used for chemical analysis. The collected stormwater samples were stored at 4 °C, and shipped to the laboratories in Brisbane on ice for analysis.

2.3. Sample processing for microbial analysis

Samples were concentrated within 24 h of collection by using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA, USA) as previously described by Hill et al. (2005). Briefly, the water sample to be concentrated was pumped with a peristaltic pump (Masterflex: Cole Parment Instrument Co, USA) in a closed loop with high-performance, platinum-cured L/S 36 silicone tubing (Masterflex; Cole Parmer Instrument Co., USA). In between sampling events, tubing was cleaned and disinfected by soaking in 1% sodium hypochlorite solution followed by washing and then sterilized by autoclaving. At the end of the concentration process, pressurized air was passed through the filter cartridge from the top to recover as much water as possible. The samples were concentrated to approximately 100 ml and further concentration of sample was carried out by JumboSep with 100 K MWCO filters (Pall, Australia) to a final concentration of approximately 10 ml (Sidhu et al., 2012).

2.4. Quantification of fecal indicator bacteria (FIB)

Quantification of FIB (*E. coli* and *Enterococcus* spp.) was performed by the membrane filtration technique (Sidhu et al., 2012). Briefly, 1 and 10 ml samples were filtered through 0.45 μm nitrocellulose (Millipore) filters (47 mm) and placed on respective selective agar plates in triplicate. *E. coli* was enumerated on Chromocult™ coliform agar (Merck, Germany) and *Enterococcus* spp. on Chromocult™ enterococci agar (Merck). Plates were incubated at 37 °C overnight and then typical colonies were counted to determine the average number of colony forming units (CFU) 100/ml of water.

2.5. Detection of MST markers

Nucleic acid was extracted from 200 μl of each concentrated sample using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) as per manufacturer instructions, and stored at −80 °C until processed. Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Australia) was used to measure DNA concentration in extracted samples. A 10 and 100 fold dilutions of extracted nucleic acid were prepared in MilliQ water prior to PCR with universal bacteria primer (Stoll et al., 2012) to detect the presence of PCR inhibition. The dilutions which retuned the lowest threshold cycle (C_t values) were used for the real time qualitative PCR assays.

A real-time PCR assays were performed for the detection of the HF183, *nifH*, HAv and HPv using previously published primers and probes (Table 2). HAv and HPv amplifications were performed in 25 μl reaction mixtures using iQ Supermix (Bio-Rad Laboratories). The PCR mixture contained 12.5 μl of Supermix, 400–500 nM each primer, 400–600 nM corresponding probe and 3 μl of template DNA. The *nifH* and HF183 gene amplifications were performed in 20 μl reaction mixtures using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, CA, USA). The PCR mixture contained 10 μl of Supermix, 300–400 nM each primer, DNase and RNase-free deionized water, and 3 μl of template DNA. Bovine serum albumin (BSA) was added to each reaction mixture to a final concentration of 0.2 μg/μl to relieve PCR inhibition (Kreader, 1996). The cycling parameters are shown in Table 2. The PCR was performed using the

Table 2
Primers and probes used in this study.

Sewage-associated markers	Primers and probes (5′–3′)	Cycling parameters	Reference
Methanobrevibacter smithii nifH	F: AAC AGA AAA CCC AGT GAA GAG R: ACG TAA AGG CAC TGA AAA ACA	92 °C for 2 min, 40 cycles of 92 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min	Ufnar et al. (2006)
Bacteroides HF183	F: ATC ATG AGT TCA CAT GTC CCG R: TAC CCC GCC TAC TAT CTA ATG	95 °C for 10 min, 45 cycles 95 °C for 30 s, 53 °C for 1 min, and 60 °C for 1 min.	Seurinck et al. (2005)
Adenovirus (HAv)	F: GCC ACG GTG GGG TTT CTA AAC TT R: GCC CCA GTG GTC TTA CAT GCA P: FAM TGC ACC AGA CCC GGG CTC AGG AGG TAC TCC GA BHQ1	10 min at 95 °C, 50 cycles of 15 s at 95 °C and 20 s at 60 °C and 20 s at 72 °C	Heim et al. (2003)
Polyomavirus (HPv)	F: SM2 AGT CTT TAG GGT CTT CTA CCT TT R: P6 GGT GCC AAC CTA TGG AAC AG P: KGJ3 (FAM)-TCA TCA CTG GCA AAC AT-(MGBNFQ)	10 min at 95 °C, 50 cycles of 15 s at 95 °C and 20 s at 55 °C and 60 s at 60 °C	McQuaig et al. (2006, 2009)

Bio-Rad iQ5 thermal cycler (Bio-Rad Laboratories). For each PCR experiment, positive controls (e.g., corresponding plasmid or genomic DNA) and negative control (e.g., sterile water) were included.

