

## Distinguishing human and possum faeces using PCR markers

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### ABSTRACT

Specificity testing of two published polymerase chain reaction (PCR) markers for the detection of human faecal pollution, revealed 100% false-positive rates to brush-tailed possum faeces ( $n = 10$ ), but low false-positive rates against other potential pollution sources. Cross-reaction with possums could be a problem with other human-specific markers; therefore, a possum PCR marker was developed for use in conjunction with human PCR markers. The possum PCR marker was based on *Bacteroidales* 16S ribosomal ribonucleic acid sequences, and was tested on 233 individual faecal samples from 11 other animal species. Sensitivity of the possum marker in possum faeces ( $n = 36$ ) was high at 83.3%. Cross-reactivity of the possum marker was limited to black swan (7/20 samples), human (2/48 samples) and rabbit (1/10) faecal samples, all at marker concentrations at least four orders of magnitude lower than possum faeces. The possum marker was not detected in human sewage or the faeces of other animal species. Specificity of the possum PCR marker, therefore, was high at 95.7%. To exclude the possibility that only possum pollution is being detected, additional testing by other faecal source tracking methods is required where the water sample is positive for both human and possum markers.

**Key words** | *Bacteroidales*, faecal source tracking, water pollution

### INTRODUCTION

The common brushtail possum (*Trichosurus vulpecula*) is a small (1.5–3.0 kg) marsupial that was introduced into New Zealand (NZ) from Australia by Europeans in the 1800s to establish a fur industry (Meyer 2000). With an absence of natural predators, possums flourished in NZ, and an estimated 70 million possums are now distributed over 90% of NZ's land area (McDowell & McLeod 2007). Possums are one of NZ's most serious mammalian pests, depleting native forests and bird species through selective browsing of tree species, and predation of eggs and nestlings of native fauna (Brown *et al.* 1993; Montague 2000). It is also recognised that possums are vectors of bovine tuberculosis, posing a threat to cattle, deer and dairy industries which are significant to the agricultural backbone of New Zealand's economy (Coleman & Livingstone 2000).

This unique situation in the NZ environment where possum populations have reached pest levels may also be

impacting NZ's water quality. The widespread distribution of possums in trees adjacent to streams (Cowan 1990) means that possums could contribute to faecal contamination in rural and urban freshwaters. This study sought to develop a faecal source tracking tool to identify pollution in waterways attributed to possum faecal inputs.

Identification of faecal contamination in water typically uses microbial indicators such as *Escherichia coli*. These microbial faecal indicators, however, are present in the faeces of all animal types; therefore, a range of other tools have been developed for identifying actual sources of pollution (Sinton *et al.* 1998). These include various chemical and microbial markers which distinguish faecal outputs from animal and bird species.

Microbial markers, based on genetic markers, can discriminate among animal, bird and human faecal inputs (Sinton *et al.* 1998; Field & Samadpour 2007). The genetic

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markers target bacteria specific to the intestinal environment of a particular animal or bird and amplify the deoxyribonucleic acid (DNA) from the bacterium using the polymerase chain reaction (PCR). PCR markers have gained interest as they out performed other methods of faecal source tracking (FST) in interlaboratory experiments (Griffith *et al.* 2003). In addition, with the advent of real-time PCR methods, genetic markers are regarded as delivering timely and cost-effective results compared with other FST methods (Field *et al.* 2003; Santo Domingo *et al.* 2007). Many PCR markers have been designed based on the bacterial order *Bacteroidales*, as these bacteria are well represented in mammalian intestines (Kildare *et al.* 2007; Savichtcheva *et al.* 2007). They are reported to be identified in higher concentrations in the gut than traditional microbial indicators such as *E. coli* (Salyers 1984). Furthermore, an important consideration for an indicator of faecal contamination is that the *Bacteroidales* are obligate anaerobes, which reduces the likelihood that they will survive when excreted into the environment.

In this study, specificity testing of two published PCR markers, HumM3 (Shanks *et al.* 2009) and HF183 (Bernhard & Field 2000a), designed to identify human pollution, found a high false-positive rate against possum faeces and a very low number of positives against a range of other animals and birds. A primer system was designed to target unique faecal bacteria harboured by possums and investigate the prevalence of possum faecal pollution in NZ surface waters. Using this novel marker allowed detection of human PCR markers in a water sample to be corroborated as human (no detection of possum marker), and/or possum (detection of possum and human marker). Validation of the novel possum PCR marker in a pilot study of NZ environmental waters suggested a low prevalence of possum faecal contamination. To exclude the possibility that only possum pollution is being detected, additional testing by other faecal source tracking methods is required where the water sample is positive for both human and possum PCR markers. An attempt could be made to design a human-specific PCR marker that was not detected in possum faeces, although this may be difficult because of the high number of false positives for possum with two human markers. These two human PCR markers target different genes, 16S rRNA (Bernhard & Field 2000a) and a

sigma factor (Shanks *et al.* 2009) suggesting that similar non-specificity may arise if designing another human PCR marker solely for the NZ environment with its uniquely high possum population. In addition, the identification of a low prevalence of possum faecal material in a pilot study of waterways may make this unnecessary.

