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Implications of faecal indicator bacteria for the microbiological assessment of roof harvested rainwater quality in Southeast Queensland, Australia

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Running title: Implications of indicator bacteria for rainwater quality monitoring

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Abstract: The study aimed to evaluate the suitability of *Escherichia coli*, enterococci and *C. perfringens* to assess the microbiological quality of roof harvested rainwater, and to assess whether the concentrations of these faecal indicators can be used to predict the presence or absence of specific zoonotic bacterial or protozoan pathogens. From a total of 100 samples tested, respectively 58%, 83% and 46% of samples were found to be positive for *E. coli*, enterococci and *C. perfringens* spores, as determined by traditional culture based methods. Additionally, in the samples tested, 7%, 19%, 1%, 8%, 17%, and 15% were PCR positive for *A. hydrophila lip*, *C. coli ceuE*, *C. jejuni mapA, L. pneumophila mip*, *Salmonella invA, and G. lamblia* β-giardin genes. However, none of the samples was positive for *E. coli* O157 LPS, VT1, VT2 and *C. parvum* COWP genes. The presence or absence of these potential pathogens did not correlate with any of the faecal indicator bacterial concentrations as determined by a binary logistic regression model. The roof-harvested rainwater samples tested in this study appear to be of poor microbiological quality and no significant correlation was found between the concentration of faecal indicators and pathogenic microorganisms. The use of faecal indicator bacteria raises questions regarding their reliability in assessing the microbiological quality of water and particularly their poor correlation with pathogenic microorganisms. The presence of one or more zoonotic pathogens suggests that the microbiological analysis of water should be performed, and appropriate treatment measures should be undertaken especially in tanks where the water is used for drinking.

Keywords: faecal indicators; enteric pathogens, roof-harvested rainwater; PCR; public health risk.

Introduction

The demand on potable water supply is increasing in line with economic growth and increase in industrial output and commerce, and population growth. This is further exacerbated by the adverse impacts of climate change on water supply. Consequently, water authorities are keen to explore alternative water sources to meet the ever increasing demand. Among the alternatives, roof-harvested rainwater (RHRW) has been considered as a potential source for potable and non-potable uses in many countries (Despins et al. 2009; Evans et al. 2007; Uba and Aghogho 2000). In Australia, the use of rainwater tanks is becoming increasingly common in most major cities in addition to rural and remote areas. Subsidies and other regulatory measures introduced in recent years to encourage rainwater tanks installation in several capital cities including Brisbane, Queensland to cope with the severe drought conditions highlight their importance as an alternative source.

The most significant issue in relation to RHRW reuse is the potential public health risks associated with microbiological pollutants (Ahmed et al. 2008; Simmons et al. 2001). Various microorganisms including pathogens could be present in the faeces of birds, insects, small mammals and reptiles. Consequently, following rain events faecal matter and other organic debris could be introduced to the tank via roof runoff. The microbiological quality of RHRW is generally assessed by monitoring faecal indicator bacteria such as faecal coliforms, *Escherichia coli* and enterococci (Appan 1997; Dillaha and Zolan 1985; Ghanayem 2001; Plazinska 2001; Vasudevan et al. 2001). There is a general community perception that rainwater is safe to drink without having to undergo prior treatment.

This is partially supported by limited epidemiological studies (Heyworth et al. 2006). Additionally, a previous research study has reported that RHRW quality is generally acceptable for drinking and household use (Dillaha and Zolan 1985), and poses no increased risk of gastrointestinal illnesses when compared with mains water (Heyworth 2006). In contrast, a number of studies have reported the presence of specific pathogens including opportunistic pathogens in RHRW (Ahmed et al. 2008; Birks et al. 2004; Crabtree et al. 1996; Lye 2002; Simmons et al. 2001; Uba and Aghogho 2000). Therefore, questions have arisen regarding the microbiological quality of rainwater and consequent public health risks.

This in turn highlights the most important limitation of faecal indicator bacteria (i.e., faecal coliforms, *E. coli*) arising from their poor correlation with pathogenic microorganisms in environmental waters (Ahmed et al. 2009; Hörman et al. 2004; McQuaig et al. 2006). This limitation is also common in sewage (Harwood et al. 2005). This is not surprising considering faecal indicator bacteria exhibit differential survival rates compared to

pathogens especially viruses and protozoans. Furthermore, faecal indicators may replicate in external environments (Anderson et al. 2005; Byappanahalli et al. 2006; Desmarais et al. 2002). Currently, there is a paucity of knowledge in relation to the occurrence and concentrations of pathogens in RHRW and their relationships with traditional faecal indicator bacteria.

