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Review

Current Status of Marker Genes of *Bacteroides* and Related Taxa for Identifying Sewage Pollution in Environmental Waters

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Abstract: Microbial source tracking (MST) endeavors to determine sources of fecal pollution in environmental waters by capitalizing on the association of certain microorganisms with the gastrointestinal tract and feces of specific animal groups. Several decades of research have shown that bacteria belonging to the gut-associated order *Bacteroidales*, and particularly the genus *Bacteroides*, tend to co-evolve with the host, and are, therefore, particularly suitable candidates for MST applications. This review summarizes the current research on MST methods that employ genes belonging to *Bacteroidales/Bacteroides* as tracers or "markers" of sewage pollution, including known advantages and deficiencies of the many polymerase chain reaction (PCR)-based methods that have been published since 2000. Host specificity is a paramount criterion for confidence that detection of a marker is a true indicator of the target host. Host sensitivity, or the prevalence of the marker in feces/waste from the target host, is necessary for confidence that absence of the marker is indicative of the absence of the pollution source. Each of these parameters can vary widely depending on the type of waste assessed and the geographic location. Differential decay characteristics of bacterial targets and their associated DNA contribute to challenges in interpreting MST results in the context of human health risks. The HF183 marker, derived from the 16S rRNA gene of *Bacteroides dorei* and closely related taxa, has been used for almost two decades in MST studies, and is well characterized regarding host sensitivity and specificity, and in prevalence and concentration in sewage in many countries. Other markers such as HumM2 and HumM3 show promise, but require further performance testing to demonstrate their widespread utility. An important limitation of the one-marker-one-assay approach commonly used for MST is that given the complexities of microbial persistence in environmental waters, and the methodological challenges of quantitative PCR (qPCR) in such samples, the absence of a given marker does not ensure the absence of fecal pollution in the source water. Approaches under development, such as microarray and community analysis, have the potential to improve MST practices, thereby increasing our ability to protect human and ecosystem health.

Keywords: microbial source tracking; *Bacteroides*; fecal pollution; fecal indicator bacteria; wastewater; water quality; next-generation sequencing

1. Introduction

Recreational and shellfish harvesting waters that are polluted with human and animal wastewater can pose a risk to human health due to the potential presence of pathogenic bacteria, protozoa, viruses, and helminths. Fecal pollution may originate from a variety of sources including defective sewage treatment plants [1], on-site septic systems [2], storm water runoff [3], and wildlife, and domestic

animals [4–6]. Identification of the source(s) of fecal pollution provides the first step in initiating remediation efforts and minimizing human health risks.

Fecal indicator bacteria (FIB) such as *Escherichia coli* and *Enterococcus* spp. occur in high concentrations in the gut of humans and other warm-blooded animals. Due to their high levels in feces, they have been considered as indicators of fecal pollution in waters for decades [7,8]. However, several studies have demonstrated that elevated levels of FIB above the recommended guidelines do not always correlate with the presence of viral and protozoa pathogens [9,10]. The presence of FIB in water does not provide information about pollution source due to their cosmopolitan nature (presence in all warm-blooded, and many cold-blooded animals) [11]. In addition, FIB can replicate in pristine beach sands [12], sediment and soil [13,14], surface waters [15], and aquatic vegetation [16].

Due to the limitations of FIB in microbial water quality monitoring, researchers developed new tools that can provide information on the potential source(s) of FIB or fecal pollution by using the association of certain microorganisms with the gastrointestinal tract of specific animal groups. These are commonly referred to as microbial source tracking (MST) tools. Initially developed MST tools were library-dependent, which required the isolation and typing, or "fingerprinting" of FIB from human and animal feces. These fingerprints were compared with those from environmental waters to identify the source(s) of FIB [17–19]. Later, numerous polymerase chain reaction (PCR) and quantitative PCR (qPCR)-based rapid MST tools (known as library-independent) were developed to identify and quantify host-specific genes (commonly referred to as "markers") targeting bacteria [20–22], protozoa [23], and viruses [24,25] in water [26].

