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Evaluation of the *nifH* Gene Marker of *Methanobrevibacter smithii* for the Detection of Sewage Pollution in Environmental Waters in Southeast Queensland, Australia

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ABSTRACT: This study aimed at evaluating the host-specificity and -sensitivity of the *nifH* gene marker of *Methanobrevibacter smithii* by screening 272 fecal and wastewater samples from 11 animal species including humans in Southeast Queensland (SEQ), Australia. In addition, environmental water samples (n = 21) were collected during the dry and wet weather conditions and tested for the presence of the *nifH* marker along with other sewage-associated markers, namely, enterococci surface protein (*esp*) found in *Enterococci faecium*, *Bacteroides* HF183, adenoviruses (AVs), and polyomaviruses (PVs). The overall host-specificity of the *nifH* marker to differentiate between human and animal feces was 0.96 (maximum value of 1), while the overall sensitivity of this marker in human sourced feces and wastewater was 0.81 (maximum value of 1). Among the 21 environmental water samples tested, 2 (10%), 3 (14%), 12 (57%), 6 (29%), and 6 (29%) were positive for the *nifH*, *esp*, HF183, AVs and PVs markers, respectively. The prevalence of the *nifH* marker in environmental water samples, however, was low compared to other markers, suggesting that the use of this marker alone may not be sensitive enough to detect fecal pollution in environmental waters. The *nifH* marker, however, appears to be sewage-specific in SEQ, Australia, and therefore, it is recommended that this marker should be used as an additional marker in combination with the HF183 or viral markers such as AVs or PVs for accurate and sensitive detection of fecal pollution in SEQ waterways.

INTRODUCTION

The source identification of fecal pollution in environmental waters is vitally important to minimize public health risks associated with the exposure to enteric pathogens. Fecal pollution tracking in environmental waters is challenging, however, due to the diffuse nature of different sources of pollution. In recent years, library-independent microbial source tracking (MST) methods have been developed to detect, and in some cases, quantify sewage and animal feces associated markers in environmental waters.[1−3] The commonly used markers include anaerobic bacterial gene markers,[4,5] bacterial toxin gene markers,[6−8] and viral markers.[2,3,9]

Ideally, a MST marker should have certain characteristics such as: (i) it should be specific to only target host group (known as host-specificity), (ii) it should be present in all members within a host group (known as host-sensitivity), (iii) it should exhibit temporal and geographical stability, and (iv) the decay rate should be similar to those of pathogens.[10,11] Among these characteristics, host-specificity and -sensitivity are considered as important traits because these could influence the false positive and negative detection of fecal pollution in environmental waters. It has been reported that certain markers are highly host-specific[8,12−14] while other markers have been reported to exhibit low host-specificity.[15,16] The host-specificity of a particular marker, especially anaerobic bacterial gene or toxin gene markers may vary across different geographical locations.[10,11,17] Because of this, validation of the MST markers against reference fecal samples has been recommended in a new geographical area before application.[11]

It has been reported that the genus *Methanobrevibacter* is found in animal intestinal tracts, decaying plants and anaerobic sludge of sewage treatment plants (STPs).[18] Among the 11 species, *Methanobrevibacter smithii* is known to specifically colonize within the human intestine and vaginal tract, and the numbers in the intestine ranged from 10^7 to 10^{10} per gram (dry weight).[19−21] Because of the host-specificity and high numbers in the human intestine, *M. smithii* may be useful as an indicator of sewage or human fecal pollution in environmental waters. A recent study reported the development of the conventional PCR based method for the detection of the sewage-associated *nifH* gene marker of *M. smithii* in recreational waters.[22]

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Table 1. PCR Positive/Negative Results for Sewage-Associated *nifH* Marker in Sewage and Animal Host-Groups in Southeast, Queensland, Australia