Direct monitoring of pathogens in water sources could be an attractive option, as it would provide invaluable information regarding public health risks. However isolation and identification of specific pathogens using traditional culture based methods could be cumbersome. In recent times, PCR based methods have been widely used for the detection/quantitative detection of various pathogens in environmental waters (Ahmed et al. 2009; Guy et al. 2003; Hörman et al. 2004; Sails et al. 2002). An important feature of the PCR based methods is that they can be used to detect and quantify pathogens which are difficult to isolate using traditional culture-based and microscopic methods. It has to be noted that, to date only a limited number of studies have applied PCR to detect specific pathogens in RHRW (Ahmed et al. 2008).

A primary aim of this study was to investigate the prevalence of faecal indicator bacteria such as *E. coli,* enterococci and *C. perfringens*, and a wide range of bacterial and protozoan pathogens in RHRW samples. Samples were collected from Brisbane, Gold Coast and Sunshine Coast Regions in Queensland, and tested for the presence of faecal indicators using traditional culture based methods and specific pathogens using PCR detection. Secondly, the study also aimed to assess whether the concentrations of faecal indicator bacteria was suitable for predicting the presence or absence of specific pathogens.

Materials and methods

Sources of samples

Initially 27 RHRW samples were collected from 27 residential houses in Brisbane in Southeast Queensland Australia, and the results have been published elsewhere (Ahmed et al. 2008). For this study, a total of 73 new RHRW samples were collected from 55 residential houses located in Brisbane, Gold Coast and Sunshine Coast regions giving a total number of 100 RHRW samples from 82 residential houses. The size of the tanks sampled ranged from 500 to 15,000 litres and were polyethylene water tanks. The end uses were (i) outdoor use (65%), including gardening and car washing; and (ii) indoor use (35%), including drinking and kitchen use. Samples were collected within 1 to 4 days after a rain event (ranging from 35 to 130 mm). Samples were collected in sterilized 10-liter containers from the outlet taps located close to the base of the tanks. Before the rainwater was

sampled, the tap was sterilised with 96% ethanol, and allowed to run for 30 to 60 s to flush out water from the tap. Samples were transported to the laboratory on ice, and processed within 8 to 10 h.

Isolation and enumeration of faecal indicators

The membrane filtration method was used to process the water samples (i.e., 100 ml) for faecal indicator bacterial enumeration. Sample serial dilutions were made and filtered through 0.45-µm-pore-sized (47-mm-diameter) nitrocellulose membranes (Advantec, Tokyo, Japan) and placed on modified mTEC agar (Difco, Detroit, MI), membrane-*Enterococcus* indoxyl-β-D-glucoside (mEI) agar (Difco), and oleandomycin-polymyxin-sulfadiazine perfringens (OPSP) agar with supplement (Oxoid, London, United Kingdom) for the isolation of *E. coli*, enterococci, and spore-forming *C. perfringens*, respectively. For the isolation of *C. perfringens* spores, water samples were heated at 60°C for 30 min before filtration. The OPSP agar plates were overlaid with 15 ml of molten OPSP agar before incubation. 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), and mEI agar plates were incubated at 41°C for 48 h (US EPA 1997). OPSP agar plates (for *C. perfringens*) were incubated anaerobically at 44°C for 24 h. The confirmatory test for *C. perfringens* was performed according to the method described previously (Wohlsen et al. 2006). For bacterial enumeration, all water samples were tested in triplicate.

DNA extraction from water samples

For PCR analysis of potential bacterial pathogens, 1L of water sample from each tank was filtered through 0.45-µm pore size nitrocellulose membrane (Advantec). In case of membrane clogging during filtration, multiple membranes were used. The membranes were immediately transferred into 15 ml screw cap tube containing 10 ml of sterile STE buffer (0.1 M NaCl, 10 mM Tris, and 1 mM EDTA [pH 7.6]. The tubes were vortexed vigorously for 8-10 min to detach the bacteria from the membranes followed by centrifugation at 8,000 g for 30 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 2 ml of sterile distilled water. DNA was extracted using DNeasy blood and tissue kit (Qiagen), and stored at -80°C until use.

