**Evidence of Avian and Possum Fecal Contamination in Rainwater Tanks as Determined by Microbial Source Tracking Approaches**

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**ABSTRACT**

Avian and marsupial fecal droppings may negatively impact roof-harvested rainwater (RHRW) water quality due to the presence of zoonotic pathogens. This study was aimed at evaluating the performance characteristics of a possum feces-associated (PSM) marker by screening 210 fecal and wastewater samples from possums (*n* = 20) and a range of non-possum hosts (*n* = 190) in Southeast Queensland, Australia. The host-sensitivity and -specificity of the PSM markers were 0.90 and 0.95 (maximum value of 1.00). The mean concentrations of the GFD markers in possum fecal DNA samples (8.8 × 107 gene copies per g of feces) were two orders of magnitude higher than non-possum fecal DNA samples (5.0 × 105 gene copies per g of feces). The host-sensitivity, -specificity and concentrations of the avian-feces associated GFD markers have been reported in our recent study (Ahmed et al. 2016). The utilities of the GFD and PSM markers were evaluated by testing a large number of tank water samples (*n* = 134) from the Brisbane and Currumbin areas. GFD and PSM markers were detected in 39 of 134 (29%) and 11 of 134 (8%) tank water samples, respectively. GFD markers in PCR positive samples ranged from 3.7 × 102 to 8.5 × 105gene copies per L, whereas the PSM markers ranged from 2.0 × 103to 6.8 × 103 gene copies per L of water. The results of this study suggest the presence of fecal contamination in tank water samples from avian and marsupial hosts. To the best of our knowledge, this is the first study that established an association between the degradation of microbial tank water quality with avian and marsupial feces. Based on the results, we recommend disinfection of tank water, especially for tanks designated for potable use.

**Keywords:** Harvested rainwater; microbial source tracking; fecal indicator bacteria; quantitative PCR; health risks; molecular markers

**INTRODUCTION**

Growing water scarcity has led to the increased reliance on alternative and decentralized potable and non-potable water resources in recent decades. Australia is the driest inhabited continent on earth, and suffered from a severe “millennium” drought from 2001 to 2009 (Dijk et al. 2013). As a result of water scarcity in this region, the use of roof-harvested rainwater (RHRW) (stored in tanks) for domestic purposes is a widely accepted practice. This is beneficial for simultaneously conserving water and reducing stormwater runoff. The presence of multiple microbial pathogens including opportunistic pathogens in rainwater tanks have been reported by several studies (Ahmed et al. 2008; Ahmed et al. 2014; Dobrowsky et al. 2014) supporting the necessity for the management of potential health risks.

Pathogens could be introduced to tanks via roof runoff containing fecal matter from birds, insects, bats, possums and reptiles. The microbiological quality of RHRW stored in tanks is generally assessed by monitoring *Escherichia coli* (*E. coli*), which are commonly found in the gut of warm-blooded animals (Albrechtsen 2002; Ahmed et al. 2008; Lee et al. 2010). The presence of *E. coli* in tank water generally indicates fecal contamination and the potential for public health risks. Drinking water guidelines have been used to assess the microbial quality of the tank water. For most guidelines, this entails the non-detection of *E. coli* in 100 mL of water (NHMRC–NRMMC 2004; WHO 2004). Even when tank water is not used for drinking, the assessment of the microbial quality is usually undertaken by monitoring *E. coli* (Ahmed et al. 2008, Ahmed et al. 2010a; Appan 1997; Dillaha and Zolan 1985). One major limitation of *E. coli* for monitoring is that they fail to predict the presence of pathogens in water sources (Hörman et al. 2004; McQuaig et al. 2009; Selvakumar and Borst 2006). In a previous study, the presence of *E. coli* did not correlate with the presence of potential pathogens including opportunistic pathogens such as *Aeromonas hydrophila*, *Campylobacter jejuni*, *Campylobacter coli*, *Legionella pneumophila*, *Salmonella* spp., and *Giardia lamblia* in tank water samples (Ahmed et al. 2010a; Ahmed et al. 2014).

