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Detection and quantification of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater

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Summary

The human-specific HF183 *Bacteriodes* 16S rRNA genetic marker can be used to detect human faecal pollution in water environments. However, there is currently no method to quantify the prevalence of this marker in environmental samples. We developed a real-time polymerase chain reaction (PCR) assay using SYBR Green I detection to quantify this marker in faecal and environmental samples. To decrease the amplicon length to a suitable size for real-time PCR detection, a new reverse primer was designed and validated on human and animal faecal samples. The use of the newly developed reverse primer in combination with the human-specific HF183 primer did not decrease the specificity of the real-time PCR assay but a melting curve analysis must always be included. This new assay was more sensitive than conventional PCR and highly reproducible with a coefficient of variation of less than 1% within an assay and 3% between assays. As the *Bacteroides* species that carries this human-specific marker has never been isolated, a bacteria real-time assay was used to determine the detection efficiency. The estimated detection efficiency in freshwater ranged from 78% to 91% of the true value with an average detection efficiency of $83 \pm 4\%$ of the true value. Using a simple filtration method, the limit of quantification was $4.7 \pm 0.3 \times 10^5$ human-specific *Bacteroides* markers per litre of freshwater. The aerobic incubation of the human-specific *Bacteroides* marker in freshwater for up to 24 days at 4 and 12°C, and up to 8 days at 28°C, indicated that the marker persisted up to the end of the incubation period for all incubation temperatures.

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Introduction

Faecal pollution of coastal and inland water resources often poses important source allocation and remediation challenges. Both human and animal faecal pollution impose risks to human health from exposure to pathogenic bacteria (Baker and Herson, 1999), viruses (Pina *et al.*, 1998) and protozoa such as *Cryptosporidium* (Lefay *et al.*, 2000). This faecal material can originate from point source discharges such as raw sewage, storm water, combined sewer overflows, effluents from wastewater treatment plants and industrial sources. In addition, non-point source discharges such as agriculture, forestry, wildlife and urban run-off can also impair water quality (Griffin *et al.*, 2001). If the origin of the faecal pollution could be correctly identified, remediation efforts could be allocated in a more cost-effective manner. One possible approach to identify faecal pollution sources is based on the microbial source tracking (MST) technologies currently under development.

A variety of methods has been proposed to identify faecal sources in water. Many of these MST-methods are based on differences in phenotypic and genotypic characteristics between populations of microorganisms, e.g. *Escherichia coli* in different faecal sources. These methods require a library of fingerprints of the target microorganism isolated from different faecal sources. Subsequently, the fingerprints of environmental isolates are compared with the library, which would indicate whether the faecal pollution in the environment is derived from a particular host group represented in the library. Phenotypic library-based methods, such as antibiotic resistance analysis (Harwood *et al.*, 2000; Wiggins *et al.*, 2003) and carbon source utilization analysis (Souza *et al.*, 1999; Hagedorn *et al.*, 2003), as well as genotypic library-based methods, such as ribotyping (Parveen *et al.*, 1999; Carson *et al.*, 2001), pulse-field gel electrophoresis (Parveen *et al.*, 2001) and repetitive extragenic

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palindromic-polymerase chain reaction PCR (Dombek *et al.*, 2000; Seurinck *et al.*, 2003), have all been developed. These library-based methods require a large number of isolates to be cultured and assessed because of non-differentiating phenotypic or genetic variations of the target bacterium (Wiggins *et al.*, 2003). To avoid these problems, non-culturing methods can be used to detect host-specific markers, such as viruses (Ley *et al.*, 2002) and bacteriophages (Calci *et al.*, 1998), *Bacteroides* 16S rRNA genetic markers (Bernhard and Field, 2000a,b) and toxin markers (Khatib *et al.*, 2002). In combination with automation, these non-culturing methods have the potential to vastly increase the throughput of MST technologies.

Bernhard and Field (2000a) identified a human-specific *Bacteroides* 16S rRNA genetic marker by screening 16S rDNA from human faeces with length heterogeneity PCR and terminal restriction fragment length polymorphism. A conventional host-specific PCR assay was developed to detect this genetic marker in environmental samples (Bernhard and Field, 2000b). The human-specific *Bacteroides* 16S rRNA genetic marker seems to be widely distributed within the USA (Field, 2002; Boehm *et al.*, 2003) and was already detected in river waters in New Zealand (Gilpin *et al.*, 2003). There is some evidence that the amount of marker present in a human individual may vary (Field, 2002). The *Bacteroides* species that carries this human-specific marker has never been isolated.

The outcome of the host-specific PCR assay indicates whether human faeces are present in the water environment. As outlined by Loge and colleagues (2002), sequence detection using PCR-based analysis is heavily dependent on the detection limit of the assay. Once detection limits are adequately characterized, quantitative methods such as real-time quantitative PCR will increase the confidence in risk-based management of faecal pollution (Loge *et al.*, 2002).

In this study, we developed a real-time PCR assay using SYBR Green I to quantify the human-specific HF183 *Bacteroides* 16S rRNA genetic marker in human faeces and environmental samples. As non-specific dsDNA may interfere with the assay when SYBR Green I detection is used (Kaiser *et al.*, 2001; Ruzsovics *et al.*, 2001), we carefully validated non-specific product formation. The precision, the limit of quantification, the limit of detection and detection efficiency of the real-time PCR assay were also assessed.