For PCR analysis of protozoan *G. lamblia* β-giardin and *C. parvum* COWP genes, 3-5 L water sample from each tank was filtered through 3-µm pore size membrane (47 mm diameter) (Advantec). After filtration, the membrane was transferred into a petri dish. DNA was extracted directly on the filter using DNeasy blood and tissue kit (Qiagen). In brief, 360 µl of buffer ATL was added to each filter paper. The filter paper was scraped very well and discarded. Each sample was transferred into 1.5-ml micro centrifuge tube, and subjected to three

cycles of freezing-thawing. After freezing-thawing, 40 µl of proteinase K was added to each tube. The tubes were then incubated overnight at 56°C. After incubation, the DNA was extracted according to manufacturer's instructions.

PCR-positive controls

Strains were purchased from the American Type Culture Collection (ATCC), as follows: *Aeromonas hydrophila* ATCC 7966, *Campylobacter coli* ATCC 43478, *Legionella pneumophila* ATCC 33152, and *Salmonella enterica* serovar Typhimurium ATCC 14028. *Escherichia coli* NCTC 12079 strain was kindly donated by Mr. Jack Tucker from the University of the Sunshine Coast, Queensland, Australia. DNA prepared from ATCC genuine cultures purchased for *Campylobacter jejuni* (33560D), *Giardia lamblia* (30888D), and *Cryptosporidium parvum* (PRA-67D).

Specificity of the PCR primers

PCR detection of pathogenic bacteria and protozoans was done using previously published primers. The primer sequence and annealing temperature for corresponding target genes are shown in Table 1. Primer specificity was determined by searching for similar sequences in microbial genomes using the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST/). This ensured that no homology was observed with known gene sequences of other pathogenic microorganisms commonly found in environmental waters. The cross reactivity of each primer set was also evaluated by testing DNA isolated from other non-target species commonly found in environmental waters. These included: (1) *A. hydrophila* (2) *B. vulgatus* (3) *C. coli* (4) *C. jejuni* (5) *C. freundii* (6) *C. perfringens* (7) *E. faecalis* (8) *E. coli* (9) *K. pneumoniae* (10) *L. pneumophila* (11) *P. aeruginosa* (12) *S. Typhimurium* (13) *S. Sonnei* (14) *C. parvum*, and (15) *G. lamblia*.

PCR detection of potential pathogenic microorganisms

Amplification was performed in 25-µl reaction mixtures using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA). 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 (i.e. target DNA), and negative controls (sterile water) were included. The PCR reactions were performed using the Rotor-Gene 6000 real-time cycler (Corbett Research, Mortlake, Australia). Cycling parameters for the *A. hydrophila lip* gene were 2 min at 50°C, 15 min at 95°C for initial denaturation, and 35 cycles of 94°C for 1 min, 62°C for 1 min for annealing, and 72°C

for 1.5 min, followed by a final extension step of 72°C for 5 min; for *C. coli ceuE* and *C. jejuni mapA* genes, 2 min at 50 ºC, 10 min at 95ºC for initial denaturation, and 40 cycles of 95ºC for 15 s, 59ºC for 30 s for annealing; for *L. pneumophila mip* gene, 2 min at 50°C, 15 min at 95°C for initial denaturation, and 35 cycles of 94°C for 30 s, 54°C for 1 min for annealing, and 72°C for 1 min, followed by a final extension step of 72°C for 5 min; for the *Salmonella invA* and *spvC* genes, 2 min at 50 ºC, 5 min at 94ºC for initial denaturation, and 45 cycles of 94ºC for 30 s, 59ºC for 35 s for annealing, and 72ºC for 2 min, followed by a final extension step of 72 ºC for 10 min; for *E. coli* O157 LPS, VT1 and VT2 genes, 2 min at 50 ºC, 10 min at 95ºC for initial denaturation, and 40 cycles of 95ºC for 30 s, 59ºC for 30 s for annealing and 72ºC for 30 s, followed by a final extension step of 72 ºC for 5 min; For *G. lamblia* β-giardin and *C. parvum* COWP genes, 2 min at 50°C, 10 min at 95ºC for initial denaturation, and 40 cycles of 94ºC for 15 s, 59ºC for 1 min for annealing.