host groups	number of samples	types of samples	volume used for DNA extraction	PCR positive results/numbers of samples tested for the *nifH* marker of undiluted and serially diluted DNA		
				undiluted DNA	diluted (10^{-1})	diluted (10^{-2})
humans						
primary influent	37	composite	10 mL	37/37		
secondary effluent	22	composite	1 L	15/22	15/22	15/22
humans	5	individual[a]	180−220 mg	0/5	0/5	0/5
animals						
birds	30	individual	50−150 mg	1/30	1/30	1/20
camels	4	individual	180−220 mg	0/4	0/4	0/4
cattle	4	individual[a]	180−220 mg	0/4	0/4	0/4
cattle	22	composite	10 mL	0/22	0/22	0/22
chickens	23	individual	150−200 mg	0/23	0/23	0/23
dogs	18	individual	180−220 mg	0/18	0/18	0/18
ducks	15	individual	150−200 mg	0/15	0/15	0/15
horses	15	individual	180−220 mg	0/15	0/15	0/15
kangaroos	17	individual	180−220 mg	0/17	0/17	0/17
pigs	20	composite	10 mL	6/20	6/20	6/20
pigs	10	individual[a]	180−220 mg	0/10	0/10	0/10
possums	30	Individual	150−220 mg	0/30	0/30	0/30

[a] Individual fecal samples were not included in composite fecal samples.

The *nifH* marker appeared to be highly host-specific and has been used to detect sewage pollution in environmental waters in California,[23] Mississippi,[22] and Florida[3] in the U.S. It has to be noted that, however, little is known regarding the host-specificity of this marker outside the U.S.

The primary aim of this study was to evaluate the host-specificity and -sensitivity of sewage-associated *nifH* marker in fecal samples from 11 host groups in Southeast Queensland (SEQ), Australia. In addition to the testing of specific host groups, environmental water samples were tested for the presence of the *nifH* marker along with the other sewage-associated markers [enterococci surface protein (*esp*) found in *Enterococcus faecium*,[8] *Bacteroides* HF183,[4,24] adenoviruses (AVs),[2] and polyomaviruses (PVs).[3] The host-specificity and -sensitivity of the sewage-associated markers along with the PCR results were then used to validate the presence of sewage pollution in SEQ environmental waters.

MATERIALS AND METHODS

Host Groups Sampling. To determine the host-specificity and -sensitivity of the *nifH* marker, 272 fecal samples were collected from 11 host groups using aseptic technique (Table 1). Human fecal samples were collected from the primary influent and secondary effluent of three sewage treatment plants (STPs). In addition, human fecal samples were also collected from individuals. Bird fecal samples were collected from the City Botanical Garden adjacent to Queensland University of Technology (QUT) and Currumbin Wildlife Sanctuary Hospital. Camel fecal samples were collected from the Wivenhoe Dam area. Individual cattle fecal samples were collected from a farm, whereas cattle and pig wastewater samples were collected from an abattoir in Brisbane. Chicken fecal samples were collected from the backyard of a household and a chicken-processing farm. Dog fecal samples were collected from a dog park. Duck fecal samples were collected from areas adjacent to ponds and lakes in the Sunshine Coast region. Horse fecal samples were collected from a horse racecourse. Kangaroo fecal samples were collected from the University of the Sunshine Coast (USC). Possum fecal samples were collected from the rooftops of various households in the Brisbane area.

Environmental Water Sampling. Environmental water samples ($n = 21$) were collected between March 2010 and July 2011 from the Fitzgibbon (FG) stormwater drain ($n = 4$), the Brisbane River (BR) ($n = 3$), Cabbage Tree Creek (CT) ($n = 3$), Oxley Creek (OC) ($n = 3$), and four different sites (i.e., WD1−WD4) from the Wivenhoe Dam (WD) ($n = 4$ sites $\times$ 2 samples = 8 samples) in SEQ. Among the 21 samples, 10 were collected during dry weather conditions and the remaining 11 were collected following wet weather events. The sampling site FG is located in a stormwater drain that receives runoff from a surrounding 290 ha residential catchment. The suspected source of fecal pollution in this site includes sewage network pipes and small numbers of horses and cattle. The site BR is located in the Brisbane River and is tidally influenced. This site receives urban runoff through stormwater drains. Sampling site CT is located in the Cabbage Tree Creek Catchment and contains residential and industrial developments and serviced by a STP. Sampling site OC is a tributary of the Brisbane River. This site is also tidally influenced and is highly populated, and characterized by industrial areas, as well as a STP. Sampling sites WD1, WD2, WD3, and WD4 are located at the WD which is located approximately 80 kms from Brisbane. The catchment area of the dam is approximately 7020 km^2 and has an average annual rainfall of 940 mm. Wivenhoe Dam provides water supply to approximately 60% the population of Brisbane and adjacent regions. The dam is surrounded by cattle farms and receives treated wastewater from

upstream towns. The dam area is also used for camping and other outdoor recreational activities such as swimming and fishing. The suspected sources of fecal pollution are native and feral wildlife, cattle grazing, and human recreational activities. Water samples were collected in sterile plastic containers at 30 cm below the water surface of all sites, and transported to the laboratory and processed within 4−8 h.