A collaborative study between Southern California Coastal Water Research Project (SCCWRP), and the U.S. EPA compared the performance of library-dependent and library-independent MST tools [27]. Reference animal fecal samples were provided to the participating researchers to construct libraries comprised of fingerprints of FIB, or to test with library-independent tools. Test isolates and seeded water samples derived from the same reference animal feces, whose source was kept unknown to the participants were analyzed by 12 library-dependent and -independent MST tools [27]. The results indicated that library-dependent tools were prone to false-positive detection (a source was identified in a sample when it was absent), whereas library-independent tools tended to have false negative detection (a source was not identified in a sample when it was present). The limitations of library-dependent tools in correctly assigning isolates to their sources have been discussed [11,28,29]. For example, development of a library comprised of fingerprints of FIB can be costly and time-consuming. The performance of a library can be affected by several factors such as geographical and temporal stability of FIB and complexity in statistical analysis [29,30]. Due to the complexity associated with library-dependent tools, the application of library-independent PCR markers became widespread among researchers and regulators.

Among the bacterial targets, members of the genus *Bacteroides* and related taxa (the order *Bacteroidales*) have potential as alternative indicators of fecal pollution owing to advantages such as short-term survival rates in water, exclusivity to the gut of warm-blooded animals, and constituents of a larger portion of fecal bacteria compared to *E. coli* or *Enterococcus* spp. [31,32]. The use of *Bacteroides* for routine water quality monitoring has been limited because of the difficulty of cultivating. However, advances in molecular assays and decreased costs are increasing the feasibility of using these anaerobes in a regulatory framework [11].

Based on the hypothesis that some species of the genus *Bacteroides* might be host-specific [33], Bernhard and Field [20] identified human- and ruminant-specific *Bacteroides-Prevotella* 16S rRNA gene markers by using length heterogeneity PCR (LH-PCR) and terminal restriction fragment length polymorphism (T-RFLP). A follow-up study concluded that PCR assays to detect these markers are useful for detecting human and ruminant fecal pollution in waters [20,34]. Subsequently, in a method comparison study, these markers were shown to produce accurate results compared to library-dependent methods [27]. As a result, researchers developed many *Bacteroides* 16S rRNA [35–37] and non-16S rRNA markers [38,39] to detect human and animal fecal pollution in water. Due to the

accuracy, precision, and quick turnaround time, PCR detection of *Bacteroides* markers emerged as a tool for MST studies on several continents such as North America, Europe, Asia, and Australia [37,40–42].

Human fecal pollution poses greater health risks than animal feces due to potential exposure to viruses that are highly specific to humans [43]. Therefore, only relevant MST studies that investigated the application of human-specific *Bacteroides* markers were included in this review. This review exclusively focuses on the host sensitivity and specificity of the currently used human-specific *Bacteroides* markers in human and non-human fecal samples. Also, the concentrations of these markers in point sources (raw sewage, septic and effluent wastewater), and receiving waters are presented. This review also sheds light on the correlation between FIB and human-specific *Bacteroides* markers, their decay in water, and the relevance to public health risks.

2. Assays Targeting Human Fecal Pollution

Several PCR/qPCR assays targeting uncultured *Bacteroides* 16S rRNA and non-16S rRNA genes have been developed (Table 1). Initially, Bernhard and Field [20] developed a qualitative PCR assay to detect human *Bacteroides* markers in water [34]. The authors designed two sewage-specific forward primers (HF134 and HF183) and a reverse primer (Bac708). The forward primer targets human-specific *Bacteroides*, while the reverse primer targets broader phylogenetic groups. Based on the host specificity and sensitivity comparison, the HF183 marker was found to be better than the HF134. Since then, HF183/Bac708 PCR assay has been used widely to detect human fecal pollution in many MST field studies.

