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Fecal Indicators and Zoonotic Pathogens in Household Drinking Water Taps Fed from Rainwater Tanks in Southeast Queensland, Australia

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In this study, the microbiological quality of household tap water samples fed from rainwater tanks was assessed by monitoring the numbers of *Escherichia coli* bacteria and enterococci from 24 households in Southeast Queensland (SEQ), Australia. Quantitative PCR (qPCR) was also used for the quantitative detection of zoonotic pathogens in water samples from rainwater tanks and connected household taps. The numbers of zoonotic pathogens were also estimated in fecal samples from possums and various species of birds by using qPCR, as possums and birds are considered to be the potential sources of fecal contamination in roof-harvested rainwater (RHRW). Among the 24 households, 63% of rainwater tank and 58% of connected household tap water (CHTW) samples contained *E. coli* and exceeded Australian drinking water guidelines of <1 CFU *E. coli* per 100 ml water. Similarly, 92% of rainwater tanks and 83% of CHTW samples also contained enterococci. In all, 21%, 4%, and 13% of rainwater tank samples contained *Campylobacter* spp., *Salmonella* spp., and *Giardia lamblia*, respectively. Similarly, 21% of rainwater tank and 13% of CHTW samples contained *Campylobacter* spp. and *G. lamblia*, respectively. The number of *E. coli* ($P = 0.78$), *Enterococcus* ($P = 0.64$), *Campylobacter* ($P = 0.44$), and *G. lamblia* ($P = 0.50$) cells in rainwater tanks did not differ significantly from the numbers observed in the CHTW samples. Among the 40 possum fecal samples tested, *Campylobacter* spp., *Cryptosporidium parvum*, and *G. lamblia* were detected in 60%, 13%, and 30% of samples, respectively. Among the 38 bird fecal samples tested, *Campylobacter* spp., *Salmonella* spp., *C. parvum*, and *G. lamblia* were detected in 24%, 11%, 5%, and 13% of the samples, respectively. Household tap water samples fed from rainwater tanks tested in the study appeared to be highly variable. Regular cleaning of roofs and gutters, along with pruning of overhanging tree branches, might also prove effective in reducing animal fecal contamination of rainwater tanks.

Roof-harvested rainwater (RHRW) has been used as a potable- and a nonpotable-water source in many countries (14, 15, 34). There is a general community feeling that RHRW is safe to drink, and this is supported by limited epidemiological evidence (18). In contrast, the presence of potentially pathogenic microorganisms, such as *Aeromonas* spp., *Campylobacter* spp., *Salmonella* spp., *Giardia* spp., and *Cryptosporidium* spp., in RHRW samples has been reported (1, 3, 12, 30, 32). The most significant issue in relation to RHRW for potable- and nonpotable-water uses is the potential health risks associated with exposure to these pathogenic microorganisms. Case-control studies established links between gastroenteritis and consumption of untreated RHRW (8, 27).

Wild animals, such as birds, mammals, and reptiles, are the most likely sources of fecal contamination in RHRW, as the animals have access to the roof surface. Consequently, fecal matter from the animals and other organic debris originating from overhanging trees could be transported to the rainwater tanks via roof runoff following rain events.

The microbiological quality of RHRW is generally assessed by monitoring fecal indicator bacteria, such as fecal coliforms, *Escherichia coli*, and enterococci, which are commonly found in the guts of warm-blooded animals, including humans (29, 31, 33). In addition, a number of studies on the microbial quality of RHRW reported the presence of zoonotic bacterial and protozoan pathogens in individual or communal rainwater tanks (1, 7, 12, 24, 30, 32). Most of these studies assessed the quality of the RHRW on the basis of the presence or absence of the specific pathogens, with little information available regarding their numbers or potential sources in RHRW.

Around 10% of Australians currently use RHRW as a major source of their drinking water, and an additional approximately 5% use RHRW as a potable replacement for showering, toilet flushing, and clothes laundering (5). However, it is usually not recommended to use RHRW for drinking where municipal water is available. For example, Queensland regulations do not prohibit the plumbing of rainwater tanks to supply drinking water. However, if a person, chooses to use rainwater for drinking or any other purpose, then that person is responsible for ensuring that the quality of the water is sufficient for its intended use. Many householders who drink RHRW use an undersink filtration (USF) system in order to reduce exposure to pathogenic microorganisms, suspended solids, and harmful chemicals.