Results

Real-time PCR range

The standard curves had a linear range of quantification from 1.4×10^7 to 1.4×10^2 human-specific HF183 *Bacteroides* 16S rRNA genetic markers per microlitre of DNA extract (Fig. 1A and B). Lower levels of the human-specific *Bacteroides* marker (14 to 1.4 human-specific *Bacteroides* markers per microlitre of DNA extract) were tested but the amplification and detection was not reproducible. The slope of the standard curves varied between –3.27 and –3.71 for all real-time PCR assays performed, and the correlation coefficient was always higher than 0.99. The corresponding melting curve analysis is shown in Fig. 1C. A peak at melting temperature $78.4 \pm 0.2°C$ was indicative for a positive and correct amplification.

Specificity and precision of the real-time PCR assay

A GenBank BLAST search using the new primer set matched mostly *Bacteroides* 16S rDNA sequences originating from human faeces (Bernhard and Field, 2000a; Hayashi *et al.*, 2002a,b; Hold *et al.*, 2002; Wang *et al.*, 2003). However, the primer set also had a 100% match with a 16S rDNA sequence from chicken caecal samples (Lan *et al.*, 2002; Zhu *et al.*, 2002) whereas chicken caecal 16S rDNA obtained in other phylogenetic studies did not match the new primer set (Gong *et al.*, 2002; Lu *et al.*, 2003). The new primer set was also evaluated on chicken faecal DNA, as well as other animal faecal DNA (horses, cows, dogs and pigs) to test if non-specific amplification occurred. Real-time PCR analysis of one chicken faecal DNA resulted in an amplification curve, but the melting curve analysis never showed a peak at $78.4 \pm 0.2°C$ (Fig. 2). Spiking of this chicken faecal DNA in the real-time PCR standard (range of 1.4×10^7–1.4×10^2 human-specific HF183 *Bacteroides* 16S rRNA genetic markers per microlitre of DNA extract) did not result in significant changes in the Ct-values of the real-time PCR standard (data not shown). Thus there was no interference with quantification of the human-specific *Bacteroides* marker. The human-specific genetic marker could not be detected in any of the other animal faecal samples (dogs, horses, cows, pigs) analysed with the conventional PCR and real-time PCR assay. Thus, melting curve analysis is required to confirm a positive result obtained with the new real-time PCR assay.

The human-specific HF183 *Bacteroides* 16S rRNA real-time assay detected between 10^5 and 10^9 *Bacteriodes* markers per gram of wet human faeces and between 10^9 and 10^{10} human-specific *Bacteroides* markers per litre of wastewater treatment plant influent (Table 1). The real-time PCR assay was more sensitive than the conventional PCR assay with 4/6 humans positive in the conventional compared to 5/6 humans positive with the real-time assay. Sequencing of the real-time PCR amplicon of human 6, not amplified in conventional PCR showed that the correct sequence was indeed amplified.

The within- and between-assay coefficient of variation (CV) of the real-time PCR assay assessed with the

