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Distributions of Fecal Markers in Wastewater from Different Climatic Zones for Human Fecal Pollution Tracking in Australian Surface Waters

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Recreational and potable water supplies polluted with human wastewater can pose a direct health risk to humans. Therefore, sensitive detection of human fecal pollution in environmental waters is very important to water quality authorities around the globe. Microbial source tracking (MST) utilizes human fecal markers (HFMs) to detect human wastewater pollution in environmental waters. The concentrations of these markers in raw wastewater are considered important because it is likely that a marker whose concentration is high in wastewater will be more frequently detected in polluted waters. In this study, quantitative PCR (qPCR) assays were used to determine the concentrations of fecal indicator bacteria (FIB) *Escherichia coli* and *Enterococcus* spp., HFMs *Bacteroides* HF183, human adenoviruses (HAdVs), and polyomaviruses (HPyVs) in raw municipal wastewater influent from various climatic zones in Australia. *E. coli* mean concentrations in pooled human wastewater data sets (from various climatic zones) were the highest (3.2×10^6 gene copies per ml), followed by those of HF183 (8.0×10^5 gene copies per ml) and *Enterococcus* spp. (3.6×10^5 gene copies per ml). HAdV and HPyV concentrations were 2 to 3 orders of magnitude lower than those of FIB and HF183. Strong positive and negative correlations were observed between the FIB and HFM concentrations within and across wastewater treatment plants (WWTPs). To identify the most sensitive marker of human fecal pollution, environmental water samples were seeded with raw human wastewater. The results from the seeding experiments indicated that *Bacteroides* HF183 was more sensitive for detecting human fecal pollution than HAdVs and HPyVs. Since the HF183 marker can occasionally be present in nontarget animal fecal samples, it is recommended that HF183 along with a viral marker (HAdVs or HPyVs) be used for tracking human fecal pollution in Australian environmental waters.

Direct monitoring of pathogenic microorganisms in water resources is likely to provide important information regarding public health risks. However, routine monitoring for a wide variety of pathogenic microorganisms can be expensive and challenging due to their uneven distribution among the host population and the affected waters. The microbiological quality of water is generally assessed by monitoring fecal indicator bacteria (FIB), such as *Escherichia coli* and *Enterococcus* spp., using culture-based methods (1, 2). These FIB are abundant in the feces of warm-blooded animals. The presence of elevated levels of FIB in environmental waters indicates not only the occurrence of fecal pollution but also the likely presence of pathogenic microorganisms that are capable of causing illnesses in exposed humans. For the remediation of polluted water bodies, it is vital for water utilities and regulators to identify the source(s) of the fecal pollution. However, monitoring FIB alone does not provide information regarding their origin due to their presence in all warm-blooded animals, including humans (3, 4). This major limitation can be resolved by application of microbial source tracking (MST) techniques, which can identify and quantify the source(s) of fecal pollution in environmental waters (5, 6).

Numerous MST techniques targeting bacteria (7–9), protozoa (10), and viruses (11, 12) have been reported in the literature. Among the bacterial targets, *Bacteroides* markers hold promise as alternative indicators of fecal pollution owing to a number of advantages, including short survival rates outside the hosts, exclusivity to the guts of warm-blooded animals, occurrence as a larger portion of fecal bacteria than to FIB, and inability to proliferate in the environment (7, 13, 14). A number of PCR- and quantitative PCR (qPCR)-based methods have been developed to detect and quantify human- and animal-associated *Bacteroides* markers in environmental waters (7, 15–18). Among the human-associated *Bacteroides* markers, HF183 has been studied extensively, and several PCR/qPCR assays have been developed to detect and quantify this marker in environmental waters (7, 19–21). Among the enteric viruses, human adenoviruses (HAdVs) and human polyomaviruses (HPyVs) have received significant attention as MST markers due to their high abundance in the feces and urine of hosts, high persistence in environmental waters, and strict host association (12, 22–24).

The successful field application of any MST marker depends on several performance characteristics, such as host specificity, host prevalence (also known as host sensitivity), evenness, and relevance to health risks (6, 25). Host specificity testing has been the focal point of many MST evaluation studies (26–29). The host

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TABLE 1 Description of the wastewater treatment plants that were selected for this study

Wastewater treatment plant	Location	Climatic conditions	Treatment process	Vol of wastewater treated per day (Ml)
A	Brisbane	Subtropical (no dry season)	Activated sludge	54
B	Perth	Mediterranean (dry summer)	Activated sludge	135
C	Hobart	Temperate (mild summer)	Activated sludge	6.6

specificity of the *Bacteroides* HF183 marker has been well studied around the globe (26-28, 30). However, host specificity is not an important issue for viral MST markers due to their strict host association (31).