Little is known regarding the prevalence of zoonotic pathogenic microorganism in wild animals, such as birds and mammals, which are most likely contaminating RHRW. Mammals can get access to the roof via overhanging trees or electricity cables or by climbing to the roof via walls or other structures attached to the

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TABLE 1 Survey results for rainwater tanks tested in the study

Household ID[a]	Size of tank (liters)	Age of tank (yr)	Presence of overhanging trees[b]	Presence of TV aerials[b]	Evidence of wildlife fecal droppings on roof[b]	First-flush diverters installed[b]	Undersink filtration[b]
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
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
H9	20,000	1	N	N	Y	Y	Y
H10	22,500	3	Y	N	N	Y	N
H11	20,000	2	N	N	N	Y	Y
H12	22,000	1	Y	N	N	Y	Y
H13	10,000	2	Y	N	N	Y	Y[c]
H14	20,000	2	N	N	N	Y	N
H15	15,000	3	N	N	Y	N	Y
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
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
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

[a] ID, identifier.
[b] Y, yes: N, no.
[c] UV installed in addition to undersink filtration.

house. Birds can get access to the roof via overhanging trees or structures mounted on the roof, such as television (TV) aerials and solar panels. Knowing the source of pathogenic microorganisms is important in order to design management strategies and reduce public health risks from exposure to pathogenic microorganisms.

The aims of this study were (i) to investigate the prevalence and numbers of fecal indicators (*E. coli* and enterococci) and zoonotic bacterial (*Campylobacter* spp. and *Salmonella* spp.) and protozoan (*Cryptosporidium parvum* and *Giardia lamblia*) pathogens in water samples from rainwater tanks and connected household taps and (ii) to investigate the prevalence of the above-mentioned pathogens in fecal samples from possums and various species of wild birds. Conventional culture-based methods were used to enumerate *E. coli* and enterococci, and quantitative PCR (qPCR) was used to obtain the numbers of zoonotic pathogens in RHRW, connected household tap water (CHTW), and animal fecal samples.

MATERIALS AND METHODS

Study area and sanitary survey. The study area, Currumbin Ecovillage, is located on the southern end of the Gold Coast, Southeast Queensland (SEQ), Australia. The Ecovillage is known for its sustainable residential developments and is often viewed as a blueprint for future urban development. Twenty-four households participated in the study. All the households use captured RHRW for drinking and other nonpotable-water uses, such as car washing, clothes laundering, showering, and gardening. A sanitary inspection was undertaken to identify factors (i.e., the presence of overhanging trees, TV aerials, and wildlife fecal contamination on the roof) that might contribute to the fecal contamination of the rainwater tanks. Information on the filtration methods for RHRW prior to drinking it was also obtained from the householders (Table 1).

Roof-harvested rainwater and connected household tap water sampling. Two water samples were collected from each household (i.e., one from the rainwater tank and one from the connected household tap), giving a total of 48 samples from the 24 households. Samples were collected within 1 to 4 days after a rain event (>100 mm) in 20-liter sterile containers. The external taps were located 15 to 20 cm from the bottoms of the rainwater tanks, and the connected household taps were located over the kitchen sinks. Before sampling, the external taps and connected household cold water taps were wiped with 70% ethanol and allowed to run for 30 to 60 s to flush water. The samples were transported to the laboratory and processed within 2 to 4 h.

Enumeration of fecal indicators. The membrane filtration method was used to process water samples for bacterial enumeration (35, 36). Sample serial dilutions were made (where necessary) and filtered through 0.45-μm-pore-size (47-mm-diameter) nitrocellulose membranes (Millipore, Tokyo, Japan). The membranes were placed on modified mTEC agar (Difco, Detroit, MI) and membrane-enterococcus indoxyl-β-D-glucoside (mEI) agar (Difco) for the isolation of *E. coli* and enterococci, respectively. 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 (36), and mEI agar plates were incubated at 41°C for 48 h (35).

Concentration of water samples. Approximately 19-liter water sample from each rainwater tank and household tap were concentrated by a hollow-fiber ultrafiltration system (HFUS), using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA) as previously described by Hill et al. (19). Briefly, each water sample was pumped with a peristaltic pump (Masterflex; Cole-Parment Instrument Co.) in a closed loop with sterile high-performance, platinum-cured L/S 36 silicone tubing (Masterflex; Cole-Parmer Instrument Co.). The tubing was sterilized

TABLE 2 Primers, probes, and cycling parameters for qPCR assays used in the study

