Health Risk Assessment of Roof-Captured Rainwater

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September 2012

Urban Water Security Research Alliance
Technical Report No. 77

Queensland Government
National Research FLAGSHIPS Water for a Healthy Country
CSIRO
Griffith UNIVERSITY
The University Of Queensland AUSTRALIA

Urban Water Security Research Alliance Technical Report ISSN 1836-5566 (Online)
Urban Water Security Research Alliance Technical Report ISSN 1836-5558 (Print)

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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Ahmed, W., Hodgers, L., Sidhu, J., Gardner, T., Richardson, K., Palmer, A. and Toze, S. (2012). *Health Risk Assessment of Roof-Captured Rainwater.* Urban Water Security Research Alliance Technical Report No. 77.

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ACKNOWLEDGEMENTS

This research was undertaken as part of the South East Queensland (SEQ) Urban Water Security Research Alliance, a scientific collaboration between the Queensland Government, CSIRO, The University of Queensland and Griffith University.

Particular thanks go to residents of SEQ who provided access to their houses for collecting samples. We thank "Peter the Possum Man" for providing possum faecal samples and Currumbin Wildlife Sanctuary Hospital for providing bird faecal samples.

Our sincere thanks to Don Begbie (UWSRA), Greg Jackson (Queensland Health) Heather Uwins (DERM), Joan Rose (Michigan State University), Declan Page (CSIRO), Ashok Sharma (CSIRO), Ted Gardner (CQU) and Vourn Lutton (QWC) and David McCarthy (Monash University) for their technical inputs in this study.

FOREWORD

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.

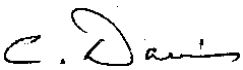
As the largest regionally focused urban water research program in Australia, the Alliance is focused on water security and recycling, but will align research where appropriate with other water research programs such as those of other SEQ water agencies, CSIRO's Water for a Healthy Country National Research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

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EXECUTIVE SUMMARY

Roof-captured rainwater (RCR) is one of the major alternative water sources that can be used in South East Queensland (SEQ) to supplement potable and non-potable water sources. An important issue in relation to RCR use is the potential public health risks associated with pathogens in rainwater tanks. Various pathogens could be present in the faeces of birds, possums, reptiles and insects. Consequently, faecal matter and other organic debris could be introduced to the tank water during rain events. There is some limited information about the occurrence of zoonotic bacterial and protozoa pathogens in rainwater tanks in the literature. This research study reports on the research outcomes on the type, prevalence, persistence and also potential sources of these pathogens in a range of rainwater tanks in SEQ.

A total of 80 rainwater tanks was selected for microbiological analysis. Of the 80 samples tested, more than 70% were positive for faecal indicator bacteria (FIB), and the numbers were above the Australian Drinking Water Guideline value of 0 *E. coli* per 100 mL of water. Of the 80 water samples, 15% and 7.5% were also positive for *Campylobacter* spp. and *G. lamblia*, respectively. To identify the sources of these pathogens, faecal samples from possums and wild birds were also collected from the SEQ region and screened for the above pathogens. These pathogens were also detected in possum and bird faecal samples.

The prevalence and numbers of zoonotic bacterial and protozoa pathogens in rainwater tanks and connected household water tap samples were also determined. Among the 24 households studied, 21% and 13% of rainwater tank samples were positive for *Campylobacter* spp., and *G. lamblia*, respectively, whilst 21% and 13% of the connected household water tap samples were also positive for *Campylobacter* spp. and *G. lamblia*, respectively.

We also investigated the inactivation rates of FIB on the roof and in the gutter of a "model" RCR system. Inactivation rates were also measured under in situ conditions using diffusion chambers in a rainwater tank. The inactivation experiments were undertaken to obtain information on the time periods between faeces being deposited on the roof and their entering the tank, and the relative health risks. FIB inactivated rapidly on the roof exposed to sunlight (T_{90} < 2 h) compared to a shaded roof where a much slower inactivation process took place (T_{90} = 120-212 h). FIB also inactivated rapidly in the gutter. It appears that FIB can survive longer (T_{90} = 5-8 days) on the roof under shaded conditions. If there is a rainfall event within a week after the deposition of faecal mater on the roof, it is highly likely that FIB and pathogens will be transported to the tank water. When introduced to the tank, a slow FIB inactivation process probably takes place (T_{90} = 10-15 days).

As high numbers of *E. coli* were detected in rainwater tank samples, we postulated a strong link between *E. coli* in tank water with animal faecal matter. To assess this link, *E. coli* isolates from rainwater tanks, possum and bird faecal samples were tested for the toxin virulence genes (VGs) using PCR methods. All strains harbouring toxin genes were also biochemically fingerprinted and compared with those strains isolated from rainwater tanks to determine whether their source was consistent with bird and possum faecal contamination. Some 33% of strains harbouring toxin genes isolated from the rainwater tanks were identical to single or multiple strains from birds. Similarly, 21% of strains from rainwater tanks were identical to single or multiple strains from possums.

The presence of pathogens along with FIB indicate a poor level of microbial water quality and represents a **potential** health risk to end users, especially those who use the water for drinking and in the kitchen. Once the microorganisms are introduced in the tank water, they may survive for a prolonged period (weeks). The presence of one or more zoonotic bacterial and protozoa pathogens in possum and bird faecal samples on roofs indicates the occurrence of possum and bird faecal contamination in rainwater tanks. This was further confirmed by *E. coli* toxin VG analysis. The detailed information being obtained through this research will allow a more detailed and accurate health risk assessment to be undertaken to determine if RCR can be used for a wider range of uses within households than currently permitted or whether the current restricted uses (i.e., toilet flushing, clothes washing) should remain.

1. INTRODUCTION AND BRIEF LITERATURE REVIEW

1.1. Introduction

The demands on potable water supply are escalating in line with increasing population growth, particularly in urban areas, along with increases in industrial output and commerce. This is further exacerbated by the adverse impacts of recent droughts on water supply sources. Consequently, water authorities around the world are keen to explore alternative water sources to meet ever-increasing demands for potable (drinking) water. Roof-captured rainwater (RCR) has been considered as a potential source for both drinking and various non-potable uses, such as irrigation, toilet flushing, car washing, showering, and clothes laundering. Countries that have investigated the potential benefits of RCR include Australia, Canada, Denmark, Germany, India, Japan, New Zealand, Thailand, and the United States (Despins *et al.,* 2009; Evans *et al.,* 2006). For example, it was estimated that 10% of Australian people use RCR as a major source of their drinking water, and an approximate additional 5% use RCR as potable replacement for showering, toilet flushing, and clothes laundering (ABS, 2007).

Many countries have provided subsidies to encourage the installation of rainwater tanks to promote the increased uptake of alternative water sources with the specific aim of decreasing the reliance and use of reticulated potable or scheme water (Ahmed *et al.,* 2010; Albrechtsen, 2002). For instance, in 2006, the Queensland State Government, Australia, initiated the "Home Water Wise Rebate Scheme," which provided subsidies to South East Queensland (SEQ) residents who installed rainwater tanks for nonpotable domestics uses. More than 260,000 householders were granted subsidies by December 2008 when the scheme was discontinued. There currently are restrictions on rainwater end uses in SEQ, which prevent it for end uses such as hot water system, or even potentially replacing potable water used for cooking and drinking. These restrictions apply to households connected to scheme water, and are principally due to uncertainty on the degree of qualitative and quantitative health risks associated with RCR, particularly from pathogens. (Ahmed *et al.,* 2008; Crabtree *et al.,* 1996; Simmons *et al.,* 2001).

A wide array of pathogens can be present in the faeces of wild birds, insects, mammals, and reptiles that have access to the roof. Consequently, following rain events, animal faecal droppings and other organic debris deposited on the roof and gutter can be transported into the tank with the roof runoff. There is a general community perception that RCR is safe to drink without having to undergo prior treatment. In support of this perception, Dillaha and Zolan (1985) reported that the quality of RCR is generally acceptable for drinking and household use. This was further supported by an epidemiological survey of gastroenteritis among 4 to 6 years old children in rural South Australia who drank rainwater or treated mains water that showed RCR poses no increased risk of gastroenteritis when compared with mains water (Heyworth *et al.,* 2006). Similar results were reported in a more sophisticated double blind intervention study by Rodrigo et al (2010). In contrast, a number of other studies on the microbial quality of RCR have reported the presence of specific zoonotic pathogens in individual or communal RCR systems (Ahmed *et al.,* 2008; Birks *et al.,* 2004; Crabtree *et al.,* 1996; Simmons *et al.,* 2001; Uba and Aghogho, 2000). Legitimate questions, therefore, have arisen from health regulators regarding the quality of rainwater and the consequent public health risks.

1.2. Literature Review

1.2.1. Faecal Indicator Bacteria (FIB)

To determine the acceptability of rainwater for drinking, it is common practice to use drinking water guidelines. For most guidelines, this entails the non-detection of the FIB such as *Escherichia coli* or *Enterococcus* spp. (usually at numbers < 1 colony forming units [CFU] per 100 mL of water) whose presence are used to indicate potential faecal contamination of the water (NHMRC–NRMMC, 2004; WHO, 2004). Even when rainwater is not used for drinking, the assessment of the microbial quality is usually undertaken by monitoring the presence of FIB (Ahmed *et al.,* 2008; Ahmed *et al.,* 2010; Appan, 1997; Dillaha and Zolan, 1985). Table 1 shows the percentage of positive samples for various FIB in water samples from rainwater tanks reported in research studies.

Table 1. Percentage of rainwater samples positive for faecal indicator bacteria (FIB).

Country	Percentage of Rainwater Samples Positive (> 1 CFU per 100 mL) for FIB (No. of samples tested)				Reference
	Faecal Coliforms	*E. coli*	*Enterococcus* spp.	*C. perfringens*	
Australia	38 (100)				Verrinder and Keleher, 2001
Australia		33 (49)	73 (49)		Spinks *et al.*, 2006
Australia		63 (27)	78 (27)	48 (27)	Ahmed *et al.*, 2008
Australia		58 (100)	83 (100)	46 (100)	Ahmed *et al.*, 2010
Australia	78 (41)	57 (67)	82 (67)	49 (67)	CRC for Water Quality and Treatment, 2006
Australia	83 (6)				Thomas and Green, 1993
Australia	63 (81)				Evans *et al.*, 2006
Canada	14 (360)				Despins *et al.*, 2009
Greece		41 (156)	29 (156)		Sazakil *et al.*, 2007
Denmark		79 (14)			Albrechtsen, 2002
Micronesia	70 (176)				Dillaha and Zolan, 1985
New Zealand	56 (125)				Simmons *et al.*, 2001
South Korea		72 (90)			Lee *et al.*, 2010
Thailand		40 (86)			Pinfold *et al.*, 1993
USA		3 (30)			Lye, 1987
US Virgin Islands	36 (45)				Crabtree *et al.*, 1996
Zambia	100 (5)				Handia, 2005

ND: Not detected; CFU: Colony forming unit; FIB: Faecal indicator bacteria

In Micronesia, 176 rainwater tank samples were surveyed for faecal coliforms. Among the samples, 30% had no measurable numbers of faecal coliforms (Dillaha and Zolan, 1985). Despite the fact that 70% of samples were not compliant with the WHO drinking water guidelines, the authors suggested that the RCR could be reasonable for drinking. Lye (1987) also reported the low occurrence of faecal coliforms in RCR samples in Kentucky, USA, where only one sample out of 30 had faecal coliforms numbers > 10 CFU per 100 mL of water. In contrast, several studies reported higher occurrence of FIB in RCR samples (Table 1). In Victoria, Australia, for example, 49 rainwater tanks surveyed for the presence of *E. coli* and *Enterococcus* spp. Spinks *et al*., (2006) found that 33% were positive for *E. coli* and 73% positive for *Enterococcus* spp., thereby exceeding the Australian Drinking Water Guidelines of 0 CFU per 100 mL (ADWG, 2011). Another recent study in South Korea reported that 72% of rainwater tank samples were positive for *E. coli* (Lee *et al.,* 2010). In all the positive samples, the numbers of these FIB were above the WHO drinking water guideline values. High numbers of *E. coli* were also found in Danish RCR systems by Albrechtsen (2002), where *E. coli* was observed to be present in 11 out of 14 systems with numbers ranging from 4 to 900 CFU per 100 mL of water. The conclusion made in this particular study was that the presence of *E. coli* indicated that the water may not be suitable for drinking (Albrechtsen, 2002).

High numbers of *E. coli* (ranging from 4 to 800 CFU per 100 mL) and *Enterococcus* spp. (5 to 200 CFU per 100 mL) were also reported in rainwater tank samples tested in SEQ, Australia (Ahmed *et al.,* 2008). In this study, of the 27 samples tested, 63%, 78%, and 48% were positive for *E. coli*, *Enterococcus* spp., and *C. perfringens*, respectively. Ahmed *et al.,* (2008) concluded that as *E. coli* could not be detected in a number of the water samples that were positive for other indicators of potential faecal origin such as *Enterococcus* spp. or *C. perfringens,* RCR samples should be tested for multiple FIB where possible to obtain multiple lines of evidence on the occurrence of potential faecal contamination. Several studies also reported that *Enterococcus* spp. are more prevalent in RCR samples compared with *E. coli* (Ahmed *et al.,* 2008; Ahmed *et al.,* 2010; CRC for Water Quality and Treatment, 2006; Spinks *et al.,* 2006), and thus may be a better indicator for assessing faecal contamination.

1.2.2. Bacterial Pathogens

To date, only a small number of studies have investigated the presence of bacterial pathogens in RCR systems (Table 2). While much of the focus on bacterial pathogens is predominantly on enteric pathogens such as *Campylobacter* spp. and *Salmonella* spp., in RCR, other non-enteric pathogens such as *Legionella* spp. are also considered to be of concern for human health. The link between water and *Legionella* infections is well known, and a recent study demonstrated a link between *Aeromonas* spp. isolated from clinical and water samples, indicating a transmission from water (Khajanchi *et al.,* 2010). In one of these studies, Simmons *et al.,* (2001) reported the presence of *Aeromonas* spp. and *Salmonella* spp. in rainwater tank samples collected in Auckland, New Zealand, with, 20% and 0.9% of the 125 samples tested as positive for *Aeromonas* spp. and *Salmonella* spp., respectively. No results on the pathogen number were reported in this study. However for a health risk assessment to be undertaken for these microorganisms, an assumption needs to be made that pathogens such as *Salmonella* spp. are present but at numbers below the detection limit of the analysis method used. Despite this, based on the positive detections obtained, the authors concluded that RCR was not suitable for drinking.

Table 2. Percentage of rainwater samples testing positive for pathogenic bacteria.

Country	Percentage of Rainwater Samples Positive for Potential Bacterial and Protozoa Pathogens (No. of samples tested)					
	***Aeromonas* spp.**	***Pseudomonas* spp.**	***Legionella* spp.**	***Campylobacter* spp.**	***Salmonella* spp.**	***Shigella* spp.**
Australia[1]	15 (27)[a]		26 (27) [a]	45 (27) [a]	11 (27) [a]	
Australia[2]	7 (100) [a]		8 (100) [a]	20 (100) [a]	17 (100) [a]	
Australia[3]	32 (56)		15 (67)	1.5 (67)	3 (67)	
Denmark[4]	14 (14)	7 (14)	71 (7)	12 (17)		
New Zealand[5]				37 (24) [a]		
New Zealand[6]	20 (125)		ND (125)	ND (125)	0.9 (125)	
Nigeria[7]		83 (6)			67 (6)	67 (6)
U.S. Virgin Islands[8]						
U.S. Virgin Islands[9]			80 (10)			

[a] Polymerase chain reaction-based methods were used for pathogen detection; ND: Not detected.
[1]: Ahmed *et al.,* 2008.
[2]: Ahmed *et al.,* 2010.
[3]: CRC for Water Quality and Treatment, 2006.
[4]: Albrechtsen, 2002.
[5]: Savill *et al.,* 2001.
[6]: Simmons *et al.,* 2001.
[7]: Uba and Aghogho, 2000.
[8]: Crabtree *et al.,* 1996.
[9]: Broadhead *et al.,* 1998.

Campylobacter spp. has also been detected in rainwater tank samples in New Zealand (Savill *et al.,* 2001). In all, 37% of the samples were positive for *Campylobacter* spp. with numbers ranging from < 0.06 to 0.56 MPN–PCR per 100 mL water. In addition to *Aeromonas* spp., other microbial pathogens such as *Pseudomonas aeruginosa*, *C. jejuni*, and *Mycobacterium* spp. have also been detected in RCR systems in Denmark (Albrechtsen, 2002). On the basis of these findings, the author concluded that connecting RCR to the drinking water systems would increase the level of risk of gastroenteritis and respiratory illness. A recent study that used PCR to detect evidence of bacterial pathogens in tank water in SEQ, Australia, found that between 1% and 19% of the samples were positive for *Aeromonas hydrophila*, *C. jejuni*, *C. coli*, and *Salmonella* spp. (Ahmed *et al.,* 2010). In addition, 8% of the samples were positive for the respiratory pathogen *L. pneumophila*. The authors also reported that RCR appears to decrease in microbiological quality after rain events.

1.2.3. Protozoa Pathogens

Despite a well-established zoonotic link, as with the presence of bacterial pathogens, the presence of protozoa pathogens in RCR has not been extensively investigated, with only a few studies examining RCR for the presence of *Giardia* spp. and *Cryptosporidium* spp. (Table 3). Crabtree *et al.,* (1996) reported that in the U.S. Virgin Islands, *Giardia* cysts and *Cryptosporidium* oocysts in tank water were

highly prevalent. They found that in 44 water samples tested from private and public rainwater systems, 45% and 23% of samples were positive for *Giardia* cysts and *Cryptosporidium* oocysts, respectively. The levels of cysts and oocysts were found to range from 1 to 10 organisms per 100 L, with one sample containing 70 oocysts per 100 L. Simmons *et al.,* (2001) also reported the presence of *Cryptosporidium* spp. in 4% of tank water samples in Auckland, New Zealand. However, unlike Crabtree *et al.,* (1996), Simmons *et al.,* (2001) were unable to detect any *Giardia* cysts. They concluded, however, that the prevalence of protozoa pathogens may have been underestimated because the standard analytical technique had low detection sensitivity. Albrechtsen (2002) reported the presence of *Cryptosporidium* spp. in Danish RCR systems. They tested 17 rainwater samples, of which six were positive for *Cryptosporidium* spp. The numbers of *Cryoptosporidium* spp. were as high as 50 oocysts per L; however, as in the study of Simmons *et al.,* (2001), *Giardia* spp. was not detected in any of the samples. In contrast, another study in SEQ, Australia, reported the presence of *G. lamblia* in 19% of rainwater tank samples tested, but none of the samples were positive for *C. parvum* (Ahmed *et al.,* 2008; Ahmed *et al.,* 2010).

Table 3. Percentage of rainwater samples testing positive for pathogenic protozoa.

Country	Percentage of rainwater samples positive for potential bacterial and protozoa pathogens (No. of samples tested)		Reference
	***Cryptosporidium* spp.**	***Giardia* spp.**	
Australia	-	19 (21) [a]	Ahmed *et al.,* 2008
Australia	ND	15 (100) [a]	Ahmed *et al.,* 2010
Denmark	35 (17)	ND (17) [a]	Albrechtsen, 2002
New Zealand	4 (125)	ND (125) [a]	Simmons *et al.,* 2001
U.S. Virgin Islands	45 (45)	23 (45) [a]	Crabtree *et al.,* 1996

[a] Polymerase chain reaction-based methods were used for pathogen detection; ND: Not detected.

1.2.4. Health Risks

To date, eight studies reported sporadic gastroenteritis associated with the consumption of untreated rainwater (Table 4). It should be noted that RCR should only be considered a potential risk from zoonotic pathogens. Other issues that can influence the level of actual risk include the type and numbers of pathogen carried by the infected animals, the time between deposition of faecal matter on the roof and pathogens being flushed in the tank water, the form of exposure (ingestion from drinking vs. exposure to droplets in the shower or toilet flushing), and the relative persistence of the different pathogens. An example of how all these factors can create a human health risk is a *Salmonella arechevalata*-related gastroenteritis outbreak reported by Koplan *et al.,* (1978) that occurred among 83 campers in Trinidad, West Indies. Epidemiological and bacteriological studies were performed to identify the source of *S. arechevalata* infection. Through patient surveys, food items were ruled out as the source of the gastroenteritis; however, water samples collected from two kitchen taps connected to a RCR system were found to be positive for *S. arechevalata*, although the bacterium could not be directly isolated from the tank. A sanitary survey revealed that the roof was covered with bird faeces, and it was postulated that rainwater washed off faecal matter containing *S. arechevalata* into the tank, leading to the gastroenteritis outbreak. To test this hypothesis, a number of intestinal samples were collected from local birds, rodents, and reptiles that were assumed to be the source of the contamination. However, *S. arechevalata* could not be isolated from the faeces of these local animals.

Another description of a potential link between pathogens and RCR reported on an outbreak of three cases of infant botulism in New South Wales, Australia, where *Clostridium botulinum* type B was isolated from soil around one house and in the rainwater tank from another house (Murrell and Stewart, 1983). *Clostridium botulinum* type A was also present in soil, dust from a vacuum cleaner, and the rainwater tanks. The presence of *C. botulinum* spores in the rainwater tanks was suggested as possibly contributing to the occurrence of the infant botulism cases. As a result of this study, consumers with infant children were advised to disinfect water for the first 6 months of the infant's lives. Another study reported the isolation of *Campylobacter fetus* from a 64 yr old febrile

neutropaenic patient, which was subsequently linked to the tank water (Brodribb *et al.,* 1995). Three sets of blood cultures from the patient were positive for *C. fetus* using PCR. The rainwater was the only source of water supply for this particular household. To identify the source of infection, a sample of the tank water was tested and *C. fetus* was isolated from as little as 200 mL of water sample. The patient was advised to boil the tank water before consumption and had no further report of the illness.

Merritt *et al.,* (1999) reported an outbreak of *Campylobacter* enteritis among 23 resort staff in Queensland, Australia, with untreated RCR. Food was initially suspected as possible sources of infection. None of the food samples were positive for the *Campylobacter* spp., but four rainwater tank samples were positive for total coliforms, with particularly high total coliform numbers found in one tank. The authors reported a strong association between gastroenteritis and consumption of water from a dispenser that had probably been filled from one of the contaminated tanks. It was hypothesised that the *Campylobacter* spp. that caused the outbreak may have been introduced into one or more of the tanks by contamination with the faeces of wild animals. Another reported outbreak of gastroenteritis with a strong link to contaminated RCR in Melbourne, Australia, involved an outbreak of *Salmonella* Typhimurium at a rural school camp served by a private rainwater supply (Franklin *et al.,* 2009). The phage type strain DT9 of *Salmonella* was found in both the faecal specimens of patients and water taps supplying the untreated rainwater that were used as a drinking water source, indicating a direct link between the RCR and the disease outbreak.

