A real-time qPCR assay for the detection of the *nifH* gene of *Methanobrevibacter smithii*, a potential indicator of sewage pollution

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Abstract

Methanobrevibacter smithii is a methanogenic archaea prevalent in the intestinal tract of humans. Due to its abundance in the human gut and low likelihood of regrowth in the environment, the *nifH* gene of *M. smithii* may be a useful marker of sewage pollution in water. This paper describes the development of a real-time quantitative polymerase chain reaction (RT-qPCR) assay to detect the *nifH* gene of *M. smithii*. Quantification standards were prepared from *M. smithii* genomic DNA dilutions and a standard curve used to quantify the target gene and calculate estimated genome equivalency units (GEUs). A competitive internal positive control was designed and incorporated into the assay to assess inhibition in environmental extracts. Testing the assay against a panel of 23 closely related methanogen species demonstrated specificity of the assay for *M. smithii*. A set of 36 blind water samples was then used as a field test of the assay. The internal control identified varying levels of inhibition in 29 of 36 (81%) samples, and the *M. smithii* target was detected in all water samples with known sewage input. Our results suggest that the quantitative polymerase chain reaction (qPCR) assay targeting the *M. smithii nifH* gene developed in this study is both sensitive and rapid, and shows promise as a reliable indicator of sewage in environmental waters.

Introduction

Sewage pollution in the environment constitutes a serious risk to human health through exposure to pathogens in recreational (Calderon *et al*. 1991, Haile *et al*. 1999) and drinking waters (MacKenzie *et al*. 1994, Fong *et al*. 2007), as well as consumption of contaminated seafood (Yamashita *et al*. 1992, Lipp and Rose 1997) and agricultural products (Straub *et al*. 1993, Armon *et al*. 2002). Human sources of faecal contamination are believed to be the most serious threat to human health because of the host specificity of pathogens (Scott *et al*. 2002). The standard methods that are currently used to detect faecal pollution, such as the enumeration of total coliforms, faecal coliforms, and enterococci, have many documented limitations (Griffin *et al*. 2001, Scott *et al*. 2002, Simpson *et al*. 2002, Meays *et al*. 2004), including their inability to identify the source of contamination (Field *et al*. 2003) and poor predictive relationships to human health risks (Horman *et al*. 2004, Boehm *et al*. 2009). As a result, current research is focused on discovering alternative indicators of faecal pollution that are host specific, display a survival pattern similar to pathogens, and have predictive correlations to human illness.

A variety of anaerobic micro-organisms have been targeted as alternative indicators of faecal pollution because of their much higher abundance in comparison with traditional indicators (Bernhard and Field 2000, Savage 2001, Eckburg *et al*. 2005), their

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limited ability to grow outside of their host, and their potential for host-specific associations (Long *et al*. 2003, Bonjoch *et al*. 2004, Dick *et al*. 2005, Ufnar *et al*. 2006). Because of the advancement and widespread use of molecular-based methods for identifying micro-organisms, it is now possible to rapidly detect and quantify anaerobes, while avoiding the difficulties associated with cultivation. Anaerobes account for the majority of the microbial community found in the gastrointestinal tract of humans. In one study, more than 90% of the 13,335 bacterial 16S rRNA genes detected in human faecal samples and intestinal mucosal tissue were identified as anaerobes (Eckburg *et al*. 2005).

Methanobrevibacter smithii is a methanogenic Euryarchaeote that is found in the gastrointestinal tract of approximately one-third of individuals living in the United States and United Kingdom (Bond *et al*. 1971, Lin and Miller 1998, Ufnar *et al*. 2006) and can comprise up to 10% of all anaerobes found in the large intestines of healthy adults (Miller and Wolin 1986, Eckburg *et al*. 2005). Although *M. smithii* is found in only ~30% of individuals, it has been demonstrated that *M. smithii* is highly prevalent in mixed sewage, with a 93% (25 of 27) detection rate in sewers sampled in Gulfport, MS (Ufnar *et al*. 2006). *Methanobrevibacter smithii* may also be host specific to the human gastrointestinal system (Miller 1984; Eckburg *et al*. 2005, Ufnar *et al*. 2006), making it a potentially useful indicator of sewage pollution in the environment. A traditional polymerase chain reaction (PCR) assay has been developed for detecting the *M. smithii nifH* gene using gel-based detection (Ufnar *et al*. 2006). The *nifH* gene was used as the target for *M. smithii* identification because it has been shown to belong to a unique group of *nifH* genes that do not encode a functional nitrogenase enzyme and are found only in methanogens (Ohkuma *et al*. 1999). In addition, there is enough sequence divergence among methanogen *nifH* genes to make it a useful target for phylogenetic discrimination among species. Ufnar *et al*. (2006) reported finding the 222-bp PCR product in 29% (20 of 70) of the human faecal samples and 93% (25 of 27) of sewage samples tested, while no product was detected in any of the nonhuman faecal samples. The *M. smithii* target was also detected in coastal waters up to 24 days after a documented sewage line break, but was absent on the 31st day, demonstrating its usefulness as an environmental indicator of sewage pollution.