2.6. Detection of CST markers

For CST marker analysis, 1 l of composite stormwater sample was processed through a 1.2 μm GF/C filter (Whatman, GE Healthcare Pty Ltd, Australia). Chemical analysis of micro-pollutants was an adaptation of US EPA method 1694 implemented by the Queensland Health Forensic and Scientific Services (EPA, 2007). Two aliquots of aqueous sample were extracted on solid phase extraction (SPE) cartridges (Phenomenex StrataX, 200 mg/3 ml), one acidified with hydrochloric acid and without pH adjustment, using a Gilson Aspec SPE system. Shimadzu UFLC chromatographic system equipped with a Phenomenex C18 Luna column coupled to an Applied Biosystems 4000QTrap® LC/MS/MS was used for detection. Internal standards and stable isotope surrogates were used for quantification. Samples with paracetamol and salicylic acid concentrations below the level of reporting (LOR) were considered as negative.

2.7. Data analysis

Prior to statistical analysis, data from all six catchments was grouped under two categories, predominantly residential (Fitzgibbon Drain, Banyan Creek and Ku-Ring-Gai) and commercial (Makerston Street, Hornsby and Smith Street). Pearson's correlation (r_p) was used to test the relationship between *E. coli* and *Enterococcus* spp. numbers in the stormwater samples. Data on *E. coli* and *Enterococcus* spp. numbers was log transformed prior to statistical analysis. A binary logistic regression analysis was performed to confirm existence of any correlation between the presence of FIB numbers and MST and CST markers (Minitab version 16, Minitab Inc., State College, PA) (Ahmed et al., 2012b). Statistical significances of the results were determined by applying a Student's *t*-test to the FIB numbers and CST marker concentrations between residential and commercial catchments. Prior to *t*-test, the FIB numbers were $\log_{10}$ transformed. The critical *P*-value for the test was set at 0.05. The null hypothesis was accepted if the *P* value was greater >0.05 and compared data was considered to be not significant.

Baye's Theorem was used to calculate the conditional probability that the detection of the HF183 and *nifH* markers in the stormwater samples originated from sewage or other sources of human feces rather than feces from the non-target host-groups that may occasionally contain the HF183 and *nifH* markers. The following equation was used to calculate the conditional probability (Kildare et al., 2007; Weidhaas et al., 2011).

$$P(H\backslash T) = \frac{P(T\ H)P(H)}{P(T\ H)P(H) + P(T\ H')P(H')}$$

$P(H\backslash T)$ is the probability (P) of human fecal contamination (H) in a water sample given a positive test result (T) for the sample.
$P(T\backslash H)$ is the true positive.
$P(H)$ is the background probability of detecting a sewage marker in a water sample.
$P(T\backslash H')$ is the false positive.
$P(H')$ is the background probability that a sewage marker was not detected in a water sample. The value of $P(H')$ is $1 - P(H)$.

The concurrence of MST and CST markers was compared pair-wise. The percentage of total concurrence was calculated by adding the percentage of concurrence (when two pair-wise markers were present) and non-concurrence (when two pair-wise markers were absent) for each pair-wise comparison.

3. Results

3.1. FIB numbers in collected water samples

The numbers of FIB in water samples collected after the storm event ranged from 4×10^1 to 7×10^3 CFU/100ml for *E. coli*, and from 1×10^3 to 3×10^4 CFU 100/ml for *Enterococcus* spp. (Table 3). A total of 48% stormwater samples had *Enterococcus* spp. numbers more than 1×10^4 CFU/100ml. There was no correlation ($P = 0.044$, $r_p = 0.24$) found between *E. coli* and *Enterococcus* spp. numbers. The numbers of *Enterococcus* spp. were generally ten-fold higher than *E. coli* across all sites. *E. coli* and *Enterococcus* spp. numbers from commercial and residential catchments did not differ significantly ($P > 0.05$) from each other.