Host prevalence is also considered an important performance characteristic because it is likely that a highly host-prevalent marker will be more frequently detected in polluted water samples. Many studies have reported the host prevalence values of the HF183, HAdV, and HPyV markers by analyzing individual fecal and wastewater samples using binary PCR (positive/negative) (8, 12, 26, 27, 32). The host prevalence value of a particular marker in the host population may vary geographically due to uneven distribution. It has been recommended that host prevalence be determined across a geographic region and verified in a new geographic region (6–9, 32, 33). In our previous studies, we determined the host prevalence (as a percentage) of the HF183, HAdV, and HPyV markers in subtropical Southeast Queensland, Australia, by testing raw wastewater samples using PCR (22, 34, 35). However, little is known regarding the concentrations of these markers in raw wastewater samples from different climatic zones in Australia. This information is important for identifying whether these makers can be reliably used for the detection of human fecal pollution in the surface waters in various climatic zones.

The aims of this study were (i) to determine the concentrations of FIB (*E. coli* and *Enterococcus* spp.) and human fecal markers (HFMs) *Bacteroides* HF183, HAdVs, and HPyVs in raw wastewater samples using qPCR assays, (ii) to examine the differences in the concentrations of FIB and HFMs in wastewater treatment plants (WWTPs) from three different climatic zones in Australia, and (iii) to determine any correlations that exist among FIB and HFMs in raw wastewater. Finally, the concentrations of HFMs in environmental water samples seeded with raw wastewater were used to support their usefulness for MST field studies across Australia.

MATERIALS AND METHODS

Human wastewater sampling. WWTPs representing three different climatic zones, Brisbane, Perth, and Tasmania, were selected for this study (Table 1). WWTP A is located in Brisbane, Queensland, and treats human wastewater from approximately 250,000 people. The treatment process consists of a primary treatment, a secondary treatment (activated sludge), and disinfection with chlorine and UV prior to discharge of the wastewater into the Brisbane River. WWTP B is located in Perth, Western Australia, and treats human wastewater from approximately 600,000 people. The treatment process is similar to that in WWTP A. However, the wastewater is not subjected to UV disinfection as in WWTP A prior to being discharged into the Indian Ocean. WWTP C is located in Hobart, Tasmania, and treats human wastewater from 35,000 people. Prior to being discharged into the Coral River or Derwent River, chlorinated wastewater is passed through a 10-μm filter.

Raw wastewater grab samples (approximately 100 ml each) were collected in sterile bottles from the influent of each WWTP. The samples were collected in triplicate using a telescopic bailer device from each of the WWTPs studied over a period of 11 weeks in early September to late November 2014. In total, 33 samples were collected from each WWTP. Samples were transported on ice to the laboratory and stored at 4°C.

DNA extraction. DNA was extracted from an aliquot of 250 μl of raw wastewater sample using the MO Bio PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA) with minor modifications (36). The extraction protocol was amended to allow the utilization of all supernatant at each step and, therefore, increased volumes of solutions C3 and C4 were added to compensate. Extracted DNA was eluted through the spin filter membrane by addition of 100 μl of solution C6, followed by storage at −80°C. Each DNA sample was quantified using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technology, Wilmington, DE).

PCR inhibition testing. To obtain information on the level of PCR inhibition, all wastewater DNA samples were spiked with 10 pg of *Oncorhynchus keta* DNA (Sigma Chemical Co., St. Louis, MO) and tested with the Sketa22 real-time PCR assay as described elsewhere (37). PCR inhibition was not detected in any of the DNA samples tested.

qPCR assays. qPCR standards for *E. coli* 23S rRNA, *Enterococcus* 23S rRNA, and HAdVs were prepared from the genomic DNA of *E. coli* ATCC 35150, *Enterococcus faecalis* ATCC 19433, and HAdV strain 41 ATCC VR-930 as described elsewhere (38, 39). qPCR standards for HF183 and HPyVs were prepared from the plasmid DNA (26, 40). Next 10-fold dilutions ranging from 1×10^6 to 1 copy per μl of genomic and plasmid DNA standards were prepared and stored at −20°C. A 3-μl template from each dilution was used to prepare a standard curve for each qPCR assay. The primer sequences and amplification conditions for the qPCR assays used in this study are shown in Table S1 in the supplemental material. The *E. coli*, *Enterococcus* spp., HAdV, and HPyV qPCR assays were performed in 20-μl reaction mixtures using 10 μl of SsoAdvanced universal probe supermix (Bio-Rad Laboratories), 800 nM each primer and 80 nM probe (*E. coli*), 500 nM each primer and 400 nM probe (*Enterococcus* spp.), 250 nM each primer and 250 nM probe (HAdV) and 250 nM each primer and 200 nM probe (HPyV), and 3 μl of template DNA. The HF183 qPCR assays were performed in 20 μl of reaction mixture using 10 μl of iQ SYBR green supermix (Bio-Rad Laboratories), 300 nM each primer, and 3 μl of template DNA. To separate the specific product from nonspecific products, including primer dimers, melting curve analysis was performed for the HF183 qPCR. During melting curve analysis, the temperature was increased from 65 to 95°C at 0.5°C increments. All qPCRs were performed in triplicate. For each qPCR assay, a negative (sterile water) control was included.