Quality control

To prevent false positive results for RHRW samples, a method blank was included for each batch (n = 10) of water samples. In brief, 1 L of distilled water sample was filtered through 0.45-μm pore size membrane (Advantec). The filter paper was washed with sterile STE buffer followed by centrifugation as described above. The supernatant was discarded, and the pellet was resuspended in sterile distilled water. DNA was extracted using DNeasy blood and tissue kit (Qiagen). To prevent false positive results during DNA extraction, a reagent blank was included for each batch (n = 10) of samples. During setting up of the PCR assays, the PCR conditions for annealing temperature were optimized by performing gradient analysis (i.e., temperature ranged from 53°C to 63°) for each target. The primer concentrations (100 nM to 500 nM) were also optimized to reduce the level of primer dimer for each target. To separate the specific product from non-specific products, DNA melting curve analysis was performed for each PCR experiment. During melting curve analysis, the temperature was increased from 57-95°C at approximately 2°C min^{-1}. Amplified products were also visualized by electrophoresis through 2% E-gel® (Invitrogen), and exposure to UV light for further confirmation (if required). Samples were considered to be positive when the visible band was the same as that of the positive control strain, and had the same melting temperature ± 0.2°C as the positive control. To minimize PCR contamination, DNA extraction, PCR set up, and gel electrophoresis were performed in separate laboratories.

PCR limit of detection

To determine the PCR lower limits of detection (LOD), known gene copies (i.e., 10^3 -10^0) of each target gene were tested by PCR. The lowest concentration of gene copies detected consistently in replicate assays was considered as PCR LOD.

PCR inhibitors

An experiment was conducted to determine the potential presence of PCR inhibitory substances in rainwater samples collected from three different tanks from the Brisbane region. Each sample (i.e., 1 L) was concentrated using the membrane filtration technique as described above. DNA was extracted using DNeasy blood and tissue kit (Qiagen), serially diluted and tested with the PCR. DNA was also extracted from ultra pure DNAse and RNAse free sterile distilled water (Invitrogen) in the same manner for comparison with the tank water. All samples (undiluted, diluted and distilled water DNA) were spiked with 10^3 gene copies of human-specific HF183 *Bacteroides* markers (Bernhard and Field 2000). The C_T values obtained for the DNA samples from spiked tank water were compared to the DNA samples from distilled water.

DNA sequencing

To verify the identity of the PCR products obtained from water samples, up to three PCR-amplified products from each target were purified using the QIAquick PCR purification kit (Qiagen) as recommended by the manufacturer's (Qiagen), and cloned, in duplicate, into the pGEM®-T Easy Vector system (Promega, Madison, WI, USA) as recommended by the manufacturer. Plasmids were extracted using the QIAprep Spin- Miniprep kit (Qiagen). Bidirectional sequences were obtained using T7 and SP6 long sequencing primer targeting sites on either side of the insert. DNA sequencing was carried out at the Australian Genome Research Facility (St Lucia, Queensland, Australia). The sequences were analysed using Bioware Jellyfish Software, and were verified with the published sequence.

Statistical analysis

The Spearman rank correlation was used to test the relationship between faecal indicator concentrations in RHRW samples. A binary logistic regression analysis was also performed to obtain correlations between the presence/absence of pathogen detection by PCR, and the concentrations of faecal indicators. Logistic regression is the technique most commonly used to model such a binary (i.e., presence/absence) response. The

presence/absence of pathogens was treated as the dependent variable (i.e., a binary variable). When a target organism was present, it was assigned the value 1, and when a target organism was absent, it was assigned the value 0. For this analysis, the concentration of faecal indicator bacteria found in 100 ml water sample was converted to 1 L (to be compared with bacterial pathogens) and 3 L (to be compared with protozoan pathogens). Minitab Release version 11.12 (State College, Pa.) software was used for the Spearman rank correlation and logistic regression analysis. In all cases, a difference was considered significant if the *P* value for the model chi square was 0.05.

RESULTS

Specificity of PCR primers

The specificity of each primer set for each target was assessed by testing a panel of other microorganisms that could be found in RHRW. The primers used in this study did not amplify any PCR products other than those that were expected.

PCR inhibitors

For the spiked distilled water, the mean C_T value for the HF183 DNA was 23.8 ± 0.4. For rainwater samples, the mean C_T value was 23.6 ± 0.4 when undiluted DNA was spiked. For 10-fold, 100-fold and 1000-fold dilutions of DNA, these values were 23.4 ± 0.3, 23.4 ± 0.1, and 23.3 ± 0.2, respectively. One-way analysis of variance (ANOVA) was performed to determine the differences between the C_T values obtained for distilled water and those obtained for rainwater samples. No significant differences were observed between the C_T values for spiked distilled water, undiluted DNA, and serially diluted thus indicating that the tested rainwater samples were free of PCR inhibitor.