Simmons and Smith, (1997) reported the isolation of *S.* Typhimurium phage type I from two of four family members who sought medical attention due to gastrointestinal symptoms. The family lived in a beachside house and used RCR for household use. The family reported that one of their cats, which spent much time on the roof and frequently defecated on it, had loose stools for several years. An investigation was undertaken to identify whether the infection was food borne or if it originated from the cat faeces. However, the infection could not be related to food or the cat faeces. *Salmonella* Typhimurium phage type I, and faecal coliforms were isolated from the houses tap water, leading the authors to conclude that RCR was the possible source of infection, although the original contamination source could not be determined.

An outbreak of Legionnaires' disease in an isolated suburb of Auckland, New Zealand, was linked to RCR using PCR method, demonstrating that the isolates of *L. pneumophila* from patient's clinical specimens were identical to the high levels of *L. pneumophila* present in the nozzle of a local marina water blaster used to clean boats. Sampling of nearby rainwater collection systems revealed that contaminated water spray from the water blaster had been carried and deposited on roof surfaces in the local area. The *L. pneumophila* within the spray were washed into rainwater tank, and users were exposed through bathroom showers (Simmons *et al.,* 2008). Since 1978, there have been eight reports of gastroenteritis associated with the consumption of RCR cited in the literature (Brodribb *et al.,* 1995; Franklin *et al.,* 2009; Koplan *et al.,* 1978; Merritt *et al.,* 1999; Murrell and Stewart, 1983; Schlech *et al.,* 1985; Simmons and Smith, 1997; Simmons *et al.,* 2008). These studies suggest that the untreated rainwater may be a contributing factor for gastroenteritis; little is known, however, regarding the actual health risks from drinking the rainwater. Several authors highlighted the need for evaluating the actual health risks from drinking RCR (Ahmed *et al.,* 2008; Lye, 1992; Simmons *et al.,* 2001).

Table 4. Reported disease cases-associated with the consumption of untreated roof-captured rainwater (RCR).

Country	Disease Causing Pathogens	Types of Diseases	No. of People Affected	Reference
Australia	*C. botulinum*	Not specified	3	Murrell and Stewart, 1983
Australia	*Campylobacter fetus*	Diarrhea, vomiting	1	Brodribb *et al*., 1995
Australia	*Campylobacter* spp.	Diarrhea, abdominal pain,	23	Merritt *et al.,* 1999
Australia	*S.* Typhimurium phage 9	Diarrhea, abdominal pain, nausea	27	Franklin *et al.,* 2009
New Zealand	*S.* Typhimurium phage I	Diarrhea	2	Simmons and Smith, 1997
New Zealand	*L. pneumophila*	Legionnaires' disease	1	Simmons *et al.,* 2008
U.S. Virgin Islands	*L. pneumophila* serogroup I	Legionnaires' disease	27	Schlech *et al.,* 1985
West Indies	*S. arechevalata*	Diarrhea, headache, fever, vomiting	48	Koplan *et al.,* 1978

An epidemiological study to identify the risk of gastroenteritis among 4 to 6 yr old children who drank RCR compared with children who drank treated mains water in South Australia noted that the consumption of RCR did not increase an observed level of gastroenteritis relative to mains water consumption (Heyworth *et al.*, 2006). The authors concluded, however, that their data could also have reflected a level of acquired immunity among regular users of RCR and therefore may not reflect the actual risk to the new users. In New Zealand, numerous cases of campylobacteriosis have been associated with the rainwater collection systems (Eberhart-Phillips *et al.,* 1997). A case control study found strong association with gastrointestinal diseases caused by *Campylobacter* spp. with the consumption of RCR. It was hypothesised that the rainwater systems in this study had been contaminated by birds roosting on the roofs. In a reported outbreak of 27 cases of Legionnaire's disease among tourists visiting the U.S. Virgin Islands, the infections were thought to have originated from inhalation of *L. pneumophila* detected in the drinking water system of a local hotel (Schlech *et al.,* 1985). Case control and microbiological studies were undertaken to identify the mode of transmission. The exact mode of transmission during this outbreak could not be determined, but the potable water was implicated as the most probable cause in the absence of other sources. The hotel obtained its potable water from a RCR system, and the identical serogroup of the *L. pneumophila* isolated from the infected patients was found in the stored RCR and as well as in hot and cold water taps. No further cases of Legionellosis were identified after the hotel water system was chlorinated.

The last of the reported incidences of RCR-acquired infections was reported a case control study in Tasmania, Australia, where single variable associations were found between drinking untreated rainwater and cases of infection with *Salmonella mississippi* (Ashbolt and Kirk, 2006). The highest risk was found to be associated with exposure to untreated RCR away from the home of the participants. These higher risk estimates probably reflected a lower level of immunity in populations not frequently exposed to this pathogen. Direct contact with native animals known to be a source of salmonellosis was determined not to be the cause of infections. The strongest predictor of infection was found to be the indirect contamination of RCR systems. It is probable that incidences of gastrointestinal associated with RCR may be under reported. It is highly likely that only a small number of people with severe gastroenteritis would seek medical attention, and most faecal specimens that were collected from patients would not be tested in the hospitals.

In Australia, it has been estimated that only between 8% and 11% of *Campylobacter*- and *Salmonella*-related food-borne gastroenteritis cases are reported (Hall *et al.,* 2006). In the United States, it has been estimated that only 10% to 33% of water related gastroenteritis are reported (Frost *et al.,* 1996). These statistics suggest that gastroenteritis from sources such as RCR would have at least similarly low reporting levels, if not even lower. Based on the common community belief that RCR is of good quality, it should be considered probable that cases of gastroenteritis would be blamed on other

sources such as food before being blamed on RCR. Another limitation is that cases of gastroenteritis due to drinking untreated rainwater could also actually be masked by the background levels of gastroenteritis from other sources such as consumption of food and community-based infections. The most credible epidemiological study to date reported that the consumption of RCR did not increase the risk of gastroenteritis as opposed to mains water (Heyworth *et al.,* 2006; Rodrigo *et al.,* 2010). However, such results should be interpreted with care due to the lack of sensitivity of the epidemiological tool to detect gastroenteritis (Craun *et al.,* 2004; Hrudey and Hrudey, 2004).

A few studies have attempted to identify the inherent risk of infection associated with the drinking and non-potable uses of RCR using Quantitative Microbial Risk Assessment (QMRA). A QMRA is a four-step probabilistic tool for estimating the human health risk associated with defined scenarios from exposure to specified pathogens (NRC, 1983). One such study was undertaken by Fewtrell and Kay (2007), who investigated the risk of infection of *Campylobacter* from toilet flushing with RCR in homes in the United Kingdom. A QMRA estimate was performed to quantify the risks of *Campylobacter* infection via ingestion of aerosols. The outcomes of this QMRA estimate concluded that any risk from flushing the toilet with harvested rainwater would be well within the acceptable range.

Another recent study used QMRA analysis to quantify the risk of infection associated with the exposure to zoonotic pathogens from drinking and non-potable uses of RCR in SEQ, Australia (Ahmed *et al.,* 2009). This study concluded that the risk of infection from *G. lamblia* and *Salmonella* spp. associated with the use of rainwater for biweekly garden hosing was below the threshold value of one extra infection per 10,000 persons per year. However, the estimated risk of infection from drinking the rainwater daily was 44 to 250 (for *G. lamblia*) and 85 to 520 (for *Salmonella* spp.) infections per 10,000 persons per year, which is above the acceptable guideline levels outlined in the Potable Reuse section of the Australian Guidelines for Water Reuse (NRMMC–EPHC–NHMRC, 2008). Despite this, the overall health risk appeared to be higher than predicted from reported incidences of gastroenteritis in the local community where the study was undertaken. The authors noted that further work is needed to improve the assumptions made in the analysis. These studies showed that health risk analyses have their place in aiding the use of RCR but are currently restricted by the lack of comprehensive work on the prevalence of pathogens in RCR. Improved levels of available data would enable accurate calculations of the level of risk and allow an assessment of the required levels of reductions in pathogens numbers for different end uses (drinking vs. non-potable uses). This can then enable more appropriate treatment measures such as filtration and ultraviolet disinfection (Daschner *et al.,* 1996; Jordan *et al.,* 2008) can be undertaken to reduce the risk of infection from RCR.

There is some background information about the occurrence of microbial pathogens in rainwater tanks based on some initial testing undertaken via a research team in Department of Environment and Resource Management, Qld and research undertaken in other States. More information is required, however, on the prevalence and range of FIB and pathogens in RCR tanks from different locations within SEQ and the persistence of FIB and pathogens in storage tanks (Ahmed *et al.,* 2010). The resulting information will be combined with the research outputs of the Decentralised Systems project to maximise the research outputs assessing the optimal uses of RCR for the maximum savings of potable and non-potable water. The information on the prevalence and persistence of pathogens will be used to highlight the qualitative health risks for RCR systems in SEQ. These information can be used for the formulation of transparent, accurate and valid policies and guidelines for the use of these alternative water sources, if necessary, valid restrictions for some uses, and management requirements for storage tanks and distribution systems.

1.2.5. Research Objectives

- **Occurrence and the numbers of faecal indicator bacteria (FIB) and pathogens in rainwater tanks in SEQ:** to investigate the numbers of FIB and pathogens in rainwater tank samples. In addition, the occurrence of bacterial and protozoa pathogens in animal faecal samples (wild birds and possums have been linked as major sources of contamination) was also investigated to determine any links between bird and possum faecal matters with rainwater tanks.

- **Incidence and prevalence of pathogens in connected household tap water samples sourced from rainwater tanks:** to investigate the prevalence and numbers of FIB and pathogens in water samples from household taps fed from rainwater tanks.
- **Inactivation of FIB:** to investigate the inactivation rates of FIB on the roof surface, in the gutter and tank water to assess the persistence of FIB and relative health risks.
- **Occurrence of *E. coli* and *Enterococcus* spp. virulence genes (VGs) in isolates from rainwater tank samples:** to investigate the presence of virulence genes (VGs) in a collection of *E. coli* isolates from rainwater tank samples, and to determine any potential public health risks associated with these *E. coli*. In addition, a collection of *Enterococcus* spp. was obtained from rainwater tank samples and their distribution into the species level and the occurrence of six VGs within the collection were determined. This was done to obtain information on the source and ecology of *Enterococcus* spp. in rainwater tank samples.
- **Tracking the sources of potential pathogenic *E. coli* in rainwater tank samples:** to identify the likely sources of *E. coli* in rainwater tanks so that faecal contamination can be minimised. *E. coli* toxin gene analysis was used as a source tracking tool to establish a link between faecal contamination in rainwater tanks with faeces from wild animals.

2. QUANTITATIVE DETECTION OF FAECAL INDICATOR BACTERIA (FIB) AND PATHOGENS IN RAINWATER TANK SAMPLES

2.1. Introduction

Direct monitoring of pathogens in water is an attractive option, as it would provide invaluable information regarding public health risk. However, there are hundreds of different types of pathogens that can be found in water due to faecal contamination. Therefore, it is not economically, technologically, and practically feasible to routinely monitor the microbiological quality of water for all possible pathogens. Alternatively, traditional and alternative FIB, such as faecal coliforms, *Escherichia coli*, *Enterococcus* spp., and *Clostridium perfringens*, have long been used as surrogates for the presence of pathogens. Epidemiological studies have established human health standards based on exposure to FIB for recreational waters (Prüss, 1998).

Most studies assess the quality of RCR based on the number of traditional FIB such as faecal coliforms and/or *E. coli* (Evans *et al.* 2006; Sazakil *et al.,* 2007; Yaziz *et al.,* 1989). However, the major limitations of using FIB as indicators are a poor correlation between the bacterial number and the presence of pathogens in environmental water samples (Hörman *et al.,* 2004) and the FIB's ability to replicate outside the host, especially in tropical environments (Desmarais *et al.,* 2002). The recent advances in molecular techniques such as quantitative polymerase chain reaction (qPCR) technology enable rapid, specific, and sensitive quantification of potential pathogens in environmental waters that are difficult and/or laborious to culture using traditional microbiological methods.

Little is known regarding the prevalence of zoonotic pathogens in animals such as wild birds and possums that are the most likely contaminating rainwater tanks. Possums can get access to the roof via over hanging trees, electricity cable or climbing the roof via walls or other structures attached to the house. Birds generally sit on the overhanging trees or mounted structures on the roof such as TV aerials and solar panels. Knowing the source(s) of FIB and pathogenic microorganisms is important for formulating efficacious risk reduction management strategies.

The specific aims of this study were (i) to investigate the numbers of FIB (*E. coli* and *Enterococcus* spp.) and pathogens (*Campylobacter* spp., *Salmonella* spp., *G. lamblia*, *C. parvum*) in rainwater tank samples; and (ii) to investigate the prevalence and numbers of these pathogens in faecal samples from wild birds and possums to establish a link between animal faecal contamination with degraded rainwater quality.

2.2. Materials and Methods

2.2.1. Location of the Rainwater Tanks

In all, 80 rainwater tanks were selected for this study representing 31 suburbs in Brisbane and Gold coast region in SEQ, Australia. The list of suburbs is shown in Appendix 1.

2.2.2. Water Sampling

A single water sample was collected from each rainwater tank, within three to seven days after a rain event. Water samples were collected in sterilised 20-L containers from the outlet taps located close to the base of the tanks (Figure 1). Before the tank was sampled, the tap was wiped with 70% ethanol and allowed to run for 30 to 60 s to flush out water from the tap. Samples were transported to the laboratory and processed within 2-4 h.

Figure 1. Rainwater Sampling.

2.2.3. Possum and Bird Faecal Sampling

Altogether 78 faecal samples were collected from various species of birds (n = 38) and possums (n = 40). Possum faecal samples were collected from a possum removal service in Brisbane (Figure 2). Bird faecal samples were collected from botanical garden, bird sanctuary and a veterinary hospital. The species of birds has been listed elsewhere (Ahmed *et al.,* 2012c). Up to three samples were collected from each species of bird. All samples were transported to the laboratory, stored at 4ºC and processed within 24 h after collection.

Figure 2. Possum and bird faecal sampling.

2.2.4. Enumeration of Faecal Indicator Bacteria (FIB)

The membrane filtration method was used to process the tank water samples for the enumeration of FIB (Ahmed *et al.,* 2008). For the isolation of *E. coli* and *Enterococcus* spp., modified mTEC agar plates were incubated at 35°C for 2 h to recover stressed cells, followed by incubation at 44°C for 22 h, and mEI agar plates were incubated at 41ºC for 48 h (Figure 3).

Figure 3. *Escherichia coli* isolation on agar plates.

2.2.5. Concentration of Water Samples

Nineteen-litre water sample from each rainwater tank was concentrated by hollow-fiber ultrafiltration system (HFUFS), using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA, USA) (Figure 4). The samples were concentrated to approximately 100 mL. Each 100 mL sample was further centrifuged at 4,500 rpm for 30 mins at 4ºC. The supernatant was discarded, and the pellet was resuspended in 4 mL of sterile distilled water.

Figure 4. Concentration of water samples using hollow fibre ultrafiltration cartridge, shown in the top of photo. The raw water sample is in the container on the right. A peristaltic pump (bottom left) supplies pressure to pass the water through the UF cartridge.

2.2.6. DNA Extraction

For PCR analysis of bacterial and protozoa pathogens, DNA was extracted from 1.5-ml of concentrated samples using Blood and Tissue Kit (Qiagen, Valencia, CA) and stored at -80ºC until use. For PCR analysis of bacterial and protozoa pathogens, DNA was extracted from fresh faeces from each individual animal faecal sample using QIAmp Stool DNA Kit (Qiagen).

2.2.7. PCR Inhibitors

An experiment was conducted to determine the potential presence of PCR inhibitory substances in DNA extracted from rainwater tank samples (Ahmed *et al.,* 2008; Ahmed *et al.,* 2012a). DNA isolated from wild bird and possum faecal samples were checked for the potential PCR inhibitors. None of the samples had PCR inhibitors.

2.2.8. qPCR Analysis

qPCR assays were performed using previously published primers, probes and cycling parameters (Appendix 2). Standards for qPCR of the *Campylobacter* spp. 16S rRNA, *Salmonella* spp. *invA*, *C. parvum* COWP and *G. lamblia* β-giardin genes were prepared from the genomic DNA. The genomic copies were calculated, and a 10-fold dilution ranging from 10^6 to 10^0 copies per µl of DNA extract was prepared from the genomic DNA, and stored at –20°C until use. For each PCR experiment, negative control (sterile water) was included.

2.3. Results

2.3.1. Numbers of Faecal Indicator Bacteria (FIB)

The number of *E. coli* in water samples (n = 80) from rainwater tanks ranged from 0 to 4,800 CFU per 100 mL of water with an average 180 CFU per 100 mL. For *Enterococcus* spp., these figures were 0 to 3,233 CFU per 100 mL with an average 246 CFU per 100 mL. Of the 80 samples tested, 28% had no measurable *E. coli* and the remaining 72% had > 1 *E. coli* per 100 mL. Similarly, 8% had no measurable *Enterococcus* spp., and the remaining 92% had > 1 *Enterococcus* spp. per 100 mL (Figure 5).

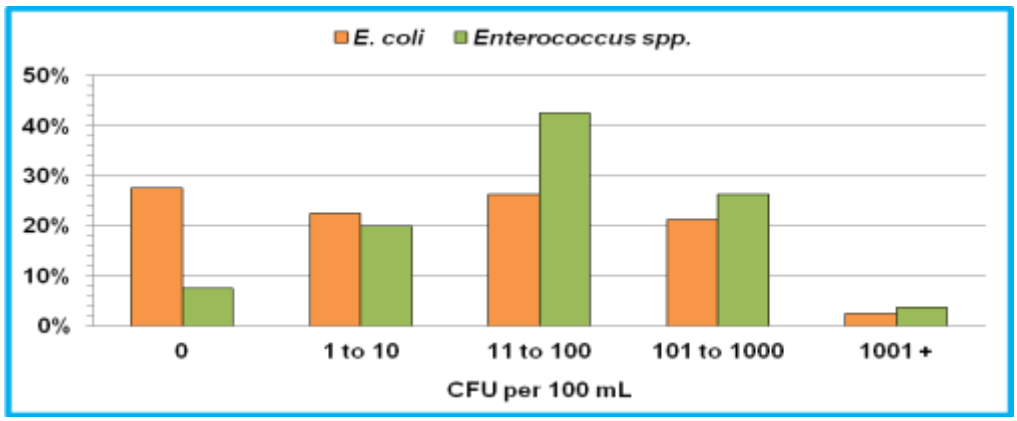


Figure 5. Percentage of rainwater tank samples positive for *E. coli* and *Enterococcus* spp.

2.3.2. Numbers of Pathogenic Bacteria and Protozoa

Among the 80 households, 15%, 1.3%, and 7.5% were positive for *Campylobacter* spp. 16S rRNA, *Salmonella invA*, and *G. lamblia* β-giardin genes, respectively (Figure 6). After conversion of number of genomic copies to number of cells, the number of *Campylobacter* spp. in rainwater tank samples ranged from 5 to 100 cells per L of water. Similarly the estimated number of *Salmonella* spp. was 7,300 cells per L of water. The numbers of *G. lamblia* cysts ranged from 120 to 580 per L of water. None of the samples were positive for *C. parvum*.

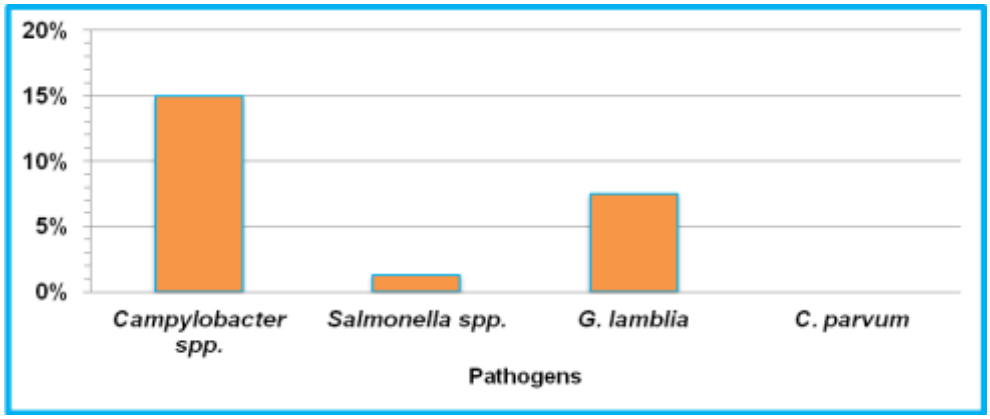


Figure 6. Percentage of rainwater tank samples positive for bacterial and protozoa pathogens.

2.3.3. Numbers of Pathogens in Animal Faecal Samples

Among the 38 bird faecal samples tested, the *Campylobacter* spp. 16S rRNA, *Salmonella invA*, *C. parvum* COWP, and *G. lamblia* β-giardin genes were detected in 24%, 11%, 13%, and 5% samples, respectively. Similarly, of the 40 possum faecal samples tested, the *Campylobacter* spp. 16S rRNA, *C. parvum* COWP, and *G. lamblia* β-giardin genes were detected in 60%, 13% and 30% samples, respectively (Figure 7).

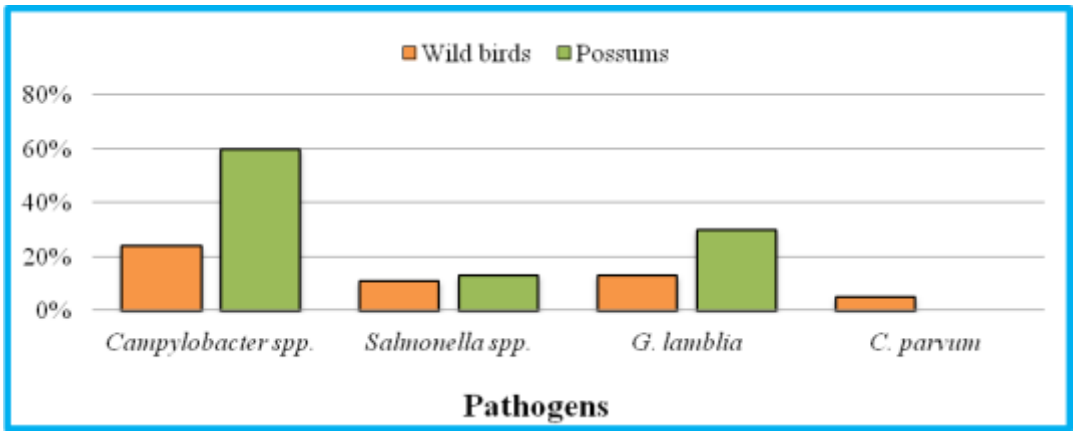


Figure 7. Percentage of faecal samples positive for bacterial and protozoa pathogens.

The numbers of qPCR quantified genomic copies were converted to equivalent cells and cysts. The number of *Campylobacter* spp. *Salmonella* spp. and *G. lamblia* organisms in bird faecal samples ranged from 6.6×10^4 to 6.6×10^6 cells (for *Campylobacter* spp.), 630 to 1,800 cells (for *Salmonella* spp.) and 1.3 to 100 cysts (for *G. lamblia*) per gm of faeces (Table 5).