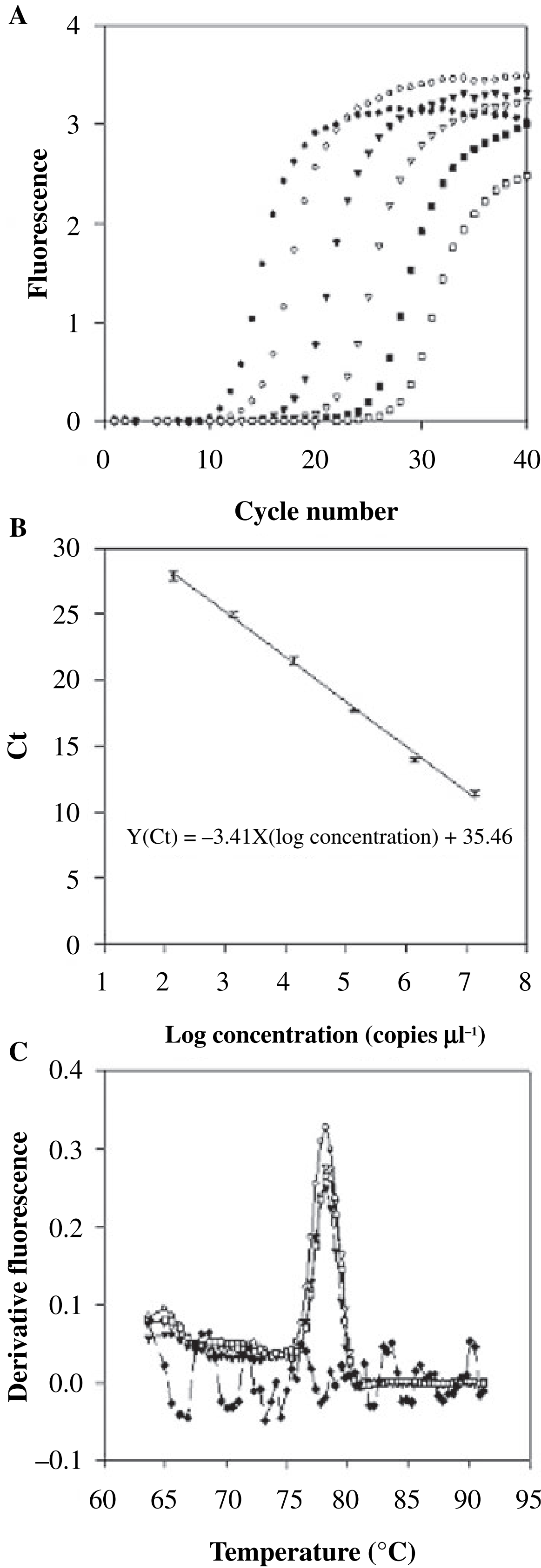

Fig. 1. A. Amplification curves of the real-time PCR standard ranging from 1.4×10^7 to 1.4×10^2 human-specific HF183 *Bacteroides* 16S rRNA genetic markers per microlitre of DNA extract (● 1.4×10^7, ○ 1.4×10^6, ▼ 1.4×10^5, ▽ 1.4×10^4, ■ 1.4×10^3, □ 1.4×10^2).
B. Standard curve of Mean Ct ± SD versus human-specific *Bacteroides* markers.
C. Corresponding melting curves of the real-time PCR standard. A peak at melting temperature 78.4 ± 0.2°C was indicative for a positive and correct amplification (● 1.4×10^7, ○ 1.4×10^6, ▼ 1.4×10^5, ▽ 1.4×10^4, ■ 1.4×10^3, □ 1.4×10^2, ◆ blank).

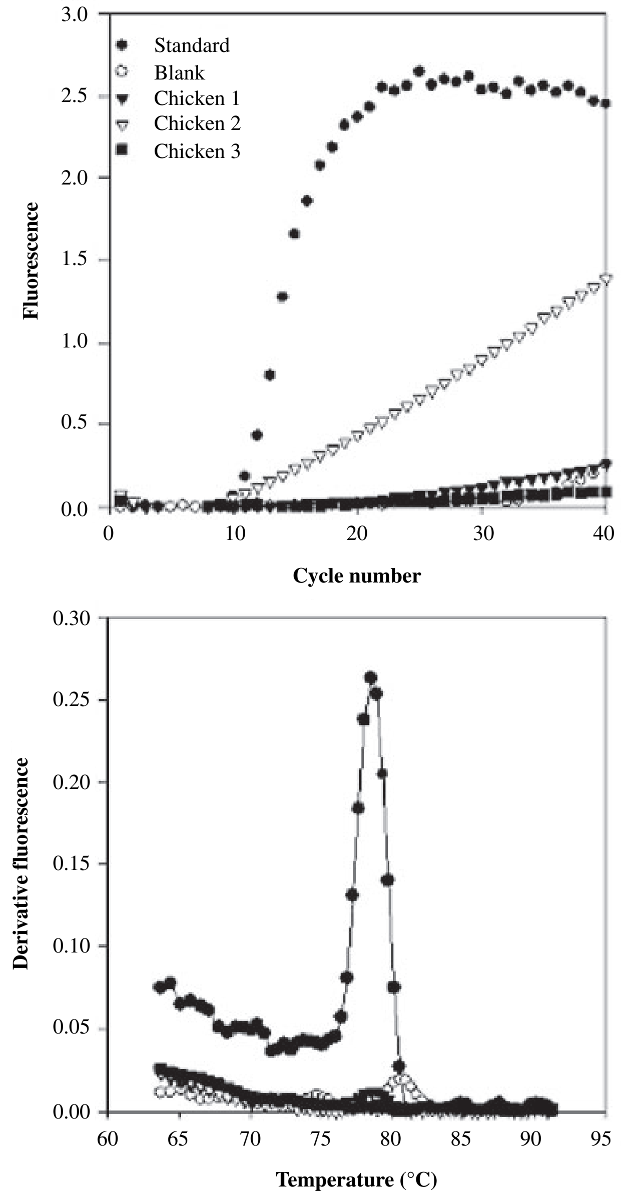

Fig. 2. Amplification curves and melting curve analysis of chicken faecal DNA compared with the standard of 1.4×10^7 human-specific *Bacteroides* markers per microlitre of DNA extract.

Table 1. Conventional PCR and real-time PCR assay results of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker in human faecal samples and raw sewage samples, collected at the same wastewater treatment plant on four consecutive days.

Sample	Conventional PCR	Human-specific *Bacteroides* markers per gram of wet faeces or per litre of influent (mean ± SD)
Human 1	+	$1.2 \pm 0.1 \times 10^7$
Human 2	+	$3.0 \pm 0.3 \times 10^7$
Human 3	+	$1.0 \pm 0.1 \times 10^7$
Human 4	ND	ND
Human 5	+	$7.2 \pm 1.1 \times 10^9$
Human 6	ND	$8.4 \pm 0.1 \times 10^5$
Human 7	+	$2.0 \pm 0.2 \times 10^9$
Raw sewage 7/14	+	$6.8 \pm 1.2 \times 10^9$
Raw sewage 7/15	+	$5.9 \pm 0.7 \times 10^9$
Raw sewage 7/16	+	$2.5 \pm 0.3 \times 10^{10}$
Raw sewage 7/17	+	$3.1 \pm 0.3 \times 10^{10}$

ND, not detected.

Table 2. The within- and between-assay coefficient of variation for the real-time PCR assay within the range of 1.4×10^7 to 1.4×10^2 human-specific *Bacteroides* markers per microlitre of DNA extract.

Quantity (copy number per microlitre of DNA extract)	Within-assay CV (%)	Between-assay CV(%)
1.4×10^7	0.85	0.78
1.4×10^6	0.76	1.12
1.4×10^5	0.98	1.67
1.4×10^4	0.85	1.88
1.4×10^3	0.68	2.22
1.4×10^2	0.83	2.09

real-time PCR standard was less than 1% and 3%, respectively, and did not increase as copy numbers decreased from 1.4×10^7 to 1.4×10^2 human-specific *Bacteroides* markers per microlitre of DNA (Table 2).