Concentration of Water Samples. Variable volumes (ranged from 9 to 19 L) of water samples from each site were concentrated by hollow-fiber ultrafiltration system (HFUS), using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA) as previously described.[25] Briefly, each water sample was pumped with a peristaltic pump (Masterflex: Cole-Parment Instrument Co. Vernon Hills, IL) in a closed loop with high-performance, platinum-cured L/S 36 silicone tubing (Masterflex; Cole-Parmer Instrument Co.). Tubing was sterilized by soaking in 10% bleach, washed with deionized (DI) water and autoclaved at 121 °C for 15 min. At the end of the sample concentration process, pressurized air was passed through the filter cartridge from the top to recover as much samples as possible. A new filter cartridge was used for each sample. The samples were concentrated to approximately 100−150 mL depending on the turbidity of the water samples. Each sample was further centrifuged at 3000*g* for 30 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 5 mL of sterile distilled water.

Isolation and Enumeration of Fecal Indicator Bacteria (FIB). The membrane filtration method was used to process environmental and the sewage spiked water samples for fecal indicator bacteria (FIB) isolation and enumeration. Sample serial dilutions were made and filtered through 0.45 mm pore size (47 mm diameter) nitrocellulose membranes (Millipore, Tokyo, Japan), and placed on modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC agar) (Difco, Detroit, MI) and membrane- Enterococcus indoxyl-D-glucoside (mEI) agar (Difco) for the isolation of *E. coli* and enterococci. 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,[26] while the mEI agar plates were incubated at 41 °C for 48 h.[27]

DNA Extraction. The primary influent samples were purified and concentrated with CentriprepYM-50 concentrator columns (Millipore, Bedford, MA). Samples were added to the CentriprepYM-50 and centrifuged at 1000*g* for 10 min, followed by removal of the sample that passed through the ultrafiltration membrane and further centrifugation at 1000*g* for 10 min to obtain a final volume of 200 μL concentrated sample. Secondary effluent samples were concentrated using the HFUS system. DNA was extracted from the concentrated samples using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). QIAmp Stool DNA Kit was used to extract DNA from fresh feces/wastewater (i.e., 100−200 mg or 10 mL) from each individual animal. For real-time PCR analysis of the *esp* marker, 500 mL of each environmental sample was filtered through a 0.45 mm pore size (47 mm diameter) nitrocellulose membrane (Millipore) and enterococci were isolated as described above. DNA was extracted from 2 mL of enriched enterococci culture using DNeasy Blood and Tissue Kit (Qiagen). For real-time PCR analysis of the *nifH*, HF183, AVs and PVs, DNA was extracted from the pellet (obtained from 1.5 mL of concentrated samples) using the PowerSoil DNA Kit (MOBIO Laboratories, Carlsbad. CA) following the manufacturer's instructions. Extracted DNA samples were stored at −20 °C until processed.

Real-Time PCR Analysis of Sewage-Associated Markers. Real-time PCR assays were performed using previously published primers, probes and cycling parameters.[2,3,8,22,24] The *nifH*, *esp*, and HF183 amplifications were performed in 20 μL reaction mixtures using Sso Fast EvaGreen Supermix (, CA). The PCR mixture contained 10 μL of Supermix, 300−400 nM each primer, DNase- and RNase-free deionized water, and 5 μL of template DNA. AVs and PVs amplifications were performed in 25-μL reaction mixtures using iQ Supermix (Bio-Rad Laboratories, Irvine, CA). The PCR mixture contained 12.5 μL of Supermix, 400−500 nM each primer, 400−600 nM corresponding probe, and 5 μL of template DNA. For each PCR experiment, positive controls (e.g., corresponding plasmid DNA or genomic DNA) and negative control (e.g., sterile water) were included. The PCR was performed using the Bio-Rad iQ5 thermal cycler (Bio-Rad Laboratories).

