Health Risk Assessment of Urban Stormwater

Edited by:

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The Urban Water Security Research Alliance (UWSRA) is a $50 million partnership over five years between the Queensland Government, CSIRO's Water for a Healthy Country Flagship, Griffith University and The University of Queensland. The Alliance has been formed to address South East Queensland's emerging urban water issues with a focus on water security and recycling. The program will bring new research capacity to South East Queensland tailored to tackling existing and anticipated future issues to inform the implementation of the Water Strategy.

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Description: Stormwater sampling at Fitzgibbon Drain site
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- Adj. Professor Ted Gardner, formerly CSIRO and DERM (now DSITIA)
- Mr Donald Begbie, Alliance Director
- Ms Vorn Lutton, Queensland Water Commission (now DEWS)

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FOREWORD BY THE URBAN WATER SECURITY RESEARCH ALLIANCE

Water is fundamental to our quality of life, to economic growth and to the environment. With its booming economy and growing population, Australia's South East Queensland (SEQ) region faces increasing pressure on its water resources. These pressures are compounded by the impact of climate variability and accelerating climate change.

The Urban Water Security Research Alliance, through targeted, multidisciplinary research initiatives, has been formed to address the region's emerging urban water issues.

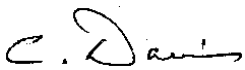
As the largest regionally focused urban water research program in Australia, the Alliance is focused on water security and recycling, but will align research where appropriate with other water research programs such as those of other SEQ water agencies, CSIRO's Water for a Healthy Country National Research Flagship, Water Quality Research Australia, eWater CRC and the Water Services Association of Australia (WSAA).

The Alliance is a partnership between the Queensland Government, CSIRO's Water for a Healthy Country National Research Flagship, The University of Queensland and Griffith University. It brings new research capacity to SEQ, tailored to tackling existing and anticipated future risks, assumptions and uncertainties facing water supply strategy. It is a $50 million partnership over five years.

Alliance research is examining fundamental issues necessary to deliver the region's water needs, including:

- ensuring the reliability and safety of recycled water systems.
- advising on infrastructure and technology for the recycling of wastewater and stormwater.
- building scientific knowledge into the management of health and safety risks in the water supply system.
- increasing community confidence in the future of water supply.

This report is part of a series summarising the output from the Urban Water Security Research Alliance. All reports and additional information about the Alliance can be found at http://www.urbanwateralliance.org.au/about.html.



Chris Davis
Chair, Urban Water Security Research Alliance

FOREWORD BY THE CRC FOR WATER SENSITIVE CITIES

It is envisaged that future water sensitive cities would secure its water supply through investment in a diversity of water sources underpinned by a range of centralised and decentralised infrastructure providing cities with the flexibility to access a 'portfolio' of water sources at optimal value and with least impact on rural and environmental water needs. Stormwater is becoming widely recognised as a significant resource in increasing the security of water supply in many Australian cities and towns. Stormwater provides an additional and abundant source of water to support the greening of cities, which in turn provides multiple benefits in creating more liveable and resilient urban environments.

It is therefore timely that a report directed at assessing the health risk of urban stormwater is published, a culmination of many years of research collaboration by the Urban Water Security Research Alliance and the Cities as Water Supply Catchments Research Program at Monash University. The latter program is now incorporated into the recently established Corporative Research Centre for Water Sensitive Cities to complete this program by 2014 and incorporate its findings to the rest of the research activities of the CRC.

The CRC for Water Sensitive Cities will deliver the socio-technical urban water management solutions, education and training programs, and industry engagement required to make Australian towns and cities water sensitive. Water sensitive cities are resilient, liveable, productive and sustainable. They: efficiently use the diversity of water resources available within towns and cities; enhance and protect the health of urban waterways and wetlands; and, mitigate against flood risk and damage. They also create public spaces that harvest, clean and recycle water, increase biodiversity and reduce urban heat island effects.

Fostering a fit-for-purpose use of all available water resources within cities and towns is a particular focus of the Cities as Water Supply Catchments program with its integrated and diverse projects across a number of disciplines. To that end, ensuring community and institution receptivity to the fit-for-purpose use of stormwater require the necessary scientific documentation of the characteristics and associated health risk of stormwater and corresponding treatment requirements for its use. This project presents a significant step towards achieving this outcome.

I commend the key researchers for their excellent work and am particularly pleased with the manner in which our respective organisations have pooled our resources to deliver this project.

Professor Tony Wong
Chief Executive

CRC for
Water Sensitive Cities

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ACRONYMS

ADWG	Australian Drinking Water Guidelines
ASTR	Aquifer storage transfer and recovery
CDF	Cumulative distribution function
CFU	Colony forming units
CST	Chemical source tracking
DALY	Disability adjusted life year
DEQ	Diuron equivalent concentration
DOC	Dissolved organic carbon
EEM	Excitation-emission matrix
EEQ	Estradiol equivalent concentration
EMC	Event mean concentration
FIB	Faecal indicator bacteria
FST	Faecal source tracking
FU	Fluorescence units
LC-OCD	Liquid chromatograph with online organic carbon detection
LOR	Level of reporting
HAv	Human adenovirus
HDPE	High density polyethylene
HPv	Human polyomavirus
ICP-OES	Inductively coupled plasma optical emission spectroscopy
MST	Microbial source tracking
PAH	Polyaromatic hydrocarbon
PCB	Polychlorinated biphenyls
PCH	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
PDU	PCR detectable units
QHFSS	Queensland Health Forensic and Scientific Services
QMRA	Quantitative Microbial Risk Assessment
qPCR	Quantitative PCR
SEQ	South East Queensland
SPE	Solid phase extraction
TEQ	Toxic equivalent concentration
TTV	Torque teno virus
VG	Virulence gene

EXECUTIVE SUMMARY

Urban stormwater is a relatively untapped alternate water source in Australia, which could be used to augment non-potable and potable water supplies within cities. Elevated levels of chemical and microbial contaminants in the storm runoff, however, can negatively impact public health if polluted surface water bodies are used for recreational purposes or under treated stormwater is used for non-potable purposes such as gardening and landscaping irrigation. The presence of chemical pollutants poses a long-term chronic health risk (if ingested), whereas, enteric pathogens present short-term acute health risks. This study was undertaken to develop an understanding on the presence and loadings of chemical pollutants and pathogens in the urban stormwater runoff with an aim to produce quantitative data which could be used to guide appropriate treatment levels, as well as assess human health risk.

All stormwater samples from Brisbane, Sydney and Melbourne were found to contain high concentrations of the faecal indicator bacteria (FIB) E*nterococcus* spp. and *Escherichia coli (E. coli),* with numbers (10^3 to $>10^4$ 100 mL^{-1}) frequently exceeding the upper limit of Australian guidelines for managing risks in recreational water for category D. Enteric bacterial pathogens such as *Salmonella enterica* (0-2400 MPN L^{-1}), *Campylobacter* spp. (0-10^3 pdu L^{-1}), and the human adenovirus (0-10^3 pdu L^{-1}), torque tenovirus (0-10^3 L^{-1}) and polyomavirus (0-10^2 L^{-1}) were also frequently detected in the storm water at all sites. An increased prevalence of diarrheagenic *E. coli* pathotypes in the surface water after storm events was also observed.

The flow finger printing of FIB, *Salmonella enterica,* and the human adenovirus, polyomavirus, and torque tenovirus during storms at the Fitzgibbon drain and Makerston Street sites demonstrated a high inter- and intra-storm variability. No correlations between the presence of FIB/somatic coliphage and *Salmonella enterica* and human adenovirus, polyomavirus, and torque tenovirus were detected in stormwater. This suggests that FIB and somatic phage are not suitable indicators of the presence of enteric virus and bacterial pathogens in stormwater.

The prevalence of microbial markers (HF183, adenovirus, polyomavirus) along with chemical markers (paracetamol, salicylic acid, acesulfame, and caffeine) in nearly all stormwater samples analysed from all site across Brisbane, Sydney and Melbourne suggests a ubiquitous contamination of stormwater by sewage. This indicates that there is potentially significant human faecal contamination, as opposed to contamination from animals, and thus has much higher public health implications. An integrated stormwater management approach to control faecal contamination is required which may involve controlling the sources of contamination such as sewage leakage, elimination of cross connections or treatment after collection (e.g. by wetlands to allow natural attenuation or other engineered solutions) prior to discharge into surface water or stormwater harvesting.

Chemical characterisation of stormwater confirmed that pollution with heavy metals can be sufficiently severe to require treatment, particularly if high human exposure scenarios are contemplated. Several metals (e.g. lead, nickel, cadmium, mercury) occurred at concentrations well above Australian Drinking Water Guidelines in untreated stormwater. Pesticides were shown to be present in all samples to varying degrees, but usually at concentrations that are considerably below guideline values for potable purposes, i.e. the Australian Water Recycling Guidelines, Phase II – Augmentation of Drinking Water Supplies. Amongst the pesticides detected, MCPA, 2,4-D, diuron, simazine and triclopyr were detected at concentrations which were 0.1% to 20% of the guideline values for indirect potable reuse.

Pharmaceuticals and personal care products were rarely found, with the exception of several substances used in high volumes in the population such as caffeine, acesulfame-K (artificial sweetener), paracetamol and salicylic acid. Prescription drugs such as venlafaxine, tramadol, dapsone, doxylamine, propranolol, sertraline or citalopram were detected above the limit of quantification in approximately 10 to 15% of the samples analysed (n = 30). Of the 57 substances analysed, only caffeine was found in untreated stormwater above the Australian guideline value for recycled water augmentation of drinking water supplies, with most substances having negligible values in comparison to these guidelines.

It can be concluded that most of these substances will not be of human health concern, but it was hypothesized that they can be employed as indicators of sewage ingress into stormwater systems and a surrogate for microbial risk. While establishing this link qualitatively seems very possible based on the simultaneous detection of multiple compounds, 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.

In-vitro bioassays were applied to complement chemical analysis of the dissolved organic pollution. Non-specific toxicity was highly variable and the baseline toxicity equivalent concentrations (TEQ) of the most polluted samples were similar to secondary treated effluent from wastewater treatment plants. Estrogenicity was generally low, with only a single sample among 20 samples showing estrogenic behaviour similar to raw sewage. Phytotoxicity effects were in good agreement with pesticide chemical analysis confirming the absence of strong herbicidal effects of unknown pesticides. Other biological effects measured were minor in comparison with other potential alternative water sources such as secondary effluent.

The high variability observed in the adenovirus, polyomavirus and *Salmonella enterica* numbers in inter and intra storm periods suggest that there is always health risks associated with the stormwater which needs management. A risk management framework is required for making informed decisions on the design of "*fit-for-purpose*" water treatment processes. The Quantitative Microbial Risk Assessment (QMRA) used the quantitative data on pathogen detection from Fitzgibbon Drain and Makerston Streets sites along with the data on adenovirus and *Salmonella enterica* inactivation to calculate the human health risks for a variety of scenarios such as irrigation, accidental ingestion, swimming and boating. The results demonstrate that the high numbers of adenovirus present in the urban stormwater resulted may result in unacceptable human health risks for municipal irrigation and accidental ingestion. Hence risk management is required to safe guard public health for and example exposure control with no public access during irrigation could be sufficient for municipal irrigation with captured stormwater. The specific treatments such as use of stormwater harvesting wetlands, bio-retention basins, and elements of water sensitive urban design used for capture and treatment may require a case by case validation to determine the treatment efficacy for removal of pathogens.

Overall the study demonstrated that microbial and chemical sewage contamination markers can be collectively used to detect recent sewage contamination in stormwater runoff, and provide a more informed level of information for decision making processes aimed at protecting human health.

The overall project conclusions indicate that:

(i) there is evidence of high levels of human faecal contamination in the stormwater;

(ii) human adenovirus and polyomavirus were regularly detected in high numbers which may present health risks;

(iii) chemicals, apart from metals, are less of a heath concern; and

(iv) some degree of treatment of captured stormwater would be required if it were to be used for non-potable purposes.

1. INTRODUCTION

Urban stormwater is a relatively untapped alternate water source in Australia (Hatt *et al.,* 2006), which can be used to augment non-potable and potable water supplies within cities and other urban areas. Stormwater is one of the last major untapped urban water resources that can be exploited as an alternative water source. In South East Queensland (SEQ), it was estimated that the urban water consumption during the 2009 transition year from high-to-medium level water restriction was 232 GL/annum (Chong *et al.,* 2011); whereas the unrestricted water consumption rate in 2004 is 450 GL/annum (Chong *et al.,* 2011). On the other hand, the estimated amount of stormwater runoff from urban catchments in SEQ for 2007 is approximately 870 GL/annum (Chong *et al.,* 2011). This underlines the potential of stormwater harvesting for meeting a substantial fraction of urban water demand and thereby improving the security of potable water supply from traditional mains water supply. At present, however there have been very few cases in Australia such as the Parafield stormwater harvesting system, city of Orange and the aquifer storage transfer and recovery (ASTR) research site (Page and Levett 2010). Stormwater is harvested and treated for beneficial and higher value end-uses such as dual reticulation third pipe to local households for toilet flushing, cold water laundry use and other external uses.

One of the potential reasons for the limited exploitation of urban stormwater as a substitution water source is the lack of understanding of the pollutants occurrences in the urban aquatic environment, and its associated environmental and public health risks. Owing to the stochastic nature in hydrology and sources contribution from different anthropogenic activities and land uses, it was reported that the concentrations of pollutants often exceed standard water quality guidelines (NCHRP 2006). A good understanding on the untreated quality of stormwater is essential as it allows for the development of risk management framework to ensure water quality excursions are avoided, as well as to make informed choices on the design of "*fit-for-purpose*" water treatment processes. A review of the stormwater quality in Australia for sediments and nutrients was carried out by Duncan (2005), but not for other emerging pollutants of concern such as heavy metals, organic matter (e.g. polycyclic aromatic hydrocarbons), pesticides, herbicides and other miscellaneous chemicals. The chemical pollutants generally results in long-term chronic risks; whereas the short-term acute health risks are related to pathogens found in urban runoff.

In Australia, the current stormwater quality guideline is the Australian Guidelines for Water Recycling: Stormwater Harvesting and Reuse which only encompasses a limited number of stormwater quality parameters (NRMMC, EPHC and NHMRC 2009). Additional stormwater quality parameters such as aforementioned chemicals as well as pathogens should be considered if stormwater is to be used for higher value end-uses (i.e. potential human contact via ingestion and cross-connection). However, the monitoring of stormwater quality is always severely affected by the (1) limited monetary budget available for monitoring to enable a full suite of water quality analysis, (2) the stochastic nature of rainfall and catchment hydrology and (3) uncertainties in pollutants occurrences in urban runoff. The combination of these factors normally yields an incomplete picture from a stormwater monitoring perspective, in terms of enumerating the potential chemical and microbiological risks in urban stormwater. There are a number of reported studies on analysing stormwater qualities using the event mean concentration (EMC) metric to estimate the average pollutants concentrations and loads from rainfall events (Kayhanian *et al.*,2007; Charbeneau and Barrett 1998), but not one study has provided a sound methodological and comprehensive risk assessment approach to accurately determine the potential environmental and public health risks in stormwater to enable a better risk management framework, water treatment and reuse scheme (Kim *et al*., 2004). This study constitutes the first in Australia to concurrently investigate the potential chemical, toxicology and microbiological indicators of raw stormwater from urban catchments in SEQ, Melbourne and Sydney.

1.1. Research Objectives

Microbial and chemical quality of collected stormwater was studied during storm events from two catchments each in Brisbane, Melbourne and Sydney. A base line study to determine the presence of enteric pathogens under base-flow conditions was also undertaken in Brisbane urban streams. The study was designed to achieve several specific research objectives, including:

- Determine the extent of microbial and chemical contamination in stormwater.
- Identify and prioritise key microbial and chemicals contaminants in storm water.
- Determine if microbial and chemical pollutants are catchment specific.
- Determine the patterns and loads of pathogens and faecal indicators in stormwater runoff over the storm hydrograph.
- Investigate the correlation between the faecal indicators to human enteric pathogen over the storm hydrograph.
- Determine the involvement of a human sewage as potential source of contamination by using human specific microbial source tracking techniques.
- Determine the frequency of occurrence of diarrheagenic *Escherichia coli (E. coli)* pathotypes in surface waters, and the impact of storm events on their prevalence and distribution.
- Use QMRA to assess the human health risk from exposure to stormwater both for irrigation reuse in urban areas and for recreation in the Brisbane River.

2. GENERAL METHODS AND MATERIALS

2.1. South East Queensland Stormwater Sampling Sites

The first catchment, Fitzgibbon, is located in the northern suburbs of Brisbane (27º20´08"S; 153º01´14"E) (Figure 1). This is a medium density residential catchment which covers a total area of 290 ha. 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%. This stormwater catchment area is situated in a growing residential hub of northern Brisbane and consists of mixed residential dwellings, commercial centres and buildings, education facilities and semi-rural areas. The sampling point for this study is located at a downstream urban stormwater drain. Site specific additional information is presented in the Table 1.

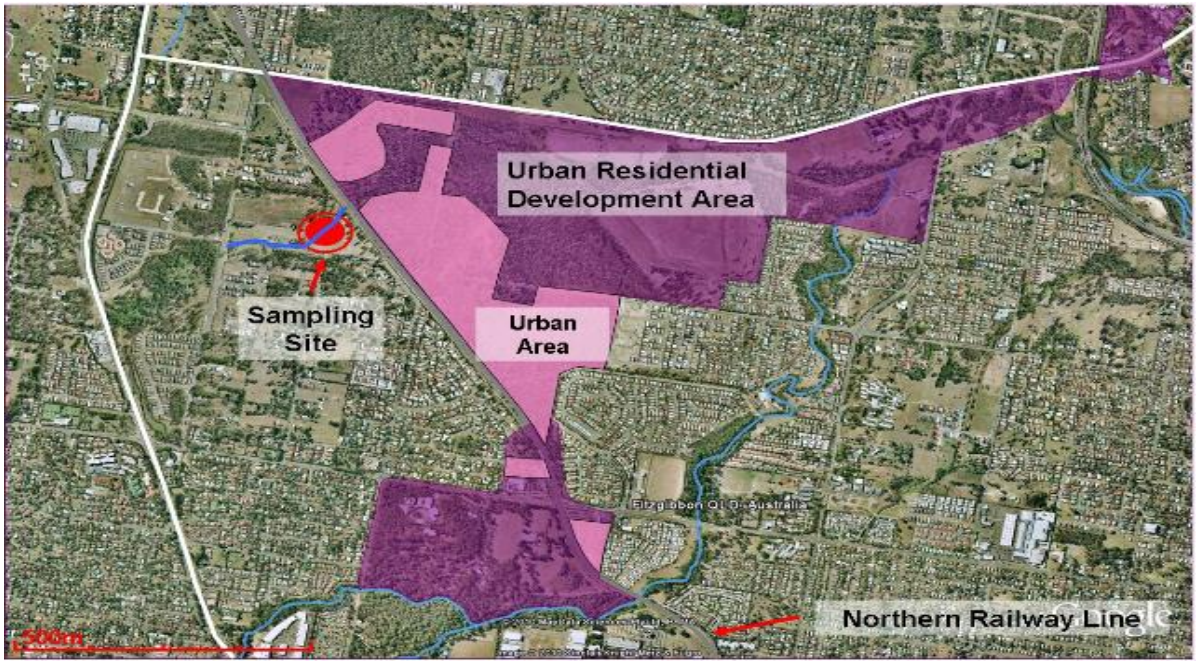

Figure 1: Aerial view of Fitzgibbon catchment.

The second stormwater sampling site, Makerston street (27º28´2.4"S; 153º1´4.5"E), in located in the central business district of Brisbane (Figure 2). This catchment covers high density commercial area of Brisbane with a total area of 30.1 ha which is mostly impervious (≈90%). The sample collection site is located in the stormwater drain at Makerston Street which discharges into the Brisbane River. Site specific additional information is presented in the Table 1.

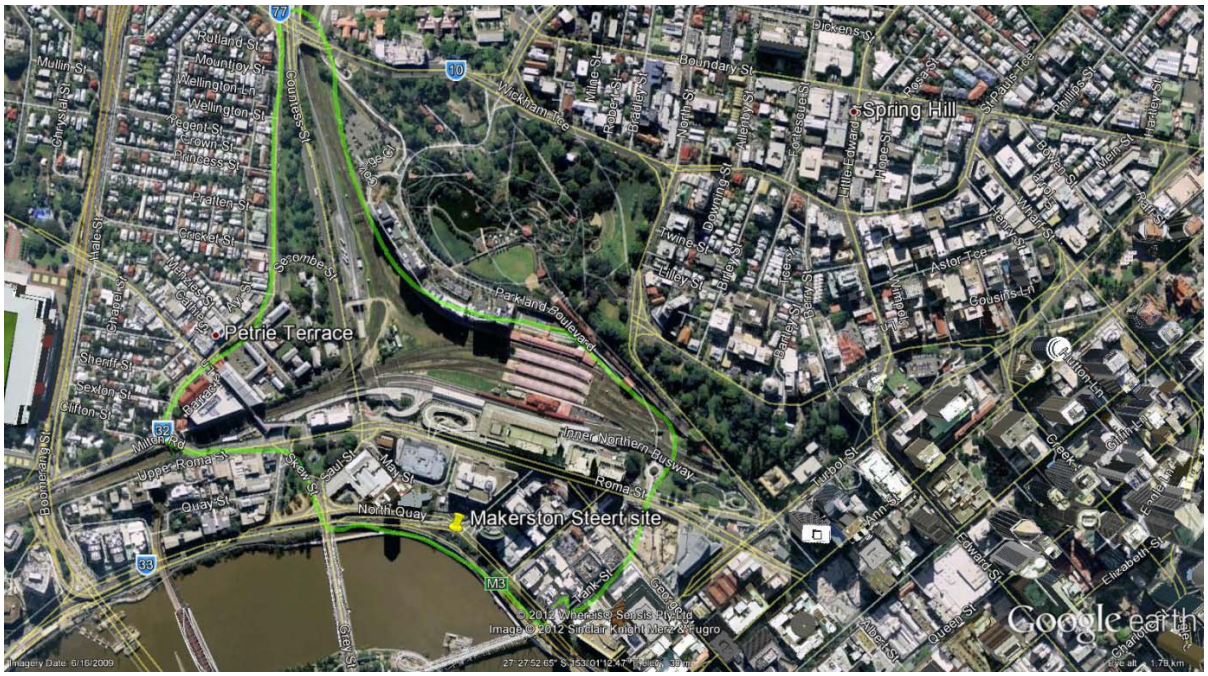

Figure 2: Aerial view of Makerston street catchment in the Brisbane CBD catchment boundry marked in green.

2.2. Melbourne and Sydney Sampling Sites

In addition to Brisbane catchments, stormwater samples collected from Banyan Reserve (south of Melbourne), Smith Street (Melbourne), which is located in the business districts, and an industrial site. Samples were also collected from Hornsby (Sydney), Ku-ring-gai (north of Sydney) and Blackman Swamp Creek, Orange, NSW. Additional information on site description and potential sources of contamination in these catchments is presented in the Table 1.

Table 1: Stormwater sites and brief site description.

Sites	Land Use	Total Area (ha)	Impervious Area (%)	Potential Source of Faecal Pollution
Carseldine drain, Fitzgibbon Brisbane	Residential, large blocks	290	30	Sewage pipe network, pets, small numbers of horses and cattle
Makerston Street, Brisbane	City, commercial	30.1	>90	Sewage pipe network
Hornsby, Sydney	Urban roads/car park	1.08	87	Sewage pipe network
Banyan Reserve, Melbourne	Residential	235	35	Sewage pipe network, pets, small number of horses and cattle
Smith Street, Melbourne	Commercial	10	>90	Sewage pipe network, commercial
Ku-ring-gai, Sydney	Residential with open space	8.8	39	Sewage pipe network, pets
Industrial site, Melbourne	Industrial	20	-	Sewage pipe network, industry land-uses
Blackman Swamp Creek, Orange City Council	Residential, commercial and agriculture	3 417	24	Sewage pipe network, wildlife, pets, livestock

2.3. Stormwater Sampling Strategy

The selection of sampling modes and frequencies was largely based on the prior knowledge of rainfall-runoff within the urban catchment. Typically, the sampling modes are highly catchment site and compound specific. Conventional water sampling was usually operated in common time- or flow-proportional modes (Ort *et al*., 2010). In the case of urban stormwater sampling (i.e. increasing frequencies in peak flows with the impervious surface coefficient), time-proportional sampling might not be the appropriate choice as it requires a rigorous calibration between the rainfall-runoff relationships to make inform the sample collection intervals. Flow-proportional sampling modes are easy to implement but still require a valid scientific justification over the sampling intervals and frequencies. At Fitzgibbon and Makerston sites, automated samplers were used to collect a number of discrete stormwater samples over the runoff hydrograph, usually over a 24-hour period. Figure 3 shows the automatic ISCO samplers setup at the Fitzgibbon site with 24 x 20 L HDPE bottles in a custom made shed.

Figure 3: Automatic samplers setup at Fitzgibbon site with 24 x 20 L HDPE bottles.

In this study, a flow-proportional sampling mode was chosen at the Fitzgibbon Drain and Makerston Street sites (Section 2.1) to pool the discrete samples collected over a storm event into a composite sample for event mean concentration (EMC) analysis. Figure 4 shows example of an event hydrograph at the Fitzgibbon site where four discrete samples were collected over the storm period.

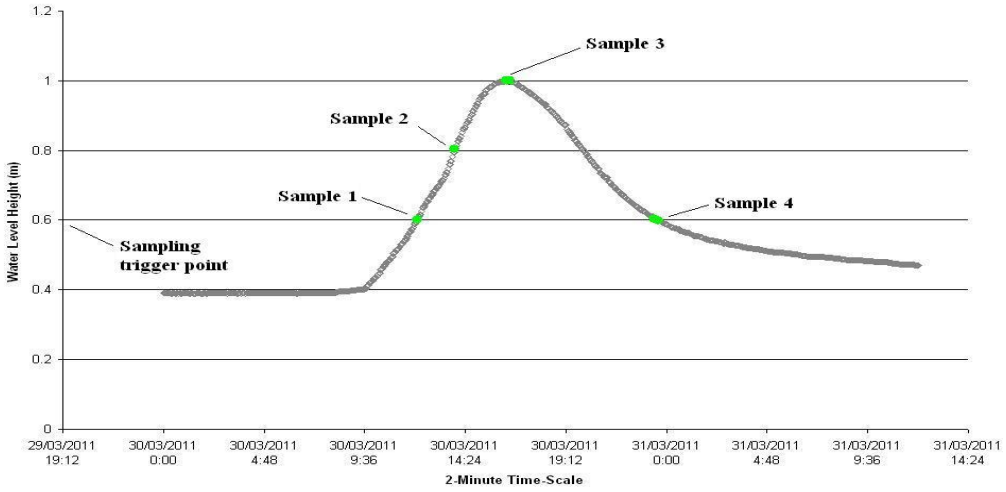

Figure 4: A storm event hydrograph measured at Fitzgibbon site, and the corresponding sampling regime.

Following this, the samples were pooled according to Equation 1 to yield a composite sample for subsequent EMC analysis.

$$EMC = \frac{\sum_{1}^{n}(Q_i \times C_i)}{\sum^{n} 1 Q_i}$$

(Equation 1)

At other stormwater sampling sites in Melbourne and Sydney, volume proportional composite samples were taken using automated pumping samplers (ISCO 6700 or equivalent) triggered by flow measurement (either using a Doppler flow-meter or a weir, depending on site characteristics). The automatic samplers were programmed to the site specific requirements ensuring the reliable EMCs via the composite samples.

2.4. Automated Stormwater Sampling

Automatic samplers (ISCO 6700 series) were used in parallel for stormwater sample collection at both Brisbane sites. These samplers were programmed to fill up to 24 x 20 L high density polyethylene containers (HDPE) (Food and Drug approved grade) during a storm event. The automatic samplers were used to capture the dynamics of stormwater flow (i.e. rising limb, peak and receding flows) where the samplers were simultaneously triggered to give a sample volume of 20 L for each preset flow threshold. A submersible Argonaut Flow Doppler (Thermo Fisher Sci) was installed to measure the in-stream stormwater flow during the wet weather events and triggered the automatic samplers for sample collection. The Argonaut Flow Doppler is capable of accurate measurement of depth and velocity, after a series of manual flow gauging and calibration procedures. A remote telemetry system was used to notify via SMS alert once the first 20 L of samples was collected at the site. This system removes the need for sample refrigeration, as the research team can attend to the site for (almost) immediate sample collection and transfer back to the laboratory for subsequent analysis. To avoid cross-contamination in the HDPE bottles, they were cleaned using sodium hypochlorite solution (1%) and rinsed with ultra-pure water (MilliQ system, Millipore) in the laboratory before replacing the used HDPE bottles at the field site.

2.5. Chemicals and Analytical Methods

Samples were refrigerated at all times during transport and storage. Samples were first filtered through a series of meshes (250 µm, 160 µm and 63 µm) and then through a 1.2 µm glass fibre filter (GF/C Whatman) to determine total solids fractions and particle size distributions of solids in the sample. The filtrate was then subjected to chemical analysis.

Particle size distribution in the colloidal fraction of the filtrate was measured by dynamic light scattering (DLS) method using a Zetasizer NanoZS (Malvern). The instrument has an option to measure specific colloids if its refractive index is known. Humic substances were selectively measured by selecting its refractive index 1.33 to represent organic colloids.

Rapid spectroscopic methods as described in Section 3.2 were used to measure the occurrence of organic matter, followed by toxicity measurement in Section 3.3. If, the toxicity measurements showed a significant peak in certain classes of toxicity (as classified by their mode of action/targeted chemicals in Table 2), a comprehensive suite of chemical analysis was performed. The composite samples were also subjected to quantification of FIB and pathogens, as discussed in Section 4. Other than the standard EMC measurements, we also measured the concentrations of chemicals and microbiological pollutants within each discrete sample collected over each storm event, in order to validate the robustness of our EMC approach.

Table 2: Summary of different bio-analytical test battery used for toxociological end-points measurement in stormwater.

Mode of Action	Assay	Targeted Chemicals
Baseline toxicity	Bioluminescence inhibition assay	All chemicals
Phytotoxicity, photosynthesis (PSII) inhibition	I-PAM (phytotox)	Triazine and phenylurea herbicides
Estrogenic effects	E-SCREEN	Estrogens, estrogenic industrial chemicals
Binding to Ah receptor	AhR CAFLUX	Polychlorinated dibenzodioxins/furans, PCB, PAH
Genotoxicity	umuC (genotox)	Aromatic amines, PAH
Oxidative Stress	AREc32	Soft electrophiles

Following further pre-filtration by 0.45 µm nylon filter, dissolved organic matter was characterised by size exclusion chromatography using a liquid chromatograph with online organic carbon detection (LC-OCD) (Huber *et al*. 2011). The LC-OCD system (DOC-LABOR) was equipped with a size exclusion chromatography column HW-50S filled with Toyopearl resin (pore size of 125 Å). UV spectroscopy was performed on a Varian Cary 50 Bio UV spectrophotometer in a 1 cm quartz cuvette.

Three-dimensional fluorescence excitation-emission matrix (EEM) data was collected using a luminescence spectrometer (PerkinElmer LS55, USA). In these studies, the EEM spectra were collected with corresponding scanning emission spectra from 280 nm to 500 nm at 5 nm increments by varying the excitation wavelength from 200 nm to 400 nm at 5 nm sampling intervals. The excitation and emission slits were maintained at 7 nm and the scanning speed was set at 1200 nm/min. A 290 nm emission cut-off filter was used in scanning to eliminate the second order Raleigh light scattering. EEM fluorescence results were interpreted using a fluorescence regional integration approach (FRI) as established by Chen *et al*. (2003) and fluorescence was corrected for inner filter effects using the procedure outlined by Lakowizc (2003).

Pharmaceuticals, pesticides and personal care products were measured by Queensland Health Forensic and Scientific Services (QHFSS, a National Association of Testing Authorities, Australia accredited laboratory) in an adaptation of US EPA method. Two aliquots of aqueous sample are extracted on solid phase extraction (SPE) cartridges (Phenomenex StrataX, 200 mg/3 mL), one acidified with hydrochloric acid and the other not pH adjusted, using a Gilson Aspec SPE system. The chromatographic system is a Shimadzu UFLC system equipped with a Phenomenex C18 Luna column coupled to an Applied Biosystems 4000QTrap® LC/MS/MS. Internal standards and stable isotope surrogates were used for quantification.

A detailed list of analytes and detection limits is provided in Appendix A1 and A2.

Heavy metals and other cations were analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES). Anions were analysed by ionic chromatography with electrochemical conductivity detection.

2.6. Microorganisms Used and Analytical Methods

The list of enteric pathogens and indicator microorganisms tested in stormwater and the analytical methods used for each microorganism is given in Table 3. Detailed methodology is provided in Section 4.

Table 3: Enteric microorganisms tested in collected stormwater samples.

Enteric Microorganism Group	Enteric Microorganism	Analytical Method
Bacteria	*E. coli* O157:H7	PCR
	Salmonella enterica	MPN with Culture on XLD agar, PCR
	Campylobacter jejuni	PCR
	Enterococcus faecalis	Culture on Chromocult Enterococci Agar
	E. coli	Culture on Chromocult Agar
Protozoa	*Cryptosporidium* oocysts	PCR, Microscopy using vital stains
	Giardia cysts	PCR, Microscopy using vital stains
Enteric virus	Adenovirus	qPCR
	Polyomavirus	qPCR
	Enterovirus	qPCR

2.7. Bioanalytical Assays Used for Toxicology

Prior to *in vitro* bioanalytical tests being performed, the sample filtered through 1.2 µm GF/C was subjected to solid phase extraction using 1 g OASIS® HLB solid phase material in 20 ml cartridges (Waters, Australia). After conditioning the cartridges with 10 mL methanol and 20 mL MilliQ water (pH<2), a known volume of sample was percolated under a vacuum. The cartridges were left to dry overnight in a vacuum prior to being eluted with 10 mL methanol and 10 mL hexane:acetone (1:1). All samples were evaporated to dryness and made up to 0.5 mL with methanol. The bioanalytical tests listed in Table 2 were performed following the procedures described in detail in Macova *et al*. (2010), with the exception of the AREc32 test, which is described in detail in Escher *et al*. (2012).

All effects were reported in terms of toxic equivalent concentrations (TEQ). The equivalent concentration represents the concentration of a reference compound that would be required to produce the same effect as the mixture of different compounds in the sample. Further details on the detailed procedures to obtain equivalent concentrations can be found in Macova *et al*. (2010).

3. CHEMICAL CONTAMINANTS IN STORMWATER

While the typical chemical composition of sewage effluent is reasonably well understood, there is potential for a broader range of contaminants likely to be present in raw stormwater particularly from urban stormwater systems. Existing stormwater quality data focuses predominately on heavy metals, physicochemical characteristics, nutrient levels and standard microbial indicators.

Factors influencing the range and concentration of pollutants collected at "ground level" in urban runoff include; the nature, condition and texture of the road surface; vehicle traffic density; tyre and brake abrasion; drip losses from vehicles (e.g. brake fluid, fuel, oil) and the efficiency of engine combustion in individual vehicles (Brown and Peake, 2006). This work focused on stormwater, recognised as 'ground level' urban runoff, as distinct from runoff from roofs. From a precautionary health risk perspective, this is considered appropriate, as stormwater reclaimed from urban runoff is considered to present a higher risk to human health than rainfall collected from roofs, as it contains higher amounts of pathogens and chemical contaminants (NRMMC *et al*., 2009). Chemical analysis (and bioanalytical tools) focuses on dissolved fractions, as solids are expected to be efficiently removed by existing stormwater treatment technologies whenever a high human exposure application in envisaged for a stormwater harvesting scheme.

In the following sections, results from the Fitzgibbon and Makerston Street catchments are presented. These are two very different examples of a peri-urban and an inner-city suburb catchment. Both catchments are located within the Brisbane City Council area. Furthermore, micropollutant data will be presented for a series of eight different catchments from different states (NSW, QLD, VIC, see also Table 5).

3.1. Particle Size Distribution

Nineteen composite samples representing event mean concentrations (11 samples from Fitzgibbon and eight samples from Makerston Street were analysed (Figure 5). The particle size distribution for Fitzgibbon samples showed a range of 329 to 86 nm with standard deviation of 64 nm. The Makerston Street sample had a range of 335 to 98 nm with standard deviation of 76 nm. The result suggests that Fitzgibbon Drain had more uniform colloidal particle range except for event eight (FTZ8) but Makerston Street had a significant variation in colloidal particle range from event to event.

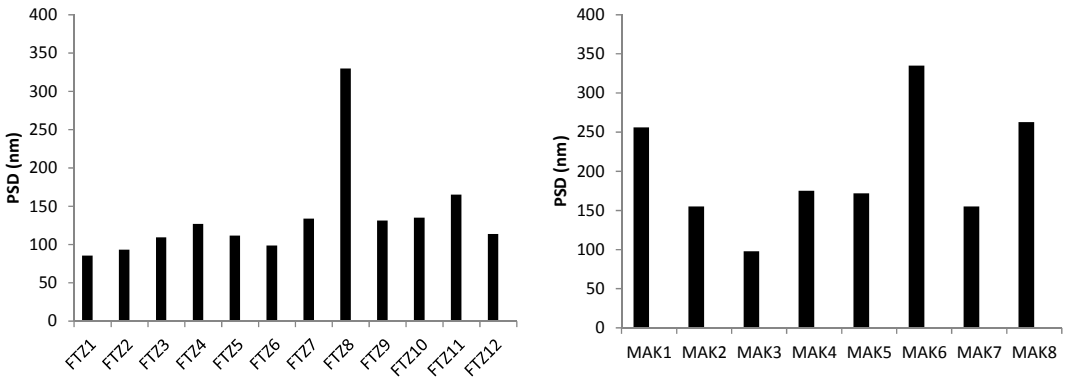

Figure 5: Colloidal particle size distribution in stormwater collected in Fitzgibbon Drain (left) and Makerston Street (right) sites as determined by employing a Malvern Nanosizer equipment after filtration through 1.2µm filter.