Another limitation of *E. coli* is that their presence does not provide information regarding their sources (Harwood et al. 2000; US EPA 2005). Identifying the source(s) of fecal contamination in tank water is critical for implementing appropriate remediation and protecting potential human health risks associated with designated water use. Water quality researchers are currently using microbial source tracking (MST) markers to detect fecal contamination in environmental waters (McQuaig et al. 2009; Prystajecky et al. 2014; Ufnar et al. 2006). However, the application of MST markers to identify the sources of fecal contamination in rainwater tank samples is rare. Previously, an attempt was made to identify the likely sources of clinically significant *E. coli* in rainwater tanks by analysing *E. coli* isolates from tank water and fecal samples from birds and possums. Biochemical phenotypes of *E. coli* strains carrying virulence genes from a small number of tank water samples were identical to a number of biochemical phenotypes of *E. coli* strains carrying the same virulence genes from bird and possum feces. These findings suggested that these animals may be the likely sources of *E. coli* strains identified in tank water samples (Ahmed et al. 2012a). However, the detection of virulence genes followed by biochemical phenotyping are time consuming, expensive and process requires the collection and fingerprinting a large number of *E. coli* isolates.

A recent study has reported the development of a qPCR assay for the identification of avian feces-associated GFD markers of unclassified *Helicobacter* spp. in various host groups in the United States, Canada and New Zealand (Green et al. 2012). The distributions of the GFD markers in avian feces across the USA, Canada and New Zealand suggested it might have broad applicability for MST studies in other parts of the world (Green et al. 2012). A recent follow up study in Australia and the USA also reported the high host-specificity of the GFD markers, and suggested that this marker could be used as a reliable marker to detect the presence of avian fecal contamination in environmental waters (Ahmed et al. 2016). Another study developed a possum PCR marker based on the *Bacteroidales* 16S rRNA sequences (Devane et al. 2013). The authors did not name the marker; here, we designate it “PSM markers”. The host-sensitivity (0.83) and -specificity (0.96) values of the PSM markers in tested possum fecal (*n* = 36) and non-possum fecal samples (*n* = 233) were high.

The primary aim of this study was to evaluate the host-sensitivity and -specificity of PSM markers by analysing fecal samples from a variety of host groups in Southeast Queensland, Australia. The host-specificity and -sensitivity values of the GFD marker were reported in a recent study for Southeast Queensland (Ahmed et al. 2016). Tank water samples (presumed to be affected with fecal contamination) were collected from the Brisbane and Currumbin Ecovillage. GFD and PSM markers were quantified using qPCR from tank water samples along with the enumeration of *E. coli* using a culture-based method. The host-sensitivity and -specificity of the GFD and PSM markers along with their distribution in tank water samples were then used to validate the presence of avian and possum fecal contamination in tank water samples.

**MATERIALS AND METHODS**

**Animal fecal and wastewater sampling.** To determine the host-sensitivity and -specificity of the PSM marker, individual and composite fecal and wastewater samples were collected from various host groups in Brisbane and Currumbin areas (Table 1).Additional information on the fecal and wastewater samples is given in the Supplementary Note S1. All samples were transported on ice to the laboratory, stored at 4°C, and processed within 24 h.

**Concentration of cattle and pig wastewater samples.** Cattle and pig wastewater samples were concentrated with Amicon® Ultra-15 (30 K) Centrifugal Filter Devices (Merck Millipore Ltd., Tokyo, Japan). In brief, 10 mL of wastewater sample was added to the Amicon Device, and centrifuged at 4,750 *g* for 10 min. Entire volumes (180-200 µL) of concentrated samples were collected from the filter device sample reservoir using a pipette (Ahmed et al. 2010b). The concentrated samples were stored at -20°C for a maximum of 24 h prior to DNA extraction.

**Study areas and sanitary inspection.** 134 rainwater tanks were sampled from various areas of Brisbane (*n* = 84) and the Currumbin Ecovillage (*n* = 50), both located in Southeast Queensland, Australia. The Ecovillage is a decentralized residential development that employs a range of strategies to conserve water and energy including a cluster-scale sewage treatment/water reclamation plant, rainwater storage tanks, solar panels, and source-separated urine usage. 100% and 20% of rainwater tanks are used for potable use in Brisbane and Currumbin, respectively, in addition to other non-potable uses such as cooking, showering and gardening. On each property, a visual sanitary inspection was undertaken to identify factors such as the presence of overhanging trees, TV aerials, wildlife fecal droppings, absence of first flush diverters that can affect the quality of RHRW stored in tanks.