3.2. Prevalence of MST markers

Among six stormwater sites tested, all sites (100%) were positive for sewage associated markers. Among 23 stormwater samples collected from all sites, eight samples (34%) were positive for all four MST markers, five samples (22%) were positive for three markers and nine samples (39%) were positive for two markers. None of the MST markers could be detected from one out of five samples collected from Banyan Creek (Table 3). *Bacteroides* HF183 was most frequently detected in 96% of stormwater samples whereas, the *nifH* gene marker was detected in 43% of samples only (Fig. 1). HAv had higher prevalence (91%) in the collected stormwater samples compared to HPv (56%).

Baye's Theorem was used to estimate the conditional probability of accurately detecting sewage contamination in storm water samples for the HF183 and *nifH* markers since these markers were detected in fecal samples from non-target host-groups in South East Queensland (Ahmed et al., 2012b). The background probabilities, $P(H)$, of detecting the HF183 and *nifH* markers in the storm water samples, therefore, were 0.96 and 0.43. The background probability that these markers were not detected in the stormwater samples were $1 - P(H)$, or 0.04 (for the HF183 marker) and 0.57 (for the *nifH* marker). $P(T\backslash H)$ is the true-positive rate of the assays and the values were calculated from the host-sensitivity assays as reported in previous studies (Ahmed et al., 2012a, 2012b). The values were 0.99 and 0.81 for the HF183 and *nifH* markers. $P(T\backslash H')$ is the false-positive rate of the assays and the values were calculated from the host-specificity assays in our previous studies. The values were 0.03 and 0.04 for the HF183 and *nifH* markers, respectively. Based on the concurrence and non-concurrence results of the HF183 and *nifH* markers in the stormwater samples and fecal samples from target and non-target host-groups, there was a 99% probability that the detection of the HF183 marker in a stormwater sample was due to the true sewage contamination and not from non-target hosts. Similarly, there was a 94% probability that the detection of the *nifH* marker in a stormwater sample was due to the true sewage contamination and not from non-target hosts.

3.3. Prevalence of CST markers

Among the 23 stormwater samples tested from six sites, 22 (96%), 21 (91%), 20 (87%), and 18 (78%) of samples were positive for acesulfame, caffeine, paracetamol and salicylic acid, respectively (Fig. 1). Acesulfame was most frequently detected in 96% of stormwater samples whereas, caffeine was detected in 91% of samples (Fig. 1). Paracetamol had higher prevalence (91%) compared to salicylic acid which was detected in 78% of stormwater samples. Caffeine had the highest concentration among all the CST markers ranging from below 0.01 (level of reporting, LOR) to 5.20 μg/l which was followed by acesulfame ranging from below 0.01 (LOR) to 0.23 μg/l. Among the pharmaceuticals, paracetamol had the highest concentration ranging from below

Table 3
Fecal indicator bacteria numbers and sewage associated markers detected in stormwater samples collected from six catchments in Australia.

Sites	Rainfall (mm)	FIB counts[a]		Microbial markers				Pharmaceuticals[b]		Food markers[b]	
		E. coli	*Enterococcus* spp.	HF183	*nifH*	Adenovirus	Polyomavirus	Paracetamol	Salicylic acid	Acesulfame	Caffeine
FG1	16	4.73×10^3	1.75×10^4	+	+	+	+	0.08	0.10	0.04	<LOR
FG2	40.4	3.60×10^3	1.67×10^4	+	−	+	+	0.02	0.10	0.03	0.03
FG3	48	1.03×10^3	1.08×10^3	+	+	+	−	0.08	0.10	0.04	<LOR
FG4	22.6	3.56×10^3	1.18×10^4	+	−	+	+	0.06	0.60	0.03	0.09
FG5	44.4	1.17×10^3	1.43×10^3	+	−	+	−	<LOR	0.10	0.07	0.10
MA1	72.4	6.66×10^3	1.80×10^3	+	−	+	−	0.03	<LOR	0.03	0.27
MA2	17.2	4.57×10^3	4.10×10^3	+	+	+	+	<LOR	<LOR	0.11	5.20
MA3	7.4	6.07×10^3	1.27×10^3	+	−	+	+	<LOR	<LOR	0.03	0.06
MA4	20.0	3.60×10^3	5.56×10^3	+	+	+	+	0.02	0.10	0.16	1.10
MA5	38.4	3.00×10^2	1.17×10^3	+	+	+	+	0.13	0.10	0.10	1.10
HN1	5.8	5.90×10^3	2.58×10^4	+	+	+	+	0.05	0.20	0.09	1.80
HN2	12	5.90×10^3	2.95×10^4	+	−	+	+	0.05	0.20	0.04	0.30
HN3	82	1.00×10^2	1.12×10^4	+	−	+	−	0.02	<LOR	0.09	1.80
HN4	21.4	2.00×10^2	1.12×10^4	+	−	+	−	0.03	0.30	0.05	0.70
NH5	14.6	4.00×10^1	1.93×10^3	+	−	+	−	0.09	0.10	0.07	2.50
BA1	11.6	3.40×10^3	1.02×10^4	+	+	+	+	0.05	<LOR	0.04	0.30
BA2	NR	1.10×10^3	1.37×10^3	−	−	−	−	0.03	0.10	0.06	0.38
BA3	9.8	7.20×10^3	2.26×10^4	+	+	+	+	0.11	0.10	0.05	0.43
BA4	9.4	1.00×10^3	1.00×10^4	+	−	+	−	0.05	0.10	0.07	0.31
SM1	39.4	6.56×10^3	1.52×10^4	+	+	+	+	0.20	0.10	0.23	3.00
SM2	15.8	8.93×10^2	7.90×10^3	+	−	+	−	0.14	0.10	0.17	1.70
KG1	17.2	9.00×10^2	9.93×10^3	+	−	+	−	0.02	0.10	0.03	0.03
KG2	NR	6.40×10^3	3.64×10^3	+	+	−	−	0.02	0.10	<LOR	0.14