Target	Primer and probe sequences (5′–3′)[a]	Cycling parameters	Amplicon size (bp)	Reference
Campylobacter spp. 16S rRNA	F: CACGTGCTACAATGGCAAT R: GGCTTCATGCTCTCGAGTT P: FAM-CAGAGAACAATCCGAACTGGGACA-BHQ1	10 min at 95°C; 45 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C	108	23
Salmonella invA gene	F: ACAGTGCTCGTTTACGACCTGAAT R: AGACGACTGGTACTGATCGATAAT	5 min at 94°C; 30 cycles of 30 s at 94°C, 35 s at 59°C, and 120 s at 72°C	244	11
G. lamblia β-giardin gene	F: CATAACGACGCCATCGCGGCTCTCAGGAA R: TTTGTGAGCGCTTCTGTCGTGGCAGCGCTAA P: FAM-AGCTCAACGAGAAGGTCGCAGAGGGCTT-TAMRA	3 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C	218	25
Cryptosporidium parvum COWP gene	F: CAAATTGATACCGTTTGTCCTTCTG R: GGCATGTCGATTCTAATTCAGCT P: HEX-TGCCATACATTGTTGTCCTGACAAATTGAAT-BHQ1	10 min at 95°C; 45 cycles of 15 s at 95°C, 1 min at 60°C	150	16

[a] F, forward primer; R, reverse primer; P, probe; FAM, 6-carboxyfluorescein, TAMRA, 6-carboxytetramethylrhodamine; HEX, hexachlorofluorescein; BHQ1, black hole quencher 1.

by soaking in 10% bleach, washed with deionized (DI) water, and autoclaved at 121°C for 15 min. At the end of the concentration process, pressurized air was passed through the filter cartridge from the top to recover as much water as possible. A new filter cartridge was used for each sample. The samples were concentrated to approximately 100 ml. Each 100-ml sample was further centrifuged at 3,000 × *g* for 30 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 5 ml of sterile distilled water.

DNA extraction. For qPCR analysis of bacterial pathogens, DNA was extracted from the pellet obtained from 1.5 ml of concentrated samples (i.e., 48 samples) using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) and stored at −80°C until use. For qPCR analysis of protozoan pathogens, DNA was also extracted from the pellet obtained from 1.5 ml of concentrated samples using the same kit with some modification. In brief, 180 μl of buffer ATL was added to each pellet and subjected to three cycles of freezing (−80°C) followed by thawing (56°C) in a water bath. After the samples underwent freeze-thaw cycles, proteinase K (20 μl) was added to each tube. The tubes were incubated overnight at 56°C. After incubation, the DNA was extracted according to the manufacturer's instructions.

Animal fecal sampling and DNA extraction. Brush tail possum fecal samples ($n = 40$) were obtained from the possum removal service in Brisbane (Peter the Possum Man). Bird fecal samples ($n = 38$) were collected from botanical gardens, bird sanctuaries, and a veterinary hospital. The bird species included plover, wood duckling, noisy minet, Pacific black duckling, blue faced honey eater, magpie, crow, ibis, seagull, topknot pigeon, crested tern, juvenile black swan, Pacific baza, fantail cuckoo, rainbow lorikeet, and tawny frogmouth. Up to three samples were collected from each species of bird. All samples were transported to the laboratory, stored at 4°C, and processed within 24 h. DNA was extracted from fresh feces (80 to 220 mg) from each individual animal using a QIAmp Stool DNA kit (Qiagen).

Positive controls and qPCR assays. Strains and prepared DNA from typed cultures were purchased from the American Type Culture Collection (ATCC) as follows: *Salmonella enterica* serovar Typhimurium (ATCC 14028), *Campylobacter jejuni* (33560D), *G. lamblia* (30888D), and *C. parvum* (PRA-67D). qPCR assays were performed using previously published primers and probes (Table 2). Standards for qPCR of the *Campylobacter* 16S rRNA, *Salmonella invA*, *C. parvum* oocyst wall protein (COWP), and *G. lamblia* β-giardin genes were prepared from the genomic DNA. The concentration of genomic DNA was determined by measuring the A_{260} using a Beckman Coulter DU 730 spectrophotometer. 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.

Salmonella qPCR amplification was performed in 20-μl reaction mixtures using Sso Fast EvaGreen Supermix (Bio-Rad Laboratories, CA). The PCR mixture contained 10 μl of Supermix, 300 nM each primer, 4.25 μl of DNase- and RNase-free deionized water, and 5 μl of template DNA (11). *Campylobacter*, *C. parvum*, and *G. lamblia* qPCR amplifications were performed in 25-μl reaction mixtures using iQ Supermix (Bio-Rad Laboratories). The PCR mixture contained 12.5 μl of Supermix, 500 nM each primer, 400 to 600 nM corresponding probe, and 5 μl of template DNA (16, 23, 25). For each PCR experiment, a negative control (i.e., sterile water) was included. The PCR was performed using the Bio-Rad iQ5 (Bio-Rad Laboratories).