qPCR performance characteristics. The qPCR standards were analyzed in order to determine the amplification efficiencies (E) and the correlation coefficient (r^2). The qPCR performance characteristics are shown in Table S2 in the supplemental material. The repeatability (intra-assay agreement) and reproducibility (interassay agreement) of each qPCR assay were assessed by determining the percent coefficient of variation (CV) (41). The CV values were calculated from the quantification cycle (C_q) values of each standard ranging from 3×10^6 to 3 gene copies. The intra-assay repeatability was calculated based on the C_q values by testing each dilution 10 times in the same qPCR run. The interassay reproducibility was calculated based on the C_q values by testing each standard on 5 different days. The mean intra-assay repeatability and interassay reproducibility CV for the qPCR assays are shown in Fig. S1 in the supplemental material. The qPCR lower limit of quantification (LLOQ) was also determined from the C_q values obtained for each standard. The smallest amount of diluted standard detected in 100% triplicate assays was consid-

ered the qPCR LLOQ. The LLOQ of the qPCR was determined to be 30 gene copies for all five assays.

Seeding experiment. A 15-liter river water sample was collected from the Brisbane River at a site located in the lower portions of the river in a highly urbanized area. River water samples were stored at 4°C for no more than 1 h before processing. In addition, a 1-liter raw wastewater sample was collected from WWTP A. For the qPCR analysis of *E. coli*, *Enterococcus* spp., and HF183, 490-ml water samples ($n = 3$) were seeded with 10-ml raw wastewater samples. Water samples were serially diluted (10^{-1}, 10^{-2}, and 10^{-3}) and filtered through 0.45-μm pore size (90-mm diameter) nitrocellulose membranes (Millipore, Tokyo, Japan). For the qPCR analysis of HAdVs and HPyVs, another batch of 490-ml water samples ($n = 3$) were also seeded with 10-ml raw wastewater samples and serially diluted (10^{-1}, 10^{-2}, and 10^{-3}). HAdVs and HPyVs were concentrated using a previously published method (40). The method began with adjustment of each water sample pH to 3.5 using 2.0 N HCl. Water samples were then passed through 0.45-μm, 90-mm diameter negatively charged HA membranes (HAWP09000; Merck Millipore Ltd., Sydney, Australia) via a glass funnel and base (Merck Millipore Ltd.). All of the membranes were then placed in 50-ml PowerMax bead solution tubes. Nucleic acid was extracted directly from the membranes using a Mo Bio PowerMax soil DNA isolation kit. Extracted bacterial and viral nucleic acid was eluted through the spin filter membranes by addition of 2 ml of solution C6 and stored at −20°C until processed. The background concentrations of *E. coli*, *Enterococcus* spp., HF183, HAdVs, and HPyVs in river water samples were enumerated using qPCR assays as described above.

Statistical analysis. The concentrations of FIB and HFMs in raw wastewater samples were not normally distributed (as determined by a Kolmogorov-Smirnov test). Therefore, the nonparametric Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's posttest was performed to determine if there were any significant differences in FIB and HFM concentrations within and among WWTPs. The nonparametric Spearman rank correlation with a two-tailed P value was also used to establish the relationship between FIB and HFM concentrations in raw wastewater samples. In general, $r = >0.7$ was considered a strong positive correlation, $r = >0.4$ but <0.7 was moderate correlation, and $r = >0.2$ but <0.4 was weak correlation. GraphPad Prism 6 was used for statistical analysis (GraphPad Software, Inc.).

Chemometric and statistical analyses were performed with SIMCA 14 (Umetrics AG, Umeå, Sweden). The cutoff level for significant features was kept at a false-discovery rate (FDR) (q value) of ≤0.1 and P values at ≤0.05. Unsupervised data were analyzed by principal-component analysis (PCA). Furthermore, in order to accommodate within-group analysis, partial least-squares discriminant analysis (PLS-DA) was employed.