## METHODS

### Sample collection

Individual faecal samples ( $n = 259$ ) were collected from various locations around NZ from 12 different animal and bird species likely to impact on water quality, including human ( $n = 48$ ), possum ( $n = 36$ ), rabbit ( $n = 10$ ), dog ( $n = 18$ ), cow ( $n = 20$ ), sheep ( $n = 20$ ), duck ( $n = 21$ ), black swan ( $n = 20$ ), gull ( $n = 20$ ), Canada goose ( $n = 20$ ), chicken ( $n = 12$ ) and horse ( $n = 14$ ). Faecal samples were collected after observation of defecation or if samples had a moist sheen on the surface of the sample indicating recent defecation. Samples were collected in a manner to reduce contamination from the underlying environmental surface and in some cases, samples (e.g. avian species) were collected from a plastic sheet that had been laid down prior to collection. Human-derived raw sewage samples ( $n = 10$ ) were also collected from municipal waste plants prior to treatment. Environmental water samples ( $n = 25$ ) were collected from areas throughout NZ to investigate the detection of the possum PCR marker in conjunction with the human PCR markers. Water samples were chosen to represent a range of environments such as estuarine, rural bush and pasture streams, and urban streams, including stormwater outfalls. Particular consideration was given to choosing locations where tree cover over streams would increase the likelihood of possum habitation in the environment surrounding the waterways.

### DNA extraction

DNA was extracted from faeces (200 mg wet weight) using the protocol of the ZR Fecal DNA Kit™ (#D6010 Zymo Research, Orange, CA, USA), which included processing faeces in a bead beater (MixMate, EppendorfAG, Hamburg,

Germany) for 5 min at 2,000 rpm. DNA was eluted in 100  $\mu$ l of elution buffer.

DNA was extracted from water samples according to the protocol of Dick & Field (2004). In brief, 100 ml water samples were filtered through a Supor 200, 0.2  $\mu$ m Polyethersulfone (PES) filter (Pall Corp., Washington Port, NY, USA), and 1 ml of guanidine isothiocyanate (GITC) buffer (5 M GITC, 0.1 M EDTA, 10% sarcosyl) was added. The filter was immersed in the GITC buffer and vortexed, after which it was frozen at  $-20^{\circ}\text{C}$ . After thawing and repeated vortexing of the filter, DNA was extracted using the Qiagen DNeasy Kit (QIAGEN, Valencia, CA, USA). Briefly, 700  $\mu$ l AL buffer (supplied by manufacturer) was added to the filter and the mixture was vortexed and incubated for 5 min at room temperature. The supernatant was added to a spin column from the DNeasy kit, and the column centrifuged for 1 min at 15,700 g. The flow-through was discarded. This step was repeated until all of the supernatant was transferred to the spin column. The filter was then washed using the kit's reagents and the DNA eluted in 100  $\mu$ l of elution buffer. During each extraction, a blank of sterile Gibco UltraPure water (Invitrogen, Paisley, UK) was extracted to monitor for potential DNA contamination.

### PCR analysis

Routine testing of the geographic specificity of previously published general faecal PCR markers using human, ruminant and bovine faecal-specific primers was performed on individual faecal samples collected from humans, cows, sheep, pigs, ducks, black swans and possums, and human sewage samples from municipal wastewater plants. Primer systems tested were the probe-based assays of GenBac3 (Siefring *et al.* 2008), human-specific HumM3 (Shanks *et al.* 2009), ruminant-specific BacR (Reischer *et al.* 2006), bovine-specific CowM2 (Shanks *et al.* 2008) and a SYBR Green assay using human-specific primers HF183 and Bac708R (Bernhard & Field 2000a). Additional SYBR Green assays using primers for general *Bacteroidetes* (Dick & Field 2004), ruminant CF128 (Bernhard & Field 2000a) and wildfowl (Devane *et al.* 2007) faecal detection were also used to test environmental water samples.

PCR amplifications were performed in a total volume of 25  $\mu$ l using 2  $\mu$ l of DNA template. PCR conditions for the SYBR Green assays were as follows, 2 $\times$  LightCycler 480 SYBR Green I Master mix (Roche Diagnostics Ltd, Penzberg, Germany), 0.25  $\mu$ M of each primer and 0.2 mg/ml of bovine serum albumin (BSA) (Sigma-Aldrich, MO, USA).

PCR conditions for the probe-based assays were as follows: 2 $\times$  LightCycler 480 Probes Master mix (Roche Diagnostics Ltd), 100 nM of probe, 500 nM of each primer and 0.2 mg/ml of BSA (Sigma-Aldrich).

All primer sets in this study used an annealing temperature of  $60^{\circ}\text{C}$  and followed the protocol outlined for amplification. The use of a single annealing temperature meant an increase in annealing temperature for HF183, CF128 ruminant (Bernhard & Field 2000a), and BacR ruminant (Reischer *et al.* 2006) (original annealing:  $59^{\circ}\text{C}$ ,  $58^{\circ}\text{C}$  and  $58^{\circ}\text{C}$ , respectively). Using the same PCR cycling parameters enabled screening of multiple assays, reducing cost and increasing efficiency of sample screening. In addition, the increase in annealing temperature for some assays may increase specificity.

Thermal cycling conditions for the LightCycler 480<sup>®</sup> (Roche Diagnostics Ltd) started with an initial denaturing cycle at  $95^{\circ}\text{C}$  for 5 min, followed by 45 cycles at  $95^{\circ}\text{C}$  for 10 s and  $60^{\circ}\text{C}$  for 10 s, and elongation at  $72^{\circ}\text{C}$  for 20 s. Each assay run included a non-template control (NTC), an extraction blank and a standard curve. The standard curve was generated from 10-fold serial dilutions of the appropriate target cloned into *E. coli* DH5 $\alpha$  (Invitrogen, Carlsbad, CA, USA) using the pGEM-T Easy cloning kit (Promega, Fitchburg, WI, USA). A NanoDrop<sup>®</sup> ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA), determined the DNA concentration and allowed for calculation of the copy number of target DNA extracts from plasmid constructs. Melting curve ( $T_m$ ) analysis of SYBR assays began with a pre-incubation step at  $95^{\circ}\text{C}$  for 5 s, then 1 min at  $65^{\circ}\text{C}$ , followed by an increase in the temperature from 65 to  $97^{\circ}\text{C}$  at a ramp rate of  $0.11^{\circ}\text{C}/\text{s}$ , and a cooling period at  $40^{\circ}\text{C}$  for 10 s. All amplicons were within  $0.3^{\circ}\text{C}$  of the plasmid standards on each LightCycler 480<sup>®</sup> run. If the  $T_m$  of duplicates was not within  $\pm 0.3^{\circ}\text{C}$  of the standard  $T_m$ , or the  $C_p$  of duplicates for the probe assays was not within  $\pm 1 C_p$ , then another replicate of the DNA extract was analysed by qPCR, and the result scored as

two out of three. Samples that registered a Cp value above 40 were considered as not detected.