qPCR reproducibility and limit of detection. The reproducibility of the qPCR was assessed by determining intra-assay repeatability and interassay reproducibility. The coefficient of variation (CV) was calculated using six dilutions (5×10^6 to 5×10^0 gene copies) of the *C. jejuni*, *S.* Typhimurium, *C. parvum*, and *G. lamblia* genomic DNAs. Each dilution was quantified in replicates. The CV for evaluation of intra-assay repeatability was calculated based on the cycle threshold (C_T) value by testing the six dilutions three times in the same experiment. The CV for interassay reproducibility was calculated based on the C_T values of six dilutions on three different days. To determine the qPCR limit of detection, known gene copies (5×10^3 to 5×10^0 gene copies) were measured from pure genomic DNA isolated from corresponding control strains and tested by qPCR. The lowest number of gene copies that was detected consistently in replicate assays was considered the qPCR limit of detection.

Testing for PCR inhibitors. An experiment was conducted to determine the potential presence of PCR inhibitory substances in DNA extracted from RHRW ($n = 3$) and CHTW ($n = 3$) samples from three different households (i.e., H1, H10, and H15). DNAs isolated from possums ($n = 3$) and birds ($n = 3$) were also checked for potential PCR inhibitors. Ten-fold serial dilutions were made, and all DNA samples (i.e., undiluted and diluted) were spiked with 10^3 gene copies of human adenovirus type 41. The C_T values obtained for the DNA samples from spiked RHRW, connected household taps, and animal fecal samples were compared to those of the DNA samples from distilled water (1).

Quality control. To prevent carryover contamination of water and fecal samples, method blank runs were performed to ensure that the disinfection procedure was effective in preventing carryover contamination between sampling events. In addition, to prevent carryover contamination during DNA extraction, reagent blanks were included for each batch of samples. No carryover contamination was observed. During the setup of the PCR assays, the PCR conditions for annealing temperature were optimized by performing gradient analysis (the temperature ranged from 53 to 63°C) for each target. During melting curve analysis, the temperature was increased from 57 to 95°C at approximately 2°C per min. Samples were considered positive when they had the same melting temperature

(±0.2°C) as the positive control. To minimize PCR contamination, DNA extraction and PCR setup were performed in separate laboratories.

Statistical analysis. Analysis of variance (ANOVA) was performed to determine the differences between C_T values obtained for DNA isolated from distilled water and those obtained from RHRW, CHTW, and fecal samples. Prior to the statistical analysis, all indicators and pathogen numbers were $\log_{10}$ transformed. A Wilcoxon signed-rank test was applied to test the significance of differences in fecal indicators and pathogen numbers between RHRW and CHTW samples. Pearson's multiple correlation was used to test the relationship between *E. coli* and enterococcal numbers in RHRW and CHTW samples. In all cases, a difference was considered significant if the *P* value was <0.05. All statistical analyses were performed with SAS version 9.2 software (SAS Institute, Cary, NJ).

RESULTS

Survey results. The sizes of the selected rainwater tanks ranged from 7,200 to 30,000 liters, and their ages were between 1 and 5 years (Table 1). Among the 24 households surveyed, 6 (25%) had overhanging trees ($n = 4$) or TV aerials ($n = 2$) mounted on the roof. Seven (29%) tanks had visible signs of fecal droppings on the roof. Twenty of the tanks (88%) had first-flush diverters installed. Among the 24 households, 10 (42%) filtered the water before it was drunk. Of the 10 households, 9 (90%) had USF (i.e., a cartridge-type filter, 0.5-μm pore size) and 1 had both USF and UV installed (Table 1).

qPCR standards, reproducibility, and limit of detection. Ten-fold dilutions of quantified *C. jejuni*, *S.* Typhimurium, *C. parvum*, and *G. lamblia* cells were analyzed in order to determine the reaction efficiencies. The standard curves had a linear range of quantification from 10^6 to 10^1 genomic copies per μl of DNA extracts. The amplification efficiencies were >95%, and the correlation coefficient (r^2) was >0.98 for all four assays. The mean intra-assay and interassay CV values and standard deviations, respectively, were 3.9% ± 1.0% and 2.3% ± 1.6% (for the *Campylobacter* assay), 1.9% ± 0.8% and 1.9% ± 1.3% (for the *Salmonella* assay), 3.9% ± 1.9% and 2.9% ± 1.6% (for the *C. parvum* assay), and 3.2% ± 1.2% and 4.5% ± 2.1% (for the *G. lamblia* assay), indicating high reproducibility. The qPCR limit of detection was five gene copies for all target pathogens.