Seurinck and colleagues [44] developed a qPCR assay using SYBR Green I to quantify the HF183 marker in human feces and environmental water samples. The authors designed a new reverse primer that was utilized in the qPCR assay in combination with the forward primer. The authors did not designate the new reverse primer; in a review paper, it was designated as SSHBac-R [26]. The SSHBac-R primer was designed to decrease the amplicon length to a suitable size (82 bp) for qPCR amplification from the consensus sequence in the HF183 marker obtained from the human fecal samples. The specificity of the newly developed assay using primer set HF183/SSHBac-R was similar to the HF183/Bac708 primer set designed by Bernhard and Field [20]. However, the authors recommended that a melting curve analysis be included in each qPCR run to discriminate between chicken and human fecal pollution. The HF183/SSHBac-R qPCR was shown to be an order of magnitude more sensitive than the HF183/Bac708 PCR assay [34,44].

Layton and colleagues [45] developed the HuBac qPCR assay targeting *Bacteroides* 16S rRNA genes. The host specificity of the HuBac markers was tested against a panel of fecal samples from bovine, swine, canine, and equine feces. HuBac markers were detected in all sewage samples but also produced a significant false positive result for swine and canine feces. Due to the low host specificity, the authors concluded that the development of more specific markers is warranted for the accurate identification of human fecal pollution. Reischer and colleagues [35] also developed the BacH qPCR assay with a TaqMan probe based on 16S rRNA gene sequences from the phylum *Bacteroidetes*. The preliminary evaluation indicated high host specificity (~99%) of the primer set when tested against 302 non-human fecal samples. The only non-specific reaction was with one cat fecal sample. The assay was sensitive enough to detect as little as 100 pg of feces in fecal suspensions. The assay was successfully field tested to determine the extent of human fecal pollution in water samples from an Alpine Karstic Spring in Austria [35].

In Japan, Okabe and colleagues [37] developed a TaqMan®-based qPCR assay known as Human-Bac1 based on the comparative 16S rRNA gene sequence *Bacteroides-Prevotella* group. The specificity of the Human-Bac1 primer set was evaluated against DNA extracted from a small number of non-human fecal samples. The Human-Bac1 primer set quantified as high as 2.0×10^{11} gene copies per *g* of human feces and 1.0×10^{9} gene copies per *g* of pig and cattle feces, suggesting poor host specificity of the Human-Bac1 marker among the fecal samples tested. In the USA, Kildare and colleagues [36] also developed a TaqMan®-based qPCR assay known as BacHum-UCD for tracking

the sources of human fecal pollution in waters. This assay was based on the amplification of fecal 16S rRNA marker sequences from uncultured cells of the order *Bacteroidales*. The BacHum-UCD assay was able to discriminate between human and cattle feces. The marker was detected in dog fecal samples but not in cat, horse, or seagull fecal samples.

Haugland and colleagues [46] developed several TaqMan®-based qPCR assays based on the 16S rRNA gene sequences of cultured *Bacteroides* rather than gene sequences from uncultured bacteria. The authors designed six new forward primers targeting six different *Bacteroides* spp., and used a previously published reverse primer (BFDrev) to develop six new species-specific qPCR assays known as BuniF2, BfragF1, BvulgF1, BsteriF1, PcoprF1, and BthetaF2. When these assays were evaluated using human and animal fecal samples, all markers were present in sewage and feces at high concentrations. However, none of these species-specific markers were completely specific to sewage. The performance of the forward primer HF183 along with a previously designed reverse primer (BFDrev) was superior to all six newly developed species-specific qPCR assays [46,47].