G. lamblia β-giardin gene was detected in nine samples, however, only seven were quantifiable. *C. parvum* COWP gene was not quantifiable and *Salmonella invA* gene could not be detected in DNA from possum faecal samples. After conversion of number of genomic copies to number of cells, the number of *Campylobacter* spp. and *G. lamblia* organisms in possum faecal samples ranged from 2.0×10^5 to 2.0×10^7 (for *Campylobacter* spp.) and 2.1 to 1,600 (for *G. lamblia*) per gm of faeces.

Table 5. Numbers of zoonotic pathogens in possum and bird faecal samples.

Samples	Range of Bacterial and Protozoa Pathogens per gm of Faeces								
	Campylobacter spp.			*Salmonella* spp.			*G. lamblia*		
	Min.	Max.	Ave.	Min.	Max.	Ave.	Min.	Max.	Ave.
Birds	6.6×10^4	6.6×10^6	3.3×10^6	630	1,800	1,215	1.3	120	40
Possums	2.0×10^5	2.0×10^7	1.0×10^7	ND	ND	ND	21	1,600	380

ND: Not detected; Min. : Minimum; Max. : Maximum; Ave. :Average.

2.4. Discussion

Roof-captured rainwater samples were tested for the numbers of traditional FIB, using conventional culture-based methods, as well as the presence of potential pathogens using qPCR. Previous studies that have reported acceptable levels of microbiological quality of RCR used conventional culture-based methods to detect traditional FIB (Coombes *et al.,* 1999; Dillaha and Zolan, 1985; Evans *et al.,* 2006). However, there are several limitations to those culture methods, including the underestimation of the bacterial concentration due to the presence of injured or stressed cells (Delgado-Viscogliosi *et al.,* 2005). Furthermore, certain microorganisms in environmental waters could be viable but not culturable (Oliver, 2000). In addition, some of the conventional test methods are time-consuming, labor-intensive, and not practical for routine monitoring of pathogens. However, the application of qPCR-based methods has generated interest in the estimation of pathogens in environmental waters. The advantages of qPCR-based methods are that they are rapid and can detect organisms that are difficult to grow using conventional culture techniques. In addition, qPCR has the potential to improve detection limits. For these reasons, qPCR-based methods have been used to detect a wide range of pathogenic microorganisms in waters (Sails *et al.,* 2002; Savichtcheva *et al.,* 2007).

In view of this, qPCR-based assays were applied for the measurement of zoonotic pathogens in samples from rainwater tanks. Before the qPCR assays were applied, the sensitivity of the assays was rigorously evaluated by amplifying known concentrations of DNA from target pathogens. The lower limits of detection ranged from 5 to 10 copies, indicating that the detection sensitivity values of the qPCR assays were comparable to the values reported in the literature (Behets *et al.,* 2007; Sails *et al.,* 2002).

Of the 80 samples tested, 73% and 93% exceeded the Australian drinking water guidelines for 0 *E. coli* and *Enterococcus* spp. per 100 mL of water (ADWG, 2011), respectively. Of the 80 samples tested, 15%, 1.3% and 7.5% were positive for the *Campylobacter* spp., *Salmonella* spp. and *G. lamblia*. To identify the likely sources of these pathogens in rainwater tank samples, wild birds and possum faecal samples were screened as these animals have access to the roof surface, and were identified as a major sources of tank water contamination in SEQ. In all, 60% possum and 24% bird faecal samples were positive for *Campylobacter* spp. All bird faecal samples were positive for *C. jejuni*. None of the possum faecal samples were positive for *C. jejuni* (data not shown). Possum and bird faecal samples were also positive for *G. lamblia* and the numbers of cysts ranged from 21 to 1,600 (for possums) and 1.3 to 120 (for birds) per gm of faeces.

Previous research studies also reported the presence of *G. lamblia* in possum and bird faeces in North Island, New Zealand (Chilvers *et al.,* 1998; Marino *et al.,* 1992). In this study, five possum and two bird faecal samples were also positive for *C. parvum*. The prevalence of *C. parvum* in possum and bird faecal samples was lower than *G. lamblia*. Chilvers *et al.,* (1998) reported similar findings and suggested that this could be because the duration of *Cryptosporidium* infection is much shorter than *Giardia* infection. It has to be noted that *Giardia* cysts were also detected in faecal samples from cats, rats and mice and therefore, these animals may also contribute *Giardia* to rainwater tanks (Chilvers *et al.,* 1998). Other animals such as lizards, frogs and flying foxes that have access to the roof cannot be ruled out as possible sources of bacterial and protozoa pathogens in rainwater tank samples.

We validated our qPCR positive results by sequencing up to three amplicons for each target and verified they were ≥ 97% identical to the published sequences (data not shown). Quantitative PCR results obtained in the study do not provide information regarding the pathogenicity of the targets. A major limitation of PCR is its inability to distinguish between viable and nonviable pathogens. Therefore, in this study, it cannot be ruled out that qPCR assays may have detected DNA from nonviable pathogens.

The presence of potential pathogens, however, along with the presence of one or more FIB in samples collected after rainfall events indicate a poor level of microbial quality of tank water samples and could represent a potential health risk to end users, especially for those who use the water for drinking and kitchen uses.

3. QUANTITATIVE DETECTION OF FAECAL INDICATOR BACTERIA (FIB) AND PATHOGENS IN HOUSEHOLD TAP WATER FED FROM RAINWATER TANKS

3.1. Introduction

It has been reported that around 10% of Australian people use RCR as a major source of their drinking water (ABS, 2007), however, it is usually not recommended to use RCR for drinking where town water is available. For example, Queensland regulations do not prohibit the plumbing of rainwater tanks to supply drinking water. If a person, however, chooses to use rainwater for drinking or any other purpose, then that person is responsible for ensuring the quality of the water is fit for its intended use. Many householders who drink rainwater generally use under sink filtration (USF) system in order to reduce the exposure to pathogenic microorganisms, suspended solids and harmful chemicals.

The aims of this study were (i) to investigate the prevalence and numbers of *E. coli* and *Enterococcus* spp., zoonotic bacterial (*Campylobacter* spp., and *Salmonella* spp.) and protozoa (*C. parvum* and *G. lamblia*) pathogens in water samples from rainwater tanks and connected household tap water (CHTW) samples. Conventional culture based methods were used to enumerate *E. coli* and *Enterococcus* spp., and qPCR assays were used to obtain the numbers of zoonotic pathogens in RCR and CHTW samples. These were done to obtain a better understanding of the magnitude of health risk associated with the potable uses of RCR.

3.2. Materials and Methods

3.2.1. Study Area

The study area "Currumbin Ecovillage" is located on the southern end of the Gold Coast, SEQ, Australia. The Ecovillage is known for its sustainable residential developments and the technology is often viewed as a blueprint for future urban developments. Twenty-four households participated in this study. These tanks were the part of the 80 tanks mentioned in section 2.2.1. All households use captured RCR for drinking and other non-potable uses such as car washing, cloth laundering, showering, gardening etc.

3.2.2. Sanitary Survey

A sanitary inspection was undertaken to identify factors (the presence of overhanging trees, TV aerials and wildlife faecal contamination on the roof) that might contribute to the faecal contamination of the rainwater tanks. Information on the filtration methods for RCR prior to drinking was also obtained from the householders.

3.2.3. Water Sampling

Two water samples were collected from each household (one from rainwater tank and one from the connected household tap) giving a total of 48 samples from 24 households. Samples were collected within one to four days after a rain event in 20 L sterile containers. Before sampling, the external taps (attached to the rainwater tank) and household taps were wiped with 70% ethanol and allowed to run for 30 to 60 s to flush water. Samples were transported to the laboratory and processed within 2-4 h.

3.2.4. Enumeration of Faecal Indicator Bacteria (FIB) and Concentration of Water Samples

The membrane filtration method was used to process water samples for bacterial enumeration (see section 2.2.4). Approximately, 19 L water sample from each rainwater tank and household tap was concentrated by HFUS, using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA, USA).

3.2.5. DNA Extraction

For qPCR analysis of bacterial and protozoa pathogens, DNA was extracted from the pellet obtained from 1.5 mL of concentrated samples using a DNeasy Blood and Tissue Kit (Qiagen), and stored at -80ºC until use.

3.2.6. qPCR Analysis

qPCR assays were performed using previously published primers, probes and cycling parameters (see Appendix 2). Standards for qPCR of the *Campylobacter* spp. 16S rRNA, *Salmonella* spp. *invA*, *C. parvum* COWP and *G. lamblia* β-giardin genes were prepared from the genomic DNA. The genomic copies were calculated, and a 10-fold dilution ranging from 10^6 to 10^0 copies per µL of DNA extract was prepared from the genomic DNA, and stored at –20°C until use. For each PCR experiment, negative control (sterile water) was included.

3.2.7. Reproducibility of qPCR and PCR Inhibitors

The reproducibility of the qPCR was assessed by determining intra-assay repeatability and inter-assay reproducibility. The qPCR assays were highly reproducible (Ahmed *et al.,* 2012a). An experiment was conducted to determine the potential presence of PCR inhibitory substances in DNA extracted from connected household tap water (CHTW) (n = 3) samples from three different households (H1, H10, and H15). The samples were PCR inhibitors free (Ahmed *et al.,* 2012a).

3.2.8. Statistical Analysis

Prior to the statistical analysis, all indicators and pathogen numbers were Log_{10} transformed. Wilcoxon signed-rank test was applied to test the significance of difference in FIB and pathogen numbers between RCR and CHTW samples. The Pearson's multiple correlation (r_p) was used to test the relationship between *E. coli* and *Enterococcus* spp. numbers in RCR and CHTW samples. In all cases, a difference was considered significant if the P value was < 0.05.

3.3. Results

3.3.1. Survey Results

The size of the selected rainwater tanks ranged from 7,200 to 30,000 L and were aged between one to five years (Appendix 3). Among the 24 households surveyed, 25% had overhanging trees (n = 4) or TV aerials (n = 2) mounted on the roof. Seven (29%) tanks had visible sign of faecal droppings on the roof. Twenty of the tanks (88%) had first flush diverter installed. Among the 24 households, 42% treated the water before drinking.

3.3.2. Numbers of Faecal Indicator Bacteria (FIB)

Of the 24 households, 62% RCR and 58% CHTW samples were positive for *E. coli*. Similarly, 92% RCR and 83% CHTW samples were positive for *Enterococcus* spp. (Table 6). The numbers of *E. coli* in positively identified samples ranged from 1 to 230 CFU per 100 mL (for RCR) and 1 to 300 CFU per 100 mL (for CHTW) of water, respectively. For *Enterococcus* spp., these numbers were 2 to 110 CFU per 100 mL (for RCR) and 1 to 110 (for CHTW) CFU per 100 mL, respectively. *Enterococcus* spp. were more frequently detected in both RCR (22 of 24 samples were positive) and CHTW (20 out of 24) than *E. coli* (15 out of 24; RCR and 14 out of 24; CHTW). Among the 24 samples tested from RCR tanks, 96% samples were positive at least one FIB and 58% were positive for both indicators. Similarly, among the 24 samples tested from connected household taps, 92% were positive at least one FIB and 50% were positive for both FIB.

Table 6. Numbers of faecal indicator bacteria (FIB) in roof-captured rainwater and connected household tap water samples.

Household ID	Mean Numbers of FIB per 100 mL of Water			
	E. coli		*Enterococcus* spp.	
	RCR	CHTW	RCR	CHTW
H1	15	20	21	13
H2	3	4	12	13
H3	1	4	91	100
H4	2	ND	3	6
H5	2	3	3	10
H6	230	67	27	ND
H7	1	2	37	82
H8	89	6	40	61
H9	ND	ND	4	1
H10	2	ND	17	2.5
H11	5	9	28	36
H12	12	6	ND	ND
H13	ND	ND	3	ND
H14	5	2	54	61
H15	12	300	75	110
H16	ND	ND	23	18
H17	ND	3	2	2
H18	ND	ND	49	41
H19	1	1	15	40
H20	ND	ND	ND	24
H23	ND	15	110	25
H25	ND	ND	5	3
H29	1	ND	2	ND
H35	ND	ND	4	3

ND: Not detected; RCR: Roof-captured rainwater; CHTW: Connected household tap water; FIB: Faecal indicator bacteria.

3.3.3. Numbers of Zoonotic Pathogens

Among the 24 households, 21%, 4%, and 13% RCR samples were positive for *Campylobacter* spp. 16S rRNA, *Salmonella invA*, and *G. lamblia* β-giardin genes, respectively. Similarly, 21% and 13% of the CHTW samples were positive for *Campylobacter* spp. 16S rRNA, and *G. lamblia* β-giardin genes, respectively. *Salmonella invA* gene could not be detected in CHTW samples. For the estimation of pathogen numbers, genomic copies (determined by qPCR) of each pathogen were converted to bacterial cells or protozoa cysts (Ahmed *et al.,* 2010; Guy *et al.,* 2003; Lee *et al.,* 2009).

After conversion of genomic copies to number of cells, the number of *Campylobacter* spp. in RCR and household tap water samples ranged from 5 to 110 (in RCR) and 11 to 19 (in CHTW) cells per L of water (Table 7). Similarly the estimated number of *Salmonella* spp.ranged from 0 to 7,300 (in RCR) cells per L of water. The numbers of *G. lamblia* cysts ranged from 120 to 580 (in RCR) and 110 to 140 (in CHTW) per L of water.

Table 7. Numbers of zoonotic pathogens in roof-captured rainwater and connected household tap water samples.

Household ID	Mean Numbers of Bacterial and Protozoa Pathogens per L of Water					
	Campylobacter spp.		*Salmonella* spp.		*G. lamblia*	
	RCR	CHTW	RCR	CHTW	RCR	CHTW
H1	ND	ND	ND	ND	120	140
H4	ND	ND	7300	ND	ND	ND
H6	110	+ [a]	ND	ND	ND	ND
H7	ND	ND	ND	ND	160	140
H10	47	ND	ND	ND	ND	ND
H11	ND	14	ND	ND	ND	ND
H12	+ [a]	11	ND	ND	ND	ND
H14	5	12	ND	ND	580	110
H15	30	19	ND	ND	ND	ND

ND: Not detected; [a]: Detected but not quantifiable; RCR: Roof-captured rainwater; CHTW: Connected household tap water.

3.3.4. Correlation Between Zoonotic Pathogens and Faecal Indicator Bacteria (FIB)

The number of FIB and pathogens were pooled for all RCR and CHTW samples to determine whether the numbers correlated between RCR and CHTW samples. The number of *E. coli* ($P = 0.78$), *Enterococcus* spp. ($P = 0.64$), *Campylobacter* spp. ($P = 0.44$), and *G. lamblia* ($P = 0.50$), in RCR did not significantly differ from those numbers in CHTW samples. The numbers of *E. coli* and *Enterococcus* spp. were analysed to determine whether the numbers within the RCR and CHTW correlated with each other. Significant correlations were observed between *E. coli* and *Enterococcus* spp. in water samples from RCR ($r_p = 0.33$; $P = 0.005$) and CHTW ($r_p = 0.28$; $P = 0.01$).

3.4. Discussion

In this study, 62% of the RCR and 58% of the CHTW samples fed from the RCR tanks exceeded Australian Drinking Water Guidelines (ADWG, 2011) of < 1CFU *E. coli* per 100 mL water. The pooled numbers of *E. coli* and *Enterococcus* spp. in the CHTW samples did not differ significantly from the numbers found in the RCR samples. It has to be noted that, 58% of households in this study did not use any treatment methods, therefore, the presence of FIB in the CHTW samples was not unexpected. Ten (42%) households had USF installed, however, these systems do not appear to be effective in removing FIB. For example, households H3, H8, H11, H12, H1, H18 and H35 had USF, however, the numbers of FIB in CHTW samples did not differ significantly from the RCR.

Campylobacter spp. was detected in 21% of the 24 RCR tanks tested in this study. H6, H10, H12 and H15 were four of the five households where there were overhanging trees (H10 and H12) or evidence of wild life faecal droppings (H6 and H15) on the roofs. Two households (H12 and H15) had USF installed, however, *Campylobacter* spp. was detected in the CHTW samples suggesting the poor efficacy of USF systems. For *Campylobacter* spp., most human infections (95%) are caused by *C. jejuni* and *C. coli* (Butzler, 2004), and therefore, all *Campylobacter* spp. PCR positive samples were further tested for the presence of *C. jejuni* and *C. coli*. Three RCR tanks and two CHTW samples were positive for *C. coli*. None of the RCR tanks and CHTW samples was positive for *C. jejuni* (data not shown). *G. lamblia* was detected in three (13%) of the RCR tanks tested in this study. H1 and H7 were two of the three households where there was evidence of wild life faecal droppings. All three CHTW samples were also positive for *G. lamblia*. It has to be noted that these households did not apply any treatment methods for rainwater purification prior to drinking. The high numbers of *G. lamblia* in both RCR and CHTW samples from households H1, H7 and H14 may pose serious health risks to the consumers because of the low infectious dose (< 10 cysts) of *Giardia.*

To obtain an insight on the magnitude of health risks, genomic copies of *G. lamblia* were converted to cysts numbers. The *G. lamblia* β-giardin gene is expressed as a single-copy gene within the nucleus of each trophozoite (Holberton and Marshall, 1995). Cysts of *Giardia* contain two trophozoites that have undergone multiple steps of nuclear division, resulting in 16 copies of total genetic information within each cyst (Bernander *et al.,* 2001) resulting 16 copies of the β-giardin gene per *Giardia* cyst (Guy *et al.,* 2003). The number of *G. lamblia* appeared to be one order of magnitude higher in rainwater samples in this study compared to our previous study (Ahmed *et al.,* 2010). It has to be noted that in this current study, 19 L of water samples were tested and whereas in the previous study, a smaller volume (2-2.5 L) of water samples was tested. Concentration of large volume of water samples may have increased the detection sensitivity (Leskinen *et al.,* 2010). *C. parvum* could not be detected in any of the samples tested, however, the presence of *Cryptosporidium* spp. in RCR samples has been reported in US Virgin Islands and Denmark (Albrechtsen, 2002; Crabtree *et al.,* 1996). *Salmonella* spp. were detected in one rainwater tank sample whereas none of the CHTW samples was positive for *Salmonella* spp.

The results of this study indicate that certain householders were potentially exposed to potential pathogenic bacteria and protozoa, however, no increase in reported cases of illnesses is evident from disease notifiable database. This could be due to the fact that there is a naturally high incidence of gastroenteritis in the community, which may mask the actual disease (Hellard *et al.,* 2001). Before the disease can be reported in the Notifiable Diseases Surveillance System, it must first be identified, and not every individual will seek medical attention if the illness is mild and lasts only for a few days. Another factor is the possibility of individuals acquiring immunity to certain pathogens due to frequent exposure.

The faecal contamination of RCR appears to be limited to improperly designed systems as well as systems that are not well maintained. It has been suggested that all RCR systems should be appropriately maintained, including ensuring the cleanliness of the systems before rainfall events, especially roofs and gutters, which should be cleaned frequently, while the receiving tanks should be cleaned at least two times per year to improve the quality of water (Cunliffe, 1998). The roof should be kept clear of overhanging trees which may provide access to wild animals. Indeed, the high numbers of bacterial and protozoa pathogens in possum and bird faecal samples indicates the need for good maintenance of roof and gutter and elimination of overhanging tree branches to minimise faecal contamination of RCR. It is evident that further information relating to the occurrence of pathogens throughout the year and the viability of pathogens in rainwater tanks is needed. In addition, more information is required on the survival of bacterial and protozoa pathogens in rainwater tanks.

4. INACTIVATION OF FAECAL INDICATOR BACTERIA (FIB) ON THE ROOF, IN THE GUTTER AND IN THE RAINWATER TANK

4.1. Introduction

The presence of FIB in water indicates the presence of potential pathogens of faecal origin. The microbiological quality of RCR in SEQ appears to be highly variable and of poor quality (reported in two previous sections). The presence of FIB, along with the potential bacterial and protozoa pathogens in rainwater tanks and related household tap water samples suggests that untreated RCR may not be suitable for drinking (Ahmed *et al.* 2012a). Recent studies have also reported the presence of clinically significant virulence genes (VGs) associated with FIB *E. coli* (Ahmed *et al.,* 2011) and *Enterococcus* spp. (Ahmed *et al.,* 2012b) in rainwater tank samples in SEQ. Under these circumstances, it is imperative to measure the inactivation rates of FIB and pathogens in various components of a RCR system such as roof, gutter and tank water.

The primary aim of this study was to investigate the persistence of *E. coli* and *Enterococcus* spp. from animal faecal droppings deposited on the roof and in the gutter of a RCR system. The specific aims of the study were to: (i) determine the potential survival time of *E. coli* and *Enterococcus* spp. on the corrugated iron roof and in the gutters; (ii) determine the influence of ambient climatic conditions on the inactivation of FIB on corrugated iron roof and gutters; and (iii) determine survival potential of FIB in the tank water. The inactivation experiments were undertaken to obtain information on the time periods between faeces deposited on the roof and entering the tank and relative health risks.

4.2. Materials and Methods

4.2.1. Study Site

This study was conducted at the Ecosciences Precinct, Dutton Park, Qld, Australia (27ºS.153ºE). A "model" RCR system comprised of 2 × 5,000 L polyethylene tanks (tanks A and B), a roof (2 m^2) constructed with colourbond corrugated iron sheets (similar to those urban RCR systems) and gutter made with colorbond zincalume and plastic downpipes leading water into both tanks was constructed to simulate urban RCR systems. Tank A was placed underneath the roof in an enclosure to create shaded tank conditions and Tank B was placed adjacent to the tank A in direct sunlight.

4.2.2. Sources of *E. coli* and *Enterococcus* spp. Used for Inactivation Experiments

E. coli and *Enterococcus* spp. were obtained from fresh, wild bird (crow, pigeon, honey eaters, magpie, tawny frogmouth) and possum faecal samples since these were the most likely sources of faecal deposition on the roof and in the gutters of dwellings in SEQ.

4.2.3. Isolation of Faecal Indicator Bacteria (FIB)

Possum and bird faeces were serially diluted with the phosphate buffer saline (PBS) and streaked on Chromocult™ coliform agar (Merck, KGaA, Darmstadt, Germany) and Chromocult™ enterococci agar (Merck) plates. Agar plates were incubated at 37°C overnight. Single isolates from agar plates were further streaked on Chromocult™ coliform and Chromocult™ enterococci agar plates to obtain pure isolates.

4.2.4. *E. coli* and *Enterococcus* spp. Inactivation on the Roof and in the Gutters

Possum faecal pellets were chosen as seeding matrix over faecal pellets from other animals (birds, lizards and bats) because of the relative ease of collection. The pellets were pooled and homogenised into approximately 900 mL slurry using sterile PBS. The background numbers of *E. coli* and

Enterococcus spp. in possum faecal slurry were determined within 24 h after collection using the spread plate method as described elsewhere (Sidhu *et al.,* 2008). The numbers were approximately 2×10^4 CFU per mL for both FIB. Since the background numbers of FIB were low in possum faecal slurry, a known numbers of *E. coli* and *Enterococcus* spp. were seeded into the possum faecal slurry to obtain a final number approximately 10^8 *E. coli* and *Enterococcus* spp. per mL of slurry.