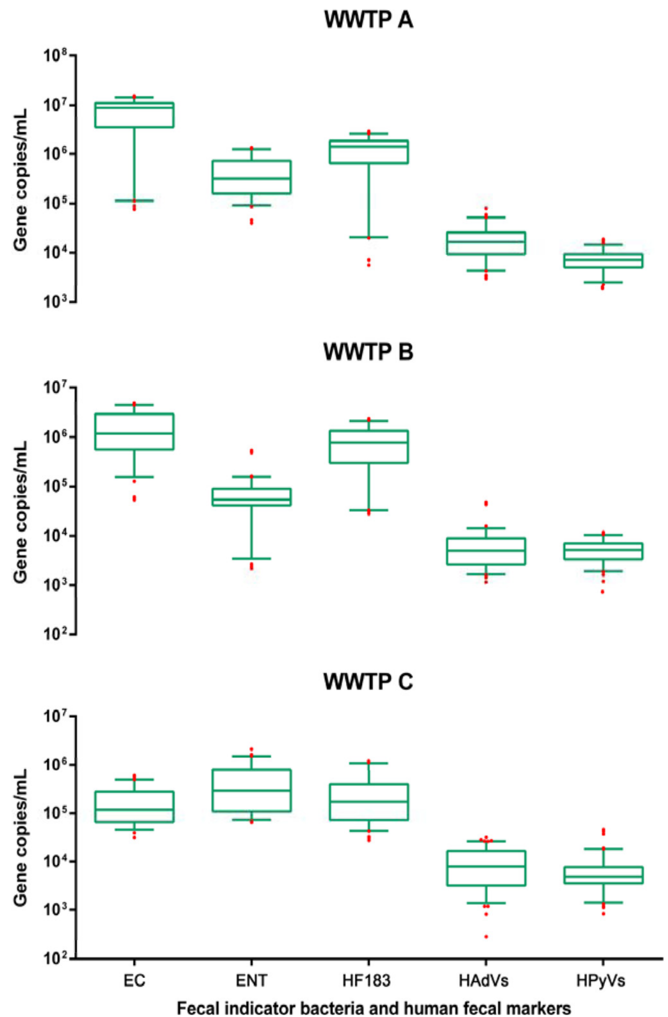

FIG 1 Box and whisker plots of the concentrations (gene copies per milliliter) of *Escherichia coli* (EC), *Enterococcus* spp. (ENT), sewage-associated *Bacteroides* (HF183), human adenoviruses (HAdVs), and human polyomaviruses (HPyVs) in raw wastewater samples collected from three wastewater treatment plants (WWTPs A, B, and C) in Australia. The upper and lower boxes denote the 75th and 25th percentiles, respectively. The upper and lower bars show the 95th and 5th percentiles, respectively, with outliers represented by red circles. Note the logarithmic vertical axis.

RESULTS

Concentrations of FIB and HFMs in raw wastewater samples. All wastewater DNA samples were determined to have concentrations of *E. coli*, *Enterococcus* spp., HF183, HAdVs, and HPyVs that were greater than the qPCR LLOQ. The concentrations of FIB and HFMs in raw wastewater samples from WWTP A, B, and C are shown in Fig. 1 in a box and whisker plot format. Among the five targets tested, the mean *E. coli* concentrations were the highest (7.6×10^6 gene copies per ml) in raw wastewater samples from WWTP A, followed by those of HF183 (1.3×10^6 gene copies per ml) and *Enterococcus* spp. (4.7×10^5 gene copies per ml). The mean concentrations of HAdVs (2.1×10^4 gene copies per ml) and HPyVs (7.7×10^3 gene copies per ml) were 2 to 3 orders of magnitude lower than those of FIB and HF183. Similar trends in FIB and HFM concentrations were also observed for WWTP B. However, the concentrations of FIB and HFMs in raw wastewater samples from WWTP C did not follow the same pattern. The mean concentration of *Enterococcus* spp. was the highest (5.2×10^5 gene copies per ml), followed by those of HF183 (2.9×10^5 gene copies per ml) and *E. coli* (1.8×10^5 per gene copies per ml). The mean concentrations of *E. coli* in pooled data sets (from three WWTPs) were the highest (3.2×10^6 gene copies per ml), followed by those of HF183 (8.0×10^5 gene copies per ml) and *Enterococcus* spp. (3.6×10^5 gene copies per ml). The HAdV and HPyV concentrations were 2 to 3 orders of magnitude lower than those of FIB and HF183.

The Kruskal-Wallis one-way ANOVA was undertaken to determine if there are any significant differences in the FIB and HFM concentrations in each WWTP. Dunn's multiple comparisons posttest indicated that the concentrations of FIB and HFMs in raw wastewater samples collected from WWTP A were significantly different ($P < 0.05$) from each other except for the results for *Enterococcus* spp. versus HF183, which were not significant ($P > 0.05$) (see Table S3 in the supplemental material). For WWTP B, the concentrations of FIB and HFMs were significantly different