### Development of the possum-specific PCR marker

DNA extracts from 35 individual possum faecal samples generated 44 amplicons by amplification with the primers Bac32F and Bac708R (Bernhard & Field 2000b). Seventeen of these amplified sequences were cloned into *E. coli* DH5 $\alpha$  (Invitrogen) using the pGEM-T Easy cloning kit (Promega). All sequencing was performed on an ABI 3130XL capillary sequencer (Applied Biosystems, Foster City, CA, USA) using the Big Dye Terminator V3.1 (Applied Biosystems) and the primers Sp6 and T7 for the 17 cloned inserts. The other 27 sequences were sequenced directly using Bac32F and Bac708R (Bernhard & Field 2000b).

All sequences were used in multiple alignments using the sequence alignment programme in Bionumerics Version 5.10 (Applied Maths, Belgium) to identify unique 16S ribosomal ribonucleic acid (rRNA) regions specific to bacteria hosted by possums. The uniqueness of potential targets was assessed by comparing them with non-target host species in GenBank. Primers were designed using Primer3Plus software (Untergasser *et al.* 2007). Putative primers were compared with nucleotide sequences using the BLASTN algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm specificity to the target sequence. Confirmation of primer specificity was provided by testing against individual faecal samples of 36 possum and 233 non-target animal and human samples. Possum-specific primers developed for a SYBR assay were P56F (5'-TGCAAGTCGAGGGGTAACAG-3') and P208R (5'-TAAGGAGACCATGCGGAATC-3').

### Determination of specificity and sensitivity of the Bacteroidales possum marker assay

Specificity was determined as  $a/(a+b)$  where  $a$  represents the total number of true PCR negatives of non-target animal and bird faecal samples, and  $b$  represents the total number of false-positive PCR results. Sensitivity was defined as  $c/(c+d)$  where  $c$  represents the total number of possum faecal extracts which were positive with the possum PCR marker and  $d$  represents the number of possum faecal extracts in which the possum marker was not detected.

### Mixed dilution experiments with human and possum faecal DNA extracts

During specificity testing of the SYBR possum assay, DNA extracts from human and dog faeces generated similar sized amplicons to those from possum faeces. Therefore, the DNA extracts from possum, human and dog faeces were amplified with primers P56F and P208R and cloned into pGEM-T Easy (Promega) according to the manufacturer's instructions. Clones were sequenced using T7 and Sp6 followed by multiple alignments to identify sequence differences among possum, human and dog faeces.

A series of dilution experiments with human and possum faecal DNA extracts investigated the events in which there was potential for mixed faecal inputs from humans and possums. The aim was to find out if the differences in melting peak ( $T_m$ ) observed between amplicons from human and possum faecal extracts assayed with the possum marker, could be used to identify a mixed faecal source of possum and human. All of the dilution series described next were assayed using the possum marker PCR conditions developed in this study.

The possum and human faecal DNA used for this mixing experiment were extracted from 200 mg of wet weight of their respective faeces. The possum faecal extract was diluted in a 10-fold serial dilution in Gibco UltraPure water (Invitrogen) and a 2  $\mu$ l volume added to the PCR reaction mix to determine the concentration of the possum marker in possum faeces and its detection level in the PCR assay. A similar dilution series was performed on the human faecal extract to determine the lowest dilution where the  $T_m$  84.5 °C (human peak) was detected.

To determine if both melting peaks could be detected when concentrations of possum and human DNA were similar, the following mixed dilution series was performed. A mixed 10-fold dilution series (down to  $10^{-5}$ ) of equal concentrations of possum and human DNA extracts was tested with the possum marker assay. The volume of DNA template added to the final PCR for each extract was 2  $\mu$ l for all assays.

The final experiment mixed equal volumes (2  $\mu$ l each) of undiluted possum DNA and decreasing concentrations of the human DNA extract in a 10-fold dilution series (down

to  $10^{-5}$ ). The inverse experiment was also performed where undiluted human faecal DNA was mixed with equal volumes of decreasing amounts of possum faecal DNA in a 10-fold dilution series (down to  $10^{-5}$ ).

### Detection thresholds of the *Bacteroidales* possum marker

The assay limits of detection and quantification were defined in relation to possum marker detection in molecular biology grade water rather than in an environmental water sample.

### Amplification efficiency

The amplification efficiency of the *Bacteroidales* possum marker assay was determined by collating the results of eight standard curves generated using 10-fold serial dilutions of known amounts of the PGem-T easy plasmid carrying the cloned unique possum sequence. The slope(s) of each of the standard curves was used to calculate the amplification efficiency (E) using the following formula:

$$E = 10^{-1/s} - 1 \quad (1)$$

### Limits of detection of the PCR assay

The lower limit of quantification (LLOQ) was determined by preparing two solutions that closely spanned above and below the concentration of the lowest standard within the linear range of the standard possum marker curve that consistently reported a positive amplification signal during the LightCycler 480<sup>®</sup> amplification runs. These two solutions and the lowest standard were quantified in eight replicate wells by amplification using the possum marker PCR. The LLOQ was determined to be the concentration which reported all eight replicates having a  $C_p \leq 40$ .