3.2. Dissolved Organic Matter Characterisation

Eleven samples from Fitzgibbon and eight samples from Makerston Street were analysed by size exclusion chromatography with organic carbon detection (LC-OCD) after filtration through 0.45 µm nylon filters. Each sample was collected and composited as previously described to measure event mean concentrations (EMC) of a different storm event.

Mean dissolved organic carbon (DOC) concentrations were 4.4 $mg \cdot L^{-1}$ (n=11, SD = 1.7 $mg \cdot L^{-1}$) and 4.0 $mg \cdot L^{-1}$ (n=8, SD = 2.1 $mg \cdot L^{-1}$) respectively for Fitzgibbon and Makerston Street. No statistically significant difference occurred between the mean DOC comparing the two catchments.

LC-OCD analysis permits to further fractionate the dissolved organic carbon and distinguish hydrophobic and hydrophilic dissolved organic carbon, and furthermore within the latter fraction distinguish biopolymers, humic substances, humic building blocks (i.e. similar chemical functionality than humic substances but lower molecular weight) and low molecular weight neutrals and low molecular weight acids (Huber *et al*. 2011). Some of the results are shown in a box-plot in Figure 6.

Biopolymers were generally not found above detection limit (usually in the range of 10 to 50 $\mu g \cdot L^{-1}$). Although the box plot shown in Figure 6 suggests that there are differences, in particular for the median values, applying t-tests shows no significant differences for the mean concentrations, except for low molecular weight acids (P=95%). However it should be noted that for small sample sizes the test becomes vulnerable to outliers.

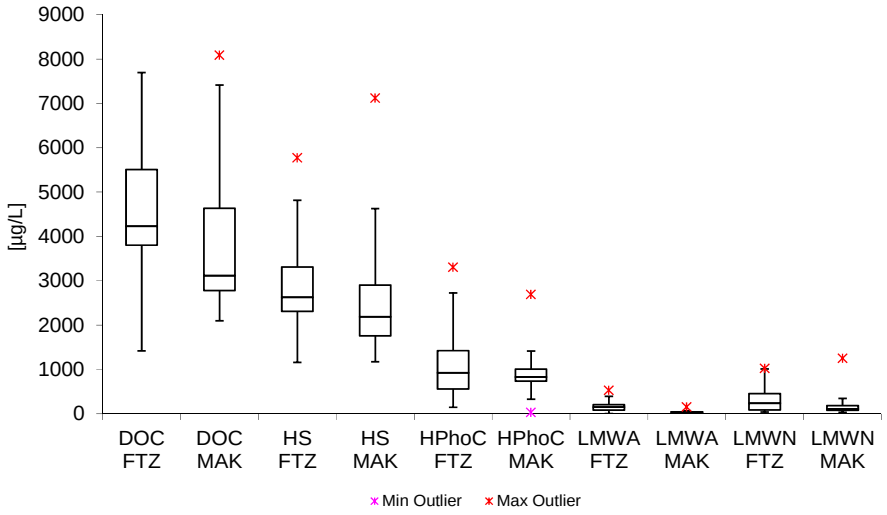

Figure 6: Fitzgibbon (FTZ, n=11) and Makerston (MAK, n=8) LC-OCD analysis results. Dissolved organic carbon (DOC), sum of humic substances and building blocks (HS), hydrophobic dissolved organic carbon (HPhoC) and low molecular weight neutral and acids (LMWN, LMWA) are shown in a box plot. The boxes represent the inner quartiles and the whiskers represent the maximum and minimum values. If maximum or minimum fall outside of 1.5 times the inner quartile range (Q3-Q1), the whiskers are plotted as 1.5 times inner quartile range and maximum or minimum falling outside this range are plotted as maximum or minimum outlier.

Fluorescence spectroscopy generating data in the form of an excitation-emission map (EEM) has been used by several researchers to obtain additional information on dissolved organic carbon. Particularly in environmental waters, the fluorescence of proteins (caused by the aromatic amino acids tyrosine and tryptophan), humic-like substances and chlorophyll A and B has been exploited in research. One widely applied methodology is the integration of fluorescence of different excitation and emission regions using the classification into five regions suggested by Chen el al (2003) in humic acids, fulvic acids, protein 1 (tyrosine-like), protein 2 (tryptophan-like) and soluble microbial by-products.

Figures 7 and 8 show the fluorescence attributed to these regions in arbitrary fluorescence units (FU) and in percentage contribution of each region to total fluorescence following the methodology introduced by the above-mentioned paper. The fluorescence is clearly higher for Fitzgibbon than for Makerston for the EEM regions attributed to humic (p=99.8%) and fulvic (p=96.7%) substances. Humic substances, due to their negative charge have often been reported to be important in the transport of environmental contaminants, especially heavy metals (Reuter and Perdue, 1977). The cause of the enhanced presence of fluorescent organic substances is presumably related to the higher proportion of pervious surface (70%) in the Fitzgibbon catchment. The fluorescence measurement, contrarily to the LC-OCD analysis, also clearly shows the existence of different origins and characteristics of the organic carbon present in the run-off of both catchments. This becomes particularly evident, when the contribution of individual EEM regions to total fluorescence is compared as in Figure 8.

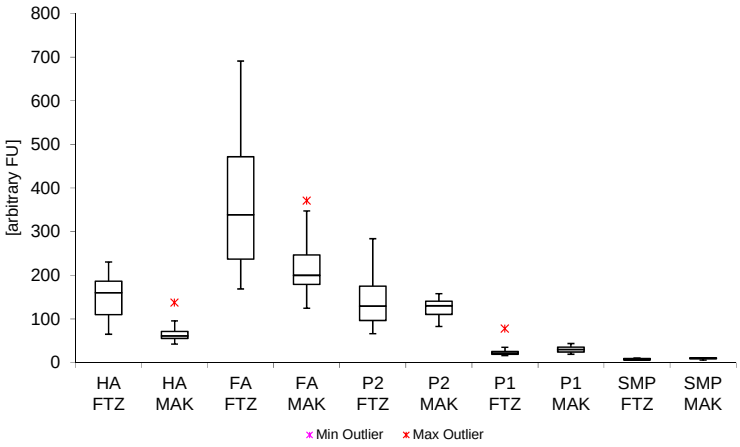

Figure 7: Average mean fluorescence in each EEM region shown for Fitzgibbon (FTZ, n=11) and Makerston (MAK, n=8). Humic and fulvic acids (HA, FA), Protein 1 and 2 (P1, P2) and soluble microbial by-products (SMP). See caption of Figure 6 for description of box plot.

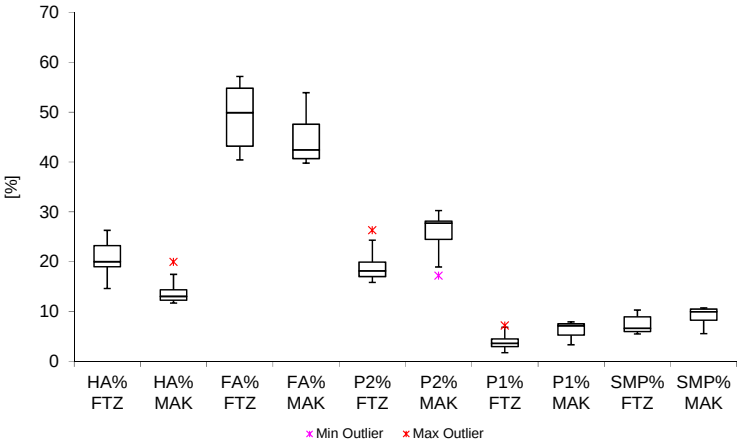

Figure 8: Contribution of each EEM region to total fluorescence for Fitzgibbon (FTZ, n=11) and Makerston (MAK, n=8). Humic and fulvic acids (HA, FA), Protein 1 and 2 (P1, P2) and soluble microbial by-products (SMP). See caption of Figure 6 for description of box plot.

3.3. Metals

The presence of metals in urban run-off is well documented (Duncan, 1999; Goebel *et al.*, 2007, Makepeace *et al*., 1995) and considerable efforts have been undertaken to investigate their fate in stormwater treatment systems, thereby improving our ability to manage this type of contaminant (Davis *et al.*, 2009, Hatt *et al.*, 2007).

In regard to their potential human health impacts when reusing stormwater, metals can be distinguished into different classes, i.e. those that are primarily of aesthetic concern (e.g. iron, aluminium, manganese, although the latter has also a health implication at higher concentrations), and those that exhibit a relatively strong toxicity and are therefore regulated at low microgram per litre levels (e.g. antimony, arsenic, cadmium, chromium, lead, mercury, nickel) in the Australian Drinking Water Guidelines. Finally, copper and zinc are two metals that have environmental and health implications but are regulated at higher levels, 2 and 3 $mg \cdot L^{-1}$ respectively, in the same regulation.

Figure 9 shows the dissolved metal concentrations for these metals measured as EMCs as previously described from different storm events from Fitzgibbon and Makerston Street along with existing ADWG limits where available. Omnipresent metals such as aluminium, iron and manganese are generally below the respective aesthetic limits. One event was observed at the Fitzgibbon site, where aluminium and iron were both particularly high, possibly related to very high rainfall preceding this particular event (180 mm two days before).

Generally, all reported concentrations fall within the ranges reported in literature (as shown on the graphs), with copper and zinc actually being rather low and far below their respective health limits. However, the concentrations of several metals that are considered toxic at low levels were close to, or above, their respective ADWG guideline values. These were cadmium, mercury, nickel, lead and antimony. It can also be noted that several of those metals that are present in bulk metal products such as steel (e.g. Co, Cr, Ni, V) are more abundant in the commercialised Makerston catchment. We have no explanation as to why the cadmium concentrations are higher in the Fitzgibbon catchment. Cadmium sources are among the least understood metal sources in urban catchments for those metals that are generally acknowledged as important pollutants (Davis *et al*., 2001). It is interesting to notice that the lead contamination found in this study (mean concentration 0.0089 $mg \cdot L^{-1}$) is considerably lower compared to the mean concentrations ranging from 0.0209 to 1.558 $mg \cdot L^{-1}$ reported in an earlier literature review (Makepeace *et al*., 1995), which suggests that source control and the change of consumer patterns (i.e. the introduction of unleaded fuels in this case) can be excellent measures for reducing contamination.

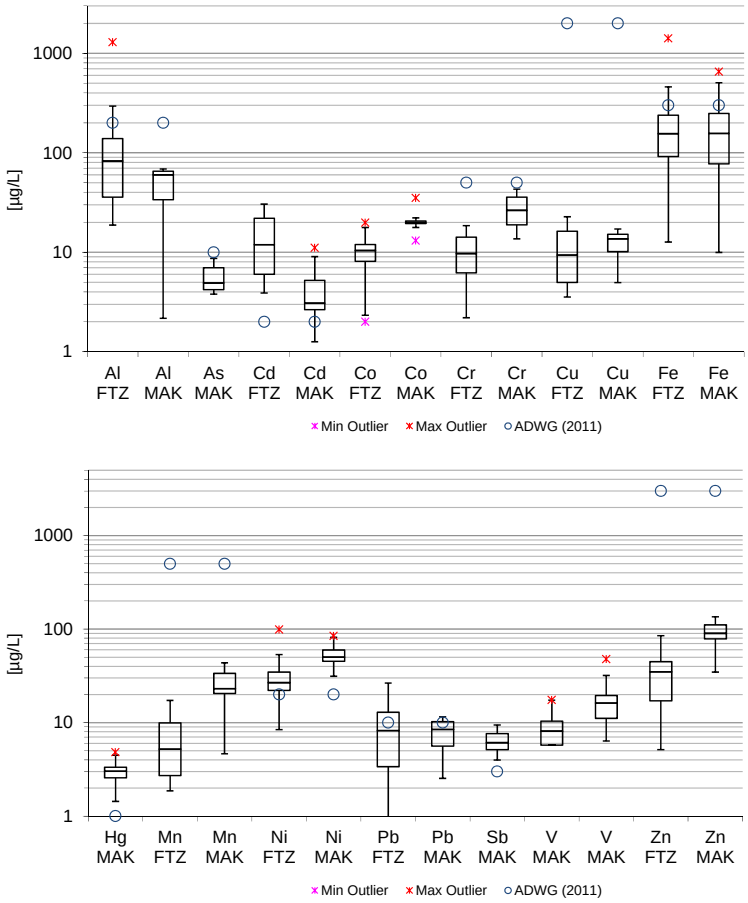

Figure 9: **Dissolved metal concentration for Fitzgibbon (FTZ, n=10) and Makerston (MAK, n=7). See caption of Figure 6 for description of box plot.**

3.4. Pesticides

Samples from the eight different catchments in Australia were measured for pesticides in filtrates through 1.2 µm GF/C filters. Each sample results given represents an EMC of a different storm event. Depending on the pesticide, 11 to 36 samples were measured with the results being shown in Table 4. The pesticides found most frequently above the level of reporting (>50% of samples) were 2,4-D, diuron, MCPA, simazine and triclopyr, all of which are herbicides. The median concentration at which those were found range from 0.01 to 0.03 $\mu g \cdot L^{-1}$. These concentrations are around 1000 times lower than their respective guideline value in the Queensland Public Health Regulation for recycled water to augment drinking water supplies, with MCPA being the only one with a factor of only 100 between median and guideline value. Even if the maximum concentration value was contemplated, the concentrations found in urban run-off were determined to be 5 to 167 times lower than their respective guideline value. The same factors for the other 12 pesticides were substantially higher.

Existing literature on the occurrence of pesticides in urban run-off is scarce. Eriksson *et al.* (2007b) reported MCPA concentrations from <0.05 – 0.13 $\mu g \cdot L^{-1}$ in Copenhagen, Denmark. From their study, similar to the present study, MCPA emerged as the pesticide of highest concern, in this case in regard to environmental health protection. Maillard *et al.* (2012) report diuron and simazine at concentrations of up to 0.32 and 0.18 $\mu g \cdot L^{-1}$, respectively, in run-off from a vineyard in France. In another study diuron, simazine and atrazine average inflow concentrations to the Parafield wetlands in Adelaide, Australia, have been estimated to be 0.192, 0.07 and 0.005 $\mu g \cdot L^{-1}$, respectively, during a study period of 28 days employing passive sampling techniques (Page *et al.*, 2010). These values are within 50% of average concentrations for the present study showing the potential applicability of passive sampling systems to stormwater treatment systems, if hydraulic conditions are assessed properly.

Table 4: Pesticide, pharmaceutical and other micropollutant concentrations determined in samples from eight catchments located throughout Australia. LOR = Level of Reporting by the analytical laboratory.

Pesticide	n	LOR [µg·L^{-1}]	n>LOR	Min [µg·L^{-1}]	$Q_{25\%}$ [µg·L^{-1}]	Median [µg·L^{-1}]	$Q_{75\%}$ [µg·L^{-1}]	$Q_{90\%}$ [µg·L^{-1}]	Max [µg·L^{-1}]	Guideline* [µg·L^{-1}]	Guideline*/ Max
2,4-D	28	0.01	23	<LOR	0.01	0.03	0.09	0.22	1.33	30	23
Ametryn	36	0.01	1	<LOR	<LOR	<LOR	<LOR	<LOR	0.01	50	5000
Atrazine	36	0.01	9	<LOR	<LOR	<LOR	<LOR	0.02	0.19	50	263
Bromacil	36	0.01	5	<LOR	<LOR	<LOR	<LOR	0.02	0.05	300	6000
Desethyl Atrazine	36	0.01	4	<LOR	<LOR	<LOR	<LOR	<LOR	0.02	40	2000
Desisopropyl Atrazine	36	0.01	8	<LOR	<LOR	<LOR	<LOR	0.03	0.04	40	1000
Diuron	36	0.01	33	<LOR	0.01	0.03	0.10	0.35	0.88	30	34
Fluometuron	36	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	50	>5000
Hexazinone	36	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	300	>30000
Imidacloprid	11	0.01	4	<LOR	<LOR	<LOR	0.01	0.02	0.02	NA	NA
MCPA	28	0.01	22	<LOR	0.01	0.02	0.05	0.09	0.39	2	5
Metolachlor	36	0.01	2	<LOR	<LOR	<LOR	<LOR	<LOR	0.02	300	15000
Prometryn	36	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	105	>10500
Simazine	36	0.01	28	<LOR	0.01	0.03	0.05	0.08	0.12	20	167
Tebuthiuron	36	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	NA	NA
Terbutryn	36	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	300	>30000
Triclopyr	28	0.01	16	<LOR	<LOR	0.01	0.03	0.10	0.23	10	43
Other											
Acesulfame K	30	0.01	29	<LOR	0.04	0.05	0.07	0.12	0.23	NA	NA
Caffeine	30	0.02	26	<LOR	0.07	0.30	1.04	1.88	5.20	0.35	0.07
DEET	30	0.01	26	<LOR	0.04	0.15	0.36	0.48	0.86	2500	2907
Paracetamol	30	0.02	21	<LOR	<LOR	0.03	0.08	0.11	0.20	175	875
Salicylic acid	30	0.10	13	<LOR	<LOR	<LOR	0.10	0.20	0.60	105	175
Venlafaxine	30	0.01	6	<LOR	<LOR	<LOR	<LOR	0.02	0.11	75	682

* Queensland Public Health Regulation 2005, Schedule 3B - Standards for quality of recycled water supplied to augment a drinking water supply

In summary, the observed concentrations in this study are consistent with concentrations reported in the literature. For the eight catchments which generated up to 36 samples (event mean concentrations) there was no single value measured that would exceed a human health guideline such as the Australian Water Recycling Guidelines for Augmentation of a Drinking Water Supply.

Figure 10 explores further the measured concentrations plotting the diuron concentration against the MCPA concentration. Both are frequently detected herbicides with similar sorption coefficients (experimentally determined K_{OW} of 2.68 and 3.25, EPISuite, www.chemspider.com). It is clearly shown that run-off concentrations, despite varying between run-off events in each catchment, are quite different for each catchment. For example, Makerston and Smith Street are consistently among the samples with the highest diuron concentrations. Curiously, these two catchments are also the only catchments located in the CBDs of capital cities. A third catchment with high diuron concentrations is the residential Banyan.

The results also show a trend that if the concentration of one pesticide is elevated, the concentration of the other pesticide also tends to be high. It is noteworthy that this type of correlation can also be established for other combinations of pesticides frequently found. This may indicate several things. First, temporal, spatial or regional usage patterns of different pesticides may be correlated. Second, the catchment characteristics are related to the run-off mechanisms. At present, the cause for this observation is undetermined, but we will hope to obtain further insights through the continuation of this work within the CRC for Water Sensitive Cities (http://watersensitivecities.org.au/).

Finally, a similar correlation can be appreciated within some of the catchments, in particular Hornsby and Fitzgibbon (see Figure 10), partly also because of the very limited number of samples per catchment. Again, this will be further investigated in future research in the CRC for Water Sensitive Cities.

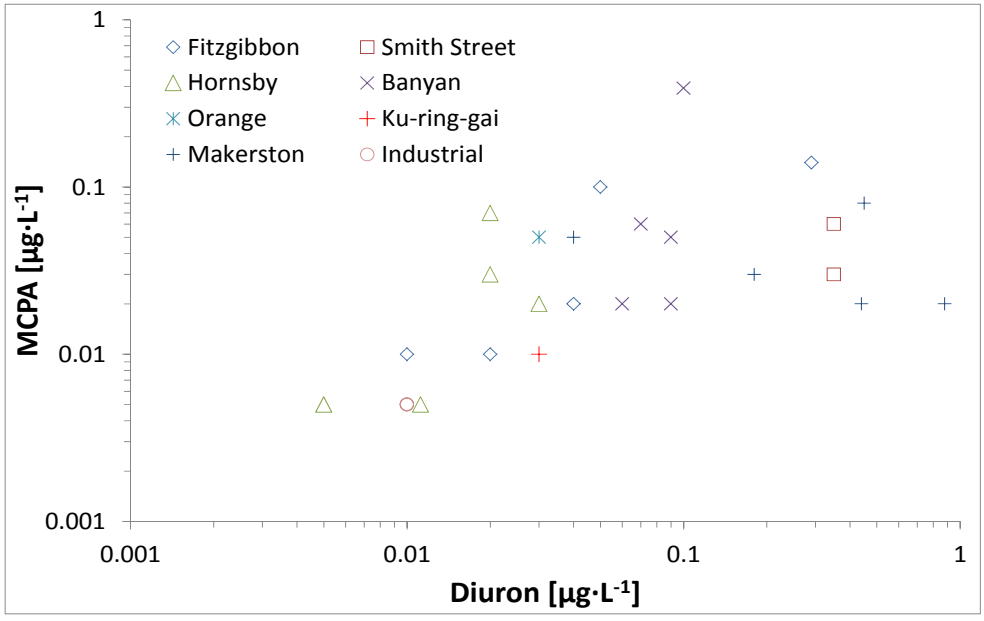

Figure 10: **Concentrations of diuron and MCPA in different catchments.**

3.5. Pharmaceuticals and Personal Care Products

The presence of pharmaceuticals and personal care products in the environment, often related to the discharge of treated or untreated sewage, has been the focus of numerous scientific studies with recent reviews by Boxall *et al.* (2012) and Kuemmerer (2009). These substances have also attracted particular attention by their detection in drinking water supplies (Benotti *et al.*, 2009) and consequently in their fate in wastewater treatment (Onesios *et al.* 2009) and water recycling (Wintgens

et al., 2008) processes. However, they have been rarely studied in stormwater with a few exceptions such as the study of Boyd *et al*. (2004), who also were the first to suggest that the presence of these types of compounds could indicate sewage contamination in stormwater and urban streams. In the following, these ideas have also been further applied and developed in studies on the impact of overflows from combined sewers (Philips *et al*., 2012). Australia however, predominately has separate sewer and stormwater systems, and sewage ingress into stormwater systems should presumably be caused by breakage of sewage pipes or connections and resulting seepage into the environment and stormwater systems, or sewer overflows during storms and resulting ingress into the stormwater pipes. The present study aims at evaluating the possibility to study sewage ingress into stormwater systems.

We evaluated 30 samples from the same eight catchments as described in Section 2 for the presence of 57 pharmaceuticals and personal care products. As with other chemical analysis these were composite samples to determine EMCs of different storm events. Appendix A1 and A2 contains a detailed list of all substances analysed including their reporting limits and their guideline values in the Queensland (Qld) Public Health Act. Of the 57 substances analysed, 28 substances could be detected at least once, whilst only six substances could be detected at least six times or more. Those six substances were acesulfame, caffeine, DEET, paracetamol, salicylic acid and venlafaxine. More details on the results for these substances are included in Table 5, with further detail on the results of all substances analysed for in Appendix A1.

To estimate if there is a potential risk from excessive exposure through ingestion of these compounds, a very simple risk coefficient can be defined by dividing the maximum concentration detected by an appropriate guideline value. We considered the Qld Public Health Act, which is essentially an implementation of the Australian Guidelines for Water Recycling. When the compound was not detected above the level of reporting, instead a minimum risk coefficient was determined. For example, the level of reporting of diclofenac is 0.01 $\mu g \cdot L^{-1}$ and the guideline value is 1.8 $\mu g \cdot L^{-1}$. Consequently, the risk coefficient, obtained by dividing one concentration by the other is ">180". The higher the risk coefficient, the lower the risk presented by a compound is.

Caffeine was the only compound that actually exceeded the guideline value in any given sample. In fact even its median value (0.30 $\mu g \cdot L^{-1}$) was close to the guideline value (0.35 $\mu g \cdot L^{-1}$). Furthermore, a single high value of acetylsalicylic acid would have presented a risk coefficient of 1.8 (i.e. be present at 55% of the guideline value), though that is likely to be due to sample contamination as the value is a clear outlier. Triclosan was encountered three times at 0.01 $\mu g \cdot L^{-1}$ (guideline value 0.35 $\mu g \cdot L^{-1}$). Citalopram and its metabolite desmethyl citalopram were encountered each four times at concentrations between 0.01 and 0.04 $\mu g \cdot L^{-1}$ (both guideline values 4 $\mu g \cdot L^{-1}$). All other compounds analysed, if found at all, were determined at concentrations that were less than 1% of the guideline value. It is worth noting that 9 of 57 substances analysed are not regulated within the guideline used here for comparative purposes, among them acesulfame K, which was found in 29 of 30 samples above the level of reporting. Further details can be found in Appendix A1 and A2.

As a general conclusion, it can be confidently stated that only a very small number of pharmaceuticals and personal care products have been detected in this study at concentrations that are within the range of the respective guideline applicable to potable reuse applications, with caffeine being the only compound that actually exceeded those guideline values. It should be emphasized that the samples collected were from stormwater catchments that were not intended for potable reuse and that the concentrations measured present unmitigated risk, i.e. concentrations in stormwater before any treatment has occurred.

As described above, contamination of environmental waters by treated or untreated sewage has been suggested to be able to be evidenced by the presence of pharmaceuticals. While this may be qualitatively true, several difficulties are associated with a quantitative determination of sewage ingress into stormwater, as described in the following paragraphs.

Previous research from Ort *et al.* (2005) discussed the variability of micropollutant data sewage according to diurnal use patterns, exacerbated if the number of applications or doses in a catchment is low. In another publication, Ort *et al.* (2010) illustrated this experimentally taking it to the extreme of showing that single toilet flushes can be important in determining the load of a compound in sewage and impact highly on the dynamics of concentrations in sewage. Size, hydraulic retention times and

number of connected users of a sewer catchment will also impact severely on the variability of concentrations in sewage. In general, concentration variability will be lower if: the usage pattern is uniform during the day; the catchment is large with long retention times; and many connected users are stochastically evening out the use, with the latter obviously being more favourable if the compound is used by a large share of the population.

Other factors of uncertainty include excretion ratio, biodegradation or sorption of compounds, and sewage flow variations either induced by diurnal use patterns or by stormwater ingress in sewers. Data from sewage treatment plants shows that, despite prevailing separate sewer systems in Australia, it is not uncommon that sewage flow at the inlet of the sewage treatment plant during wet weather increases by 50% to 150% compared to average dry weather flow. Finally, for a number of compounds, there are uncertainties around the source specificity for several compounds. For example caffeine in stormwater could well also stem from a caffeinated soft drink spilled on the street. This is difficult to assess objectively for many compounds and Sercu *et al.* (2011) proposed tracer studies with rhodamine-WT instead to determine sewage ingress into stormwater systems. Nevertheless, such tracer studies are laborious to execute, and require accurate knowledge about the sewer network. As such an alternative method to detect sewage ingress in stormwater based on substances inherently present in sewage would be preferable.

There is little publicly available Australian data on trace contaminant concentrations in raw sewage. Some related work has been performed by Ort *et al.* (2010), LeCorre *et al.* (2012), Trinh *et al.* (2012) and further data was obtained by O'Brien and Mueller (2012). Internationally, more data has been reported and recently reviewed by Ort *et al.* (2011) and Ratola *et al.* (2012). The first noteworthy aspect is that the same chemical can be reported at concentrations differing by 10 to 100 fold between studies. This observation was based on literature from influent sampling at sewage treatment plants (i.e. fairly large catchments) and most likely will be worse for more localized sampling in the sewer system. Within individual studies, the variability observed in repeated samplings is generally lower but can still be around a factor of 10.

Generally, most trace organic compounds are typically present in sewage at concentrations lower than 1 $\mu g \cdot L^{-1}$ and, given the limits imposed by the analytical detection (typical 10-100 $ng \cdot L^{-1}$), are therefore unsuitable for source tracing at high dilution in environmental waters. Among the remaining, according to the sources cited above, acesulfame K, caffeine, paracetamol, salicylic acid and ibuprofen can be expected to be present typically in a concentration of 10-100 $ug \cdot L^{-1}$. Others like carbamazepine, cephalexin, codeine, diclofenac, gemfibrozil, iopromide, tramadol and venlafaxine, however, are typically found in the 1-10 $ug \cdot L^{-1}$ range, some of them tending towards the higher and others the lower end of those ranges. Also, for the abovementioned additional factors an even higher variability can be expected for the short-term frequency concentration upstream in a sewage catchment, where a potential ingress of sewage into the stormwater conveyance system would happen. If the expected range of concentration in sewage is divided by the level of reporting (LOR, compare Appendix A1), it will give an estimate of the level of dilution a contamination by sewage could possibly be detected through this compound, always supposing that this will be the only source of contamination. For example, if the concentration of paracetamol is expected to be 10-100 $ug \cdot L^{-1}$ and the LOR is 0.01 $ug \cdot L^{-1}$, it can be estimated that paracetamol can be detected, if the dilution is less than a factor 1000-10000 (i.e. more than 0.01-0.1% of the stormwater volume is sewage derived).

From the components in this study, the pharmaceutical and food components that seem most suitable for sewage tracing in stormwater from these calculations are:

- quantifiable at an expected dilution > 1000-10000: acesulfame K, caffeine and paracetamol – these were mostly found.
- quantifiable at an expected dilution > 500-5000: salicylic acid, venlafaxine, tramadol – salicylic found relatively frequently, venlafaxine and tramadol less so.
- quantifiable at an expected dilution > 100-1000: codeine, ibuprofen, gemfibrozil, cephalexin, iopromide – not found in stormwater with the exception of cephalexin and codeine on rare occasions.

- quantifiable at an expected dilution of approx. >50-500: diclofenac, carbamazepine – not found in stormwater in this study.

Khan (2010, chapter 6) comprehensively reviewed the practice of assessing chemical concentrations by using log normal distributions. One of the main advantages of this procedure is that values below detection limit are taken into account in a statistical correct manner. Other common practices such as assuming a non-detect as measured at 100% of the detection limit or e.g. 50% of the detection limit essentially can be conservative assumptions, which is often in the spirit of public health productions, but they can also skew probability density functions of observed concentrations and lead to wrong conclusions.

Figure 11. shows the concentrations of five compounds (acesulfame K, caffeine, paracetamol, salicylic acid and venlafaxine) plotted in a probability chart. This report followed the procedures outlined by Khan (2010) using the Blom formula (Equation 2) for assigning the plotting positions in the probability chart. The observable linear trends in the figure show that a log normal distribution can be assumed to assess and model probability density functions of the data presented.

$$p_i = \frac{i - 0.375}{n + 0.25} \qquad \text{(Equation 2)}$$

where p_i is the associated probability value of a sample, i is the sample's rank (ranked from lowest to highest value) and n the number of total observation.

Modelling the associated probability density function and cumulative distribution function (Equation 3) would then allow estimating concentrations associated to e.g. 90% percentile, 50% (median) or 25% percentile, even if those values may actually be below LOR.

$$CDF(x, \mu, \sigma) = \frac{1}{2}\left[1 + erf\left(\frac{x - \mu}{\sqrt{2\sigma^2}}\right)\right] \qquad \text{(Equation 3)}$$

where CDF is the value of the cumulative distribution function as function of x, the concentration value, μ and σ, the mean and standard deviation of the natural logarithm of the values of x, respectively.

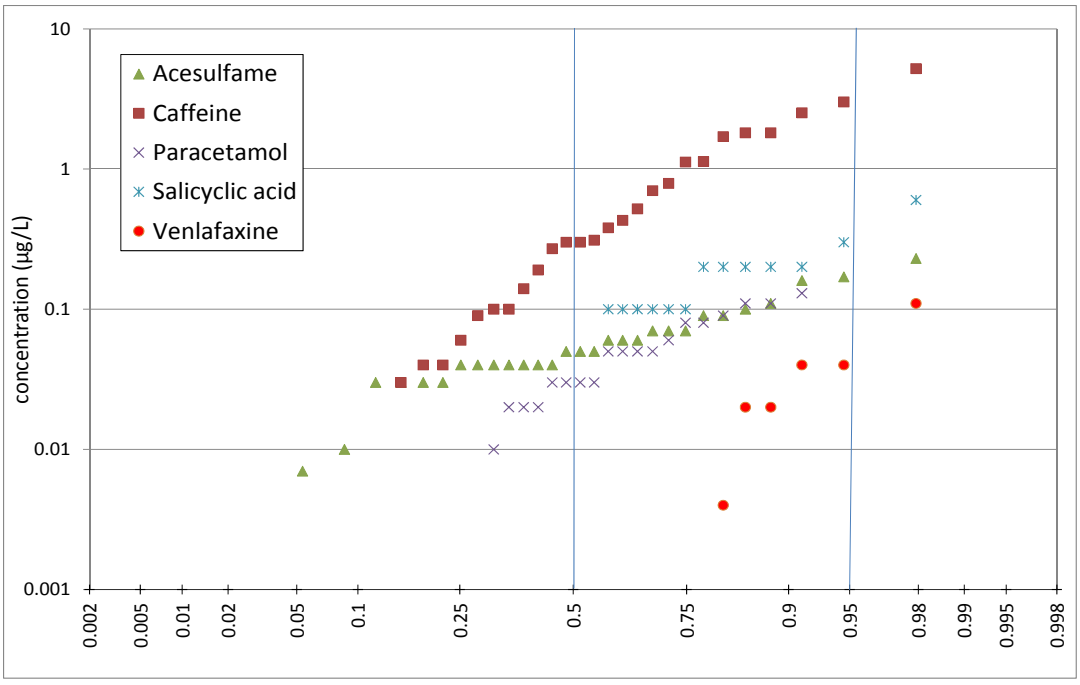

Figure 11: **Concentrations of acesulfame K, caffeine, paracetamol, salicylic acid and venlafaxine shown in a probability plot (n=30).**

In this study, CDF has been modelled by curve fitting minimizing the sum of squared errors between modelled CDF and measured concentrations. The curve fitting process has been done by fitting μ and σ simultaneously with the solver function of Microsoft Excel® applying the GRG nonlinear algorithm. These fitted CDF functions are compared to real data for caffeine, acesulfame K and venlafaxine in Figure 12. Using the modelled CDF, concentration values for different percentile (25%, 50%, 75% and 90%) have been calculated for the five substances acesulfame K, caffeine, paracetamol, salicylic acid and venlafaxine and are reported in Table 5.

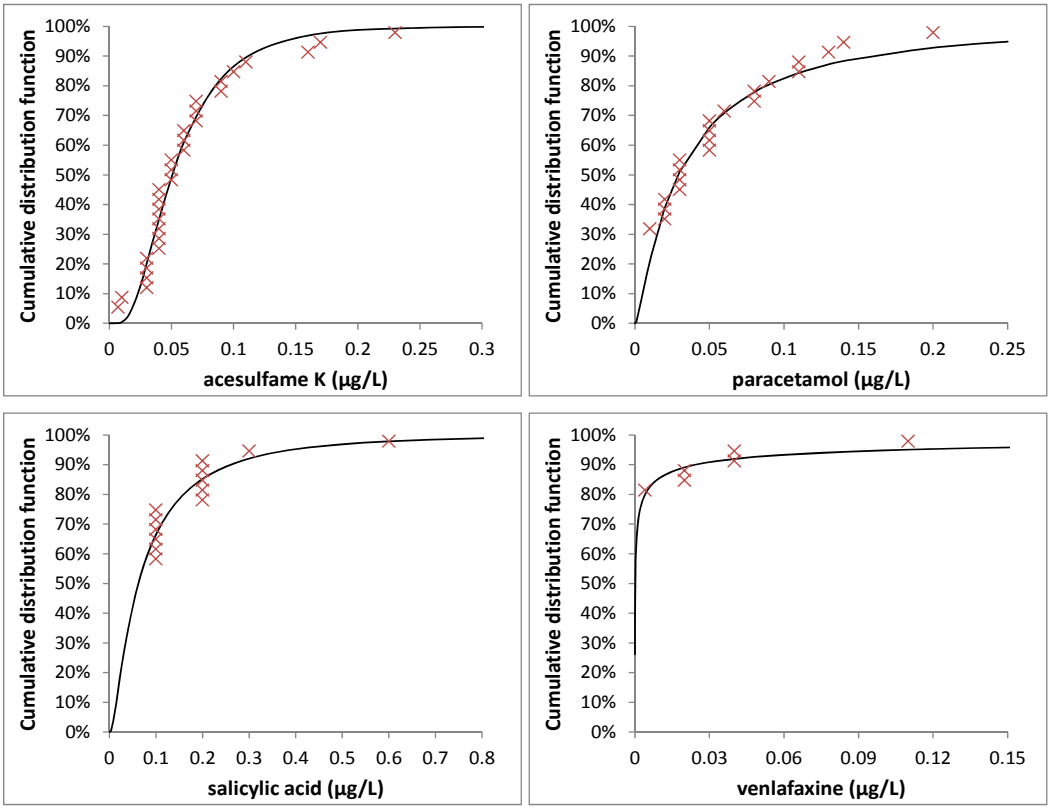

Figure 12: **Examples of cumulative distribution functions of acesulfame, paracetamol, salicylic acid and venlafaxine. The experimental data (n=30) was fitted as a log normal distribution. See text for further details.**

Table 5: **Measured and calculated concentrations for 25%, 50% (median), 75% and 90% percentile for 5 pharmaceuticals or food ingredients and possible ranges of sewage ingress.**

		n	LOR [µg·L^{-1}]	>LOR	$Q_{25\%}$ [µg·L^{-1}]	Median [µg·L$_{-1}$]	$Q_{75\%}$ [µg·L^{-1}]	$Q_{90\%}$ [µg·L^{-1}]
Measured	Acesulfame K	30	0.01	29	0.04	0.05	0.07	0.12
	Caffeine	30	0.02	26	0.07	0.30	1.04	1.88
	Paracetamol	30	0.02	21	<LOR	0.03	0.08	0.11
	Salicylic acid	30	0.1	13	<LOR	<LOR	0.10	0.20
	Venlafaxine	30	0.01	6	<LOR	<LOR	<LOR	0.02
Calculated from CDF	Acesulfame K				0.03	0.05	0.08	0.11
	Caffeine				0.07	0.25	0.96	3.2
	Paracetamol				0.01	0.03	0.07	0.16
	Salicylic acid				0.03	0.06	0.13	0.26
	Venlafaxine				0.000009	0.00013	0.002	0.024
Estimated Sewage Contribution to Stormwater					$Q_{25\%}$	Median	$Q_{75\%}$	$Q_{90\%}$
					[% contribution]			
	Acesulfame K	Min Max			0.08 – 1.5%	0.13 – 2.5%	0.20 – 4.0%	0.28 – 5.5%
	Caffeine	Min Max			0.07 – 1.4%	0.25 – 5.0%	0.96 – 19%	3 – 60%
	Paracetamol	Min Max			0.003 – 0.10%	0.010 – 0.30%	0.023 – 0.7%	0.05 – 1.6%
	Salicylic acid	Min Max			0.03 – 0.6%	0.06 – 1.2%	0.13 – 2.6%	0.26 – 5.2%
	Venlafaxine	Min Max			0.0002 – 0.004%	0.003 – 0.07%	0.05 – 1.0%	0.60 – 12%

NOTE: Possible ranges of sewage ingress were estimated assuming the following concentration ranges: acesulfame K 2-40 µg·L^{-1}, caffeine 5-100 µg·L^{-1}, paracetamol 10-300 µg·L^{-1}, salicylic acid 5-100 µg·L^{-1}, venlafaxine 0.2-4 µg·L^{-1}. A minimum % and a maximum % of sewage contamination was calculated using the higher and the lower value of these concentrations ranges for sewage, which were estimated based on data reported in the literature as well as the study by O'Brien and Mueller (2012). Refer to the text for further interpretations of the data presented as there are numerous assumptions made to calculate these values, whose validity is debated there.