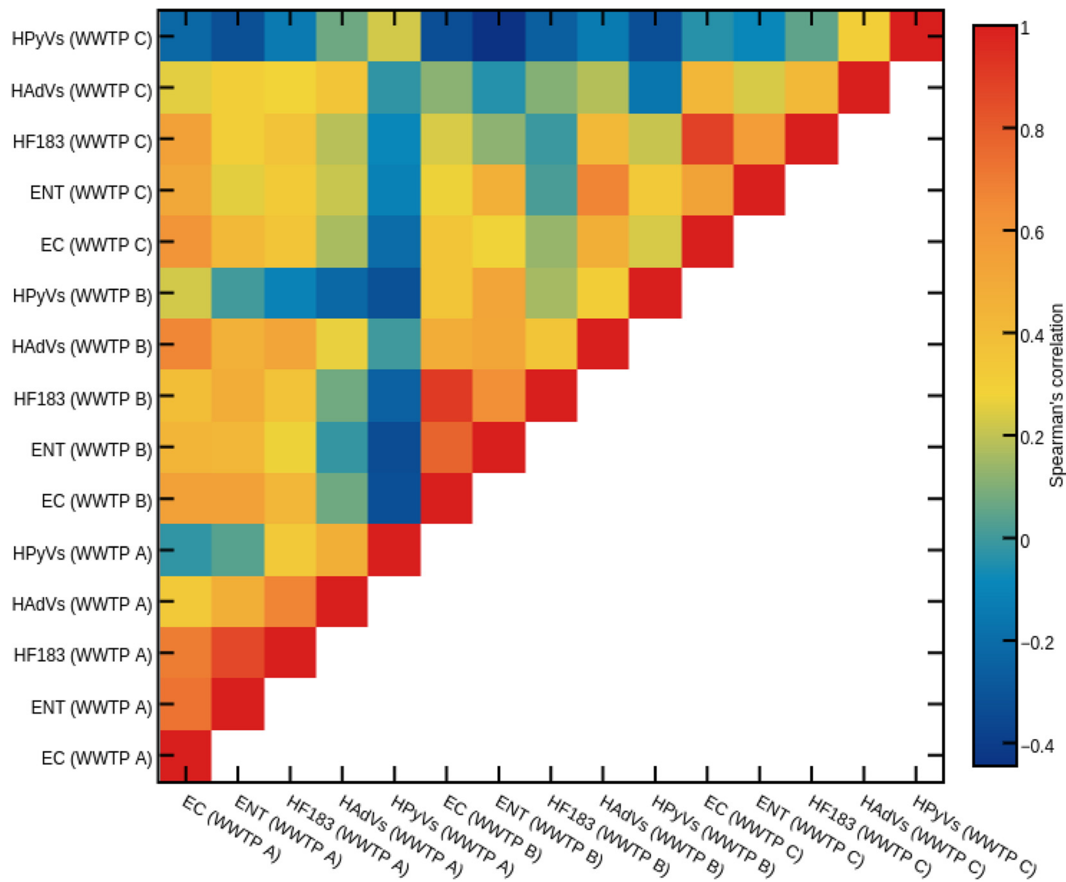

FIG 2 Spearman correlations among *E. coli* (EC), *Enterococcus* spp. (ENT), *Bacteroides* HF183, human adenoviruses (HAdVs), and human polyomaviruses (HPyVs) in raw wastewater samples collected from three wastewater treatment plants (WWTP A, B, and C).

($P < 0.05$) for most of the comparisons except for *E. coli* versus *Enterococcus* spp. ($P > 0.05$) and *E. coli* versus HF183 ($P > 0.05$), which yielded nonsignificant results ($P > 0.05$). Similar results were also observed for WWTP C except for both FIB versus HF183 ($P > 0.05$) and HAdVs versus HPyVs ($P > 0.05$), which yielded nonsignificant results ($P > 0.05$). For the pooled data sets, the concentrations of FIB and HFMs were significantly different ($P < 0.05$) from each other except for *Enterococcus* spp. versus HF183, which yielded nonsignificant results ($P > 0.05$).

An ANOVA was also undertaken to determine if there are any significant differences in the FIB and HFM concentrations across the three WWTPs. The concentrations of *E. coli*, HF183, and HAdVs were significantly different across the WWTPs ($P < 0.05$). The concentrations of *Enterococcus* spp. were significantly different between WWTPs A and B ($P < 0.05$) and between WWTPs B and C ($P < 0.05$). No significant difference was observed between WWTPs A and C ($P > 0.05$) in terms of *Enterococcus* concentrations. The concentrations of HPyVs were significantly different between WWTPs A and B ($P < 0.05$) and between WWTPs A and C ($P < 0.05$). No significant difference was observed between WWTPs B and C in terms of HPyV concentrations ($P > 0.05$).