### Sample limit of detection ( $S_{LOD}$ ) in an environmental water sample

Sample limit of detection ( $S_{LOD}$ ) was performed by modifying the method of [Fremaux \*et al.\* \(2009\)](#). Three faecal

samples from individual possums were suspended separately (0.2 g wet weight per 200 ml) in local river water which had tested negative for the possum marker. Ten-fold serial dilutions were performed on each sample ranging from  $10^{-1}$  to  $10^{-9}$  and DNA was extracted from each dilution (100 ml) as previously described. PCR analysis was performed on each dilution series using the possum-specific primers. The  $S_{LOD}$  was determined as the lowest concentration (g/100 ml) of each faecal sample, which reported a signal for the melting peak of interest.

### Conditional probability analyses of confidence in faecal detection based on Bayes' Theorem

By applying modifications of the method of [Kildare \*et al.\* \(2007\)](#), Bayes' Theorem was used to calculate the probability that a positive result for detection of the possum marker in a water sample was the result of a true faecal event associated with the target species. Modifications applied by [Lamendella \*et al.\* \(2009\)](#) were included as the prior probability of detecting a host-specific marker was unknown and relied upon surveys of the catchment under study to determine the likelihood of specific faecal sources.

The following formula estimated the posterior probability that a positive result generated by the possum PCR marker was likely to be the result of a true faecal input by possums in the waterway under investigation:

$$P(Po \setminus T) = \frac{P(T \setminus Po) * P(Po)}{P(T \setminus Po) * P(Po) + P(T \setminus Po') * P(Po')} \quad (2)$$

where  $P(T \setminus Po)$  is the proportion of possum faecal samples that are positive for the possum PCR marker, and  $P(T \setminus Po')$  is the proportion of non-possum faecal samples that are positive for the possum PCR marker (these are the false-positive PCR results for non-target species),  $P(Po)$  is the prior probability that there is possum faecal contamination in the water sample. This aspect of the equation is unknown although it can be estimated by a survey of the environment surrounding the waterway. The equation, therefore, was modified according to [Lamendella \*et al.\* \(2009\)](#) and calculated by using a range of values for the prior probability

(0 to 1.0) and consequently for the parameter  $P(\text{Po}')$  which is defined as  $1 - P(\text{Po})$ .

## RESULTS AND DISCUSSION

Some studies have recognised the importance of re-evaluating PCR markers when they are applied to geographical environments removed from the area for which they were first developed (Ahmed *et al.* 2009; Fremaux *et al.* 2009). This re-evaluation of marker specificity is required because animal and bird species endemic to the region of interest may not have been tested in the original study. In addition, geographical and climatic differences may impact the bacterial composition of the gut of host animals and birds, reducing the sensitivity of the PCR marker or leading to false-positive detection in non-target species.

In this study, specificity testing of possum faecal extracts identified a 100% false-positive rate for the human assays HumM3 (Shanks *et al.* 2009) and HF183 (Bernhard & Field 2000a) (Table 1). In both assays, high arithmetic mean copy numbers of  $10^7$  per g faeces, suggest that the presence of possum faeces in a sample would result in it being incorrectly identified as human faecal contamination. The ruminant-specific assay (Reischer *et al.* 2006) also had a 100% false-positive rate with possum faeces (Table 1).

Faecal extracts from other animals known to impact on faecal pollution in NZ waters had a true negative rate of 100% with these same human markers (Table 1). Therefore, these two human markers which target different genes, 16S rRNA (Bernhard & Field 2000a) and a sigma factor (Shanks *et al.* 2009), are potentially very useful for the identification of human-derived pollution in the NZ environment. As reviewed by Ahmed *et al.* (2009), the high specificity of the HF 183 assay has been confirmed in 10 trials conducted in seven countries. Specificity testing reported values ranging from 85–100%, with six of those trials reporting 100% specificity. To ensure that we were aware of any interference from possum faeces, we designed a possum PCR marker to confirm the presence of human pollution by the non-detection of possum pollution. This is similar to the approach used by Gourmelon *et al.* (2007), who found the ruminant CF128 marker also reacted with pig faeces, and where pig and ruminant pollution was

Table 1 | Specificity testing of animal and human PCR assays

PCR assay	Number of positive PCR results/total sample number of specific faecal types tested (mean copy number per gram faeces) <sup>a</sup>							
	Possum	Human	Sewage	Cow	Sheep	Pig	Duck	Black swan
GenBac3	10/10 ( $2.1 \times 10^9$ )	16/16 ( $4.5 \times 10^9$ )	4/4 ( $2.3 \times 10^7$ )	20/20 ( $1.8 \times 10^9$ )	20/20 ( $3.0 \times 10^9$ )	10/10 ( $4.0 \times 10^9$ )	10/10 ( $1.5 \times 10^9$ )	9/10 ( $1.6 \times 10^8$ )
HumM3	10/10 ( $9.8 \times 10^7$ )	10/16 ( $6.8 \times 10^7$ )	2/4 ( $3.3 \times 10^5$ )	0/20	0/20	0/10	0/10	0/10
HF183	10/10 ( $6.8 \times 10^7$ )	11/16 ( $4.3 \times 10^7$ )	2/4 ( $3.0 \times 10^5$ )	0/20	0/20	0/10	0/10	0/10
Ruminant-specific BacR	10/10 ( $6.3 \times 10^4$ )	0/10	2/4 ( $8.8 \times 10^3$ )	20/20 ( $6.5 \times 10^7$ )	20/20 ( $1.3 \times 10^8$ )	0/10	1/10 ( $8.3 \times 10^2$ )	2/10 ( $6.8 \times 10^2$ )
Bovine-specific CowM2	0/10	0/16	0/4	12/20 ( $1.9 \times 10^7$ )	0/20	0/10	0/10	0/10

<sup>a</sup>Arithmetic mean copy number calculated on the number of positive results rather than the total number of faeces tested.

possible, the use of both ruminant and pig marker assays was required.