A multi-laboratory study tested a variety of MST assays against samples blinded to the testing laboratories that were spiked with fresh feces from animals and/or human feces, sewage, or septic system effluent [48]. Samples contained a single source (singleton) or two sources of waste (doubletons). All doubletons included one human waste source and one nonhuman source. One of the several papers generated by this study focused on human-associated fecal anaerobes [49]. Among the lessons learned in the detailed analysis was that the means of normalizing the qPCR signal strongly affected the apparent performance of the assays. Normalization strategies included wet mass, ng DNA, culturable FIB (*E. coli* and *Enterococcus* spp.), and FIB genes (23S rRNA of *Enterococcus* spp., general *Bacteroidales* (GenBac3). The authors made the comparisons among all methods by normalizing to ng total DNA, in part because this value accounted for the extensive variability in fecal load samples. An example of the effect of normalization on apparent performance is that of BacH, whose sensitivity was 100% when normalized to 1 mg or 15 mg wet mass, 42% when normalized to 5000 copies BacUni-UCD, 75% when normalized to 1 ng DNA, and 92% normalized to 10 ng DNA. Following the same order as the previous sentence, the specificity of BacH was 77% for both wet masses, and 100%, 88% and 85% for the other units. Apparent specificity of the HF183 TaqMan® was similarly affected by normalization procedure, with values of 62%, 42%, 96%, 96% and 73%, respectively.

Another important finding from this study was that method performance on a presence/absence basis is greatly affected by the treatment of measurements in which the MST marker is detected, but not quantifiable (DNQ). This phenomenon occurs when the measurement (C_T value) is lower than that of the no-template control but falls outside the lowest level of standard that can be reliably quantified. Counting the DNQ measurements as positive increased sensitivity for most methods, but decreased specificity. For example, BacH sensitivity in singleton samples was 100% if DNQ values were counted as positive, but only 75% if DNQs were considered negative. Conversely, BacH specificity was 77% if DNQs were called positive, but rose to 85% if DNQs were considered negative. This dichotomy was consistent across the methods for which the comparison could be made. The series of studies used a benchmark of 80% sensitivity and 80% specificity for adequate method performance [48]. HF183 TaqMan® normalized to 1 ng total DNA consistently exceeded the benchmarks, and was designated the best-performing method in the study. It is worth noting, however, that all nine of the markers tested showed some level of cross-reactivity with nonhuman feces, particularly deer, although their concentrations per ng total DNA were generally 1–2 orders of magnitude below that seen in septage or sewage.

The TaqMan® HF183/BFDrev qPCR assay developed by Haugland and colleagues [46] routinely forms nonspecific PCR amplification products [50]. Because of this, Green and colleagues [50] modified the TaqMan® assay by designing a new reverse primer (BacR287) that alleviates this problem. Comparison of assays' performance characteristics such as primer dimer formation, standard curve parameters, limit of detection, inter- and intra-assay variability, multiplexed IAC performance, and the DNA target distribution in raw sewage samples showed that the newly developed assay is superior to

the HF183/BFDrev assay. The cross-reactivity of the primer set for non-human fecal samples, however, has not yet been thoroughly evaluated and warrants further investigation.

Many studies have reported the cross-reactivity of *Bacteroides* 16S rRNA assays by testing non-human fecal samples [49,51–55]. The 16S rRNA region is highly conserved between different species of Bacteria and Archaea. Therefore, cross-reactivity may occur when this region is targeted for MST marker development [56]. It has been suggested that genes directly involved in host–microbe interactions may provide more host specificity than the highly conserved 16S rRNA gene, and may, therefore, be suitable targets for marker development [57]. Several researchers developed qPCR assays targeting *Bacteroides* genes other than those for 16S rRNA. Shanks and colleagues [38] developed two TaqMan® qPCR assays for the detection of human-specific *Bacteroidales* markers, one targeting a hypothelial protein (designated HumM2) and the other targeting a putative RNA polymerase extracytoplasmic function type sigma factor (HumM3). Both assays were highly sensitive in detecting the markers in individual human fecal and raw sewage samples from 20 wastewater treatment plants across the USA. The assays also exhibited a host specificity value >97% when tested against a large number of non-human fecal samples. Between the two assays, HumM2 had a slightly better host specificity value than the HumM3 assay.