PCR inhibitors. For spiked distilled water, the mean C_T value for human-specific adenovirus DNA was 25.6 ± 0.4 (Table 3). For RHRW samples ($n = 3$), CHTW samples ($n = 3$), and possum ($n = 3$) and bird ($n = 3$) fecal samples, the mean C_T values were 28 ± 0.3, 27 ± 1.0, 27 ± 1.1, and 26 ± 0.4, respectively, when undiluted DNA was spiked. The values obtained for 10-fold and 100-fold dilutions are shown in Table 3. One-way ANOVA was performed to determine the differences between the C_T values obtained for distilled water and those obtained for RHRW samples, CHTW samples, and possum and bird fecal samples. No significant differences were observed between the C_T values for spiked distilled water, undiluted DNA, and serially diluted DNA, indicating that the tested samples were free of PCR inhibitors.

Numbers of fecal indicators in roof-harvested rainwater and connected household tap water samples. Among the 24 households, *E. coli* was cultured from 15 (62%) RHRW and 14 (58%) CHTW samples. Similarly, 22 (92%) RHRW and 20 (83%) CHTW samples contained cultured enterococci (Table 4). The numbers of *E. coli* bacteria in these samples ranged from 1×10^0 to 2.3×10^2 per 100 ml (for RHRW) and 1×10^0 to 3.0×10^2 CFU per 100 ml (for CHTW) of water. For enterococci, these numbers were 2×10^0 to 1.1×10^2 CFU per 100 ml (for RHRW) and 1×10^0 to 1.1×10^2 (for CHTW) CFU per 100 ml. Enterococci were more frequently detected in both RHRW (22 of 24 samples contained enterococci) and CHTW (20 out of 24) than *E. coli* (15 out of 24 for RHRW and 14 out of 24 for CHTW). Among the 24 samples from RHRW tanks tested, 96% contained at least one fecal indicator and 58% contained both indicators. Similarly, among the 24 samples from the connected household taps tested, 92% contained at least one fecal indicator and 50% were positive for both indicators.

TABLE 3 Evaluation of PCR inhibition on the PCR detection of spiked sewage-associated adenoviruses in RHRW, CHTW, and animal fecal samples as opposed to spiked distilled water samples

Sample	C_T value (mean ± SD) for PCR		
	Undiluted DNA	10-fold dilution	100-fold dilution
Distilled water	25.6 ± 0.4		
RHRW			
H1	27.1 ± 0.3	26.4 ± 0.3	26.9 ± 0.3
H10	27.2 ± 0.6	26.3 ± 0.4	26.6 ± 0.5
H15	26.7 ± 0.2	26.5 ± 0.5	26.7 ± 0.4
CHTW			
H1	26.8 ± 0.1	26.8 ± 0.2	27.9 ± 0.3
H10	28.4 ± 0.3	26.3 ± 0.6	26.0 ± 0.4
H15	26.4 ± 0.4	25.9 ± 0.4	25.8 ± 0.6
Possum feces			
P3	26.0 ± 0.3	25.8 ± 0.6	25.8 ± 0.7
P14	26.0 ± 0.6	25.4 ± 0.8	25.9 ± 0.3
P26	28.0 ± 0.1	25.9 ± 0.5	25.9 ± 0.6
Bird feces			
B1	26.0 ± 0.4	26.0 ± 0.4	25.9 ± 0.9
B5	26.4 ± 0.5	25.6 ± 0.7	25.4 ± 0.4
B11	25.6 ± 0.2	25.2 ± 0.8	25.2 ± 0.6

Numbers of zoonotic pathogens in roof-harvested rainwater and connected household tap water samples. Among the 24 households, 5 (21%), 1 (4%), and 3 (13%) RHRW samples contained *Campylobacter* sp. 16S rRNA, *Salmonella invA*, and *G. lamblia* β-giardin genes, respectively (Table 4). Similarly, 5 (21%) and 3 (13%) of the CHTW samples contained *Campylobacter* 16S rRNA and *G. lamblia* β-giardin genes, respectively. The *Salmonella invA* gene could not be detected in CHTW samples. For the estimation of pathogen numbers, the numbers of genomic copies (determined by qPCR) for each pathogen were converted to numbers of bacterial cells or protozoan cysts (2, 16, 21).

After the conversion of numbers of genomic copies to numbers of cells, the numbers of *Campylobacter* cells in RHRW and household tap water samples ranged from 5×10^0 to 1×10^2 (in RHRW) and 1×10^1 to 1.9×10^1 (in CHTW) cells per liter of water. Similarly the estimated number of *Salmonella* cells was 7.3×10^3 (in RHRW) per liter of water. The numbers of *G. lamblia* cysts ranged from 1.2×10^2 to 5.8×10^2 (in RHRW) and 1.1×10^2 to 1.4×10^2 (in CHTW) per liter of water.