### Development of the possum PCR assay

To improve the likelihood of comparability with existing assays, the possum marker targets members of the *Bacteroidales* order, which is prevalent in possum faeces. Partial 16S rRNA sequences were determined by amplifying possum DNA with primers Bac32F and Bac708R (Bernhard & Field 2000b) and cloning the products into pGEM-T Easy (Promega). Examination of these sequences within the region with which the human indicative HF183 primer binds, identified an exact match in sequence. Alignment of possum 16S rRNA sequences with GenBank database sequences identified regions of potential specificity to possums, in which primers P56F and P208R were designed. Partial 16S rRNA sequences from possums have been uploaded to GenBank under Accession numbers JX418318 and JX418319.

Initial specificity tests of primers P56F and P208R identified amplification from human and dog faecal extracts. The PCR products from each of these host faecal extracts were cloned into pGEM-T Easy and sequenced using T7 and Sp6 to identify sequence differences among the amplicons from possum, human and dog. The primer pair produced a slightly smaller product from amplification of possum faecal extracts (152 bp) compared with the 156 bp sequence amplified from human and dog extracts. Dog and human sequences were identical over the region sequenced. The possum sequence differed in three areas, including a

deletion of four bases. The melting point, therefore, between the two amplicons was different to the possum amplicon ( $T_m = 86.13^\circ\text{C}$ ,  $\text{SD} \pm 0.21$ ) being higher than that observed from 40 human faecal extracts ( $T_m = 84.52^\circ\text{C}$ ,  $\text{SD} \pm 0.22$ ) and three dog faecal extracts ( $T_m = 84.3^\circ\text{C}$ ). These non-specific amplicons with lower melting points also tended to be present at lower levels in non-target faeces. Sequences for the amplicons generated by the possum primer set are available on request.

These DNA extracts from human and dog faeces had equivalent initial faecal mass (200 mg wet weight) as the possum extracts, with their amplification being an average of 10 cycles later (at least a three log reduction) compared with possum amplicons.

The non-specific melting point at  $T_m = 84.5^\circ\text{C}$ , did not interfere with detection of possum faecal contamination and suggested the possibility that the possum marker might allow concurrent detection of human and possum pollution. Therefore, mixed dilution experiments of DNA extracts from human and possum faeces were undertaken to establish whether the two peak signals would be identified in a single possum marker assay and could be useful for the detection of a mixed faecal event (Table 2). Each DNA extract was derived from 200 mg of faeces with the undiluted possum DNA extract having a threshold cycle ( $C_p$ ) of 21.2 for the possum marker at  $T_m = 86.1^\circ\text{C}$ , and the human DNA extract having a similar  $C_p$  of 21.1 but a different melting point peak of  $T_m = 84.5^\circ\text{C}$  for the possum marker. The initial concentration of the possum marker in the possum faecal extract used for this mixing experiment was determined to be

**Table 2** | Detection of target melting peaks using the possum PCR marker when there is a mix of human and possum faecal DNA present in a water sample

		Dilution series of possum faecal DNA						
		0	Undiluted possum DNA <sup>a</sup>	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>
Dilution series of human faecal DNA	0	ND	$T_m = 86.1^b$	86.1	86.1	86.1	86.1	86.1
	Undiluted human DNA <sup>a</sup>	$T_m = 84.5^c$	86.1	84.3 <sup>c</sup> and 86.0 <sup>b</sup>	84.3	84.3	84.3	
	10 <sup>-1</sup>	84.5	86.1	86.1				
	10 <sup>-2</sup>	84.5	86.1		86.1			
	10 <sup>-3</sup>	84.5	86.1			86.1		
	10 <sup>-4</sup>	84.5	86.1				86.1	
	10 <sup>-5</sup>	84.5						NT <sup>d</sup>

<sup>a</sup>Faecal mass extracted 200 mg (wet).

<sup>b</sup>Melting peak for possum faeces ( $86.1 \pm 0.3^\circ\text{C}$ ).

<sup>c</sup>Melting peak for human faeces ( $84.5 \pm 0.3^\circ\text{C}$ ).

<sup>d</sup>Not tested.

$5.7 \times 10^5$  copies per assay which equates to  $1.4 \times 10^8$  copies per g of wet faeces. Serial dilution of the same DNA extract in UltraPure water (Invitrogen) allowed detection to a level of  $1.8 \times 10^3$  copies per g of wet faeces. There was no cloned standard developed for the human assay at  $T_m$  84.5 °C, consequently there was no standard curve to determine copy number. The melting peak at  $T_m$  84.5 °C, however, was detected to the same  $10^5$  dilution as the possum melting peak at  $T_m$  86.1 °C, suggesting a similar level of detection.

Exclusive detection of the melting peak specific to the possum ( $T_m$  86.1 °C) occurred in all samples with equal concentrations of possum and human faecal DNA, and when undiluted possum DNA was mixed with serial dilutions of the human faecal DNA (Table 2). The only time a double melting peak of  $T_m$  84.3 °C (human associated) and  $T_m$  86.1 °C (possum specific) was observed, occurred when undiluted human faecal DNA was mixed with a  $10^{-1}$  dilution of the possum DNA. Thereafter, for the mix of undiluted human plus diluted possum faecal extracts, the peak associated with human DNA at  $T_m$  84.3 °C dominated the assay with no signal detected from the possum marker peak at  $T_m$  86.0 °C.

These results demonstrate that the possum assay is not able to identify a mixed pollution event where human and possum faecal inputs occur in the same waterway. The exception to this would be where the possum contamination was present at a concentration that was 10-fold less than the human input. This mixing experiment showed that in an environment of mixed human and possum pollution, the possum PCR preferentially amplifies the possum-derived *Bacteroidales* DNA target. In addition, the melting curves for possum and human amplicons remained clearly differentiated throughout this experiment and within a tight boundary of  $\pm 0.2$  for each  $T_m$  as evidenced when the human melting peak showed a slight shift from  $T_m$  84.5 °C to  $T_m$  84.3 °C after mixing with diluted possum faecal DNA (Table 2).