For the roof and gutter inactivation experiments, 5 mL of faecal slurry was poured into a series of 50 mm petri dishes (without lids), and placed on the corrugated iron roof and in the gutter of the experimental structure. The petri dishes were exposed to diurnal cycles of insolation receiving equivalent amounts of sunlight. For the roof experiment, 30 petri dishes were kept directly under sunlight and another 30 were kept in the shade. The shade on the roof was created artificially by placing a tarpaulin over the dishes. Enough air space was provided between the petri dishes and the tarpaulin to ensure that free airflow could still occur. For the gutter experiment, 30 petri dishes were placed in the clean segment of the gutter (free from vegetation and organic debris), and another 30 petri dishes were placed in the dirty gutter (Figure 8). The gutter was made dirty by filling it with moist sediment (similar to dirty urban household gutters) with vegetation and organic debris.

Figure 8. Possum faecal slurry in the gutter.

Triplicate petri dishes containing FIB were randomly collected from the dish population after 0, 1, 2, 3, 4, 6, 8, 24, 48 and 72 h exposure time for the roof and respective gutter segments, and the numbers of surviving FIB were enumerated. The collected petri dishes were placed on ice, transported to the laboratory and processed within 2-4 h. The volume of slurry was adjusted to 5 mL with PBS in petri dishes where desiccation was observed in order to correct for evaporation loss. Rehydrated materials were scraped carefully from the petri dishes and transferred into 15 mL tubes. Enumeration of *E. coli* and *Enterococcus* spp. was then performed in triplicate. Serial dilutions were made for each replicate, and 50 µL of each serial dilution was spreaded on Chromocult™ coliform agar (Merck) and Chromocult™ enterococcus agar (Merck) plates for the isolation of *E. coli* and *Enterococcus* spp., respectively. The plates were incubated at 37ºC for 24 and 48 h. Plates with numbers 20-200 colonies were enumerated.

4.2.5. Inactivation Experiments in Tank Water

The FIB inactivation experiments in tank water were undertaken using diffusion chambers as previously described elsewhere (Toze *et al.* 2004).

Prior to setting up diffusion chambers, rainwater sample from the tank B was collected in a sterile 1 L bottle, and stored at 4ºC. *E. coli* and *Enterococcus* spp. numbers were determined in the broth cultures and added to the rainwater sample matrix to final numbers approximately 3.6×10^6 *E. coli* and 1.4×10^7 *Enterococcus* spp. per mL of water.

All of the assembled diffusion chambers were suspended in the tank B at a depth of 1 meter below the water level. Triplicate diffusion chambers containing the *E. coli* and *Enterococcus* spp. were collected on days 1, 2, 4, 6, 10, 17, 24, day 34 after deployment. Sample serial dilutions were made and the numbers of *E. coli* and *Enterococcus* were enumerated using spread plate method as described above.

4.2.6. Meteorological Data and Data Analysis

Ambient temperature, rainfall, evaporation, relative humidity, wind speed and solar exposure data were collected from Australian Bureau of Meteorology (BOM) web site during each of the inactivation experiments. Temperature data loggers (HOBO devices; Onset Computer Corporation, Pocasset, Mass.) were placed on the roof and in the gutter adjacent to the petri dishes to record the temperature at 1 h intervals for the duration of roof and gutter experiment. HOBO device was also suspended into the tank water to record the temperature at 4 h intervals for the duration of tank water experiment.

For each die off experiment the FIB, numbers in each replicate at each sampling occasion were converted to $\log_{10}$ values and plotted over time. The following formula was then used to estimate the first order kinetics:

$C/C_0 = e^{-k.t}$

where C_0 is the initial numbers of *E. coli* and *Enterococcus* spp. (CFU per mL), C is the number (CFU per mL) at times t, k is the first order inactivation kinetics (1/h) and t is time (hour).

A regression line was then fitted to the plot. The inactivation kinetics (kh) and related standard error was determined from the slope of the regression line. The results were also reported as a T_{90} time (time for a 90% or 1 log reduction of each FIB) which was determined as $1/kh$. Statistical significances of the results were determined by applying a student's t-test to the T_{90} values. 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 compared data to be non significant.

4.3. Results

4.3.1. Climatic Conditions and Roof Inactivation Experiment

Roof, gutter and tank inactivation experiments of FIB were undertaken at different time periods (Table 8). The average minimum temperature and maximum temperature during the experiments ranged from 11.6ºC (dirty gutter) to 18.0ºC (shaded roof) and 20.4ºC (dirty gutter) to 30.2ºC (tank water), respectively. The evaporation rates were high throughout the inactivation experiments with values higher during the roof experiments compared to the gutter and tank water experiments. The relative humidity ranged from 75% to 79%) during the roof and gutter experiments. The wind speed was higher (34.5 km/h) during the roof experiments compared to other experiments where wind speed was relatively stable ranging from 21.6 to 23.6 km/h. Similarly daily solar radiation was higher (16.9 MJ m^{-2} per day) during the roof sunlight experiment compared to roof shade, gutter and tank water experiments.

The tank water temperature (measured by data logger) ranged from 21.4ºC to 28.2ºC during the tank inactivation experiment. The temperature of the corrugated iron roof and gutter was also measured using the data loggers. For the roof experiment, the temperature of the unshaded roof ranged from 23.7ºC to 41.3ºC. For the roof shade experiment, the temperature ranged from 19.3ºC to 26.0ºC. Gutter temperature ranged from 24.5ºC to 48ºC during the gutter inactivation experiment.

4.3.2. Roof Inactivation Experiment

The inactivation rates of FIB in possum faecal slurry placed on the roof were evaluated under full sunlight and shaded conditions at ambient meteorological conditions (Appendix 4). The numbers of FIB contained in the faecal slurry consisted of the background (2×10^4 CFU per mL for both FIB) already present in the faecal slurry and as well as the known number of seeded FIB. Under direct

sunlight, *E. coli* rapidly inactivated (T_{90} = 1.7 h) compared to the shaded treatment where slow, non-linear (biphasic) inactivation (T_{90} = 111 h and 9 h) rate was observed (Table 8). A slow inactivation (< 1log) was observed in the first 50 h followed by a rapid decline. Similar inactivation results were also obtained for *Enterococcus* spp. which was faster under direct sunlight (T_{90} = 1.5 h) compared to shaded condition, where slow biphasic inactivation (T_{90} = 200 h and 11.5 h) was also observed (Fig. 4.2a). No significant differences were observed in *E. coli* inactivation compared to *Enterococcus* spp. for full sunlight (paired t-test, P = 0.059) and shaded (paired t-test, P = 0.140) conditions. Significant differences, however, were observed between sunlight and shaded conditions for both *E. coli* (paired t-test, P = 0.024) and *Enterococcus* spp. (paired t-test, P = 0.013).

Table 8. Inactivation kinetics (T_{90} in hours) of *Escherichia coli* and *Enterococcus* spp. on the roof and gutter surface and in the tank water.

Faecal Indicator Bacteria (FIB)	Experiments	Experimental Conditions	Inactivation Kinetics (k_h)	T_{90} (h)
E. coli	Roof surface	Sunlight	0.581	1.7 h
		Shaded	0.009; 0.111	111 h; 9 h
	Gutter surface	Clean	0.021; 0.345	48 h; 2.9 h
		Dirty	0.025; 0.162	40 h; 6.2 h
	Tank water		0.138; 0.003	72 h; 273 h
Enterococcus spp.	Roof surface	Sunlight	0.689	1.5 h
		Shaded	0.005; 0.087	200 h; 11.5 h
	Gutter surface	Clean	0.502	2 h
		Dirty	0.179	5.6 h
	Tank water		0.026 h; 0.005	38 h; 195 h

4.3.3. Gutter Inactivation

The inactivation rates of FIB in possum faecal slurry were evaluated in the gutters under direct sunlight. *E. coli* showed biphasic inactivation rates under both clean and dirty gutter conditions (T_{90} = 48 h and 2.9 h for the clean gutter and T_{90} = 40 h and 6.2 h for the dirty gutter). *Enterococcus* spp., however, showed relatively rapid inactivation (T_{90} = 2 h for the clean gutter and T_{90} = 5.6 h for the dirty gutter) compared to *E. coli* (Fig. 1b). No significant differences were observed for *E. coli* (paired t-test, P = 0.534) and *Enterococcus* spp. (paired t-test, P = 0.676) inactivation between clean and dirty gutters.

4.3.4. *E. coli* and *Enterococcus* spp. Inactivation Rates in Tank Water

The inactivation rates of *E. coli* and *Enterococcus* spp. were determined under in situ condition in the rainwater tank B. The *E. coli* showed non-linear biphasic inactivation (T_{90} = 72 h and 273 h). Similarly, the estimated T_{90} value for *Enterococcus* spp. was 38 h and 195 h (Fig. 4.2c). No differences were observed in *E. coli* inactivation compared to *Enterococcus* spp. (paired t-test, P = 0.167).

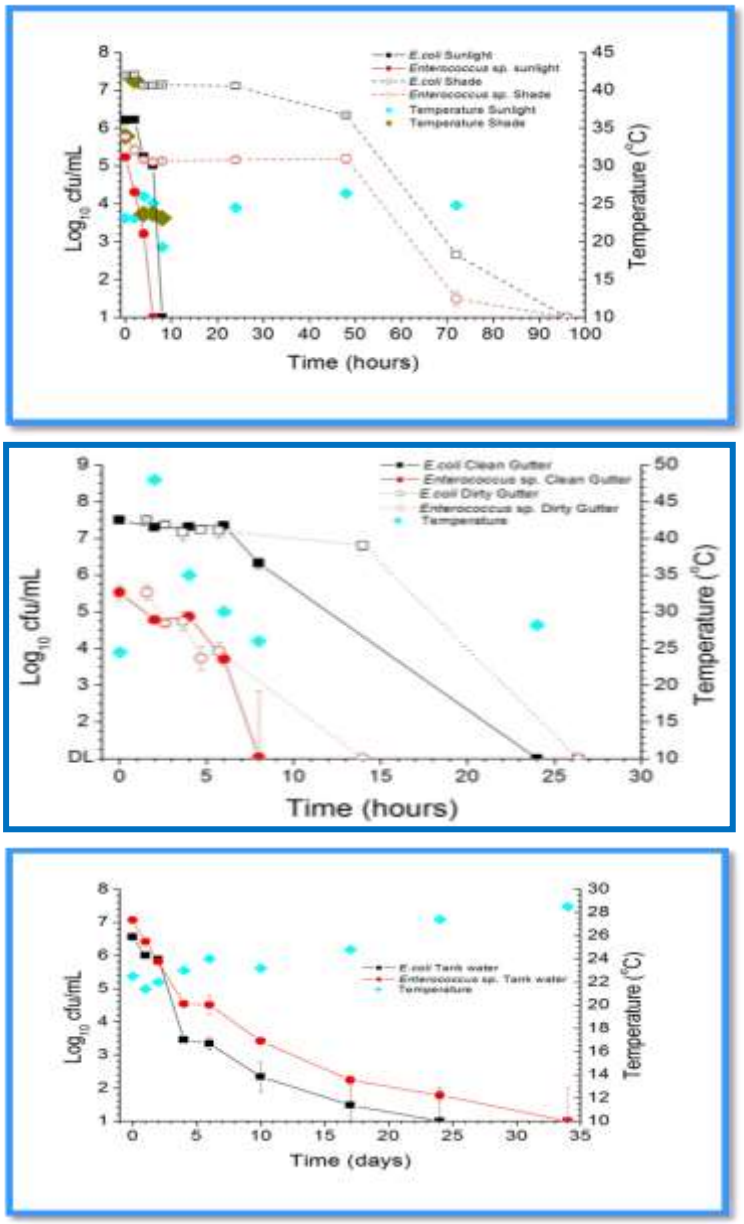


Figure 9. Log_{10} inactivation of *Escherichia coli* and *Enterococcus* spp. on the (a) roof surface (b) roof gutter and (c) in tank water.

4.4. Discussion

A significant numbers of rainwater tank samples in SEQ reported to have faecal indicators above the Australian drinking water guideline value (Ahmed *et al.,* 2008; Ahmed *et al.,* 2010). Furthermore, PCR analysis of clinically significant VGs associated with *E. coli* and *Enterococcus* spp. indicated the presence of wide array of VGs in *E. coli* and *Enterococcus* spp. isolated from rainwater tank samples (Ahmed *et al.,* 2011; Ahmed *et al.,* 2012b). Certain *E. coli* strains harboring VGs were identified to be originated from possum and bird faeces (Ahmed *et al.,* 2012c). Pathogens such as *Campylobacter* spp., *Salmonella* spp., *G. lamblia*, and *Cryptosporidium* spp. were also detected in rainwater tank samples (Ahmed *et al.* 2010). Little or no information is available on the inactivation rates of *E. coli* and *Enterococcus* spp. or pathogens on the roof, in the gutter or in the tank water.

Survival potential of *E. coli* and *Enterococcus* spp. was investigated in this study due to their wide prevalence in RCR systems in SEQ and the former is recommended FIB for monitoring microbiological quality of RCR in SEQ, Australia (Ahmed *et al.,* 2010). *E. coli* and *Enterococcus* spp. became inactivated more rapidly on the roof under sunlight conditions compared to shaded conditions. The average ambient daily minimum and maximum temperature over the sampling period for sunlight and shaded conditions were similar, and, therefore, appeared not to have played any significant role in FIB inactivation. FIB are the part of normal gut-flora of warm-blooded animals and the optimum growth temperature is 35^{o}C for most enteric bacteria, although growth can occurs at higher and lower temperatures (Sinton *et al.*, 2002). Hence temperature < 35°C may have little impact on the inactivation (Klein *et al.,* 2011; Sinton *et al.,* 2007).

A biphasic inactivation was observed under shaded conditions for both FIB with slow inactivation rates for the first 2 days followed by rapid losses. Loss of moisture through rapid evaporation (5.4 mm per day) may have been a significant factor leading to the rapid inactivation observed for sunlight conditions compared to shaded conditions as moist conditions are essential for the viability of metabolically active bacteria (Sinton *et al.,* 2002; Ward *et al.,* 1981). Under full sunlight, complete desiccation of the slurry was observed to occur in 2 h compared to shade where the complete desiccation occurred in 8 h. Moriarty *et al.*, (2011) reported significant increase in inactivation of FIB in cow pats when the moisture content of the pats decreased from 80% to 40%. Another factor leading to the rapid inactivation under sunlight could be high intensity of ultraviolet radiation associated with direct sunlight (16.9 MJ m^{-2} per day) compared to shade (12.6 MJ m^{-2} per day) where the faecal slurry was sheltered from the direct sunlight (Moriarty *et al.,* 2011). In this study, the roof experiment was undertaken on the corrugated iron roof, which can have extremely high surface temperature under sunlight (Bretz *et al.,* 1998). The temperature logger kept on the roof under sunlight recorded temperature as high as 41.3ºC during the first few hours of experiment. The combination of direct sunlight and high roof temperatures may have lead to a rapid inactivation of FIB. Data obtained in this study should be interpreted with care as the inactivation rates of FIB may yield variable results on other roof types such as concrete or tiled roof, which were not included in this study.

For the gutter experiment, it was hypothesised that FIB would survive longer in the dirty gutter compared to the clean gutter due to micro-climatic factors such as moisture, nutrients and protection from UV in the leaf debris. However, in this study, there was no difference observed between FIB inactivation rates in the dirty gutter compared to clean gutter. The results suggested that organic matters and vegetation placed in the dirty gutter (Figure 8) did not influence the inactivation rates as hypothesised. This could be due to the fact that a rapid desiccation (< 3 h) of FIB was noted in both types of gutters. *Enterococcus* spp. in both dirty and clean gutters survived relatively shorter periods of time than *E. coli*. We acknowledge that clean and dirty gutter experiments were undertaken under sunlight conditions only. It is highly likely that FIB would inactivate more slowly in the dirty gutter under shaded conditions similar to that roof shade experiment. Nonetheless, gutters should remain free of debris and vegetation because over time dirty gutters may provide sufficient shelter to FIB and pathogens from inactivation factors such as increased temperature or desiccation (Cunliffe, 1998).

The most important component of the RCR system is the storage tank. It is therefore imperative to understand inactivation rates of FIB in tank water that is used for potable or non-potable purposes. The survival of FIB in water can be affected by complex physical, chemical and biological factors (Rhodes and Kator, 1998). Creating these factors in laboratory microcosms can be difficult (McFeters and Terizieva, 1991). In view of this, the FIB inactivation assay was undertaken *in-situ* in order to mimic the real world scenario as closely as possible. For spiking, a mixture of wild strains from bird and possum faeces were used because these isolates represent likely faecal sources that are frequently implicated in contamination of rainwater tanks in SEQ (Ahmed *et al.*, 2012c).

When comparing the two FIB groups, the results indicated that *Enterococcus* spp. became inactivated more rapidly than *E. coli*. Faecal coliforms have greater persistence in freshwater than *Enterococcus* spp. (Sinton *et al.,* 2002). Slower inactivation rates were observed for both indicators in the tank water compared to the roof and gutter experiments. This is not unexpected, considering the fact that FIB in tank water were not exposed to the harsh meteorological conditions of the roof and gutter experiments. It is possible that certain strains of FIB survived better than others in the tank water, on the roof and in the gutter since faecal strains were used for spiking in this study. Biphasic inactivation rates were

observed for roof, gutter and tank experiments suggesting perhaps certain strains of FIB survived better than others. Studies have shown that *E. coli* persistence in the environment is strain dependent (Topp *et al.*, 2003; Whittam, 1989). Several studies have also documented slow inactivation of FIB of faecal origin similar phenomenon reported in this study (Noble *et al.,* 2004; Sinton *et al.,* 2002; Sommer *et al.,* 1997). We acknowledge that the inactivation experiment was undertaken in tank B, which was kept under sunlight. Many urban rainwater tanks are located in the shade or underneath the house, and in such conditions the FIB indicators inactivation rates may not be similar to the results obtained in this study.

In conclusion, it appears that FIB can survive longer (T_{90} = 5-8 days) on the roof under shaded conditions. If there is a rainfall event within a week after the deposition of faecal maters on the roof, it is highly likely that FIB would be transported to the tank water. When introduced to the tank, a slow inactivation process may take place (T_{90} = 10-15 days). Further research is required to understand the persistency of bacterial and protozoa pathogens on the roof and in tank water relative to FIB because pathogens are known to be more persistent in the environment than FIB. Maintenance of good roof and gutter hygiene and elimination of overhanging tree branches and other mounted structures on the roof where possible to prevent the roosting of possums and birds should be considered to minimise chances of faecal contamination on the roof and in the gutter.

5. OCCURRENCE OF *ESCHERICHIA COLI* HARBOURING VIRULENCE GENES IN RAINWATER TANK SAMPLES

5.1. Introduction

E. coli is often characterised as a commensal or harmless bacterium (Hartl *et al.,* 1984). However, certain strains of *E. coli* can be pathogenic and responsible for both intestinal and extraintestinal infections (Kaper *et al.,* 2004; Nataro and Kaper, 1998). It has been reported that faeces of some warm-blooded animals may contain high numbers of *E. coli* carrying virulence genes (VGs) (Ishii *et al.,* 2007). These VGs allow pathogenic *E. coli* to cause a wide array of infections such as diarrhoea, urinary tract infections (UTI), neonatal meningitis, soft tissue infections and bacteremia (Anastasi *et al.,* 2010; Johnson *et al.,* 2002; Kaper *et al.,* 2004).

Pathogenic *E. coli* strains that are capable of causing diseases in humans and animals can be categorised as (I) intestinal pathogenic *E. coli* (InPEC) and (II) extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000). *E. coli* pathotypes that are responsible for intestinal infections are known as: enterotoxigenic (ETEC), enteropathogenic (EPEC), shiga-toxigenic (STEC), enteroinvasive (EIEC), enteroaggregative (EaggEC) and diffusely adherent (DAEC) *E. coli* (Nataro and Kaper, 1998).

These pathotypes contain various combinations of VGs for attachment and elaboration of hemolysins and enterotoxins (Bertin *et al.,* 2001). ExPEC *E. coli* strains have special ability to cause extraintestinal infections such as UTIs, neonatal meningitis and neonatal sepsis and wound infections which can lead to serious complications including death (Law, 2000; Orskov and Orskov, 1985). ExPEC strains possess VG combinations that are distinctive from those that cause intestinal infections.

Furthermore, *E. coli* strains belong to four main phylogenetic groups (A, B1, B2, and D) (Clermont *et al.,* 2000). InPEC and commensal strains belong to phylogenetic group A and B1 (Clermont *et al.,* 2000). ExPEC strains derive predominantly from phylogenetic group B2 and, to a lesser extent, group D (Clermont *et al.,* 2000; Johnson and Stell, 2000). Detailed information about InPEC and ExPEC can be found in review articles (Johnson and Stell, 2000; Johnson *et al.,* 2002; Nataro and Kaper, 1998).

Despite increasing evidence that *E. coli* strains from several animal hosts contain VGs and some have been shown to cause intestinal and extraintestinal diseases in humans (Nataro and Kaper, 1998), none of the studies have determined whether *E. coli* found in rainwater tanks carry VGs which potentially can cause intestinal and extraintestinal infections in humans. We detected high numbers of *E. coli* in rainwater tank samples and it seems likely there could be a link between *E. coli* contamination in rainwater tanks and animal faeces. To determine such a link, a preliminary investigation was undertaken on the presence of 20 VGs associated with InPEC and ExPEC pathotypes in a collection of *E. coli* isolates from rainwater tank samples in SEQ, Australia.

5.2. Materials and Methods

5.2.1. Survey of Rainwater Tanks

In all, 30 rainwater tanks (subset of the 80 tanks mentioned in Section 2) were selected for this study representing seven suburbs in Brisbane and the Gold Coast region in SEQ, Australia. These tanks were located in peri-urban and urban areas and were selected on the basis of the end uses. Among the 30 tanks, 24 were used for both potable and non-potable uses and the remaining 6 were used for non-potable purposes. A sanitary survey was undertaken to identify physical characteristics of the RCR systems such as size of the tanks, age of the tanks, tank material and factors that may contribute to the faecal contamination of the tanks such as the presence of overhanging trees on the roof. The roofs were also surveyed for the presence of possible wild life faecal contamination.

5.2.2. Water Sampling

A single water sample was collected from each rainwater tank, within three to seven days after a major rain event (> 80 mm). Water samples were collected in sterilised 20-litre containers from the outlet taps located close to the base of the tanks. Before the tank was sampled, the tap was wiped with 70% ethanol and allowed to run for 30 to 60 s to flush water from the tap to minimise cross contamination. Samples were transported to the laboratory and processed within 2-4 h.

5.2.3. Enumeration and Isolation of *E. coli*

The membrane filtration method was used to process water samples for *E. coli* isolation. The modified mTEC agar (Difco, Detroit, MI) was used for the isolation of *E. coli*. Modified mTEC agar plates were incubated at 35°C for 2 h to recover stressed cells, followed by incubation at 44°C for 22 h.