PCR limit of detection

The LOD assays were performed by analysing purified genomic DNA from bacterial and protozoan strains containing corresponding target genes. To determine the reproducibility of the assay, several replicates (n = 10) were tested. The PCR detection limits were as low as five gene copies for *A. hydrophila lip*, *C. coli ceuE*, *Salmonella invA, Salmonella spvC,* and *L. pneumophila mip* genes. For *C. jejuni mapA*, and *E. coli* O157 LPS, VT1 and VT2 genes, the detection limits were 10 gene copies. For *G. lamblia* β-giardin and *C. parvum* COWP

genes, the detection limits were seven and ten gene copies respectively. Lower levels (i.e., one copy) were tested for these targets, but the results were not reproducible for all replicates.

Prevalence of faecal indicator bacteria

The concentration of *E. coli* in water samples from RHRW ranged from < 1 to 3060 ± 456 CFU $100ml^{-1}$ of water. For enterococci and *C. perfringens* spores, these figures were < 1 to 3400 ± 700 CFU $100ml^{-1}$, and < 1 to 200 ± 30 CFU $100ml^{-1}$, respectively. Of the 100 samples tested, 42% samples had < 1 *E. coli* 100 ml^{-1} (Table 2). Similarly, 17% and 54% of samples had < 1 enterococci and *C. perfringens* spores, respectively. Enterococci were more frequently detected (83 out of 100 samples were positive) in water samples compared to *E. coli* (58 out of 100 samples), and *C. perfringens* spores (46 out of 100 samples). Of the 100 samples tested, 89% were positive for at least one faecal indicator, 62% were positive for at least two indicators and 36% were positive for all three indicators tested in this study. The concentrations of fecal indicators were pooled for all tanks, and were analysed to determine if the concentrations correlated with each other. Significant correlations were observed between *E. coli* vs. enterococci (Spearman's $r_s = 0.57$; $P = 0.0001$), and enterococci vs. *C. perfringens* spores ($r_s = 0.22$; $P = 0.0258$). However, the concentrations of *E. coli* did not correlate with *C. perfringens* spores ($r_s = 0.10$; $P = 0.3056$).

Prevalence of pathogenic microorganisms

Of the 100 samples tested, 7% of samples were positive for *A. hydrophila lip* gene (Table 3). *C. jejuni mapA* gene was detected only in one sample. However, *C. coli* were more prevalent, and 19% of the samples were positive for *C. coli ceuE* gene. *L. pneumophila mip* and *Salmonella invA* genes were detected respectively in 8% and 17% of the samples. *Salmonella spvC*, *E. coli* O157 LPS, VT1 and VT2 genes were not detected in any samples tested in this study. Additionally, 15% of the samples were positive for *G. lamblia* β-giardin gene. However, none of the samples were positive for *C. parvum* COWP gene. Most of the pathogens were detected in samples collected from the Brisbane region followed by the Gold Coast region. None of the samples from the Sunshine Coast region was positive for any pathogens tested. Of the 100 samples tested, 1% was positive for at least the four target genes, 8% was positive for at least three target genes, 18% was positive for at least two target genes, and 40% was positive for at least one target gene. However, none of the potential pathogens were detected in 60% of RHRW samples.

Correlation between faecal indicator and pathogenic microorganisms

Discrepancies were observed in terms of the occurrence of faecal indicators and zoonotic pathogens. For example, 12% of samples had < 1 *E. coli* but were positive for one or more target pathogens. Similarly 6% and 19% of samples had < 1 enterococci and *C. perfringens* spores, respectively but were positive for one or more target pathogens. Binary logistic regression was used to test the hypothesis that faecal indicator concentrations can predict the presence or absence of pathogens in samples collected from RHRW tanks. PCR results of bacterial and protozoan pathogens (those only gave positive and negative signals) were converted to binary data. When a pathogen was present, it was assigned the value 1, and when a pathogen was absent, it was assigned the value 0. The presence or absence of pathogens did not correlate with any of the indicator bacterial concentrations (Table 4).