Lee and Lee [39] developed a qPCR assay targeting a single copy *gyrB*, a gene that encodes the B-subunit of DNA gyrase, of *B. fragilis*. The authors designed a *B. fragilis* specific primer set by aligning 322 *gyrB* genes. The Bf904F/Bf958R assay was tested under end-point and qPCR conditions. The end-point assay did not cross-react with cow or dog fecal samples but was positive for one of 10 pig fecal samples. Based on the results, the authors concluded that the *gyrB* assay might serve as a complementary tool for 16S rRNA-based assays when they fail to separate closely related bacteria (*Bacteroides* and *Prevotella*) in human wastewater. Yampara-Iquise and colleagues [58] developed a qPCR assay targeting a single-copy putative α-1-6-mannanase gene based on the complete genomic sequence of *Bacteroides thetaiotaomicron* VP1 5482. The markers were highly prevalent (1.4×10^8 cells per L) in raw sewage samples. The assay was also highly specific (100%) when tested against a panel of pooled non-human fecal samples.

Although numerous PCR/qPCR assays have been developed, and there is a growing interest in the application of these assays to detect sewage pollution in water, there was no standardized protocol available until recently. Shanks and colleagues [59] generated qPCR data for HF183/BacR287 and HumM2 assays across 14 laboratories in the USA to establish assay performance benchmarks. Each laboratory used a standardized protocol with the same DNA, extraction kit, reagents, and samples to generate comparable data. The authors established proficiency metrics that included replicate sample testing within a laboratory, and random error for PCR inhibition, processing controls, extraneous DNA contamination assessments, and the performance of the standard curve. To demonstrate the implementation of proposed standardized protocols and data acceptance criteria, data from two additional laboratories were compared. Data acceptance criteria established in this study should aid researchers and regulators to evaluate the technical quality of MST data against the recommended benchmark [59].

An ideal MST marker should meet certain performance criteria, *i.e.*, it should be highly specific to its host, and broadly distributed in the feces of individuals within an animal group. The concentration of the marker should be high enough, and it should be evenly distributed in the feces of the host with little or no temporal or geographical variations. The persistence of the marker in the environment should be similar to FIB and pathogens, and the presence should be correlated with human health risks [11,60]. The following sections discuss some of these performance criteria of human-specific *Bacteroides* markers.

Table 1. Primer and probe sequences, concentrations, and annealing temperature for the sewage-associated *Bacteroides* 16S rRNA and non-16S rRNA PCR/qPCR assays.

Assays	Primer Sequences	Primer and Probe Concentrations	Annealing Temperature (°C)	References
16S rRNA-based assays				
HF183	HF183 F:ATCATGAGTTCACATGTCCG Bac708 R:CAATCGGAGTTCTTCGTG	10 µM 10 µM	63	[34]
	HF183 F:ATCATGAGTTCACATGTCCG SSHBac R:TACCCCGCCTACTATCTAATG	250 nM 250 nM	53	[44]
	HF183 F:ATCATGAGTTCACATGTCCG BFDRev:CGTAGGAGTTTGGACCGTGT BFDFam:FAM-CTGAGAGGAAGGTCCCCCACATTGGA-TAMRA	1 µM 1 µM 80 nM	60	[34,46]
	HF183 F:ATCATGAGTTCACATGTCCG BacR287:CTTCCTCTCAGAACCCCTATCC BacP234:FAM-CTAATGGAACGCATCCC-MGB	1 µM 1 µM 80 nM	60	[50]
HF134	HF134 F:GCCGTCTACTCTTGGCCA Bac708 R:CAATCGGAGTTCTTCGTG	10 µM 10 µM	63	[34]
HuBac	HuBac566F:GGGTTTAAAGGGAGCGTAGG HuBac692R:CTACACCACGAATTCCGCCT HuBac594P:FAM-TAAGTCAGTTGTGAAAGTTTGCGGCTC-BHQ1	15 pmol 15 pmol 5 pmol	60	[45]
BacHum-UCD	BacHum160F:TGAGTTCACATGTCCGCATGA BacHum241R:CGTTACCCCGCCTACTATCTAATG BacHum1930P:FAM-TCCGGTAGACGATGGGGATGCGTT-TAMRA	400 nM 400 nM 80 nM	60	[36]
BacH	BacHF:CTTGGCCAGCCTTCTGAAAG BacHR:CCCCATCGTCTACCGAAAATAC BacHPC:MGBNFQ-GTCCTACCCTAGTACT-FAM BacHPT:MGBNFQ-GTTCTACCGTAGTACT-FAM	200 nM 200 nM 100 nM 100 nM	61	[35]
Human-Bac1	qHS601F:GTTGTGAAAGTTTGCGGCTCA qBac725R:CAATCGGAGTTCTTCGTGATATCTA qHS624P:FAM-CGTAAAATTGCAGTTGA-MGB	900 nM 900 nM 200 nM	62	[37]