In the current study, we developed a TaqMan-based RT-qPCR assay for the detection and quantification of the *M. smithii nifH* gene that included a competitive internal positive control (CIPC). Real-time qPCR provides a faster, more sensitive detection method than traditional PCR, allowing for simultaneous quantification of the target without the need for gel-based detection of the product. The specificity, range of quantification, and detection limit of the *M. smithii* qPCR assay were determined by analyzing a quantitative standard and testing the assay on various methanogens, environmental samples, and samples spiked with sewage. A major limitation of PCR-based detection methods is the presence of inhibitors commonly found in environmental samples that can lead to underestimation of the intended target or prevent amplification all together (Wilson 1997, Hoorfar *et al*. 2004). The CIPC is a synthetic double-stranded deoxyribonucleic acid (DNA) fragment that was developed to assess inhibition in the real-time PCR assay and used in this investigation to remove PCR bias in environmental samples. The CIPC, added to each reaction and co-amplified with the target gene, can be used to determine whether a negative result is a true negative and to calculate a correction factor for quantification of a partially inhibited sample.

Methods

Methanogen Cultures and DNA Extracts

Live *M. smithii* cultures were obtained from the American Type Culture Collection (ATCC 35061; ATCC, Manassas, VA) and the German Collection of Microorganisms and Cell Cultures (DSM 861; DSMZ, Braunschweig, Germany). Deoxyribonucleic acid was extracted directly from these cultures using the DNeasy Tissue kit following the manufacturer's protocol for bacteria cells (Qiagen, Valencia, CA). Additionally, DNA was recovered from 23 different methanogens that were extracted and stored on 2-mm punches from Whatman FTA Classic Cards (Florham Park, NJ) as previously reported (Ufnar *et al*. 2006). The genomic DNA was recovered from the FTA cards by incubating for 5 minutes in a 35µl of 0.1 N NaOH, 0.3 mmol/L ethylenediamine triacetic acid (EDTA; 13.0 pH) at room temperature, followed by the addition of 65 µl of 0.1 mmol/L Tris–HCl (7.0 pH). The punch was incubated in this solution at room temperature for an additional 10 minutes while mixing by vortex every 1 minute. The FTA card punch was removed

from the resulting 100-µl volume of genomic DNA in TE buffer (66 mmol/L Tris–HCl, 0.1 mmol/L EDTA). The recovered DNA was quantified by spectrophotometry using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA), and serial dilutions were made for each methanogen from 1 to 0.001 ng of total DNA.

Primers and Probes

A TaqMan-based qPCR assay was developed to detect the *nifH* gene of *M. smithii*, and PCR primers (Mnif 202F and Mnif 353R) and a hybridization probe were designed to target a 151-bp segment from published *M. smithii nifH* gene sequences (Table 1). The qPCR assay targets a smaller portion of the *nifH* gene originally used by Ufnar *et al*. (2006). The primers and probe were designed using NetPrimer (Premier Biosoft, Palo Alto, CA), and a BLAST search was used to determine homology between previously sequenced genes. The target-specific TaqMan probe was labelled with the 5' reporter fluorophore FAM (6-carboxyfluorescein) and the 3' quencher dye black hole quencher 1 (BHQ-1). A second probe was designed to detect the CIPC template and labelled with the 5' reporter fluorophore Cy5 (carbocyanine) and 3' quencher dye black hole quencher 2 (BHQ-2) to distinguish the CIPC signal from the *M. smithii* target signal (Table 1). Thermal cycling and fluorescence detection were carried out in the iQ5 Real-Time PCR detection system (Bio-Rad, Hercules, CA). The optimized reaction was performed in a total volume of 25 µl containing 1X PCR buffer (50 mmol/L KCl, 20 mmol/L Tris–HCl, 8.4 pH; Bio-Rad), 5 mmol/L $MgCl_2$ (Bio-Rad), 800 µmol/L deoxynucleotide triphosphates (dNTPs; Bio-Rad), 800 nmol/L primers (Integrated DNA Technologies, Coralville, IA), 240 nmol/L each of the Mnif and CIPC probes (Integrated DNA Technologies), 200 copies of the CIPC template, 2.3 U Taq DNA polymerase (Bio-Rad), and 3 µl of the *M. smithii* quantification standard or DNA extract. All samples were run in triplicate with a five-point standard curve that also served as positive controls, and PCR-grade water was evaluated in separate reactions as a template for negative controls. A temperature gradient was used to determine the optimal annealing temperature for the primer pair. The final cycling conditions were an initial denaturation for 10 minutes at 95°C, 50 cycles of denaturation for 10 seconds at 95°C, and annealing⁄extension for 30 seconds at 57°C. Real-time fluorescence measurements were collected by the iQ5 instrument beginning after the first three cycles to prevent any residual bubbles from causing background fluorescence signal. Background well factors were collected from the experimental plate, and the fluorescent thresholds were set automatically by the iQ5 software in the PCR baseline subtracted curve fit analysis mode. The cycle thresholds (C_T) where sample fluorescence exceeds background fluorescence were recorded for the extracted samples, quantification standards, and CIPC. The numbers of *M. smithii nifH* gene targets were interpolated from the standard curve generated from the quantification standards in relation to their C_T.