Table 5 reports the concentration values calculated by applying the process outlined above, and then estimates the theoretical sewage contributions assuming ranges of concentrations in raw sewage reported in the literature, and the assumption that the source of the compounds is fully sewage derived. For example, if the median concentration of acesulfame is 0.05 µg L^{-1} and the acesulfame concentration in raw sewage is estimated to be 2 µg L^{-1} this would mean that sewage ingress is 2.5 %, whereas assuming an acesulfame concentration of 40 µg L^{-1} would lead to an estimated sewage ingress of only 0.13%.

From the table, it is apparent that caffeine seems to have additional sources to sewage, since the median concentration of caffeine detected in stormwater would indicate a median sewage contamination of stormwater of 0.25 – 5%. However, the other substances clearly point towards a lower sewage contamination. Also, a sewage contamination higher than 1% would be picked up by other parameters such as TOC, which it usually was not. For the other 4 substances shown in Table 5, the median concentrations suggest a range of 0.003 to 2.5% sewage contamination. Similarly for the 90% percentile concentrations, a range of sewage contamination of 0.05 – 12% would be suggested. When all these uncertainties are taken into account, we argue that using pharmaceutical or food ingredients concentrations in stormwater is not a very practical or accurate way to estimate sewage contamination in stormwater. Nonetheless, the presence of these substances points qualitatively towards the fact that sewage contamination of stormwater is frequently present to some degree. Also, while ascertaining source specificity is an issue, other observations such a significant correlation among paracetamol and acesulfame K concentrations support the finding that the origin of the contamination is at the very least partially from the same source and is likely to be sewage.

3.6. In Vitro Bioanalytical Tools

Bioanalytical tools have been used as first tier screening for water quality assessment ranging from sewage, treated effluent to drinking water and recycled water (Macova *et al*., 2010 and 2011, Reungoat *et al*., 2010). No studies have used bioanalytical tools to study the toxicological profile of raw urban stormwater. This information is useful for identifying the mode of action toxicity, and also to guide chemical analysis as there are too many chemicals to quantify each individually. Here, for the first time in the literature, we demonstrate how bioassays can show the temporal and spatial differences of toxicity across various sampling sites with different land use characteristics (Tang *et al*., 2012).

3.6.1. Methodology for the Bioassays

Stormwater samples were concentrated for the bioassays as outlined in section 1.1.6. A brief over view of the bioanalytical test battery is provided in the Table 2. Macova *et al.* (2010) and Escher *et al*. (2012) provide further details on the methodology.

3.6.2. Baseline Toxicity

Baseline toxicity was measured using a Microtox 30 minute assay with a marine bacteria *Vibrio fischeri*. This non-specific toxicity assay is widely recognised in the field of ecotoxicology as the standard assay to measure acute cytotoxicity (Johnson, 2005; Farré *et al*., 2006). This assay measures the relative decrease in light output from the naturally bioluminescent marine bacteria, *Vibrio fischeri*, following its exposure to a toxicant. Damage occurring at any level of cellular organisation, including the disruption of membranes, the electron transport chain, enzymes and cytoplasm composition, can result in decreased light output. Hence, the assay reflects the general "energy status" of the bacteria and can indicate the toxic potency of a broad spectrum of compounds with different modes of action. We consider this assay to represent baseline toxicity and to respond non-specifically to all compounds present in the sample. As such, it is integrative, while the other selected bioassays respond specifically to a defined mode of toxic action. Note that baseline toxicity is the underlying toxicity of every compound, and one needs to take precautions at higher effect levels of the specific bioassays that the specific effect is not influenced by non-specific baseline toxicity. The effect is expressed as baseline toxicity equivalent concentration (baseline – TEQ). Most stormwater samples in Figure 13 showed baseline toxicity around the level of secondary treated effluent reported in a previous study (Macova *et al*., 2010). Levels similar to surface water were found in Fitzgibbon samples on 22 May and 30 May 2011 as well as 25 January and 9 February 2012. The highest baseline toxicity was observed on 14 April 2012.

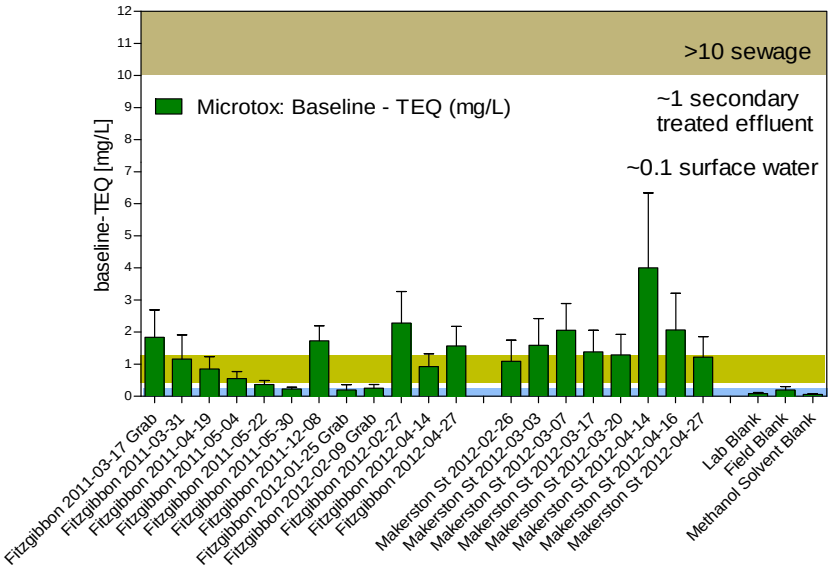

Figure 13: **Baseline toxicity measured by Microtox {Escher, 2011 #827} for Fitzgibbon (n=12) and Makerston (n=8).**

3.6.3. Phytotoxicity

Phytotoxicity was quantified via inhibition of photosystem II (PSII), a key target mode of action of herbicides. PS II herbicides disrupt the electron flow, and excitation energy is re-emitted as fluorescence rather than driving photochemical processes. The I-PAM assay is a fluorescence-based photosynthetic yield analysis technique that uses *Pseudokirchneriella subcapitata*. It has developed as a rapid tool for the detection of chemicals such as phenylurea and triazine herbicides, which bind to and inhibit PS II (Schreiber *et al*., 2007). Chemicals which directly inhibits photosynthesis can be measured by the quantum yield after two hours and the results are expressed as diuron equivalent concentration (DEQ). (Escher *et al*., 2008; Vermeirssen *et al*., 2010). The highest level was found on 17 March 2012 for Makerston St drain (Figure 14) and we suspect this is due to the runoff from the nearby Roma Street Parkland, as herbicides are unlikely to be found in urban CBD area. Fitzgibbon has levels around that of surface water and two samples in May 2011 were similar to that of secondary treated effluent.

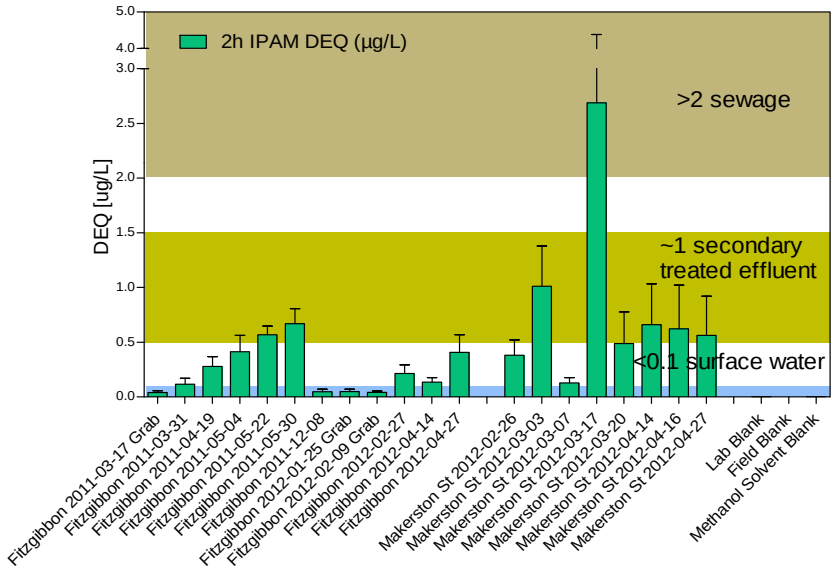

Figure 14: **Phytotoxicity measured by the IPAM test for Fitzgibbon (n=12) and Makerston (n=8).**

3.6.4. Estrogenicity

The E-SCREEN assay was developed by Soto *et al*. (1995) and modified by Körner *et al*. (1999). It responds specifically to estrogenic and xeno-estrogenic compounds that can mimic the activity of the natural female sex hormone estradiol. Similar to estradiol, pseudo-estrogens induce the proliferation of human breast cancer cells. The results of this bioassay are reported as estradiol equivalent concentrations (EEQ). Limited studies used chemical analysis or bioassays to measure the endocrine disrupting compounds in stormwater. In this study, we found one occurrence of a high level of estrogenicity at Fitzgibbon (Figure 15), which may be due to sewer overflow or contamination. Microbial analysis from this study (discussed in Section 3) also found human sewage bacterial indicators in Fitzgibbon, suggesting that together with these markers we can use these tools as source tracking in stormwater drains.

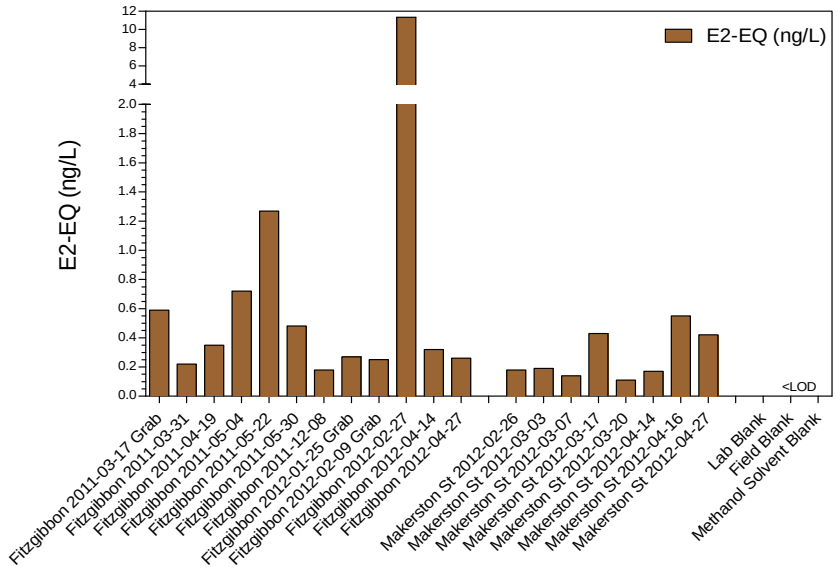

Figure 15: Estrogenicity measured by the E-SCREEN test for Fitzgibbon (n=12) and Makerston (n=8).

3.6.5. Genotoxicity

Low levels at or below detection limits were found for the genotoxicity test, using either metabolic activation with rat S9 enzyme or without metabolic activation (Figure 16). These agree with the chemical analysis results showing low levels of polyaromatic hydrocarbons (PAH).

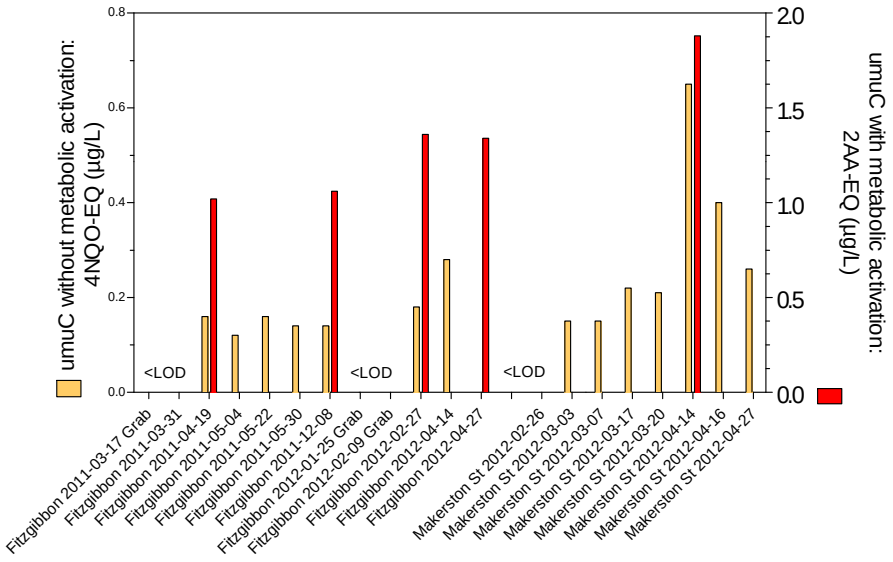

Figure 16: Genotoxicity measured by the umuC test with and without metabolic activation for Fitzgibbon (n=12) and Makerston (n=8).

3.6.6. Ah Receptor Binding

The AhR-CAFLUX (Chemically Activated FLUorescent gene eXpression) assay was developed by the Denison group (Nagy *et al*., 2002; Zhao and Denison, 2004). It responds specifically to compounds that bind to the arylhydrocarbon receptor (AhR), such as dioxin-like compounds. The AhR-CAFLUX assay makes use of a rat hepatoma cell line (H4IIE) stably transfected with a vector containing the gene of an enhanced green fluorescent protein (pGreen1.1) under the control of the dioxin-responsive elements (DREs) (Zhao and Denison, 2004). Upon binding, the AhR-ligand complex is activated and translocated to the nucleus where it specifically binds to dioxin-responsive elements and induces or inhibits the transcription of genes under control of DREs. In this study the sample extracts tested in the AhR-CAFLUX assay was without acid silica gel clean-up, which would otherwise destroy all but persistent compounds such as polychlorinated dibenzodioxins/furans and biphenyls. Therefore, the TCDDEQ reported in this study represented the sum of all compounds present in the sample that can bind to the arylhydrocarbon receptor, i.e., the persistent compounds as well as the less persistent group e.g. polycyclic aromatic hydrocarbons (PAHs). Similar to previous genotoxicity results, only low levels of dioxin-like compounds were found in this bioassay (Figure 17).

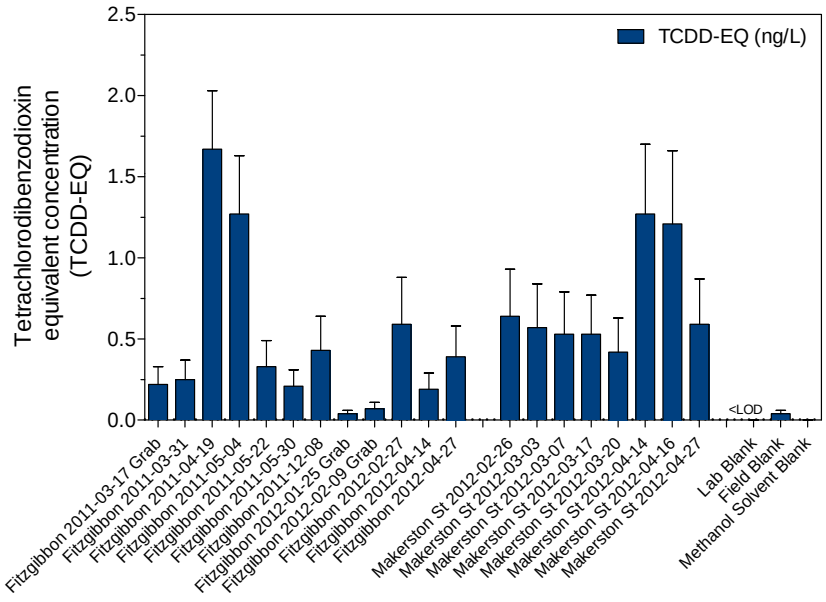

Figure 17: Ah receptor binding measured by the AhR CAFLUX test for Fitzgibbon (n=12) and Makerston (n=8).

3.6.7. Oxidative Stress

We recently established a reporter gene assay AREc32 based on the induction of the Nrf2 mediated oxidative stress response pathway (Escher *et al*., 2012). The Nrf2-ARE pathway is responsive to many chemicals that cause oxidative stress, among them a large number of pesticides and skin irritants. The results are expressed as tert-butylhydroquinone equivalent concentration (tBHQ-EQ). All samples showed responses similar to a surface water. The current results (Figure 18) demonstrated the applicability of the AREc32 bioassay to be applied for water quality assessment which serves the endpoint as an early indicator of oxidative stress.

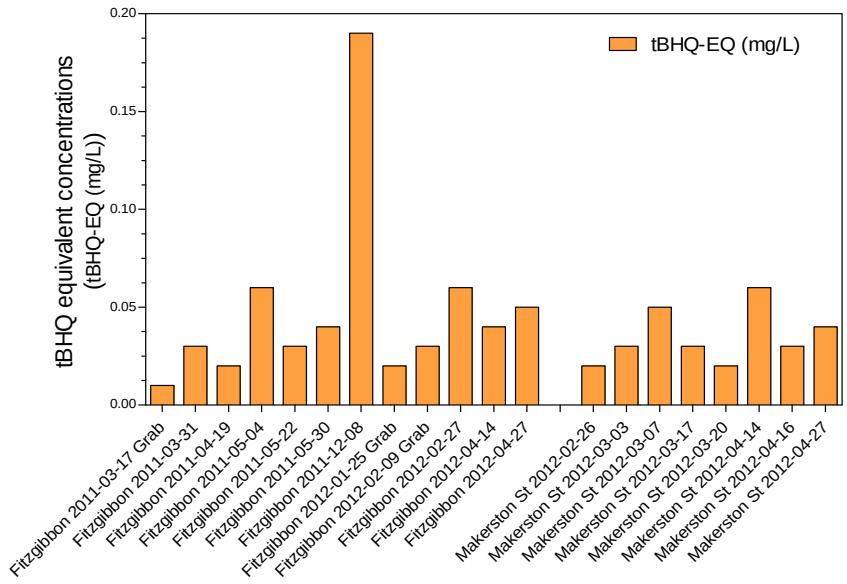

Figure 18: tBHQ equivalent concentrations (tBHQ-EQ) for AREc32 bioassay for Fitzgibbon (n=12) and Makerston (n=8).

4. MICROBIAL QUALITY OF STORMWATER

Human enteric pathogens can find their way into stormwater and subsequently surface water through leaking sewer systems, sewer pumping station overflows, seepage from septic systems, agricultural runoff and discharge of treated wastewater into aquatic environments (Gaffield *et al*., 2003; Noble *et al*., 2006; Rajal *et al*., 2007). High numbers of enteric viruses, bacteria and protozoa have been reported in stormwater runoff indicating the presence of sewage pollution (Noble *et al*., 2006; Rajal *et al*., 2007; Sercu *et al*., 2009; Cizek *et al*., 2008). Concerns over this issue are heightened by the reported links between the occurrence of storm events, microbial inputs from non-point sources and increase in the incidence of waterborne diseases (Curriero *et al*., 2001; Gaffield *et al*., 2003). Thus, it is important to understand the presence and environmental loadings of pathogens in the receiving waters, especially during and after storm events. Hence, monitoring for faecal pollution in stormwater is required to make an assessment of health risks from exposure to harvested stormwater and the extent of treatment required prior to its use as alternative water.

The aims of this section are to:

(i) conduct a preliminary survey of the presence of enteric bacterial and viral pathogens in the stormwater runoff in Brisbane, Australia;

(ii) develop an understanding of the extent of increase in pathogens and indicator numbers in surface water bodies after the storm event; and

(iii) determine the involvement of a human sewage as potential source of contamination by using human specific MST techniques.

4.1. Baseline Pathogens and Indicators

Recent advances in molecular techniques have made it possible to rapidly and easily detect enteric pathogens in the surface water (Rajal *et al*., 2007; Muscillo *et al*., 2008, Dyke *et al*., 2009; Mull and Hill 2009). Thus direct surveillance for pathogens such as enteric viruses in addition to faecal indicator bacteria (FIB) may be a better approach for risk identification and protection of public health. Human adenovirus and polyomavirus were reported to be present in higher numbers (10^5 to 10^6 L^{-1}) in the wastewater and also found to be highly prevalent in rivers and coastal waters (Muscillo *et al*., 2008, Bofil-Mas *et al*., 2006; Jiang, 2006). Thus by inference these viruses may be suitable indicators of human specific enteric viral pollution in the stormwater runoff. However, very limited information is available on the presence of adenovirus, polyomavirus and bacterial pathogens of concern such as *Campylobacter* spp. and *Salmonella enterica* in stormwater runoff. Recently, microbial source tracking (MST) methods have been developed to identify potential sources of stormwater contamination (Parker *et al*., 2010; Sauer *et al*., 2011). Use of MST techniques in conjunction with monitoring for pathogens and indicators has the potential to provide information on the extent of faecal pollution and potential sources of contamination.

4.1.1. Material and Methods

4.1.1.1 Study Sites

Six sites in Brisbane (Brisbane River, Oxley Creek, Cabbage Tree Creek, Fitzgibbon site, Enoggera Creek and Pine River in Samford) were selected to determine microbial contaminant numbers in the base-flow and in stormwater runoff from November 2010 to May 2011. The sites were selected to cover a broad range of land use and urbanisation and site description along with GPS coordinates are provided in Table 6. In this study, two samples were collected from each site during the dry period (no rain within 48 hours prior to sampling) and two samples were collected after >10 mm precipitation.

Table 6: **Sampling sites description and location around Brisbane.**

Site Name	Description	GPS Coordinates
Cabbage Tree Creek	Medium density urban residential and industrial developments, serviced by a wastewater treatment plant.	27º20´59.7"S; 153º02´06.6"E
Fitzgibbon site	Low density urban areas, some animal faecal input from cattle, horses and other domestic animals.	27º20´08.7"S; 153º.01´14.5"E
Oxley Creek	Major tributary of Brisbane River, industrial area close by, medium density Urban population, serviced by a wastewater treatment plant.	27º32´07.8"S; 152º59´31.4"E
Enoggera Creek	Moderately populated urban area, some animal faecal input from cattle, horses and other domestic animals.	27º26´41.96"S; 152º57´16.90"E
Samford site	Rural area with large block size, animal faecal input such as cattle, horses and sheep	27º22´37.45"S; 152º.59´54.1"E
Brisbane River	Stormwater drain outlet from urban area, dilution effect due to tidal influence.	27º28´50.05"S; 152º.59´53.84"E

4.1.1.2 Stormwater Sample Collection and Concentration

Approximately 20 L water samples were collected in sterile carboy containers (Nalgene) and transferred to the laboratory for storage at 4°C prior to processing. Collected samples were concentrated for the detection of enteric pathogens which are often found in low numbers within six hours of collection by using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA, USA) as previously described by Hill *et al*., (2005) (Figure 19).

Figure 19: Stormwater sample concentration set up using Hemoflow HF80S dialysis filter.

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 were cleaned and disinfected by soaking in 1% bleach followed by washing and then sterilized by autoclaving. At the end of the concentration process, pressurised 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 100K MWCO filters (Pall, Australia) to a final volume of approximately 10 mL.

4.1.1.3 Quantification of Faecal Indicator Bacteria

Quantification of faecal indicator bacteria (FIB) (*E. coli* and *Enterococcus* spp.) was performed by the membrane filtration technique. 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) and *Enterococc*us 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^{-1}.

4.1.1.4 Molecular Detection of Pathogens and Indicators

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 prior to analysis. Nested PCR was used for the detection of pathogens from concentrated water samples. Briefly, first amplification was carried out with 5 µL of DNA template extracted from the sample with primer sets followed by detection of target genes with TaqMan probes from 1 µL of amplified product. Adenovirus, polyomavirus, *Salmonella enterica*, *Campylobacter* spp. and *Bacteroides* HF183 gene were detected with the published primer and probe sets described in Appendix B1.

4.1.1.5 PCR Standards

Standards for PCR amplification were prepared from the genomic DNA of standard cultures of *C. jejuni*, *C. coli, Salmonella enterica* serovar Typhimurium, human adenovirus type 41, and human polyomavirus (JC). Concentration of the standard adenovirus genomic DNA was measured by using a NanoDrop ND-1000 spectrophotometer. After calculation of genomic copy numbers, a serial 10-fold dilution (10^6 to 10^0 DNA copies μL^{-1}) was prepared. The aliquots of these serial dilutions were stored at –80°C for subsequent use. To determine the potential presence of PCR inhibitory substances in the DNA extracted from water samples, each sample was spiked with 10^3 gene copies of adenovirus. The cycle threshold (C_T) values obtained for stormwater samples spiked with adenovirus were compared to those of MilliQ water (control) spiked with adenovirus DNA.

4.1.1.6 PCR Amplification

PCR reactions were performed on a Bio-Rad iQ5 (Bio-Rad Laboratories, California, USA), using iQ supermix (Bio-Rad) or Sso Fast™ EvaGreen® Supermix (Bio-Rad). For the first round PCR, 12.5 µL of iQ Supermix, 200 nM of each primer, and 5 µL of template DNA was used for all target microorganisms. A second round of nested PCR was carried out with 10 µL of Sso Fast EvaGreen® Supermix, 200 nM of each primer and 1 µL of template DNA for the detection of *Bacteroides* HF183, *esp* gene, and *Salmonella enterica*. Melt curves were also added after the PCR step to eliminate false-positive results. Whereas, the second round of nested PCR detection of adenovirus, polyomavirus, *Campylobacter* spp., *C. jejuni, and C. coli* was performed using 12.5 µL of Supermix, 350 nM of each primer, 250-350 nM corresponding TapMan probes and 1 µL of template DNA from the first round of PCR amplification with iQ Supermix. Bovine serum albumin (BSA) was added to each reaction mixture to a final concentration of 0.2 µg μL^{-1} to reduce PCR inhibition (Kreader, 1996). For each PCR run, a corresponding positive (i.e., target DNA) and negative (sterile water) controls were included. Thermal cycling conditions for PCR reactions to detect microorganism are presented in Appendix B1.

4.1.1.7 Statistical Analysis

Prior to the statistical analysis, all determined FIB numbers were Log_{10} transformed. A student's *t-test* was performed to compare *E. coli* and *Enterococcus* spp. numbers during the wet and dry conditions. The critical *P*-value for the *t-test* was set at 0.05 and all tests were considered significant if the *P* value was < 0.05. Pearson correlation analysis was carried out on the Log_{10} transformed FIB numbers to determine the existence of correlation between *E. coli* and *Enterococcus* spp. during dry and wet weather.

4.1.2. Results

4.1.2.1. FIB Numbers in Collected Water Samples

Collected samples were analysed for FIB numbers, and the results were plotted after $\log_{10}$ transformation of raw data (Figure 20). Mean of $\log_{10}$-transformed numbers from dry and wet weather samples from all sites were compared. Both *E. coli* and *Enterococcus* spp. numbers during the wet period were significantly higher ($P<0.05$) than the dry period. The combined mean of *E. coli* numbers from all sites was higher (3.54 log $100mL^{-1}$) during the wet period than the dry period (2.32 log $100mL^{-1}$). Similarly, the combined mean of *Enterococcus* spp. numbers from all sites was 2.43 and 3.58 log $100mL^{-1}$ respectively (Figure 20). In general, *E. coli* numbers from all sites varied between 8 $x10^1$ to 1 $x10^3$ $100mL^{-1}$ between dry and wet period whereas, corresponding numbers for *Enterococcus* spp. varied between 4 $x10^1$ to 3 $x10^4$ $100mL^{-1}$ (Table 7). A better correlation ($R^2 = 0.84$) between *E. coli* and *Enterococcus* spp. numbers was found during the dry period when data from all six sites was pooled. Whereas, a better correlation was observed during the dry period compared to wet period ($R^2 = 0.42$). Samples collected from Cabbage Tree Creek and the Fitzgibbon site have significantly higher ($P<0.05$) counts of both *E. coli* and *Enterococcus* spp. after the storm events compared to the other four Brisbane sites tested.

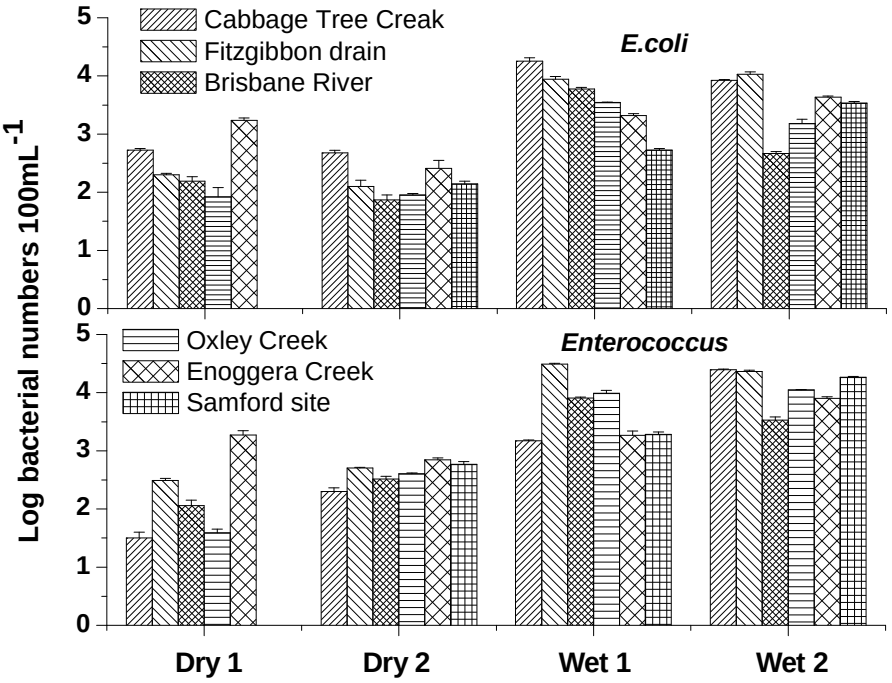

Figure 20: *E. coli* and *Enterococcus* spp. numbers during two dry and wet sampling events at six sites across Brisbane.

4.1.2.2. PCR Inhibition Assessment

To test for the PCR inhibitory effect, 10^3 gene copies of adenovirus were seeded into the extracted DNA from the water samples. Variable degree of PCR inhibition was observed in nearly all samples, with samples collected from Brisbane River showing the least inhibition and samples collected from the Enoggera and Fitzgibbon sites showing highest inhibition with no amplification of seeded adenovirus at 100 fold dilution of extracted nucleic acid (data not shown). To improve detection limit in the presence of PCR inhibition, nested PCR amplifications were carried out to detect presence of pathogens in the collected water samples.

4.1.2.3. Pathogen Detection in Water Samples

All samples tested positive for the presence of *Campylobacter* genus specific primer set. After further investigation the presence of *C. jejuni* or *C. coli* was detected in 17 and 12 samples respectively, out of the 22 samples (Table 7). Some samples also tested for the presence of both *Campylobacter* spp. *Salmonella* spp. was also detected in eight samples primarily after the storm events. Human specific adenovirus and polyomavirus were also detected in the collected water samples after the storm events. Adenovirus was detected more frequently (15 samples) than polyomavirus (12 samples) during the dry and wet weather.

4.1.2.4. Human Sewage Specific Markers Detection in Water Samples

Human-specific *Bacteroides* HF183 marker was widely detected in 16 samples out of the 21 tested, during both dry and wet weather conditions at all six sites. Whereas, *E. faecium esp* gene was found in only eight samples out of the 23 tested. Unlike HF183, the *esp* gene was found to be absent in the water samples, even those containing high FIB count and enteric virus after the storm events (Table 7).

Table 7: **PCR analysis of collected water samples for pathogens and human faecal markers.**

Site/Event	*E. coli* (100 mL^{-1})*	*Enterococcus* spp. (100 mL^{-1})*	*Campylobacter* spp.	*C. jejuni*	*C. coli*	*Salmonella enterica*	Adenovirus	Polyomavirus	*esp*	HF183	Rainfall (mm)
EC dry	1.73×10^3	1.93×10^3	NT	NT	NT	NT	NT	NT	-	NT	0
EC dry	2.83×10^2	7.10×10^2	+	-	-	-	+	NT	-	+	0
EC wet	2.10×10^3	1.90×10^3	+	-	-	-	+	-	+	+	21.4
EC wet	4.33×10^3	8.00×10^3	+	+	-	+	+	+	+	+	18.4
CC dry	4.83×10^2	2.03×10^2	+	+	+	-	NT	NT	-	+	0
CC dry	5.33×10^2	4.57×10^2	+	+	+	+	+	+	-	+	2.6
CC wet	8.37×10^3	2.50×10^3	+	+	+	+	+	+	-	+	19.4
CC wet	1.82×10^4	1.48×10^3	+	+	-	-	NT	NT	+	NT	134.6
FD dry	3.60×10^2	5.10×10^2	+	+	-	-	NT	NT	-	+	0.4
FD dry	1.33×10^2	1.27×10^2	+	+	-	-	NT	NT	-	-	2.6
FD wet	8.93×10^3	2.23×10^3	+	-	+	-	+	-	-	+	19.4
FD wet	1.07×10^4	3.11×10^3	+	-	+	+	+	+	+	+	134.8
BR dry	1.60×10^2	1.20×10^2	+	-	-	-	+	+	-	-	0
BR dry	7.67×10^1	3.30×10^2	+	+	-	-	+	+	-	-	0
BR wet	6.00×10^3	8.10×10^3	+	+	-	-	+	+	+	+	4.6
BR wet	4.67×10^2	3.43×10^3	+	-	+	+	+	-	+	+	10.2
OX dry	9.33×10^1	4.00×10^1	+	-	+	-	-	-	-	-	0
OX dry	9.00×10^1	4.03×10^2	+	+	-	+	-	-	+	+	0
OX wet	3.50×10^3	9.93×10^3	+	+	+	-	+	+	-	+	7
OX wet	1.57×10^3	1.12×10^1	+	+	+	-	+	NT	-	+	15
SS dry	1.40×10^2	5.93×10^2	+	+	+	-	-	-	-	-	0
SS wet	5.33×10^2	1.93×10^3	+	+	+	-	+	-	+	+	40
SS wet	3.43×10^3	1.84×10^4	+	+	+	+	+	-	-	+	33.2

EC= Enoggera Creek; CC= Cabbage Tree Creek; FD= Fitzgibbon drain; BR= Brisbane River; OX= Oxley Creek; SS= Samford site * = Mean bacterial counts: NT = sample not tested

4.1.3. Discussion

In this study, we tested surface water samples collected during the dry period and after storm event for the presence of traditional FIB along with selected human pathogens and faecal contamination markers with PCR. This approach could improve our ability to identify and prioritize sources that contribute pathogens to surface waters.

Enterococcus spp. numbers detected in water samples collected from all sites during the dry period were generally higher than the recommended limits for category D (<501 *Enterococci* per 100 mL^{-1}) under Australian guidelines for managing risks in recreational water, and higher by several orders of magnitude after the storm events (NHMRC, 2008). High FIB numbers observed after the rain events were also similar to what had been previously reported in the literature (Brownell *et al.*, 2007; Parker *et al*., 2010).