Real-Time PCR Sample Limit of Detection (SLOD) of the *nifH* Marker in Sewage and Sewage Spiked Environmental Water Samples. To determine the PCR sample limit of detection (SLOD) of the *nifH* marker in sewage, three primary influent samples (1 L each) were collected and serial dilutions (10^{-1} to 10^{-8}) were made for each sample. The numbers of *E. coli* and enterococci were enumerated for each dilution and DNA was extracted from each diluted sewage sample in order to determine the SLOD of the *nifH* marker in comparison with *E. coli* and enterococci numbers. To determine the SLOD of the *nifH* marker for environmental water samples ($n = 3$), primary influent samples ($n = 3$) were suspended in environmental water samples to a final concentration of 1−9 L of water. The serial dilutions (10^{-1} to 10^{-8}) were made for each sample and the numbers of *E. coli* and enterococci were enumerated for each dilution. DNA extraction was performed for each dilution and tested with the real-time PCR as described earlier. Total culturable *E. coli* and enterococci colonies were compared with the positive and negative results of the PCR to estimate the minimum colony forming units (CFU) and the amount of sewage that must be present in a sample for the detection of the *nifH* marker.

Testing for PCR Inhibitors in Environmental Water Samples. An experiment was conducted to determine the effects of PCR inhibitors on the detection of sewage-associated markers in environmental water samples. 10-fold serial dilutions were made and all DNA samples (undiluted, 10-fold and 100-fold dilutions) were spiked with 10^3 gene copies of the *Campylobacter jejuni mapA* gene. The threshold cycle (C_T) values obtained for the DNA samples from the spiked environmental water samples were compared to those of the DNA samples from distilled water.

Data Analysis. The sensitivity and specificity of the *nifH* marker were determined as: sensitivity = $a/(a + c)$ and specificity = $d/(b + d)$, where 'a' is true positive (samples were positive for athe marker of its own species), "*b*" is false positive (samples positive for the PCR marker of another species), "*c*" is false negative (samples were negative for the marker of its own species), "*d*" is true negative (samples were negative for the PCR marker of another species) (Gawler et al. 2007). One-way analysis of variance (ANOVA) was performed to determine the differences between the C_T values obtained for distilled water and those obtained for DNA isolated from environmental water samples. Results were considered significant at $P < 0.05$. Mann−Whitney's nonparametric test was used to test the significance differences of FIB numbers in dry and wet conditions (Graph Pad, Instat Version 3.1). Pearson's correlation was used to test the relationship between FIB (e.g., *E. coli* vs enterococci)

Table 2. Numbers of *Escherichia coli* and Enterococci and the Occurrence of the *nifH* Marker in Sewage and Sewage Spiked Environmental Water Samples

		numbers of fecal indicator bacteria		
sample	dilutions (amount of sewage in each dilution)	*E. coli*	enterococci	PCR positive results for the *nifH* marker
sewage	10^{-1} (10^{2} mL)	2.0×10^{7}	1.3×10^{7}	+
	10^{-2} (10^{1} mL)	2.3×10^{6}	9.9×10^{5}	+
	10^{-3} (10^{-1} mL)	2.6×10^{5}	1.0×10^{5}	+
	10^{-4} (10^{-2} mL)	2.3×10^{4}	1.2×10^{4}	+
	10^{-5} (10^{-3} mL)	1.9×10^{3}	1.1×10^{3}	+
	10^{-6} (10^{-4} mL)	2.1×10^{2}	9.8×10^{1}	ND
	10^{-7} (10^{-5} mL)	2.0×10^{1}	1.0×10^{1}	ND
	10^{-8} (10^{-6} mL)	ND	ND	ND
environmental water spiked with sewage	10^{-1} (10^{2} mL)	1.7×10^{6}	9.8×10^{5}	+
	10^{-2} (10^{1} mL)	2.1×10^{5}	8.7×10^{4}	+
	10^{-3} (10^{-1} mL)	1.9×10^{4}	7.3×10^{3}	+
	10^{-4} (10^{-2} mL)	1.8×10^{3}	8.0×10^{2}	ND
	10^{-5} (10^{-3} mL)	2.3×10^{2}	7.0×10^{1}	ND
	10^{-6} (10^{-4} mL)	1.1×10^{1}	$9.0 \times 10^{\circ}$	ND
	10^{-7} (10^{-5} mL)	ND	ND	ND
	10^{-8} (10^{-6} mL)	ND	ND	ND