**Tank water sampling**. The tap/spigot connected directly to the rainwater tank was wiped with 70% ethanol, and the water was run for 15 s prior to filling a 10 L sterile container. In the absence of a tap, samples were collected directly from openings in the top of the tank. Samples were transported to the laboratory, kept at 4°C, and processed within 6-72 h.

**Enumeration of *Escherichia coli*.** Colilert® (IDEXX Laboratories, Westbrook, Maine, USA) Test kits were used to determine the concentrations of *E. coli* in 100 mL of each tank water sample. Test kits were incubated at 37 ± 0.5°C for 18-24 h as per the manufacturer’s recommendation.

**Concentration of rainwater samples.** Approximately 10 L water sample from each tank was concentrated by a hollow-fiber ultrafiltration system (HFUF) using Hemoflow FX 80 dialysis filters (Fresenius Medical Care, Bad Homberg, Germany) as previously described (Hill et al. 2007). Briefly, 1 g of sodium hexametaphosphate (NaPP, Sigma-Aldrich, St. Louis, Missouri, USA) was added to each 10 L rainwater sample to achieve a concentration of 0.01% (w/v). Each water sample was pumped with a peristaltic pump (Adelab Scientific, South Australia, Australia) in a closed loop with sterile high-grade Norprene® A60 f tubing (Adelab Scientific). The tubing was sterilized by soaking in 3% bleach, washing with deionized water, and autoclaving at 121°C for 15 min. The sample was concentrated to approximately 150-200 mL, depending on turbidity. 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. To improve recovery, after each sample was processed through the HFUF, 500 mL of elution solution [0.5% Tween 80, (Sigma-Aldrich, St. Louis, Missouri, USA) 0.01% NaPP, and 0.001 Antifoam A (Sigma-Aldrich, St. Louis, Missouri, USA)] was recirculated through the filter for 5 min, and then concentrated in the same manner as the sample to 150 mL. This elution solution was added to the concentrated sample to achieve a final volume of approximately 300-400 mL and stored at -4°C. A new filter cartridge was used for each sample. The combined concentrate was filtered through a 0.45 µm cellulose filter paper (Advantec, Tokyo, Japan), and stored at -80°C until DNA extraction. In case of filter clogging, multiple filter papers were used for each sample.

**DNA extraction.** DNA was extracted from the concentrated cattle and pig wastewater samples using DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA). A QIAamp Stool DNA Kit (Qiagen, Valencia, California, USA) was used to extract DNA from 100-220 mg of fresh animal feces and 250 µL raw human wastewater samples. A PowerSoil® Max DNA Kit (Mo Bio, Carlsbad, California, USA) was used to extract DNA from tank water samples according to the manufacturer’s instructions and stored at -80°C until use. The kit was modified slightly with 2 mL of DNA eluted buffer C6 instead of 5 mL (Gyawali et al. 2015). DNA concentrations were determined using NanoDrop spectrophotometer (ND-1000, NanoDrop Technology, Wilmington, Delaware, USA).

**PCR inhibition.** An experiment was conducted to determine the presence of PCR inhibitory substances in tank water DNA samples using a Sketa22 real-time PCR assay (Haugland et al. 2005). Of the 134 samples, 23 (17%) had the sign of PCR inhibition. These inhibited samples were 10-fold serially diluted, and further tested with the Sketa22 real-time PCR assay. The results indicated the relief of PCR inhibition. Based on the results, neat DNA samples (PCR uninhibited samples) and 10-fold diluted (PCR inhibited) samples were tested with GFD and PSM qPCR assays.

**Preparation of qPCR standards.** Standards for the GFD qPCR assay were prepared from a gene fragment amplified from bird feces and cloned into the pGEM-T Easy vector system II (Promega, Madison, Wisconsin, USA). Plasmid DNA was isolated using the Plasmid Mini Kit (Qiagen, Valencia, California, USA). Standards for the PSM qPCR assay were designed using IDT custom gene synthesis to construct plasmids inserted with a gene fragment containing 152 bp target (TGC AAG TCG AGG GGT AAC AGG GCC TAG CAA TAG GCC GCT GAC GAC CGG CGC ACG GGT GAG TAA CAC GTA TCC AAC CTG CCG ATA ACT CGG GGA TAG CCT TTC GAA AGA AAG ATT AAT ACC CGA TAG CAT AAG GAT TCC GCA TGG TCT CCT TA) from the known sequence produced by Integrated DNA Technologies (pIDTSmart with ampicillin), and cloned into a vector followed by plasmid extraction (IDTDNA.com; Coralville, Iowa, USA). The purified recombinant plasmids were serially diluted to create standards ranging from to 1 × 106 to 1 copies per µL of DNA extract. A 3 µL template from each serial dilution was used to prepare a standard curve for each qPCR assay. For each standard, the genomic copies were plotted against the cycle number at which the fluorescence signal increased above the quantification cycle value (*Cq* value). The amplification efficiency (*E*) was determined by analysis of the standards and was estimated from the slope of the standard curve as *E* = 10-1/slope.