Correlations between FIB and HFMs in raw wastewater samples. FIB (*E. coli* versus *Enterococcus* spp.) and HFMs (HF183 versus HAdVs versus HPyVs) and FIB versus HFMs showed significant cross-correlations with each other within and across WWTPs. A heat map of the Spearman rank order correlation matrix is shown in Fig. 2. Among the 105 bivariate comparisons, 11 (10.5%) showed strong positive correlations ($r = 0.728$ to 0.950; $P < 0.0001$) and 41 (39%) showed no or negative correlations ($r = -0.023$ to 0.171; $P < 0.05$) (see Table S4 in the supplemental material). Strong positive ($r > 0.7$) to weak ($r > 0.2$ but < 0.4) correlations were observed among FIB, HF183, and HAdVs in raw wastewater samples from WWTPs A and C. HPyVs did not correlate with FIB. Strong positive to weak correlations were also observed among FIB and HFMs in raw wastewater samples from WWTP B. For the pooled data sets, strong positive to weak correlations were observed among FIB, HF183, and HAdVs. However, HPyVs did not correlate with FIB (see Table S4).

In order to explore the differences between the three WWTPs, a chemometric analysis of FIB and HFMs was undertaken. First, an unsupervised PCA plot was created and was observed to not discriminate samples. (No clusters or groups were observed, as evident in Fig. S2 in the supplemental material.) DCrit (critical value of DModX), derived from the F-distribution, calculates the size of the observational area under analysis. As illustrated in Fig. S3 in the supplemental material, the DModX plot of the PCA data indicates that there are no samples that exceed the threshold for rejecting a sample. The threshold for a moderate outlier is considered when the sample DModX value is twice the DCrit at 0.05, which in this instance was 3.425.

In order to discriminate among the WWTPs further and investigate within-group variations, a PLS-DA model was applied to

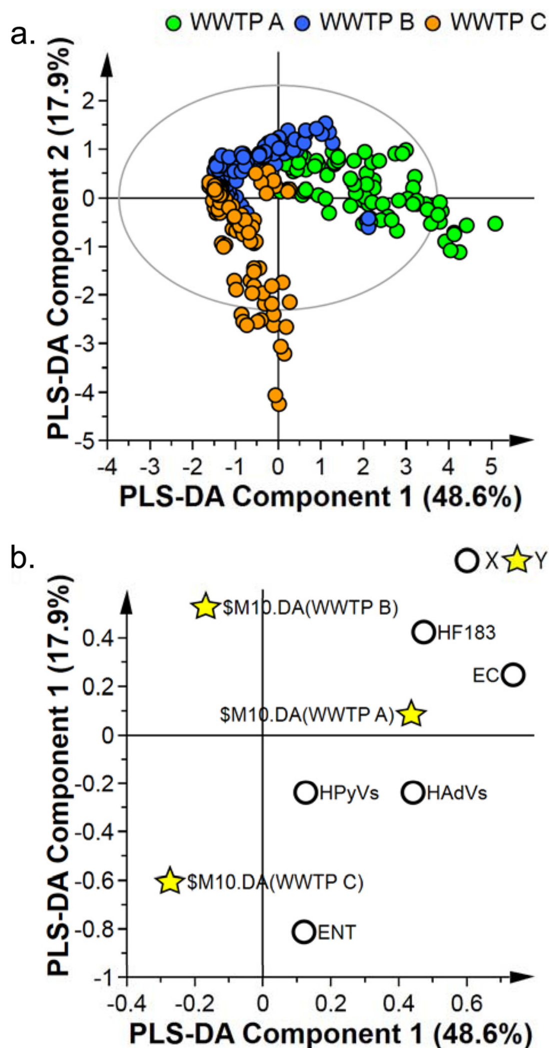

FIG 3 Partial least-squares discriminant analysis (PLS-DA) of the concentrations (gene copies per milliliter) of *Escherichia coli* (EC), *Enterococcus* spp. (ENT), sewage-associated *Bacteroides* (HF183), human adenoviruses (HAdVs), and human polyomaviruses (HPyVs) in raw wastewater samples collected from three WWTPs (A, B, and C). (a) PLS-DA score scatter plot; (b) PLS-DA loading scatter plot. Each point on the scatter plot refers to a single sample, with R^2X (cumulative) = 72.7%, R^2Y (cumulative) = 51.4%, and Q^2 (cumulative) = 50.3%. The ellipse represents the 95% confidence level.

the same data set. The subsequent PLS-DA score scatter plot and PLS-DA loading scatter plot are presented in Fig. 3 and illustrate clear separation between the treatment plants, with R^2X, R^2Y, and Q^2 values of 0.727, 0.514, and 0.503, respectively. This result is indicative of a model that moderately fits the data (R^2X of >0.7) but has a poor predictive capability (Q^2). As evident in the loading plot (Fig. 3b; see also Fig. S4 in the supplemental material) and subsequent one-way ANOVA ($P < 0.05$) with an applied Fisher least significant difference (LSD) *post hoc* analysis using a false-discovery rate (FDR) value (*q* value) of 0.1, it was observed that WWTP A > WWTP B > WWTP C in regard to *E. coli* concentrations (P = 1.283e−53 and FDR of 6.415e−53). Furthermore, WWTP C had elevated values of *Enterococcus* spp. (P = 3.5376e−17 and FDR of 4.4219e−17) and HAdVs (P = 2.0262e−17 and FDR of 3.77e−17) in comparison to those of WWTPs A and B. In addition, WWTPs A and B had elevated levels of HF183 compared to those of WWTP C (P = 7.5847e−23 and FDR of 1.8962e−22). Last, WWTP A was found to have slightly elevated levels of HPyVs in comparison to those of WWTP B, while WWTP C had greater HPyV concentrations than WWTP B (P = 0.04789 and FDR of 0.00479).