NR = not recorded, FG = Fitzgibbon Drain, MA = Makerston Street, HN = Hornsby Site, BA = Banyan Reserve, SM = Smith Street KG = Ku-Ring-Gai. LOR (limit of reporting) for caffeine, acesulfame and salicylic acid was 0.01 μg/l, whereas, paracetamol was 0.02 μg/l.
[a] CFU 100/ml.
[b] μg/l.

0.02 (LOR) to 0.2 μg/l of water. Salicylic acid concentration varied from below 0.1 (LOR) to 0.60 μg/l (Table 3). Student's *t*-test was applied to compare the concentration of CST markers between commercial and residential catchments to determine if the prevalence of CST markers was significantly different (Table 4). Concentration of caffeine was significantly higher ($P < 0.05$) in the commercial catchments compared to residential catchments. Whereas, the differences in the concurrence of other CST markers tested in this study were statistically not significant ($P < 0.05$). A moderate correlation between caffeine and acesulfame ($P = 0.001$, $r_p = 0.64$) was observed whereas, there were no correlations among other CST markers tested.

3.4. Concurrence between MST and CST markers

The concurrence of FIB, MST and CST markers was compared pair-wise for all the stormwater samples. The percentage of total concurrence was calculated by adding the percentage of concurrence and non-concurrence for each pair-wise comparison. MST marker HF183 had high concurrence with HAv (96%) and acesulfame (92%) as shown in Table 5. Similarly, CST marker caffeine had high concurrence with HF183 and acesulfame (87%) and HAv (83%). Both acesulfame and HAv had the highest concurrence of 96%. Paracetamol and salicylic acid also had 87% concurrence in stormwater samples. The HF183 had the highest total concurrence (76%) with other sewage contamination markers followed by HAv (76%) whereas, HPv and *nifH* markers had a total concurrence of 58% and 46% respectively with the seven other markers. Acesulfame, paracetamol and caffeine had a good concurrence of 75%, 71% and 70%, respectively. A binary logistic regression was used to identify whether any correlation existed between the numbers of FIB and the presence/absence of results for sewage-associated MST and CST markers. The presence/absence of the BFA markers sewage markers was found to not correlate with the FIB numbers (Supplementary Table S1).

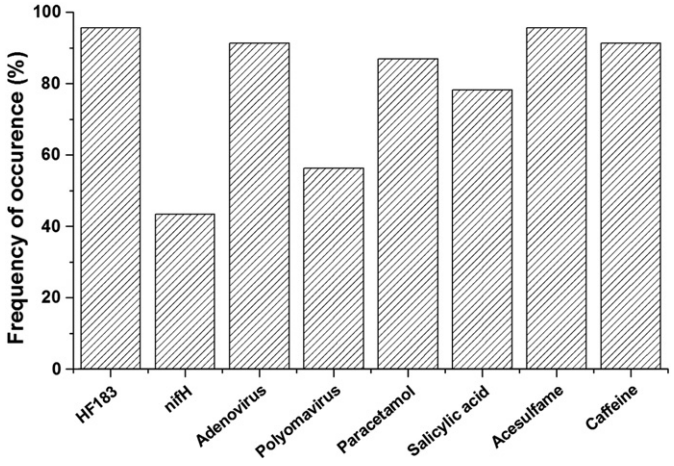

Fig. 1. Frequency of concurrence of microbial source tracking (MST) and chemical source tracking (CST) markers in stormwater samples ($n = 23$) collected from six catchments across Australia.