**qPCR assays**. qPCR assays were performed using previously published primers, probes, and cycling parameters (see Supplementary Table S1 for more details). GFD and PSM qPCR amplifications were performed in a 20 µL reaction mixture using Sso FastTM EvaGreen Supermix (Bio-Rad Laboratories, California, USA). The qPCR mixtures contained 10 µL of Supermixes, 100 nM of each primer (GFD assays), 250 nM of each primer (PSM assay) and 3 µL of template DNA. To separate the specific product from non-specific products, including primer dimers, a melting curve analysis was performed for each qPCR run. During melting curve analysis, the temperature was increased from 65 to 95°C at 0.5°C increment. Melting curve analysis showed a distinct peak at temperature 84.0°C ± 0.2°C (for GFD assay) and 85.5°C ± 0.2°C (for PSM assay), indicating positive and correct amplifications. Standards (positive controls) and sterile water (negative controls) were included in each qPCR run. All qPCR reactions were performed in triplicate using a Bio-Rad® CFX96 thermal cycler.

**qPCR performance characteristics.** qPCR standards were analysed in order to determine the amplification efficiencies (*E*) and the correlation coefficient (*r*2). The qPCR lower limit of quantification (LLOQ) was also determined from the standard series. The lowest concentration of gene copies from the standard series detected in all triplicate samples was considered qPCR LLOQ.

**Quality control.** Method blank runs were performed to ensure that the disinfection procedure was effective in preventing carryover contamination between sampling events. In addition, to prevent DNA carryover contamination, reagent blanks were included for each batch of DNA samples. No carryover contamination was observed. To minimize qPCR contamination, DNA extraction and qPCR setup were performed in separate laboratories.

**Statistical analysis.**

The host-sensitivity and -specificity of the PSM marker were determined as follows: host-sensitivity = a/(a + b) and -specificity = c/(c + d), where a is true positive (possum fecal samples were positive for the PSM marker), b is false negative (non-possum fecal samples were negative for the PSM marker), c is true negative (non-possum fecal samples were negative for the PSM marker), and d is false positive (non-possum fecal samples were positive of the PSM marker) (Ahmed et al. 2016). Samples were considered quantifiable when the PSM marker levels were above the qPCR LLOQ. Samples that fell below the LLOQ level were considered as positive but not quantifiable. The concentrations of *E. coli*, GFD and PSM markers in tank water samples were not normally distributed (as determined by a Kolmogorov-Smirnov and Shapiro-Wilk normality tests). Therefore, non-parametric Spearman rank correlation with a two-tailed *P* value was also used to establish the relationship between *E. coli* and markers (GFD and PSM) in tank water samples.

**RESULTS**

**Sanitary inspection results.** Roofs connected to the rainwater tanks from Brisbane had more overhanging trees present (17%) compared to Currumbin (7%) (Fig. 1). Sanitary inspection also identified more wildlife droppings on Brisbane roofs (63%) than Currumbin (43%). 81% Currumbin tanks had first flush diverters installed, whereas, only 29% Brisbane tanks had the first flush devices. Brisbane roofs also had more TV aerials (76%) installed compared to Currumbin (26%).

**qPCR performance characteristics and lower limit of quantification (LLOQ).** qPCR standards were analysed in order to determine the performance characteristics such as slope, amplification efficiencies, and correlation coefficient values. The standards had a linear range of quantification from 1 × 106 to 1 gene copies per µL of DNA extracts. qPCR performance characteristics for individual assays within the values prescribed by the MIQE Guidelines (Bustin et al. 2009) (see Supplementary Table S2). The lowest amount of diluted gene copies detected in triplicate samples was considered qPCR LLOQ. qPCR LLOQ was determined to be 3 gene copies for the GFD assay, 30 gene copies for the PSM assay.