To further investigate within-group comparisons, three additional within-group PLS-DA models were prepared to compare WWTP A to B (model 1), WWTP A to C (model 2), and WWTP B to C (model 3). In this analysis, it was observed that *E. coli* had fold changes of 4.4653 (P = 3.9544e−25), 42.752 (P = 1.77e-36), and 9.5742 (6.7289e−22) for model 1, model 2, and model 3, respectively. *Enterococcus* spp. had fold changes of 6.1651 (P = 2.6326e−20) and 0.14385 (4.7049e−15) for model 1 and model 3, respectively. HAdVs had fold changes of 3.0163 (P = 7.4878e−14) and 2.0539 (P = 4.3253e−9) for model 1 and model 2, respectively. Last, HF183 had fold changes of 4.2878 (P = 1.6386e−25) and 2.8671 (3.9245e−13) for model 2 and model 3, respectively.

Concentrations of FIB and HFMs in Brisbane River water samples seeded with raw wastewater. HAdVs and HPyVs were not detected in ambient Brisbane River water samples. However, the samples were PCR positive for *E. coli*, *Enterococcus* spp., and HF183, but the results were not quantifiable (less than the LLOQ of 30 gene copies). The concentrations of FIB and HFMs in 3-μl river water (seeded with raw wastewater) DNA samples are shown in Table 2.

The mean concentration of *E. coli* was 1.1×10^2 gene copies per 3 μl of DNA at the dilution 10^{-3} (represents 10 μl of raw wastewater seeded into 490-ml water samples). However, at this dilution, *Enterococcus* spp. were not detected. Among the three HFMs, the mean concentration of HF183 was 1.8×10^2 gene copies per 3 μl of DNA at the dilution 10^{-3}, whereas HAdV and HPyV concentrations were, respectively, 2.4×10^1 and 8.1×10^1 gene copies per 3 μl of DNA at the dilution 10^{-1} (represents 1 ml of sewage). HAdVs and HPyVs were detected at the dilution 10^{-2} (represents 100 μl of sewage) but the concentrations were not quantifiable.

DISCUSSION

Host prevalence is generally expressed as a percentage of samples from a given host that test PCR positive for a given marker (12, 27, 34, 42, 43). The closer the values are to 100%, the greater the prevalence and the better the performance of a marker. However, knowing the concentration of a marker in its host is important because it is likely that a marker whose concentration is high will be consistently and more easily detected in polluted water samples. In our previous studies, we have determined the host prevalences of the HF183, HAdV, and HPyV markers in a small number of individual fecal samples and in septic, raw, and treated wastewater samples collected from Southeast Queensland, Australia using binary PCR (22, 26, 35). In the current study, qPCR assays were used to determine the concentrations of HF183, HAdVs, and HPyVs along with two FIB in raw wastewater samples collected from three different climatic zones in Australia for 11 sampling events.

Notably, the concentrations of the *E. coli* 23S rRNA gene in WWTP C were 1 to 2 orders of magnitude lower than those in WWTPs A and B. This might be attributed to the fact that WWTP C treats a much lower volume of human wastewater from a smaller population than WWTPs A and B. It is also possible that

TABLE 2 Concentrations of fecal indicator bacteria and human fecal markers in raw wastewater-seeded Brisbane River water DNA samples

Amt of raw wastewater seeded	Concn (mean ± SD gene copies) of FIB and MST marker per 3-μl DNA sample[a]				
	FIB		HFMs		
	E. coli	*Enterococcus* spp.	*Bacteroides* HF183	HAdVs	HPyVs
10 ml	$9.4 \times 10^4 \pm 1.9 \times 10^4$	$2.8 \times 10^4 \pm 2.8 \times 10^3$	$3.0 \times 10^4 \pm 4.8 \times 10^3$	$1.5 \times 10^3 \pm 1.5 \times 10^3$	$9.6 \times 10^2 \pm 1.1 \times 10^2$
1 ml (10^{-1})	$7.8 \times 10^3 \pm 8.3 \times 10^2$	$2.1 \times 10^3 \pm 5.7 \times 10^2$	$2.2 \times 10^3 \pm 9.5 \times 10^1$	$2.4 \times 10^1 \pm 1.6 \times 10^1$	$8.1 \times 10^1 \pm 3.4 \times 10^1$
100 μl (10^{-2})	$1.0 \times 10^3 \pm 1.1 \times 10^2$	$2.8 \times 10^2 \pm 5.6 \times 10^1$	$4.5 \times 10^2 \pm 6.7 \times 10^1$	NQ	NQ
10 μl (10^{-3})	$1.1 \times 10^2 \pm 2.2 \times 10^1$	ND	$1.8 \times 10^2 \pm 2.5 \times 10^1$	ND	ND