Detection efficiency of the real-time PCR assay in freshwater

Ideally, the detection efficiency of the real-time PCR assay would have been determined by spiking freshwater with known amounts of the *Bacteroides* species carrying the human-specific HF183 *Bacteroides* 16S rRNA genetic marker. However, as the *Bacteroides* species that carries this human-specific marker has never been isolated, it was not possible to use the developed real-time PCR assay to determine the detection efficiency in freshwater. Instead, a bacteria real-time assay was used to quantify spiked *Bacteroides vulgatus* ATCC 8482 (Boon *et al*., 2003). The estimated detection efficiency in freshwater ranged from 78% to 91% of the true value with an average detection efficiency of 83 ± 4% of the true value (Table 3). Statistical analyses showed that there was an interacting effect between the matrix and spiked concentration, and that the detection efficiencies were significantly ($P < 0.05$) different between spiked concentrations for all matrices. Detection efficiencies varied substantially between all spiked concentrations in the non-filtered and filtered deionized water matrices but not for the freshwater matrix. The effect of filtration on the estimated detection efficiency was rather small as the detection efficiencies between non-filtered and filtered deionized water did not differ much.

Limit of detection (LOD) and limit of quantification (LOQ) in freshwater

The linear range of quantification of the real-time PCR assay in freshwater was from dilution 10^{-2} up to dilution 10^{-5} for the replicate dilutions (Fig. 3), resulting in the freshwater LOQ of $4.7 \pm 0.3 \times 10^5$ human-specific *Bacteroides* markers per litre of freshwater. In contrast, the *Bacteroides* marker was only detectable up to dilution 10^{-3} with conventional PCR for the replicate dilutions, corresponding with $1.3 \pm 0.4 \times 10^7$ human-specific *Bacteroides* markers per litre of freshwater. The freshwater LOD could not be deduced from the dilution experiment as the human-specific HF183 *Bacteroides* 16S rRNA genetic marker was still detectable in the highest dilution (10^{-9}) but the response was not proportional to the amount of *Bacteroides* markers present in the water sample. The level of human-specific *Bacteroides* markers per gram of wet faeces added to the freshwater, resulted in the detection of human faecal pollution up to 1 ng of wet faeces per litre of freshwater with the real-time PCR assay.

Persistence of the human-specific HF183 Bacteroides *16S rRNA genetic marker in freshwater*

The human-specific HF183 *Bacteroides* 16S rRNA genetic marker persisted in freshwater up to the end of

Table 3. Detection efficiency with real-time PCR assay for the three different matrices (non-filtered and filtered autoclaved deionized water, and autoclaved freshwater) spiked with known concentrations of an early stationary phase *B. vulgatus* culture.

	Detection efficiency ± SE (%)		
	Deionized water		
Spiked cells (cells l^{-1})	Non-filtered	Filtered	Freshwater
$8.96 \pm 1.98 \times 10^6$	55 ± 1	50 ± 7	78 ± 1
$8.96 \pm 1.98 \times 10^7$	88 ± 2	71 ± 5	91 ± 2
$8.96 \pm 1.98 \times 10^8$	106 ± 0	94 ± 3	81 ± 2

Spiking of the different matrices was performed in triplicate for each concentration ($n = 3$). Mean detection efficiency (%) ± SE are reported.

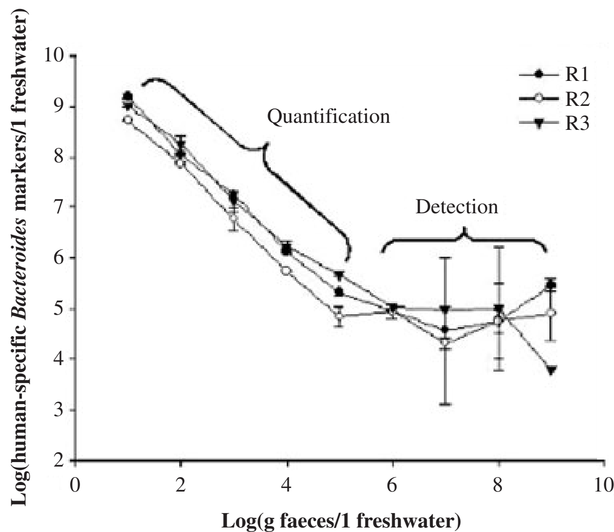

Fig. 3. Assessment of the limit of detection (LOD) and limit of quantification (LOQ) in freshwater. The quantification results of the human-specific *Bacteroides* marker in the different dilution of R1, R2 and R3, are plotted against the corresponding faecal concentration (gram of faeces per litre of freshwater). In some cases, the error bars were too small to illustrate.

the incubation period for all three temperatures (Fig. 4). The amount of human-specific *Bacteroides* markers per litre of freshwater did not decrease significantly at 4°C throughout the incubation period of 24 days. The amount of human-specific *Bacteroides* markers per litre of freshwater decreased one log-unit after 10 days of incubation at 12°C, and two log-units after 6 days of incubation at 28°C.