numbers in both dry and wet conditions (Graph Pad). A binary logistic regression (BLR) (SPSS Version 19.0) analysis was also performed to obtain correlations between the presence of the sewage-associated markers and FIB numbers. BLR is a technique commonly used to model the binary (presence/absence) responses from environmental water samples. The presence/absence of sewage-associated markers was treated as the dependent variable (i.e., a binary variable). When a marker was present, it was assigned the value 1, and when a marker was absent, it was assigned the value 0. Nagelkerke's R square value, which ranges from 0.0 to1.0, denotes the effect size (the strength of the relationship) where stronger associations have values closer to 1.0. Relationships were considered significant when the P value for the model chi-square was < 0.05 and the confidence interval for the odds ratio did not include 1.0. Greater odd ratios indicate a higher probability of change in the dependent variable with a change in the independent variable.

RESULTS

Host-Specificity and -Sensitivity. Among the 64 human fecal samples tested, 52 (81%) were PCR positive for the *nifH* marker (Table 1). Seven of 22 secondary effluent samples and all five individual human fecal samples were PCR negative. Secondary effluent and five human fecal DNA samples which gave negative signals, were serially diluted, and further tested with the PCR to rule out the possibility of the presence of PCR inhibitors that could potentially mask the amplification of the *nifH* marker. No discrepancies were observed between undiluted and diluted DNA samples (e.g., 10-fold and 100-fold) indicating the samples were free of PCR inhibitors. Among the 188 animal fecal DNA samples tested, 181 samples (undiluted DNA, 10-fold and 100-fold dilutions) were negative for the *nifH* marker. Six pig wastewater and one bird fecal DNA samples, however, were positive for the *nifH* marker. The overall specificity of the *nifH* marker to differentiate between human and animal fecal samples was 0.96 (maximum value of 1) and the overall sensitivity of this marker in human wastewater was 0.81 (maximum value of 1).

Real-Time PCR Sample Limit of Detection (SLOD). For sewage, the SLOD assay resulted in the detection of the *nifH* marker up to dilution 10^{-5}. At this dilution, the numbers of *E. coli* and enterococci were 1.9×10^{3} and 1.1×10^{3} CFU, respectively (Table 2). For environmental waters spiked with sewage, the SLOD assay resulted in the detection of the *nifH* marker up to dilution 10^{-3}. At this dilution, the numbers of *E. coli* and enterococci were 1.9×10^{4} and 7.3×10^{3} CFU, respectively.

PCR Inhibitors in Environmental Water Samples. DNA isolated from environmental water samples (n = 21) were checked for the potential presence of PCR inhibitory substances. For the spiked distilled water, the mean C_T value for the *mapA* gene was 32.4 ± 0.6. For environmental water samples, the mean C_T values were 32.4 ± 0.8 (for undiluted DNA), 31.8 ± 1.0 (for 10-fold dilutions) and 31.3 ± 0.7 (for 100-fold dilutions). One-way ANOVA was performed to determine the differences between the C_T values obtained for distilled water and those obtained for surface water samples. No significant difference ($P > 0.05$) were observed among the C_T values for spiked distilled water, undiluted, 10-fold and 100-fold diluted DNA indicating the environmental samples were potentially free of PCR inhibitors.

Numbers of Fecal Indicator Bacteria (FIB) and the Prevalence of Sewage-Associated Markers in Environmental Water Samples. The numbers of FIB in water samples collected during dry conditions ranged from 0 to 5.3×10^{2} (for *E. coli*) and from 4.7×10^{1} to 5.1×10^{2} (for enterococci) (Table 3). For wet conditions, these figures were 0 to 8.4×10^{3} (*E. coli*) and 7.2×10^{1} to 2.5×10^{4} (enterococci). The numbers of both *E. coli* and enterococci were generally 1−2 orders of magnitude higher during the wet conditions compared to dry conditions. The numbers of both *E. coli* and enterococci in the dry conditions