The amount of rainfall prior to sampling appeared to have some influence on the FIB numbers at the Cabbage Tree Creek and Fitzgibbon sites as the highest numbers of *E. coli* and *Enterococcus* spp. were observed after the highest rainfall event (135 mm), which was several fold higher than the other wet periods (20 mm). In contrast, Parker *et al*., (2010) found no link between the FIB numbers and the amount of precipitation. The increase in FIB numbers observed after the storm event could be due to the movement of fresh faecal pollution from sewage leakage and animal sources into the surface water. In addition, the storm event might also have stirred up creek sediments which could have led to the re-suspension of bacteria bound to particles. A significant proportion of bacteria (15 to 30%) in the stormwater have been reported to be attached to particulate matter (Cizek *et al*., 2008; Krometis *et al*., 2010). The sediments in the stormwater conveyance system may also act as reservoir for enteric pathogens, hence better understanding of pathogen presence and persistence in the sediments is required for a proper health risk assessment. In general, *Enterococcus* spp. numbers observed in this study was higher than the *E. coli* at all sites. This is in agreement with previously reported numbers of *Enterococcus* spp. in stormwater runoff (Parker *et al*., 2010; Krometis *et al*., 2010).

Campylobacter spp. was detected in all water samples collected, showing their wide prevalence at all sites. The *Campylobacter* spp. specific primer set used in this study primarily detect *C. jejuni*, *C. coli* and *C. lari* (Lund *et al*., 2004) which are known to cause Campylobacteriosis in humans. There are a number of documented evidences on the extensive presence of *Campylobacter* spp. in surface water (Savill *et al*., 2001; Dorner *et al*. 2007; Dyke *et al*., 2009). This is not unexpected as, apart from human input, domestic animals and birds are known reservoirs of *Campylobacter* genus (Dyke *et al*., 2009). We also detected the presence of *C. jejuni* and *C. coli* which are known to cause human infections. *Salmonella enterica* was found to be less prevalent than *Campylobacter* with only seven positive samples out of the 22 tested. However, *Salmonella* was detected more frequently in the stormwater runoff than in the water samples collected during the dry weather. A similar increase in the prevalence of *Salmonella* after storm events has been previously reported (Krometis *et al*., 2010). Apart from the human faecal input from the sewage leakages, domestic animals such as cattle, dogs, horses, sheep, poultry and birds may also contribute to bacterial pathogen loads in the stormwater runoff (Dyke *et al*., 2009; Lemarchand and Lebaron, 2003).

Human adenovirus and polyomavirus were detected in the stormwater runoff and dry weather samples from all sites, suggesting a wide prevalence of human faecal contamination in these waterways. Adenovirus was found to be more prevalent than polyomavirus, which was mostly detected in water samples collected after the storm events. This corroborates with previous findings on the presence of human adenovirus and polyomavirus in the surface water and stormwater (Sauer *et al*., 2011; Hamza *et al*., 2009; Muscillo *et al*., 2008; Rajal *et al*., 2007). They are also known to be present in high numbers (10^5 to 10^6 L^{-1}) in wastewater (Bofill-Mas *et al*., 2006). Both human adenovirus and polyomavirus numbers in surface water and stormwater have been reported in the range of 1x 10^2 to 10^5 L^{-1} (Sauer *et al*., 2011; Hamza *et al*., 2009; Muscillo *et al*., 2008). Hence, the presence of these viruses in the environment is not unexpected Thus, if there is sewage contamination, then there is a high probability of adenovirus and polyomavirus being present in the stormwater runoff. The detection of adenovirus and polyomavirus is also an indication that other human pathogens including protozoa could be in the water, further increasing the potential health risks. The potential use of adenovirus and

polyomavirus as indicators of human faecal contamination needs to be further investigated as they can provide a better health risk assessment than faecal indicators due to their exclusive human source.

E. faecium surface protein gene (*esp*) and human specific *Bacteroides* HF183 gene, have been previously used to detect the presence of human faecal pollution in aquatic environment with a high degree of reliability (Scott *et al*., 2005; Seurink *et al*., 2005). In this study, human specific *Bacteroides* HF183 genetic marker was detected in all stormwater runoff samples and frequently in the water samples collected during the dry period, again confirming the ubiquitous presence of sewage pollution in the urban environment. Prevalence of faecal pollution in surface water from urban catchments is reported to be wide spread in the literature (Sauer *et al*., 2011; Brownell *et al*., 2007). The relatively low prevalence of the *E. faecium esp* gene compared to the *Bacteroides* HF183 gene in the water samples could be due to its low prevalence in sewage (Lund *et al*., 2006; Scott *et al*., 2005).

The wide spread prevalence of human specific faecal markers along with human adenovirus and polyomavirus in the water samples collected during the dry period suggests that sources other than sewage, such as point sources and sediments, may act as reservoir for pathogens. Sediment carried by stormwater has been shown to contain pathogens and FIB which are reported to survive for longer period of time in the sediments (Cizek *et al*., 2008; Haller *et al*., 2009). The results of this study also suggested that the presence of high numbers of FIB in surface water after the storm events cannot be attributed to non-human sources alone, as human enteric pathogens were also found at these sites. This observation has important ramifications for stormwater runoff management, as testing for pathogens such as human adenovirus at known "FIB hot spots" may be a more effective approach for rigorous health risk assessment as well as identification and abatement of sources of contamination.

Molecular methods offer the opportunity for accurate and rapid detection of a wide range of pathogens which could be used as alternative indicators for risk assessment from stormwater runoff. However, concentration of water samples is often required to enable detection of pathogens which are intermittently present, often in low numbers. This concentration step also results in the co-purification of PCR inhibitory compounds, which can then impact on the quantification of pathogens with PCR. The approach used in this study appears to provide good results for the first tier assessment (ie present/absent) of pathogens of concern in the stormwater. However, its suitability for quantitative PCR detection of pathogens needs to be validated. Some techniques such as virus adsorption and elusion from charged membranes have been reported to perform better than the concentration approach using ultrafiltration due to very limited co-extraction of PCR inhibitors (Katayama *et al*., 2002; Hamza *et al*., 2009). However, this extraction approach is not suitable for the detection of bacterial and protozoan pathogens. Hence, a careful consideration is required in determining the volume of water to be concentrated, as well as PCR methodologies used for the detection of pathogens of interest.

4.1.4. Conclusions

The presence of human specific bacteria and viruses in the stormwater suggests that aging sewage infrastructure combined with numerous non-point pollution sources present a significant risk of human faecal contamination of stormwater. The results of this study suggest that in addition to monitoring for FIB, testing for specific pathogens and faecal pollution markers with sensitive and specific approaches would improve our ability to detect pathogens and improved health risk assessments which are based on pathogen prevalence. This study has shown that the use of simple and rapid molecular detection methods can provide additional information on health risks of urban stormwater runoff. Further research is needed to determine the prevalence of other enteric pathogens in stormwater runoff, especially enteric viruses and protozoa and any correlation to alternative indicators along with a quantitative assessment of human health risks.

4.2. Enteric Pathogens and Indicators in Stormwater Runoff

Contamination of stormwater with enteric pathogens result from point and non-point source such as leaking sewer systems, sewer pumping station overflows, seepage from septic systems and agricultural runoff (Gaffield *et al*., 2003; Noble *et al*., 2006; Rajal *et al*., 2007). There are a number of reasons for the variation in pathogen numbers across catchments which include specificities of the catchments, event peaks and seasons. It is important to quantify pathogen numbers during both the baseline and peak flows during storm events. Peak pathogen loads after heavy rainfall events have been linked to disease outbreaks (Curriero *et al*., 2001; Signor *et al*. 2005). Hence, understanding of the loadings and extent of variations in enteric pathogen in stormwater runoff across catchments with different land use patterns and sources of contamination is important, as it will influence the extent of treatment required and the health risk associated with stormwater reuse.

In this study, we selected six catchments from different geographical areas across Australia; these catchments differed with respect to the size of their drainage area, impervious area and land use. We aimed to determine the extent of presence of human pathogens and variation in pathogen numbers across catchments. The specific aims were to:

(i) determine the presence of enteric bacterial, viral and protozoan pathogens in the stormwater runoff from six catchments across Australia;

(ii) develop an understanding of the magnitude of the increase in pathogens and indicator numbers after storm event; and

(iii) determine if prevalence of enteric pathogens is catchment specific.

4.2.1. Materials and Methods

4.2.1.1. 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 Reserve (south of Melbourne) and Ku-ring-gai (north of Sydney), represented medium density residential catchments covering 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 reamaining three sites, Makerston Street, (Brisbane), Hornsby (Sydney) and Smith Street, (Melbourne) are located in the business districts, and covers high density commercial areas. The Makerston Street catchment covers a total area of 30.1 ha, Hornsby 1.08 ha and Smith Street 10 ha. Impervious area in each of these catchments is > 90%.

4.2.1.2. Stormwater Sample Collection and Concentration

Flow-proportional sampling was carried across all six sites as mentioned previously (Section 2.3). Collected stormwater samples were concentrated with Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA, USA) as previously described in section 4.2.3 and shipped to CSIRO Brisbane Laboratory with overnight courier on freezer blocks.

4.2.1.3. Pathogen and FIB Quantification

Selected group of enteric pathogens and FIB were quantified from collected stormwater as out lined previously under Sections 4.1.2.3 and 4.1.2.4.

4.2.2. Results

4.2.2.1. Stormwater Quality at Fitzgibbon Drain

All eight samples from Fitzgibbon drain had high numbers of FIB, which varied from 6.66 x 10^2 to 1.07 x 10^4 100mL^{-1} for *E. coli* and 1.08 x 10^4 to 2.36 x 10^5 100mL^{-1} (ten-fold higher) for *Enterococci* spp. (Table 8). *Campylobacter* spp. were detected in seven out of eight samples tested. *S. enterica* was detected in eight out of nine samples tested at Fitzgibon Drain. Human adenovirus and polyomavirus were detected in nine and eight samples respectively out of nine samples tested. *Giardia lamblia* was detected with PCR in two samples. Sewage contamination markers, *esp* and HF183, were detected in all stormwater samples analysed. In addition, *Enterococci* spp. *esp* gene was analysed initially and three samples out of four tested were positive. In the subsequent sampling events, *M. smithii nifH* gene was detected in four samples out of six analysed. Microscopic examination for the detection of *G. lamblia* in PCR positive samples did not yield any information due to the interference of particulate matter which accumulated during the concentration of stormwater samples with Hemoflow HF 80S filters.

Table 8: Microbiological analysis undertaken on the stormwater samples collected from the Fitzgibbon Drain.

Sample ID	*E. coli* (100 mL^{-1})	*Enterococci* (100 mL^{-1})	*Campylobacter* spp.	*Salmonella*	Adenovirus	Polyomavirus	*esp*	HF183	*nifH*	*G. lamblia*
RA12	8.93 x 10^2	2.23 x 10^4	+	+	+	+	+	+	+	-
RA13	1.07 x 10^4	3.11 x 10^4	+	+	+	+	+	+	+	+
RA14	8.93 x 10^3	2.36 x 10^5	+	+	+	+	+	+	+	-
RA16	4.73 x 10^3	1.75 x 10^4		+	+	+	ND	+	+	+
RA17	3.60 x 10^3	1.67 x 10^4	-	-	+	+	ND	+	+	-
RA18	3.00 x 10^2	1.81 x 10^4	+	+	+	+	ND	+	-	-
RA19	6.66 x 10^2	3.92 x 10^3	+	+	+	+	ND	+	-	-
RA110	1.03 x 10^3	1.08 x 10^3	+	+	+	-	ND	+	+	-
RA111	3.56 x 10^3	1.18 x 10^4	+	+	+	+	ND	+	+	-

ND= not done; **+** = detect; **-** = non detect.

4.2.2.2. Stormwater Quality at Makerston Street Site

All tested samples drain had high *E. coli* and *Enterococci* spp, which varied from 3.46 x 10^3 to 6.66 x 10^3 100mL^{-1} for *E. coli* and 1.26 x 10^3 to 8.33 x 10^3 100mL^{-1} (Table 9). *Campylobacter* spp. was detected in all six samples tested. *S. enterica* was detected in four out of five samples tested. Human adenovirus and polyomavirus were detected in five and four samples respectively out of five samples tested. All five samples were also tested for the presence of norovirus and enterovirus and both were not detected. *Giardia lamblia* was detected with PCR in two samples. Sewage contamination markers HF183 and *nifH* gene were detected in all stormwater samples analysed. *Cryptosporidium parvum* was not detected in any of the samples tested with PCR.

Table 9: Microbiological analysis done on the stormwater samples collected from the Makerston Street.

Sample ID	*E. coli* (100 mL^{-1})	*Enterococci* (100 mL^{-1})	*Campylobacter* spp.	*Salmonella*	Adenovirus	Polyomavirus	*Norovirus*	Enterovirus	HF183	*nifH*	*C. parvum*
RA81	6.66 X 10^3	1.8 X 10^3	+	-	+	-	-	-	+	+	-
RA82	4.56 X 10^3	4.1 x 10^3	+	+	+	+	-	-	+	+	-
RA87	6.06 X 10^3	1.26 x 10^3	+	+	+	+	-	-	+	+	-
RA88	3.46 X 10^3	8.33 x 10^3	+	+	+	+	-	-	+	+	-
RA89	3.60 X 10^3	5.5 x 10^3	+	+	+	+	-	-	+	+	-

ND= not done; **+** = detect; **-** = non detect.

4.2.2.3. Stormwater Quality at Smith Street Site

Composite stormwater samples (20-40 L) collected on nine separate occasions were analysed at CSIRO Laboratory in Brisbane. All samples from Smith Street site had high numbers of FIB, which varied from 1.70 x 10^3 to 8.33 x 10^4 100mL^{-1} for *E. coli* and 1.48 x 10^4 to 1.52 x 10^5 100mL^{-1} (ten-fold higher) for *Enterococci* spp.(Table 10). *Campylobacter* spp. were detected in the range of 8.17 x 10^1 to 1.08 x 10^3 pdu (PCR detectable units) L^{-1}. Human adenovirus was detected in all samples with numbers ranging from 1.81 x 10^2 to 1.34 x 10^3 pdu L^{-1}. *Giardia lamblia* was not detected with PCR in three samples analysed. Sewage contamination markers *esp* and HF183 were detected in all stormwater samples analysed. In addition, *nifH*, another sewage contamination marker, was detected in last two samples analysed for.

Table 10: Microbiological analysis done on the stormwater samples collected from the Smith Street.

Sample ID	*E. coli* (100 mL^{-1})	*Enterococci* (100 mL^{-1})	*Campylobacter* (L^{-1})	Adenovirus (L^{-1})	*esp*	HF183	*nifH*	*G. lamblia*
RA21	1.70 x 10^3	1.60 x 10^4	3.78 x 10^2	1.38 x 10^3	+	+	ND	ND
RA22	1.90 x 10^3	1.48E+04	8.78 x 10^1	6.06 x 10^2	+	+	ND	ND
RA23	ND	ND	1.08 x 10^3	1.34 x 10^3	+	+	ND	ND
RA24	ND	ND	1.05 x 10^2	3.35 x 10^2	+	+	ND	ND
RA25	3.00 x 10^3	8.70 x 10^4	3.69 x 10^2	1.81 x 10^2	+	+	ND	ND
RA26	8.33 x 10^3	2.40 x 10^4	7.98 x 10^2	2.31 x 10^3	+	+	ND	ND
RA27	6.56 x 10^4	1.52 x 10^5	+	PCR +	+	+	+	-
RA28	NN	7.90 x 10^3	-	PCR +	+	+	+	-
RA29	NN	2.12 x 10^4	+	PCR +	+	+	+	-

ND= not determined; NN= no number; **+** = detect; **-** = non detect.

4.2.2.4. Stormwater Quality at Hornsby Site

All stormwater runoff samples collected from Hornsby site had high numbers of FIB, which varied from 4 x 10^1 to 5.90 x 10^4 100mL^{-1} for *E. coli* and 1 x 10^3 to 9.83 x 10^4 100mL^{-1} (ten-fold higher) for *Enterococci* spp.(Table 11). *Campylobacter* spp. and *Salmonella enterica* were detected in half of the samples with nested PCR, but due to presence of PCR inhibition, numbers were not quantifiable for either pathogen. Human adenovirus and polyomavirus were detected in majority of the samples with adenovirus more prevalent than polyomavirus. However, due PCR inhibition problems numbers were not quantifiable. *Giardia lamblia* was not detected with PCR in three samples analysed. Sewage contamination marker HF183 was detected in all but one samples tested whereas, *nifH* was less frequently detected. *Enterococci* gene *esp* was detected on 3 out of 4 samples tested.

Table 11: Microbiological analysis done on the stormwater samples collected from the Hornsby site.

Sample ID	*E. coli* (100 mL^{-1})	*Enterococci* (100 mL^{-1})	*Campylobacter* spp.	*Salmonella*	Adenovirus	Polyomavirus	*esp*	HF183	*nifH*	*G. lamblia*
RA41	4.10 x 10^3	1.25 x 10^4	+	+	+	+	+	+	+	-
RA42	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
RA43	9.00 x 10^2	1.00 x 10^3	+	-	-	-	-	+	-	-
RA44	5.90 x 10^4	2.58 x 10^4	+	+	+	+	+	+	+	-
RA45	5.90 x 10^4	2.95 x 10^4	+	+	+	+	+	+	+	-
RA46	1.97 x 10^4	9.83 x 10^4	+	+	+	+	ND	-	-	-
RA47	2.00 x 10^3	9.80 x 10^3	-	-	+	-	ND	+	-	-
RA48	1.00 x 10^2	1.12 x 10^4	-	-	+	+	ND	+	+	-
RA49	2.00 x 10^2	1.12 x 10^4	-	-	+	+	ND	+	-	-
RA410	1.00 x 10^2	2.09 x 10^4	-	-	+	-	ND	+	-	-
RA411	4.00 x 10^1	1.93 x 10^3	-	-	+	+	ND	+	-	-

ND= not determined; NN= no number; **+** = detect; **-** = non detect.

4.2.2.5. Stormwater Quality at Ku-Ring-Gai Site

Four stormwater runoff samples were collected from Ku-ring-gai site with *E. coli* numbers varying form from 9 x 10^2 to 6.40 x 10^3 100mL^{-1} and *Enterococci* spp numbers varied between 3.50 x 10^3 to 9.93 x 10^3 100mL^{-1} (Table 12). *Campylobacter* spp. was detected in two samples and *Salmonella enterica* as not detected. Due to the high organic content in the concentrated samples, PCR inhibition was an issue and quantification of numbers was not possible. Human adenovirus was detected on two occasions whereas, polyomavirus was not detected; however, due PCR inhibition problems numbers were not quantifiable. *Giardia lamblia* was not detected with PCR in three samples analysed. Sewage contamination marker HF183 was detected in all three samples tested whereas, *nifH* was detected in two out of three samples.

Table 12: Microbiological analysis done on the stormwater samples collected from Ku-ring-gai.

Sample ID	*E. coli* (100 mL^{-1})	*Enterococci* (100 mL^{-1})	*Campylobacter spp*	*Salmonella*	Adenovirus	Polyomavirus	HF183	*nifH*	*G. lamblia*
RA71	9.00 x 10^2	9.93 x 10^3	-	-	+	-	+	-	-
RA72	6.40 x 10^3	3.64 x 10^3	+	-	-	-	+	+	-
RA73	3.20 x 10^3	3.50 x 10^3	+	-	+	-	+	+	-

+ = detect; **-** = non detect.

4.2.2.6. Stormwater Quality at Banyan Reserve Site

Six stormwater runoff samples were collected from Banyan Reserve site with *E. coli* numbers varying form from 5 x 10^2 to 1.62 x 10^5 100mL^{-1} and *Enterococci* spp numbers varied between 1 x 10^3 to 1.01 x 10^5 100mL^{-1} (Table 13). *Campylobacter* spp. and *Salmonella enterica* was detected in four out of six samples. However, due to PCR inhibition, quantification of numbers was not possible. Human adenovirus was detected in five out of six samples whereas, polyomavirus was detected in four samples; however, due to PCR inhibition problems, numbers were not quantifiable. *Giardia lamblia* was detected in one sample with PCR. Sewage contamination marker HF183 was detected in all samples tested whereas, *esp* and *nifH* gene was detected in four and three samples respectively.

Table 13: Microbiological analysis done on the stormwater samples collected from the Banyan Reserve.

Sample ID	*E. coli* (100 mL^{-1})	*Enterococci* (100 mL^{-1})	*Campylobacter spp.*	*Salmonella*	Adenovirus	Polyomavirus	*esp*	HF183	*nifH*	*G. lamblia*
RA51	1.62 x 10^5	1.01 x 10^5	+	+	+	+	+	+	+	-
RA52	3.40 x 10^3	1.02 x 10^4	-	+	+	+	+	+	+	-
RA53	1.10 x 10^3	1.37 x 10^3	-	-	-	-	-	+	-	-
RA54	8.00 x 10^3	1.10 x 10^3	+	-	+	+	-	+	-	+
RA55	7.20 x 10^3	2.26 x 10^4	+	+	+	+	+	+	+	-
RA56	5.00 x 10^2	1.00 x 10^3	+	+	+	-	+	+	-	-

+ = detect; **-** = non detect.

4.2.3. Discussion

Enterococcus spp. numbers detected in stormwater samples collected from all six sites were generally higher by several orders of magnitude after the storm events than the recommended limit for category D (<501 *Enterococci* per $100mL^{-1}$) under Australian guidelines for managing risks in recreational water and (NHMRC, 2008). High FIB numbers observed after the rain events were also similar to what had been previously reported in the literature (Brownell *et al.*, 2007; Parker *et al*., 2010). Similarly, *E. coli* numbers were higher in the range of 10^2 to 10^4 $100mL^{-1}$ across all sites. This suggests that stormwater water could be a potential source of infections to human if untreated harvested stormwater is used for non-potable purposes such as gardening and landscaping irrigation.

The clinically most important *Campylobacter* spp. are the members of the thermophilic group, *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* (Allos and Blaser 1995). The primer set used in this study detects all of these *Campylobacter* spp. (Lund *et al*., 2004). Thermophilic *Campylobacter* spp. were detected in urban stormwater samples collected from all sites, showing their wide prevalence of human pathogenic campylobacters in stormwater.

There are a number of documented evidences on the extensive presence of *Campylobacter* spp. in surface water (Savill *et al*., 2001; Dorner *et al*. 2007; Dyke *et al*., 2009). This is not unexpected as, apart from human input, domestic animals and birds are known reservoirs of *Campylobacter* genus (Dyke *et al*., 2009). The presence of *Campylobacter* spp. in surface water is a cause of concern which needs further investigation to assess their numbers and the infectivity status of this bacterium in stormwater runoff. *Salmonella enterica* was found to be less prevalent than *Campylobacter* spp. Prevalence of *S. enterica* in stormwater has also been previously reported (Krometis *et al*., 2010). Apart from the human faecal input from the sewage leakages, domestic animals such as cattle, dogs, horses, sheep, poultry and birds may also contribute to *Campylobacter* spp. and *Salmonella enterica* in the stormwater runoff (Dyke *et al*., 2009; Lemarchand and Lebaron, 2003).

Concentrated stormwater samples were tested for the prevalence of *G. lamblia* and *C. parvum* using PCR, which is a more sensitive detection method compared to fluorescent microscopy. A small proportion of samples tested positive for the presence of *G. lamblia*. However, due to interference from particulate matter in the concentrated samples, fluorescent microscopy did not yield any results. Samples collected from the Makerston Street site were tested for the presence of *C. parvum* and none of the samples tested positive with PCR. Although all samples tested with PCR were not positive, the presence of *C. parvum* and *G. lamblia* cannot be ruled out as PCR inhibition in the concentrated samples was major issue, as discussed in Section 4.4. The presence of *Giardia* and *Cryptosporidium* in low numbers in storm runoff have been reported in the literature (Cizek *et al*., 2008). Mean cryptosporidium oocysts numbers of 176 $10\ L^{-1}$ in stormwater have been reported from Australia (NRMMC-EPHC–NHMRC 2009). Further development of methodology for the quantitative detection of both protozoan pathogens from the stormwater which contains high concentration of suspended solids need to be carried out.

Human adenovirus and polyomavirus were frequently detected in the stormwater from all sites, suggesting a wide prevalence of human faecal contamination in these waterways. In stormwater samples from Smith Street, adenovirus were quantifiable and numbers ranged from 1.81×10^2 to 1.34×10^3 pdu L^{-1}. Adenovirus was found to be more prevalent than polyomavirus across all sites. This corroborates previous findings on the presence of human adenovirus and polyomavirus in the surface water and stormwater (Sauer *et al*., 2011; Hamza *et al*., 2009; Muscillo *et al*., 2008; Rajal *et al*., 2007). Both human adenovirus and polyomavirus numbers in surface water and stormwater have been reported in the range of $1x\ 10^2$ to $10^5\ L^{-1}$ (Sauer *et al*., 2011; Hamza *et al*., 2009; Muscillo *et al*., 2008).

Human specific *Bacteroides* HF183 genetic marker was detected in nearly all stormwater samples collected across all sites, which suggested the ubiquitous presence of sewage pollution in the urban environment. Presence of human specific *Bacteroides* HF183 and *nifH* genes have been previously used to detect the presence of human faecal pollution in aquatic environment with a high degree of accuracy (Scott *et al*., 2005; Seurink *et al*., 2005; Ahmed *et al*., 2012).

Stormwater samples can have a high amount of particulate matter and compounds that interfere with PCR amplification of DNA. In this study, we observed a variable degree of PCR inhibition which appeared to be site specific. Similar PCR inhibition issues in the stormwater water have been reported previously (Rajal *et al*., 2007). For example, the sample concentrates from the Hornsby and Fitzgibbon drain sites have a dark brown appearance suggesting the presence of humic substances which are known to inhibit PCR (Wintzingerode *et al*., 1997). The primary source of humic acids at these sites is most likely decomposing plant material as there is more vegetation around these sites. The results of this study suggest that PCR inhibition may lead to under reporting of pathogen numbers or false negative results, hence, internal controls should be used to overcome this problem.

4.2.4. Conclusions

The wide presence of adenovirus and polyomavirus in the urban stormwater runoff indicates that there is significant human faecal contamination, as opposed to contamination from animals, and thus has much higher public health implications. Detection of *Campylobacter* and *Salmonella enterica* suggests a strong likelihood of the presence of other pathogens (*Cryptosporidium* and enteric virus) based on evidence of human faecal contamination. Although PCR inhibition prevented quantitative detection of enteric pathogens in many of the samples, the relatively high numbers (10^2 to 10^3 L^{-1}) of adenovirus measured at Smith Street strongly suggest adenovirus will be regularly present at the other sites. Consequently, some degree of treatment of captured stormwater prior to its reuse for potable and non-potable purposes would be required for public health risk mitigation.

4.3. Pathogen Behaviour during Storm Events

Storm events have been reported to negatively impact surface water quality due to mobilisation and transport of microbial contaminants from point and non-point sources in a number of studies (Ackerman and Weisberg 2003; Sercu *et al.,* 2009;Surbeck *et al*., 2006; Rajal *et al.,* 2007; Sidhu *et al*., 2012). Only a limited number of studies (Surbeck *et al*., 2009; Krometis *et al.,* 2007; Stumpf *et al*., 2010) have looked into the extent of variations in the number of microorganisms through the duration of storms (ie over the storm hydrograph). Hence, there is paucity of information on how the loading rates of microbial pathogens change between storm events and in catchments with different characteristics. Understanding of the loadings and variations in enteric pathogen numbers in stormwater is important, as it will influence the extent of treatment required and the health risk associated with stormwater reuse. There are a number of reasons for the variation in pathogen numbers which include specificities of the catchments, event peaks and seasons. It is important to quantify pathogen numbers during both baseflow and peak flows during storm events. Peak pathogen loads after heavy rainfall events have been linked to disease outbreaks (Curriero *et al*., 2001; Signor *et al*. 2005).

This component of the study had the following objectives:

(i) examine the patterns and loads of enteric virus (adenovirus and polyomavirus), *Salmonella* enteric, conventional faecal indicators (*E. coli*, *Enterococcus* spp. and somatic coliphages) in stormwater over the storm hydrograph;
(ii) examine the variability in faecal pollution indicators and pathogens over the storm hydrograph across two catchments with different characteristics during multiple storm events;
(iii) use quantitative polymerase chain reaction (qPCR) based methods to assess patterns of adenovirus and polyomavirus in stormwater; and
(iv) Compare the hydrologic conditions and numerical results of faecal indicators to human enteric pathogen and assess patterns of contamination.

4.3.1. Materials and Methodology

4.3.1.1. Study Sites

Stormwater samples were collected from two catchments in Brisbane; Fitzgibbon drain and Makerston Street. Detailed information on catchment characteristics is previously provided in Table 1.

4.3.1.2. Automated Sampling during Storm Events

Fourty stormwater samples were collected from the two stormwater sampling sites over six storm events using automatic pumping samplers (ISCO 6700 series).

4.3.1.3. Quantification of Faecal Indicator Bacteria (FIB) in Stormwater

Quantification of FIB (*E. coli* and *Enterococcus* spp.) was performed by the membrane filtration technique as outlined previously under Sections 4.1.2.3 and 4.1.2.4.

4.3.1.4. Quantification of FIB, and *S. enterica*

Most Probable Number (MPN) technique, with three-tube MPN was used for the quantification of *Salmonella* enteric, from concentrated stormwater samples. Briefly, for the detection of *S. entrica* form the concentrated water sample, sample equivalent to 1 L, 100, 10 and 1 mL sample was enriched in Buffered peptone water (Oxoid) at 37ºC overnight. Selective enrichment was carried out in the Rappaports Vassiliadis media (Oxoid) for the detection of *S. entrica* as outlined in Sidhu *et al*., (2001). Enriched samples were stored at -20ºC for PCR detection.

4.3.1.5. HAv, HPv, Torque Teno Virus, and Somatic Coliphage Quantification from Stormwater

Stormwater samples (1 L) were passed through negatively charged HA type membrane (Millipore, Australia) with a 0.45 µm pore size and 90-mm diameter after addition of $MgCl_2$ as described previously (Katayama *et al*., 2002). Briefly, 5 mL $MgCl_2$ (2.5 M) was added to the one litre stormwater sample to obtain a final concentration of 25 mM. After mixing water sample was passed through the negatively charged HA type membrane placed on vacuum filtration funnel. Then the membrane was rinsed with 200 mL of 0.5 mM H_2SO_4 (pH 3.0) to remove excess $MgCl_2$ followed by elution of captured virus with 5 mL of elution buffer (1 mM NaOH at pH 10.8). The pH of eluent was then adjusted to neutral with pH 100 mM H_2SO_4 (pH 1.0) and 100 µL of Tris–EDTA buffer (pH 8.0) was added. Further concentration of the virus was carried out by centrifugation using a Centriprep (Millipore) at 2500 rpm for 10 min to obtain a final volume of one mL as outlined in Sidhu *et al*. (2012c). This method is based on the capture of virus on a charged membrane and then releasing it with NaOH based elution buffer is significantly different approach used in the previous chapters which resulted in co-concentration of PCR inhibitory compounds from large volumes of wastewater.

4.3.1.6. PCR Detection of Viral and Bacterial Pathogens

Nucleic acid was extracted from each of the samples (200 µL) using the Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA) as per manufacturer instructions and stored at -80°C prior to analysis.

Human adenovirus (HAv), human polyomavirus (HPv), Torque teno virus (TTV), somatic coliphages (*Microviridae* family), and *Salmonella* enteric were detected with the published primer and probe sets given in the Table 2. Quantitative PCR (qPCR) reactions were performed on Bio-Rad iQ5 (Bio-Rad Laboratories, California, USA), using iQ Supermix (Bio-Rad) real-time PCR kit. Each 25 µL PCR reaction mixture contained 12.5 µL of SuperMix, 300 nM of each primer, 200-250 nM corresponding TapMan probes 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^{-1} to relieve PCR inhibition (Kreader, 1996). Thermo-cycling conditions are presented in Appendix B1.

4.3.1.7. PCR Standards

Standards for PCR amplification were prepared from the genomic DNA of standard culture of *S. enterica* serovar Typhimurium (ATCC 14028). The concentration of genomic DNA was measured by using a NanoDrop ND-1000 spectrophotometer. After calculation of genomic copy numbers, a serial 10-fold dilution (10^6 to 10^0 DNA copies μL^{-1}) was prepared. The aliquots of these serial dilutions were stored at –80°C for subsequent use.

The PCR amplified products of HAv, HPv, TTV and somatic coliphage were purified using the QIAquick PCR purification kit (Qiagen), and cloned into the pGEM®-T Easy Vector System (Promega Madison, WI, USA), transferred into *E. coli* JM109 competent cells and plated on LB agar ampicillin, IPTG (isopropyl-β-D-thio-galactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β- D-galactopyranoside) as recommended by the manufacturer. Plasmid was purified using plasmid mini kit (Qiagen). Purified plasmid DNA containing HAv, HPv, and somatic coliphage were quantified using a spectrophotometer (NanoDrop ND-1000). Plasmid copies were calculated, and a ten-fold serial dilution was prepared in MilliQ water to a final concentration ranging from 10^0 to 10^6 copies/µL and aliquots were stored at -80ºC until use. Three microlitre template from of each dilution was used to prepare standard curves for qPCR.

4.3.1.8. PCR Reproducibility and Limit of Detection

The reproducibility of the qPCR was assessed by determining intra-assay repeatability and inter-assay reproducibility. The Coefficient of Variation (CV) was calculated using six dilutions (10^6 to 10^0 gene copies) of the HAv, and HPv, plasmid DNA. Each dilution was tested in triplicate. The CV for evaluation of intra-assay repeatability was calculated based on the C_T value by testing the six dilutions six times in the same experiment. The CV for inter assay reproducibility was calculated based on the

C_T value of six dilutions on six different days. To determine the qPCR limit of the detection, known gene copies (i.e., 10^6 to 10^0) of HAv, and HPv, plasmid DNA and somatic coliphage were detected in triplicates in separate qPCR runs. The lowest numbers of gene copies (1-10) that were detected consistently in replicate assays was considered as the qPCR limit of detection.

4.3.1.9. Recovery Efficiency of HA Type Membranes

The effectiveness of the virus concentration procedure with negatively charged HA type membrane was determined by adding adenovirus to stormwater samples collected from both catchments. Briefly, one litre stormwater samples (n=6) collected from Fitzgibbon (n=3) and Makerston street (n=3) were spiked with known numbers of adenovirus ($1x10^6$ pdu mL^{-1} sample) and containers were mixed well after addition of $MgCl_2$. Water samples were passed through negatively charged HA type membrane as mentioned previously to capture adenovirus. MilliQ water sample (1L) was also seeded with adenovirus and used as control to determine recovery efficiency. The recovery efficiency was calculated as follows:

% Recovery = (Total pdu of adenovirus in the recovered /seeded pdu of adenovirus in the 1 L inoculated) X 100.

Note: pdu = PCR Detectable Units.

4.3.1.10. Statistical Analysis

All statistical analyses were conducted using SPSS version 12.0. Data normality test on both physical parameters and microbial numbers was performed by the Kolmogorov-Smirnov test, which determined that distribution was not normal. Therefore, a non-parametric Spearman rank order correlation was performed to test the relationship between indicators (*E. coli*, *Enterococci* spp., somatic coliphages) and pathogen (HAv, HPv and *Salmonella* enteric) numbers in stormwater. The Spearman rank order correlation does not require the variables to be assigned as independent and dependent or normal distribution; it only measures the strength of association between the variables. The Spearman rank order correlation coefficient is calculated by ranking all values of each variable and then computing a Pearson product moment correlation coefficient of the ranks. Prior to the statistical analysis, all determined FIB and pathogen numbers were Log_{10} transformed due to non-normal distribution.

A binary logistic regression was performed to determine whether indicator organism numbers predicted the probability of the occurrence of pathogens in the stormwater samples. The dependent variable (pathogen) was treated as a binary variable. When a pathogen was present, a score of 1 was assigned. When it was not detected, a score of 0 was assigned. The independent variables (indicators) were continuous. A student's *t-test* was performed to compare *pathogens and FIB* numbers across two catchments. The critical *P*-value for the *t-test* was set at 0.05 and all tests were considered significant if the *P* value was < 0.05.

4.3.2. Results

4.3.2.1. Adenovirus Recovery Rates with HA Filters

The recovery rates of adenovirus seeded into 1 L stormwater samples collected from Makerston Street (n=2) and Fitzgibbon Drain (n=3) are presented in Table 14. Recovery rates from Makerston Street samples with HA type filters were 80% or higher whereas, very low recovery rates (0-14%) were observed in samples from the Fitzgibbon Drain. However, after 10-fold dilution of the extracted DNA much better recovery rates (32-78%) were observed showing the influence of PCR inhibitors in the stormwater samples from the Fitzgibbon Drain site. Hence, for the quantitative PCR, extracted DNA from the Fitzgibbon site was 10-fold diluted prior to setting up PCR reactions.

Table 14: Recovery rates of seeded adenovirus with HA type membrane filters.

Sample ID	Seeded Virus Numbers (pdu mL^{-1})	Recovered Virus Number (pdu mL^{-1})	Recovery Rate (%)
MilliQ water	1.77×10^5	1.77×10^5	100
Makerston Street			
M1	1.77×10^5	1.49×10^5	84
M2	1.77×10^5	1.44×10^5	81
Fitzgibbon Drain			
F1	1.77×10^5	5.69×10^4	32
F2	1.77×10^5	8.86×10^4	50
F3	1.77×10^5	1.38×10^5	78

Note: pdu = PCR Detectable Units.

4.3.2.2. FIB Numbers during the Storm Events

The distribution of *E. coli* and *Enterococcus* spp. numbers at Fitzgibbon Drain and Makerston Street sites during the three separate storm events is presented in Figure 21. The mean FIB numbers in water samples collected after the storm event ranged from 3.51 to 3.91 Log_{10} L^{-1} for *E. coli* and 4.07 to 4.92 Log_{10} L^{-1} for *Enterococcus* spp. In general, median *E. coli* (3.79 Log_{10}) and *Enterococcus* spp. (5.03 Log_{10}) numbers were higher at Fitzgibbon than Makerston Street which were 3.60 Log_{10} and 5.03 Log_{10} respectively. The *E. coli* and *Enterococcus* spp. numbers during the storm event generally followed similar trend and were up to certain extent dependent upon the intensity of the storm with higher numbers observed at peak flow rates (Figures 24 and 25).