4. Discussion

Urban stormwater has been reported to contain high numbers of FIB and enteric pathogens (Cizek et al., 2008; Noble et al., 2006; Sercu et al., 2009; Sidhu et al., 2012). Leakages in aging sewage infrastructure, especially in older cities, and cross connections are under-recognized sources of sewage contamination in stormwater (Marsalek and Rochfort, 2004; O'Shea and Field, 1992). This study attempted to assess the extent and frequency of concurrence of sewage contamination in stormwater by comparative analysis of data on both MST and CST markers from six urban catchments across Australia.

High numbers of *E. coli* and *Enterococcus* spp. were observed in the stormwater runoff across all sites (Table 3), which is most likely due to the presence of fresh fecal contamination from sewage leakage and animal sources. A spike in the numbers of FIB after storm events has been previously reported in the literature (Brownell et al., 2007; Parker et al., 2010). *Enterococcus* spp. numbers detected in the stormwater samples collected across all sites were generally higher by

Table 4
Range of FIB and chemical source tracking (CST) markers for sewage contamination across residential and commercial catchments.

	Residential catchments[c]				Commercial catchments[d]			
	Mean	Median	Max	Range	Mean	Median	Max	Range
E. coli[a]	3.28	3.53	3.86	2.15–3.86	3.25	3.61	3.82	2.00–3.82
Enterococcus spp.[a]	3.81	4.00	4.35	3.03–4.35	3.75	3.07	4.47	3.07–4.47
Paracetamol[b]	0.05	0.05	0.11	0–0.11	0.06	0.04	0.20	0–0.20
Salicylic acid[b]	0.14	0.10	0.60	0–0.60	0.10	0.10	0.30	0–0.30
Acesulfame[b]	0.04	0.04	0.07	0–0.07	0.10	0.09	0.23	0.03–0.23
Caffeine[b]	**0.17**	0.1	0.43	0–0.43	**1.63**	1.40	5.20	0.06–5.20

Bold faced = statistically significant.
[a] Log_{10}/l.
[b] μg/l.
[c] Fitzgibbon Drain, Banyan Creek and Ku-Ring-Gai.
[d] Makerston Street, Hornsby and Smith Street.

a couple of orders of magnitude than the recommended limits for the lowest water quality category D (<501 *Enterococci* per 100/ml) under the Australian guidelines for managing risks in recreational water (NHMRC, 2008). This suggests that further assessment of health risks and identification of sources of contamination of stormwater is required prior to its reuse in urban environment.

Sewage associated *Bacteroides* HF183 and *M. smithii nifH* markers, were detected in 96% and 43% of stormwater samples respectively. The prevalence of the *nifH* marker was low in stormwater samples compared to the HF183, potentially due to low prevalence of *M. smithii* in human sewage (Ahmed et al., 2012b) or due to higher decay rate in environment compared to *Bacteroides*. The HF183 and *nifH* markers have been previously shown to be human sewage specific (Seurinck et al., 2005; Ufnar et al., 2006) and have been used to detect the presence of sewage contamination in aquatic environments in South East Queensland (Ahmed et al., 2012a, 2012b). However, it is highly unlikely that a bacterial marker would be absolutely host-specific due to their presence in non-target host groups (Kildare et al., 2007). Baye's Theorem has been used by several researchers to overcome the issue of host specificity with certain MST markers (Kildare et al., 2007; Ryu et al., 2012; Weidhaas et al., 2011). In this study, based on the Baye's Theorem, there was a 99% probability that the detection of the HF183 marker in stormwater samples was due to the true sewage contamination and not from fecal contamination originating from non-target hosts such as dogs, chickens and cat fecal samples where these markers were occasionally detected (Ahmed et al., 2012a). Similarly, there was 94% probability that the detection of the *nifH* marker in stormwater samples was also due to the true sewage contamination and not due to fecal contamination from non-target host. This suggests that sewage contamination is a major source of pollution in stormwater.