[a] FIB, fecal indicator bacteria; HFM, human fecal markers; HAdVs, human adenoviruses; HPyVs, human polyomaviruses; ND, not detected; NQ, not quantifiable.

WWTP C is located in a cool temperate region in Tasmania compared to WWTPs A and B, which are located in subtropical and Mediterranean-like climatic zones, respectively. *E. coli* cells are able to grow outside the intestine, especially in environmental waters in warmer tropical and subtropical climatic zones (44, 45). Given the potential for growth ability in tropical and subtropical climatic zones, *E. coli* concentrations can be artificially elevated above the expected level from fecal inputs alone. The concentrations of *E. coli* in WWTPs A and C were 1 to 1.5 orders of magnitude higher than those of *Enterococcus* spp. However, the *E. coli* concentrations in WWTP C were 0.5 order of magnitude lower than those of *Enterococcus* spp. The reason for such a discrepancy is not well understood; however, climatic variations may play a role.

Little is known regarding the effects of temporal and climatic variations on the concentrations of HFMs in raw wastewater samples in Australia. It has been reported that climatic variability may influence the prevalence and concentrations of *Bacteroides* markers (27, 46). If the concentration of a marker is highly variable in human wastewater, then it is likely that it may not be detected in environmental waters in the presence of human fecal pollution. The mean concentrations of the HF183 and HAdV markers across all three WWTPs were 8.0×10^5 and 1.3×10^4 gene copies per ml of raw wastewater, respectively. Similar levels of gene copies of these markers have been reported in raw wastewater samples from Spain, Japan, Italy, and the United States (26, 30, 47–50).

Little information on the concentrations of HPyVs in raw wastewater has been documented. McQuaig and colleagues (12) reported that the concentrations of HPyVs in raw wastewater might be as high as 4.7×10^4 gene copies per ml, which is comparable to the concentrations obtained in this study. The concentrations of all three HFMs in raw wastewater samples showed small or no temporal variations over the course of the study (see Table S5 in the supplemental material). Taken together, the high concentrations and small temporal variations of HFMs in raw wastewater samples from all three WWTPs across different climatic zones indicate that they might be useful for detecting human wastewater fecal pollution across Australia.

In this study, the correlations between FIB and HFMs in raw wastewater samples were determined. These are particularly important for establishing the fact that the concentrations of FIB can predict the concentrations of HFMs or vice versa. Strong positive correlations were observed between FIB and HF183 in raw wastewater samples from all three WWTPs. HPyVs showed no correlation or a negative correlation with *E. coli* or *Enterococcus* spp. McQuaig and colleagues (12) determined the correlations between FIB (fecal coliform bacteria, *E. coli*, and *Enterococcus* spp.) and HPyVs for human, disinfected, and septic wastewater samples. HPyVs were poorly or negatively correlated with all three FIB tested. Several factors such as the dilution effect, turbidity, differences in analytical methods, and decay may account for the lack of correlations observed. Poor correlations between FIB and viral markers may not necessarily hinder their application as MST tools if the objective of the study is to determine the sources of fecal pollution for the purpose of mitigation.

Sensitive detection of human fecal pollution in environmental waters is important for protecting public health risks because such pollution can impose a direct risk to humans. To identify the most sensitive marker of human fecal pollution, raw wastewater was seeded into river water samples and analyzed for FIB and HFMs. HF183 was quantifiable in the presence of 10 μl of sewage seeded into 500 ml of water. In contrast, HAdVs and HPyVs were quantifiable in the presence of 1 ml of sewage seeded into environmental waters. Both HAdVs and HPyVs were detected (but were not quantifiable) in the presence of 100 μl of sewage. The results of this study also indicate that the HF183 marker is the most sensitive marker compared to HAdVs and HPyVs. This was expected because the concentrations of HF183 in raw wastewater were 2 to 3 orders of magnitude higher than those of HAdVs and HPyVs. Although HF183 is more sensitive, HPyVs and HAdVs have the advantage of greater host specificity in Australia (22, 26, 35). Since the HF183 marker can occasionally be present in nontarget animal fecal samples (26), it is recommended that HF183 along with HAdVs or HPyVs should be used for human fecal pollution tracking in surface waters in Australia.

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