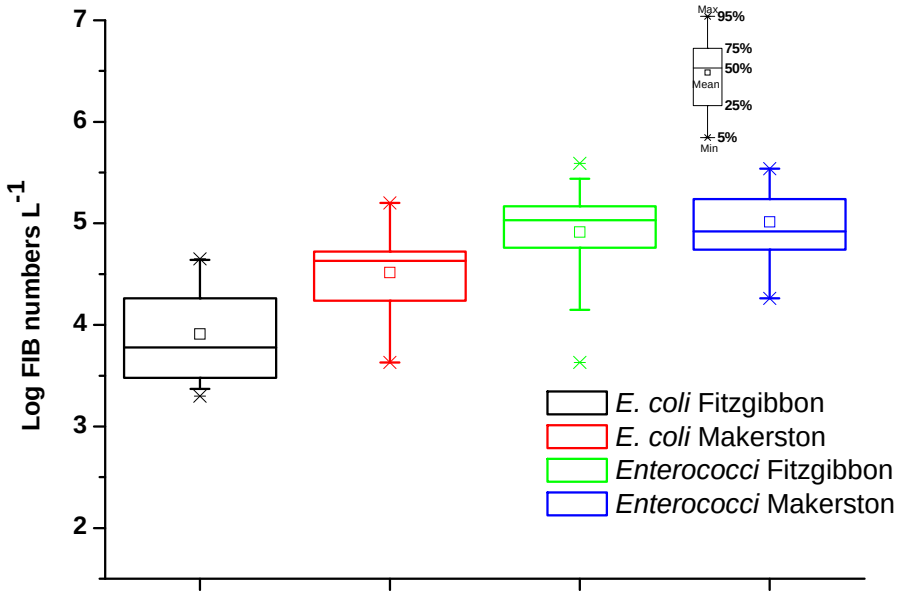

Figure 21: Distribution of FIB numbers in the stormwater samples collected from Fitzgibbon Drain and Makerston Street sites. The vertical line represents range of data, black circle represent mean, small horizontal lines at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

4.3.2.3. Distribution of FIB, HAv, HPv, TTV, Somatic Coliphage and *S. enterica*

In total, 41samples of stormwater were collected through the hydrograph from Fitzgibbon Drain and Makerston Street sites during three storm events. Data on comparative prevalence of FIB, HAv, HPv, TTV, somatic coliphage and *Salmonella enterica* are presented in Figures 22 and 23 for Fitzgibbon Drain and Makerston Street, respectively. All microbial numbers were $\log_{10}$ transformed prior to plotting. *S. enterica* number varied between 0 to 2.38 $\log_{10}$ L^{-1} (10 to 2400 MPN L^{-1}) across both sites. The median *S. enterica* numbers were similar (1.38 $\log_{10}$ L^{-1}) in stormwater samples collected from Fitzgibbon Drain and Makerston Street. HAv numbers varied between 1 to 3.96 $\log_{10}$ L^{-1}, whereas, HPv numbers varied between 1 to 3.90 $\log_{10}$ L^{-1}. Somatic coliphages (Microviridae) varied between 1 to 3.94 $\log_{10}$ L^{-1} across both sites. TTV numbers were higher than at Fitzgibbon site and varied between 1 to 3.84 $\log_{10}$. The median HAv and somatic coliphages numbers at Makerston Street were higher (3.06 and 3.29 $\log_{10}$ L^{-1} respectively), compared to Fitzgibbon Drain (2.00 and 2.48 $\log_{10}$ L^{-1} respectively), whereas, HPv numbers were similar (1 $\log_{10}$ L^{-1}) at both sites.

No significant correlation was found between the occurrence of FIB and any enteric virus tested in this study. Similarly there was no significant correlation between the occurrence of somatic coliphages and any of enteric virus.

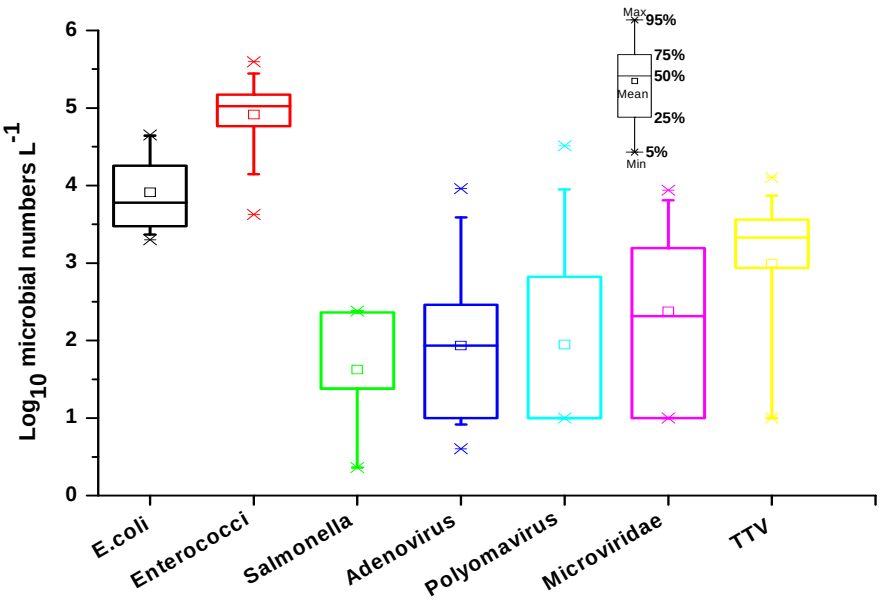

Figure 22: Comparative distribution of *E. coli*, *Enterococci* spp., *S. enterica*, HAv, HPv, TTV and somatic coliphages in the stormwater samples collected during three storm events at Fitzgibbon Drain. The vertical line represents range of data, black circle represent mean, small horizontal lines at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

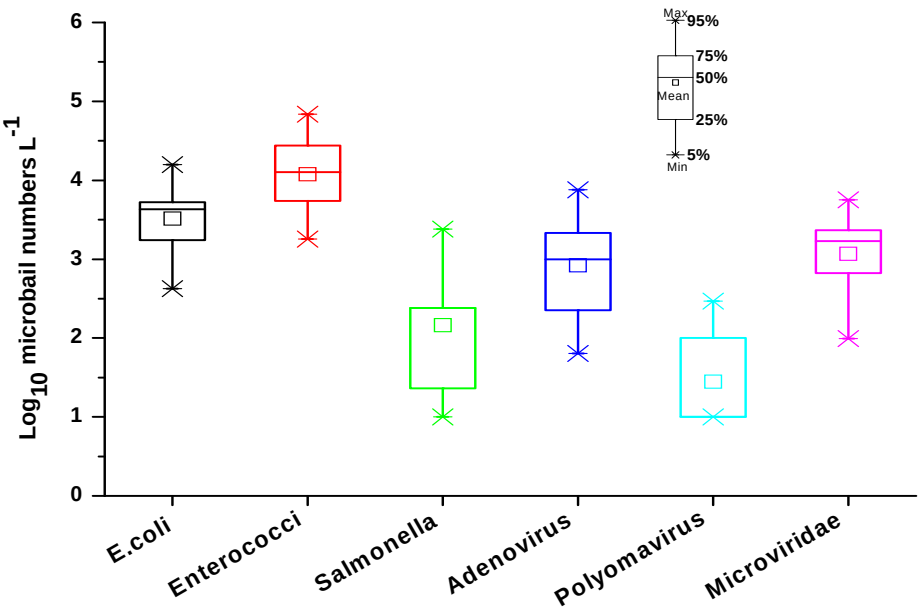

Figure 23: Comparative distribution of *E. coli*, *Enterococci* spp., *S. enterica*, HAv, HPv, and somatic coliphages in the stormwater samples collected during three storm events at Makerston Street. The vertical line represents range of data, black circle represent mean, small horizontal lines at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

4.3.2.4. Flow Finger Printing FIB and Pathogens

The plot between distribution of FIB and pathogens over the storm hydrographs from both storm water sampling sites is presented in Figures 24 and 25. Makerston Street catchment has > 90% impervious area compared to 30% at Fitzgibbon Drain and, as a result, much higher storm flow was observed during all three storm events, even though the catchment area was much smaller. FIB bacteria numbers increased at the onset of the storm event and remained high (>10^3 L^{-1}) during the storm event. Although *Enterococci* spp. numbers were generally higher than *E. coli*, but there was no obvious trend of increase or decrease in numbers at both sites during the storm events. Both *E. coli* and *Enterococcus* spp. showed no dependency on the stream flow. *Salmonella enterica* numbers were several fold lower compared to FIB and no dependency on the stream flow was observed at either site. Adenovirus showed some relationship with the streamflow where high numbers were detected at the peak of storm events (Figures 24 and 25). No relationship was observed between FIB and pathogens. Similarly, no correlation was detected among any of the enteric virus or somatic coliphages at both sites.

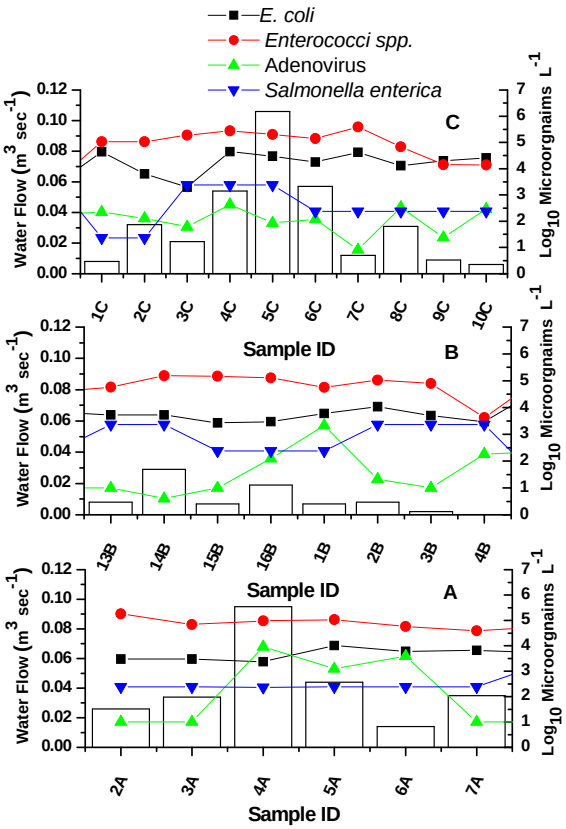

Figure 24: Hydrograph (bars) and distribution of pathogens and FIB during three different storm events (A, B and C) at Fitzgibbon Drain. Bars represent stream flow at individual sampling events.

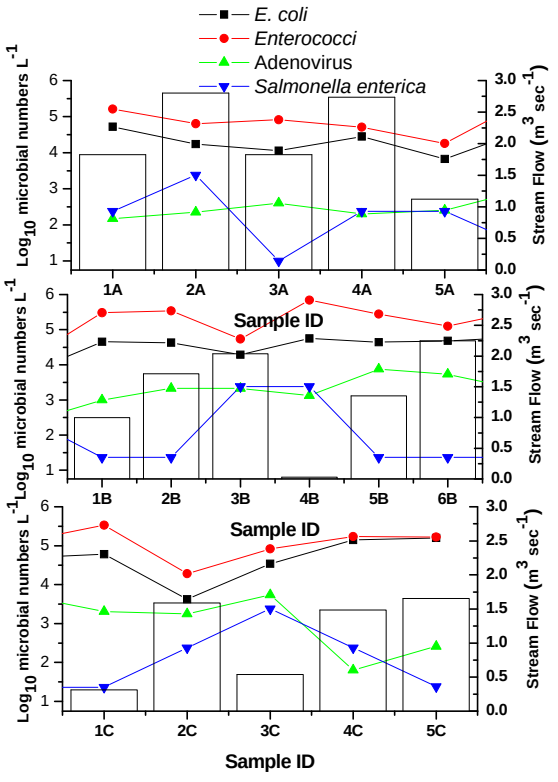

Figure 25: Hydrograph (bars) and distribution of pathogens and FIB during three storm events (A, B and C) at Makerston Street. Bars represent stream flow at individual sampling events.

4.3.3. Discussion

4.3.3.1. FIB and Pathogen Numbers in Collected Water Samples

E. coli and *Enterococcus* spp. numbers at Fitzgibbon Drain and Makerston Street sites were generally higher than the recommended limits for category D (<501 *Enterococci* per 100 mL^{-1}) under Australian guidelines for managing risks in recreational water and by further several orders of magnitude after the storm events (NHMRC, 2008). High FIB numbers observed in the stormwater runoff were also similar to data previously reported in the literature (Brownell *et al.*, 2007; Parker *et al*., 2010). *S. enterica* was frequently detected in the stormwater runoff from both sites suggesting likely presence of other pathogenic bacteria such as *Campylobacter* spp. An increased prevalence of *Salmonella* in urban water shed after storm events has been previously reported (Krometis *et al*., 2010). In addition to human sources of *Salmonella* and other bacterial pathogens, domestic animals such as cattle, dogs, horses, sheep, poultry and birds may also contribute to contamination of stormwater (Dyke *et al*., 2009; Lemarchand and Lebaron, 2003).

Recovery rates of seeded adenovirus in the stormwater samples collected from Makerston Street and Fitzgibbon Drain suggests variable recovery rates (Table 14). The observed variable recovery rates was probably due to the presence of PCR inhibitory substances as better recovery (32-78%) of extracted DNA was observed after 10-fold dilution. Human adenovirus, torque teno virus, polyomavirus and somatic coliphages were detected in the stormwater runoff from both sites during all three events at each site. HAv median numbers were generally higher than the HPv and was more frequently detected in the stormwater runoff. Presence of human adenovirus and polyomavirus in the surface water and stormwater has been reported in the previous studies (Sauer *et al*., 2011; Hamza *et al*., 2009; Muscillo *et al*., 2008; Rajal *et al*., 2007). HAv and HPv virus numbers (10- 10^3 pdu L^{-1}) detected at both sites comparable to the numbers (1x 10^2 to 10^5 L^{-1}) reported in surface water and stormwater in other studies (Sauer *et al*., 2011; Hamza *et al*., 2009; Muscillo *et al*., 2008; Hamza *et al*., 2011).

Human torque teno virus (TTV) are prevalent worldwide in the general population, due to its environmental stability it has been also suggest as an indicator of faecal contamination and process indicator for drinking water industry (Haramoto *et al.,* 2005; Diniz-Mendes *et al.,* 2008). In this study, TTV were regularly detected in the stormwater runoff from Fitzgibbon site with numbers ranging from 10 to 6.85 x 10^3 pdu L^{-1}. These numbers are similar to 103 pdu L^{-1} reported from river water in Germany and Japan (Hamza *et al*., 2011; Haramoto *et al*., 2010). Much higher number of TTV (7.4 x 10^6 pdu L^{-1}) in river water been reported from Brazil (Diniz-Mendes *et al.,* 2008).

Somatic coliphages are non-enveloped viruses structurally similar to enteric virus, and thus been proposed as indicators of enteric viral presence and behaviour in the surface water, drinking water and wastewater matrix (Moce-Llivina *et al.,* 2005). Somatic colipahge belonging to the Microviridae family have been reported to be widely prevalent in the sewage contaminated surface waters (Lee 2009). In this study, we tested the prevalence of somatic coliphages (Microviridae family) with an aim to determine if there prevalence is correlated to the presence of other enteric virus. Although the somatic coliphage numbers were comparable to adenovirus numbers, no correlation was observed with adenovirus or other enteric virus tested in this study. This suggests monitoring for the adenovirus might be a better option form public health point of view as they more prevalent in stormwater runoff and are known to be among the most thermally stable and are resistant to ultraviolet light (Gerba *et al*., 2002; Meng and Gerba, 1996).

A careful approach must also be adopted while using the data of this study for risk assessment purpose as PCR based techniques overestimate pathogen survival times and hence an over estimation of posed risk by the viruses such as rotavirus. PCR based techniques are very sensitive and specific in detection of virus genome however, there can be a difference between the loss of infectivity and complete degradation of viral genome (Charles *et al.* 2009, de Roda Husman *et al*. 2009). Conversely, cell culture based detection methods are able to detect infectious virus particles. However, cell culture based assays tend to underestimate infectious virus particles as each virus has different affinity for different cell lines. Human norovirus and rotavirus cannot be cultured and most of the adenovirus

strains require trained cell lines for infectivity assays. In addition, infectivity in a cell assay depends upon a number of variables such as duration of exposure to host cells, age of the cells and cell toxicity resulting from environmental samples (Rodriguez *et al.* 2009). Until methodologies for the detection of difficult to culture viruses such as rotavirus and norovirus in environmental samples are well established, an overestimation of risk is considered preferable to an underestimation obtained via cell culture based methodologies.

4.3.3.2. Pathogen Numbers vs Streamflow Relationships

This study was conducted to characterise the numbers of enteric pathogens vs streamflow relationships (flow fingerprints) in stormwater runoff from Fitzgibbon Drain and Makerston Street sites during three storm events. No relationship between the streamflow and FIB or enteric pathogens was observed at either site. This finding is in agreement with previously reported finding of no correlation between streamflow and FIB (Subreck *et al*., 2006). A general trend between adenovirus and stream flow was observed where higher number of adenovirus was detected at the peak of storm events, however, further research need to be carried out to explore the relationship between enteric pathogen numbers and streamflow. The results of this study also suggest that highest numbers of pathogens are not necessarily related to the intensity of the storm. It is possible that the duration of dry period between the storm events may also play a role in transporting microbial contaminants from the land surface to the stormwater runoff which need to be investigated further.

Accurate quantification of enteric pathogen numbers is essential to determine the extent of stormwater treatment required to mitigate public health risk if captured water is to be used for non-portable purposes. Since no correlation was found between the enteric pathogens numbers and stream flow, it is highly likely that pathogens numbers cannot be accurately determined with one off sampling. Consequently, composite sampling which covers the whole hydrograph may provide more accurate information on the pathogen numbers in the stormwater. Further research is required to establish the validity of composite sampling to accurately determine pathogen numbers in stormwater.

4.3.4. Conclusions

The results of this study suggest that enteric virus and bacteria frequently occur in the stormwater runoff. HAv, HPv and TTV were found to be present in the stormwater runoff in significant numbers. Flow finger printing of FIB, *Salmonella enterica* and human adenovirus, polyomavirus, and torque tenovirus during the storm at the Fitzgibbon drain and Makerston Street sites demonstrated a high inter and intra storm variability. No correlation was found between the enteric pathogens numbers vs streamflow. The flow independent nature of presence of enteric pathogens and FIB in stormwater runoff is consistent with the idea that these microbial contaminants are ubiquitously present in the urban environment.

The distribution of faecal pathogen numbers in stormwater was found to be catchment dependant with higher contamination observed in the Makerston Street site. A stormwater catchment risk management approach would be essential to minimise human faecal pollution with stormwater quality monitoring programs focused on the identifying the expected sources of pathogens in the catchment and the storm events that lead to increased human health risks.

4.4. Assessment of Human Faecal Contamination in Stormwater

Urban stormwater have been reported to contain high numbers of faecal indicator bacteria (FIB) and enteric pathogens (Noble *et al.,* 2006; Sercu *et al.,* 2009; Cizek *et al.*, 2008; Sidhu *et al.,* 2012). Generally, agricultural runoff, domestic animals, wildlife and pets are considered major sources of faecal pollution. However, there is 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). Hence, it is important to differentiate between human and animal sources of faecal pollution in stormwater, so that better assessment on potential health risks from exposure to stormwater could be made.

Both microbial and chemical faecal source tracking (FST) methods have been used to identify the potential sources of faecal pollution in environmental waters (Parker *et al.*, 2010; Sauer *et al.*, 2011; Glassmeyer *et al.,* 2005; Nokada *et al.,* 2008). Microbial FST 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 *Methanobrevibacter smithii nifH* markers which are known to be highly host specific in surface water (Ahmed *et al.* 2010; Ahmed *et al.* 2012; McQuaig *et al.* 2009; Sercu *et al.* 2011; Seurinck *et al.* 2006; Ufnar *et al*. 2006). Human adenovirus (HAv) and polyomavirus (HPv) are reported to be highly prevalent (10^5 to 10^6 L^{-1}) in sewage and also reported in surface waters in the range of 1x 10^2 to 10^5 L^{-1} (Sauer *et al.,* 2011; Hamza *et al.,* 2009; Muscillo *et al.,* 2008).

During the past decade, a number of studies have extensively surveyed prevalence of pharmaceutical and personal care products in sewage effluent and aquatic environment (Glassmeyer *et al*., 2005; Benotti and Brownawell 2007; Nokada *et al*., 2008). A number of chemical markers including faecal sterols/stanols (Gregor *et al*., 2002; Isobe *et al*., 2004), caffeine (Siegener and Chen 2000; Buerge *et al.,* 2003), artificial sweeteners (Nakada *et al*., 2008; Scheurer *et al*., 2011) pharmaceuticals and personal care products (Clara *et al*., 2004; Fono and Sedlack, 2005; Glassmeyer *et al*., 2005) have been proposed for sewage ingression. Persistent markers such as acesulfame are useful for tracing the pathways of treated sewage, whereas, labile compounds such as caffeine are indicators of untreated wastewater ingression due to sewer overflow in the fresh water (Buerge *et al*. 2006).

Each of the FST methods described in the literature to date has advantages and disadvantages. The consequence of inaccurate source tracking may lead to expensive infrastructure improvements that may not improve the water quality. It has been recommended that a "toolbox" approach involving a number of FST markers is generally recommended for an accurate identification of polluting sources (Ahmed et *al.,* 2012; Boeh *et al.,* 2003; Noble *et al.,* 2006; Mauffret *et al.,* 2012).

To date, most published studies on the characterising of faecal pollution of stormwater are limited to microbiological source tracking markers (Ahmed *et al.,* 2007; Noble *et al.,* 2010; Sauer *et al.*, 2011; Surbeck *et al.,* 2006;), To our knowledge, no study have been published on the use of both chemical and microbiological markers for assessment of sewage pollution. In this study, we assessed the extent of sewage pollution in stormwater outfalls from six residential and commercial catchments in Brisbane, Sydney and Melbourne, using both chemical and microbiological markers of sewage contamination. Stormwater samples were collected over a year period from all six catchments in a flow proportional sampling method and subsequently (combined) mixed to get an event mean concentration EMC. In particular, this Section is focused on the following objectives:

(i) determine the frequency of occurrence of sewage related chemical and biological markers in stormwater outfalls from urban and commercial catchments;

(ii) compare the prevalence pattern of chemical and biological markers in relation to human adenovirus (HAv) and human polyomavirus (HPv); and

(iii) evaluate the performance of FST markers to detect sewage pollution in stormwater outfalls.

4.4.1. Material and Methods

4.4.1.1. Stormwater Sampling Sites

Stormwater samples were collected from three medium density residential catchments, Fitzgibbon, (north of Brisbane), Banyan Reserve (south of Melbourne) and Ku-ring-gai (north of Sydney) and three high density commercial areas, Makerston Street, (Brisbane), Hornsby (Sydney) and Smith Street. Site details are provided previously in Table 1.

4.4.1.2. Stormwater Sampling Strategy

Composite samples (20-40 L) were collected from each of the six sites with automatic samplers (ISCO 6700 series) in 20 L high density polyethylene containers (HDPE) during the storm events as outline previously in the Section 2.1.3.

In total, 23 stormwater events were analysed; Fitzgibbon (FG) stormwater drain (n=5), Makerston Street (MA) (n=5), Hornsby site (HN) (n=5), Banyan Reserve (BA) (n=4), Smith Street (SM) (n=2) and Ku-ring-gai (KG) (n=2). The collected samples from each event were mixed on the flow proportion basis to provide a composite sample to represents the EMC. Approximately 20 L of composite sample was used for microbiological analysis. 1 L sample was used for chemical analysis. The collected stormwater samples were stored at 4°C and shipped to Brisbane on ice for processing.

4.4.1.3. Quantification of Faecal Indicator Bacteria

Quantification of FIB (*E. coli* and *Enterococcus* spp.) was performed by the membrane filtration technique as outlined previously in section 3.1.1.2.

4.4.1.4. Detection of Microbial Source Tracking 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. Real-time polymerase chain reaction (PCR) assays were performed for the detection of *Bacteroides* HF183, *nifH*, HAv and HPv using previously published primers and probes (Heim *et al.,* 2003; McQuaig *et al.,* 2009; Seurinck *et al.,* 2005; Ufnar *et al.,* 2006). 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 n*ifH* and HF183 gene amplifications were performed in 20-µl reaction mixtures using Sso Fast™ 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^{-1} to relieve PCR inhibition (Kreader 1996). The cycling parameters are shown in Table B3. The PCR was performed using the Bio-Rad iQ5 thermal cycler (Bio-Rad Laboratories). For each PCR experiment, positive controls (e.g. corresponding plasmid DNA or genomic DNA) and negative control (e.g. sterile water) were included.

4.4.1.5. Detection of Chemical Markers

Prevalence of two pharmaceuticals (paracetamol and salicyclic acid) along with the food chemicals caffeine and acesulfame (artificial sweetener) was assessed in stormwater samples. One litre 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 Queensland Health Forensic and Scientific Services (US EPA 2007). Two aliquots of aqueous sample were extracted on solid phase extraction (SPE) cartridges (Phenomenex StrataX, 200 mg 3 mL^{-1}), one acidified with hydrochloric acid and without pH adjustment, using an 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.

4.4.1.6. Data Analysis

Prior to statistical analysis, data from all six catchments was grouped under two categories, predominantly residential (Fitzgibbon Drain, Banyan Reserve 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. A binary logistic regression analysis was performed to confirm existence of any correlation between the presence of FIB numbers and FST markers (Minitab version 16, Minitab Inc., State College, PA). Binary logistic regression is a commonly used to model the binary (presence/absence) responses. In this study, presence/absence of FST markers was treated as the dependent variable (binary variable). When a FST marker was present, it was assigned the value 1, and when a FST marker was absent, it was assigned the value 0. Relationships were considered significant when the *P* value for the model chi-square was < 0.05 and the confidence interval for the odds ratio did not include 1.0. Greater odd ratios indicate a higher probability of change in the dependent variable with a change in the independent variable. Statistical significances of the results were determined by applying a Student's *t*-test to the FIB numbers and chemical markers 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 than (0.05) and the compared data was considered to be not significant.

Bayes' Theorem was used to calculate the conditional probability that the detection of sewage associated HF183 and *nifH* markers in the stormwater samples originated from sewage rather than faeces from the non-target host-groups that may occasionally contain the HF183 and *nifH* markers. Equation 4 has been used to calculate the conditional probability (Kildare *et al.,* 2007; Weidhaas *et al.,* 2011).

$$P(H \setminus T) = \frac{P(T\setminus H)P(H)}{P(T \setminus H)P(H) + P(T \setminus H')P(H')} \qquad \text{(Equation 4)}$$

P(H\T) is the probability (*P*) of bovine faecal contamination (*H*) in a water sample given a positive test result (*T*) for the sample.

P(T\H) is the true positive.

P(H) is the background probability of detecting a sewage marker in a water sample.

P(T\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)*.

4.4.2. Results

4.4.2.1. FIB Numbers in Collected Water Samples

The numbers of FIB in water samples collected after the storm event ranged from 40 to 7,200 cfu 100 mL^{-1} for *E. coli*, and from 1,930 to 29,500 cfu 100 mL^{-1} for *Enterococcus* spp. (Table 15). 48% of stormwater samples had *Enterococcus* spp. numbers more than 10,000 cfu 100 mL^{-1}. 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.

Table 15: **Faecal indicator bacteria numbers and sewage associated markers detected in stormwater samples collected from six catchments in Australia. These are EMC data calculated for the various storm events,.eg 5 events for Fitzgibbon.**

Sites	FIB Counts*		Pharmaceuticals		Food Markers		Microbial Markers			
	E. coli	*Enterococcus* spp.	Paracetamol	Aspirin	Acesulfame	Caffeine	HF183	*nifH*	Adenovirus	Polyomavirus
FG1	4733	17500	0.08	0.10	0.04	BDL	+	+	+	+
FG2	3600	16700	0.02	0.10	0.03	0.03	+	+	+	+
FG3	1033	1075	0.08	0.10	0.04	BDL	+	+	+	-
FG4	3560	11800	0.06	0.60	0.03	0.09	+	+	+	+
FG5	1166	1432	BDL	0.10	0.07	0.10	+	+	+	-
MA1	6660	1800	0.03	BDL	0.03	0.27	+	+	+	-
MA2	4566	4100	BDL	BDL	0.11	5.20	+	+	+	+
MA3	6066	1266	BDL	BDL	0.03	0.06	+	+	+	+
MA4	3600	5560	0.02	0.10	0.16	1.10	+	+	+	+
MA5	300	1166	0.13	0.10	0.10	1.10	+	+	+	+
HN1	5900	25800	0.05	0.20	0.09	1.80	+	+	+	+
HN2	5900	29500	0.05	0.20	0.04	0.30	+	+	+	+
NH3	100	11200	0.02	BDL	0.09	1.80	+	+	+	-
NH4	200	11200	0.03	0.30	0.05	0.70	+	+	+	-
NH5	40	1930	0.09	0.10	0.07	2.50	+	+	+	-
BA1	3400	10200	0.05	BDL	0.04	0.30	+	+	+	+
BA2	1100	1366	0.03	0.10	0.06	0.38	-	-	-	-
BA3	7200	22600	0.11	0.10	0.05	0.43	+	+	+	+
BA4	1000	10000	0.05	0.10	0.07	0.31	+	+	+	-
SM1	6560	15200	0.20	0.10	0.23	3.00	+	+	+	+
SM2	893	7900	0.14	0.10	0.17	1.70	+	+	+	-
KG1	900	9933	2.00	1.00	1.00	2.00	+	+	+	-
KG2	6400	3640	0.02	0.10	BDL	0.14	+	+	-	-

FIB counts* cfu $100mL^{-1}$, μg/L (1 ng =0.001 μg) FG= Fitzgibbon stormwater drain, MA= Makerston street drain, HN= Hornsby site, BA= Banyan Reserve, SM= Smith street KG= Ku-ring-gai.

4.4.2.2. Prevalence of Microbial Markers

Among six stormwater sites tested, all sites (100%) were positive for sewage associated markers. Among 23 (composite) 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 microbial source tracking (MST) markers could be detected in one sample from Banyan Reserve (Table 3). *Bacteroides* HF183 was most frequently detected in 96% of stormwater samples whereas, *M. smithii nifH* gene marker was detected in 43% samples only (Figure 26). HAv had higher prevalence (91%) in the collected stormwater samples compared to HPv (52%).

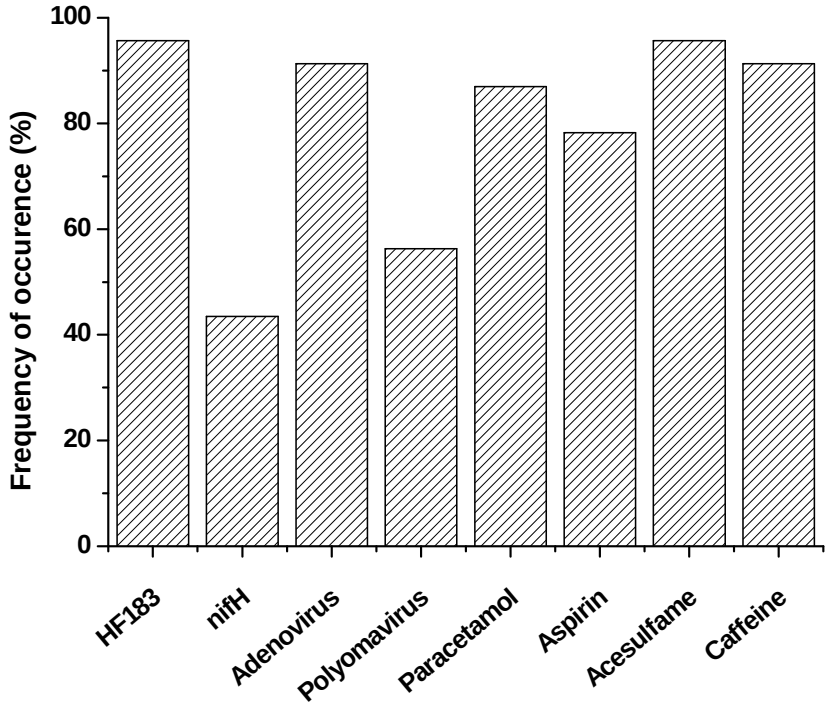

Figure 26: Frequency of occurrence of FST makers in composite stormwater samples (n=23) collected from six catchments across Australia.

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 faecal samples from non-target host-groups in SEQ (Ahmed *et al.,* 2012). The background probabilities, $P(H)$, of detecting the HF183 and nifH markers in the storm water samples, 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.,* 2012; Ahmed *et al.,* 2012 b). 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 occurrence and non-occurrence results of the HF183 and *nifH* markers in the stormwater samples and faecal 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 storm water sample was due to the true sewage contamination and not from non-target hosts.

4.4.2.3. Prevalence of Chemical Markers

Among the 23 stormwater samples tested from six sites, 22 (96%), 21 (91%), 20 (87%), and 18 (78%) samples were positive for acesulfame, caffeine, paracetamol and salicyclic acid, respectively (Figure 26). Acesulfame was most frequently detected in 96% of stormwater samples whereas, caffeine was detected in 91% of samples. Paracetamol had higher prevalence (91%) compared to salicyclic acid which was detected in 78% of stormwater samples. Caffeine had the highest concentration among all the chemical source tracking (CST) markers ranging from below 0.01 (level of reporting, LOR) to 5.20 µg L^{-1} which was followed by acesulfame ranging from below 0.01 (LOR) to 0.23 µg L^{-1}. Among the pharmaceuticals, paracetamol had the higher concentration ranging from below 0.02 (LOR) to 0.2 µg L^{-1} of water. Salicylic acid concentration varied from below 0.1 (LOR) to 0.60 µg L^{-1} (Table 15). A comparison between the concentration of CST markers between commercial and residential catchments was made to determine if the prevalence of CST markers was significantly different (Table 14). Concentration of caffeine was significantly higher ($P < 0.05$) in the commercial catchments compared to residential catchments, whereas the differences in the occurrence of other CST markers 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.

Table 16: Concentration range of FIB and chemical source tracking (CST) markers for sewage contamination across residential and commercial catchments.

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

FIB numbers in $\log_{10}$, chemical markers in μg L^{-1}

4.4.2.4. Concurrence between MST and CST Markers

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 (Table 17). MST marker HF183 had high concurrence with HAv (96%) and acesulfame (92%). 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 salicyclic 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 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 MST and CST markers sewage markers was found not to correlate with the FIB numbers (Appendix B1).

Table 17: A correlation matrix showing the concurrence among microbial source tracking (MST) and chemical source tracking (CST) markers.

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

Markers showing degree of concurrence above 80% are bold faced.

4.4.2.5. Correlation among Faecal Indicators with FST Markers

Binary logistic regressions were used to identify existence of correlation between the numbers of FIB and the presence/absence of results for sewage-associated microbial and chemical markers. The presence/absence of the BFA sewage markers did not correlate with the FIB numbers Appendix B1.

4.4.3. 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 (O'Shea and Field, 1992; Marsalek and Rochfort, 2004). This study attempted to assess the extent to which stormwater acts as a conduit for sewage contamination 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 generally observed in the stormwater samples across all sites (Table 15), which is most likely due to the presence of fresh faecal contamination from sewage leakage and animal sources into the stormwater. A spike in the numbers of FIB after storm events has been previously reported in the literature (Brownell al., 2007; Parker *et al*., 2010). *Enterococcus* spp. numbers detected in the stormwater samples collected across all sites were generally higher by couple of orders of magnitude than the recommended limits for lowest water quality category D (< 501 *Enterococci* per 100 mL^{-1}) under Australian guidelines for managing risks in recreational water (NHMRC 2008).

Sewage associated *Bacteroides* HF183 and *M. smithii nifH* genes, were detected in 96% and 46% of stormwater samples tested respectively. The prevalence of the *nifH* marker was low in storm water samples compared to the HF183. The low prevalence of the *nifH* marker in stormwater samples could be due to the fact that these markers either have different decay rates in environment compared to other markers or because of their low prevalence in sewage (Ahmed *et al*., 2012). The HF183 and *nifH* markers have been previously shown to be sewage specific (Seurink *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*., 2008; Ahmed *et al*., 2012b). However, it is highly unlikely that a bacterial marker would be absolutely host-specific (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; Weidhass *et al*., 2011; Ryu *et al*., 2012). 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 had not originated from non-target hosts such as dogs, chickens and cat faecal samples where these markers were occasionally detected (Ahmed *et al*., 2012). Similarly, there

was a 94% probability that the detection of the *nifH* marker in stormwater sample was due to the true sewage contamination and not due to faecal contamination from non-target host.

HAv was found to be more prevalent (91%) than HPv (57%) in the stormwater samples. The presence of these human specific viruses in the environment was not unexpected as they are known to be present in high numbers (10^5 to 10^6 L^{-1}) in sewage with HPv numbers generally lower than HAv (Bofill-Mas *et al*., 2006). This corroborates with previous findings on the wide prevalence of HAv and HPv in surface water and stormwater (Rajal *et al*., 2007; Muscillo *et al*., 2008; Hamza *et al*., 2009; 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 viruses and protozoa could also be present, thus confirming the potential health risks.