Correlation between zoonotic pathogens and indicators in roof-harvested rainwater and connected household tap water samples. The numbers of fecal indicators and pathogens were

TABLE 4 Numbers of fecal indicators and zoonotic pathogens in roof-harvested rainwater and connected household tap water samples

	No. (mean) of fecal indicators per 100 ml of water				No. (mean) of bacterial cells and protozoan cysts per liter of water					
	E. coli		Enterococci		*Campylobacter* spp.		*Salmonella* spp.		*G. lamblia*	
Household ID	RHRW	CHTW	RHRW	CHTW	RHRW	CHTW	RHRW	CHTW	RHRW	CHTW
H1	1.5×10^1	2×10^1	2.1×10^1	1.3×10^1	ND[a]	ND	ND	ND	1.2×10^2	1.4×10^2
H2	3×10^0	4×10^0	1.2×10^1	1.3×10^1	ND	ND	ND	ND	ND	ND
H3	1×10^0	4×10^0	9.1×10^1	1×10^2	ND	ND	ND	ND	ND	ND
H4	2×10^0	ND	3×10^0	6×10^0	ND	ND	7.3×10^3	ND	ND	ND
H5	2×10^0	3×10^0	3×10^0	1×10^1	ND	ND	ND	ND	ND	ND
H6	2.3×10^2	6.7×10^1	2.7×10^1	ND	1.1×10^2	+[b]	ND	ND	ND	ND
H7	1×10^0	2×10^0	3.7×10^1	8.2×10^1	ND	ND	ND	ND	1.6×10^2	1.4×10^2
H8	8.9×10^1	6×10^0	4.0×10^1	6.1×10^1	ND	ND	ND	ND	ND	ND
H9	ND	ND	4×10^0	1×10^0	ND	ND	ND	ND	ND	ND
H10	2×10^0	ND	1.7×10^1	2.5×10^1	4.7×10^1	ND	ND	ND	ND	ND
H11	5×10^0	9×10^0	2.8×10^1	3.6×10^1	ND	1.4×10^1	ND	ND	ND	ND
H12	1.2×10^1	6×10^0	ND	ND	+[b]	1.1×10^1	ND	ND	ND	ND
H13	ND	ND	3×10^0	ND	ND	ND	ND	ND	ND	ND
H14	5×10^0	2×10^0	5.4×10^1	6.1×10^1	5×10^0	1.2×10^1	ND	ND	5.8×10^2	1.1×10^2
H15	1.2×10^1	3×10^2	7.5×10^1	1.1×10^2	3×10^1	1.9×10^1	ND	ND	ND	ND
H16	ND	ND	2.3×10^1	1.8×10^1	ND	ND	ND	ND	ND	ND
H17	ND	3×10^0	2×10^0	2×10^0	ND	ND	ND	ND	ND	ND
H18	ND	ND	4.9×10^1	4.1×10^1	ND	ND	ND	ND	ND	ND
H19	1×10^0	1×10^0	1.5×10^1	4×10^1	ND	ND	ND	ND	ND	ND
H20	ND	ND	ND	2.4×10^1	ND	ND	ND	ND	ND	ND
H23	ND	1.5×10^1	1.1×10^2	2.5×10^1	ND	ND	ND	ND	ND	ND
H25	ND	ND	5×10^0	3×10^0	ND	ND	ND	ND	ND	ND
H29	1×10^0	ND	2×10^0	ND	ND	ND	ND	ND	ND	ND
H35	ND	ND	4×10^0	3×10^0	ND	ND	ND	ND	ND	ND

[a] ND, not detected.
[b] Not quantifiable.

pooled for all RHRW and CHTW samples to determine whether the numbers were correlated between RHRW and CHTW samples. The numbers of *E. coli* ($P = 0.78$), *Enterococcus* ($P = 0.64$), *Campylobacter* sp. ($P = 0.44$), and *G. lamblia* ($P = 0.50$) cells in RHRW did not significantly differ from the numbers in CHTW samples as determined by Wilcoxon's signed-rank test. The numbers of *E. coli* and enterococcal cells were analyzed to determine whether the numbers within the RHRW and CHTW correlated with each other. Significant correlations were observed between *E. coli* and enterococci in water samples from RHRW ($r_p = 0.33$; $P = 0.005$) and CHTW ($r_p = 0.28$; $P = 0.01$) as determined by Pearson's multiple correlation.