Table 1. Primer and probe sequences. Primer sites homologous to the *M. smithii nifH* gene are shown in bold. Sequences homologous to the CIPC probe (or the reverse compliment) are italicized.

Oligonucleotide	Sequence	Location*
Mnif 202F†	5′-**GAA AGC GGA GGT CCT GAA**-3′	202–219
Mnif 353R†	5′-**ACT GAA AAA CCT CCG CAA AC**-3′	334–353
Mnif Probe†	5′-[FAM]-CCG GAC GTG GTG TAA CAG TAG CTA-[BHQ-1]-3′	236–259
CIPC Probe‡	5′-[Cy5]-*TGT GCT GCA AGG CGA TTA AGT TGG GT*-[BHQ-2]-3′	
CIPC F Oligo†	5′-**GAA AGC GGA GGT CCT GAA** CCG GGT GTT GCT TCG CTA TTA CGC CAG CTG GCG AAA GGG GGA *TGT GCT GCA AGG CGA TTA AGT TGG GTA* ACG-3′	
CIPC R Oligo†	5′-**ACT GAA AAA CCT CCG CAA AC**A TTC AAT TCG AGG CAA CGT CGT GAC TGG GAA AAC CCT GGC GTT *ACC CAA CTT AAT CGC CTT GCA GCA CA*T-3′	

BHQ-1, black hole quencher 1; BHQ-2, black hole quencher 2.
*Nucleotide position in reference to the *Meth. smithii nifH* gene sequence (accession no. ABO19138).
†Sequences designed in this study.
‡Sequence taken directly from Kleiboeker (2003) and Gregory *et al.* (2006).

Quantification Standards

Genomic DNA extracted from a culture of *M. smithii* (DSM 861) was used to establish quantification standards for generating a standard curve. The DNA was extracted using the Qiagen DNeasy Tissue kit following the manufacturer's protocol for bacteria cells (Qiagen) and purified using the PowerClean DNA Clean-Up kit (MO BIO, Carlsbad, CA). Each DNA concentration was determined. by spectropho-

tometry using a Nanodrop ND-1000 spectrophotometer. Standards were diluted in nuclease-free water and stored in single-use aliquots at -80°C. A five-point tenfold serial dilution of the *M. smithii* genomic DNA (10 to 100,000 fg) was run in triplicate with each set of reactions to generate the standard curve.

CIPC

The CIPC designed in this study was modified for DNA-based qPCR assays from methods previously employed in viral qRT-PCR assays using competitor RNA (Kleiboeker 2003, Gregory *et al*. 2006). The CIPC was added to each reaction, co-amplified with the *M. smithii* target using the same primer set, and detected using a distinct TaqMan probe. Forward and reverse DNA oligonucleotides, each 90 nucleotides in length, were synthesized (Integrated DNA Technologies) with the Mnif 202F and Mnif 353R primer sequences incorporated into the 5' ends (Table 1). The 3' ends of each oligonucleotide were designed with complementary sequences so that they would hybridize over their final 30 bp to create a 150-bp double-stranded DNA product. The oligonucleotides were designed such that the sequence between the 5' primer sites is homologous to the pCRII-TOPO T7 vector with no similarity to naturally occurring sequences in GenBank. This sequence provided a unique probe site for detection of the CIPC.

The reaction designed to anneal and extend the oligonucleotides to form the double-stranded DNA product was performed in a total volume of 50 µl containing 1X PCR buffer (50 mmol/L KCl, 20 mmol/L Tris–HCl, 8.4 pH, 1.5 mmol/L, $MgCl_2$, 400 µmol/L dNTPs), 800 nmol/L of each oligonucleotide, and 1.25 U Taq DNA polymerase. The optimized conditions for creating the double-stranded CIPC product were an initial denaturation for 10 minutes at 95°C, 5 cycles of denaturation for 30 seconds at 95°C, annealing for 1 minute at 72°C, and a final 6-minute extension at 72°C. The resulting product was confirmed by electrophoresis on a 12% polyacrylamide gel, stained with ethidium bromide, and visualized under UV transillumination using an Alpha Imager (Alpha Innotech, San Leandro, CA). The product was excised and purified using the Wizard SV Gel and PCR clean-up system (Promega Corp., Madison, WI) and quantified by spectrophotometry using a Nanodrop ND-1000 spectrophotometer.