Caffeine has been shown to be a suitable marker for sewage contamination in surface water and known to degrade rapidly during wastewater treatment and in the aquatic environment (Burge *et al*., 2003; Benotti and Brownawell 2007; Heberer *et al*., 2002). Caffeine concentrations in raw sewage ranging from 20 to 300 µg L^{-1} reducing to 0.1 to 20 µg L^{-1} in treated effluents has 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^{-1} have been reported (Buerge *et al*., 2003). The background levels of caffeine, origination from naturally occurring plant sources is usually negligible (Peeler *et al*., 2006). In this study, caffeine was frequently detected (91%) in the stormwater outfalls with concentration several times higher than reported for aquatic ecosystems (0.14 µg L^{-1} 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*., 2009; Scheurer *et al*., 2009). The typical entrance pathway of AS to stormwater is via wastewater (Scheurer *et al*., 2011). Acesulfame is known to be present in raw wastewater and treated effluent (12-46 µg L^{-1}) as it is not removed during the wastewater treatment and known to persist in surface water (Burge *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^{-1}. This also suggests sewage contamination as the main source of its origin in stormwater. However due to its persistence, it may not necessarily stem from recent sewage contamination but could in principle also indicate prior contamination of the catchment.

Pharmaceuticals can be good alternative markers for the verification of sewage contamination of stormwater. Analgesics, paracetamol (acetaminophen) and salicyclic acid (acetylsalicylic acid) are in the top ten most commonly dispensed (by weight) pharmaceuticals in Australia and are often detected in the wastewater and treated effluent at µg L^{-1} levels (Khan and Ongerth 2004; Al-Rifai *et al*., 2007). In the stormwater samples from all six sites, both paracetamol (0.03 to 2.00 µg L^{-1}) and salicylic acid (0.10 to 0.60 µg L^{-1}) were detected in high concentrations, which suggest ubiquitous sewage contamination. Moreover, both paracetamol and salicyclic acid are biodegradable and have very high removal rates during the wastewater treatment process (Kasprzyk-Hordern *et al*., 2009) and in the environment. Therefore, presence of both pharmaceuticals especially at µg levels in the stormwater points towards recent contamination from human sewage.

The results from both MST and sewage associated CST markers demonstrate that human sewage input was the major source of contamination of storm runoff as commercial catchments, with very limited number of animal sources of contamination, were equally contaminated as residential catchments. Furthermore, sewage contamination of stormwater may not be limited only to sewer overflows as other sources such as leakages from sewage infrastructure and cross connections also need to be managed. The concentration of caffeine was significantly higher ($P < 0.05$) in the stormwater runoff from commercial catchments as compared to residential catchments from Brisbane, Sydney and Melbourne (Table 16). This is potentially due to a higher per capita consumption of caffeine containing beverages in the commercial areas compared to the residential areas. Conversely, the median concentrations of paracetamol and salicylic acid were similar from residential and 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^{-1} levels, in stormwater suggests more recent contamination from raw sewage which is probably the cause high prevalence of adenovirus observed in this study. The results from this study, demonstrate very good concurrence (> 80%) between the occurrence of *Bacteroides* marker (HF183), adenovirus, acesulfame, paracetamol and caffeine (Table B2). MST marker HF183 had high concurrence with HAv (96%) and acesulfame (92%), which suggests that prevalence of HAv could be accurately predicted if HF183 is present in the stormwater. Similarly, acesulfame had very high concurrence of 96% with HAv again suggesting it is a useful indicator of the presence of adenovirus and potentially other enteric virus in stormwater. Caffeine, which is readily biodegradable (Buerge *et al*., 2006), also appears to be a good marker of sewage contamination and potential indicator for the presence of adenovirus. Consequently, monitoring for these FST markers in stormwater could provide accurate information on the presence of enteric virus which could be used towards prioritizing remediation projects.

4.4.4. Conclusions

In conclusion, this study demonstrates that human sewage input is likely to be the major source of enteric pathogen contamination of stormwater. An integrated stormwater management approach to control faecal contamination is required which may involve controlling the sources of contamination such as sewage leakage, elimination of cross connections and/or treatment after collection (e.g. by wetlands to allow natural attenuation or other engineered solutions) prior to discharge into surface water or stormwater harvesting. This study highlights the need of employing a "toolbox approach" which could include monitoring for HF183 and adenovirus along caffeine and paracetamol which will not only provide information of the presence of sewage contamination but also if it was a recent contamination. The collective use of MST and CST markers could provide a higher level of information toward decision making processes to protect human health.

4.5. Prevalence of *E. coli* Virulence Genes in the Stormwater

Escherichia coli and *Enterococcus* spp. commonly found in mammalian faeces have been traditionally used as indicators of faecal pollution in fresh and marine waters (NHMRC 2008, US EPA 1986). After storm events, several fold increase in the faecal indicator bacteria (FIB) numbers occur in the surface waters (Brownell *et al*. 2007, Parker *et al*., 2010, Sidhu *et al*., 2012). There may be several sources of *E. coli* that contribute to sudden increases in numbers of this bacterium in waterways including, sewage over flows, farm animals, pets and birds. The elevated microbial contaminants in storm runoff (Parker *et al*., 2010, Sauer *et al*., 2011) and subsequently in receiving waters bodies may pose a serious public health risks. Disease outbreaks related exposure to contaminated freshwater are well documented (Ackman *et al*., 1997, Shelton *et al*., 2006, Chalmers *et al*., 2000, Olsen *et al*., 2002) Exposure to recreational water has been linked to high numbers (21 out of 31) of reported *E. coli* O157:H7 disease outbreaks in the USA from 1982-2002 (Rangel *et al*., 2005). Despite the significant disease burden linked to contaminated water exposure, the prevalence of *E. coli* pathotypes in the urban aquatic environment is not well characterised.

The majority of *E. coli* strains are harmless; however, some strains have acquired specific virulence attributes that allow them to cause a wide spectrum of intestinal and extra-intestinal infections, such as diarrhea, urinary tract infection, meningitis and septicemia (Kaper *et al*., 2004, Nataro *et al*., 1998). Diarrheagenic *E. coli* have been classified into five well-described groups: enterotoxigenic *E. coli* (ETEC) strains, which are associated with traveler's diarrhea, porcine and bovine diarrhea; enteropathogenic *E. coli* (EPEC) strains, which cause diarrhea in children; enterohemorrhagic *E. coli* (EHEC) strains, which are associated with hemorrhagic colitis and hemolytic-uremic syndrome in humans; enteroaggregative *E. coli* (EAEC) strains, which are associated with persistent diarrhea in humans; and enteroinvasive *E. coli* (EIEC) strains, which are involved in invasive intestinal infections, watery diarrhea, and dysentery in humans and animals (Kaper *et al*., 2004, Nataro *et al*., 1998).

The main aim of this study was to determine the frequency of occurrence of diarrheagenic *E. coli* pathotypes in surface waters and the impact of storm runoff on their prevalence and distribution. The specific objectives of the study were to:

(i) determine the frequency of occurrence of potentially pathogenic *E. coli* strains in the surface water in sub-tropical Brisbane, Australia;

(ii) characterize the virulence gene (VG) profile of *E. coli* isolates to determine most common pathotypes; and

(iii) assess the influence of storm events and runoff on the distribution of potentially pathogenic *E. coli* strains. This was done to determine the extent of potential human health risks posed by the prevalence of diarrheagenic *E. coli* pathotypes in surface waters used for potable, non-potable and recreational purposes.

4.5.1. Materials and Methods

4.5.1.1. Water Sampling and Enumeration of *E. coli.*

One liter grab samples were collected in sterile Nalgene® containers from six sites in Brisbane, prior to, and after storm events representing a diverse faecal pollution sources ranging from high density urban areas to a relatively unpolluted site. A brief site description of sites are provided in Table 1. Samples were collected 1m from the stream bank and at a depth of about 0.5 m below water surface with a telescopic water sampler following a dry period (no rain fall in 48 h prior to sampling) and 10-12 h after a storm event (> 20 mm rain fall). Samples were transported to the laboratory on ice and processed within six hours of collection.

Standard membrane filtration method was used for the quantification of *E. coli* from the collected water samples (62). Briefly, 1 and 10 ml samples were filtered through 0.45 µm nitrocellulose (Millipore) filters (47 mm) and placed on Chromocult^{TM} coliform agar (Merck). Plates were incubated at 37^{o}C overnight and typical *E. coli* colonies were counted to determine the average number of colony forming units (cfu) per 100 ml.

4.5.1.2. *E. coli* Isolation and Extraction of DNA

Individual well-isolated typical *E. coli* colonies were picked from the Chromocult™ coliform agar plates and streaked on fresh Chromocult agar plates. During the dry period, 90 wild *E. coli* colonies were isolated from six sites in two sampling events with an aim of collecting 10 isolates each time from each sample, with no more than 2-3 colonies per plate. Similarly, during two wet period sampling events from same six sites, around 15 isolates per site were collected, resulting in 210 wild *E. coli* isolates. After purification (twice), single colonies were picked from agar plates and inoculated into 2 ml centrifuge tubes containing 1.5 ml nutrient broth (Oxoid). Inoculated tubes were incubated over night at 37°C in the shaking platform incubator at 100 rpm. All isolated *E. coli* were stored at -80°C in nutrient broth and 15% (vol/vol) glycerol. At the time of DNA extraction, *E. coli* isolates were grown in 5 ml of nutrient broth at 37°C overnight. One millilitre of overnight cell culture from each isolate was centrifuged at 10,000 g for 3 min. The resulting supernatants were removed and the cell pellets resuspended in 200 µL of sterile water by vortexing. DNA was extracted from the cell pellets by using InstaGene™ Matrix according to the manufacturer's instruction (Bio-Rad Laboratories). Presumptive *E. coli* isolates were confirmed by PCR amplification of the *uidA* gene as described previously.

4.5.1.3. PCR Positive Controls

E. coli ATCC 9637 was used as a positive control (*uidA* gene) in PCR assays to confirm presumptive *E. coli*. *E. coli* O157:H7 (ATCC 35150) was used as a positive control for the *eaeA*, stx_1 and stx_2, genes. *Shigella sonnei* (ATCC 29930) was used as a positive control for the *ipaH* gene *E. coli* strain belonging to serotypes O138 of porcine origin was used as positive control for ST and LT genes. Clinical isolates containing remaining target genes were used as positive controls.

4.5.1.4. PCR Detection of Toxin and Other Virulence Genes

The list of VGs and the corresponding pathotypes tested in this study is shown in Table 2. PCR confirmed *E. coli* isolates were screened for the presence of 11 diarrheagenic *E. coli* VGs by using previously published primers sets (Appendix B3). PCR reactions were performed on a Bio-Rad iQ5 thermocycler system (Bio-Rad Laboratories, California, USA), using iQ Supermix (Bio-Rad). Each 25 µL PCR reaction mixture contained 12.5 µL of SuperMix, 120 nM of each primer, and 3 µL of template DNA. For each PCR experiment, corresponding positive (i.e., target DNA) and negative (sterile water) controls were included. Thermal cycling conditions are presented in Table 3. A melt curve analysis was performed after each PCR run to differentiate between actual products and primer dimmers, and to eliminate the possibility of false-positive results. The melt curve was generated using 80 cycles of 10 s each starting at 55°C and increasing in 0.5°C intervals to a final temperature of 95°C. The Tm for each amplicon was determined using the iQ5 software (Bio-Rad).

4.5.1.5. Statistical Analysis

The difference in VGs distribution among the six sites, and existence of variation among VGs patterns was determined by analysis of variance (ANOVA) on the pooled *E. coli* data from the dry and wet weather isolates with significance defined as $p < 0.05$. All data on *E. coli* numbers from all sites was $\log_{10}$ transformed prior to statistical analysis. The student's *t-test* was performed to compare the significance of difference between *E. coli* numbers across sites and during the dry and wet period. The critical *p*-value for the *t-test* was set at 0.05 and all tests were considered significant if the *p* value was < 0.05. A linear regression analysis was applied to investigate existence of any correlation between *E. coli* numbers and VGs during the dry and wet weather.

4.5.2. Results

4.5.2.1. Prevalence of *E. coli* during Dry and Wet Period

The $\log_{10}$ transformed results for the detection of *E. coli* are shown in Figure 27. In general, *E. coli* numbers in water samples from all sites varied between 1.8 to 4.2 $\log_{10}$ per 100 ml between dry and

wet periods. The mean *E. coli* numbers after the storm events were significantly higher (p 0.05) than the dry period. Samples collected from Cabbage Tree Creek and the Fitzgibbon drain sites had significantly higher ($p<0.05$) *E. coli* counts after the storm events compared to the other four sites tested.

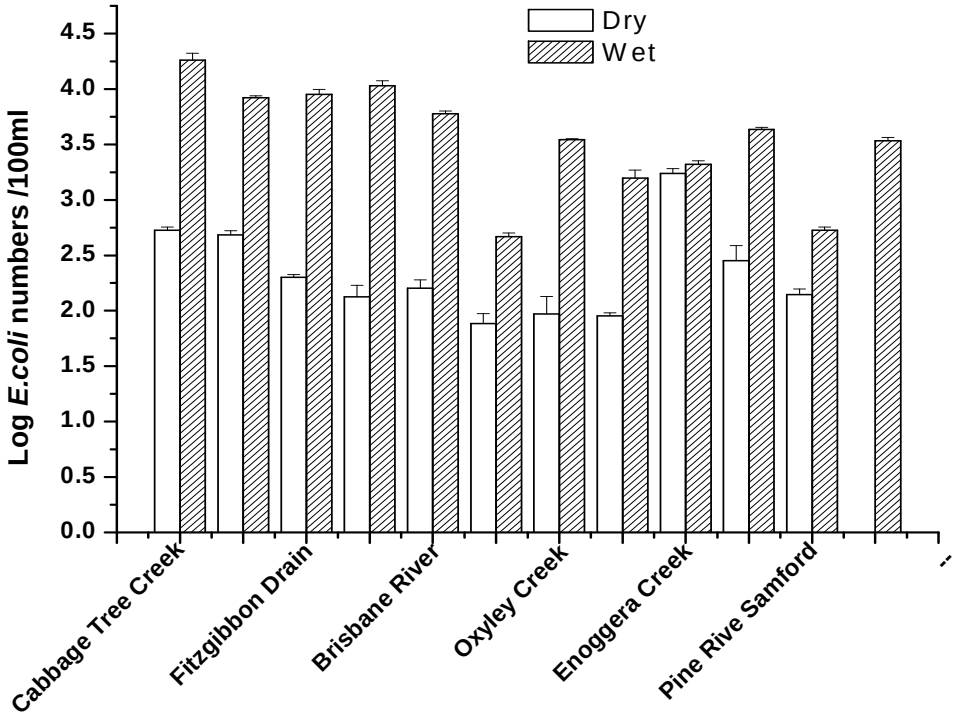

Figure 27: Comparative *E. coli* numbers during the dry and wet weather conditions at the six sampling sites. Note 2 sets of data are reported for each site.

4.5.2.2. Prevalence of VGs among *E. coli* Isolates

Among the 300 *E. coli* isolates tested, 256 (85%) carried at least one VG, with no VGs detected in 44 isolates (15%). During the dry period, 72 out of 90 isolates (80%) carried 1 to 3 VGs and only 7 isolates (8%) carried > 4 VGs (Figure 28). However, after the storm, 145 isolates (82%) were found to harbor 1 to 3 VGs and a further 32 (18 %) carried > 4 VGs. The prevalence of multiple VGs in the *E. coli* isolates was higher after the storm events compared to dry periods. However, no correlation was observed between the total number of *E. coli* and E. coli that were carrying VGs in water samples during the dry periods or the storm events.

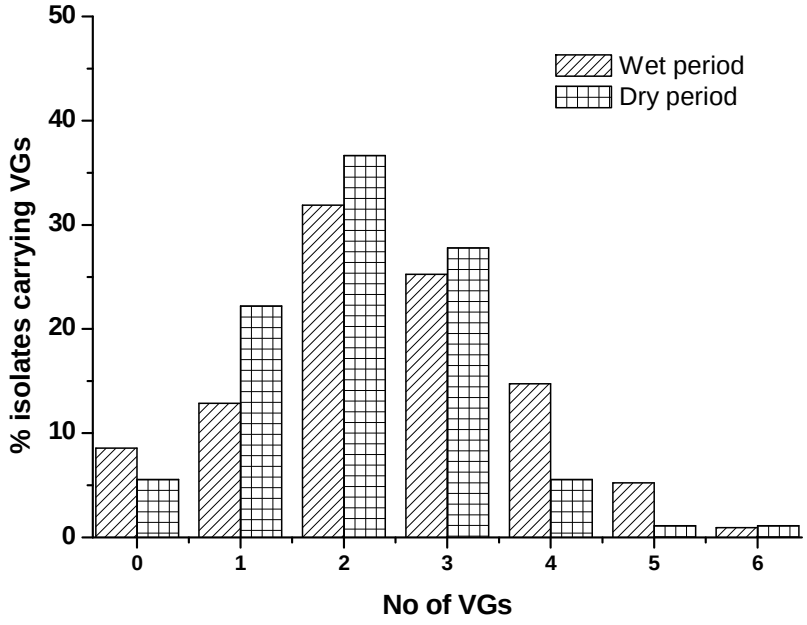

Figure 28: Comparative distribution of the numbers of virulence genes (VGs) carried by individual *E. coli* isolates.

Among the adhesion and invasion VGs, *eaeA* which codes for intimin protein in both EHEC and EPEC pathotypes was the single most prevalent gene (65%) (Table 18). Among 300 *E. coli* isolates, nine (3%) carried only *eaeA* gene and these isolates were classified as atypical EPEC. The EAEC transcriptional regulator gene *agg*R was detected in 34% of the isolates which was followed by the EPEC bundle forming pilli gene *bfp* (28%) and the EIEC invasion plasmid antigen gene *ipaH* (16%). The gene *eaeA* was detected more frequently (73%) in *E. coli* isolates after the storm events compared to the dry periods (48%). Similarly, the *bfp* and *ipaH* genes were detected more frequently, 32% and 21%, respectively after the storm events compared to dry periods where their prevalence was 19% and 6%, respectively. In contrast, the *agg*R gene had a higher prevalence (41%) during the dry periods compared to wet periods (31%).

Table 18: Occurrence of virulence genes (VGs) in *Escherichia coli* isolated from surface water samples across six sampling sites in Brisbane.

Number of Isolates	*No. of E. coli* Carrying Virulence Gene and % Distribution (in brackets)										
	eaeA*	***stx*$_1$**	***stx*$_2$**	**LT1**	**ST**	***bfp***	***cdtB****	***ipaH***	***aggR***	***astA***	***ehxA****
Dry period (90)	38 (42)	4 (5)	12 (13)	2 (2)	0 (0)	15 (17)	8 (9)	5 (6)	32 (36)	67 (75)	9 (10)
Wet period (210)	129 (61)	15 (7)	18 (9)	4 (2)	12 (6)	56 (27)	17 (8)	37 (18)	55 (26)	139 (66)	29 (14)
Total (300)	167 (56)	19 (6)	30 (10)	6 (2)	12 (4)	71 (24)	25 (8)	42 (14)	87 (29)	206 (69)	38 (13)

* genes shared by more than one *E. coli* pathotype

Among the toxin genes, the enteroaggregative heat-stable enterotoxin (*east1*) coded by the *astA* gene carried primarily by EAEC but also by EHEC was the single most prevalent gene (80%). The gene e*hxA* primarily carried by EHEC was the second most commonly detected toxin gene (15%) with a slight increase in detection from 11 to 16% from dry to wet period. The EPEC toxin gene *cdtB* was detected in 10% of isolated during both dry periods and after storm events. The EHEC toxin gene *stx*$_2$ (12%) was more frequently detected than *stx*$_1$ (7%) with both genes showing very little variation

between dry and wet periods. The heat stable toxin gene (ST), generally carried by ETEC was only detected in *E. coli* isolates during the wet period (7%). In contrast to all the other toxin genes the heat labile toxin (LT gene) was detected in small number of isolates (2%) during both dry and wet periods (Table 18).

In order to further explore the distribution of the 11VGs among all six sites, an ANOVA was performed on the pooled data of wet and dry periods. There was highly significant difference ($p < 0.001$) between the occurrence of *eaeA*, ST and LT genes in *E. coli* isolates. Similarly, a highly significant difference ($p < 0.001$) was observed in the occurrence of *astA* gene and stx_1, ST, LT and *cdtB* genes. A highly significant difference ($p < 0.001$) was also observed between the occurrence of *aggR* gene, ST and LT genes. The difference between the occurrence of *ipaH* and *astA*, ST and *bfp*, stx_2 and *astA*, *eaeA* and stx_1 genes was also significant ($p < 0.01$).

4.5.2.3. Comparative Prevalence of *E. coli* Pathotypes

The percentages of *E. coli* isolates with defined pathotypes from the six sampling locations during the dry and the wet periods are shown in Figure 29. On the basis of combinations of VGs, approximately 53% of the *E. coli* isolates could be placed into five main pathotypes (EHEC, EAEC, EIEC, ETEC and EPEC) during the dry periods and a further 4% of isolates were observed to have combinations of genes from both EHEC and EAEC pathotypes. During the dry periods, isolates belonging to the EAEC pathotype were the most commonly detected (23%) followed by EHEC (11%) and EPEC (11%). Nearly 40% of the dry period isolates carrying VGs could not be placed under defined categories due to random distribution of single or multiple genes from different defined pathotypes.

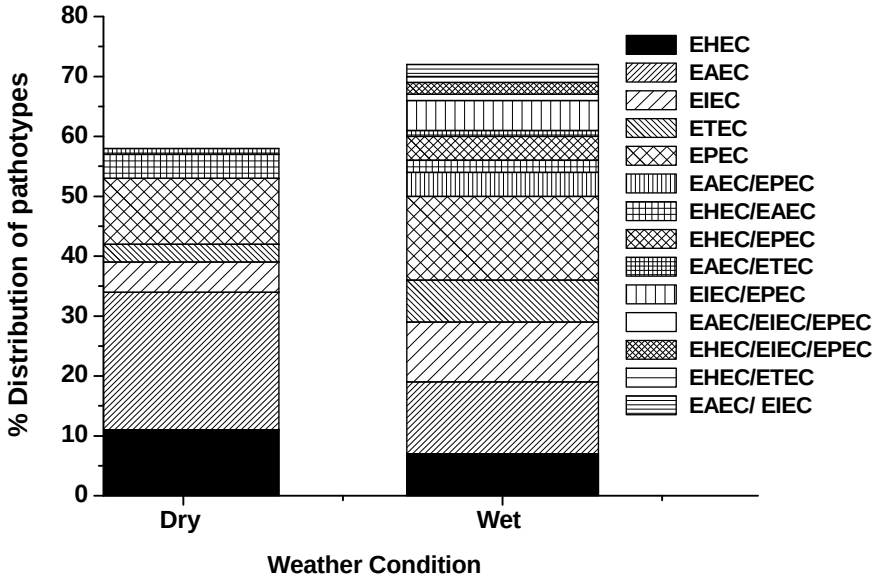

Figure 29: Comparative distribution of *E. coli* pathotypes during dry and wet weather conditions from all six sites in Brisbane, Australia.

Approximately 50% of the *E. coli* isolates collected after storm events could be placed under five main pathotypes, however, the distribution of pathotypes was more uniform compared to the dry periods. The pathotypes EPEC (14%), EAEC (12%) and EIEC (10%) were more commonly detected compared to EHEC (7%) and ETEC (7%). In addition, due to a more common occurrence of multiple VGs in the *E. coli* isolates collected after the storm events, nearly 20% of *E. coli* isolates could be placed under more than one pathotype. Approximately 9% of the isolates carried a combination of EPEC, EIEC and EAEC VGs. The remaining 30% of the isolates carrying single or multiple VGs could not be classified into known pathotypes due to a random distribution of genes from more than one pathotype.

4.5.2.4. Comparison of *E. coli* VGs Profile from Six Sites

A comparative analysis of the distribution of VGs across all six sites during the dry periods and after the storm events is presented in Figure 30. In general, the frequency of occurrence of VGs in *E. coli* isolates collected after the storm events conditions was higher than the dry period. However, at the Oxley Creek site, apart from the *eaeA* gene all other genes were detected in higher frequency in isolates from the dry periods (Figure 30 E). The Enoggera Creek site has the lowest overall frequency of occurrence of VGs (Figure 30 F). In contrast, the Fitzgibbon drain has the highest occurrence of VGs. The VGs *astA* and *eaeA* were most frequently detected across all the sites, whereas the LT and ST genes were infrequently detected (Figure 30 A). Among the toxin genes screened in this study, e*hxA* and stx_2 genes were the two most commonly detected genes across all sites after *astA* gene which was highly prevalent (>60%). Out of the six sites, the toxin gene *cdtB* was only detected in more that 10% isolates from the Oxley Creek and Enoggera Creek sites.

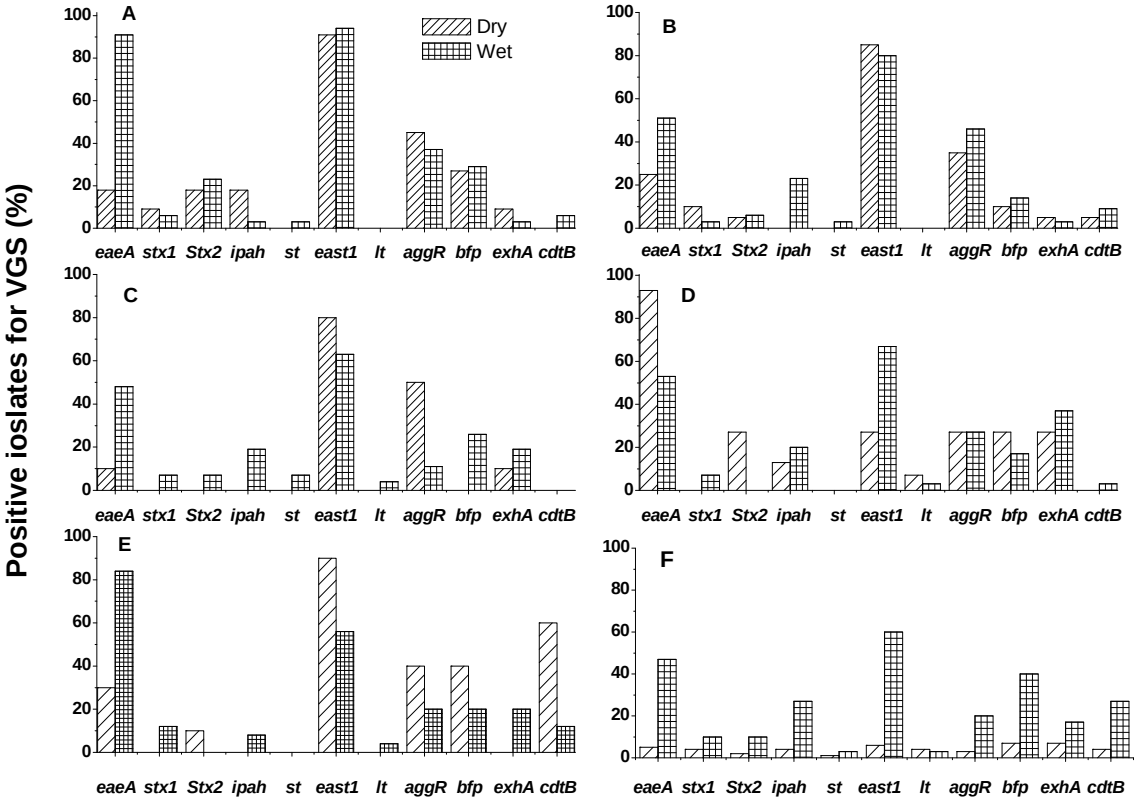

Figure 30: Comparative distribution of VGs in *E. coli* isolates at the six sites during the dry periods and after storm events (A) Fitzgibbon drain, (B) Cabbage Tree Creek, (C) Brisbane River, (D) Pine River, (E) Oxley Creek and (F) Enoggera Creek.

A comparison between sites was made (ANOVA) to determine if the sites were similar or different on the basis of occurrence of VGs. The Enoggera Creek site was significantly different ($p < 0.05$) from the Brisbane River site, whereas, differences between the other sites was found to be statistically non significant. In the *E. coli* isolates from Brisbane River, the toxin genes stx_1, stx_2, ST, LT and adhesion genes *ipah* and *bfp* were only detected after the storm events. In contrast, at the Enoggera Creek site all these genes were detected during both dry and wet periods with a slight increase in frequency after rain fall.

4.5.3. Discussion

Storm runoff may lead to an increased prevalence of microbial pathogens including diarrheagenic *E. coli* pathotypes in the surface water bodies due to transport of faecal contamination from land (Sidhu *et al*., 2012). This study compared the distribution and frequency of occurrence of the potentially diarrheagenic *E. coli* pathotypes in surface water prior to and after storm events to assess if stormwater runoff could lead to elevated health risks.

The results show a significant increase ($P < 0.05$) in the *E. coli* numbers after rain fall at all sites. A potential cause could be fresh human input from sewage leakage and overflow, other likely sources include input from animal sources (Davies *et al*., 2000) mobilization of *E. coli* surviving in the soil (Brennan *et al*., 2010), sediments Czajkowska *et al*., 2005) and aquatic environment (Lothigius *et al*., 2010). These findings are in agreement with previously reported observation of several fold increase in FIB numbers in the surface water bodies after storm events (Brownell *et al*., 2007, Parker *et al*., 2010, Sidhu *et al*., 2012). In this study, an increase in the VGs distribution among *E. coli* isolates was observed after the storm events, however, no correlation could be found between the *E. coli* numbers and the occurrence of VGs during dry period or storm events. This demonstrates the potential risk of infection from *E. coli* carrying VGs in stormwater even when *E. coli* numbers in water are not high.

The presence of a single or multiple VGs in an *E. coli* strain does not necessarily indicate that a strain is pathogenic unless that strain has the appropriate combination of VGs to cause disease in the host (Boerlin *et al*., 1999). The pathogenic *E. coli* use a complex multi-step mechanism of pathogenesis involving a number of virulence factors depending upon the pathotype, which consists of attachment, host cell surface modification, invasin, variety of toxins and secretion systems which eventually lead toxins to the target host cells (Kaper *et al*., 2004). Thus VGs are ideal targets for determining the pathogenic potential of a given *E. coli* isolate (Kuhnert *et al*., 2006).

Enteroaggregative *E. coli* (EAEC) cause persistent diarrhoea in children and adults and are defined by the presence of heat-stable enterotoxin-1 (*east1*) along with *aggR* (Kaper *et al*., 2004). In this study, *astA* gene encoding the *east1* enteroaggregative *E. coli* heat-stable enterotoxin-1 *astA* was found to be widely distributed in *E. coli* isolates from all sites (Table 18). The prevalence of *astA* gene was statistically higher (ANOVA, $P<0.001$) compared to the stx_1, ST, LT and *cdtB* genes. The high prevalence of *E. coli* carrying *astA* gene in fresh and estuarine water has also been reported previously (Masters *et al*., 2011) and could potentially be due to its reported presence in many commensal *E. coli* isolates (Menard *et al*., 2002). The results of this study showing the presence of *astA* gene in diarrheagenic *E. coli* pathotypes EAEC, EHEC and EPEC are in agreement with the previously reported wide distribution of this gene among diarrheagenic *E. coli* isolates from humans and animals (Yamamoto *et al*., 1997, Savarino *et al*., 1996, Yatsuyanagi *et al*., 2003). The high prevalence of the toxin gene *astA* in the *E. coli* isolates from the stormwater is a cause of concern as *E. coli* strains carrying *astA* toxin gene alone have been shown to cause diarrhea in studies from Japan and Spain (Itoh *et al*., 1997, Viljanen *et al*., 1990). Since the *astA* gene is also reported to be carried by many commensal *E. coli,* the implications of the wide prevalence of this gene in surface water remains an open question (Savarino *et al*., 1996). All *E. coli* isolates were screened for the presence of *aggR* gene to determine if they belong to the EAEC pathotype and *E. coli* carrying both *astA* and *aggR* genes were classified as typical EAEC. The *aggR* gene was detected in isolates from both dry (36%) and wet period (26%) with slightly higher frequency suggesting relatively high prevalence of this pathotype.

The *eaeA* gene which codes for intimin protein was the second most prevalent gene (56%) in the *E. coli* isolates from stormwater. This gene is necessary for intimate attachment to host epithelial cells in both the EHEC and EPEC pathotypes. The frequency of occurrence of this gene was statistically higher at all times (ANOVA, $P<0.001$) compared to ST, LT and other VGs which were infrequently detected and had a noticeably higher prevalence in the isolates collected after the storm event (61%) compared to dry periods (42%). This observation is in agreement with previously reported finding of significantly higher prevalence of the *eaeA* gene (up to 96%) in surface water (Shelton *et al*., 2006, Masters *et al*., 2011). EHEC causes hemorrhagic colitis and hemolytic uremic syndrome in humans and key virulence factors include intimin (*eae*A gene) and shiga-toxins (stx_1 and stx_2 genes) (Boerlin *et al*., 1999). The relatively high occurrence of stx_2 gene (10%) compared to stx_1 (6%) in the stormwater *E. coli* isolates suggests that *E. coli* carrying a combination of the *eae*A and stx_2 genes is more

common than the *eae*A and stx_1 combination. This observation is of concern as the former combination of genes is known to cause more severe diarrhea in humans (Paton and Paton 1998; Boerlin *et al*., 1999). Typical EPEC strains carry the LEE pathogenicity island, which encodes for several virulence factors, including intimin (*eaeA*) and the plasmid encoded bundle forming pilus (*bfp*) which mediates adhesion to intestinal epithelial cells (Kaper *et al*., 2004, Ishi *et al*., 2007). Therefore, all isolates were further tested for the presence of the *bfp* gene to determine if they belong to the EPEC pathotype. In this study, noticeably higher prevalence of the *bfp* gene in isolates from stormwater (27%) compared to dry period isolates (17%) suggests that a higher prevalence of the EPEC pathotype could be expected in the surface water bodies after storm events.

In addition to the presence of *eaeA* gene *in* both EHEC and EPEC pathotypes, it was also detected in isolates which lacked other typical genes from both groups and 3% of isolates carried only *eaeA* gene. This suggests that there is a wide prevalence of this gene in *E. coli* found in aquatic ecosystems. Similarly, high prevalence of *eaeA* gene in surface water has been reported in other studies (Shelton *et al*., 2006, Master *et al*., 2011). This is a cause of concern as atypical EPEC pathotype which lacks the *bfp* gene but carry the *eaeA* gene have been found to be a major cause of gastroenteritis worldwide (Hernandes *et al*., 2009) and in patients suffering from community acquired gastroenteritis in Melbourne, Australia (Robins-Browne *et al*., 2004), and from children with diarrhea in Germany (Kozub-Witkowski *et al*., 2008).

The toxin gene *ehxA* which is carried by EHEC and non-Shiga toxin producing *E. coli* pathotypes (Ishii *et al*., 2007, Kaper *et al*., 2004, Paton and Paton 1998, Paton and Paton 2002) was the second most commonly detected toxin gene (13%) with a slight increase in the detection frequency from 11 to 16% from dry to wet periods. Enteroinvasive *E. coli* (EIEC) carry the invasion plasmid antigen H (*ipaH*) which has been used for identification of isolates belonging to this pathotype (Guion *et al*., 2008). The frequency of occurrence of this gene was noticeably higher during the wet periods (18%) compared to dry periods (6%), which suggests noticeable movement of this pathotype into surface water from storm runoff.

The results of this study show that diarrheagenic *E. coli* pathotypes occur at each of the sampling sites during both dry and wet periods (Figure 29). During the dry periods, a high percent (53%) of isolates could be grouped under five main diarrheagenic *E. coli* pathotypes. EAEC which causes persistent diarrhea in children and adults were the single most common pathotype (23%). This was expected as the occurrence of the heat-stable enterotoxin-1 (*astA*) along with the *aggR* genes which define this pathotype (Kaper *et al*., 2004) were two of the most frequently detected genes. This is in agreement with the previously reported high prevalence (up to 80%) of EAEC pathotype in fresh and estuarine samples (Masters *et al*., 2011). The high prevalence of this pathotype is of concern as EAEC strains are the second most common agent of traveller's diarrhea after ETEC, with food and water being the most likely means of transmission (Huang *et al*., 2006, Mohamed *et al*., 2007). EHEC and EPEC were the second and third most common pathotypes detected in this study with each group represented by 11% of isolates. This suggests that the three pathotypes EAEC, EHEC and EPEC occur widely in the surface water at all sites.

A more uniform distribution of *E. coli* pathotypes was observed in *E. coli* isolates after the storm events with EPEC (14%), EAEC (12%), EIEC (10%) being the three most commonly detected pathotypes followed by EHEC (7%) and ETEC (7%). Furthermore, the frequency of occurrence of EAEC pathotypes declined noticeably from 23 to 12% between the dry and wet period. The observed decline in EAEC pathotypes and more uniform distribution of *E. coli* pathotypes after rainfall could possibly be due to mobilization of *E. coli* from point sources such as wastewater treatment plant discharge and non-point sources such as animal sources (Nataro and Kaper, 1998; Paton and Paton, 1998; Lyautey *et al*., 2010; Levine 1987) *E. coli* surviving in the soil (Brennan *et al*., 2010), sediments (Czajkowska *et al*., 2005) and aquatic environment (Lothigius *et al*., 2010). This could also be a possible explanation of the observed increase (5 to 20%) in the frequency of occurrence of isolates which could be placed under more than one pathotype. The occurrence of unusual combinations of VGs in *E. coli* isolates observed in this study could be explained on the basis of horizontal gene transfer between cells which enables the exchange of genetic material located on mobile elements (transposons, integrons or plasmids) among related or unrelated bacterial species (Davison, 1999).