5.2.4. DNA Extraction and Confirmatory Test

Up to ten *E. coli* isolates were selected from replicate mTEC agar plates giving a total number of 200 *E. coli* isolates from 22 rainwater tanks. *E. coli* could not be isolated from the remaining 8 tanks. DNA was extracted from 1 mL of pure colony culture using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Strains were confirmed as *E. coli* by PCR amplification of the *uidA* gene as described elsewhere (Frahm and Obst, 2003).

5.2.5. Animal Faecal Sampling and DNA Extraction

Altogether 40 faecal samples were collected from fresh faecal droppings of brush tail possums (n = 20) and various species of birds (n = 20) from Brisbane and the Gold Coast Region in SEQ. Up to two samples were collected from each species of bird. All samples were transported to the laboratory, stored at 4ºC and processed within 24 h. DNA was extracted from fresh faeces from each individual animal by using QIAmp Stool DNA kit (Qiagen).

5.2.6. Phylogenetic Group Classification and Detection of Virulence Genes

Confirmed *E. coli* isolates from rainwater tank samples were tested for phylogenetic groups using multiplex PCR with the *chuA* and *yjaA* genes and the DNA fragment TSPE4.C2 according to the method described by Clermont *et al.,* (2000). All isolates were further tested for the presence of 20 *E. coli* VGs associated with intestinal and extraintestinal diseases. DNA extracted from animal faecal samples was also tested for the presence of seven *E. coli* toxin VGs associated with intestinal and extraintestinal diseases (Table 9). PCR detection of *uidA* gene (Frahm and Obst, 2003), phylogenetic group classification (Clermont *et al.,* 2000), and VGs (Johnson and Stell, 2000; Paton and Paton, 2002; Ram *et al.,* 2007) was undertaken using previously published primers and PCR protocols. The primer sequences of the VGs are shown in Appendix 5.

5.2.7. PCR Detection

PCR amplification of VGs associated with InPEC was performed in 25 µL reaction mixtures using SYBR Green iQ Supermix (Bio-Rad Laboratories, Calif). The PCR mixture contained 12.5 µL SuperMix, 300 nM of each primer, and 2 µL of template DNA. For each PCR experiment, corresponding positive (target DNA) and negative controls (sterile water) were included.

Table 9. ***Escherichia coli*** **pathotypes and associated virulence genes (VGs) tested in this study.**

Pathotypes	Virulence Genes (VGs)					
	Adhesins	**Toxins[a]**	**Invasins**	**Sidephores**	**Capsule synthesis**	**Additional genes**
STEC	*eaeA**	stx_1				
		stx_2				
		*exhA**				
ETEC		LT1				
		ST1				
EPEC	*eaeA**	*cdtB**				
		*exhA**				
ExPEC	*bmaE*	*cdtB**	*ibeA*	*iutA*	*kpsMT*III	PAI
	papG alleleII	*cvaC*			*kpsMT* K1	*traT*
	papG allele III					
	papAH					
	papEF					
	focG					

*Indicates genes shared by more than one *E. coli* pathotype.
[a] Animal faecal samples were tested for these genes.

5.3. Results

5.3.1. Survey Results

The size of the tanks ranged from 5,000 to 30,000 litres and aged between one to 20 years (Appendix 6). Among the 30 tanks surveyed, 12 (40%) had overhanging trees on the roof and 18 (60%) tanks had visible sign of faecal droppings on the roof. Among the 30 tanks, 16 (53%) had first flush diverter installed, 13 (43%) treated the water before consumption and 22 (73%) were never desludged since installation (1 to 20 years). Of the 30 tanks, 24 (80%) were used for both potable and non-potable uses and the remaining 6 (20%) were used only for non-potable purposes.

5.3.2. Numbers of *E. coli* in RCR

Among the 30 rainwater tank samples tested from the rainwater tanks, 73% were found to be positive for *E. coli*. The number of *E. coli* in positive samples ranged between 2 to 986 CFU per 100 mL of water. In all, 27% tanks had < 1 CFU *E. coli* per 100 mL of water. All eight of these tanks had either first flush diverters installed (reduce the contamination level by by-passing the first 20 litres or so of rainfall) or were not characterised either by visible signs of faecal droppings or trees overhanging the roof. The number of *E. coli* exceeded that specified by Australian drinking water guidelines of 0 CFU per 100 mL for 53% of 30 samples used for drinking.

5.3.3. Phylogenetic Group Classification

Samples from the 22 rainwater tanks that were positive for *E. coli* were further tested for phylogenetic groups. Among the 22 samples, 77% and 50% contained *E. coli* belonging to phylogenetic groups A and B1, respectively. Similarly, 45% and 72% contained *E. coli* belonging to phylogenetic groups B2 and D, respectively. Of the 200 isolates tested from these 22 tanks, 32%, 16%, 22.5%, and 29.5% belonged to groups A, B1, B2 and D, respectively (Figure 10).

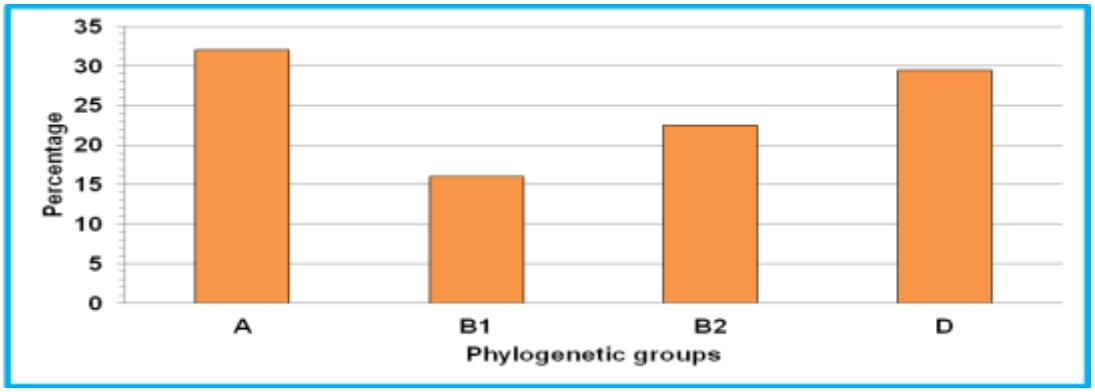


Figure 10. Distribution of *E. coli* (*n* = 200) into phylogenetic groups.

5.3.4. Occurrence of InPEC and ExPEC VGs

Among the 20 VGs tested, 10 (50%) genes were detected in 17 (77%) of the 22 rainwater samples that were positive for *E. coli*. These included *eaeA*, ST1, *cdtB*, *cvaC*, *ibeA*, *kpsMT*III, *kpsMT*K, PAI, *papAH* and *traT*. The remaining VGs *exhA*, stx_1, stx_2, *bmaE*, *focG*, *iutA*, *papG* alleleII, *papG* alleleIII, and *papEF* could not be detected in any of the 200 isolates tested from 22 rainwater tanks. *eaeA* belonging to EPEC and STEC and ST1 belonging to ETEC pathotype were detected in eight (36%) and five (23%) of 22 tanks. VGs belonged to ExPEC were detected in 15 (68%) of 22 tanks. Among the 200 isolates tested, 30 (15%) and eight (4%) were positive for *eaeA* and ST1 VGs. Among ExPEC VGs, *kpsMT*III was most prevalent (17.5%) followed by *papAH* (13.5%), *ibeA* (13%), and *traT* (12.5%) (Figure 11).

Among the 30 *eaeA* positive isolates, 18, seven, three and two belonged to phylogenetic groups B2, D, A, and B1 respectively. Similarly, of the eight ST1 positive isolates, three, two, two and one belonged to groups D, B2, B1 and A, respectively. In all, 79 isolates were positive for one or more ExPEC VGs. Of these, 56% belonged to group D and 35% belonged to B2. The remaining 6% isolates belonged to either B1 or A. Of the 96 VGs positive isolates, 42% were carrying a single VG, 37.5% were carrying two VGs, 18% were carrying three VGs, and 3% had 4 or 4+ VGs.

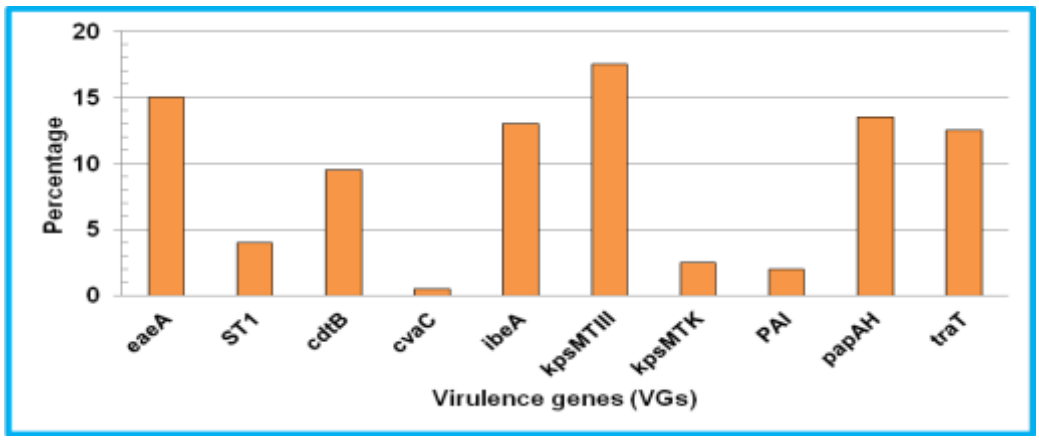


Figure 11. Occurrence of virulence genes (VGs) in *E. coli* (*n* = 200) from rainwater tanks.

5.3.5. Occurrence of InPEC and ExPEC Toxin VGs in Animal Faecal Samples

Among the 20 possum faecal samples tested, one (5%) and two (10%) were positive for *stx*$_2$ and *cdtB* genes, respectively (Figure 12). *Stx*$_1$, *exhA*, LT1, ST1 and *cvaC* genes could not be detected in any of the possum faecal samples. Among the 20 bird faecal samples, three (15%), one (5%), one (5%), and three (15%) were positive for *stx*$_2$, *stx*$_1$ ST1, and *cdtB* toxin genes, respectively. *exhA*, LT1 and *cvaC* genes could not be detected in any of the bird faecal samples tested.

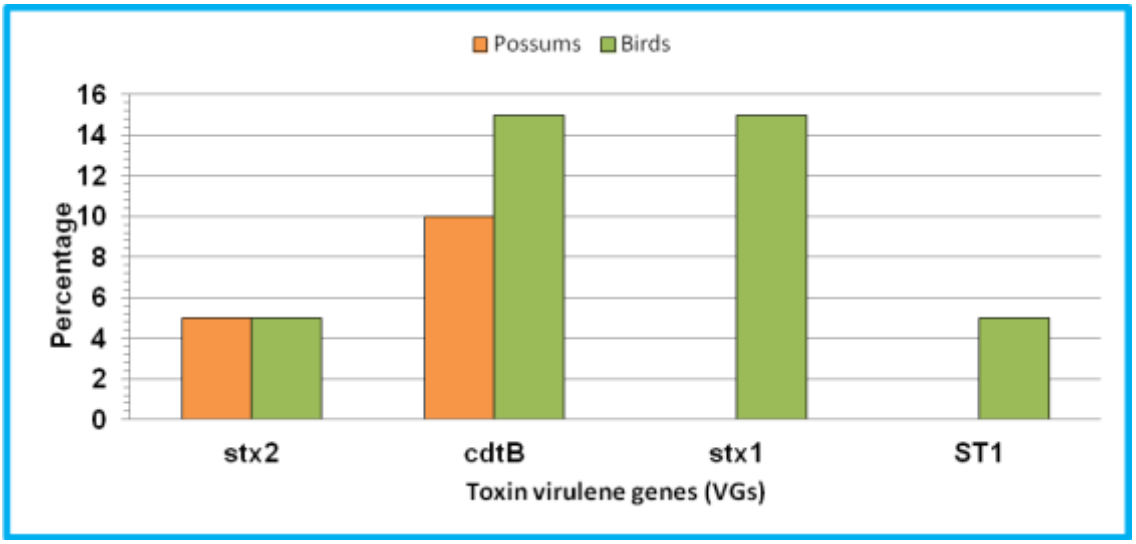


Figure 12. Occurrence of *E. coli* toxin virulence genes (VGs) in possum (*n* = 20) and bird (*n* = 20) faecal samples.

5.4. Discussion

Of the 30 rainwater samples tested, 73% exceeded the Australian drinking water guidelines of no *E. coli* per 100 mL of water (NRMMC-EPHC-NHMRC, 2008), and indicated strongly that faecal contamination had occured. It has to be noted that the occurrence of faecal contamination in most of rainwater tanks was expected in samples collected after rainfall events. Roof-run-off would have introduced to the tanks, faecal matter present on the roof or in gutters, most likely originating from birds, possums, lizards, or other animals that have access to the roof. Several other research studies reported the high detection frequencies and numbers of *E. coli* in RCR (Ahmed *et al.,* 2008; Sazakil *et al.,* 2007; Spinks *et al.,* 2006). However, to our knowledge, none of the studies in the research literature investigated the presence of InPEC and ExPEC VGs in water samples from rainwater tanks.

E. coli pathotypes such as STEC, EPEC, ETEC, ExPEC and their associated VGs were selected in this study as other studies reported the presence of these pathotypes in environmental waters originating from sewage and/or animal faeces (Gilmore and Ferretti, 2003; Hamelin *et al.,* 2006; Ram *et al.,* 2007). Overall, 30 (15%) of 200 isolates from eight tanks were positive for the *eaeA* gene which belongs to STEC or EPEC (Hamelin, 2007). However, none of the isolates was positive for *stx*$_1$ and *stx*$_2$ genes. *E. coli* O157:H7 are known to harbour *stx*$_1$, *stx*$_2$ or both toxin genes along with the *eaeA* gene (Boerling *et al.,* 1999; Mead *et al.,* 1998; Pinfold *et al.,* 1993). All *eaeA* positive strains were further tested for the presence of *bfpA* (bundle forming pilus) gene which indicates whether the strains are EPEC or not (Hamilton *et al.,* 2010). None of these strains were positive for *bfpA* gene, and therefore, these strains can be classified as atypical EPEC (aEPEC) as suggested by Kaper *et al.* (Johnson *et al.,* 2002). The role of aEPEC is not well understood in terms of pathogenesis of InPEC. It has been suggested that these strains likely derive from STEC that have lost bacteriophages carrying *stx*$_1$ or *stx*$_2$ genes or strains that have lost the *bfpA*-encoding EAF plasmid (Bielaszewska *et al.,* 2008; Kaper *et al.,* 2004).

Overall, 4% isolates from five tanks were positive for ST1 toxin gene associated with ETEC pathotype. However, none of the isolates was positive for LT1 toxin gene. ETEC strains carrying both LT and ST genes or the ST gene along with colonisation factors (CFs) which allow the organisms to readily colonise the small intestine, have been shown to cause relatively more severe disease than those carrying the LT gene alone (Qadri *et al.,* 2005). A number of ExPEC associated VGs with the potential to cause urinary tract infections (UTIs), neonatal meningitis and neonatal sepsis were also detected in this study. Amongst these genes, the *cdtB* toxin gene was detected in 19 (9.5%) isolates from seven tanks. *E. coli* carrying the *cdtB* gene are known to cause extraintestinal as well as intestinal infections (Johnson and Russo, 2002).

Furthermore, 13% isolates from nine tanks were positive for the *ibeA* gene which is associated with neonantal meningitis, endothelial cell invasion and has an essential role in UTIs (Frahm and Obst, 2003). ExPEC associated *E. coli* VGs such as *kpsMT*III, *kpsMT*K1, PAI, *papAH* and *traT* that cause UTIs or septicemia have also been detected in this study (Ishii *et al.,* 2007). However, none of the tested isolates contained complete sets of *pap* family genes. Overall, ExPEC associated genes were more prevalent than STEC or ETEC. The high prevalence of ExPEC in the environmental waters has been reported previously (Gilmore and Ferretti, 2003; Hamelin *et al.,* 2006).

In this study, STEC associated VGs stx_1 and stx_2 could not be detected, these genes have been detected in *E. coli* isolated from wild birds (Hörman *et al.,* 2004; Silva *et al.,* 2009). The presence of ST1 gene in *E. coli* isolates from pigeon/geese faeces has been reported in the literature (Ewers *et al.,* 2005; Silva *et al.,* 2009). It is acknowledged that, in this study, we did not investigate the presence of toxin genes in *E. coli* isolates from possum and bird faecal samples. However, we investigated the presence of toxin genes in total faecal DNA isolated from a small number of possum ($n = 20$) and bird ($n = 20$) faecal samples.

A number of samples were positive for stx_1, stx_2, ST1 and *cdtB* toxin genes. Ewers *et al.,* (2005) reported the presence of ExPEC associated *pap*, *ibeA*, and *traT* along with other VGs in the faeces of chickens and wild birds. Avian isolates carrying VGs are known as avian pathogenic *E. coli* (APEC). These VGs were detected in this study suggesting the presence of APEC strains in RCR tanks. The sanitary survey indicated the presence of faecal droppings (60% roofs surveyed) or overhanging trees (40% roofs surveyed) on the roof. Indeed, in this study, a number of possum and bird faecal DNA samples were positive for *E. coli* toxin VGs.

All *E. coli* isolates collected from the rainwater tank samples were also tested for the phylogenetic classification as proposed by Clermont *et al.,* (2000). In this study, most commensal *E. coli* (not carrying any VGs) belonged to group A or B1. STEC and EPEC associated isolates harbouring the *eaeA* gene belonged mainly to the B2 group. *E. coli* pathotype such as STEC also grouped within the A or B1 groups, whereas EPEC distributed across all the four groups (Escobar-Paramo *et al.,* 2004; Johnson and Oswald, 2002). Of the eight ST1 positive isolates, four belonged to group A or B1 and the remaining four belonged to group D. Most (74 isolates of 79) of the ExPEC isolates in this study belonged to either group B2 or D. The remaining five isolates belonged to either group B1 or A, although these isolates were carrying ExPEC associated VGs. It has to be noted that classification of *E. coli* into phylogenetic groups may vary and change over time due to factors such as dietary habits and better level of hygiene (Escobar-Paramo *et al.,* 2004).

PCR analysis of VGs in this study has revealed that *E. coli* isolates from rainwater tanks may carry one or more VGs. However, mere possession of a single or a few VGs does not endow a strain with pathogenic status unless that strain has acquired the appropriate combination of VGs to cause diseases. (Gilmore and Ferretti, 2003). VG acquisitions, however, can reequip such isolates with the potential to develop into a pathogen (Chapman *et al.,* 2006).

In conclusion, this is the first study that reports the presence of InPEC and ExPEC VGs associated with *E. coli* strains in rainwater tank samples in SEQ, Australia. The significance of these *E. coli* strains in causing illnesses remains unknown and needs to be assessed by comparing strains from rainwater tanks and faeces of householders drinking rainwater. In addition, the source identification of these strains is required by using microbial source tracking (MST) methods so that the magnitude of faecal contamination can be minimised.

6. TRACKING THE SOURCES OF *ESCHERICHIA COLI* HARBOURING TOXIN GENES IN RAINWATER TANK SAMPLES

6.1. Introduction

The presence of InPEC and ExPEC associated *E. coli* VGs in rainwater tanks in SEQ has been reported in the previous chapter. Of the 20 VGs tested, *eaeA*, ST1, *cdtB*, *cvaC*, *ibeA*, *kpsMT* allele III, *kpsMT* allele K1, PAI, *papAH* and *traT* were detected in *E. coli* strains from 77% of the 22 rainwater tank samples. Among the 200 *E. coli* isolates tested, 4%, 9.5%, and 0.5% strains were positive for the ST1, *cdtB*, and *cvaC* toxin genes, respectively. The local public health authority has expressed concerns in terms of the presence and possible sources of these *E. coli* in rainwater tanks.

The primary aim of this study was to identify the likely sources of *E. coli* strains harbouring toxin genes in rainwater tanks so that potential public health risks can be minimised. In our previous study (Ahmed *et al.,* 2011), 7 toxin genes (*stx*$_1$, *stx*$_2$, *hlyA*, LT1, ST1, *cdtB* and *cvaC*) were tested, and in this study, the list was extended to 10 toxin genes by incorporating 3 additional toxin genes (*ehxA*, *east1* and *cnf1*). *E. coli* isolates from bird and possum faecal samples were also tested for the toxin genes, and all strains harbouring toxin genes were **biochemically fingerprinted** and compared with those strains isolated from rainwater tanks to determine whether a causal link could be established with bird and possum faecal contamination.

6.2. Materials and Methods

6.2.1. Sources of *E. coli*

A total of 200 *E. coli* isolates were collected from 22 rainwater tanks from Brisbane and Gold Coast region in SEQ (these tanks were part of the 80 tanks and isolates mentioned in sections 2.2.1 and 5.2.4). In addition, a total of 428 *E. coli* isolates were also collected from fresh faecal droppings of 38 birds (n = 214) and 40 possums (n = 214) from Brisbane and Gold Coast region. All samples were transported to the laboratory, stored at 4°C, and processed within 24 h.

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

The membrane filtration method was used to isolate *E. coli* from rainwater samples (Ahmed *et al.,* 2011). Possum and bird faecal samples were mixed with PBS and streaked on modified mTEC agar plates for the isolation of *E. coli*. All modified mTEC agar plates were incubated at 35°C for 2 h to recover stressed cells, followed by incubation at 44°C for 22 h (US EPA, 2002). DNA was extracted from 1 mL of pure colony culture using a DNeasy Blood and Tissue Kit (Qiagen).

6.2.3. PCR Detection of InPEC and ExPEC Toxin Genes

E. coli isolates from rainwater tank, possum and bird faecal samples were tested for the presence of ten *E. coli* toxin genes associated with intestinal and extra-intestinal diseases. The list of toxin genes and the corresponding pathotypes tested in this study is shown in Table 10.

Table 10. *Escherichia coli* pathotypes and associated toxin genes.

Pathotypes	Toxin Genes	Description/Function
EHEC	*stx*$_1$	Shiga toxin I
	stx$_2$	Shiga toxin II
	hlyA [a]	α-Hemolysin
	ehxA [a]	Enterohemolysin
ETEC	LT1	Heat labile toxin 1
	ST1	Heat Stable toxin 1
EPEC	*cdtB* [a]	Cytolethal distending toxin
	ehxA [a]	Enterohemolysin
EaggEC	*east1*	EaggEC heat-stable enterotoxin
ExPEC	*cdtB* [a]	Cytolethal distending toxin
	cnf1 [a]	Cytotoxic necrotizing factor 1
	cvaC	Colicin V, conjugative plasmids
	hlyA [a]	α-Hemolysin

[a] Indicates genes shared by more than one *E. coli* pathotype.

PCR detection of uidA gene (Frahm and Obst, 2003) and toxin genes (Johnson and Stell, 2000; Paton and Paton, 2002; Ram *et al*., 2007; Yamamoto *et al*., 1996) was undertaken using previously published primers and cycling parameters.