Detecting Sample Inhibition with CIPC

The amount of delay in the detectable CIPC signal as measured by a difference in C_T values is used to assess the degree of PCR inhibition and can be used to correct the estimates of *M. smithii* genome equivalents per reaction. This ΔC_T value is calculated by subtracting the mean CIPC C_T value for the uninhibited control reactions from the CIPC C_T recorded in the environmental or test samples. The ΔC_T value is applied to the equation $(E + 1)^{\Delta CIPC\ CT}$, where E is the amplification efficiency calculated from the standard curve using the equation $E = [10^{(-1/slope)}] - 1$. This calculation provides an estimate of the factor by which the corresponding *M. smithii* genome equivalent estimate must be multiplied to reflect the actual number of genome equivalents that would have been obtained by the qPCR if no inhibition had occurred.

A cut-off value of 3.3 for the CIPC ΔC_T was considered inhibited. This value was used because a decrease in the C_T of the CIPC of 3.3 cycles corresponds to a log decrease in the number of CIPC targets detected based on 100% amplification efficiency, representing a substantial reduction in amplification of the CIPC target (Gregory *et al*. 2006). Samples that were negative for *M. smithii* and had a ΔC_T <3.3 for the CIPC were considered true negatives or below the detection limit of the qPCR assay. Samples that resulted in a positive *M. smithii* signal and with a ΔC_T <3.3 for the CIPC were considered positive, and genome equivalent estimates were corrected using the $(E + 1)^{\Delta CIPC\ C_T}$ correction factor. If the sample resulted in a $\Delta C_T \geq 3.3$ for the CIPC and was either positive or negative for the *M. smithii* target, the reaction was considered significantly inhibited, and the amount of extract added to the reaction was decreased from 3 to 1 µl in an attempt to reduce inhibition through dilution. In samples which the CIPC signal did not cross the threshold of detection, the DNA extracts were diluted in the same fashion and reanalysed.

Detection Limits

To determine the minimum number of *M. smithii nifH* gene targets required for detection, genomic DNA extracted from a pure culture of *M. smithii* was used to create a series of dilutions with a lower limit of 2 fg, or approximately a single genome equivalent based on the genome size of 1853 160 bp (Samuel *et al*. 2007). The genomic DNA dilutions were analysed with the qPCR assay with and without the

CIPC template added. In addition, a series of water samples spiked with various amounts of sewage influent from the Orange County Sanitation District were tested with the assay to assess the lower limits of detection in more complex sample matrices.

Assay Validation

The qPCR assay was first tested on a panel of closely related methanogen species to ensure that there was no cross-reactivity between genetically similar organisms (Table 2). To assess the sensitivity and specificity of the qPCR assay to detect sewage pollution in environmental water samples, a set of blind samples was processed in partnership with the Southern California Coastal Water Research Project (SCCWRP) during a methods evaluation study (Griffith *et al*. 2009). A total of 36 blind samples with duplicates from several locations on the California coast consisting of negative controls, ambient seawater, seawater spiked with bird guano, and seawater with known or spiked sewage inputs (Table 3) were processed for DNA extraction. All sewage-spiked samples were inoculated with inflow from the Orange County Sanitation District, which has a service population of three million people. A subset of these samples contained dilutions of spiked sewage in seawater with targeted Enterococcus faecalis concentrations ranging from 50 to 10,000 CFU/100 ml.

The volume of water sample filtered was dependent on turbidity and ranged from 50 to 500 ml. Each water sample was filtered through a 0.2-µm Supor-200 membrane (Pall Corporation, Port Washington, NY), and the DNA was extracted using the MO BIO Powersoil DNA isolation kit (MO BIO) according to the manufacturer's protocol. The DNA template was added to the qPCR at an initial volume of 3 µl. In samples that were determined to have inhibition, the volume was decreased to 1 µl to reduce inhibition through dilution.

Table 2. Methanogens tested for primer specificity using the qPCR assay.