Further screening of the *E. coli* isolates with these unusual VGs patterns in tissue culture or animal models would be required to demonstrate their pathogenicity.

In this study, we collected water samples from areas with diverse human population density and land use to determine if these factors influence the distribution of VGs (Figure 30). The results of this study did not show any clear pattern of occurrence of VGs across the sites apart from a noticeable difference of occurrence of *cdtB* gene (>10% isolates) at Oxley Creek and Enoggera Creek. This suggests that, overall, the contamination sources (point and non-point) were potentially similar across sites. There was difference in the overall occurrence of VGs with the Fitzgibbon Drain having a higher occurrence and Enoggera Creek site with one of the lowest occurrence of VGs. However, the difference in occurrence was statistically significant (ANOVA, $P < 0.05$) only between the Enoggera Creek and Brisbane River sites, with the later site showing prevalence of stx_1, stx_2, ST, LT, *ipah* and *bfp* genes only after rainfall, unlike the former site which had a prevalence of these genes during both dry period and after the storm events.

A better understanding of the prevalence and distribution of *E. coli* pathotypes in water sources used for potable, non-potable or recreation purposes could be an important tool in the development of public health risk mitigation strategies. Pathotyping of *E. coli* isolates may also provide useful information to identify potential sources of pollution, as the principal reservoirs of EAEC, EIEC and EPEC pathotypes are humans, whereas bovine intestinal tract is the main source of EHEC pathotype (Levine 1987, Kaper *et al*., 2004). The lower prevalence of EHEC pathotype compared to other pathotypes suggests that human faecal contamination of the waterways is the main source of diarrheagenic *E. coli* pathotypes in the surface water as opposed to contamination from animals. This underscores the importance of managing municipal wastewater sources such as sewage leaks and over flows and wastewater treatment plant discharge in aquatic environments. Although the frequency of occurrence of certain VGs and pathotypes clearly increased after the rainfall, the presence of these genes could not be attributed to stormwater runoff alone. The prevalence of VGs in the *E. coli* isolates collected during the dry periods suggests that there is always presence of pathogenic *E. coli* in the surface water. The results demonstrate that the risk of contacting infection however, may increase after the storm event.

Since this study was focused on the detection of *E. coli* pathotypes carrying VGs, it is plausible to assume that actual distribution of these VGs in surface water could be higher. While the ability of *E. coli* isolates described in this study to cause human diarrheal diseases was not demonstrated, a high proportion of isolates carried a full set of VGs linked to known pathotypes. Further screening for other VGs along with serotype testing and other assays may provide further information on pathogenicity of these isolates.

4.5.4. Conclusions

In conclusion, we found *E. coli* bacteria with defined pathotypes which appear to be mainly originated from human sources, as opposed to contamination from animals, in surface water samples. This underscores the importance of controlling sources of human faecal pollution such as managing municipal wastewater sources to reduce potential risks to human health. This also highlights the need for some degree of treatment of captured stormwater prior to its reuse for potable and non-potable purposes for public health risk mitigation. The results of this study suggests that there is a potential risk of infection from *E. coli* carrying VGs in stormwater even when *E. coli* numbers in water are not high. This study clearly indicate that there is a need to develop a better understanding of public health implications of occurrence of *E. coli* carrying VGs in water sources used for potable, non-potable and recreational purposes.

4.6. Pathogen Survival Potential in Stormwater

Microbial pathogens, enteric viruses, protozoa and bacteria, have been shown to persist in reclaimed water (Rose *et al.*, 1996; Gennaccaro *et al.*, 2003) and storm water (Pitt *et al.,* 1999). In general, microbial pathogens lose viability in the aquatic environment with time (Noble *et al.,* 2004; Sidhu *et al*., 2010) and a number of factors have been implicated in inactivation of pathogens. In particular, temperature (Yates *et al.,* 1990), dissolved oxygen (Jansons *et al.,* 1989), sunlight (Sinton *et al.,* 2002) the activity of autochthonous microorganisms (Gordon and Toze, 2003) and organic carbon concentration (Feng *et al.,* 2003) has been reported to influence the decay of enteric pathogens. Inactivation of pathogens in aquatic environment is also type specific. Many of the factors implicated in the inactivation of pathogens can be site specific (Sobsey *et al.,* 1995, Sidhu *et al.,* 2010), hence there is a need for localised data on the pathogen survival potential. Further development of the understanding on the fate and behaviour of pathogens in stormwater is essential for an accurate evaluation and prediction of health risks associated with stormwater reuse. In order to determine site specific inactivation rates of pathogens, *in-situ* pathogen inactivation study was carried out at Fitzgibbon site. The aim was to produce inactivation rate data which could then be used for a quantitative microbial risk assessment for stormwater reuse as outlined in the Section 4.7.

4.6.1. Materials and Methods

4.6.1.1. Study Site

In-situ pathogen inactivation study was carried out at Fitzgibbon Drain during the summer (November to January 2011). Detailed information on the site description is presented in Table 1.

4.6.1.2. In-situ Pathogen Inactivation Studies

The pathogens and indicator microorganisms used in this study are commonly found in the wastewater. *E. coli* and *E. faecalis* are commonly used indicators to monitor inactivation of bacterial pathogens. Human adenovirus and coxsackievirus were used as representative DNA and RNA virus. *E. coli* (ACM 1803) was cultured in Nutrient Broth (Oxoid) and *Enterococcus faecalis* (ACM 2517) in Brain Heart Infusion Broth (Oxoid). All bacterial cultures were incubated at 37^{o}C overnight in a shaking incubator. Adenovirus (type 3) and coxsackievirus B1 (ATCC VR-28), were cultured and prepared as described previously (Gordon and Toze 2003).

Pathogen inactivation studies in the stormwater drain was undertaken using diffusions chambers as outline previously in Sidhu *et al*., 2012b. Briefly, teflon chambers were made with 25 mm, 250 k MWCO (~0.025 μm) cellulose esters (VSWP) (Millipore, Australia). This pore size is sufficient to exclude the passage of the enteric viruses but still allow passage of water and nutrients across the membrane and through the diffusion chamber. A water sample (500 mL) was collected from the stormwater drain at Fitzgibbon site in sterile borosilicate bottle. The collected water sample was split into two sub-samples. Each of the sub-samples (250 mL) was then seeded with either bacterial strains or adenovirus and coxsackievirus to achieve a final number of approximately 10^4 to 10^7 mL^{-1}. Diffusion chambers were then activated by placing 7 mL of seeded water with bacteria or enteric virus into each chamber.

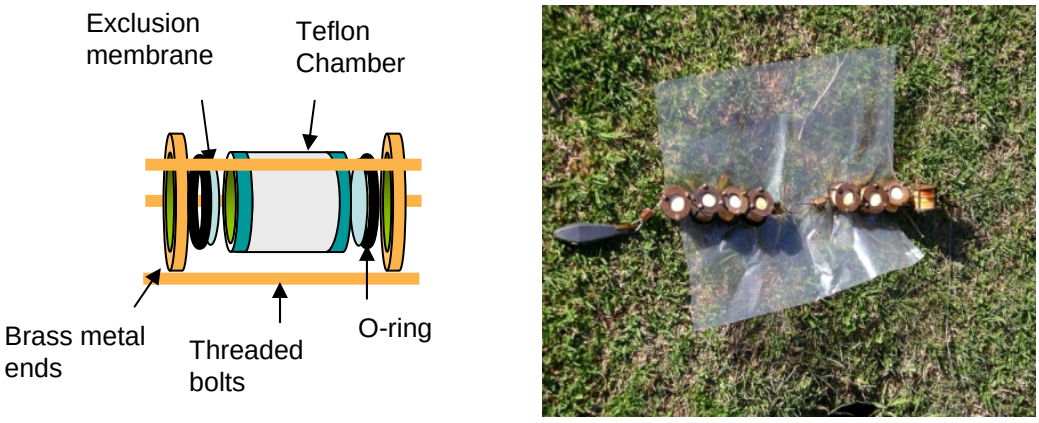

Figure 31: Diffusion chambers used for *in-situ* pathogen inactivation studies.

Figure 32: Diffusion chamber on stainless steel wire tied between star pickets.

The assembled chambers were then suspended on a stainless steel wire tied to star pickets in the stormwater drain at Fitzgibbon Drain site so that the uppermost chambers were suspended at approximately 800 mm to 1 m below the water level. On each sampling occasion, three chambers each for bacteria and enteric virus were removed from the steel wire and brought to laboratory on ice packs in an esky for the quantification. Chambers were collected at time 0, then on days 5, 7, 13, 20, 27, 41, 48 and 55 days after deployment for adenovirus and coxsackievirus. *E. coli* and *E. faecalis* chambers were collected at time 0, then on days 1, 3, 5, 7, 13, 20.

4.6.1.3. Quantification of Microorganisms during Inactivation Study

All analyses for the quantification of *E. coil* and *E. faecalis* were performed by spread plating 100 µL of appropriate serial dilutions with five replicates on the selective agar plates as outlined in the Section 4.1.1.3.

Viral RNA/DNA was extracted from the samples using BD biosciences Clonetech NucleoSpin® nucleic acid extraction kit as per manufacturer instructions and stored at -80°C prior to analysis. Virus numbers in the samples were quantified via real-time PCR in triplicate as outlined in the Section 4.3.1.6. The cloned material from both coxsackievirus and adenovirus were used for preparing standards for qPCR. Quantitative RT-PCR for the detection of coxsackievirus was run on a BioRad

iQ5, using 12.50 µL of iScript one step RT-PCR kit with SYBR Green (BioRad) 200 nM each primer and 1 µL of RNA template. Similarly, PCR reactions for the detection of adenovirus were carried out with 10 µL of Sso Fast EvaGreen® Supermix (BioRad), 200 nM each primer and 1 µL of DNA template. Both adenovirus, and coxsackievirus were quantified using published primer sets (Heim *et al.*, 2003; Abbaszadegan and Delong, 1997).

4.6.1.4. Data Analysis

Microbial quantification data on pathogen inactivation experiments was log transformed and plotted against time. A rapid decline in bacterial numbers was observed during the initial stage (0-4day) which was followed by a slower decline in numbers up to 12 days. Whereas, slow gradual decline in the numbers of seed coxsackievirus and adenovirus was observed. In order, to derive an average inactivation rate coefficient (k_d) linear regression lines were fitted to the plots. The results were also reported as a T_{90} time in days (one log loss).

4.6.2. Results

4.6.2.1. *In-situ* Pathogen Inactivation

In-situ pathogen inactivation experiment was conducted during the summer at Fitzgibbon Drain. The water temperature measured with *in-situ* temperature probe installed in the drain varied between 19 to 29ºC during the duration of the decay study (Figure 33). All microorganisms tested in this study were observed to decay in the stormwater drain (Figures 34 and 35). A comparative one log reduction times (T_{90}) of the tested microorganisms is presented in Table 19. The decay rates of seeded adenovirus, coxsackievirus, *E. coli* and *Enterococci* spp. were determined by applying liner regression to the log transformed data. The results showed that all tested bacteria had one $\log_{10}$ reduction time of <2 days. Rapid decay of seeded *E. coli* and *Enterococci* spp. was observed with one $\log_{10}$ reduction time of 2 and 1 day respectively. Inactivation times (T_{90}) of 14 and 18 days were observed for coxsackievirus and adenovirus respectively.

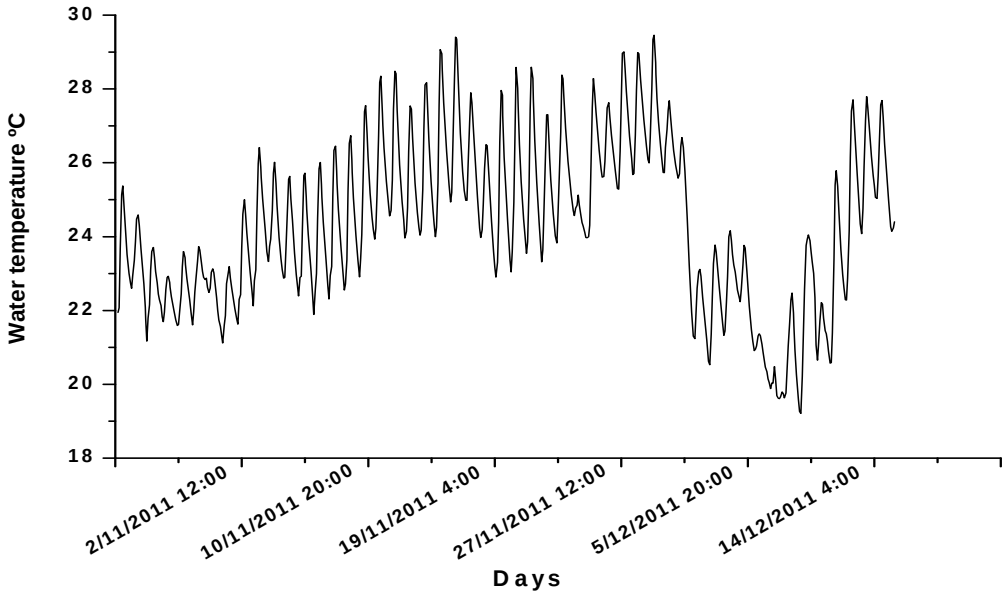

Figure 33: Water temperature during the duration of the pathogen decay study.

Table 19: Decay rates of target microorganism in stormwater.

Microorganisms	Inactivation Rate (K_d)	One Log_{10} Reduction (T_{90}) (days)	R^2
E. coli	0.37	2	0.78
Enterococci spp.	1.44	1	0.78
Adenovirus	0.054	18	0.75
Coxsackievirus	0.070	14	0.75

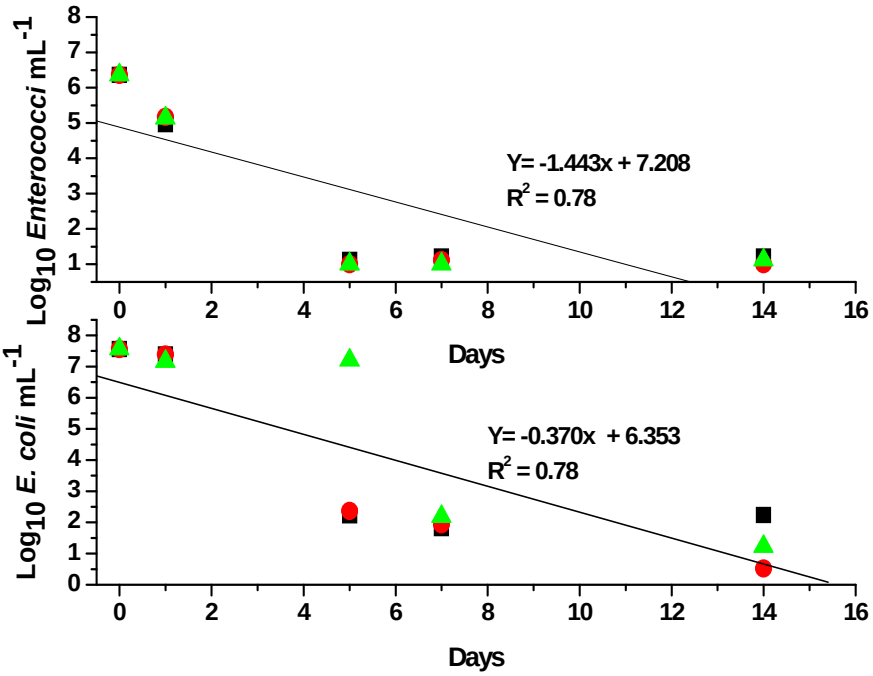

Figure 34: Decay of *E. coli* and *Enterococci* spp. in the stormwater. Square, circle and triangle represent replicate data collected from three diffusion chambers.

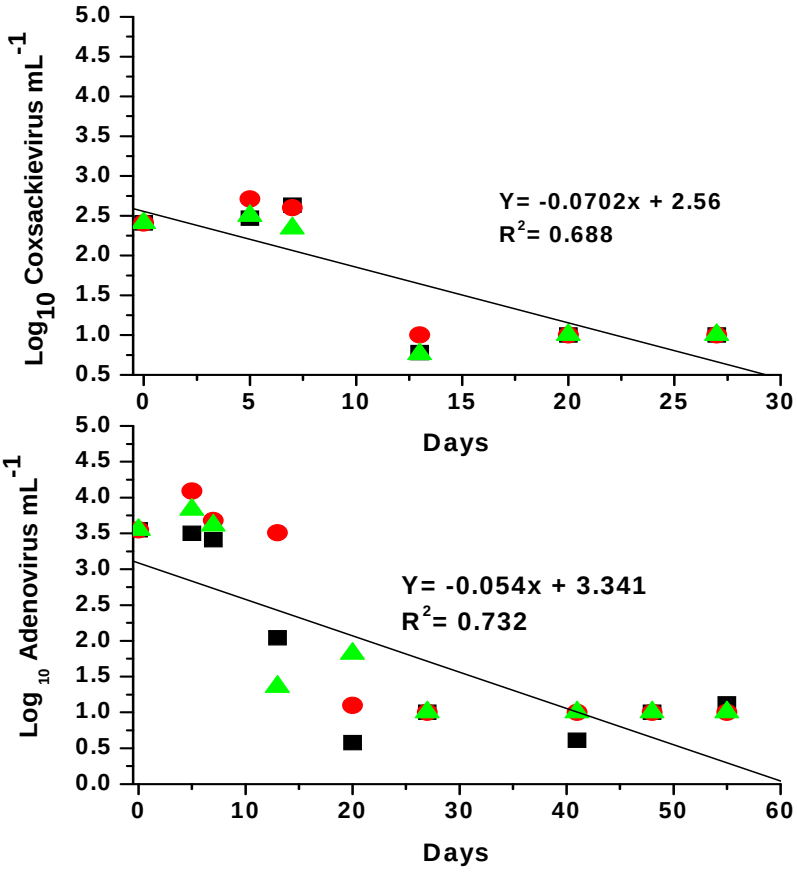

Figure 35: Decay of adenovirus and coxsackievirus in the stormwater. Square, circle and triangle represent replicate data collected from three diffusion chambers.

4.6.3. Discussion

All the bacteria tested in this study showed survival times of less <2 days, suggesting limited survival potential for the bacterial pathogens in the stormwater under the ambient conditions. The observed rate of bacterial inactivation (T_{90} <2 days) in the stormwater drain are comparable to previously reported rates in the groundwater (Gordon and Toze, 2003; Sidhu *et al.,* 2010). Also, similar one $\log_{10}$ reduction time of 2 to 4 days for faecal coliforms and *S. typhimurium* have been reported from a laboratory based study with wastewater (Karim *et al.*, 2004).

In this study, average water temperature was above 20ºC during the duration of study which resulted in rapid inactivation of both indicator bacteria whereas, slightly slower decay rate for *E. coli* and *E. faecalis* (T_{90} = 4-6 days) in reed-beds was observed in our previous study at 10.5ºC water temperature. One $\log_{10}$ reduction time of 18 and 14 days observed for adenovirus and coxsackievirus respectively was much shorter than the decay time for adenovirus (T_{90}=33 days) reported in reed-beds in our previous study. Water temperature is a significant factor, which influence decay of pathogens in aquatic environment (Sidhu *et al*., 2008). The inactivation study was carried out during the summer months when water temperature was high varying from 20 to 29°C. Lower inactivation rates could be expected if water temperature is low. In a microcosm based study with groundwater stored at 12°C, adenoviruses have been reported to survive and remain infectious for up to 364 days (Charles *et al.*, 2009). Ultra violet radiations from sunlight are also known to increase the rate of inaction of enteric pathogens in aquatic environment (Sinton *et al*., 2002). Sinton *et al.* (2002) reported that at 14°C under direct sunlight, the inactivation of enteric pathogens in fresh water was 10 times higher than in the absence of sunlight. Therefore, it is possible that a higher rate of inactivation of enteric pathogens than what was observed in this study could occur during the summer months (higher water temperature) under direct sunlight.

As compared to bacterial pathogens slower rate of inactivation was observed for enteric virus (Table 17). The results of this study suggest that for 90% reduction in number of adenovirus and coxsackievirus up to 18 days of retention time of stormwater is required. Enteric viruses and bacteria can also be bound to settelable particles within the wetland (Davies *et al*. 2003; Cizek *et al*. 2008). This in turn could considerably increase the removal of pathogens than the results of this decay study suggest. No assessment was made on the influence of adsorption and re-suspension on pathogens on the inactivation rates. The pathogen removal times seen in this study can be assumed to be on the conservative side as higher removal rates are likely in the reed-beds and due to adsorption and sedimentation of pathogens. The results of this study suggest that enteric virus present much higher public health risk as compared to bacterial pathogens due to their limited survival potential.

Viruses have also been reported to penetrate deeper into the sediment (Quinonez-Diaz *et al*., 2001). Re-suspension of sediments has been shown as a source of pathogen contamination in the wetland (Evanson and Ambrose 2006). Further, research work needs to be carried out to access survival potential of virus, bacteria and protozoan pathogens in the sediments.

Removal of enteric microorganisms in stormwater stored in wetland depends upon hydraulic retention time (Quinonez-Diaz *et al*., 2001; Sidhu *et al*., 2010) with higher removal expected with increased retention time. The other factors that can also influence inactivation of microorganisms in stormwater stored the wetland, in particular enteric virus, is the capture of particle bound microorganisms by the plants within the reed bed (Jackson and Jackson, 2008). Bacteria and viruses can also be bound to small settelable particles within the wetland (Cizek *et al.,* 2008; Davies *et al.,* 2003). This in turn could considerably increase the removal of pathogens than the results of this decay study suggest. In this study, we have specifically focused on the inactivation of pathogens in the water column inside the diffusion chambers. No assessment was made on the influence of adsorption and re-suspension on pathogens on the inactivation rates. Further research work is needed on the survival potential of enteric pathogens in sediments and role of adsorption and re-suspension on pathogens on the inactivation rates in the stormwater stored in wetlands The pathogen removal times seen in this study can be assumed to be on the conservative side as higher removal rates are likely in the wetland or storage ponds where adsorption and sedimentation of pathogens is likely to enhance removal of pathogens.

It is evident from the results of this study that further treatment of stormwater is required to protect public health if captured stormwater is to be reused. The extent of treatment required will depend upon the intended use for non-portable or portable purposes. Lagoon storage of effluent have been reported to result in 1-5 $\log_{10}$ reduction in bacterial numbers and 1-4 $\log_{10}$ reduction in enteric virus numbers (NRMMC-EPHC-AHMC 2006). Consequently, storage of captured stormwater in lagoons or storage basins might be an option depending upon the availability of storage spaces. Higher reduction in pathogen numbers could be achieved with UV disinfection as it has good efficiency against *Cryptosporidium* and enteric viruses such as rotavirus with up to 4.0 $\log_{10}$ removal with the use of standard low and medium pressure lamps (Hijnen *et al.* 2006).

4.6.4. Conclusions

The results of this study indicate that pathogen inactivation occurs in the urban stormwater with inactivation rates being pathogen type specific. The results of this study suggest that enteric virus present much higher public health risk as compared to bacterial pathogens due to their limited survival potential. In general, pathogens are expected to survive in the stormwater especially at low ambient and water temperatures in wetlands where protection from UV light might be provided by the vegetation. Consequently, adequate withholding in string basins or ponds prior to reuse may remain a requirement to minimise potential public health risks.

4.7. Assessment of Potential Health Risks from Stormwater Reuse

There are several scenarios where exposure to stormwater can cause potential human health problems. These include exposure to stormwater contaminants during reuse of stormwater for non-portable purposed in urban environment such as irrigation with handheld hoses, washing cars, swimming areas affected by stormwater discharges, boating and drinking water supplies contaminated by stormwater discharges. As evident from the section 4.4 inappropriate sanitary and other wastewaters along with the surface runoff are the major sources of introduction of human pathogens into stormwater. The intermittent presence of enteric pathogens in stormwater runoff makes it difficult to identify specific cause of contamination and resulting health implications. Therefore, human health risk assessments associated with stormwater exposure are generally theoretical evaluations based on particular risk scenarios combined with microbiological quality of stormwater rather than actual population studies.

Quantitative Microbial Risk Assessment (QMRA) is a widely adopted tool for investigating, evaluating and managing microbial risks associated with water systems (WHO 2004; EPHC-NHMRC-NRMMC 2008; Page *et al*., 2010c). The process of implementing QMRA requires quantifying pathogen numbers in the source water, their removal during treatment or environmental attenuation, through to potential exposure during water use (Page *et al*., 2010c). The QMRA assessments generally assume that multiple or recurring exposures constitute independent events with identical distributions of contamination. In most cases when this type of model has been employed, it has been implicitly assumed that secondary (person-to-person or person-to-environment-to-person) transmission and immunity are negligible (Soller and Eisenberg, 2008). To be effective, it requires quantitative input data for pathogen occurrence in the source stormwater and their removal through treatment barriers. When an assessment of pathogen occurrence at the end use is combined with exposure potential and pathogen dose–response relationships, the human disease burden, expressed as disability adjusted life years (DALYs), can be estimated (NRMMC-EPHC-AHMC, 2006).

This section presents a summary of the human health effects of stormwater under a number of scenarios.

4.7.1. Materials and Methods

4.7.1.1. Source Stormwater Quality and QMRA Data Collection

Stormwater samples from Fitzgibbon Drain and Makerston Street sites were monitored for the presence of selected group of microorganism to generate quantitative data which could be used for QMRA (section 3.4). The *in-situ* pathogen inactivation study was also carried out at Fitzgibbon Drain during the summer (November to January 2011) to determine site specific potential survival times as outlined in the previous section 3.6.

4.7.1.2. QMRA Methodology

Assessments using QMRA for evaluating microbial risk focus on estimating the probability of infection to an individual as a result of a single exposure event. These assessments generally assume that multiple or recurring exposures constitute independent events with identical distributions of contamination and that in most cases when this type of model has been employed, it has been implicitly assumed that secondary (person-to-person or person-to-environment-to-person) transmission and immunity are negligible.

For a QMRA to be effective, it requires quantitative input data for pathogen occurrence in the source water and their removal through any treatment barriers such as natural attenuation. When an assessment of pathogen occurrence at the end use is combined with exposure potential and pathogen dose–response relationships, the human disease burden, expressed as disability adjusted life years (DALYs), can be estimated (NRMMC-EPHC-AHMC, 2006). The basic principle of the DALY is to weight each health impact in terms of severity within the range of zero for good health to one for death. The weighting is then multiplied by the duration of the effect and the proportion of people affected (Page 2010a). In the case of death, duration is regarded as the years lost in relation to normal

life expectancy. DALYs have been previously used extensively by agencies such as the World Health Organization (WHO) to assess disease burdens (WHO 2011) and is the approach adopted in this study.

A QMRA was undertaken using the methodology described in the Australian Water Recycling Guidelines (NRMMC-EPHC-AHMC, 2006), using quantitative data on pathogen numbers in stormwater along with inactivation data from in-situ study carried out at Fitzgibbon site. In this chapter, human adenovirus was used to assess the health risks from enteric viruses as described in WHO (2004) and EPHC-NHMRC-NRMMC (2008).

The QMRA framework was applied to evaluate the level of treatment necessary at each site in order to meet the desired health target. A log-normal distribution was fitted to the observed numbers of adenovirus in the stormwater samples collected at Fitzgibbon drain and Makerston Street sites. Exposure to stormwater was assessed for four scenarios: municipal irrigation with inhalation via aerosols; municipal irrigation with a single annual accidental high volume exposure; weekly swimming in the Brisbane River using the mean exposure volumes derived by Stone *et al*. (2008); and weekly boating on the Brisbane River with an assumed exposure volume of 1 mL. A summary of the exposure assumptions for the different end uses is provided in Table 18.

The last step in the risk assessment was to integrate information from the hazard identification, dose–response and exposure assessments to determine the magnitude of risk, presented in terms of DALYs. DALYs have been used extensively by agencies such as the World Health Organization (WHO) to assess international disease burdens associated with a broad range of environmental hazards. The ingestion-infection dose–response models and the conversion to DALYs (DALYs per infection) used in this study for the hazards identified above are extensively detailed in the World Health Organization (2004) and NRMMC-EPHC-AHMC (2006). As the risk estimates are probability distribution functions, the mean, median and 95th percentile were routinely calculated for each pathogen risk. The tolerable mean risk adopted is 10^{-6} DALYs per person per year (WHO, 2004).

The static QMRA models were developed to facilitate Monte Carlo simulation, which entails generating hypothetical scenarios in terms of the values attributed to the factors in the risk characterisation step (Table 20). Ten thousand iterations were performed for each simulation, using Latin Hypercube sampling, with @RISK Industrial v4.5 [Palisade, Newfield, NY] and Microsoft Excel [Microsoft Corp., CA] software. The outcome was recorded as the mean, median and 95th percentile values.

Table 20: Exposure assessments used in this study.

Exposure Table	Frequency (n/yr)	Ingestion Volume (mL)	Reference
Boating	52	1	
Irrigation (aerosol)	90	1	AGWR (2006)
Irrigation (accidental ingestion)	1	100	AGWR (2006)
Swimming	52	3.54	Stone *et al.* (2008)

4.7.2. Results

4.7.2.1. FIB Numbers in Stormwater

The numbers of FIB in water samples collected after the storm event ranged from 40 to 7,200 cfu 100 mL^{-1} for *E. coli* and from 1,930 to 29,500 cfu 100 mL^{-1} for *Enterococcus* spp. (Figure 36). The numbers of *Enterococcus* spp. were generally ten-fold or more, higher than *E. coli* across all sites. The observed mean *E. coli* and *Enterococci* spp. numbers were higher at Fitzgibbon drain as compared to Makerston Street site (Figure 36, Table 19). The correlation between *E. coli* and *Enterococcus* spp. numbers from commercial and residential catchments did not differ significantly ($P > 0.5$). *Salmonella enterica* numbers varied between 10 to 2.4 x 10^3 MPN L^{-1} at both Makerston Street and Fitzgibbon drain sites. Whereas, mean *Salmonella enterica* numbers were slightly higher at Fitzgibbon drain as compared to Makerston street site (Figure 37, Table 19).

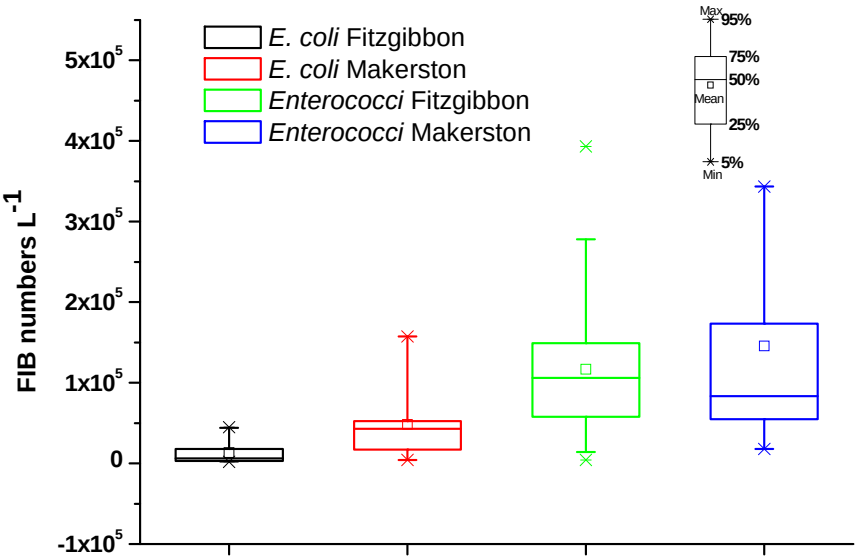

Figure 36: FIB number from two study sites. The vertical line represents range of data, black circle represent mean, small horizontal lines at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

Table 21: Distribution of FIB and pathogens in stormwater samples collected from Fitzgibbon Drain and Makerston Street sites.

Site	Mean	Median	Min	Max
Fitzgibbon				
Adenovirus (L^{-1})	7.71E+02	1.02E+02	1.00E+01	9.12E+03
Salmonella enterica (L^{-1})	9.21E+01	2.40E+01	2.30E+00	2.40E+02
E. coli (L^{-1})	1.33E+04	6.50E+03	2.00E+03	4.50E+04
Enterococci spp. (L^{-1})	1.17E+04	1.06E+04	1.40E+03	3.93E+04
Makerston				
Adenovirus (L^{-1})	1.90E+03	1.17E+03	6.37E+01	7.57E+03
Salmonella enterica (L^{-1})	6.90E+01	2.40E+01	1.00E+01	2.40E+02
E. coli (L^{-1})	4.97E+04	4.37E+04	4.23E+03	1.58E+05
Enterococci spp. (L^{-1})	6.49E+03	4.22E+03	7.00E+02	1.83E+04

4.7.2.2. Human Adenovirus Occurrence at Sampling Sites

Human adenovirus was used to assess the health risks of enteric viruses from reuse of stormwater. Distribution of HAv in 40 stormwater samples collected during three events from Fitzgibbon drain and Makerston street sites is presented in Figures 37. Adenovirus numbers varied between 6.37×10^{1} to 7.57×10^{3} L^{-1} at Makerston Street and between 10 to 9.12×10^{3} L^{-1} at Fitzgibbon drain site. Mean adenovirus numbers were higher at Makerston Street then Fitzgibbon drain site (Figure 37).

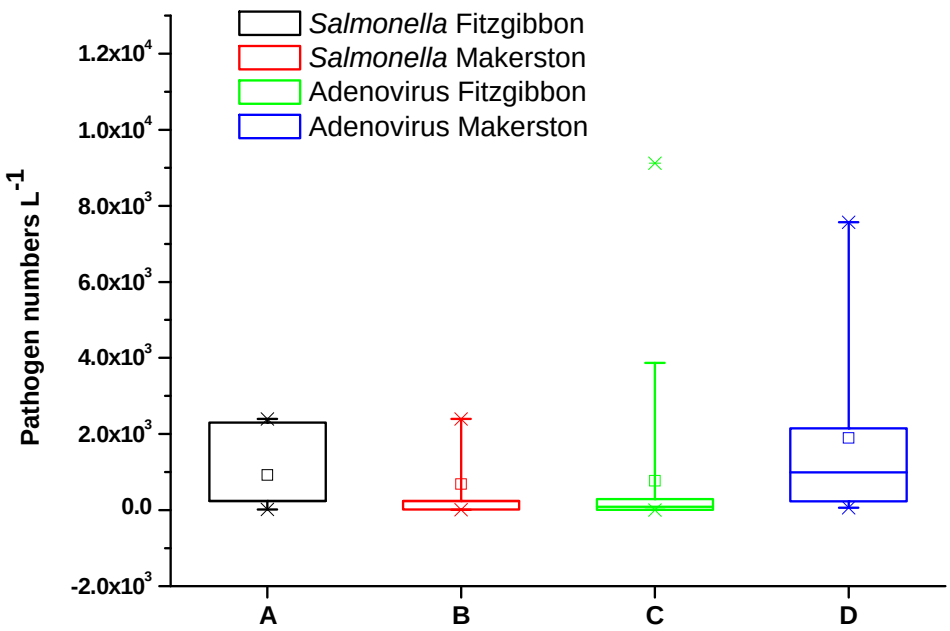

Figure 37: Comparative distribution of *Salmonella enterica* and adenovirus numbers during the three storm events at Fitzgibbon drain and Makerston Street. The vertical line represents range of data, squares represent mean, small horizontal lines (wisker) at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

A log-normal distribution was then fitted to the observed numbers of adenovirus from both sites to determine distribution range which was then used in the QMRA analysis. In this study, HAv numbers were reported as PCR detectable units. PCR-based techniques are very sensitive and specific in detection of virus genome however, there can be a difference between the loss of infectivity and complete degradation of viral genome (Charles *et al.* 2009, de Roda Husman *et al.* 2009). Choi and Jiang (2005) reported 7% detection of adenoviruses by real-time PCR, with numbers ranging from 10^2 to 10^4 viruses per litre from 114 environmental samples. However, a cell culture assay using two human tissue culture cell lines yielded negative results, suggesting that adenoviruses detected by real-time PCR might be non infectious. Similarly, He and Jiang (2005) reported that for adenovirus numbers of 10^5 /L only 0.1% were infectious. In the current study, a conservative number of 1% infectious viruses have been applied.

Figure 38: Observed and fitted pathogen data for adenovirus numbers at Fitzgibbon drain (left) and Makerston Street (Right).

4.7.2.3. QMRA Results

Analysis of monitoring results demonstrated that adenovirus and *Salmonella enterica*, were consistently present in the water at both stormwater harvesting sites. As shown in Table 19, pathogen and indicator numbers can vary over a wide range. There was no observable direct correlation between pathogen and indicator numbers in both at Fitzgibbon and Makerston Street catchments. The results from both the Fitzgibbon Drain and Makerston Street sites demonstrate that the high numbers of adenovirus and *Salmonella enterica* present in the urban stormwater may result in DALY estimates well above health targets without treatment.

Comparison of the annualised probability of infection estimates with the 10^{-6} DALYs benchmark (NHMRC-EPHC, 2006) indicated that viral pathogens were the primary concern, and that for all exposure scenarios, the median probability of infection exceeded the benchmark. While acknowledging that conservative assumptions were applied in this QMRA as described in the Australian Guidelines for Water recycling, a risk management plan that includes investigations for the possible presence of sewage at both sites is warranted. Critical required treatment limits were estimated for event pathogen numbers for a range of exposure scenarios for risk mitigation for adenovirus (Table 20).

Table 22: Adenovirus pathogen inactivation credits (Log_{10}) required for different exposure scenarios.