The possum assay was therefore defined as generation of an amplicon using primers P56F and P208R with a  $T_m = 86.1 \text{ °C} \pm 0.3$ .

### Performance of the possum PCR assay

Given our DNA extraction methods and the volume of DNA extract used in the PCR, the LLOQ was 22 copies per PCR reaction or  $5.5 \times 10^3$  gene copies per g of possum faeces,

which was the minimum concentration that could be measured and reported with 95% confidence that the possum marker concentration was greater than zero. Extraction blanks and non-template controls were monitored for each PCR assay and did not produce any amplicons at or near the target  $T_m$ .

The possum PCR marker was detected in 83.3% of the possum faeces tested ( $n = 36$ ), and at levels of up to  $10^9$  copies per g of faeces. The possum marker was not detected in the 10 samples of raw human sewage or in eight of the animal species tested (Table 3). However, false-positive amplification was observed with this marker from black swan faeces (7/20 samples), rabbits (1/10 samples) and human faeces (2/48 samples). Very late amplification was observed from two human and one rabbit sample (Cp 37 and higher), reporting levels of 10, 20 and 8 copies, respectively, which were below the LLOQ of 22 copies per PCR reaction (Table 3). The low prevalence in these sources and the non-detection of the human marker in sewage coupled with dilution effects in a waterway should minimise the influence of these as false-positive PCR results for the possum marker. The black swan false-positive results are less readily dismissed, with 7 of the 20 faecal samples containing the possum marker. Detection of the possum marker in a water sample should therefore consider the likelihood of black swans being present in the sampling vicinity.

Overall, specificity of the possum marker at  $T_m$   $86.1 \text{ °C} \pm 0.3$  was high at 95.7% when all the true negatives for the total number of non-possum species were taken into account. The average amplification efficiency of the PCR assay was excellent at 92.1%, as was the coefficient of determination ( $r^2 \geq 0.99$  for all assays).

Three possum faecal samples, which tested positive at similar concentrations for the possum marker, were individually diluted in river water to determine the  $S_{LOD}$  in a field situation. The samples were diluted in river water that had been previously tested to ensure it reported no signal for the possum marker. In addition, this river water was likely to be heavily impacted by biological material from plants overhanging and inhabiting the waterway. The possum marker was detected in dilutions down to  $10^{-7}$  g of possum faeces per 100 ml of water. These  $S_{LOD}$  values are similar to those reported by Bernhard & Field (2000a) for the cow CF128 assay, and slightly lower than those



**Table 3** | Sensitivity and specificity of possum PCR marker

Animal/bird species	Numbers tested	Percentage positive (sample number)	Melting peak	Mean copy number <sup>a</sup> in PCR assay (range)	Mean copy number per g of faeces
Possum	36	83.3 ( <i>n</i> = 30)	86.08 (SD <sup>b</sup> ± 0.21)	8.3 × 10 <sup>5</sup> (16–1.0 × 10 <sup>7</sup> )	2.1 × 10 <sup>8</sup>
Human	48	4.2 ( <i>n</i> = 2)	86.30 and 86.16	15 (10–20)	3.8 × 10 <sup>5</sup>
Raw human sewage	10	0			
Rabbit	10	10.0 ( <i>n</i> = 1)	85.96	8	2.0 × 10 <sup>3</sup>
Dog	18	0			
Cow	20	0			
Horse	14	0			
Sheep	20	0			
Duck	21	0			
Swan	20	35 ( <i>n</i> = 7)	85.83 (SD ± 0.05)	127 (23–470)	3.2 × 10 <sup>4</sup>
Gull	20	0			
Canada goose	20	0			
Chicken faeces	12	0			

<sup>a</sup>Arithmetic mean copy number calculated on the number of positive PCR results rather than the total number of faeces tested.

<sup>b</sup>Standard deviation.

reported by [Fremaux \*et al.\* \(2009\)](#) for human, cow and pig assays.

Therefore, these results suggest that the possum PCR marker is a sensitive and specific assay for detection of possum faecal contamination in waterways. Inhibition of water samples by chemical and biological constituents derived from organic matter such as humic acid, have been shown to affect amplification of DNA targets. In the case of humic acid, which is commonly encountered in water samples, inhibition appears to be due to binding of the humic acid to the DNA template, which causes a shift in the melt curve temperature ([Opel \*et al.\* 2010](#)). Inhibition by humic substances can be reduced by the method of extraction ([Dick & Field 2004](#)) and addition of BSA to the PCR reaction mix. These actions were followed for this study reducing the likelihood of interference by humic substances in environmental samples. The average  $T_m$  for all three individual possum faecal samples diluted into river water to determine the  $S_{LOD}$  was  $T_m$  86.02 °C ± 0.08 (*n* = 39 melting curves). The tight boundaries for the melting curve analysis in river water samples likely to contain humic substances, is well within the defined assay boundaries of  $T_m$  = 86.1 °C ± 0.3 and confirms the ability of the possum marker to identify possum

faecal pollution in an environmental water sample ([Opel \*et al.\* 2010](#)).