Numbers of zoonotic pathogens in animal fecal samples. Among the 40 possum fecal samples tested, *Campylobacter* 16S rRNA, *C. parvum* COWP, and *G. lamblia* β-giardin genes were detected in 60%, 13%, and 30% of samples, respectively (Table 5). After conversion of the numbers of genomic copies to numbers of cells, the number of *Campylobacter* cells in possum fecal samples ranged from 2×10^5 to 2×10^7. *G. lamblia* was detected in 12 samples; however, only 7 were quantifiable. The numbers of *G. lamblia* cells in possum fecal samples ranged from 2.1×10^1 to 1.6×10^3 cysts per g of feces. The *C. parvum* COWP gene was not quantifiable, and the *Salmonella invA* gene could not be detected in DNA from possum fecal samples.

Among the 38 bird fecal samples tested, the *Campylobacter* sp. 16S rRNA, *Salmonella invA*, *C. parvum* COWP, and *G. lamblia* β-giardin genes were detected in 24%, 11%, 5%, and 13% of samples, respectively.

The numbers of *Campylobacter*, *Salmonella*, and *G. lamblia* organisms in bird fecal samples ranged from 6.6×10^4 to 6.6×10^6

TABLE 5 Numbers of zoonotic pathogens in possum and bird fecal samples

		Campylobacter spp.		*Salmonella* spp.		*C. parvum*		*G. lamblia*	
Sample	No. of samples tested	No. (%) of PCR-positive samples	Range of bacterial cells and protozoan cysts per g of feces	No. (%) of PCR-positive samples	Range of bacterial cells and protozoan cysts per g of feces	No. (%) of PCR-positive samples	Range of bacterial cells and protozoan cysts per g of feces	No. (%) of PCR-positive samples	Range of bacterial cells and protozoan cysts per g of feces
Possums	40	24 (60)	2×10^5 to 2×10^7	ND[a]		5 (13)	−[b]	12 (30)	2.1×10^1 to 1.6×10^3
Birds	38	9 (24)	6.6×10^4 to 6.6×10^6	4 (11)	6.3×10^2 to 1.8×10^3	2 (5)	−[b]	5 (13)	1.3×10^0 to 1.2×10^2

[a] ND, not detected.
[b] Not quantifiable.

and 6.3×10^2 to 1.8×10^3 bacteria and 1.3×10^0 to 1.0×10^2 cysts per g of feces, respectively.

The *C. parvum* COWP gene was not quantifiable.

DISCUSSION

In this study, 62% of the RHRW and 58% of the CHTW samples fed from the RHRW tanks exceeded Australian drinking water guidelines (28) of <1CFU *E. coli* per 100 ml water. The pooled numbers of *E. coli* cells and enterococci in the CHTW samples did not differ significantly from the numbers found in the RHRW samples. It should be noted that 58% of households in this study did not use any filtration methods; therefore, the presence of fecal indicators in the CHTW samples was not unexpected. Ten (42%) households had USF installed; however, these systems do not appear to be effective in removing fecal indicators. For example, households H3, H8, H11, H12, H15, H18, and H35 had USF; however, the numbers of fecal indicators in CHTW samples did not differ significantly from those in rainwater samples.

Five (21%) of the 24 RHRW tanks tested in this study contained *Campylobacter* spp. H6, H10, H12, and H15 were four of the five households where there were overhanging trees (H10 and H12) or evidence of wildlife fecal droppings (H6 and H15) on the roofs. Two households (H12 and H15) had USF installed; however, *Campylobacter* spp. were detected in the CHTW samples, suggesting the poor efficacy of USF systems. For *Campylobacter* spp., most human infections (i.e., 95%) are caused by *C. jejuni* and *Campylobacter coli* (9), and therefore, All *Campylobacter* PCR-positive samples were further tested for the presence of *C. jejuni* and *C. coli*. Three RHRW tank and two CHTW samples contained *C. coli*. None of the RHRW tank and CHTW samples, however, contained *C. jejuni* (data not shown). *G. lamblia* was detected in three (13%) of the RHRW tanks tested in this study. H1 and H7 were two of the three households where there was evidence of wildlife fecal droppings. All three CHTW samples contained *G. lamblia*. It should be noted that these households did not apply any filtration methods for rainwater purification prior to drinking. The high numbers of *G. lamblia* cells in both RHRW and CHTW samples from households H1, H7, and H14 may pose serious health risks to the consumers because of the low infectious dose of *Giardia.*

To obtain insight into the magnitude of the health risks, the numbers of genomic copies of *G. lamblia* were converted to cyst numbers. The *G. lamblia* β-giardin gene is expressed as a single-copy gene within the nucleus of each trophozoite (20). 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 (6) and 16 copies of the β-giardin gene per *Giardia* cyst (16). The number of *G. lamblia* cysts appeared to be 1 order of magnitude higher in rainwater samples in this study than in our previous study (2). It should be noted that in the current study, 20 liters of water samples was tested whereas in the previous study a smaller volume (i.e., 2 to 2.5 liters) of water samples was tested. The concentration of a large volume of water samples may have increased the detection sensitivity (22). *C. parvum* could not be detected in any of the samples tested; however, the presence of *Cryptosporidium* spp. in RHRW samples has been reported in the U.S. Virgin Islands and Denmark (3, 12). *Salmonella* spp. were detected in only one rainwater tank, and none of the CHTW samples were positive for *Salmonella* spp.