Discussion

We developed a real-time PCR assay using SYBR Green I detection to quantify the human-specific HF183 *Bacteroides* 16S rRNA genetic marker in human faeces and freshwater samples. The use of the dsDNA binding dye SYBR Green I for the detection of PCR products allows protocols already in use for conventional PCR to be applicable with only slight modifications for real-time PCR (Egygor *et al.*, 2002). As non-specific dsDNA may interfere with the assay when SYBR Green I detection is used (Kaiser *et al.*, 2001; Ruzsovics *et al.*, 2001), we carefully validated non-specific product formation. A GenBank BLAST search using the new primer set matched mostly *Bacteroides* 16S rDNA sequences originating from human faeces (Bernhard and Field, 2000a; Hayashi *et al.*, 2002a,b; Hold *et al.*, 2002; Wang *et al.*, 2003) but a match was also obtained with a 16S rDNA sequence from chicken caecal samples (Lan *et al.*, 2002; Zhu *et al.*, 2002). Lan and colleauges (2002) and Zhu and colleagues (2002) found that the relative percentage of *Bacteroides* sequences was less than 2% (21 sequences) and 4% (12 sequences), respectively, and did not specify how many of these *Bacteroides* 16S rDNA clones contained the *Bacteroides* HF183 sequence. Moreover, other comparable phylogenetic studies on chicken caecal samples found even higher percentages of *Bacteroides* sequences but do not report a match with the HF183 sequence (Gong *et al.*, 2002; Lu *et al.*, 2003). Real-time PCR analysis of one chicken faecal DNA resulted in a linear amplification curve, but the melting curve analysis never showed a peak at 78.4 ± 0.2°C. The human-specific genetic marker could not be detected in any of the other animal faecal samples (dogs, horses, cows, pigs). So, the use of the newly developed reverse primer in combination with the forward human-specific HF183 primer did not decrease the specificity of the real-time PCR assay but a melting curve analysis must always be included in the real-time PCR assay.

The real-time PCR proved to be an order of magnitude more sensitive than the conventional PCR. Samples that were negative according to the conventional PCR were amplified using the real-time PCR assay and sequencing of the corresponding amplicons indicated that the amplification product was the desired human-specific HF183 *Bacteroides* 16S rRNA fragment. The difference of sensitivity between the conventional PCR and the real-time PCR assay could be attributed to a combination of factors. Besides a different reverse primer, the number of PCR cycles also differed between the conventional and the real-time PCR, namely 35 and 40 cycles respectively. Moreover, the real-time PCR assay uses a Hot Start DNA polymerase compared with the routinely used *Taq* polymerase in the conventional PCR. Kwiatek and colleagues (2003) showed that applying hot start PCR to detect

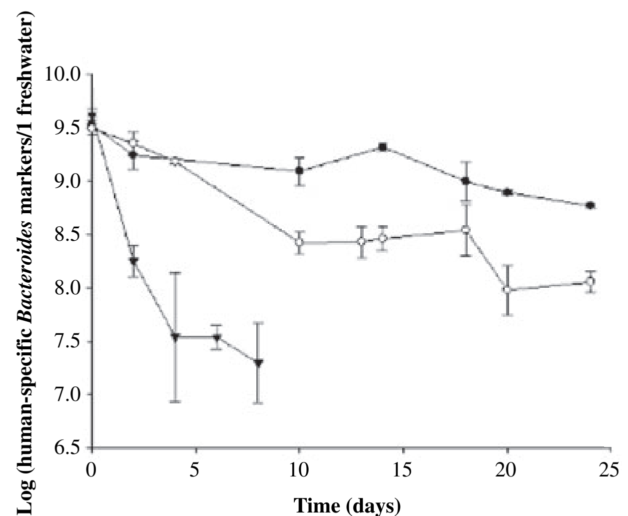

Fig. 4. Persistence of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker incubated at 4°C (●), 12°C (○), and 28°C (▼). Three series were set up per incubation temperature ($n = 3$). Mean human-specific *Bacteroides* markers per litre of freshwater ± SD are reported. In some cases, the error bars were too small to illustrate.

Listeria monocytogenes increased the sensitivity of the reaction compared with the routinely used *Taq* DNA polymerase. Finally, several researchers observed that SYBR Green I stain used in the real-time PCR assay is much more sensitive than the traditional dye ethidium bromide used in the conventional PCR (Schneeberger *et al*., 1995; Rabiya *et al*., 1999; Rengarajan *et al*., 2002). The sensitivity of this assay could be improved with high-volume filtration systems or an enrichment step prior to filtration when the conditions of enrichment of the *Bacteroides* species carrying the human-specific HF183 *Bacteroides* 16S rRNA genetic marker would be known. Panicker and colleagues (2004) found that the detection limit of the real-time assay of 10^2 *Vibrio vulnificus* per millilitre could be improved to single cell level in enriched samples. The real-time PCR assay was highly reproducible. The within- and between-assay coefficient of variation of less than 1% and 3%, were comparable or sometimes better than real-time PCR assays developed for medical purposes (Jardi *et al*., 2001; Boyle *et al*., 2003; Ito *et al*., 2003).