### Environmental water sampling

Twenty-five waters that had elevated concentrations of *E. coli* (>260 CFU/100 mL) from locations throughout the two main islands of NZ were tested with a range of PCR markers ([Table 4](#)). Samples were chosen from areas where the tree cover over streams increased the likelihood of possum habitation. Samples were tested with PCR assays for the detection of a general faecal marker, and source specific assays for human, herbivore, wildfowl and the possum marker developed in this study ([Table 4](#)). While the general faecal indicator was detected in all samples, no specific sources of faecal pollution inputs were identified in seven of the sampling locations. Nine of the environmental water samples were positive for the two human PCR markers and one was positive only for the human PCR marker developed by [Shanks \*et al.\* \(2009\)](#). The possum faecal marker was only detected in one water sample, which was also positive for human and wildfowl pollution markers. For the other samples positive with human markers, these results confirm that the positive

**Table 4** | Field evaluation of environmental waters tested with faecal source tracking markers and reported as copy number per 100 ml of water

Water samples	Possum this paper	General faecal indicator Dick & Field (2004)	Human Shanks <i>et al.</i> (2009)	Human Bernhard & Field (2000a)	Herbivore Reischer <i>et al.</i> (2006)	Wildfowl Devane <i>et al.</i> (2007)
Estuary	ND <sup>a</sup>	$5.3 \times 10^{4b}$	ND	ND	ND	ND
Estuary	ND	$3.4 \times 10^{4b}$	ND	ND	ND	ND
Estuary	ND	$1.9 \times 10^{4b}$	ND	ND	ND	ND
Estuary	ND	$6.6 \times 10^{4b}$	ND	ND	ND	ND
Estuary	ND	$2.5 \times 10^{4b}$	ND	ND	$5.1 \times 10^2$	ND
Estuary	ND	$8.8 \times 10^{3b}$	ND	ND	ND	ND
Estuary <sup>d</sup>	$3.4 \times 10^3$	$7.9 \times 10^7$	$1.6 \times 10^5$	$1.1 \times 10^5$	ND <sup>c</sup>	$4.0 \times 10^4$
Park Stream <sup>d</sup>	ND	$9.5 \times 10^8$	$2.2 \times 10^6$	$2.0 \times 10^6$	ND <sup>c</sup>	$2.1 \times 10^4$
Park Stream <sup>d</sup>	ND	$2.0 \times 10^9$	$2.2 \times 10^6$	$3.0 \times 10^6$	ND <sup>c</sup>	$8.5 \times 10^4$
Park Stream <sup>d</sup>	ND	$1.6 \times 10^{10}$	$1.7 \times 10^7$	$5.6 \times 10^6$	ND <sup>c</sup>	$9.0 \times 10^3$
Park Stream <sup>d</sup>	ND	$5.3 \times 10^7$	$1.4 \times 10^5$	NT <sup>e</sup>	ND <sup>c</sup>	$6.0 \times 10^4$
Urban stream	ND	$4.3 \times 10^9$	ND	ND	NT	$7.3 \times 10^3$
Urban stream	ND	$5.5 \times 10^9$	$7.5 \times 10^2$	$2.0 \times 10^4$	NT	$1.1 \times 10^4$
Urban stream	ND	$4.5 \times 10^9$	$5.0 \times 10^2$	$7.8 \times 10^3$	NT	$6.8 \times 10^3$
Urban stream	ND	$1.7 \times 10^9$	$6.5 \times 10^2$	$3.7 \times 10^4$	NT	ND
Beach stream	ND	ND	ND	ND	ND	ND
Stream	ND	ND	ND	ND	ND	ND
Stream	ND	$9.0 \times 10^{10}$	ND	ND	ND	$4.1 \times 10^3$
Middle beach stream	ND	$2.6 \times 10^7$	ND	ND	ND <sup>c</sup>	ND
Beach stream	ND	$1.9 \times 10^8$	ND	ND	$8.1 \times 10^{5c}$	$3.9 \times 10^3$
Stormwater outfall	ND	$9.8 \times 10^{5b}$	$1.1 \times 10^3$	$1.3 \times 10^3$	ND	ND
Bush stream	ND	$3.9 \times 10^{4b}$	ND	ND	ND	ND
Rural pasture stream	ND	$3.2 \times 10^{5b}$	$4.6 \times 10^2$	ND	ND	ND
Stormwater outfall	ND	$1.6 \times 10^{6b}$	ND	ND	ND	ND
Coastal freshwater stream	ND	$1.8 \times 10^{10}$	ND	ND	ND	$1.4 \times 10^4$
Coastal freshwater stream	ND	$3.3 \times 10^{10}$	ND	ND	ND	$1.5 \times 10^4$
Coastal freshwater stream	ND	$3.1 \times 10^{10}$	ND	ND	ND	ND

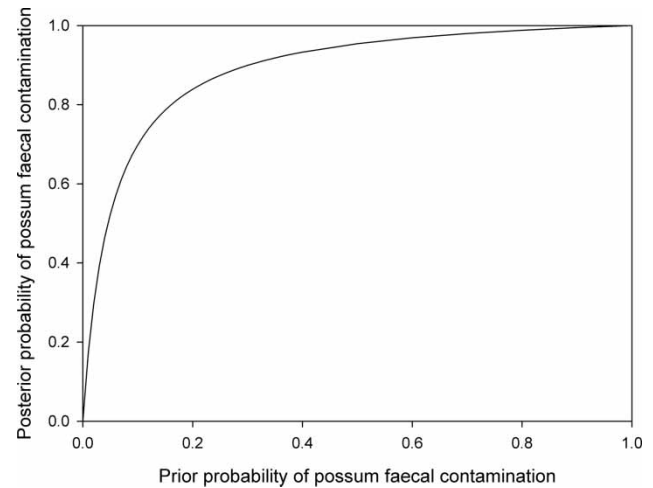
<sup>a</sup>Not detected.<sup>b</sup>Tested using the GenBac3 marker (Siefiring *et al.* 2008).<sup>c</sup>Tested using the ruminant marker, Bernhard & Field (2000a).<sup>d</sup>Catchment surveys indicated broken sewer pipes in the vicinity of these locations.<sup>e</sup>Not tested.

results with human PCR markers are due to the presence of human faecal pollution, and are not the result of possum faeces. It is only samples positive for both human and possum markers that need further testing to confirm a human pollution source. Additional tests could include other human PCR markers such as *Bifidobacterium adolescentis* (Matsuki *et al.* 2004), and chemical tests including faecal sterol analysis (Gilpin *et al.* 2003, 2011). In this study,

testing of the possum marker positive water sample with faecal sterol analysis confirmed the presence of human pollution (data not shown). Specificity testing also identified low level cross-reaction of the possum marker with swan faeces. The single water sample positive for the possum marker in this study had no known swans in the vicinity, suggesting this sample was likely to contain both human and possum faecal material.