Negative methanogen species	
Methanobrevibacter woesei	OCM 815
Methanobrevibacter gottschalkii	OCM 813
Methanobrevibacter arboriphilus	OCM 147
Methanobrevibacter thaueri	DSM 11995
Methanobrevibacter filiforms	DSM 11501
Methanobrevibacter cuticularis	DSM 11139
Methanobrevibacter acididurans	OCM 804
Methanobrevibacter wolinii	OCM 814
Methanobrevibacter curvatus	DSM 11111
Methanobrevibacter oralis	DSM 7256
Methanobrevibacter ruminantium	OCM 146
Methanobacterium bryantii	DSM 863
Methanosarcina barkeri	DSM 800
Methanofollis liminatans	DSM 4140
Methanocorpusculum aggregans	DSM 3027
Methanomicrococcus blatticola	DSM 13328
Methanocalculus pumilus	DSM 12632
Methanosphaera stadmanae	DSM 3091
Methanolobus oregonensis	DSM 5435
Methansaeta concilii	DSM 2139
Methanocaldococcus infernus	DSM 11812
Methanomicrobium mobile	DSM 1539
Methanococcus maripaludis	DSM 2067
Positive methanogen species	
Methanobrevibacter smithii	ATCC 35061, DSM 861

Results

The primers used to amplify the *M. smithii nifH* gene produced the expected 151-bp product under the optimized reaction conditions. When combined with the probe in the qPCR and run on log dilutions of the *M. smithii* quantification standards (10 to 100,000 fg), the resulting standard curve had an amplification efficiency of 95% and r^2 value of 0.99 (Figure 1). The range of quantification spanned the entire 4 logs of concentrations tested, from 10 to 100,000 fg of genomic DNA, or approximately 5 to 50,000 *M. smithii* genome equivalents. The qPCR assay was able to detect as little as 5 fg of genomic DNA (approximately 2.5 *M. smithii* genome equivalents) with 200 copies of the CIPC template added, although the signal failed to cross the detection threshold in 6 of the 12 replicates (50%) and the standard deviation of the C_T values were much higher at 5 fg (3.43) than at 10 fg (0.98). Therefore, 10 fg is the lower limit of reliable detection.

The CIPC template was successfully generated from the two oligonucleotide templates to form the expected 150-bp product. The incorporation of the Mnif 202F and 353R primer sites in the CIPC allows both the *M. smithii nifH* gene target and the CIPC template to be amplified simultaneously in the PCR using a single primer set. The CIPC template is detected using a distinct probe (Table 1), corresponding to bp 436 through 461 of the pCRII-TOPO T7 vector. When amplified in the presence of both the CIPC probe and *M. smithii nifH* gene probe with no *M. smithii* target template and vice versa, there was no cross-reactivity observed between the respective probes and target templates.

Table 3. Results of the *M. smithii* qPCR assay on blind samples from a methods evaluation study that included negative controls, ambient water, water spiked with bird guano, and water with known or spiked sewage inputs. The qPCR results are shown along with traditional indicators.

		3 μl analysed per reaction			1 μl analysed per reaction				Traditional indicator measurements‡	
Sample type	Sample	Avg. C_T	GEU* 100 ml^{-1}	CIPC ΔC_T	Avg. C_r	GEU 100 ml^{-1}	CIPC ΔC_T	Corrected GEU 100 ml^{-1}†	*Escherichia coli* CFU 100 ml^{-1}	*Enterococcus faecalis* CFU 100 ml^{-1}
Known sewage input	Seawater with sewage (50 ent)	30·48	336	0·69				533	24	100
	Seawater with sewage (50 ent)	29·75	361	0·15				399	26	22
	Seawater with sewage (150 ent)	32·62	81	0				81	58	46
	Seawater with sewage (150 ent)	34·86	12	0				12	32	68
	Seawater with sewage (500 ent)	31·3	130	0				130	280	120
	Seawater with sewage (500 ent)	27·83	1964	0				1964	380	50
	Seawater with sewage (1000 ent)	30·26§	389	0				389	860	1090
	Seawater with sewage (1000 ent)	29·75	361	>3·3	32·61	329	0	329	No data	No data
	Seawater with sewage (10 000 ent)	27·33	1783	>3·3	29·81	2058	0·08	2168	5000	5000
	Seawater with sewage (10 000 ent)	28·03	1123	1·85				3812	8000	6000
	Doheny pond with sewage	32·55	85	>3·3	32·13	933	>3·3	N/A¶	>2000	>2000
	Doheny pond with sewage	31·83	101	>3·3	32·89	569	>3·3	N/A	>2000	>2000
	Doheny wavewash with sewage	29·4	474	0·78				808	650	340
	Doheny wavewash with sewage	30·9	254	1·89				897	510	300
	Tijuana River	26·62	4370	>3·3	26·78	7207	>3·3	N/A	4200 000	3400 000
	Tijuana River	26·37	3360	>3·3	26·51	8609	>3·3	N/A	1900 000	3400 000
Ambient water	Offshore seawater	29·19	522	0·9				946	<2	<2
	Offshore seawater	34·4§	25	0·43				33	<2	<2
	Doheny pond			>3·3			>3·3		11 400	13 800
	Doheny pond			>3·3			>3·3		14 000	12 100
	Doheny wavewash			>3·3			1·11		28	48
	Doheny wavewash			1·72					56	18
	Imperial Beach wavewash			0					<2	<2
	Imperial Beach wavewash			0·78					<2	<2
	Surfrider Beach wavewash			0					6	8
	Surfrider Beach wavewash			0·46					4	14
	Malibu Creek	30·68	210	>3·3	31·8	265	0·07	278	900	100
	Malibu Creek			>3·3			>3·3		700	<100
	Ballona Creek			0·58					190	630
	Ballona Creek			0·72					60	720