Table 3. Number of *Escherichia coli* and Enterococci and PCR Positive/Negative Results of Sewage Associated Markers in Water Samples Collected from the FitzGibbon, Brisbane River, Cabbage Tree Creek, Oxley Creek and Wivenhoe Dam in Southeast Queensland, Australia[a]

				fecal indicators CFU/100 mL		*nifH* marker along with other sewage-associated PCR marker results				
sampling site	event	location	sampling condition (rainfall)	*E. coli*	Enterococci	*nifH*	*esp*	HF183	AVs	PVs
FG	1	27°20′08.7″ S; 153°.01′14.5″E	dry (0.4 mm)	3.6×10^2	5.1×10^2	ND	ND	+	ND	ND
	2		wet (49.8 mm)	4.7×10^3	1.8×10^3	+	ND	+	ND	ND
	3		dry (2.6 mm)	1.3×10^2	1.3×10^2	ND	ND	+	ND	ND
	2		wet (14.8 mm)	3.4×10^3	2.3×10^4	ND	ND	ND	+	ND
BR	1	27°28′49.1″ S; 152°.59′54.1″E	dry (0 mm)	7.7×10^1	3.3×10^2	ND	ND	+	ND	+
	2		wet (10.2 mm)	4.7×10^2	3.4×10^3	ND	+	+	ND	ND
	3		wet (19.4 mm)	6.0×10^3	8.1×10^3	+	+	+	+	+
CT	1	27°20′59.7″ S; 153°02′06.6″E	dry (0 mm)	4.8×10^2	2.0×10^2	ND	ND	+	ND	ND
	2		dry (2.6 mm)	5.3×10^2	4.6×10^2	ND	ND	+	ND	+
	3		wet (19.4 mm)	8.4×10^3	2.5×10^4	ND	ND	+	+	ND
OC	1	27°32′07.8″ S; 152°59′31.4″E	wet (15 mm)	1.6×10^3	1.1×10^4	ND	ND	ND	+	ND
	2		dry (0 mm)	9.0×10^1	4.0×10^2	ND	+	+	ND	ND
	3		wet (7 mm)	3.5×10^3	9.9×10^3	ND	ND	ND	+	+
WD1	1	27°34′97.9″ S; 152°.53′82.1″E	wet (17 mm)	ND	1.6×10^2	ND	ND	+	ND	ND
	2		dry (0 mm)	ND	3.2×10^1	ND	ND	ND	ND	+
WD2	1	27°34′84.3″ S; 152°.54′80.7″E	wet (17 mm)	5.4×10^1	1.6×10^2	ND	ND	ND	ND	ND
	2		dry (0 mm)	$5.0 \times 10^{\circ}$	1.0×10^2	ND	ND	ND	ND	ND
WD3	1	27°33′61.8″ S; 152°.53′98.9″E	wet (17 mm)	ND	7.2×10^1	ND	ND	ND	ND	ND
	2		dry (0 mm)	ND	4.8×10^1	ND	ND	ND	ND	+
WD4	1	27°33′64.6″ S; 152°.55′05.4″E	wet (17 mm)	1.0×10^2	7.2×10^1	ND	ND	+	+	ND
	2		dry (0 mm)	$7.0 \times 10^{\circ}$	4.7×10^1	ND	ND	ND	ND	ND

[a] FG: Fitzgibbon; BR: Brisbane River; CT: Cabbage Tree Creek; OC: Oxley Creek; WD: Wivenhoe Dam.

differed significantly ($P < 0.001$, *E. coli*; $P < 0.001$, enterococci) from the wet conditions. Among the 21 samples tested during the dry and wet conditions, 11 (52%) and 21 (100%) samples exceeded the Australian and New Zealand Environment and Conservation Council (ANZECC) water quality guidelines of 150 fecal coliforms and 35 enterococci per 100 mL of water for primary contact. Pearson's correlation was used to test the relationship between *E. coli* and enterococci numbers. The numbers of *E. coli* and enterococci correlated significantly during the wet ($r_p = 0.81$; $P < 0.0001$) and dry ($r_p = 0.92$; $P < 0.0001$) conditions.

Among the 21 samples tested, 2 (10%), 3 (14%), 12 (57%), 6 (29%), and 6 (29%) were positive for the *nifH*, *esp*, HF183, AVs, and PVs markers, respectively. The *esp*, HF183, and PVs were detected in samples collected during the dry and wet conditions. The *nifH* and AVs, however, were detected in samples collected during the wet conditions. Among the 21 samples tested, 17 (81%) were positive for at least one marker, six (29%) were positive for at least two markers, one (5%) was positive for all five markers tested in this study. The presence of sewage pollution in environmental waters could not be detected in 15 (71%) samples, if the *nifH* marker was used alone in this study. Similarly, sewage pollution could not be detected in 14 (66%), 5 (24%), 11 (52%), and 11 (52%) of samples, if the *esp*, HF183, AVs, and PVs markers, respectively, were used alone.