Little information is known about the abundance and distribution of possum faecal pollution in waterways in NZ. Their arboreal lifestyle means that they are less likely to contribute to significant pollution through direct faecal inputs to rivers and streams. Land runoff during heavy rainfall is the most likely scenario that may result in possum faecal pollution to waterways, but their contribution may be minor compared with other agricultural faecal inputs. Our small survey supports this suggestion. Future studies of faecal contamination that employ PCR assays incorporating the possum marker will increase knowledge about the likelihood of possum faecal pollution in rural and urban surface waters in NZ.

To understand the probability of a marker correctly identifying a faecal source, Kildare *et al.* (2007) applied Bayes' Theorem to the interpretation of true versus false-positive results for PCR markers (Equation (3)). Their equation takes into account the prevalence of the marker in its host target and the number of false-positive PCR results in non-host faecal specimens. The distribution of posterior probabilities for the possum marker was based on the cumulative false-positive rate ( $P(T \setminus Po') = 0.043$ ) derived from amplification of the possum marker with black swan (0.030), human (0.009) and rabbit (0.004) faecal extracts. The proportion ( $P(T \setminus Po)$ ) of possum faecal samples that were positive for the possum PCR marker was determined to be 0.833 (Table 2). These true versus false-positive rates estimate the likelihood of a true positive PCR result based on a priori knowledge of the watershed under investigation. Lamendella *et al.* (2009) further developed this concept by calculating the probability of a marker correctly identifying a faecal source when up to three independent PCR markers were used to determine the faecal source. In addition, they recognised that in many locations the prior knowledge of the probability of a faecal source in a waterway is unknown until extensive testing has been performed. Therefore, they produced a probability curve based on the full range of prior probabilities, and this is the procedure followed by this study and presented in Figure 1. The prior probability ( $P(Po)$ ) of possum pollution in the surrounding environment influences the confidence placed in the PCR marker returning a true positive result for detection of possum derived contamination in the water sample. The likelihood of possum faecal contamination ( $P(Po)$ ) in a waterway can be



**Figure 1** | Posterior probability that a positive result for detection of the possum PCR marker in a water sample was the result of a true faecal event associated with possums. The prior probability is based on the likelihood of possum faecal contamination being present in the surrounding environment as determined by a survey of the target area.

estimated by conducting a qualitative site survey of the likely faecal inputs to the waterway under investigation. From Figure 1, it can be determined that it would require only a 20% likelihood of possum faecal inputs into a waterway to have 80% confidence in the result from the possum marker assay.

Researchers have investigated mathematical methods to predict the presence of pathogens such as viruses and protozoa in water by using less expensive water quality parameters as input data to their modelling scenarios (Black *et al.* 2007; Neelakantan *et al.* 2001). Black *et al.* (2007) suggested that a combination of biological and chemical indicators that target the faecal source, the faecal age and the faecal loading of the pollution provided the best fit for predicting the presence or absence of viable viruses in waterways. Future investigations for predictive modelling of pathogen presence could include PCR markers such as the possum marker described in this study.

The possum marker developed in this study will add value to the correct interpretation of results from PCR markers employed to identify faecal contamination in the waterways of New Zealand. The finding of extensive (up to 100%) false-positive rates for possum faeces against various human and ruminant genetic markers is an interesting dilemma, because these same markers produce no amplification from other animal and bird faeces.

Obviously, these genetic markers have a high degree of specificity except where possum faeces is concerned, therefore, their use should not be negated for detection of their target pollution source. This is particularly true in light of the findings of this study that pollution from possums does not appear to be widespread in NZ environmental waters; however, further validation of this interim conclusion requires ongoing testing. Continued use of the human PCR markers is therefore suggested with the caveat that the possum marker should be employed in all genetic testing of water samples in NZ. Where a positive result is obtained from human and possum markers, further discrimination will require expenditure on additional FST tools to confirm human faecal contamination.

## CONCLUSIONS

- A significant cross-reaction between possum faeces (100%) and previously published human PCR markers was identified during an evaluation of specificity for the human markers in the NZ environment.
- Based on *Bacteroidales* 16S ribosomal sequences, a possum PCR marker was developed. While prevalent in possum faeces, some false-positive reactions were observed with faeces from black swans, humans and rabbits, although at much lower prevalence and at marker concentrations at least four orders of magnitude lower than possum faeces.
- Where brushtail possums are present in the environment, all assays of the human PCR markers should be accompanied by testing of the possum marker to confirm the absence of possum faeces in a water sample. Where an environmental water sample reports a positive result for both the human and possum PCR markers, then additional testing by other FST methods, such as faecal sterol analysis, would be required to exclude the possibility that only possum faecal pollution is present in the waterway.
- Development of a human-specific PCR marker that was not detected in possum faeces could be a useful future step, but in light of the low prevalence of possum faecal material in water this is probably not necessary.
- This study highlights that when using PCR assays in a new geographical area, it is important to perform

specificity testing against the animals and birds that could contribute to faecal inputs in waterways.

- Incorporation of the faecal source tracking PCR markers into mathematical models for the prediction of viable pathogens in a water sample could be investigated in future studies.

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