**Host-specificity and -sensitivity of the GFD and PSM markers.** The host-sensitivity and -specificity values of the GFD marker have been reported in a previous study (Ahmed et al. 2016) using the same set of fecal and wastewater samples analysed for the PSM marker in this study. The host-sensitivity of the GFD marker in avian feces (*n* = 36) was 0.58 (maximum value of 1). The host-specificity of the GFD marker was also high (0.94) for non-avian fecal samples (*n* = 190). In this study, among the 20 possum fecal samples tested, 18 were PCR positive for the PSM marker (Table 1). Therefore, the host-sensitivity of the PSM marker in DNA samples from possum feces was 0.90 (maximum value of 1). Among the 190 non-possum fecal DNA samples tested, 180 samples were negative for the PSM marker, yielding a host-specificity value of 0.95. Small numbers of cat (*n* = 4), deer (*n* = 2), sheep (*n* = 3) and waterfowl (*n* = 1) fecal DNA, however, were positive for the PSM marker. Several horse, human, koala, and emu fecal DNA samples also showed PCR amplifications for the PSM marker, however, had different melting peaks of < 84.5°C or > 86.5°C compared to the correct melting peak of 85.5°C for the PSM marker.

**Concentrations of PSM markers in possum and non-possum fecal samples.** The concentrations of the PSM marker in possum fecal DNA samples (from Brisbane) were highly variable per gm of feces (Fig. 2). The mean concentrations in these samples ranged from 1.7 × 105 to 1.1× 109 gene copies per g of feces. The mean concentration of the PSM marker in non-possum host groups ranged from 1.1 × 104 to 6.4 × 104 (cat), 3.7 × 104 to 1.1 × 105 (deer), 1.5 × 104 to 2.5 × 105 (sheep) and 4.3 × 106 (waterfowl) gene copies per g of feces. The overall mean concentration of the PSM marker in possum was 8.8 × 107, two orders of magnitude higher than the non-possum host groups (5.0 × 105). A t-test for equal means indicated that the mean concentration of PSM markers in possum feces was significantly different (*P* = 0.02) than non-possum feces.

**Concentrations of *E. coli*, GFD and PSM markers in tank water samples.** Of the 84 tank water samples tested from Brisbane,70% were positive for *E. coli*, whereas, of the 50 tank water samples tested from Currumbin Ecovillage,34 (68%) were positive for *E. coli*. Concentrations of *E. coli* in positive samples are shown in Fig.3. Concentrations of *E. coli* ranged from 1 to > 2,420 MPN per 100 mL for Brisbane tank water samples and from 1 to 435 MPN per 100 mL of water for Currumbin tank water samples. *E. coli* concentrations were significantly (*P* = 0.03) higher in Brisbane tank water samples than Currumbin. A t-test for equal means indicated that the mean concentration of *E. coli* in Brisbane tank water samples was significantly different (*P* = 0.01) than Currumbin.

Of the 84 tank water samples tested from Brisbane, 27 (32%) and 5 (6%) were PCR positive for the GFD and PSM markers, respectively. Similarly, of the 50 tank water samples tested from Currumbin, 12 (24%) and 6 (12%) were PCR positive for the GFD and PSM markers, respectively. Of the 84 tank water samples from Brisbane, 31 (37%) contained at least one marker and 1 (1%) tanks contained both markers. Of the 50 tank water samples from Currumbin 16 (32%) contained at least one marker and 2 (4%) tanks contained both markers. GFD markers were more prevalent in both Brisbane and Currumbin areas than the PSM markers. Concentrations of GFD and PSM markers in positive tank water samples are shown in Fig. 4. GFD markers ranged from 9.3 × 102to 3.0 × 105 gene copies per L of water (Brisbane) and 3.7 × 102to 8.5 × 105(Currumbin). PSM markers ranged from 2.7 × 103to 6.8 × 103 gene copies per L (Brisbane) and 2.0 × 103to 6.1 × 103(Currumbin) per L of water. The t-test for equal means indicated that the mean concentration of the GFD markers in tank water samples from Brisbane was significantly different (*P* = 0.007) than Currumbin. However, the PSM marker concentration did no differ significantly in Brisbane and Currumbin tank water samples. Pearson's correlation was used to test the relationship between *E. coli* concentrations with the GFD and PSM marker concentrations. The concentrations of the GFD markers negatively correlated with concentrations of *E. coli* (*rp* = -0.07, *P* = 0.04). The concentrations of the PSM markers also did not correlate with concentrations of *E. coli* (*rp* = 0.09, *P* = 0.25).