BLR was used to assess the correlation between FIB numbers and the presence/absence of sewage-associated markers. *E. coli* ($P < 0.007$ and Nagelkerke's $R^2 = 0.48$) and enterococci ($P < 0.001$ and Nagelkerke's $R^2 = 0.78$) numbers significantly correlated with AVs. The presence of the *nifH*, *esp*, HF183, and PVs, however, did not correlate with the FIB numbers.

DISCUSSION

Host-specificity and -sensitivity are two important characteristics of molecular markers because markers with low specificity and sensitivity may result in false positive and false negative detection of sewage pollution in environmental waters.[11,28] It is desirable that a marker should be highly host-specific (value of 1), however, the specificity of a particular marker may vary from study to study.[3,4,13,29] The U.S. EPA recommended that a marker with specificity <0.8 may not be useful for MST studies.[30] Several published studies reported the specificity of marker(s) > 0.9 and therefore, marker(s) showing a value above 0.9 could be considered as suitable for MST studies.[3,12,13,31−34]

It has been recommended that the specificity and sensitivity of MST markers need to be tested prior to their application for environmental studies especially for geographical locations where the specificity has never been tested.[10,11,30] In this study, the specificity of the *nifH* marker was rigorously evaluated by screening 188 fecal samples from a wide range of animal species. For each animal species except camels, more than 10 fecal samples were included in the specificity testing as recommended.[30] Care was taken to prevent PCR false positive results. DNA extracted

from animal feces and wastewater was serially diluted, and tested with the PCR to confirm that inhibitors did not mask the amplification. To prevent PCR cross contamination, animal fecal and wastewater samples were processed before sewage samples. The PCR cycling parameters were kept the same as the study that reported the development of the *nifH* marker.[22]

The *nifH* marker could not be detected in 181 (96%) of 188 fecal and wastewater samples and had a specificity value of 0.96. The marker was detected in a bird (e.g., seagull) fecal sample and six composite pig wastewater samples. Interestingly, the *nifH* marker could not be detected in a fecal sample from an individual pig. Since, the marker was detected in composite pig wastewater samples, it was not possible to estimate the prevalence of this marker in individual pig feces. The high (a value of 1.0) specificity of the *nifH* marker in Southern Mississippi in the U.S. has been reported.[22] However, subsequent studies reported the presence of the *nifH* marker in feces of a small number of cattle,[3,9,35] seagull, red-neck wallaby, rat,[36] and dog.[35] None of the sewage-associated bacterial markers reported in the research literature was shown to be absolute host-specific. For example, the *esp* and the HF183 markers were detected in nontarget fecal samples.[13,29,35−38]

The sensitivity of the *nifH* marker in this study was determined to be 0.81, which is comparable to other host-specific markers such as *esp*,[12] HF183,[4,13,29,39] AVs[31] and PVs.[14,32] All wastewater samples tested from the primary influent were positive for the *nifH* marker. The *nifH* marker, however, could not be detected in seven secondary effluent and five individual fecal samples. The absence of the *nifH* marker in individual human fecal samples is not unexpected because it has been reported that approximately 29% humans contained gut-associated *M. smithii*.[22] Recent studies also reported very low prevalence (e.g., 0−10%) of the *nifH* marker in individual human fecal samples[36,40]

The *E. coli* and enterococci numbers in environmental water samples collected during the wet conditions were significantly ($P < 0.001$, *E. coli*; $P < 0.001$, enterococci) higher than those in dry conditions. This is in agreement with previous studies and not unexpected because, after rainfall, waterways receive fecal pollution from various point and nonpoint sources.[32,41] The number of *E. coli* and enterococci in water samples from the WD sites (i.e., WD1-WD4) was low compared to FG, BR, CT and OC sites. Sampling sites from the WD were subjected to less point and nonpoint sources of fecal pollution compared to other sites. The WD has a total storage capacity of 2.61 km^3 and the surface area is 109.4 km^2. It is possible that the large water body and flow may have diluted the numbers of FIB.