The level of human-specific *Bacteroides* markers per gram of wet faeces added to the freshwater, resulted in the detection of human faecal pollution up to 1 ng of wet faeces per litre freshwater with the real-time PCR assay. Bernhard and Field (2000b) could detect up to 10 μg of wet faeces per litre of filter-sterilized bay water. However, the level of human faecal pollution in gram of wet faeces per litre of freshwater will not be correlated with the level of human-specific *Bacteroides* markers per litre of freshwater as we observed that the amount of human-specific *Bacteroides* markers per gram of wet faeces varies between different individuals. Therefore it is more correct to express human faecal detection levels in units of human-specific *Bacteroides* markers per litre of freshwater. If the number of 16S rRNA operons of the *Bacteroides* species carrying the marker is determined, the real-time PCR results can be expressed as marker *Bacteroides* cells per litre of freshwater by dividing the amount of human-specific *Bacteroides* markers by the number of 16S rRNA operons. Bernhard and Field (2000b) assume an average of five 16S rRNA operons per *Bacteroides* cell [rRNA Operon Copy Number Collection (http://rrndb.cme.msu.edu/rrndb)]. Using this assumptions the LOQ of the real-time PCR assay was $9.4 \pm 0.6 \times 10^4$ marker *Bacteroides* per litre of freshwater. The LOQ reported in this paper should be seen as a proof of concept, and probably will need to be determined for other matrices (e.g. seawater) in which this method will be applied. To date no correlation studies between this human-specific *Bacteroides* marker and the traditional indicatororganisms have been performed. Bradley and colleagues (1999) monitored *Bacteroides fragilis*, total coliforms and thermotolerant coliforms in recreational waters and found no correlation between this *Bacteroides* species and the indicatororganisms. In that sense, it is not possible to evaluate if the LOQ of the real-time assay is low enough for implementation in the field. Therefore, follow-up studies should focus on extensive field testing of this real-time assay in combination with monitoring of the traditional indicatororganisms. We anticipate that further refinements in bacteria collection will result in an improvement of the sensitivity of the real-time assay.

Ideally, the detection efficiency, and the LOD and LOQ of the real-time PCR assay should be determined by spiking with known amounts of the *Bacteroides* species carrying the human-specific HF183 *Bacteroides* 16S rRNA genetic marker. However, this could not be done as the *Bacteroides* species that carries this *Bacteroides* marker has never been isolated. Instead, the detection efficiency was estimated by spiking *Bacteroides vulgatus* ATCC 8482 in different matrices and use a bacteria real-time assay to quantify the spiked *B. vulgatus* cells (Boon *et al*., 2003). Statistical analysis showed that the matrix type and spiked concentration interacted. The freshwater matrix did not display a marked concentration effect and the average estimated detection efficiency was approximately 83% of the true value. The estimated detection efficiency values are comparable with the results of Brinkman and colleagues (2003). Their real-time PCR assay to quantify *Candida* cells in water samples had a detection efficiency of 50–200% of the true value. We postulate that the detection efficiency of the quantification of the spiked *B. vulgatus* cells was higher in the freshwater matrix because of the higher suspended solids present in the autoclaved freshwater compared with the autoclaved deionised water. According to Feng and colleagues (2003) who found higher recoveries of *Cryptosporidium* oocysts from spiked turbid reservoir water than from spiked tap water, attachment of the *Cryptosporidium* oocysts to the suspended particles enhanced the retainment by the filter. As matrix and spiked concentration interacted, the detection efficiency must be assessed for all matrices in which this method will be applied. Once the *Bacteroides* species carrying the human-specific HF183 *Bacteroides* 16S rRNA genetic marker is cultured, the detection efficiency can be exactly determined. However, we believe that our estimated detection efficiency was determined in the best manner possible at this point.

Survival of *Bacteroides* cells depends primarily on temperature and predation (Kreader, 1995), and these bacteria can survive for up to 6 days under oxygen stress condition (Avelar *et al*., 1998). To simulate environmental conditions, we incubated the human-specific HF183 *Bacteroides* 16S rRNA genetic marker aerobically in natural freshwater at 4°C and 12°C for 24 days, and at 28°C for 8 days. The human-specific *Bacteroides* marker persisted in freshwater up to the end of the incubation period for all three temperatures. This is longer persistence than that observed by Kreader (1995) who detected *Bacteroi-*

des distasonis with a species-specific PCR in freshwater up to 1 day at 28°C, and up to 5 days at 14°C. The long persistence we observed indicates that under the simulated conditions the human-specific *Bacteroides* marker indicative of human faecal pollution will be detected for a long period after the discharge event. The persistence of low concentrations of faecal material may differ from that observed here. Other authors have indicated that factors such as sunlight and organic matter play an important role in faecal material persistence (Rozen and Belin, 2001; Sinton *et al*., 2002). This assay will allow future investigators to study ambient human faeces persistence.

In conclusion, the human-specific HF183 *Bacteroides* 16S rRNA genetic marker developed by Bernhard and Field (2000a) is even more widely spread than previously considered, as the human-specific *Bacteroides* marker was successfully detected in human individuals from Belgium (Europe). To our knowledge, this is the first assay that can provide quantitative estimates of this human-specific *Bacteroides* marker in freshwater. Future studies should focus on extensive field testing of this real-time assay, which should increase the confidence in risk-based management of faecal pollution.

Experimental procedures

Sample collection

Faecal samples. Human faecal samples (approximately 50 g) were collected from seven different adult humans. Dog faecal samples were collected at five different households (Gent, Belgium), horse faecal samples were collected at five different maneges (Gent, Belgium), cow faecal samples were taken from four different dairy cows on the same farm (Diksmuide, Belgium), chicken faecal samples were collected at three different henhouses (Gent, Belgium), and pig manure was collected from two different pig farms (Belgium). The faecal samples were collected in sterile recipients and stored at −20°C.