Human adenovirus and polyomavirus were detected in the stormwater runoff from all sites with HAv more prevalent (91%) than HPv (56%). Due to the detection of PCR inhibition in nucleic acid extracted from a number of concentrated stormwater samples only binary PCR was undertaken. Therefore, the numbers of HAv and HPv in the stormwater remain unknown. However, a wide spread prevalence of human specific viruses in stormwater suggest potential health risks which need to be quantified further. In order to quantify health risks from waterborne enteric viruses in stormwater reuse scenario, it is essential to obtain quantitative numbers and as well as information on the infectivity status of adenovirus and other enteric viruses. The presence of HAv and HPv in the stormwater runoff is not unexpected as they are known to be present in sewage (Bofill-Mas et al., 2006; Sidhu et al., 2013) in high numbers (10^5 to 10^6/l) and hence in sewage contaminated water. This corroborates with previous findings of wide prevalence of HAv and HPv in surface water and stormwater (Hamza et al., 2009; Muscillo et al., 2008; Rajal et al., 2007; Sauer et al., 2011; Sidhu et al., 2012). Frequent detection of HAv and HPv in stormwater is also an indication that other human pathogens such as other enteric viruses and protozoan pathogens such as *Cryptosporidium* could also be present, thus further increasing potential health risks.

Caffeine has been shown to be a suitable marker for sewage contamination in surface water and is known to degrade rapidly during wastewater treatment and in the aquatic environments (Benotti and Brownawell, 2007; Buerge et al., 2003; Heberer et al., 2002). Caffeine concentrations in the raw sewage ranging from 20 to 300 μg/l and 0.1 to 20 μg/l in treated effluents have been reported (Buerge et al., 2003; Heberer et al., 2002). In comparison, much lower concentrations in rivers, lakes and seawaters in the range of 3 to 1,500 ng/l have been reported (Buerge et al., 2003). The background levels of caffeine, originating from naturally occurring plant sources are also usually negligible (Peeler et al., 2006). In this study, caffeine was frequently detected (91%) in the stormwater runoff with concentration several times higher than reported for aquatic ecosystems (0.14 μg/l median value) which confirms a widespread contamination of urban stormwater by human sewage.

Artificial low-calorie sweeteners (AS) such as acesulfame, saccharin and sucralose are used in beverages, food, pharmaceuticals and certain consumer products such as mouthwashes and toothpaste (Scheurer et al., 2009). They are reported to be reliable markers for sewage contamination in surface water (Buerge et al., 2003; Scheurer et al., 2009). The typical entrance pathway of AS to

Table 5
A matrix showing the concurrence among microbial source tracking (MST) and chemical source tracking (CST) markers.

Markers	HF183	*nifH*	HAv	PAv	Paracetamol	Salicylic acid	Acesulfame	Caffeine
HF183	100							
nifH	47	100						
HAv	**96**	43	100					
PAv	61	70	61	100				
Paracetamol	**83**	48	78	52	100			
Salicylic acid	70	43	70	43	**82**	100		
Acesulfame	**92**	39	**96**	61	**87**	74	100	
Caffeine	**87**	35	**83**	42	78	70	**87**	100

Maximum possible concurrence is 100, markers showing concurrence above 80% are bold faced.

stormwater is via wastewater (Scheurer et al., 2011). Acesulfame is known to be present in raw wastewater and treated effluent (12–46 μg/l) as it is not removed during the wastewater treatment and known to persist in surface water (Buerge et al., 2009). In this study, acesulfame was detected in 96% of sample tested with concentrations ranging from 0.03 to 1.00 μg/l. This also suggests sewage contamination as the main source of its origin in stormwater, however, due to its persistent nature, it may not necessarily stem from recent sewage contamination but could, in principle, also indicate prior or on-going contamination of the catchment.

Pharmaceuticals can be good alternative markers for the verification of sewage contamination in stormwater. In this study, we monitored the presence of analgesics, paracetamol (acetaminophen) and aspirin (acetylsalicylic acid) as they are most commonly dispensed pharmaceuticals in Australia and other parts of the world and hence often detected in the wastewater at μg/l levels (Al-Rifai et al., 2007; Khan and Ongerth, 2004; Verlicchi et al., 2012). In addition, both paracetamol and salicylic acid are biodegradable and have very high removal rates (up to 100%) during the wastewater treatment process (Kasprzyk-Hordern et al., 2009; Verlicchi et al., 2012). In the collected stormwater samples from all six sites, both paracetamol (0.03 to 2.00 μg/l) and salicylic acid (0.10 to 0.60 μg/l) were detected in high concentrations, which again suggest ubiquitous sewage contamination. Since both pharmaceuticals are highly biodegradable, their presence at μg/l levels in the stormwater suggests recent contamination from human sewage, which might be occurring during the storm events.