6.2.4. Biochemical Fingerprinting

The principle of the biochemical fingerprinting method using the PhPlate system (PhPlate AB, Stockholm, Sweden) has been previously described (Ahmed *et al.,* 2005; Möllby *et al.,* 1993). This system uses quantitative measurements of the kinetics of several biochemical reactions of bacteria in microtiter plates with dehydrated substrates (Möllby *et al.,* 1993). The typing reagents used in this method are specifically chosen for different groups of bacteria to give an optimal discriminatory power and reproducibility. *E. coli* colonies were selected with sterile toothpicks directly from the agar plates and suspended into the first well of each row containing 350 µ of growth medium. Using a multichannel pipette, aliquots of 25 µl of bacterial suspension were transferred into each of other 11 wells containing 150 µl of growth medium. Plates were then incubated at 37ºC, and A_{620} was measured at 7, 24, and 48 hours using a flatbed scanner. For each bacterial isolate, it yielded a biochemical fingerprint made of several quantitative data which are used with the PhPlate software to calculate the level of similarity between the tested isolates.

6.2.5. Data Analysis

After the final reading, the mean value for all three readings was calculated for each strain to obtain a biochemical phenotype (BPT) (Kühn *et al.,* 1991). The BPTs were compared pair-wise and the resulting similarity matrix was clustered according to the unweighted pair group method (Sneath and Sokal, 1973). An identity (ID) level of 0.95 was established for the system based on testing 10 strains in duplicate. BPTs showing similarity to each other above the ID-level were regarded as identical. All data handling, including optical readings, calculations of similarities among fingerprints of strains as well as clustering and printing dendrograms, was performed using the PhPlate software version 4001 (Bactus AB, Stockholm, Sweden).

6.3. Results

6.3.1. Occurrence of InPEC and ExPEC Toxin Genes

Among the 10 toxin genes tested, four genes (ST1, *east1*, *cdtB* and *cvaC*) were detected in 43 of 200 *E. coli* strains from 59.1% of the 22 rainwater tank samples (Table 11). The remaining toxin genes

stx$_1$, *stx*$_2$, *hlyA*, *ehxA*, LT1 and *cnf1* could not be detected in any of isolates tested from any of the 22 rainwater tanks. ST1 belonging to the ETEC pathotype was detected in *E. coli* strains from 22.7% of the 22 tanks. The toxin gene *east1* belonging to the EaggEC was detected in strains from 31.8% of 22 tanks. Toxin genes *cdtB* and *cvaC* belonging to EPEC/ExPEC were detected in strains from 31.8% and 4.5% of the 22 tanks, respectively. Among the 200 isolates tested, 4%, 12.5%, 9.5%, and 0.5% strains were positive for the ST1, *east1*, *cdtB*, and *cvaC*, respectively.

Table 11. Occurrence of *Escherichia coli* harbouring toxin genes in rainwater tanks, bird and possum faecal samples.

Samples	No. of *E. coli* Tested	No. of *E. coli* Isolates Harbouring Toxin Genes (%)	Distribution of *E. coli* Harbouring Toxin Genes into Intestinal and Extra-Intestinal Pathotypes (%)			
			ETEC	EaggEC	EPEC/ExPEC	ExPEC
			ST1	*east1*	*cdtB*	*cvaC*
Rainwater tanks	200	43 (21.5)	8 (4)	25 (12.5)	19 (9.5)	1 (0.5)
Birds	214	55 (25.7)	ND	30 (14.0)	11 (5.1)	18 (8.4)
Possums	214	74 (34.6)	ND	74 (34.6)	ND	ND

ND: Not detected; ETEC: Enterotoxigenic *E. coli*; EaggEc: Enteroaggregative *E. coli*; EPEC: Enteropathogenic *E. coli*; ExPEC: Extraintestinal pathogenic *E. coli*.

6.3.2. Occurrence of InPEC and ExPEC Toxin Genes in Possum and Bird Faecal Samples

Among the 10 toxin genes tested, three genes (*east1*, *cdtB*, *cvaC*) were detected in 55 *E. coli* strains from 42% of the 38 bird faecal samples (Table 11). The remaining toxin genes could not be detected. Toxin gene *east1* (belonging to EaggEC) *cdtB* (belonging to EPEC/ExPEC) and *cvaC* (belonging to ExPEC) were detected in strains from 13.1%, 7.9% and 28.9% of 38 bird faecal samples, respectively. Among the 214 isolates tested from birds, 14%, 5.1% and 8.4% strains were harbouring *east1*, *cdtB*, and *cvaC* toxin genes, respectively. *E. coli* toxin genes were, however, less prevalent in possum faecal samples. Only *east1* toxin gene was detected in *E. coli* strains from 42.5% of the 40 possum faecal samples. The remaining toxin genes could not be detected in any of the isolates tested from possum faecal samples. Among the 214 isolates tested, 34.6% strains were harbouring the *east1* toxin gene.

6.3.3. Biochemical Fingerprinting Analysis

A number of strains from rainwater tanks (n = 43), bird (n = 55) and possum (n = 74) faecal samples harbouring toxin genes were typed with the biochemical fingerprinting method. *E. coli* strains harbouring toxin genes in rainwater tanks were more diverse (Di = 0.92) than those found in bird (Di = 0.90) and possum (Di = 0.81) faecal samples (see appendix 7, 8 & 9). Cluster analysis was used to compare the BPTs of the 43 strains from rainwater tanks with those isolated from bird and possum faecal samples. Of the 43 BPTs, 32.6% strains from seven rainwater tanks were identical to single or multiple BPTs from birds with 6 BPTs harbouring the same VGs (Table 12). Similarly, of the 43 BPTs, 20.9% strains from six rainwater tanks were identical to single or multiple BPTs of possums with 4 BPTs harbouring same VGs. Four BPTs from the 3 rainwater tanks (T42, T48 and T49) were identical to both bird and possum BPTs. In contrast, 51.2% BPTs from rainwater tanks were not identical to BPTs from either birds or possums.

Table 12. Comparison of biochemical phenotypes (BPTs) of *Escherichia coli* isolates harbouring toxin genes in rainwater tank samples, possum and bird faecal samples.

Rainwater Tanks ID	BPTs ID	Rainwater BPTs Identical to Bird and Possum BPTs		Unknown BPTs
		Bird BPTs ID	Possum BPTs ID	
T1	54 [c]			54 [c]
	56[c]			56[c]
	58[c]			58[c]
T10	7[b]	B-78[c]		
	8[b]	B-78[c]		
	9[b]			9[b]
	11[b]	B-89[c]		
T12	13[c]			13[c]
	15[c]			15[c]
	18[c]			18[c]
T15	**140**[b]	B-34 [c] **B-46**[b], **B-106** [b], B-116 [c], B-123 [c], B-164 [d]		
	148[b]			148[b]
T33	103[b,c]			103[b,c]
	105[b,c]			105[b,c]
T34	110[b,c]			110[b,c]
	112[b,c]			112[b,c]
	113[b,c]			113[b,c]
	114[b,c]			114[b,c]
	115[b,c]			115[b,c]
	116[b,c]			116[b,c]
	117[b,c]			117[b,c]
	119[b,c]			119[b,c]
T42	150[b]			150[b]
	154[a]			
	155[b]	**B-27**[b], **B-37**[b]	**P-86**[b], **P-95**[b], **P-105**[b], **P-115**[b], **P-139**[b], **P-140**[b]	
	156[b]	**B-27**[b], **B-37**[b]		
	157[b]	B-181[d]		
	158[b]	**B-27**[b], **B-37**[b]		
	159[b]			159[b]
T44	171[c,d]	B-78 [c]		
T45	**185**[b]		**P-71**[b]	
	187[b]		**P-71**[b]	
T46	198[a]		P-71[b]	
T47	**199**[b]		**P-86**[b], **P-95**[b], **P-105**[b], **P-115**[b], **P-139**[b], **P-140**[b]	
	200[a]	B-181[d]	P-71[b]	
	122[c]	**B-34**[c], **B-43**[c], B-46[b], B-106[b], **B-116**[c], **B-123**[c], B-164[d], B-169[d]		
	123[c]	**B-34**[c], **B-43**[c], B-46[b], B-106[b], **B-116** [c], **B-123**[c], B-164[d], B-169[d]		
T48	137[a]	B-24[b], B-32[b], B-40[b], B-69[b], B-72[c]	P-71[b]	
	140[c]			
T49	162[a]	B-31[b], B-101[b], B-102[b], B-182[d], B-189[d]	P-181[b], P-191[b]	
	163[a]			163[a]
	165[a]			165[a]
	167[a]		P-18[b], P-19[b]	
n = 13	*n* = 43	14/43 (32.6%)	9/43 (20.9%)	22/43(51.2%)

[a] ST1 toxin gene; [b] *east1* toxin gene; [c] *cdtB* toxin gene; [d] *cvaC* toxin gene; BPTs: biochemical phenotypes.
Identical BPTs and toxin genes found in isolates from rainwater tanks, possum and bird faecal samples are bold faced.

6.4. Discussion

A number of *E. coli* strains from rainwater tanks tested in this study were positive for InPEC and ExPEC pathotypes associated toxin genes. The toxin gene *east1* (25 strains from 7 tanks) and *cdtB* (25 strains from 7 tanks) were more prevalent among *E. coli* strains than ST1 (8 strains from 5 tanks) toxin genes. We acknowledge that in the absence of any *in vivo* study, it was not possible to determine whether strains harbouring toxin genes in rainwater tanks were in fact capable of expressing themselves and because of that these strains should only be considered as potential pathogenic strains.

Among the 214 isolates tested from the 38 bird faecal samples, 14%, 5.1% and 8.4% strains were indeed harbouring *east1*, *cdtB*, and *cvaC* toxin genes, respectively. Toxin gene *east1* was detected only in *E. coli* strains from 17 of the 40 possum faecal samples. Among the 214 isolates tested, 34.6% strains were harbouring the *east1* toxin gene. Although, toxin genes stx_1, stx_2, *hlyA*, *ehxA*, LT1 and *cnf1* could not be detected in any of the tested isolates from rainwater tanks and animal faecal samples (birds and possums), these toxin genes have been detected in *E. coli* isolated from avian (Hughes *et al.,* 2009; Rodriguez-Siek *et al.,* 2005; Silva *et al.,* 2009). The presence of ST1 gene in *E. coli* isolates from pigeon/geese faeces has been reported by others (Silva *et al.,* 2009; Ewers *et al.,* 2005). The presence of stx_1, stx_2, and ST1 in total DNA isolated from bird and possum faecal samples has also been reported (Ahmed *et al.,* 2011).

The biochemical fingerprinting method used in this study has a high discriminatory ability and reproducibility and is shown to be comparable with genotypic methods in comparative studies (Kühn *et al.,* 1995; Kühn *et al.,* 1997). This method has been successfully used to identify the sources of *E. coli* and *Enterococcus* spp. in environmental waters in SEQ, Australia (Ahmed *et al.,* 2005). In this study, it was postulated that BPTs of *E. coli* strains from bird and possum faecal samples and rainwater samples harbouring clinically significant toxin genes can be compared with each other to identify the likely sources of these strains in rainwater tank samples. One important feature of such an approach is that the analysis is focused on strains carrying toxin genes rather than commensal *E. coli* of little public health significance. In addition, identical BPTs along with the presence of single or multiple toxin genes in two or more compared strains can increase the confidence level that the sources in rainwater tanks may have been correctly identified.

Of the 43 strains from rainwater tank samples, 14 (from 7 tanks) and 9 (from 6 tanks) had identical BPTs to those found in bird and possum faecal samples, respectively. Five strains from 4 rainwater tanks were identical to those isolated from both bird and possum faecal samples. The remaining 22 strains could not be identified. This may be due to the fact that the number of bird and possum faecal samples tested in this study did not capture the diversity of *E. coli* in rainwater tanks. This is partially supported by the fact that the *E. coli* BPTs were more diverse in rainwater tanks compared to those found in bird and possum faecal samples (Appendix 7, 8 and 9). It is also possible that a portion of these unidentified BPTs might have originated from other sources such as rats, lizards, frogs or fruit bats which were not tested in this study. A recent study also reported the presence of *E. coli* and pathogenic microorganisms in both airborne particulate matter and in water samples from rainwater tank in the tropical atmosphere in Singapore, which may account for another potential source of unidentified BPTs in rainwater tanks in sub-tropical SEQ (Kaushik *et al.,* 2011). While 9 strains from 4 rainwater tanks (T15, T42, T45, and T47) had identical BPTs and similar toxin genes to those isolated from bird and possum faecal samples suggesting these animals may be the likely sources of these strains in rainwater tanks. Other strains isolated from rainwater tanks had identical BPTs to possum or birds isolates but were carrying different toxin genes. For example, strains from T10 were harbouring *east1* toxin gene which had identical BPTs to strains from birds which were harbouring *cdtB* toxin gene. This could be due to the fact that some toxin genes are carried on plasmid and therefore, these genes can be lost or gained. It is also possible that strains carrying similar VGs have different biochemical fingerprints.

The rainwater tanks were also surveyed for physical characteristics. Of the 13 tanks that contained *E. coli* harbouring toxin genes, 12 tanks had either visible faecal droppings on the roof or overhanging trees or the both (Appendix 10). It has been suggested that rainwater tanks should be appropriately maintained, including cleaning the roofs and gutters periodically, while the receiving tanks should be

cleaned at least two times per year to improve the quality of water (Cuniffe, 1998). Our MST data suggest the presence of potential clinically significant *E. coli* in rainwater tanks may have been originated from bird and possum faeces. Combination of toxin *E. coli* toxin gene analysis and fingerprinting technique has the potential to pinpoint the sources of faecal contamination. The results of this study also highlight the need for good maintenance of roof and gutter and elimination of overhanging tree branches to minimise potential public health risks.

7. CHARACTERISATION OF *ENTEROCOCCUS* SPP. IN RAINWATER TANK SAMPLES

7.1. Introduction

Enterococcus spp. are widely accepted as FIB to determine the microbiological quality of environmental waters (US EPA, 1986). This group of bacteria is commonly found in the gastrointestinal tracts of warm-blooded animals including humans (Harwood *et al.,* 2004; Lanthier *et al.,* 2010). The presence of *Enterococcus* in environmental waters indicates the occurrence of faecal contamination from animal or human associated wastewater. Although *Enterococcus* has been used as FIB, certain *Enterococcus* spp. can potentially be pathogenic and have become one of the primary causes of urinary tract infections, bacteremia and hospital-acquired infection (Fisher and Phillips, 2009; Kayser *et al.,* 2003). Among the 19 species, *Enterococcus faecalis* and *Enterococcus faecium* are highly prevalent in human wastewater and among clinical isolates (Gelsomino *et al.,* 2003; Manero *et al.,* 2002). Other *Enterococcus* spp. such as *Enterococcus durans*, *Enterococcus avium*, *Enterococcus gallinarum* and *Enterococcus casseliflavus* are also reported to be associated with various human infections, however, at a lesser extent than *E. faecalis* and *E. faecium* (Moellering *et al.,* 1992). Treatment of *Enterococcus* spp. related infections has been further complicated due to their high level of intrinsic and acquired antibiotic resistance (Kainer *et al.,* 2007; Shankar *et al.,* 2002).

Several VGs have been identified in particular with *E. faecalis* associated infection (Gilmore *et al.,* 1994; Qin *et al.,* 2001; Shankar *et al.,* 2001) and their effects have been demonstrated in animal models and cultured cells (Huycke *et al.,* 1992; Kreft *et al.,* 1992; Olmsted *et al.,* 1994). For example, the *cyl* gene which is involved in the production of cytolysin, lyses a broad range of eukaryotic and prokaryotic cells and it reported to enhance the virulence factors of *E. faecalis* in animal models (Jett *et al.,* 1992; Chow *et al.,* 1993). The aggregation substance (AS) gene is a surface protein that is encoded by sex pheromone plasmids and promotes donor and recipient cell aggregation, leading to conjugative transfer of plasmids (Waters and Dunny, 2001). Other surface proteins such as *ace* (collagen-binding protein), *efaA* (a cell surface protein associated with endocarditis strains), *esp* (an enterococcus surface protein involved in adhesion) have been described. The role of these genes, however, in the pathogenesis of *Enterococcus* spp. is not clearly understood. These genes are assumed to be involved in mechanisms by which the *Enterococcus* cells adhere to biotic and abiotic surfaces and in biofilm formation (Shankar *et al.,* 2001; Lowe *et al.,* 1995). Another possible virulence factor is *gelE* gene (a metalloproteinase that targets biomolecules) which is generally associated with endocarditis strains (Singh *et al.,* 1998).

E. coli has traditionally been used as indicator of faecal contamination in rainwater tanks (Ahmed *et al.,* 2008; Sazakil *et al.,* 2007; Spinks *et al.,* 2006). A recent study, however, reported that *E. coli* may be of limited use to perform through assessment on the microbial quality of rainwater tank samples due to the fact that a number of samples yielded culturable *Enterococcus* spp., but no *E. coli* (Ahmed *et al.,* 2010). The authors suggested that rainwater tank samples should be tested for multiple indicators (where possible) to obtain multiple lines of evidence on the occurrence of faecal contamination (Ahmed *et al.,* 2010; Ahmed *et al.,* 2011). Several studies also reported that *Enterococcus* spp. is more prevalent in rainwater tank samples than *E. coli* (Ahmed *et al.,* 2008; Spinks *et al.,* 2006; CRC, 2006) and thus may be a better indicator for assessing faecal contamination.

Despite increasing evidence that *Enterococcus* is more prevalent and may be a better indicator for assessing faecal contamination in rainwater tanks, none of the studies has characterised *Enterococcus* spp. in rainwater tank samples. In this present study, a collection of *Enterococcus* isolates were obtained from rainwater tank samples in SEQ, Australia. The distribution of these *Enterococcus* isolates into the species level and the occurrence of six VGs within the collection were determined. This was done to obtain information on the source and ecology of *Enterococcus* spp. isolated from rainwater tank samples.

7.2. Materials and Methods

7.2.1. Survey of Rainwater Tanks

In all, 27 rainwater tanks were selected for this study representing 7 suburbs in Brisbane and the Gold Coast region in SEQ, Australia (these tanks were selected randomly and were part of the 80 tanks as mentioned in section 2.2.1.) These tanks were located in peri-urban and urban areas and were selected on the basis of the end uses. Among the 27 tanks, 20 were used for both potable and non-potable uses and the remaining seven were used for non-potable purposes. A sanitary survey was undertaken to identify physical characteristics of the rainwater tank systems such as size of the tanks, age of the tanks, tank material and factors that may contribute to the faecal contamination of the tanks such as the presence of overhanging trees on the roof. The roofs were also surveyed for the presence of possible wild life faecal contamination.

7.2.2. Water Sampling

A single water sample was collected from each rainwater tank, within three to seven days after a major rain event (> 80 mm). Water samples were collected in sterilised containers from the outlet taps located close to the base of the tanks. Before the tank was sampled, the tap was wiped with 70% ethanol and allowed to run for 30 to 60 s to flush water from the tap. Samples were transported to the laboratory and processed within 2-4 h.

7.2.3. Enumeration and Isolation of *Enterococcus* spp.

The membrane filtration method was used to process the water samples for *Enterococcus* enumeration. Sample serial dilutions were made with phosphate buffer saline (PBS) and filtered through 0.45 µm pore sized (47 mm diameter) nitrocellulose membranes (Millipore, Tokyo, Japan), and placed on membrane-enterococcus indoxyl-β-D-glucoside (mEI) agar (Difco, Detroit, MI) for the isolation of *Enterococcus*. Agar plates were incubated at 41ºC for 48 h (US EPA, 1997).

7.2.4. DNA Extraction

Up to 10 *Enterococcus* isolates were selected from replicate agar plates and purified. This gave a total number of 212 *Enterococcus* isolates from 23 rainwater tank samples. *Enterococcus* could not be isolated from the remaining 4 tanks. DNA was extracted from 1 ml of pure colony culture using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA).

7.2.5. PCR Speciation and Detection of Virulence Genes

Enterococcus isolates were identified to the species level using PCR according to the method described elsewhere (Jackson *et al.,* 2004). *Enterococcus* spp. tested in this study included *E. faecalis*, *E. faecium*, *E. casseiliflavus*, *E. durans*, *E. hirae*, *E. avium* and *E. mundtii*. All isolates were further tested for the presence of 6 *Enterococcus* associated VGs namely AS, *ace*, *gelE*, *efaA*, *esp*, and *cylA*. PCR speciation (Jackson *et al.,* 2004) and detection of VGs (Mannu *et al.,* 2003; Shankar *et al.,* 1999; Vankerckhoven *et al.,* 2004) were undertaken using previously published primers (Appendix 11).

7.3. Results

7.3.1. Survey Results

The size of the tanks ranged from 5,000 to 30,000 litres and aged between 1 to 5 years (Appendix 12). Among the 27 tanks surveyed, 37% had overhanging trees and 52% tanks had visible sign of faecal droppings on the roof. Among the 27 tanks, 52% had first flush diverter installed, 33% treated the water before consumption, and 74% were never desludged since installation (1 to 5 years). Among the 27 tanks, 74% were used for both potable and non-potable uses and the remaining 26% were used for only non-potable purposes.

7.3.2. Numbers of *Enterococcus* spp. in Rainwater Tank Samples

Among the 27 rainwater tank samples tested, 85% had culturable *Enterococcus* spp. The numbers of *Enterococcus* in these samples ranged between 2 to 450 CFU per 100 mL of water. Eight (35%) tanks had < 10 CFU *Enterococcus* per 100 mL of water (Appendix 13). All these tanks had either first flush diverters installed or were not characterised either by visible signs of faecal droppings or trees overhanging the roof.

7.3.3. Distribution of *Enterococcus* spp. to the Species Level in Rainwater Tank Samples

E. faecalis and *E. mundtii* were the most prevalent in rainwater tank samples (Figure 13). Among the 23 rainwater tank samples, 87% and 44% samples yielded *E. faecalis* and *E. mundtii*, respectively. *E. casseiliflavus* (30% tanks), *E. faecium* (22% tanks) and *E. hirae* (17%) were more prevalent than *E. avium* (9% tanks) and *E. durans* (4% tanks). Among the 23 rainwater tank samples, 26% samples yielded at least one of seven *Enterococcus* spp. tested in this study. The remaining 8 (2 *Enterococcus* spp., 35%), 8 (3 *Enterococcus* spp., 35%), and 1 (4 *Enterococcus* spp., 4%) samples yielded multiple *Enterococcus* spp. Among the 212 *Enterococcus* isolates from 23 rainwater tanks, 93% were identified to the species level and the remaining 7% isolates could not be identified.