The *nifH* marker was detected in two samples from the sites FG and BR indicating potential sewage pollution. Both these sites receive stormwater through urban runoff. The presence of the *nifH* marker in stormwater runoff has been reported in recent studies.[23,40,42] The *esp* marker was detected in three samples from the sites BR (two samples) and OC (one sample). Overall, the HF183 was more frequently detected in water samples than other markers. Three of four samples from the site FG were PCR positive for the HF183, suggesting high prevalence of this marker in stormwater runoff. Viral markers AVs and/or PVs were also detected in several samples suggesting the presence of sewage pollution in the tested environmental water samples. Only one sample from site BR was positive for all five markers. It has to be noted that this site receives urban runoff through stormwater drains and possibly exposed to more human fecal matters compared to other sites. Four samples from WD did not yield any culturable *E. coli*, however, were PCR positive for the HF183 and PVs markers. This could be due to the fact that, for fecal indicator analysis, a small volume of water samples (i.e., 100 mL) were tested compared to sewage-associated markers where a large volume of water samples (i.e., 9−19 L) were tested.

The prevalence of the *nifH* marker was low in environmental samples compared to the *esp*, HF183, AVs, and PVs. The sewage pollution could not be detected in 15 (71%) samples if the *nifH* marker was used alone in this study. The presence of PCR inhibitors in environmental water samples masking the *nifH* detection can be ruled out as all water samples were spiked with low gene copies (i.e., 10^3) of *C. jejuni mapA* gene. The amplification of the *mapA* gene indicated the samples were free from PCR inhibitors. Larger volumes (9−19 L depending on the turbidity) of water samples were tested in this study for the sensitive detection of the sewage-associated markers in environmental waters. A recent study reported the application of HFUS combined with the PCR detection of the MST markers in fresh and estuarine water in the U.S.[43] and determined that the HFUS method with the PCR detection was more sensitive compared to the membrane filtration method with the PCR detection.

The low prevalence of the *nifH* marker in environmental samples could be due to the fact that these markers either have different decay rates in environmental waters compared to other markers or because of their low prevalence in sewage. The PCR SLOD assay for sewage resulted in the detection of the *nifH* marker up to dilution 10^{-5} (equivalent to 10 μL of raw sewage). At this dilution, the numbers of *E. coli* and enterococci were 1.9×10^3 and 1.1×10^3 CFU, respectively. Such data suggest that the prevalence of the *nifH* marker in raw sewage could be 1−2 orders of magnitude lower than FIB numbers. For environmental water samples spiked with raw sewage, the SLOD assay resulted in the detection of the *nifH* marker up to dilution 10^{-3} (equivalent to 1 mL of raw sewage). At this dilution, the numbers of *E. coli* and enterococci were 1.9×10^4 and 7.3×10^3 CFU, respectively, suggesting that high numbers of sewage-associated FIB need to be present in environmental waters for the detection of the *nifH* marker.

In a previous study, for freshwater and seawater spiked with sewage, the SLOD assay resulted in the detection of the *esp*, AVs and PVs markers up to dilution 10^{-4} (equivalent to 6.25 μL of raw sewage). These figures for the HF183 markers were 10^{-8} (equivalent to 0.001 μL raw sewage). The prevalence of the *nifH* markers also appeared to be low in raw sewage in SEQ, Australia compared to the HF183 marker. A recent study also reported the low prevalence of the *nifH* marker in two urban watersheds in California compared to the HF183 markers.[23] The authors modified the *nifH* protocol into a two round PCR for increased sensitivity. Despite that, the HF183 marker was more frequently detected in environmental samples compared to the *nifH* marker.[23] The absence of the *nifH* marker, however, in an environmental water sample does not rule out the presence of sewage pollution. For the accurate and sensitive identification of human fecal pollution, it is recommended that multiple markers should be used to obtain confirmatory results.[14,31] The findings of the present study also suggest that multiple markers should be used in environmental studies to reduce uncertainties associated with a particular marker that fails to detect fecal pollution in environmental waters.

In conclusion, the *nifH* marker appears to be sewage-specific in SEQ, Australia, however, the application of the *nifH* marker alone may not be sensitive enough to provide the evidence of sewage pollution. The *nifH* gene can be useful as an additional marker in

combination with the HF183 or viral markers for source tracking studies. Further research is required to investigate the correlation between the *nifH* marker and pathogens in environmental waters.

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