DISCUSSION

Most of the past research studies have assessed microbiological quality of RHRW by monitoring traditional faecal indicators, namely, faecal coliforms and *E. coli* (Dillaha and Zolan 1985; Evans et al. 2006; Yaziz et al.1989). Only a limited numbers of research studies to-date have investigated the presence of specific pathogens in RHRW. These studies have invariably found limitations relating to the suitability of traditional faecal indicators (Ahmed et al. 2008; Lye 2002; Simmons et al. 2001).

Detection of specific pathogens using traditional culture based methods is laborious and lack sensitivity (Toze et al. 1999). Due to these limitations, we used PCR assays for the rapid detection of specific pathogens. Before application, the specificity of each primer and the PCR detection limit for each assay was rigorously evaluated. One major problem associated with PCR detection of pathogens in water is PCR inhibitors. Environmental waters generally contain organic and inorganic substances with the potential to inhibit PCRs (Wilson 1997). The influence of such inhibitory substances on PCR detection was evaluated by spiking rainwater DNA samples with known concentrations of human-specific *Bacteroides* HF183 marker (Bernhard and Field 2000). Human-specific HF183 *Bacteroides* marker was chosen for spiking because it is unlikely that the source of faecal contamination in rainwater tanks would be of human rather than animal origin. Only 5% samples contained PCR inhibitory substances, and a 10-fold serial dilution of DNA was required to remove the inhibitory effects.

In the 100 samples tested, 58% samples had > 1 CFU *E. coli* 100 ml^{-1} of water, and exceeded the Australian Drinking Water Guidelines. The concentrations of *E. coli* and enterococci were highly variable in the water samples, and some rainwater tanks (i.e., 5%) had > 1000 CFU *E. coli* and enterococci 100 ml^{-1} of water suggesting high levels of faecal pollution. It has to be noted that samples were collected within 1-4 days after rainfall which is when faecal and other organic matter deposited on the roof enters tanks via roof runoff. This was done to obtain information regarding the magnitude of faecal pollution in the worst case scenario. Enterococci were more prevalent than *E. coli,* and of the 100 samples tested, 83% were positive for enterococci compared to *E. coli* (58%).

A number of samples (i.e., 25/100 samples) were positive for culturable enterococci but were negative for culturable *E. coli*. This could be due to the fact that enterococci persist in the water longer than *E. coli* (McFeters et al. 1974). The study results highlight the importance of testing multiple indicators for rainwater quality monitoring. The absence of a single indicator (i.e., *E. coli* alone) does not necessarily rule out the presence of faecal pollution and microorganisms of public health significance. In the 100 samples tested 54% had < 1 CFU *C. perfringens* spores 100 ml^{-1} water. In all, 46 (85%) out of 54 samples were positive for either *E. coli* or enterococci or both. This data clearly indicates that *C. perfringens* spores may not provide reliable information regarding faecal pollution in RHRW tanks. However, it may provide additional information regarding the magnitude of faecal pollution in RHRW samples.

In the 100 samples tested, 19%, 17%, 15%, 8%, 7% and 1% were PCR positive for *C. coli*, *Salmonella* spp., *G. lamblia*, *L. pneumophila*, *A. hydrophila*, and *C. jejuni* respectively. Samples from Brisbane and Gold Coast regions were positive for one or multiple pathogens. However, none of the samples tested from the Sunshine Coast region was positive. Of the 16 samples tested, five were negative for all three indicators. The remaining 11 samples were positive for at least one indicator. The concentrations of fecal indicators and occurrence of pathogens in samples from Sunshine Coast was relatively low compared to Brisbane and Gold Coast regions. The samples from the Sunshine Coast region were collected from a new sub-division where none of the residential houses had as yet any overhanging trees or antennas on the rooftop which could eliminate the high possibility of bird fecal pollution. However, with time this situation is likely to change.