From the results of this study, it was possible to establish a qualitative link between CST markers and sewage ingress into stormwater, based on the simultaneous detection of multiple compounds. However, it remains uncertain whether a quantitative relationship of sewage ingress volumes is possible due to the large temporal and spatial variations in raw sewage pollutant concentrations, and ingress-dependent storm characteristics. Similar problems also exist for the MST markers such as HF183 and HAv.

The results from both MST and sewage associated CST markers demonstrate that human sewage input was the major source of contamination in urban catchments. Testing for contamination of stormwater runoff for animal sources was not under taken in this study and hence animal fecal as a source cannot be completely ruled out. However, the stormwater runoff collected from the commercial catchments with limited chances of the presence of animal sources of contamination was found to be contaminated with human sewage. The sewage contamination of stormwater may not be limited only to sewer overflows as other sources such as leakages from sewage infrastructure and cross connections are other likely source of contamination. The mean concentration of caffeine was significantly higher ($P < 0.05$) in the stormwater runoff samples from the commercial catchments as compared to residential catchments from Brisbane, Sydney and Melbourne (Table 4). This is potentially due to more consumption of caffeine containing beverages and foods in the commercial areas as compared to the residential areas. Conversely, the median concentrations of paracetamol and salicylic acid were similar from residential and commercial catchments. Further, research is needed to evaluate the potential use of caffeine for tracking sewage contamination in commercial catchments.

Caffeine, paracetamol and salicylic acid are labile indicators of the presence of untreated wastewater in stormwater. The presence of these CST markers, especially at μg/l levels in stormwater suggests more recent contamination from raw sewage which is potentially also the cause of high prevalence of HAv and HPv observed in this study.

The results from this study, demonstrate very good concurrence (>80%) between the concurrence of *Bacteroides* marker (HF183), HAv, acesulfame, paracetamol and caffeine (Table 5) which suggest good likelihood of detection of other markers if samples tested positive for one of the markers. This also suggests that all four markers are reliable markers for the detection of human sewage contamination in stormwater. MST marker HF183 had high concurrence with HAv (96%) and acesulfame (92%), which suggests that samples positive for HF183 or acesulfame in this study also contained HAv. Similarly, acesulfame had very high concurrence of 96% with HAv again suggesting it is also a useful indicator for the presence of HAv in stormwater. However, further research is required with more samples from different catchments to validate the results of this study.

The results of the current study suggest that a combination of MST and CST markers could be used for an accurate public health risk assessment. Use of multiple assays is expected to add to the monitoring costs. Conversely, more accurate information could be available with the use of a carefully selected set of markers for risk assessment rather than merely relying on FIB, or attempting to directly detect individual microbial pathogens in stormwater.

The results of this study suggest that stormwater runoff is frequently contaminated with human sewage. The most likely sources of human sewage contamination include leakages in aging sewage infrastructure, especially in older cities, and cross connections between sewage and stormwater networks. An integrated stormwater management approach to control fecal contamination is required, which may involve controlling the sources of contamination such as sewage leakage, elimination of cross connections and treatment after collection of stormwater. Low cost treatment options could include withholding period in ponds or collection of stormwater runoff in wetlands to allow natural attenuation prior to discharge into surface water or stormwater harvesting. Disinfection of stormwater with ultraviolet (UV) radiation could also be effective in the removal of pathogens prior to reuse in the urban environment.

5. Conclusions

This study demonstrates that human sewage input could be the major source of enteric pathogen contamination of stormwater. Human sewage pollution poses a greater health risk due to exposure to a wide array of enteric pathogens. This study underscores the value of employing a set of markers which could include monitoring for HF183 and adenovirus along with chemical markers caffeine and paracetamol, which will not only provide information on the presence of sewage contamination and potential risks from enteric viruses but also confidence in detection of recent contamination. Consequently, monitoring for selected MST and CST markers in stormwater could provide more accurate information on the presence of enteric virus and accurate assessment of public health risks.

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Conflict of interest statement

The authors have no potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence this research work.

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