End use	Fitzgibbon Drain			Makerston Street		
	50th	Mean	95th	50th	Mean	95th
Boating	1.2	2.1	2.6	2.2	3.0	3.5
Irrigation (aerosol)	1.4	2.3	2.9	2.5	3.2	3.8
Irrigation (accidental ingestion)	1.5	2.4	2.9	2.5	3.2	3.8
Swimming	1.8	2.6	3.2	2.8	3.5	3.1

System-specific data on pathogen concentrations were used, as an alternative to the default values in the Australian Water recycling Guidelines to calculate performance targets using these same formulae. Table 20 shows that there was little differences in treatment removal requirements for different uses of stormwater considered. As expected, swimming has the highest requirements; all uses required some form of treatment or exposure control.

Table 20 shows that site specific inactivation credits were very similar to the default values. Irrigation required 2.9 compared to 1.9 log in the guidelines and reflects the much higher virus numbers detected at the Queensland locations.

Reduction of pathogen numbers could be achieved by a number of factors including: exposure control, withholding periods (1 log_{10}) for irrigation scenarios; dilution in the Brisbane River (not quantified in this study); engineered treatment; and natural attenuation. Natural attenuation and dilution are likely to be a key mechanism to reduce pathogen numbers in the Brisbane River. The microbial inactivation credits (log_{10}) reduction as a function of time is shown in Figure 38 which was calculated from the *in-situ* inactivation rates observed in the inactivation experiment conducted in the Fitzgibbon Drain.

Figure 38 shows that faecal indicator bacteria *E. coli* decays very rapidly compared to both adenovirus and coxsackievirus. As a result, it is expected that for pathogenic bacteria such as *Salmonella enterica* sufficient microbial inactivation credits could be achieved after 20 days for all scenarios. However, for adenovirus, up to 85 days of storage of stormwater would be required to achieve 4 log_{10} reduction in numbers.

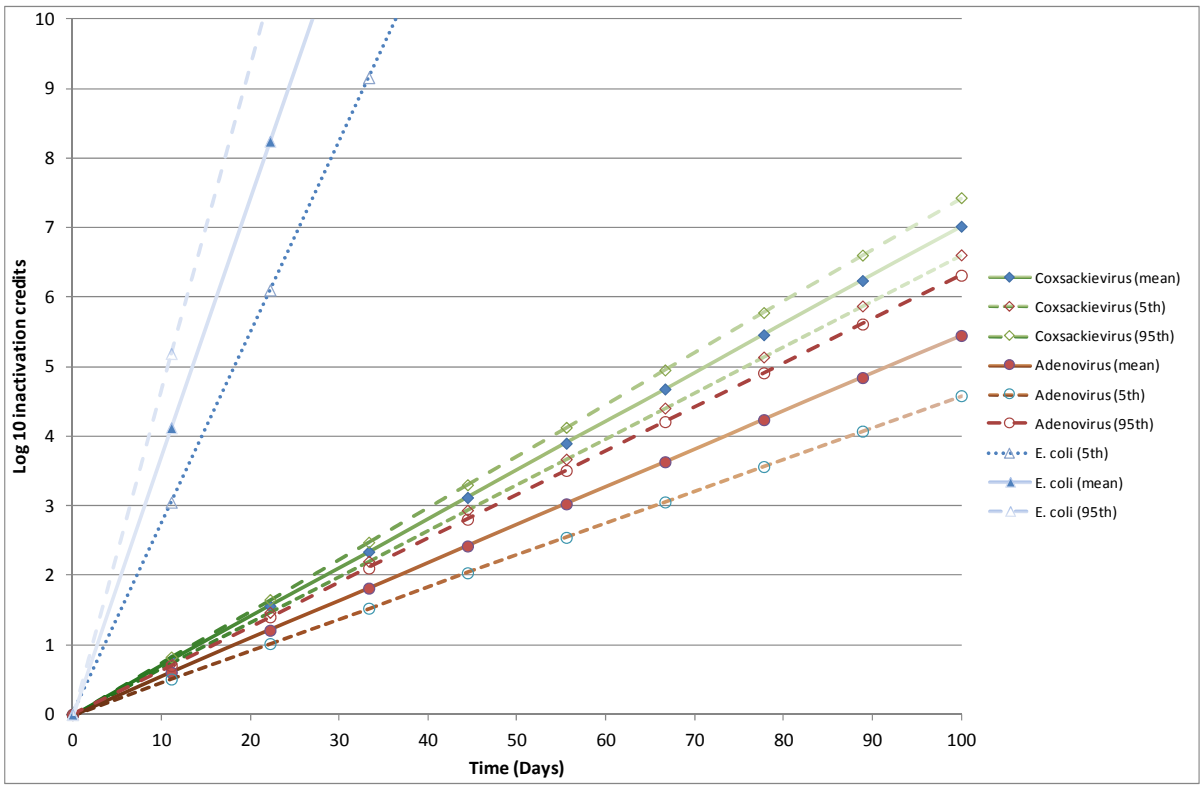

Figure 39: Pathogen inactivation credits as a function of time.

4.7.3. Discussion

4.7.3.1. Microbial Risk Assessment for Stormwater Reuse

QMRA is a widely adopted tool for investigating, evaluating and managing microbial risks associated with water systems. The process of implementing QMRA requires quantifying pathogen numbers in the source water, removal during treatment or environmental attenuation, through to potential exposure during stormwater reuse. As with any QMRA, there were several potential sources of uncertainty in this analysis. Firstly, the exposure assessments are assumptions. The irrigation and boating scenarios are based on best available estimates and remain to be validated. Further for the boating and swimming scenarios the assumption that human contact occurred shortly after a storm events, and that no dilution or die off occurred is conservative. The levels of exposure to pathogens following stormwater discharge would be expected to decline with the passage of time. This, in turn, could result in lower risks of pathogen-related disease if contact occurred during a prolonged dry period. The relatively rapid attenuation of *Salmonella enterica* and faecal indicator bacteria such as *E. coli* could mask the risks posed by viruses with their much slower decay rates (Figure 38) to achieve sufficient inactivation credits for reuse.

The health risks posed by stormwater harvesting and reuse and discharges to recreational waters have been recognised through the adoption of risk-based guidelines in the NWQMS. From a QMRA perspective, it is clear that the release of pathogens into the Brisbane River via stormwater drains remains an impediment to achieving a consistent level of water quality. Clearly, stormwater harvesting and reuse reduce the discharge of stormwater to the Brisbane River but additional treatments (Table 21 and 22) would be required to meet the microbial health based targets.

4.7.3.2. Preventive Measures to Manage Microbial Risk

The Australian Drinking Water Guidelines (ADWG) (NHMRC–NRMMC 2011) specifies the indicative $\log_{10}$ reductions of treatment processes for enteric pathogens (Table 22). The values from this table could be used to determine a suitable treatment option once the intended use of captured water is known.

Table 23: Indicative $\log_{10}$ removals of enteric pathogens for different exposure controls* and treatment processes (after Table A 1.8 NHMRC-NRMMC 2011).**

Treatment Process	Virus	Protozoa	Bacteria*
Dual media filtration with coagulation	1.0	1.5	0.5
Ultrafiltration	2.0	3.0	>4.0
Chlorination	3.0	0.0	>4.0
Ozonation	3.0	2.0	>4.0
UV disinfection	3.0	4.0	>4.0
Witholding period (1-4 hours)	1.0	1.0	1.0
No public access during irrigation	2.0	2.0	2.0
Buffer zones	1.0	1.0	1.0
Drip irrigation	4.0	4.0	4.0

* Based on the Australian Guidelines for Water Recycling; *Based on the Australian Drinking Water Guidelines (NHMRC–NRMMC 2011).

The specific treatments such as use of stormwater harvesting wetlands, bio-retention basins, and elements of water sensitive urban design used for capture and treatment require a case by case validation to determine the treatment efficacy for removal of pathogens. In general, the following assessments of risk can be determined for the different stormwater use options.

For open space irrigation, > 1.3 $\log_{10}$ reduction for reduction of viruses and *Cryptosporidium* is required under the stormwater harvesting guidelines (NRMMC-EPHC–NHMRC 2009b). This can potentially be managed using exposure controls as evident from $\log_{10}$ reduction values in Table 22. In order to use stormwater inside the house for toilet flushing and washing machine 1.9 $\log_{10}$ reduction is required (NRMMC-EPHC–NHMRC 2009b). This could be achieved through UV and chlorination. However, cross connections are the largest risk in dual reticulation systems. Exposure can be reduced using additional preventative measures such as certified plumbing schemes. Domestic garden irrigation, municipal irrigation, street cleaning, road making, car washing all require less than 2.0 $\log_{10}$ reduction and can potentially be managed using chlorination and exposure controls (Page and Levett 2010). Drinking water use requires the highest microbial health-based targets be met which would involve significant treatment, 5.5 $\log_{10}$ for using the default values from the guidelines. This can be achieved either by combinations of membrane filtration, UV and chlorine disinfection. Risks from viruses drive the microbial health-based targets for options of stormwater reuse, if sufficient treatment or exposure controls are introduced to reduce the risks from viruses, then risks from bacteria and protozoa will usually also be controlled.

For a comparison, with existing stormwater reuse schemes at the City of Orange the treatment of stormwater include settling and chemical assisted sedimentation before pumping into Suma Park Dam, the water supply reservoir. The water is then passed through the water supply treatment plant which includes ozonation and biological activated carbon and chlorination. The combined estimated $\log_{10}$ removals for viruses, protozoa and bacteria are >6.0, >6.0 and >9.0 $\log_{10}$, exceeding the target $\log_{10}$ removals for drinking water.

At the aquifer storage and recovery (ASTR) research site the treatment of stormwater included settling basin and wetland treatment, prior to recharge to the aquifer (Page *et al.* 2010). After 250 days storage, sufficient decay of pathogens had been demonstrated (Sidhu *et al.* 2010) that the recovered water required only UV disinfection and chlorination to meet the microbial health-based targets and be suitable for drinking.

4.7.4. Conclusions

The approach used for human health and environmental risk assessment of urban stormwater reuse is consistent with all of the Australian Guidelines for Water Recycling. The results of QMRA demonstrate that there are unacceptable level of health risks associated with the stormwater reuse and recreational activities. Although natural attenuation of pathogens occurs in aquatic environments, this alone might not be sufficient when ambient climatic conditions favour prolonged survival (low temperature and adhesion to suspended particles). Sewage contamination was detected at Fitzgibbon drain and Makerston Street sites during all storm events, which suggest that there is always a risk of presence of human pathogens in stormwater. Consequently, some degree of treatment depending upon the intended use is required. Potential public health risks from viruses and can be reduced by either controlling sources of contamination or/and with additional treatment, such as stormwater harvesting wetlands, bio-retention basins or engineered systems, such as chlorination and UV treatment.

5. OVERALL PROJECT CONCLUSIONS

- Urban stormwater from all sites in Brisbane, Melbourne and Sydney was contaminated with pathogens and chemicals.
- Of the dissolved inorganic and organic pollutants measured in stormwater in this study (heavy metals, pesticides, pharmaceuticals), the heavy metals were frequently present in concentrations around or above Australian guideline values for potable uses (e.g. Australian Drinking Water Guidelines, Australian Guidelines for Water Recycling, Phase 2: Augmentation of Drinking Water Supplies). Pharmaceuticals and pesticides were typically found much below their respective guideline values, with the exception of caffeine. Other chemicals reported in literature may, though, be present at relevant levels as well (e.g. benzo(a)pyrene) and can present other human health risks through chemicals in untreated stormwater.
- The present study showed that the presence of a number of pharmaceuticals and food ingredients can qualitatively indicate sewage ingress into stormwater. Nonetheless, the study also highlighted a number of difficulties in establishing a quantitative link between the presence of micropollutants and sewage contamination of stormwater. Further studies with selected microbial and chemical markers are required to establish if sewage ingress in stormwater could be quantified.
- *In-vitro* bioassays performed on the dissolved phase of stormwater showed non-specific baseline toxicity to be variable with the range of values measured comparable to previously observed results for secondary treated effluent. Estrogenic activity was consistently low with the exception of one sample among twenty. Phytotoxicity was in good agreement with the results of chemical analysis and confirmed the absence of other strong phytotoxic effects. Other bioassays showed generally a lesser biological effect compared to other alternative water sources such as secondary treated effluent.
- *Enterococcus* spp. numbers detected in storm runoff collected from all eight sites (3×10^4 $100mL^{-1}$) were generally higher by several orders of magnitude than the recommended limits for category D (<501 *Enterococci* per 100 mL^{-1}) under Australian guidelines for managing risks in recreational water. *E. coli* numbers were also consistently higher than 3×10^4 $100mL^{-1}$ in samples collected from all sites.
- Presence of bacterial pathogens (*Salmonella enterica* and *Campylobacter* spp.) and viral pathogens (adenovirus and polyomavirus) indicate that contamination of human faecal matter is occurring routinely in the stormwater. The widespread presence of human viruses in urban stormwater runoff indicates that there is significant human faecal contamination, as opposed to contamination from animals, and thus has much higher public health implications. This suggests that aging sewage infrastructure combined with numerous non-point human sewage pollution sources are the main causes of microbial contamination.
- This study illustrates that chemical and microbiological FST markers can be used to detect extent of sewage contamination in stormwater runoff and provide a higher level of information toward decision making processes to protect human health.
- A very good consensus (>80%) between the occurrence of the human adenovirus, the human specific bacteria HF183, acesulfame, paracetamol and caffeine was observed suggesting that they are very good markers of presence of human sewage contamination in stormwater.
- The prevalence of *E. coli* bacteria with defined pathotypes in stormwater which have a strong association with human sources underscores the importance of controlling sources of human faecal pollution such as managing municipal wastewater sources to reduce potential risks to human health.

- Flow finger printing of FIB, *Salmonella enterica* and human adenovirus, polyomavirus, and torque teno virus during the storms at the Fitzgibbon drain and Makerston Street sites demonstrated a high inter- and intra-storm variability. Therefore, grab sampling will not be able to capture EMC values adequately.
- No correlation between the presence of FIB/somatic coliphage and *Salmonella enterica* and human adenovirus, polyomavirus, and torque teno virus in urban stormwater was detected, making surrogate measurements of questionable value.
- The results of this study suggest that, in addition to monitoring for specific pathogens and faecal pollution markers, sensitive and specific approaches to locate human faecal pollution hot spots would improve our ability to detect and control sources of faecal pollution.
- The results of the *in-situ* study suggest that natural die-off of pathogens in stormwater occurs with time and enteric virus present much higher public health risk than bacterial pathogens due to the limited survival potential of bacterial pathogens. Consequently, natural attenuation of pathogens would occur during storage of stormwater in wetlands and bioretention basins.
- Results from both Fitzgibbon drain and Makerston Street sites demonstrate that the high numbers of human adenovirus and *Salmonella enterica* present in urban stormwater result in DALY estimates well above health targets either for non-potable use or recreational activities without treatment.
- An integrated stormwater management approach to control faecal contamination is required, which may involve controlling sources of contamination such as sewage leakage, elimination of cross connections and temporary withholding of captured water in basins to allow natural attenuation prior to reuse. In addition, some degree of treatment of captured stormwater prior to its use for potable and non-potable purposes would be required for public health risk mitigation.

6. RECOMMENDATIONS FOR FUTURE RESEARCH

- Further research is needed to determine the prevalence of other enteric pathogens in stormwater runoff, especially enteric viruses and protozoa and any correlation to alternative indicators along with a quantitative assessment of human health risks.
- This study demonstrated that human faecal contamination is the major source of enteric pathogens such as adenovirus and polyomavirus. Further research is required to develop understanding on partitioning behaviour of pathogens and role of sediments in the capture and transport of enteric pathogens.
- Additional research is also needed on survival potential of pathogens in sediments and long term sources of contamination of stormwater.
- Detection and quantification of enteric virus and protozoan pathogens from stormwater runoff is often hindered by the presence of high suspended solid content. Further research is required to develop methodologies for the detection and quantification of these pathogens in water with high turbidity.
- The present research directed its efforts on chemical analysis of the dissolved phase. Future investigation should take into account analysis of the particulate phase. This may be important to, for example, avoid misjudging the role of sediments trapped in water sensitive urban design elements such as bio-filters and potential leaching. Sediments and colloids may also be crucial in the transport of microbes.
- Further research is needed to determine the key chemical markers in stormwater derived from catchment of various land use type that can act as representative of sewage contamination. Investigations should answer the question whether a quantitative link can be established between chemical markers and sewage contamination.
- Investigations are needed to understand the role of dissolved organic matter in microbial transport for stormwater derived from catchments of various land use type to determine if dissolved organic carbon and its fraction could be used as a surrogate for presence of microbes in stormwater.
- The microbial health risk assessment remains difficult due to the presence of wide variety of pathogens in stormwater and monitoring costs, in this regard research should target two areas
  - Further investigations should direct towards the identification of virus surrogates that can help assess human health risk, in particular for operational monitoring and treatment validation, where that may be required.
  - Protocols for effective and efficient pre-site development catchment monitoring should be elaborated that allow site developers to assess raw water risks and propose effective risk mitigation strategies in line with accepted risk management frameworks.

APPENDICES

A. 1. Pharmaceutical and Other Micropollutant Concentrations

Pharmaceutical and other micro-pollutant concentrations determined in samples from eight catchments. LOR = Level of reporting by the analytical laboratory.

	n	LOR [µg·L^{-1}]	>LOR	Min [µg·L^{-1}]	$Q_{25\%}$ [µg·L^{-1}]	Median [µg·L^{-1}]	$Q_{75\%}$ [µg·L^{-1}]	$Q_{90\%}$ [µg·L^{-1}]	Max [µg·L^{-1}]	Guideline* [µg·L^{-1}]	Guideline/Max
Acesulfame K (sweetener)	30	0.01	29	<LOR	0.04	0.05	0.07	0.12	0.23	NA	NA
Acetylsalicylic acid	30	0.01	1	<LOR	<LOR	<LOR	<LOR	<LOR	16.00	29	1.8
Atenolol	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	25	>2500
Atorvastatin	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	5	>500
Caffeine	30	0.02	26	<LOR	0.07	0.30	1.04	1.88	5.20	0.35	0.07
Carbamazepine	30	0.01	1	<LOR	<LOR	<LOR	<LOR	<LOR	0.01	100	10000
Cephalexin	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	35	>3500
Chloramphenicol	30	0.1	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	175	>17500
Ciprofloxacin	30	0.15	1	<LOR	<LOR	<LOR	<LOR	<LOR	0.01	250	25000
Citalopram	30	0.01	4	<LOR	<LOR	<LOR	<LOR	0.01	0.04	4	100
Codeine	30	0.1	3	<LOR	<LOR	<LOR	<LOR	<LOR	0.01	50	6250
Cyclophosphamide	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	3.5	>350
Dapsone	30	0.01	4	<LOR	<LOR	<LOR	<LOR	0.10	0.47	NA	NA
DEET	30	0.01	26	<LOR	0.04	0.15	0.36	0.48	0.86	2500	2907
Desmethyl Citalopram	30	0.01	4	<LOR	<LOR	<LOR	<LOR	0.01	0.02	4	200
Desmethyl Diazepam	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	3	>300
Diazepam	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	2.5	>250
Diclofenac	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	1.8	>180
Doxylamine	30	0.01	4	<LOR	<LOR	<LOR	<LOR	0.01	0.49	NA	NA
Erythromycin	28	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	17.5	>1750
Erythromycin anhydrate	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	17.5	>1750
Fluoxetine	30	0.01	2	<LOR	<LOR	<LOR	<LOR	<LOR	0.04	10	250
Fluvastatin	30	0.01	1	<LOR	<LOR	<LOR	<LOR	<LOR	0.01	NA	NA
Frusemide	30	0.01	1	<LOR	<LOR	<LOR	<LOR	<LOR	0.01	10	1000
Gabapentin	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	NA	NA
Gemfibrozil	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	600	>60000

	n	LOR [µg·L^{-1}]	>LOR	Min [µg·L^{-1}]	$Q_{25\%}$ [µg·L^{-1}]	Median [µg·L^{-1}]	$Q_{75\%}$ [µg·L^{-1}]	$Q_{90\%}$ [µg·L^{-1}]	Max [µg·L^{-1}]	Guideline* [µg·L^{-1}]	Guideline/Max
Hydrochlorothiazide	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	12.5	>12500
Ibuprofen	30	0.07	1	<LOR	<LOR	<LOR	<LOR	<LOR	0.09	400	4444
Ifosfamide	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	3.5	>350
Indomethacin	30	0.01	1	<LOR	<LOR	<LOR	<LOR	<LOR	0.02	25	1250
Iopromide	30	0.2	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	750	>37500
Lincomycin	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	3500	>350000
Metoprolol	30	0.01	1	<LOR	<LOR	<LOR	<LOR	<LOR	0.02	25	1250
Naproxen	30	0.1	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	220	>2200
Norfloxacin	30	0.05	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	400	>8000
Oxazepam	28	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	15	>1500
Oxycodone	30	0.01	1	<LOR	<LOR	<LOR	<LOR	<LOR	0.01	10	1000
Paracetamol	30	0.02	21	<LOR	<LOR	0.03	0.08	0.11	0.20	175	875
Phenytoin	30	0.01	2	<LOR	<LOR	<LOR	<LOR	<LOR	0.41	NA	NA
Praziquantel	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	70	>7000
Primidone	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	NA	NA
Propranolol	30	0.01	3	<LOR	<LOR	<LOR	<LOR	<LOR	0.05	40	800
Ranitidine	30	0.01	1	<LOR	<LOR	<LOR	<LOR	<LOR	0.11	26	236
Roxithromycin	30	0.02	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	150	>7500
Salicylic acid	30	0.1	13	<LOR	<LOR	<LOR	0.10	0.20	0.60	105	175
Sertraline	30	0.01	3	<LOR	<LOR	<LOR	<LOR	<LOR	0.40	NA	NA
Sulfasalazine	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	500	>50000
Sulfadiazine	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	35	>3500
Sulfamethoxazole	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	35	>3500
Sulfathiazole	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	35	>3500
Temazepam	27	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	5	>500
Tramadol	30	0.01	4	<LOR	<LOR	<LOR	<LOR	<LOR	0.04	NA	NA
Triclosan	27	0.01	3	<LOR	<LOR	<LOR	<LOR	<LOR	0.01	0.35	35
Trimethoprim	30	0.01	4	<LOR	<LOR	<LOR	<LOR	0.01	0.07	70	1000
Tylosin	30	0.02	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	1050	>52500
Venlafaxine	30	0.01	6	<LOR	<LOR	<LOR	<LOR	0.02	0.11	75	682
Warfarin	30	0.01	0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	1.5	>150

* Queensland Public Health Regulation 2005, Schedule 3B - Standards for quality of recycled water supplied to augment a drinking water supply

A. 2. Pharmaceutical and Other Micropollutant Concentrations Determined in Samples from Eight Catchments

Pharmaceutical and other micropollutant concentrations determined in samples from eight catchments. LOR = Level of reporting by the analytical laboratory. All values are given in [$\mu g \cdot L^{-1}$]. A gap in the data matrix means that the compound was not found above the reporting limit. Few values are quantified below the standard reporting limit of the analytical laboratory, courtesy to QHFSS. While they do not pass the standard QA/QC criteria of QHFSS, the authors of the report considered these to be valuable information to be included.

			Acesulfame K (sweetener)	Acetylsalicylic acid	Caffeine	Carbamazepine	Ciprofloxacin	Citalopram	Codeine	Dapsone	DEET	Desmethyl Citalopram	Doxylamine	Fluoxetine	Fluvastatin	Frusemide	Ibuprofen	Indomethacin	Metoprolol	Oxycodone	Paracetamol	Phenytoin	Propranolol	Ranitidine	Salicylic acid	Sertraline	Tramadol	Triclosan	Trimethoprim	Venlafaxine
		LOR	0.01	0.01	0.02	0.01	0.15	0.01	0.1	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.07	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.1	0.01	0.01	0.01	0.01	0.01
QLD	Fitzgibbon	30/05/2011	0.04							0.14					0.01					NA	0.08									
QLD	Fitzgibbon	7/12/2011	0.03		0.03					0.47										NA										
QLD	Fitzgibbon	27/01/2012	0.04								0.14					0.01					0.08									
QLD	Fitzgibbon	14/04/2012	0.03		0.09				0.005		0.46										0.06				0.6					
QLD	Fitzgibbon	17/04/2012	0.04		0.04						0.02										0.01				0.1					
QLD	Fitzgibbon	27/04/2012	0.07		0.1			0.04	0.008		0.86	0.02	0.49	0.04					0.02	0.01			0.05	0.11	0.1	0.02	0.04		0.07	0.11
VIC	Smith St	28/02/2012	0.23		3						0.33										0.2									
VIC	Smith St	27/02/2012	0.17		1.7						0.26										0.14									
NSW	Hornsby	26/10/2011	0.09		1.81		0.01			0.37	0.1										0.05				0.2			0.01		
NSW	Hornsby	17/11/2011	0.04		0.3						0.1										0.05				0.2			0.01		
NSW	Hornsby	15/01/2012	0.09		1.81			0.01			0.37	0.01	0.1								0.02		0.01						0.01	0.02
NSW	Hornsby	23/01/2012	0.05	16	0.7						0.48										0.03				0.3					
NSW	Hornsby	31/01/2012	0.07		2.514						0.39										0.09				0.1					
NSW	Hornsby	12/02/2012	0.04		0.79						0.48										0.02				0.1					
VIC	Banyan	8/11/2011	0.04		0.3					0.1											0.05				0.2			0.01		
VIC	Banyan	1/12/2011	0.06		0.38	0.01					0.12										0.03									
VIC	Banyan	5/02/2012	0.05		0.43						0.43										0.11									

			Acesulfame K (sweetener)	Acetylsalicylic acid	Caffeine	Carbamazepine	Ciprofloxacin	Citalopram	Codeine	Dapsone	DEET	Desmethyl Citalopram	Doxylamine	Fluoxetine	Fluvastatin	Frusemide	Ibuprofen	Indomethacin	Metoprolol	Oxycodone	Paracetamol	Phenytoin	Propranolol	Ranitidine	Salicylic acid	Sertraline	Tramadol	Triclosan	Trimethoprim	Venlafaxine
VIC	Banyan	27/02/2012	0.06		0.52						0.24										0.11				0.1					
VIC	Banyan	28/04/2012	0.07		0.31						0.04										0.05				0.1					
NSW	Orange	2/02/2012	0.05		0.19						0.07										0.03									
NSW	Ku-ring-gai	31/01/2012	0.007		0.14						0.26										0.02									0.004
QLD	Makerston	26/02/2012	0.03		0.27						0.15										0.03						0.01			
QLD	Makerston	3/03/2012	0.11		5.2						0.33						0.09					0.03								
QLD	Makerston	7/03/2012	0.03		0.06						0.86											0.41				0.4				
QLD	Makerston	16/04/2012	0.16		1.13			0.03			0.28	0.02	0.01					0.02											0.01	0.04
QLD	Makerston	29/04/2012	0.1		1.12			0.02	0.005		0.12	0.02	0.01	0.03							0.13		0.01			0.01	0.008		0.02	0.04
VIC	Industrial	28/04/2012	0.06		0.1						0.05																			0.02
VIC	Industrial	27/05/2012	0.04		0.04						0.02														0.2		0.02			
VIC	Industrial	5/06/2012	0.01								0.02														0.2					

B. Methodology for Detection of Pathogens

Table B1. Primers, probes and cycling parameters for PCR assays.

Target	Primer Sequence (5'-3')	Cycling Parameters	Amplicon Size (bp)	Reference
Adenovirus	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 20s at 72°C	132	Heim *et al*., 2003
Polyomavirus	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 60s at 60°C	176	McQuaig *et al*., 2009
Campylobacter spp. 16S rRNA	F : CAC GTG CTA CAA TGG CAT AT R: GGC TTC ATG CTC TCG AGT T P: FAM CAG AGAA CAA TCC GAA CTG GGA CA BHQ1	10 min at 95°C, 50 cycles of 15 s at 95°C and 20 s at 58°C and 30s at 72°C	108	Lund *et al*., 2004
Coxsackievirus	F : CCTCCGGCCCCTGAATG R: ACCGGATGGCC AATCCAA P: FAM TACTTTGGGTGTCCGTGTTTC BHQ1	30 min at 50ºC, initial inactivation of reverse transcriptase at 95ºC for 5 min, then 45 cycles at 95ºC for 30 sec, 60ºC for 30 sec and 72ºC for 30 sec	197	Schwab *et al*., 1995
Campylobacter coli ceuE gene	F- AGA TAC TTT CCA TGC CCT AAG ACT R- TTG CTT TGG CGT CCG GAA ATG A P- FAM-AAC GAT AAA GTT GCA GGA GTT CCA GCT BHQ1	10 min at 95°C, 45 cycles of 15 s at 95°C and 20 s at 60°C and 20s at 72°C	180	This study
Campylobacter jejuni mapA gene	F - GCT AGA GGA ATA GTT GTG CTTGAC AA R - TTA CTC ACA TAA GGT GAA TTT TGA TCG P-FAM GCG ATG TTG GAA TTC AAT GTT GTG CCA BHQ1	10 min at 95°C, 45 cycles of 15 s at 95°C and 20 s at 60°C and 20s at 72°C	72	This study
Salmonella invA gene	F: ACA GTG CTC GTT TAC GAC CTGAAT R: AGA CGA CTG GTA CTGATC GAT AAT	10 min at 95°C, 40 cycles of 30 s at 94°C, 35 s at 59°C, and 120 s at 72°	244	Chiu and Ou, 1996
E. faecium esp gene	F-TAT GAA AGC AAC AGC ACA AGT T R- ACGTCG AAA GTT CGA TTT CC	10 min at 95°C, 45 cycles of 45 s at 95°C, 45 s at 59°C, and 45s at 72°C	870	Scott *et al*., 2005
Bacteroides HF183	F- ATC ATG AGT TCA CAT GTC CCG R:-TAC CCC GCC TAC TAT CTA ATG	95ºC for 10 min, 45 cycles 95ºC for 30 s, 53ºC for 1 min, and 60ºC for 1 min	570	Seurinck *et al*., 2005
TtV	F: CGG GTG CCG **D**AG GTG AGT TTA CAC R:GAG CCT TGC CCA T**R**G CCC GGC CAG P:FAM-AGTC AAG GGG CAA TTC GGG CTCG GGA-TAMRA	10 min at 95°C, 50 cycles of 20 s at 95°C and 20 s at 62°C and 20s at 72°C	79	Tokita *et al*., 2002
PP7	F:CCA AAA CCA TCG TTC TTT CG R: GTC CGC CTG ATC CAG TTT TA P: FAM-CGC ACT CTG ACT GAG ATC CA-TAMRA	10 min at 95°C, 50 cycles of 20 s at 95°C and 20 s at 60°C and 20s at 72°C	188	This study
Microviridae	F: TAC CCT CGC TTT CCT GC R: GCG CCT TCC ATG ATG AG P: FAM-CAT TGC TTA TTA TGT TCA TCC CG-TAMRA	10 min at 95°C, 50 cycles of 20 s at 95°C and 20 s at 61°C and 20s at 72°C	100	Lee, 2009.

Abbreviations: F: Forward primer; R: Reverse primer; P: Probe, FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine, Single-letter code: D= G+A+T; R= A+G.

Table B2. Correlations among faecal indicator bacteria (FIB) and FST markers using binary logistic regression analysis.

Comparison	Concordance (%)	Odds Ratios	*P* Value[a]
E. coli vs. *HF 183*	63.6	1.00	0.605
E. coli vs. *nifH*	55.4	1.00	0.665
E. coli vs. HAv	69	1.00	0.288
E. coli vs. HPv	43.8	1.00	0.842
E. coli vs. Paracetamol	86.7	1.00	0.249
E. coli vs. Salicyclic acid	56.7	1.00	0.622
E. coli vs. Acessulfame	72.7	1.00	0.388
E. coli vs. Caffeine	45.2	1.00	0.823
Enterococcus spp.vs. *HF 183*	63.4	1.00	0.519
Enterococcus spp. vs. *nifH*	58.5	1.00	0.451
Enterococcus spp.vs. HAv	64.3	1.00	0.362
Enterococcus spp.vs. HPv	66.2	1.00	0.291
Enterococcus spp.vs. Paracetamol	50	1.00	0.693
Enterococcus spp.vs. Salicyclic acid	64.4	1.00	0.357
Enterococcus spp. vs. Acessulfame	95.5	1.00	0.202
Enterococcus spp.vs. Caffeine	38.1	1.00	0.942

[a]*P* value for the model chi-square was < 0.05, and the confidence interval for the odds ratio did not include 1.0.

Table B3. Primers used for the detection of *E. coli* virulence genes.

Target Genes	Primer Sequence (5'-3')	Cycling Parameters	Amplicon Size	Reference
***stx*$_1$**	F: CTGGATTTAATGTCGCATAGTG R: AGAACGCCCACTGAGATCATC	10 min at 95°C, 45 cycles of 15 s at 95°C and 15 s at 59°C and 15s at 72°C	150	39
***stx*$_2$**	F: GGCACTGTCTGAAACTGCTCC R: TCGCCAGTTATCTGACATTCTG	10 min at 95°C, 45 cycles of 15 s at 95°C and 15 s at 59°C and 15s at 72°C	255	39
eaeA	F: ATGCTTAGTGCTGGTTTAGG R: GCCTTCATCATTTCGCTTTC	10 min at 95°C, 45 cycles of 15 s at 95°C and 15 s at 55°C and 15s at 72°C	248	67
hlyA	F: GCATCATCAAGCGTACGTTCC R: AATGAGCCAAGCTGGTTAAGCT	10 min at 95°C, 45 cycles of 30 s at 95°C and 30 s at 55°C and 30s at 72°C	1177	54
LT	F: GCACACGGAGCTCCTCAGTC R: TCCTTCATCCTTTCAATGGCTTT	10 min at 95°C, 45 cycles of 20 s at 95°C and 20 s at 59°C and 20s at 72°C	218	63
ST	F: TTTCCCCTCTTTTAGTCAGTCAA F: TGCTAAACCAGTAGAGTCTTCAAAA R: GCAGGATTACAACACAATTCACAGCAG	10 min at 95°C, 45 cycles of 20 s at 95°C and 20 s at 59°C and 20s at 72°C	159, 138	21
aggR	F: GTATACACAAAAGAAGGAAGC R: ACAGAATCGTCAGCATCAGC	10 min at 95°C, 45 cycles of 20 s at 95°C and 20 s at 59°C and 20s at 72°C	254	64
ipaH	F: GTTCCTTGACCGCCTTTCCGATACCGTC R: GCCGGTCAGCCACCCTCTGAGAGTAC	10 min at 95°C, 45 cycles of 30 s at 95°C and 30 s at 59°C and 30s at 72°C	619	60
bfp	F: GGAAGTCAAATTCATGGGGGTAT R: GGAATCAGACGCAGACTGGTAGT	10 min at 95°C, 45 cycles of 20 s at 95°C and 20 s at 59°C and 20s at 72°C	300	63
astA	F: CCA TCA ACA CAG TAT ATC CGA R: GGT CGC GAG TGA CGG CTT TGT	10 min at 95°C, 45 cycles of 20 s at 95°C and 20 s at 59°C and 20s at 72°C	111	69
cdtB	F: AAATCACCAAGAATCATCCAGTTA F: AAATCTCCTGCAATCATCCAGTTTA R: GAAAGTAAATGGAATATAAATGTCCG R: GAAAATAAATGGAACACACATGTCCG	10 min at 95°C, 45 cycles of 30 s at 95°C and 30 s at 63°C and 30s at 68°C	430	33

PUBLICATIONS ARISING FROM THIS REPORT

Journal Publications

Sidhu J.P.S., Hodgers L., Ahmed W., Chong M.N., Toze S. 2012. Prevalence of human pathogens and indicators in stormwater runoff in Brisbane, Australia. *Water Research.* 46(20):6652-6660.

Ahmed W., Sidhu J.P.S., Toze S. 2012. Evaluation of the *nifH* gene marker of *Methanobrevibacter smithii* for the detection of sewage pollution in environmental waters. *Environmental Science and Technology*, 46, 543-50.

Sidhu J.P.S., Ahmed W., Hodgers L., Toze S. 2013. Occurrence of Virulence Genes Associated with Diarrheagenic Pathotypes in *Escherichia coli* Isolates from Surface Water**.** *Applied and Environmental Microbiology.* 79(1):328-335.

Chong M.N, Sidhu J, Aryal R,Tang J, Gernjak, W, Escher, B. I.,Toze S. 2012. Urban stormwater harvesting and reuse: A probe into the chemical, toxicology and microbiological contaminants in water quality. *Environmental Monitoring and Assessment.* DOI 10.1007/s10661-012-3053-7.

Sidhu J.P.S., Ahmed W., Toze S. 2013. Application of Microbial Source tracking toolbox to identify sewage contamination in stormwater runoff in Brisbane**.** *AWA Water* 40(3):81-85.

Sidhu J.P.S., Ahmed, W., Gernjak, W., Aryal, R., McCarthy, D., Palmer, A., Kolotelo, P., Toze S. 2013. Evaluation of microbial and chemical source tracking markers for the detection of sewage contamination in stormwater. *Science of The Total Environment* (In Press).

Tang JYM, Aryal R, Deletic A, Gernjak W, Glenn E, McCarthy D, Escher BI. 2012. Toxicity characterization of urban stormwater with bioanalytical tools. *Water Research* (In Press).

Sidhu J.P.S., Palmer A., Ahmed W., Toze S. Enteric Pathogens and Faecal Indicators Variability Over the Storm Hydrograph in two Urban Catchments. *Environmental Science and Technology* (Under Preparation).

Sidhu J.P.S., Page D. Palmer A., Toze S. Assessment of Potential Health Risks from Stormwater Reuse. *Science of The Total Environment* (Under Preparation).

Conference Proceedings

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