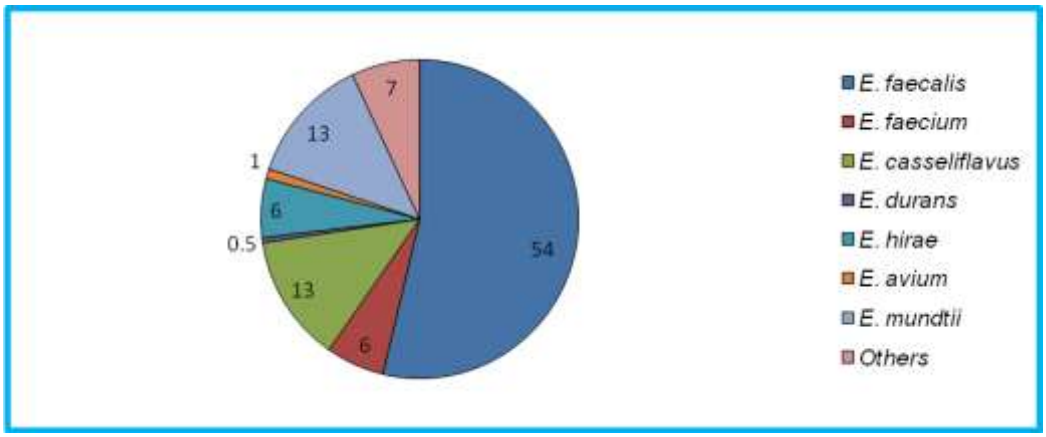


Figure 13. Distribution of *Enterococcus* spp. (*n* = 212) isolated from rainwater tanks into the species level.

7.3.4. Occurrence of *Enterococcus* spp. Associated Virulence Genes

All six VGs were detected in 87% of the 23 rainwater tank samples that yielded culturable *Enterococcus* isolates. VGs *gelE* and *efaA* were most prevalent; detected in 83% and 78% of 23 samples, respectively. VG ace was also detected in 61% samples followed by *AS*, *esp* and *cylA* genes which were detected in 13%, 9% and 4% samples, respectively (Figure 14). Among the 212 *Enterococcus* isolates tested from 23 rainwater tank samples, 57% *Enterococcus* isolates from 20 tanks were harbouring one or multiple VGs. Of the 20 tank water samples, *Enterococcus* from 25% samples were harbouring a single VG, 65% samples were harbouring 2 VGs, 85% samples were harbouring 3 VGs and 5% sample were harbouring 4 VGs. The VGs were mostly detected in *E. faecalis* strains. Among the 115 *E. faecalis* strains, 97%, 96%, 50%, 10%, 4% and 2% were harbouring *gelE*, *efaA*, *ace*, AS, *esp* and *cylA* genes. One *E. casseliflavus* and one *E. mundtii* strains were harbouring the *esp* and *gelE* genes. *E. faecium*, *E. durans*, *E. avium*, and unidentified strains did not harbour any VGs tested.

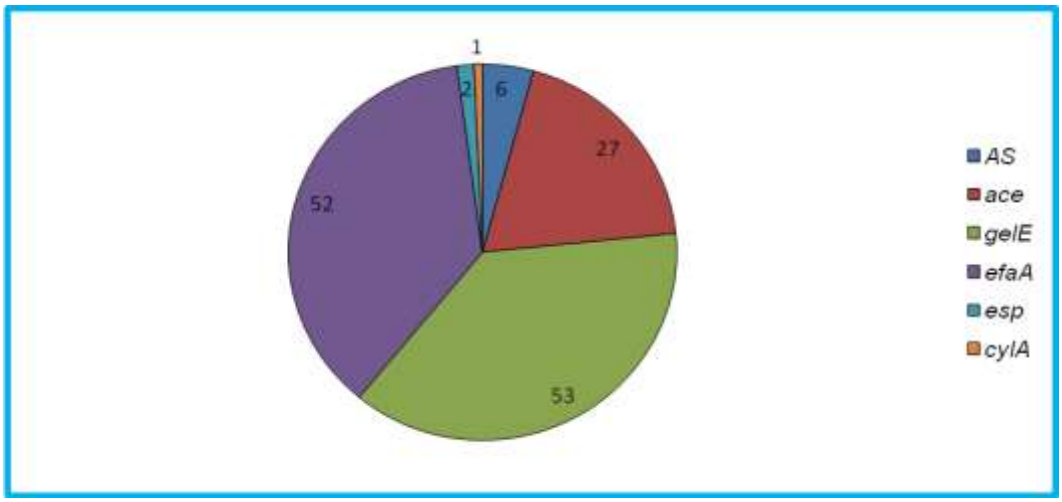


Figure 14. Occurrence of virulence genes (VGs) in *Enterococcus* spp. (*n* = 212) isolated from rainwater tanks.

7.4. Discussion

In this study, *Enterococcus* isolates were identified to the species level to obtain information on their potential sources and ecology in rainwater tank samples in SEQ, Australia. Among the 212 isolates from the 23 rainwater tanks, the predominant *Enterococcus* spp. identified was *E. faecalis*, followed by *E. mundtii*, *E. casseilaflavus* and *E. faecium*. The presence of high numbers of *E. faecalis* (20 out of 23 tanks) and a lesser extent *E. faecium* (5 out of 23 tanks) in rainwater tanks suggests the presence of faecal strains due to their high prevalence in warm blooded animals (Willey *et al.,* 1999). It has been reported that *E. faecalis* and *E. faecium* are predominant in human faeces and because of that, several authors have proposed these two species as potential candidate for microbial source tracking (MST) studies in environmental waters (Harwood *et al.,* 2004; Wheeler *et al.,* 2002; Kuntz *et al.,* 2004).

The presence of *E. faecalis* in rainwater tank samples suggests faecal contamination from wildlife or other non-point sources as the chance of human wastewater contaminating rainwater tanks is minimal. The high prevalence of *E. faecalis* in rainwater tank samples, however, suggests that these two species may be ubiquitous in nature or perhaps *E. faecalis* may not have limited host specificity as previously reported (Wheeler *et al.,* 2002) *E. faecalis* are reported to be present in the faeces of nonhuman animals including wildlife (Aarestrup *et al.,* 2002; Devriese *et al.*,1987; Layton *et al.,* 2010). During the sanitary survey, we identified wild animals such as birds, possums and reptiles are the most likely sources of faecal contamination in rainwater tanks in SEQ.

The presence of *E. faecalis* and *E. faecium* in bird faecal samples has been reported (Middleton *et al.,* 2005). Among the 40 bird faecal samples tested, 15% and 25% samples carried *E. faecalis* and *E. faecium*, respectively. Other research studies have also reported the presence of *E. faecalis* in owl, seagulls and pelicans (Kuntz *et al.,* 2004; Proucher *et al.,* 1991). The species distribution of *Enterococcus* in possum faecal samples is not known. In this study, we did not speciate the *Enterococcus* spp. isolated from possum and bird faecal samples. However, we investigated the presence of *E. faecalis* and *E. faecium* in total faecal DNA isolated from a small number of possum (*n* = 20) and bird (*n* = 20) faecal samples. Among the 20 possum faecal samples tested, 3 (15%) and 2 (10%) were positive for the *E. faecalis* and *E. faecium*, respectively. Similarly, among the 20 bird faecal samples, 7 (35%) and 6 (30%) were positive for the *E. faecalis* and *E. faecium*, respectively. The prevalence of *E. faecalis* and *E. faecium* in possum and bird faecal samples indicating these animals may have contributed *Enterococcus* in rainwater tanks. It is also possible that a portion of these *E. faecalis* and *E. faecium* might have originated from other sources such as rats, lizards, frogs or fruit bats which were not tested in this study. A recent study also reported the presence of *E. coli* and

pathogenic microorganisms in airborne particulate matter and in water samples from rainwater tank in the tropical atmosphere in Singapore, which may account for another potential source of *Enterococcus* in rainwater tanks in sub-tropical SEQ (Kaushik and Balasubramanian, 2011). The presence of *E. mundtii* and *E. casseiliflavus* in a number of rainwater tanks (n = 10; *E. mundtii* and n = 7; *E. casseiliflavus*) samples is not unexpected due to their documented association with plants, soil, and non-human animal hosts (Pinto *et al.,* 1999). In this study, these environmental associated species comprised 26% of all isolates tested and were detected along with *E. faecalis* and *E. faecium* in most of the tanks. Such results indicate the importance of identification of *Enterococcus* into species level as FIB.

VGs *gelE* and *efaA* were most prevalent in rainwater tank samples followed by *ace*. Among the 23 tanks, 83%, 78% and 61% were positive for *gelE*, *efaA* and *ace*, respectively. Other VGs AS, *esp* and *cylA* were detected in small number of tanks. The tested VGs were mostly detected in *E. faecalis* strains. Among the 115 *E. faecalis* strains, 97%, 96%, 50%, 10%, 4% and 2% were harbouring *gelE*, *efaA*, *ace*, AS, *esp* and *cylA* genes. The other *Enterococcus* spp. were negative for the VGs tested except one *E. casseliflavus* and one *E. mundtii* strains were positive for the *esp* and *gelE* genes, respectively. Our findings are in accordance with previous studies that *E. faecalis* strains harbour most of the VGs (Eaton *et al.,* 2001) where as other species carried a few VGs (Mannu *et al.,* 2003). The high prevalence of VGs *gelE*, *efaA*, in *E. faecalis* in rainwater tank samples indicates that some of these strains can be clinically significant (Eaton *et al.,* 2001) since these VGs have been reported to be associated with clinical *E. faecalis* isolates (Eaton *et al.,* 2001; Coque *et al.,* 1995; Nallapareddy *et al.,* 2002). We acknowledge that in the absence of *in vivo* study, it was not possible to determine whether strains harbouring VGs were in fact capable of expressing themselves and because of that these strains can be considered as potential pathogenic strains. A recent study reported the widespread presence of *cylA*, *efaA*, and *gelE* genes in domesticated mammals and birds suggesting possum and bird faeces may have contributed these *E. faecalis* in rainwater tanks. The rainwater tanks that contained *E. faecalis* harbouring VGs were surveyed. Of the 19 tanks, 14 tanks had either visible faecal droppings on the roof or overhanging trees. It has been suggested that rainwater tanks should be appropriately maintained, including ensuring the cleanliness of the roofs and gutters periodically, while the receiving tanks should be cleaned at least two times per year to improve the quality of water. Such results demonstrate the need for good maintenance of roof and gutter and elimination of overhanging tree branches to protect potential public health risks.

In conclusion, this is the first study that reports the distribution and as well as the occurrence of VGs of *Enterococcus* spp. isolated from rainwater tanks in SEQ. The high prevalence of *E. faecalis* and associated VGs may pose a health risk mainly to users who use rainwater for drinking. The significance of these strains in terms of health implications needs to be assessed by further characterising them for multiple drug resistance. The potential sources of these strains need to be identified using MST technique for the better management of water quality It is recommended that *Enterococcus* spp. should be used as additional FIB in conjunction with *E. coli* for the microbiological assessment of rainwater tanks.

8. CONCLUSIONS

- Rainwater tanks are increasingly being used in urban settings for restricted uses to save mains water (about 40 to 50 kL/hh/yd in SEQ). Their adoption is encouraged by regulatory sustainability requirements in many States. In addition, there are many Australians (peri urban and rural) who use rainwater as their exclusive potable water source. Further substantial gains in mains water savings could be achieved if rain water uses could be extended to bathroom uses such as hot water and possibly cold water showering. However, a major regulatory concern is whether expanded rainwater use for internal purposes is safe from a public health perspective. The evidence is ambiguous because the most credible epidemiological study from SA suggests no increased health risk of gastroenteritis by ingesting rainwater as compared to mains water, yet the literature is full of examples of moderately serious diseases outbreaks that seem to point to consumption of contaminated rainwater as the cause. Our study is the most comprehensive ever undertaken to characterise the incidence of faecal indicator bacteria (FIB) and zoonotic pathogens in domestic rainwater tanks. We also studied the survival of FIB from roof to tank, as well as the effectiveness of under sink filters in removing FIB in kitchen taps supplied by rainwater. Moreover, we measured the incidence of virulence genes (VGs) in *E.coli* isolates and the results indicated the presence of clinically significant *E. coli* strains in rainwater tanks.
- In this study, 80 rainwater tank samples were tested for FIB in a mixture of urban and peri urban settings. Most tank water samples exceeded the Australian Drinking Water Guideline for potable use. Certain tank water samples also contained pathogenic bacteria and protozoa at levels that should cause health concerns.
- An attempt was taken to identify the sources of the FIB and pathogens by measuring their numbers in possums and bird faecal samples as well as using microbial source tracking based on detection of VGs in *E coli* and undertaking biochemical fingerprinting. Overall, the evidence strongly indicated birds and possums as the contaminating sources but there are still many fingerprints (> 50% of isolates) that could not be identified. Perhaps the strains used were not diverse, as *E. coli* from frogs, lizards and flying foxes were not included in the analysis.
- The presence of VGs in *E coli* isolated from rainwater tank samples was also measured. The results indicated a high incidence of intestinal and extraintestinal VGs genes which could cause severe clinical disease symptoms. However, no such health incidences have been reported in the households of study collaborators. The significance of these *E. coli* strains in causing illnesses remains unknown and needs to be assessed by comparing strains from rainwater tanks and faeces of householders drinking rainwater.
- We looked at paired roof captured rainwater and connected household tap water samples and found high numbers of FIB as well as bacterial and protozoa pathogens irrespective of treatment using under sink filtration units. Clearly the disinfection methods used appear not to be effective. However, again we found no reported heath effects not withstanding the infective dose of pathogens present.
- Absence of adverse heath effects could have a range of explanations (acquired immunity, mild symptoms, non-reporting of illnesses etc) but it is possible that PCR method may have detected non-viable cells, a widely acknowledged limitation of the qPCR technique. Hence, we are still unresolved as to the real health effects of tanks notwithstanding the pathogen numbers and the presence of *E. coli* VG evidence.
- Based on the evidence of animal faecal sources, we explored the survival of FIB on a “model” roof, gutter and tank system. As expected, FIB die off (as measured by the T_{90} value) occurred quickly on unshaded roofs, but extended to days/weeks for shaded roofs, gutters with organic debris, and in the tank water. The results suggest that rapid die off mechanisms are not occurring and therefore do not provide adequate protection for the householders.
- As it stands, our evidence cannot recommend the extension of rainwater for other potable substitution uses such as drinking and showering unless there is an effective disinfection process in place.
- We agree with common sense roof hygiene practices such as cleaning gutters, first flush devices and reducing roof attractiveness for birds/possums, but believe it is misleading to suggest that these will have a significant effect in reducing FIB and pathogen numbers in rainwater tanks.

- Overall, the study is one of the most comprehensive and sophisticated reported in the literature to date. But even then, it does not unambiguously answer the relative health risks of ingesting rainwater via various end uses, including drinking. Further research work to resolve the apparent nil health effects from epidemiological studies and pathogen measurements from this study will need to encompass much more time consuming methods to see if PCR and VG data does translate into increased disease burden.

9. RECOMMENDATIONS

- It is recommended that roof captured rainwater should be disinfected using effective treatment procedures such as filtration, ultraviolet disinfection or simply boiling prior drinking.
- Maintenance of good roof and gutter hygiene and elimination of overhanging tree branches and other mounted structures where possible to prevent the flocking of possums and birds should be considered to minimise faecal contamination on the roof and in the gutter.
- During dry periods, faecal matter or other organic debris are deposited and accumulated on the roof and in the gutter. Exposure to harsh meteorological conditions (ultraviolet light, extreme heat, desiccation) on the rooftop would inactivate most of the bacteria. However, some will be transported to the tank depending on the climatic conditions.
- The significance of InPEC and ExPEC virulence genes (VGs) associated with *E. coli* strains in terms of health implications needs to be assessed by comparing strains from rainwater tanks and faeces of householders drinking rainwater. In addition, better characterisation of these strains is required by serotyping, genotyping or testing for multiple drug resistance from rainwater and stools from the householders. The significance of *Enterococcus* spp. strains harbouring VGs in terms of health implications needs to be assessed by further fingerprinting them. The potential sources of these strains need to be identified for the better management of water quality.
- It is recommended that *Enterococcus* spp. should be used as additional faecal indicator bacteria (FIB) in conjunction with *E. coli* for the microbiological assessment of rainwater tanks.
- In addition, better characterisation of these strains is required by serotyping, genotyping or testing for multiple drug resistance from rainwater and stools from the householders.

APPENDIX 1. List of Suburbs

1. Currumbin Ecovillage
2. Bridgeman downs
3. East Mt. Gravatt
4. Ashgrove
5. Eaton Hill
6. The Gap
7. Holland Park
8. Beaudesert
9. Versdale
10. Gleneagle
11. St. Lucia
12. Yeronga
13. Coochiemudlo Island
14. Yeerongpilly
15. Brookefield
16. Marburg
17. West Mount Cotton
18. Eastern Heights
19. Tingalpa
20. Algester
21. West End
22. Kenmore
23. McDowell
24. Annerley
25. Mitchelton
26. Coorparoo
27. New Market
28. Bellbird Park
29. Sherwood
30. Indooroopilly
31. Auchenflower
32. Graceville
33. Jamboree Heights, and
34. Albany Creek

APPENDIX 2. Primers, Probes and Cycling Parameters

Target	Primer Sequence (5'-3')	Amplicon Size	Reference
Campylobacter spp. 16S rRNA	F : CACGTG CTA CAA TGG CAT AT R: GGC TTC ATG CTC TCG AGT T P: FAM-CAG AGAA CAA TCC GAA CTG GGA CA-BHQ1	108	Lund *et al.* 2004
Salmonella invA gene	F: ACA GTG CTC GTT TAC GAC CTGAAT R: AGA CGA CTG GTA CTGATC GAT AAT	244	Chiu and Ou 1996
Giardia lamblia β-giardin gene	F: CAT AAC GAC GCCATCGCGGCTCTCAGGAA R: TTT GTG AGC GCT TCT GTC GTG GCA GCG CTAA P : FAM-AGC TCA ACG AGA AGG TCG CAG AGG GCTT-TAMRA	218	Mahbubani *et al.* 1992
Cryptosporidium oocyst wall protein (COWP) gene.	F: CAAATTGATACCGTTTGTCCTTCTG R: GGCATGTCGATTCTAATTCAGCT P: HEX-TGC CAT ACA TTG TTG TCC TGA CAA ATT GAA T-BHQ1	150	Guy *et al.* 2003

F: Forward primer; R: Reverse primer; P: Probe

APPENDIX 3. Survey Results of Roof-Captured Rainwater Tanks

Household ID	Size of the Tank (L)	Age of the Tanks (yrs)	Overhanging Trees (Y/N)	TV Aerials (Y/N)	Wild Life Faecal Droppings on the Roof (Y/N)	First Flush Diverters Installed (Y/N)	Treatment Before Drinking (Y/N)	Types of Treatment Methods Used
H1	20,000	2	N	N	Y	Y	N	-
H2	20,000	5	N	N	N	N	N	-
H3	22,500	2	N	N	N	Y	Y	USF
H4	22,500	1	N	N	N	Y	N	-
H5	22,500	2	N	N	N	Y	N	-
H6	20,000	1	N	Y	Y	Y	N	-
H7	20,000	2	N	N	Y	Y	N	-
H8	30,000	1	N	N	N	Y	Y	USF
H9	20,000	1	N	N	Y	Y	Y	USF
H10	22,500	3	Y	N	N	Y	N	-
H11	20,000	2	N	N	N	Y	Y	USF
H12	22,000	1	Y	N	N	Y	Y	USF
H13	10,000	2	Y	N	N	Y	Y	UV and USF
H14	20,000	2	N	N	N	Y	N	-
H15	15,000	3	N	N	Y	N	Y	USF
H16	15,000	1	N	N	N	Y	N	-
H17	10,000	3	N	N	Y	Y	N	-
H18	7,200	2	N	N	Y	Y	Y	USF
H19	20,000	3	N	N	N	Y	N	-
H20	20,000	2	N	N	N	Y	N	-
H23	22,000	2	Y	N	N	Y	Y	USF
H25	20,000	3	N	N	N	N	N	-
H29	20,000	1	N	Y	N	Y	N	-
H35	18,000	2	N	N	N	Y	Y	USF

USF: under sink filtration

APPENDIX 4. Average Meteorolgical Data During Inactivation Experiments

Meteorological Data	Experiments				
	Roof		Gutter		Tank water[d]
	Sunlight[a]	Shaded[b]	Clean[c]	Dirty[c]	
Temperature minimum (ºC)	15.5	18.0	16.2	11.6	15.6
Temperature maximum (ºC)	25.3	26.6	24.9	20.4	30.2
Rainfall (mm)	3.40	7.40	0.40	0.40	14.6
Evaporation (mm)	5.40	4.06	3.32	3.32	4.12
Relative humidity (%)	74.5	79.3	77.2	77.2	65.3
Wind speed (km/h)	34.5	23.6	21.6	21.6	22.1
Solar exposure (MJ m^{-2})	16.9	12.6	10.3	10.3	12.3

[a]12-04-2012
[b]17-04-2012 to 20-04-2012
[c]23-04-2012 to 24-04-2012
[d]19-1—2011 to 25-11-2011

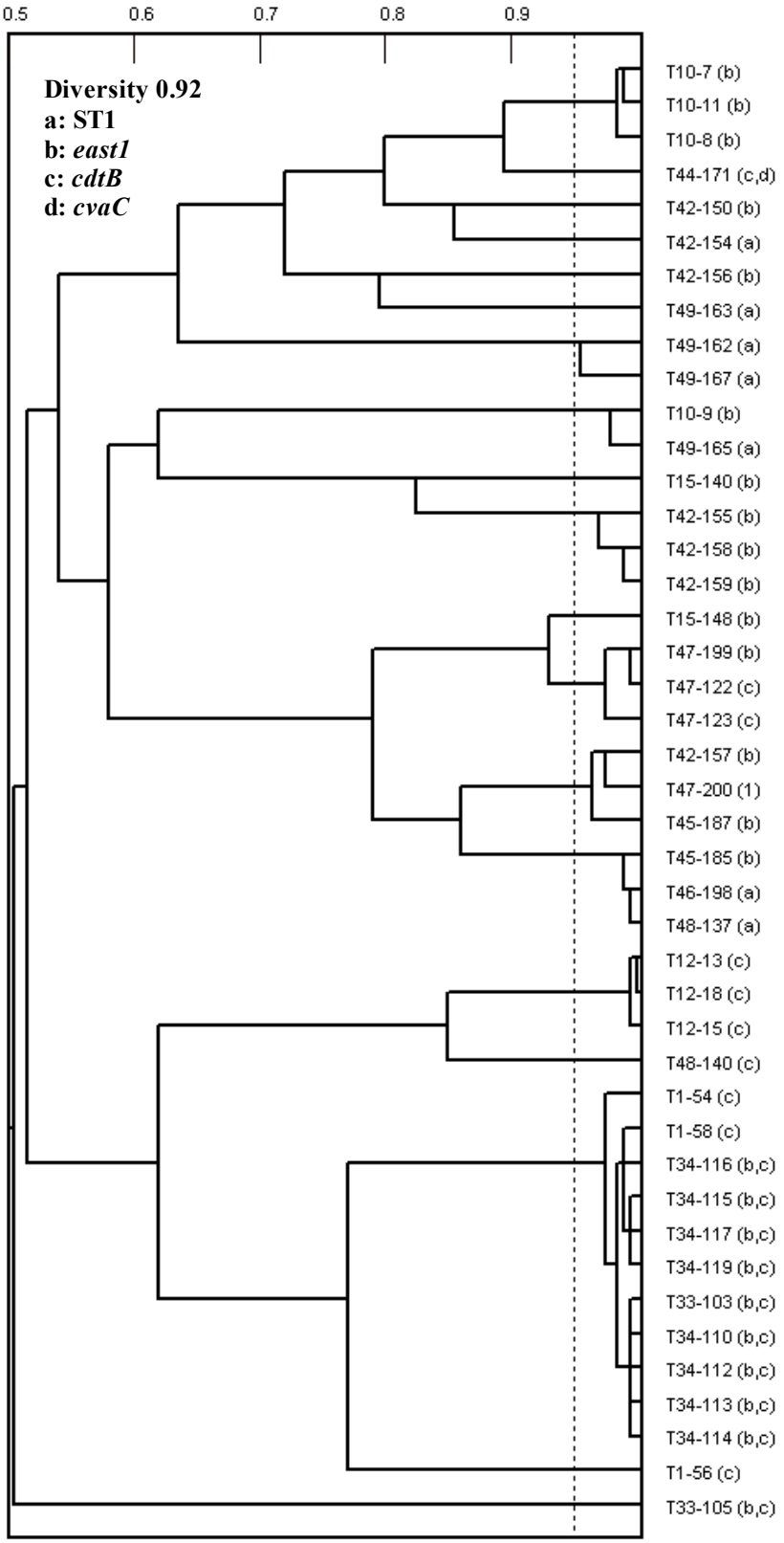
APPENDIX 5. Primers Used to Detect Virulence Genes (VGS)