Water, septic and raw sewage samples. Water samples were taken from a freshwater canal (Coupure, Gent, Belgium), septic samples were taken from septic waste collecting trucks and raw sewage samples were taken from the influent of a domestic wastewater treatment plant (Ossemeersen, Gent, Belgium). The same wastewater treatment plant was sampled on four consecutive days (14/7/03–17/7/03). Samples (1 l) were collected in sterile recipients, stored at 4°C and analysed within 24 h after collection. Water samples were filtered through a 0.22-μm filter (Type GS, Millipore, Brussels, Belgium).

DNA extraction

DNA was extracted from faeces with the QIAamp DNA Stool Mini Kit (QIAGEN, West Sussex, UK) according to the manufacturer's instructions.

Total DNA extraction from water (directly from the filters), septic (2 ml) and raw sewage (2 ml) samples was based on the protocols described previously (el Fantroussi *et al*., 1999; Boon *et al*., 2000).

Conventional PCR

The human-specific HF183 *Bacteroides* 16S rRNA genetic marker was amplified by using the human-specific HF183 forward primer (5′-ATCATGAGTTCACATGTCCG-3′) and the Bac708 reverse primer (5′-CAATCGGAGTTCTTCGTG-3′) as developed by Bernhard and Field (2000b). Polymerase chain reaction (PCR) was carried out in a volume of 25 μl, which contained 2 μl of template DNA. The PCR mixture contained 0.2 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10 μl of Thermophilic DNA Polymerase 10× Reaction Buffer ($MgCl_2$-free), 2.5 U of *Taq* DNA Polymerase (Promega, Madison, WI, USA), 5 μg of bovine serum albumin (Boehringer Ingelheim GmbH, Ingelheim, Germany) and DNase and RNase free filter sterile water (Sigma-Aldrich Chemie, Steinheim, Germany) to a final volume of 100 μl. The PCR was initiated with 4 min at 95°C, followed by 35 cycles of 30 s at 94°C; 1 min at 60°C; and 2 min at 72°C. The final cycle was followed by an additional 10 min at 72°C. All PCR experiments contained a positive control (DNA-mixture from different human faecal DNA extractions) and a negative control (no DNA).

DNA cloning and sequencing

We verified whether the sequence of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker found in our human faecal samples was identical to the marker sequence found by Bernhard and Field (2000a). The amplified 500-bp products from the conventional PCR on three human faecal samples with the human-specific HF183 forward primer and the Bac708 reverse primer were cloned into the PCR-TOPO 2.1 cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA sequencing was carried out by IIT Biotech-Bioservice (Bielefeld, Germany). In total, the sequence of the human-specific HF183 marker was determined from human faeces from three different individuals. These sequence data have been submitted to the GenBank database under the accession numbers: AY618281–AY618283.

Primer design

To decrease the amplicon length to a suitable size for real-time PCR detection a new reverse primer was designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). This new primer (5′-TACCCCGCCTACTA TCTAATG-3′) was designed from the consensus sequence in the human-specific HF183 *Bacteroides* 16S rRNA genetic marker obtained from the human faecal samples. The combination of the two primer sequences was blasted against GenBank, and the primer set was tested against animal faecal DNA (horses, cows, dogs, chickens and pigs) to test if non-specific amplification occurred.

Real-time PCR assay

The human-specific HF183 *Bacteroides* 16S rRNA genetic marker was amplified by using the human-specific HF183 for-

ward primer and the newly developed reverse primer (5′-TACCCCGCCTACTATCTAATG-3′). The new amplicon had a length of 82 bp. The real-time PCR was based on the principle of Heid and colleagues (1996). For quantification of the HF183 marker by real-time PCR, amplification was performed in 25-μl reaction mixtures by using buffers supplied with the qPCRTM core kit for Sybr® Green I (Eurogentec, Liège, Belgium). The PCR mixture contained 0.25 μM of each primer, 200 μM of each deoxynucleoside triphosphate with dUTP, 2.0 mM $MgCl_2$, 10 μl of real-time PCR 10× Buffer ($MgCl_2$-free), 2.5 U of Hot GoldStar DNA Polymerase, 3 μl Sybr® Green I 1/10, and DNase and RNase free filter sterile water (Sigma-Aldrich Chemie) to a final volume of 100 μl. The reactions were performed in MicroAmp Optical 96-well reaction plates with optical caps (Applied Biosystems). The PCR temperature programme was initiated with 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 1 min at 53°C, and 1 min at 60°C. Subsequently, during the melting curve analysis, the temperature increases from 60°C to 95°C at approximately 0.4°C per minute. The template DNA in the reaction mixtures was amplified and monitored with an ABI Prism SDS 7000 instrument (Applied Biosystems). Within each real-time PCR run all samples were analysed in triplicate.

Preparation of PCR standard for use in real-time PCR assay

A PCR standard containing the human-specific HF183 sequence was constructed by growing the recombinant *E. coli* strain carrying the recombinant TOPO 2.1 plasmid with the human-specific HF183 *Bacteroides* 16S rRNA genetic marker in Luria–Bertani broth plus ampiciline (50 mg l^{-1}) at 37°C for 24 h. Plasmid DNA was extracted from the culture with the High Pure Plasmid Isolation Kit (Roch Diagnostics GmBH, Mannheim, Germany) according to the manufacturer's instructions. The plasmid DNA concentration was determined spectrophotometrically, using a lambda DNA standard (Invitrogen). A tenfold dilution standard was prepared from the plasmid DNA extract, ranging from 1.4×10^7 to 1.4×10^1 human-specific *Bacteroides* markers per microlitre of DNA extract, stored at −20°C.