Table 3 *(Continued)*

Sample type	Sample	3 μl analysed per reaction: Avg. C_T	3 μl analysed per reaction: GEU* 100 ml^{-1}	3 μl analysed per reaction: CIPC ΔC_T	1 μl analysed per reaction: Avg. C_T	1 μl analysed per reaction: GEU per 100 ml^{-1}	1 μl analysed per reaction: CIPC ΔC_T	Corrected GEU 100 ml^{-1}†	Traditional indicator measurements‡: *Escherichia coli* CFU 100 ml^{-1}	Traditional indicator measurements‡: *Enterococcus faecalis* CFU 100 ml^{-1}
Spiked bird guano	Seawater with rehab centre guano			1·21					>2000	>2000
	Seawater with rehab centre guano	30·57§	225	1·48				619	>2000	>2000
	Doheny wavewash with Doheny guano			1·2§					>200 000	>200 000
	Doheny wavewash with Doheny guano	27·99	1153	1·87				3966	>200 000	>200 000
Negative control	Sterile phosphate-buffered saline			1·51					<1	<1
	Sterile phosphate-buffered saline			0·25					<1	<1

CIPC, competitive internal positive control.

*GEU, genome equivalency units based on the 1 853 160 bp genome size of *Meth. smithii.*

†Corrected GEUs for observed inhibition were determined using the CIPC, samples with a CIPC $\Delta C_T \geq 3{\cdot}3$ were rerun with 1 μl of extracted DNA added per reaction to relieve inhibition.

‡Traditional indicators were quantified using US EPA membrane filtration method 1603 for *E. coli* and 1600 for *Enterococcus.*

¶Inhibition remained after dilution (CIPC $\Delta C_T > 3{\cdot}3$), therefore the correction factor could not be used.

§One of three replicates failed to cross the threshold of detection.

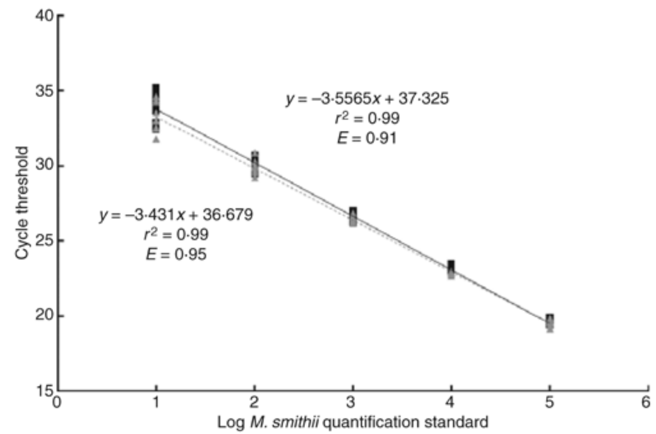

Figure 1. *M. smithii* genomic DNA was diluted to make a series of quantification standards that were used to generate a standard curve. Log dilutions of 10 to 100,000 fg of total genomic DNA, corresponding to approximately 5 to 50,000 *M. smithii* genome copies, were run with (■) and without (▲) 200 copies of the CIPC; n = 12 for all data points. The presence of the CIPC only altered the curve near the lower limit of detection, and slightly decreased the amplification efficiency from 0.95 to 0.91.

The CIPC was added to the optimized qPCR assay at various concentrations (100, 150, 200, 225 and 250 copies) to obtain the most consistent co-amplification at the lowest possible concentration so that the CIPC would not interfere with amplification of the *M. smithii nifH* target over the greatest possible range. It was determined that 200 copies of the CIPC template added was sufficient for consistent detection of the amplicon over a concentration range of 3 logs of co-amplification with the *M. smithii* quantification standard (10 to 10,000 fg; Figure 2). The C_T of the CIPC over this range did not significantly differ from the C_T of the CIPC observed in the no template controls as determined by a two-tailed t-test (P >0.05). At higher concentrations of the CIPC, the *M. smithii* target failed to amplify in the lower range of detection, while with lower concentrations of the CIPC, the *M. smithii* target was able to outcompete the CIPC target and a lag in the CIPC C_T was observed (data not shown). The standard curves and amplification efficiencies of the *M. smithii* quantification standard with and without the CIPC template at 200 copies were very similar (Figure 1).