Wild animals, such as birds, mammals, and reptiles, are the most likely sources of fecal contamination in RHRW, as they have access to the roof surface. In all, 60% of possum and 24% of bird fecal samples contained *Campylobacter* spp. All bird fecal samples contained *C. jejuni*. None of the possum fecal samples contained *C. jejuni* (data not shown). Possum and bird fecal samples also contained *G. lamblia*, and the numbers of cysts ranged from 2.1×10^1 to 1.6×10^3 (for possums) and 1.3×10^0 to 1.2×10^2 (for birds) per g of feces. Previous research studies also reported the presence of *G. lamblia* in possum and bird feces in North Island, New Zealand (10, 26). In this study, five possum and two bird fecal samples were also positive for *C. parvum*. The prevalence of *C. parvum* in possum and bird fecal samples was lower than that of *G. lamblia*. Chilvers et al. (10) reported similar findings and suggested that this could be because the duration of *Cryptosporidium* infection is much shorter than that of *Giardia* infection. It should be noted that *Giardia* cysts were also detected in fecal samples from cats, rats, and mice, and therefore, these animals may also contribute *Giardia* to rainwater tanks (10). 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 protozoan pathogens in rainwater tanks.

Around 10% of the population in Australia currently use RHRW as a major source of their drinking water (5). To date, several disease outbreaks and clinical cases associated with rainwater consumption have been reported (4, 8, 27). In contrast, an epidemiological study of young children in South Australia reported that the consumption of RHRW did not increase the risk of gastroenteritis as opposed to municipal water (18). The results of the current study indicate that certain householders were potentially exposed to pathogenic bacteria and protozoa; however, no increase in reported cases of illnesses was evident. 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 (17). 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. It is acknowledged that the qPCR methods used in the study do not provide information on what fraction of PCR-detected cells or cysts were viable and infective. This is one of the major limitations of PCR-based methods. A number of possum ($n = 5$) and bird ($n = 3$) fecal samples were tested for the presence of *Campylobacter* spp. using both conventional and PCR-based methods. Four possum and all three bird fecal samples were positive for *Campylobacter* spp. with both methods (data not shown).

The fecal contamination of RHRW appears to be limited to improperly designed systems, as well as systems that are not well maintained. It has been suggested that all RHRW 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 the water (13). The roof should be kept clear of overhanging trees, which may provide access for wild animals. Indeed, the high numbers of bacterial and protozoan pathogens in possum and bird fecal samples indicates the need for good maintenance of roofs and gutters and elimination of overhanging tree branches to minimize fecal contamination of

RHRW. 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 protozoan pathogens in rainwater tanks. In a previous study, after estimating the health risks associated with rainwater use, it was suggested that rainwater be disinfected before using it as potable water, especially for drinking (2). The householders were asked to provide information on the types of filters installed in their USF systems, as well as information on the maintenance regimes. Certain householders did not follow the manufacturer's instructions, and therefore, the presence of fecal indicators and pathogenic microorganisms in CHTW samples was not unexpected. The quality of the RHRW and CHTW can be improved by implementing effective point-of-use treatment procedures, such as filtration followed by disinfection by UV treatment, ozone disinfection, or ultramembrane filtration.

In conclusion, household tap water fed from rainwater tanks in Currumbin Ecovillage appears to be highly variable and of poor microbiological quality. The presence of one or more fecal indicators, along with the potential bacterial and protozoan pathogens, suggests that RHRW may not be suitable for drinking. Although 42% of householders filtered RHRW prior to drinking it, the poor microbiological quality suggests the inefficacy of the filtration methods that are being used. In view of this, it is recommended that RHRW should be disinfected using effective treatment procedures prior to drinking. The high prevalence of bacterial and protozoan pathogens in possum and bird fecal samples indicates these animal species may be the sources of fecal contamination in rainwater tanks. Therefore, maintenance of good roof and gutter hygiene and elimination of overhanging tree branches and other structures should be considered where possible to prevent the flocking of possums and birds.

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