In all, 40% of the samples were positive for at least one target pathogen, and of these 18 tanks were used for drinking. The presence of *Aeromonas* spp., *Campylobacter* spp., *Legionella* spp. and *Giardia* spp. in samples from RHRW has been reported in the United States, New Zealand and in the tropics (Broadhead et al. 1998; Savill et al. 2001; Simmons et al. 2001). In this study, *Salmonella spvC*, *E. coli* O157 LPS, VT1 and VT2 and *C. parvum* COWP genes were not detected. To our knowledge, enterohaemorrhagic *E. coli* has not been previously isolated from RHRW samples. However, *Cryptosporidium* spp. has been found in rainwater cisterns in the U.S. Virgin Islands (Crabtree et al. 1995). It has to be noted that a larger volume of water samples (i.e., 100 L) were screened for the detection of *Cryptosporidium* spp. in the USA. However, in this study up to 5 L of water samples were screened for PCR assay. Therefore, the assay used in this study could have underestimated the concentrations of *Cryptosporidium* spp. In this study, different volumes of water samples were tested for fecal indicators enumeration, and the occurrence of bacterial and protozoan pathogens. It has to be noted that this is a common practice for microbiological water quality monitoring because the concentration of pathogens may vary depending on the magnitude of fecal pollution and their persistence in the water. In addition, different concentration of fecal indicators and pathogens are shed in the feces of warm-blooded animals. Therefore, large volume of water samples needs to be analysed to detect pathogens. One major limitation of PCR based methods is that it does not provide information regarding the viability and infectivity of target pathogens. Nonetheless, the presence of these zoonotic pathogens is a cause for concern especially in tanks where the water is used for drinking.

A binary logistic regression was performed to identify the correlations between faecal indicator bacterial concentrations, and the PCR presence and absence of *A. hydrophila*, *C. jejuni*, *C. coli*, *L. pneumophila*, *Salmonella* spp*.,* and *G. lamblia*. None of the faecal indicator concentrations correlated with the presence/absence of pathogens. Some samples had no measurable concentration of fecal indicators although they were positive for one or more target pathogens. For example, 10 samples had < 1 CFU *E. coli* 100 ml^{-1}. However, all these samples were positive for one or more target pathogens. Similarly six samples (for enterococci) and 14 samples (*for C. perfringens* spores) had < 1 CFU *E. coli* and *C. perfringens* spores 100 ml^{-1} respectively but were positive for one or more target pathogens. These results suggest that pathogens could be present in tank water samples in the absence of faecal indicator bacteria, and raise serious questions regarding the reliability of employing faecal indicators to assess the microbiological quality of water. In this study, a one-off sample was collected from most of the tanks immediately after rain events. Therefore, limited data is available regarding the

persistence of these pathogens. Such information is valuable for health risk assessment. We are currently undertaking a longitudinal study to investigate the prevalence and concentrations of these pathogens using quantitative PCR methods. In addition, we are also using a suite of methods (quantitative PCR and culture based) to obtain information how many quantified pathogens are indeed viable. This information will be used to quantify microbial risk associated with the use of RHRW for potable and non-potable uses.

In conclusion, the RHRW samples tested in this study appears to be of poor microbiological quality. A specific number of water samples tested in this study contained high levels of *E. coli*, enterococci and *C. perfringens* spores. A significant number of samples were also positive for zoonotic bacterial and protozoan pathogens. The use of faecal indicator bacteria raises questions regarding their reliability in assessing the microbiological quality of water and particularly their poor correlation with pathogenic microorganisms. The presence of one or more zoonotic pathogens suggests that the microbiological analysis of water should be performed, and appropriate treatment measures such as under sink filtrations units, ultra violet disinfection units or simply boiling the water should be undertaken especially in tanks where the water is used for drinking.

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Table 1: Target genes and primers used for pathogen detection

Target	´- 3´)	Amplicon size (bp)	Reference
A. hydrophila lip gene	AAC CTG GTT CCG CTC AAG CCG TTG [a]	760	Cascon *et al.* 1996
	TTG CTC GCC TCG GCC CAG CAG CT [b]		
C. coli ceuE gene	CAA GTA CTG CAA TAA AAA CTA GCA CTA CG [a]	67	Price *et al.* 2006
	AGC TAT CAC CCT CAT CAC TCA TAC TAA TAG [b]		
C. jejuni mapA gene	GCT AGA GGA ATA GTT GTG CTT GAC AA [a]	72	Price *et al.* 2006
	TTA CTC ACA TAA GGT GAA TTT TGA TCG [b]		
E. coli O157 LPS gene	CGG ACA TCC ATG TGA TAT GG [a]	259	Pass *et al.* 2000
	TTG CCT ATG TAC AGC TAA TCC [b]		
E. coli verocytotoxin gene 1 (VT1)	ACG TTA CAG CGT GTT GCT GGG ATC [a]	121	Pass *et al.* 2000
	TTG CCA CAG ACT GCG TCA GTT AGG [b]		
E. coli verocytotoxin gene 2 (VT2)	TGT GGC TGG GTT CGT TAA TAC GGC [a]	102	Pass *et al.* 2000
	TTG CCA CAG ACT GCG TCA GTT AGG [b]		
L. pneumophila mip gene	GCA ATG TCA ACA GCAA [a]	159	Wilson *et al.* 2003
	CAT AGC GTC TTG CATG [b]		
Salmonella invA gene	ACA GTG CTC GTT TAC GAC CTG AAT [a]	244	Chiu and Ou 1996
	AGA CGA CTG GTA CTG ATC GAT AAT [b]		
Salmonella spvC gene	ACT CCT TGC ACA ACC AAA TGC GGA [a]	571	Chiu and Ou 1996
	ACA GTG CTC GTT TAC GAC CTG AAT [b]		
Cryptosporidium oocyst wall protein (COWP) gene	CAA ATT GAT ACC GTT TGT CCT TCTG [a]	150	Guy *et al.* 2003
	GGC ATG TCG ATT CTA ATT CAG CT [b]		
G. lamblia β-giardin gene	CCT CAA GAG CCT GAA CGA TCTC [a]	74	Guy *et al.* 2003
	AGC TGG TCG TAC ATC TTC TTC CTT [b]		