The specificity of the primer and probe set was tested on 23 species of methanogens including 11 within the Methanobrevibacter genus (Table 2). No cross-reactivity was observed at any of the concentrations of the genomic DNA from any of the

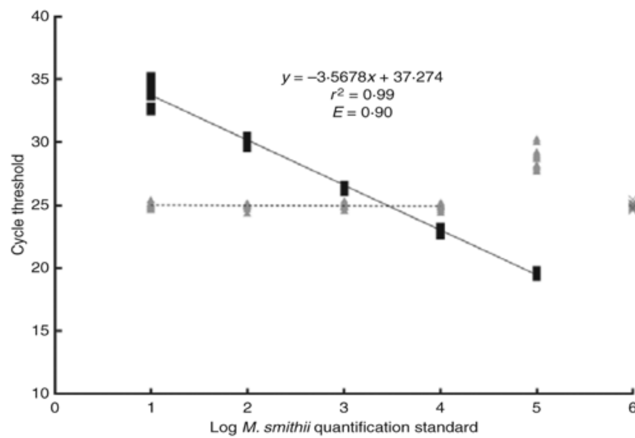

Figure 2. Co-amplification of the *M. smithii* quantification standards (■) and 200 copies of the CIPC (▲); n = 12 for all data points. Amplification of the CIPC over a concentration range of three logs of the quantification standards was nearly identical to amplification of the CIPC in the no template controls (X). At the highest quantification standard concentration, detection of the CIPC signal was delayed by approximately 3 cycles, representing a log difference in amplification and was deemed unreliable for assessing inhibition in the presence of >10,000 fg of the *M. smithii* target.

methanogens tested, *M. smithii* was the only species that gave a positive signal. Additionally, the assay was tested on 36 blind water samples that were designed to assess the ability of the method to identify sewage contamination in the environment and to assess inhibition using the CIPC. The *M. smithii* target was detected in all environmental water samples (16⁄16) with known or spiked sewage inputs (Table 3). Positive signals were also detected in three ambient water samples and two water samples spiked with bird guano. No signal was detected in the remaining ambient water samples (11), water spiked with bird guano (2), and sterile phosphate-buffered saline (2).

The CIPC indicated a wide range of inhibition in the samples, from no inhibition to complete inhibition in which the CIPC signal failed to cross the detection threshold. There were 11 samples out of the 36 in which the CIPC ΔC_T was ≥3.3. Only 4 of the 11 inhibited samples were initially scored as negative using the assay. The samples with a CIPC ΔCT ≥3.3 were reanalysed using 1 µl of DNA extract added to the reaction. This resulted in decreasing the inhibition in four of the 11 samples, 3 of which were positive for the *M. smithii* target. For the 17 diluted and undiluted samples with a CIPC ΔC_T <3.3, the CIPC was used to calculate a corrected number of gene targets. Inhibition persisted in the rest of the samples that were reanalysed (CIPC ΔC_T ≥3.3), and the correction factor was not used. Overall, there were no changes in the qualitative results in the post-dilution analysis.

Discussion

Identifying and monitoring sources of faecal pollution continues to be a significant challenge despite the longterm use of traditional faecal indicator bacteria. Because pathogens are often found in low concentrations in the environment and monitoring for all potential pathogenic micro-organisms is currently impractical, indicator organisms are used as surrogates to identify faecal pollution and the associated potential human health risks. Because of many documented limitations associated with using conventional faecal indicator bacteria (Griffin *et al*. 2001, Scott *et al*. 2002, Simpson *et al*. 2002, Field *et al*. 2003, Horman *et al*. 2004, Meays *et al*. 2004, Boehm *et al*. 2009) studies using molecular-based methods for detecting anaerobic micro-organisms as alternative indicators of faecal pollution have gained importance (Bonjoch *et al*. 2004, Dick and Field 2004, Savichtcheva and Okabe 2006).

The qPCR assay developed in this study is a sensitive and rapid method for the detection of the *M. smithii nifH* gene. The *nifH* genes of methanogens have previously been used for phylogenetic classification because of their conserved nature in methanogens and divergence from other bacteria and archaea (Kirshtein *et al*. 1991, Ueda *et al*. 1995, Ohkuma *et al*. 1999, Ufnar *et al*. 2006). Previous studies have shown that members of the *Methanobrevibacter* genus predominantly colonize the gastrointestinal systems of animals and that *M. smithii* is the most abundant archaeal methanogen found in the human intestinal system (Lin and Miller 1998, Ufnar *et al*. 2006, Dridi *et al*. 2009). Because of the potential host specificity and high concentration of *M. smithii* shed in faeces, this organism may serve as a useful indicator of human-specific sewage pollution in the environment. Although this indicator is found in only one-third of individuals, the abundance of *M. smithii* found in sewage makes it an applicable target for real world pollution detection.