Virulence Genes (VGs)	Primers	Amplicon Size	Reference
eaeA	F: GAC CCG GCA CAA GCA TAA GC R: CCA CCT GCA GCA ACA AGA GG	384	Paton and Paton 2002
stx_1	F: ATA AAT CGC CAT TCG TTG ACT AC R: AGA ACG CCC ACT GAG ATC ATC	180	Paton and Paton 2002
stx_2	F: GGC ACT GTC TGA AAC TGC TCC R:TCG CCA GTT ATC TGA CAT TCTG	255	Paton and Paton 2002
hlyA	F: GCA TCA TCA AGC GTA CGT TCC R: AAT GAG CCA AGC TGG TTA AGCT	534	Paton and Paton 2002
LT1	F: TCT CTA TGT GCA TAC GGA GC R: CCA TAC TGA TTG CCG CAAT	322	Ram et al. 2007
ST1	F: CTT TCC CCT CTT TTT AGT CAG R: TAA CAT GGA GCA CAG GCA GG	175	Ram et al. 2007
cdtB	F: AAA TCA CCA AGA ATC ATC CAG TTA R: AAA TCT CCT GCA ATC ATC CAG TTTA F: GAA AGT AAA TGG AAT ATA AAT GTC CG R: GAA AAT AAA TGG AAC ACA CAT GTC CG	430	Johnson and Stell 2000
bmaE	F: ATG GCG CTA ACT TGC CAT GCTG R: AGG GGG ACA TAT AGC CCC CTTC	507	Johnson and Stell 2000
papG allele II	F: GGG ATG AGC GGG CCT TTG AT R: CGG GCC CCC AAG TAA CTCG	190	Johnson and Stell 2000
papG allele III	F: GGC CTG CAA TGG ATT TAC CTGG R: CCA CCA AAT GAC CAT GCC AGAC	258	Johnson and Stell 2000
papAH	F: ATG GCA GTG GTG TCT TTT GGTG R: CGT CCC ACC ATA CGT GCT CTTC	720	Johnson and Stell 2000
papEF	F: GCG CAT TTG CTG ATA CTG TTG R: CAT CCA GAC GAT AAG CAT GAG CA	336	Johnson and Stell 2000
focG	F: CAG CAC GGC AGT GGA TAC GA R: GAA TGT CGC CTG CCC ATT GCT	360	Johnson and Stell 2000
cvaC	F: CAC ACA CAA ACG GGA GCT GTT R: CTT CCC GCA GCA TAG TTC CAT	680	Johnson and Stell 2000
ibeA	F: AGG CAG GTG TGC GCC GCG TAC R: TGG TGC TCC GGC AAA CCA TGC	170	Johnson and Stell 2000
iutA	F: GGC TGG ACA TCA TGG GAA CTGG R: CGT CGG GAA CGG GTA GAA TCG	300	Johnson and Stell 2000
*kpsMT*III	F: GCG CAT TTG CTG ATA CTG TTG R: CAT CCA GAC GAT AAG CAT GAG CA	392	Johnson and Stell 2000
*kpsMT*K1	F: TAG CAA ACG TTC TAT ATT GGT GC R: CAT CCA GAC GAT AAG CAT GAG CA	153	Johnson and Stell 2000
PAI	F: GGA CAT CCT GTT ACA GCG CGCA R: TCG CCA CCA ATC ACA GCC GAAC	930	Johnson and Stell 2000
traT	F: GGT GTG GTG CGA TGA GCA CAG R: CAC GGT TCA GCC ATC CCT GAG	290	Johnson and Stell 2000

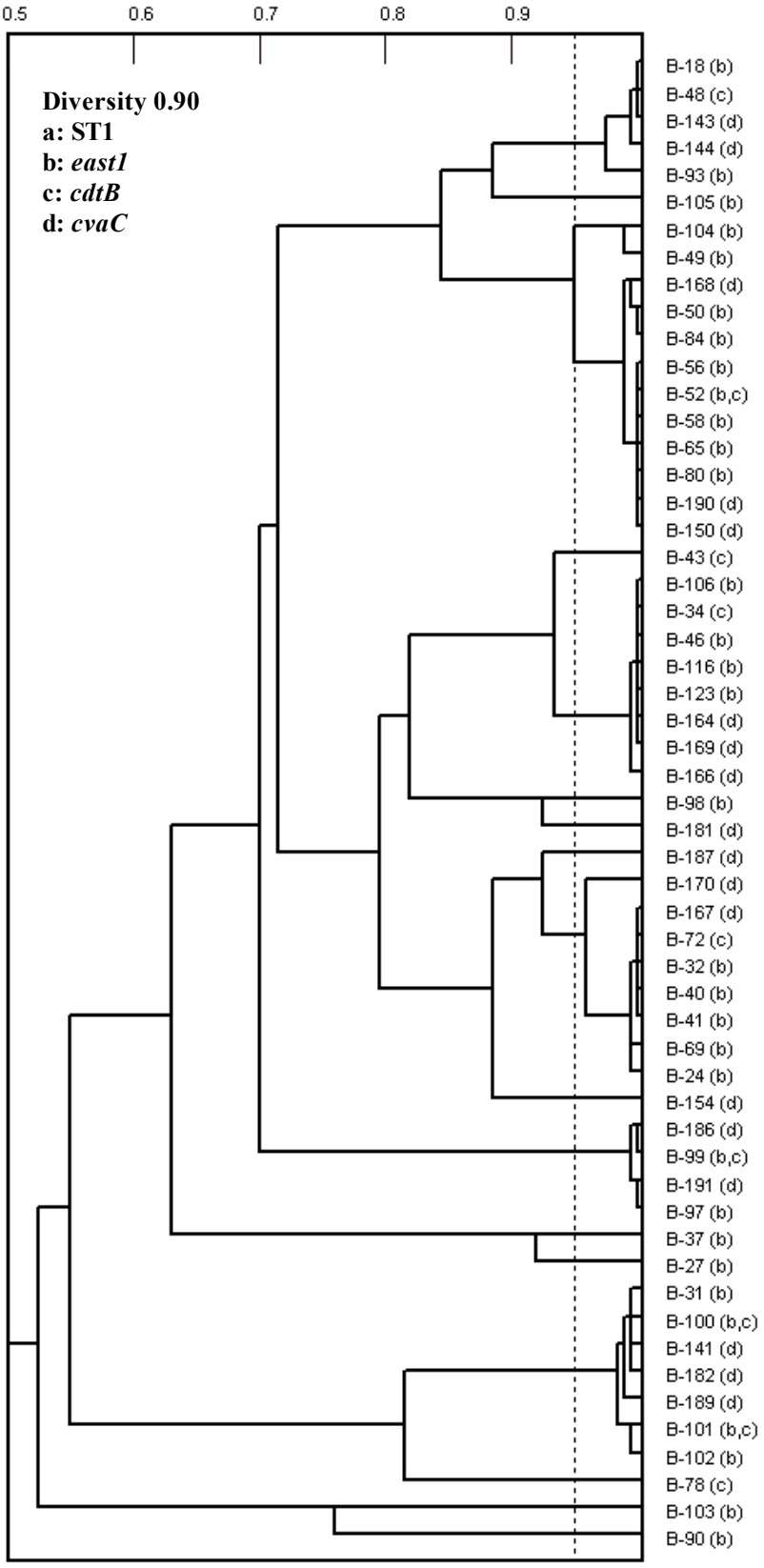
APPENDIX 6. Characteristics and End Uses of Rainwater Tanks

Tank ID	Location of Tank	Size of Tank (L)	Age of Tank [yr(s)]	Presence of Overhanging Trees (Y/N)	Evidence of Wild Life Droppings on Roof (Y/N)	Time since Desludged	End Uses
T1	Peri-urban	20,000	2	N	Y	Never	Potable and non-potable
T2	Peri-urban	20,000	5	N	N	4 years	Potable
T5	Peri-urban	22,500	2	N	N	Never	Potable and non-potable
T6	Peri-urban	22,500	1	N	N	Never	Potable and non-potable
T8	Peri-urban	30,000	1	N	N	Never	Potable and non-potable
T9	Urban	20,000	1	N	Y	Never	Potable
T10	Peri-urban	22,500	3	Y	N	Never	Potable and non-potable
T12	Peri-urban	22,500	1	N	N	Never	Potable and non-potable
T13	Peri-urban	10,000	2	Y	N	Never	Potable and non-potable
T14	Peri-urban	20,000	2	N	N	Never	Potable and non-potable
T15	Peri-urban	15,000	3	N	Y	1 year	Potable and non-potable
T16	Peri-urban	15,000	1	N	N	Never	Potable and non-potable
T18	Peri-urban	7,200	2	N	Y	3 months	Potable and non-potable
T20	Peri-urban	20,000	2	N	N	Never	Potable and non-potable
T21	Peri-urban	22,500	2	N	Y	3 months	Potable and non-potable
T22	Peri-urban	22,500	2	N	N	Never	Potable and non-potable
T23	Peri-urban	22,000	2	Y	Y	Never	Potable and non-potable
T31	Urban	5,000	3	N	N	1 month	Non-potable
T32	Urban	20,000	4	Y	Y	Never	Potable
T33	Urban	10,000	3	Y	Y	1 year	Non-potable
T34	Urban	5,000	3	Y	Y	1 year	Non-potable
T37	Urban	5,000	3	Y	Y	Never	Non-potable
T42	Peri-urban	15,000	1.5	N	Y	Never	Non-potable
T43	Peri-urban	5,000	7	N	Y	Never	Potable and non-potable
T44	Peri-urban	20,000	10	Y	Y	Never	Potable and non-potable
T45	Peri-urban	1,000	25	Y	Y	1 year	Potable
T46	Peri-urban	5,000	12	Y	Y	5 years	Potable
T47	Peri-urban	10,000	0.5	Y	Y	Never	Potable and non-potable
T48	Peri-urban	12,000	4	Y	Y	Never	Non-potable
T49	Peri-urban	10,000	20	N	Y	Never	Potable and non-potable

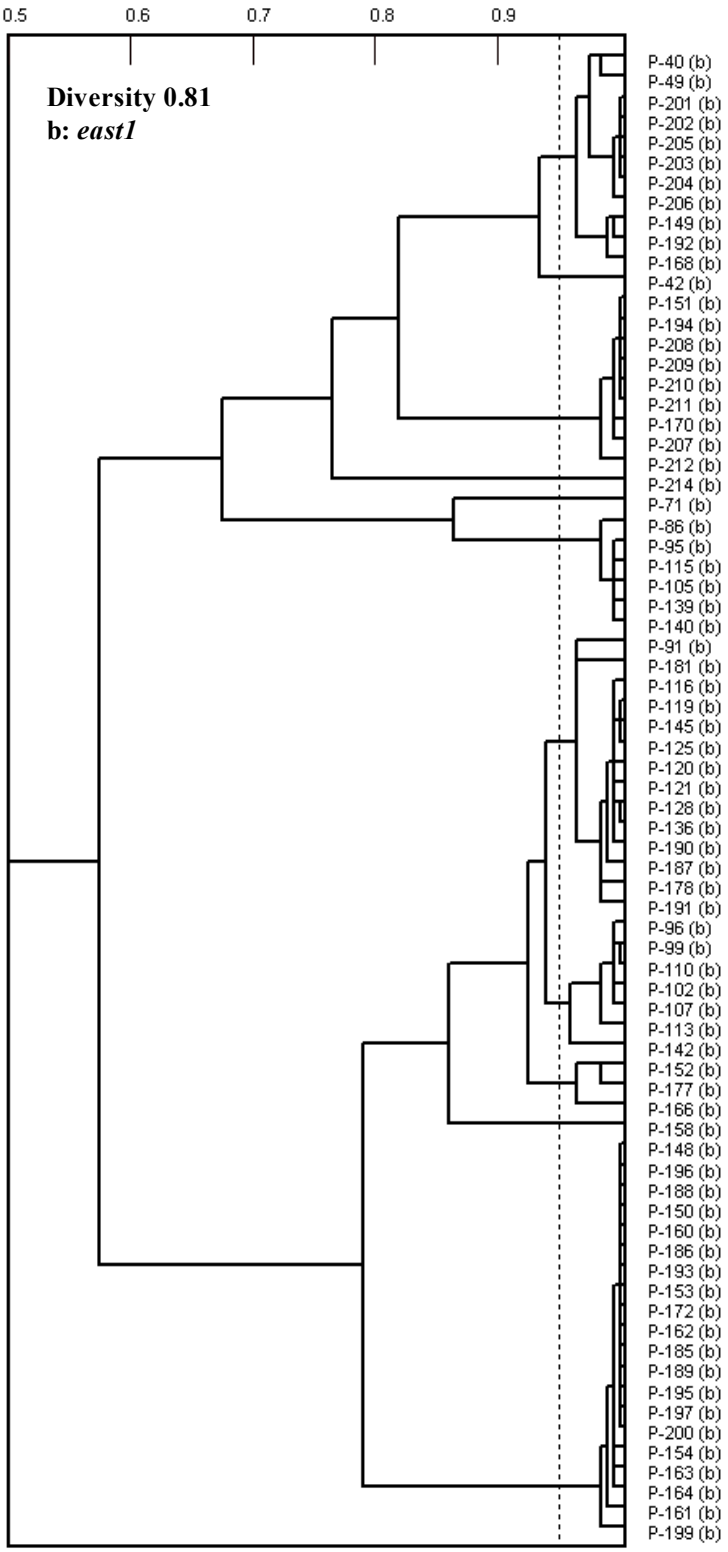
APPENDIX 7. An UPGMA Dendrogram Showing Biochemical Phenotypes (BPTS) of Escherichia Coli Isolates Harbouring Toxin Genes in Rainwater Tank Samples



APPENDIX 8. An UPGMA Dendrogram Showing Biochemical Phenotypes (BPTS) of Escherichia Coli Isolates Harbouring Toxin Genes in Bird Faecal Samples



APPENDIX 9. An UPGMA Dendrogram Showing Biochemical Phenotypes (BPTS) of Escherichia Coli Isolates Harbouring Toxin Genes in Possum Faecal Samples



APPENDIX 10. Sanitary Survey of Rainwater Tanks

Tank ID	Presence of Overhanging Trees (Y/N)	Evidence of Wild Life Droppings on Roof (Y/N)
T1	N	Y
T10	Y	N
T12	N	N
T15	N	Y
T33	Y	Y
T34	Y	Y
T42	N	Y
T44	Y	Y
T45	Y	Y
T46	Y	Y
T47	Y	Y
T48	Y	Y
T49	N	Y

APPENDIX 11. Primers used for the Identification of Enterococcus to the Species Level and Virulence Genes (VGS) Associated with Enterococcus SPP. Isolated from Rainwater Tanks

Targets	Primers	Amplicon Size (bp)	Melting Temp ºC
E. faecalis	F: ACT TAT GTG ACT AAC TTA ACC R: TAA TGG TGA ATC TTG GTT TGG	360	83.0
E. faecium	F: GAA AAA ACA ATA GAA GAA TTAT R: TGC TTT TTT GAA TTC TTC TTTA	215	81.5
E. casseliflavus	F: TCC TGA ATT AGG TGA AAA AAC R: GCT AGT TTA CCG TCT TTA ACG	288	84.0
E. hirae	F: CTT TCT GAT ATG GAT GCT GTC R: TAA ATT CTT CCT TAA ATG TTG	187	81.9
E. durans	F: CCT ACT GAT ATT AAG ACA GCG R: TAA TCC TAA GAT AGG TGT TTG	295	83.0
E. avium	F: GCT GCG ATT GAA AAA TAT CCG R: AAG CCA ATG ATC GGT GTT TTT	368	83.9
E. gallinarum	F: TTA CTT GCT GAT TTT GAT TCG R: TGA ATT CTT CTT TGA AAT CAG	173	83.0
E. mundtii	F: CAG ACA TGG ATG CTA TTC CAT CT R: GCC ATG ATT TTC CAG AAG AAT	98	78.5
AS	F: CCA GTA ATC AGT CCA GAA ACA ACC R: TAG CTT TTT TCA TTC TTG TGT TTG TT	406	81.5
ace	F: AAA GTA GAA TTA GAT CCA CAC R: TCT ATC ACA TTC GGT TGCG	320	78.5
gelE	F: AGT TCA TGT CTA TTT TCT TCAC R: CTT CAT TAT TTA CAC GTT TG	402	80.5
efaA	F: CGT GAG AAA GAA ATG GAG GA R: CTA CTA ACA CGT CAC GAA TG	499	82.0
esp	F: TTA CCA AGA TGG TTC TGT AGG CAC R: CCA AGT ATA CTT AGC ATC TTT TGG	913	82.0
cylA	F: ACT CGG GGA TTG ATA GGC R: GCT GCT AAA GCT GCG CTT	688	80.5

F: Forward primer; R: Reverse primer.

APPENDIX 12. Characteristics and End Uses of Rainwater Tanks

Tank ID	Location	Size (litres)	Age (yr)	Material	Presence of Overhanging Trees[b]	Evidence of Wild Life Droppings on Roof[b]	Desludging	End Uses
T1	Peri-urban	20,000[a]	2	Galvanized steel	N	Y	Never	Potable, non-potable
T2	Peri-urban	20,000	5	Galvanized steel	N	N	4 yr ago	Potable
T3	Peri-urban	22,500[a]	1	Galvanized steel	Y	Y	Never	Potable, non-potable[c]
T4	Peri-urban	22,500	1	Galvanized steel	Y	N	Never	Potable, non-potable
T5	Peri-urban	22,500[a]	2	Galvanized steel	N	N	Never	Potable, non-potable
T6	Peri-urban	22,500[a]	1	Galvanized steel	N	N	Never	Potable, non-potable
T8	Peri-urban	30,000[a]	1	Colorbond	N	N	Never	Potable, non-potable[c]
T9	Peri-urban	20,000[a]	1	Galvanized steel	N	Y	Never	Potable[c]
T10	Peri-urban	22,500[a]	3	Polyethylene	Y	N	Never	Potable, non-potable
T11	Peri-urban	20,000[a]		Galvanized steel	N	N	Never	Potable, non-potable[c]
T12	Peri-urban	22,500[a]	1	Galvanized steel	N	N	Never	Potable, non-potable[c]
T13	Peri-urban	10,000[a]	2	Galvanized steel	Y	N	Never	Potable, non-potable[c]
T14	Peri-urban	20,000[a]	2	Polyethylene	N	N	Never	Potable, non-potable
T15	Peri-urban	15,000	3	Colorbond	N	Y	1 year	Potable, non-potable[c]
T17	Peri-urban	10,000[a]	3	Galvanized steel	N	N	Never	Potable, non-potable
T25	Peri-urban	15,000	3	Galvanized steel	N	Y	Never	Potable, non-potable
T28	Urban	10,000	3	Polyethylene	N	Y	2 yr ago	Non-potable
T29	Peri-urban	20,000[a]	1	Galvanized steel	N	Y	Never	Potable, non-potable
T30	Urban	5,000	3	Polyethylene	N	Y	Never	Non-potable
T31	Urban	5,000	3	Polyethylene	N	N	1 mo ago	Non-potable
T32	Urban	20,000	4	Polyethylene	Y	Y	Never	Potable[d]
T33	Urban	10,000	3	Polyethylene	Y	Y	1 yr ago	Non-potable
T34	Urban	5,000	3	Polyethylene	Y	Y	1 yr ago	Non-potable
T36	Urban	5,000	3	Polyethylene	Y	Y	Never	Non-potable
T37	Urban	5,000	3	Polyethylene	Y	Y	Never	Non-potable
T38	Urban	22,000[a]	5	Galvanized steel	Y	N	2 yr ago	Potable, non-potable[c]
T39	Urban	10,000	5	Polyethylene	N	Y	3 yr ago	Potable, non-potable[c]

[a] First flush diverter installed.
[b] Y, yes; N, no.
[c] Under sink filtration and UV installed.

APPENDIX 13. Numbers of Enterococcus SPP. in Rainwater Tanks

Tank	No. of *Enterococcus* (CFU/100 mL ± S.D)
T1	21 ± 1
T2	12 ± 1
T3	91 ± 6
T4	3 ± 1
T5	3 ± 1
T8	40 ± 8
T9	4 ± 1
T10	17 ± 5
T11	28 ± 5
T13	3 ± 2
T14	54 ± 14
T17	2 ± 1
T25	5 ± 3
T28	4 ± 2
T29	2 ± 1
T30	13 ± 4
T31	16 ± 4
T33	16 ± 4
T34	450 ± 43
T36	29 ± 5
T37	123 ± 40
T38	44 ± 10
T39	60 ± 25
Total [a]	

[a] $n = 23$.

JOURNAL PUBLICATIONS ARISING FROM THIS REPORT

W. Ahmed, K. Richardson, J.P.S. Sidhu and S. Toze. 2012. *Escherichia coli* and *Enterococcus* spp. in rainwater tank samples: comparison of culture-based methods and 23S rRNA gene quantitative PCR assays. *Environmental Science & Technology*. **46:** 11370-11376.

W. Ahmed, J.P.S. Sidhu and S. Toze. 2012. Speciation and Frequency of virulence genes of *Enterococcus* spp. isolated from rainwater tank samples in Southeast Queensland, Australia. *Environmental Science & Technology.* **46:** 6843-6850.

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W. Ahmed, L. Hodgers, N. Masters, J.P.S. Sidhu, M. Katouli and S. Toze. 2011. Occurrence of intestinal and extraintestinal virulence genes in *Escherichia coli* isolates from roof-harvested rainwater in Southeast Queensland, Australia. *Applied and Environmental Microbiology.* **77:** 7394-7400.

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K. Richardson, W. Ahmed, J.P.S. Sidhu. P. Jagals and S. Toze. 2012. Inactivation of faecal indicator bacteria in a roof-captured rainwater system under ambient meteorological conditions (under review).

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