Table 1. *Cont.*

Assays	Primer Sequences	Primer and Probe Concentrations	Annealing Temperature (°C)	References
Non-16S rRNA-based assays				
HumM2	Hum2F:CGTCAGGTTTGTTTCGGTATTG Hum2R:TCATCACGTAACTTATTTATATGCATTAGC HumM2P:FAM-TATCGAAAATCTCACGGATTAACTCTTGTGTACGC-TAMRA	1 µM 1 µM 80 nM	60	[38]
HumM3	Hum3F:GTAATTCGCGTTCTTCCTCACAT Hum3R:GGAGGAAACAAGTATGAAGATAGAAGAATTAA HumM2P:FAM-AGGTCTGTCCTTCGAAATAGCGGT-TAMRA	1 µM 1 µM 80 nM	60	[38]
B. thetaiotaomicron, α-1.6-mannanase	BtHF:CATCGTTCGTCAGCAGTAACA BtHR:CCAAGAAAAAGGGACAGTGG BtHP:FAM-ACCTGCTG-NFQ	900 nM 900 nM 250 nM	60	[58]
gyrB	Bf904F:GGCGGTCTTCCGGGTAAA Bf958R:CACACTTCTGCGGGTCTTTGT Bf923P:FAM-TGGCCGACTGCTC-MGB	500 nM 500 nM 250 nM	60	[39]

3. Host-Sensitivity and Host-Specificity

3.1. Host Sensitivity

Host sensitivity is one of the key performance indicators for MST markers. It is expressed as the percentage of samples from a targeted host that are positive for the chosen marker. The greater the sensitivity and increased performance, the closer to 100% host sensitivity will be. It is expressed by the formula $\frac{a}{a+c} \times 100$, where a is the number of true positive results (a fecal or wastewater sample tests positive for the marker of its own), and where c is the number of false negatives resulting (fecal and wastewater samples are negative for the marker of its own) [61].

Host sensitivity of a human marker is determined by testing individual human fecal samples, wastewater influent or raw sewage, septic wastewater, and, to a lesser extent, effluent wastewater [26]. However, there is no consensus on the number of samples, types, and volumes needed to determine the host sensitivity of the marker. Many studies have analyzed 10–50 samples. In general, the *Bacteroides* markers are highly prevalent in composite wastewater samples (raw, septic, and effluent wastewater) compared to feces from an individual human. In this review, we calculated the overall host sensitivity values for the *Bacteroides* markers by compiling host sensitivity values reported in the relevant research articles (see Supplementary Tables S1–S10). Figure 1 shows the overall host sensitivity values for each human *Bacteroides* 16S rRNA and non-16S rRNA markers.