**Agreement and disagreement between the presence of markers and sanitary inspection results.** In all, 47 of 134 tank water samples from the Brisbane and Currumbin had either the GFD or the PSM markers.The presence/absence of GFD and PSM markers, and visual sanitary inspection results were compared pairwise for these tank water samples. From the pairwise comparison, the percentage of agreement (co-occurrence) (i.e., presence of a marker in the presence of a factor potentially affecting the quality tank water) and agreement (non-co-occurrence) (i.e., absence of a marker in the absence of a factor) were calculated. The percentage of total disagreement (i.e., presence of a marker in the absence of a factor or absence of a marker in the presence of a factor) for each pair wise comparison was also calculated by subtracting the percentage of agreement (co-occurrence and non-co-occurrence) from 100%. On average 36% (co-occurrence) and 17% (non-co-occurrence) agreements were found for the GFD markers with sanitary inspection results. These values for the PSM markers were 7% and 36% for co-occurrence and non-co-occurrence, respectively (Fig. 5). The GFD markers and the presence of wildlife droppings and TV aerial had the highest percentage (47%) of co-occurrence agreement followed by 38% co-occurrence agreement for first flush diverters. The PSM markers and the presence of TV aerial and wildlife droppings had 11% and 9% of co-occurrence agreements, respectively.

**DISCUSSION**

The numbers of rainwater tanks as a source of water for urban and rural households around the world are increasing. For example, 26% of Australian households used a rainwater tank as a source of water in 2010 compared with 19% in 2007 and 17% in 2004 (ABS 2010). There was a marked increase in the proportion of households with a rainwater tank in Queensland, Australia (17% in 2004 to 36% in 2010). In our previous studies, we have reported the presence of potential bacterial pathogens including opportunistic pathogens and protozoa in rainwater tank samples from Southeast Queensland, Australia (Ahmed et al. 2012b; Ahmed et al. 2014). If the untreated tank water is used for drinking, there are potential disease risks for people consuming this water. Therefore, it is essential to obtain information on the sources of fecal contamination in order to design management strategies and minimize public health risks from exposure to these pathogens.

In this study, we investigated the potential sources of fecal contamination in a large number of tank water samples from urban (Brisbane) and peri-urban (Currumbin Ecovillage) settings in Southeast Queensland, Australia. During the visual sanitary inspection, fecal droppings were spotted on the roofs and gutters for certain tanks. Possums, bats and avian (different species of birds) were identified as potential sources of fecal contamination on the roofs by the residents. Since monitoring *E. coli* does not provide definitive information on the sources of fecal contamination, two newly developed MST markers targeting avian and possum hosts were chosen for this study (Green et al. 2012; Devane et al. 2013). The performance characteristics of the GFD markers were evaluated in a recent study (Ahmed et al. 2016). Although, the GFD marker exhibited high host-specificity (0.94), the host-sensitivity value (0.58%) was low. On the other hand, little is known regarding the host-specificity and -sensitivity of the PSM marker.

The host-sensitivity (0.83) and -specificity (0.96) of the PSM markers were reported to be high in the original study that developed this marker by screening 36 possum and 233 non-possum fecal samples in New Zealand (Devane et al. 2013). The authors recommended that host-sensitivity and -specificity of the PSM markers need to be tested prior to field application in a new location since bacterial markers do not often exhibit absolute host-specificity and -sensitivity (Carson et al. 2005; McQuaig et al. 2009; Devane et al. 2013). In this study, the host-sensitivity and -specificity of the PSM marker determined to be 0.90 and 0.95, respectively, which were well within the recommended guidelines by the US EPA (US EPA 2005), and also similar to the values reported by Devane and Colleagues (2003).

The concentrations of the PSM marker in individual possum fecal sample varied 3-4 orders of magnitude. However, the mean concentration (8.8 × 107 gene copies per g) obtained in this study was similar to the range (1.6-1.0 × 107) reported by Devane and colleagues (2003). The variation of the PSM marker in individual possum fecal samples could be attributed to factors such as diet, which may vary both regionally and seasonally (Shanks et al. 2011; Turnbaugh et al. 2009). This has implications because a marker with variable and/or low concentrations in its host(s) can be difficult to detect in waters due to factors such as dilution, and inactivation potential (Ahmed et al. 2015). Further study would be required to shed light on the variability of this marker in a large number of possum fecal samples in order to identify factors that may be responsible for such variability.