[a:] Forward primer [b:] Reverse primer.

Table 2: Percentage of roof-harvested rainwater samples positive for faecal indicators

CFU 100 ml^{-1}	Percentage of samples		
	E. coli	Enterococci	*C. perfringens*
< 1	42	17	54
1-10	18	17	21
11-100	17	36	22
101-500	14	14	3
501-1000	4	7	0
> 1000	5	9	0

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39 **Table 3:** PCR positive results for pathogens in roof-harvested rainwater samples

Location	No. of samples positive by PCR/number of samples tested										
	Pathogenic bacteria									Pathogenic protozoan	
	A. hydrophila *lip* gene	*C. jejuni* *mapA* gene	*C. jejuni* *ceuE* gene	*E. coli* O157 gene	*E. coli* verocytotoxin gene 1 (VT1)	*E. coli* verocytotoxin gene 2 (VT2)	*L. pneumophila* *mip* gene	*Salmonella* *invA* gene	*Salmonella* *spvC* gene	*Cryptosporidium* oocyst wall protein (COWP) gene	*G. lamblia* β-giardin gene
Brisbane	7/66	1/66	16/66	0/66	0/66	0/66	8/66	15/66	0/66	0/66	13/66
Gold Coast	0/18	0/18	3/18	0/18	0/18	0/18	0/18	2/18	0/18	0/18	2/18
Sunshine Coast	0/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16
Total	7/100	1/100	19/100	0/100	0/100	0/100	8/100	17/100	0/100	0/100	15/100

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50 **Table 4:** The relationships between faecal indicators and the presence and absence of pathogens in water
51 samples collected from rainwater tanks using binary logistic regression.
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Indicators vs. pathogenic microorganisms	Concordance (%)	*P*-value [a]	Odd ratio
E. coli vs. *A. hydrophila*	6.50	0.972	1.00
E. coli vs. *C. jejuni*	94.9	0.376	1.00
E. coli vs. *C. coli*	22.0	0.597	1.00
E. coli vs. *L. pneumophila*	22.7	0.544	1.00
E. coli vs. *Salmonella* spp.	32.0	0.096	1.00
E. coli vs. *G. lamblia*	34.9	0.131	1.00
Enterococci vs. *A. hydrophila*	59.4	0.092	1.00
Enterococci vs. *C. jejuni*	12.1	0.887	1.00
Enterococci vs. *C. coli*	44.4	0.240	1.00
Enterococci vs. *L. pneumophila*	11.5	0.974	1.00
Enterococci vs. *Salmonella* spp.	44.1	0.172	1.00
Enterococci vs. *G. lamblia*	32.1	0.490	1.00
C. perfringens vs. *A. hydrophila*	51.3	0.580	1.00
C. perfringens vs. *C. jejuni*	5.10	0.948	1.00
C. perfringens vs. *C. coli*	36.9	0.415	1.01
C. perfringens vs. *L. pneumophila*	34.8	0.463	1.00
C. perfringens vs. *Salmonella* spp.	51.3	0.580	1.00
C. perfringens vs. *G. lamblia*	34.0	0.807	1.00

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54 [a] *P*-value for the model chi square was < 0.05 and the confidence interval for the odds ratio did not include 1.0.
55 Greater odds ratios indicate a higher probability of change in the dependent variable with a change in the
56 independent variable.

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