Precision of the real-time PCR assay

The within- and between-assay CV was determined by means of the PCR standard analysed in each real-time PCR run. The amount of human-specific *Bacteroides* marker ranged from 1.4×10^7 to 1.4×10^2 human-specific *Bacteroides* markers per microlitre of DNA extract. The within-assay CV was calculated based on the Ct-data within one run. Every sample within one run was analysed six times ($n = 6$). The between-assay CV was calculated based on the Ct-data of four consecutive runs ($n = 4$). The Ct-value of a sample is the threshold cycle at which the fluorescent signal is first recorded as statistically significant above the background fluorescence.

Detection efficiency of the real-time PCR assay in freshwater

Ideally, the detection efficiency of the real-time PCR assay would have been determined by spiking freshwater with known amounts of the *Bacteroides* species carrying the human-specific HF183 *Bacteroides* 16S rRNA genetic marker. However, as the *Bacteroides* species that carries this human-specific marker has never been isolated, it was not possible to use the developed real-time PCR assay to determine the detection efficiency in freshwater. Instead, a bacteria real-time assay was used to quantify *Bacteroides vulgatus* ATCC 8482 (Boon *et al.*, 2003). In order to simulate the growth phase conditions of bacterial cells when discharged into the aquatic environment, the early stationary growth phase was used. An overnight culture of *B. vulgatus* ATCC 8482 was inoculated into Brain–Heart Infusion broth (37 g l^{-1}) (BHI) (Oxoid, CM0225, Hampshire, England) supplemented with 100 mg l^{-1} Kanamycin, 750 μg l^{-1} Vancomycin, 5 mg l^{-1} Hemin (Quelab Laboratories, Montreal, Canada), 10 mg l^{-1} Vitamin K (Quelab Laboratories), and 50 ml l^{-1} Laked Horse Blood (Oxoid, SR0048C), and incubated anaerobically in gaspak jars at 37°C ($n = 3$). Samples were taken at 5, 16, 19, 24 and 48 h after inoculation and cell concentrations were determined by means of flow cytometry (Cyan, DakoCytomation, Heverlee, Belgium) to assess the growth curve of *B. vulgatus* ATCC 8482. Early stationary phase was reached after 17 h of incubation (data not shown), and the average concentration at that point was $4.5 \pm 1.09 \times 10^8$ cells per microlitre. Non-filtered and filtered autoclaved deionized water and autoclaved freshwater (canal Coupure, Ghent, Belgium) were spiked with this early stationary phase *B. vulgatus* ATCC 8482 culture to a final concentration of 9.0×10^8 cells per litre, 9.0×10^7 cells per litre, and 9.0×10^6 cells per litre. Spiking of the different matrices was performed in triplicate for each concentration ($n = 3$). Spiked water was filtered as described previously, except only 250 ml was filtered, and the DNA extraction was performed directly on the filters. All DNA extracts were 10 times diluted to obtain real-time PCR amplification for all three spiked concentrations and analysed with the bacterial real-time PCR protocol. A 10-fold dilution series of DNA extract from an early stationary phase *B. vulgatus* ATCC 8482 culture with a known amount of cells was used as standard for the bacterial real-time PCR assay. Detection efficiency was calculated as follows: detection efficiency (%) = $(10^{\log(\text{quantified})/\log(\text{spiked})}/10) \times 100$.

Limit of detection (LOD) and limit of quantification (LOQ) in freshwater of the real-time PCR assay

Fresh human faeces was suspended in freshwater (canal Coupure, Ghent, Belgium) to a final concentration of 1 g faeces per litre of water. Dilution series of 10-fold (10^{-1}–10^{-9}) of the suspension were made in the same freshwater ($n = 3$). Fifty millilitres of the 1 g l^{-1} suspension, 500 ml of the 10^{-1} dilution and 900 ml of the 10^{-2}–10^{-9} dilutions were filtered and DNA extraction was performed on the filters. Both, human-specific conventional PCR and real-time PCR were performed. Non-spiked freshwater did not contain the marker as determined with the real-time PCR assay (data not shown). The LOD was defined as the lowest concentration of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker still detectable with real-time PCR in freshwater com-

pared with background fluorescence. The LOQ was defined as the lowest concentration of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker still within the linear range of quantification of the real-time PCR assay in freshwater. We report the highest LOD and LOQ obtained from the replicate dilution experiments as the freshwater LOD and LOQ of the real-time PCR assay.

Persistence of the human-specific HF183 Bacteroides *16S rRNA genetic marker in freshwater*

Fresh septic waste (100 ml) was suspended in 1 l of freshwater (canal Coupure, Gent, Belgium). Three series were set up per incubation temperature ($n = 3$). Ten-milliliter aliquots of the suspension were incubated at 4, 12 and 28°C in aerobic conditions. Samples were taken at regular intervals, and incubated up to 24 days at 4 and 12°C and up to 8 days at 28°C. After incubation the samples were centrifuged at 5000 *g* for 10 min and 8 ml of the supernatant was discarded. Total DNA was extracted with the protocol used for water and raw sewage samples. The samples were analysed with the real-time PCR assay.

Statistical analyses

Statistical analyses were performed using SPSS version 11.5 (SPSS, Chicago, Ill). General Linear Model univariate analysis was performed on the detection efficiencies obtained for the different combinations of matrix and spiked concentration. The mean difference was tested at the 0.05 level of significance. Multiple comparisons were performed with the Tamhane T2 test if the assumption of equal variance between groups was not valid, and with the Tukey HSD test if the assumption of the variance between groups was equal.

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