In this study, approximately 70% tank water samples tested, exceeded the Australian Drinking Water Guideline of zero *E. coli* per 100 mL (ADWG 2004). The frequency of detection and concentrations of *E. coli* were significantly higher in Brisbane tank water samples than Currumbin. This could be due to the fact that the Currumbin Ecovillage is a new sub-division, and most of the rainwater tanks installed here are relatively new compared to tanks in Brisbane. Since all Currumbin tanks are used for drinking, residents put more effort in maintaining the quality of water by installing first flush diverters, and other cleanliness practices such as trimming of overhanging trees and cleaning the gutters more frequently. Such practices were not observed for Brisbane area as only 20% tanks are used for potable use. These factors collectively may have contributed to the high frequency of detection and concentrations of *E. coli* in Brisbane tank water samples.

Overall, the concentrations of *E. coli* were highly variable ranging from 1-2,420 MPN per 100 mL of water, suggesting the occurrence of fecal contamination. The results were in accordance with the facts that 29% and 8% of 134 tank water samples from the Brisbane and Currumbin were PCR positive for the GFD and PSM markers, respectively. GFD markers were more frequently detected in tank water samples than the PSM marker. This could be due to the fact that *Helicobacter* spp. associated GFD markers may have better survival ability in the tank environment than the *Bacteroides* associated PSM marker, which is an obligate anaerobe. The frequencies of GFD and PSM markers detection were higher for Brisbane tank water samples than Currumbin. Again this could be related to the poor maintenance practices of Brisbane tanks.

The GFD (Ahmed et al. 2016) and PSM markers (this study) were detected in small numbers of cat, dog, deer, kangaroo, sheep, and waterfowl fecal DNA samples. Their presence in dog, deer, kangaroo and sheep may not be problematic due to the fact that roof contamination with feces from these animals is unlikely. During the sanitary inspection, we did not observe any cats or waterfowls on the roof. However, tank water contamination from these sources cannot be ruled out. This phenomenon may not be a critical issue as long as the concentrations of the GFD and PSM markers remain low in non-avian and non-possum host groups. The mean concentrations of the GFD and PSM marker in non-avian and non-possum fecal DNA samples were 2-3 orders of magnitude lower than those in avian and possum fecal DNA samples. The concentrations of the markers in certain tank water samples were as high as 8.5 × 105 (GFD) and 6.8 × 103 (PSM) per L of water. This has public health implications as bird and possum feces are known to contain *Campylobacter* spp., *Cryptosporidium* spp., *Giardia* spp., and clinically significant *E. coli* (Marino et al. 1998; Chilvers et al. 1998; Ahmed et al. 2012a; Ahmed et al. 2012b).

Overall 70% and 90% tank water samples were PCR negative for the GFD and PSM markers. Presence of low levels of avian and possum fecal contamination in these tank water samples cannot also be ruled out because the lower limit of detection (LLOD) of the qPCR assays ranged from 1 to 3 gene copies per µL DNA which translates to approximately 67- 200 gene copies per L of water sample that would need to be present for qPCR detection. 43% tank water samples had > 1 *E. coli* per 100 mL of water, however, these samples were negative for the GFD and PSM markers. The sources of fecal contamination in these tanks may have originated from bats, insects, frogs, and lizards. Since, both the GFD and PSM markers did not show absolute sensitivity when tested against avian and possum hosts, it is also possible that avian and possum fecal contamination may be occurring in certain tanks, but the markers were absent in the feces of those animals contaminated tank water samples, and, therefore, and could not be detected with qPCR assays.

Concentrations of *E. coli* did not correlate with the concentrations of the GFD and PSM markers in tank water samples from both Brisbane and Currumbin. Therefore, this study is also in accordance with findings that *E. coli* monitoring is not likely to be a reliable surrogate for general fecal contamination and presence of pathogens as previously reported (Ahmed et al. 2010; Ahmed et al. 2014). Lack of a relationship between *E.* coli and MST markers likely reflects differences in methodologies, where *E. coli* analysis provides viable concentration of *E. coli* and MST marker detection/quantification provides the information on the presence/absence of host-specific fecal contamination and its magnitude. In addition, MST markers come from a specific host group, whereas, *E. coli* come from all warm-blooded animals. Furthermore, the fate (inactivation) of the GFD and PSM marker could be different than *E. coli* (Lu et al., 2011). A similar lack of correlation has been reported for other MST marker concentrations with *E. coli* concentrations (Lee et al. 2013; McQuaig et al. 2012).