Development of a qPCR assay advances the work by Ufnar *et al*. (2006), providing a sensitive and rapid method that allows for quantification of

the gene target. In silico analysis of the primers and probe using an NCBI BLAST search indicated they were specific to the *M. smithii nifH* gene target. This was later experimentally confirmed by determining that there was no crossreactivity when using DNA extracted from 23 closely related methanogen species as template for the qPCR. The quantification standards used in the assay provide an accurate assessment of *M. smithii* genome concentrations present in a sample over a wide range. The lower limit of detection for the assay with the CIPC included in the reaction was 10 fg, or approximately 5 GEUs for the *M. smithii* genomic DNA. The minimal amount of genomic DNA required to generate a visible product using traditional PCR and gelbased detection was 1000 fg (Ufnar *et al*. 2006). All samples subsequently tested in this study fell within the range of quantification dictated by the quantification standards.

Assay sensitivity was given priority over a greater range of quantification when determining the optimal concentration for the CIPC. At 100,000 fg of *M. smithii* genomic DNA, the average C_T for the CIPC was approximately three cycles later, representing a log difference in amplification (Figure 2). The CIPC was therefore deemed unreliable for detecting inhibition at the highest target concentration (100,000 fg) in the standard curve. However, over a range of three logs of the quantification standard, the CIPC did not significantly alter the sensitivity or reproducibility of the qPCR assay.

The qPCR assay detected the *M. smithii* target in all samples with known or spiked sewage inputs. The *M. smithii* target did not show a close correlation with culturable *E. faecalis* and Escherichia coli results in the seawater spiked sewage dilutions, although there was a general trend of increasing numbers with increased sewage concentration. This result is not surprising as many putative alternative indicators and human pathogens have been shown to have poor correlations with traditional indicator bacteria (Horman *et al*. 2004, Savichtcheva and Okabe 2006, Boehm *et al*. 2009). Differences in enteric microbial populations, numbers of micro-organisms shed in faeces, and survival patterns of individual species likely contribute to these observed results. In a separate study that sampled secondary treated wastewater off the southeastern coast of Florida, *M. smithii* numbers correlated poorly with culturable enterococci (Rosario *et al*. 2009). The *M. smithii* target was detected in all (6⁄6) outfall sites, while enterococci were found in low concentrations at half the sites (3⁄6). However, detection of the *M. smithii* target coincided well with norovirus, *Cryptospiridium*, and *Giardia*, each of which were detected in 5⁄6 outfall sites. These results indicate that the *M. smithii* gene target is present at detectable levels after sewage treatment and may correlate well with pathogens that are not removed through the treatment process.

In the current study, the *M. smithii* target was also detected in both samples of offshore seawater, a single sample from Malibu Creek, and two different samples of seawater spiked with seagull guano. Although these samples did not have known human faecal inputs, it is possible that the *M. smithii* target was present either from human sources or other unknown sources. For instance, it is possible that *M. smithii* colonization of seagulls may have occurred during the uptake of material during feeding at sewage treatment plants or landfills. It has been shown that *E. faecalis* isolates in seagulls and humans show an extremely high degree of similarity (Genthner *et al*. 2005), and methanogen colonization of the caecum of gallinaceous birds, such as chickens, was shown to be more dependent on the broiler house in which the chicken was raised rather than the birds' type of digestive system (Ufnar 2006). The *M. smithii nifH* gene has also previously been detected in offshore seawater located near point sources of secondary treated wastewater outfalls (Rosario *et al*. 2009). It is possible that the offshore seawater samples were collected near or down current from such a point source. Further, no target was detected in the sterile phosphate-buffered saline or the majority of the ambient water samples in this study. These results indicate that the *M. smithii* qPCR assay is a useful and sensitive method for detecting human sewage pollution in environmental water samples.

The CIPC was able to successfully detect a range of inhibition in the blind water samples. The majority of samples had little to moderate inhibition, and the CIPC was used to calculate a correction factor that was used to modify the estimated target concentrations. While no qualitative changes were observed in the results through reanalysis of the diluted DNA extracts, the usefulness of the CIPC was demonstrated in identifying inhibition and correcting estimated target concentrations in moderately inhibited samples.

While improvements have been made in the direct detection of pathogens in environmental samples, we are still heavily reliant on the use of indica-

tor organisms. Although validation of the qPCR assay is in its preliminary stages and extensive testing on different faecal sources across a variety of environmental sample types is necessary and currently underway, this research has demonstrated a rapid and sensitive method for detecting the *M. smithii nifH* gene in environmental samples. The qPCR assay developed in this study has contributed to the development of *M. smithii* as an alternative indicator for human-specific sources of sewage pollution. In addition, the assay includes an internal control that can be used to assess inhibition, prevent the reporting of false negative results, and more accurately estimate the concentration of targeted gene copies present in a sample. The simple design and inexpensive construction of the CIPC allows for universal applicability in qPCR assays that often do not account for inhibition.

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