An attempt was taken to determine what sanitary factors might have contributed GFD and PSM markers in tank waters. The data indicated that wildlife droppings and the presence of TV aerials had the highest percentage of co-occurrence with the presence of both GFD and PSM markers. However, this data should be interpreted with care because it is difficult to differentiate among factors that may have contributed GFD and PSM markers into the tank water. For example, roof connected to the tank T62 (see Supplementary Table S3) had overhanging trees, fecal droppings, no first flush diverter, and TV aerial. The presence of the GFD in the tank water samples could be associated with one or more of these factors. 38% and 6% agreements were observed for the GFD and PSM markers with the presence of first flush diverters suggesting that this device may not be effective in removing microbial contaminants.

The presence of *E. coli* along with the presence of GFD and PSM markers suggests the occurrence of fecal contamination in tank water samples from avian and marsupial hosts. To the best of our knowledge, this is the first study that established a potential link between the degradation of microbial quality of tank water with avian and marsupial feces. Such findings can pose a significant health risk for those residents solely depend on tank water for potable use, especially in Brisbane and Currumbin areas. However, the potential risk would be much lower for non-potable uses such as car washing, gardening etc. There are several potential factors that may have contributed to the avian and possum fecal contamination in tank water samples. Therefore, maintenance of good roof and gutter hygiene and elimination of overhanging tree branches, blocking off possum access points by placing timber or mesh or placing fake predators like owls and hawks on the roofs may be clever way to trick birds staying off the roof.

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**TABLE 1** Percentage of possum and non-possum fecal DNA samples PCR positive for the possum marker

|  |  |  |  |
| --- | --- | --- | --- |
| Host groups | No. of samples | Sources of samples | No. of PCR positive samples (%) |
| Bird | 10 | Wild, farm | 0 (0) |
| Cat | 13 | Veterinary hospital | 4 (31) |
| Cattle feces | 4 | Farms | 0 (0) |
| Cattle wastewater | 8 | Abattoir | 0 (0) |
| Deer | 12 | Sanctuary | 2 (17) |
| Dog | 13 | Veterinary hospital and parks | 0 (0) |
| Emu | 14 | Emu park | 0 (0) |
| Goat | 10 | Veterinary hospital | 0 (0) |
| Horse | 19 | Horse racecourse | 0 (0) |
| Humanfeces | 3 | Human | 0 (0) |
| Human wastewater | 20 | Wastewater treatment plants | 0 (0) |
| Kangaroo | 10 | Sanctuary | 0 (0) |
| Koala | 12 | Sanctuary | 0 (0) |
| Pig wastewater | 20 | Abattoir | 0 (0) |
| Possum | 20 | Wild | 18 (90) |
| Sheep | 14 | Veterinary hospital | 3 (21) |
| Waterfowl | 8 | Parks | 1 (13) |
| Host-sensitivity |  |  | 0.90 |
| Host-specificity |  |  | 0.95 |

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**FIG 1** Sanitary inspection results for rainwater tanks from Brisbane (*n* = 84) and Currumbin (*n* = 50) in Southeast Queensland, Australia.

**FIG 2** Concentration of possum (PSM) markers in possum and non-possum fecal DNA samples in Southeast Queensland, Australia.

**FIG 3** Box and whisker plots of the concentrations (MPN per 100 mL) of *Escherichia coli* in positive tank water samples from Brisbane and Currumbin. The inner box lines represent the medians, while the outer box lines represent the 5th and 95th data percentiles, and the whiskers extend to the range.

**FIG 4** Box and whisker plots of the concentrations (gene copies per L) of avian feces-associated GFD and possum feces-associated PSM markers in positive tank water samples from Brisbane and Currumbin. The inner box lines represent the medians, while the outer box lines represent the 5th and 95th data percentiles, and the whiskers extend to the range.

**FIG 5** Percentage agreement and disagreement between the presence of avian feces-associated GFD and possum feces-associated PSM markers and sanitary inspection results.