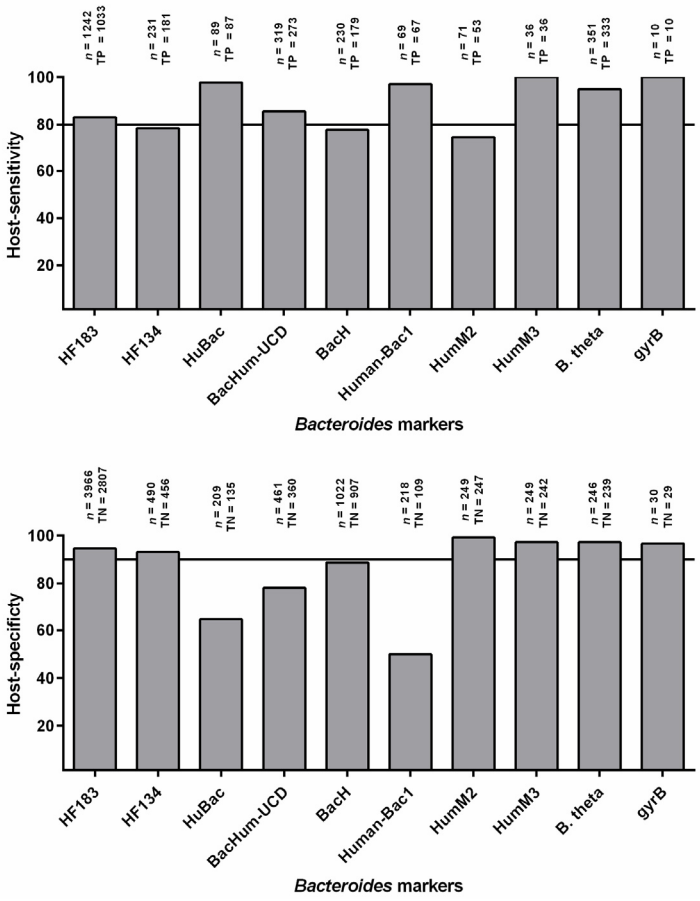

Figure 1. Host sensitivity and specificity values compiled from published studies for human *Bacteroides* 16S rRNA and non-16S rRNA markers. *n* represents a total number of human (for host sensitivity), and non-human (for host specificity) fecal samples tested between 2000 and 2016. TP and TN represent total numbers of human fecal samples that were positive and negative, respectively. The solid lines represent benchmark values (80% for host-sensitivity and 90% for host-specificity).

Currently, there is no universal benchmark for a host sensitivity measure of a marker, but a sensitivity value >80% is generally considered acceptable [48]. A marker with sensitivity values of <80% may still be useful if the specificity value is >90%. Among the human-specific *Bacteroides* markers, more information is available on the host sensitivity values of the HF183 marker compared to others. Since the development of the HF183 assay, 1242 individual human fecal, raw sewage, septic wastewater, and treated wastewater samples were screened with PCR/qPCR assays for the presence of this marker (Supplementary Table S1). Among these samples, 1033 were PCR positive for the HF183 marker, yielding an overall host sensitivity value of 83.1%. The BacHum-UCD marker also had a similar overall sensitivity (85.6%). However, a much smaller number of human fecal and wastewater samples (n = 319) were screened for the presence of this marker (Supplementary Table S4). The host sensitivity values of the HF134 and BacH were 77.8% and 78.4%, respectively. A total of 181 and 179 fecal and wastewater samples were screened for the presence of these markers (Supplementary Tables S2 and S5). The HumM2 marker had the lowest overall host sensitivity value (74.6%) due to its low prevalence in individual human fecal samples (Supplementary Table S7).

The overall sensitivity values of the HF183, HF134, BacHum-UCD, and BacH markers were affected because PCR positive/negative results for individual human fecal samples from several studies were also included in the calculations, rather than just composite fecal samples such as sewage. Not all individual human fecal samples are reported to be PCR positive for the *Bacteroides* markers, thereby reducing the overall sensitivity value [42,44,61–63]. It is not clear why the *Bacteroides* markers are not present in all individual human fecal samples; however, other host-associated markers have also shown <100% distribution in their hosts [21,25]. In reality, the absence of the marker in a small percentage of the human population is not an issue in most cases. In the developed nations, the chance of sewage reaching the environment through defective septic systems, leaky sewer pipes or pipe breaks, and combined sewer overflows is much higher than that of feces from an individual.

In contrast, the reported sensitivity values of the HuBac, Human-Bac1, HumM3, *B. thetataiomicron*, and *gyrB* (Supplementary Tables S3, S6, and S8–S10) were >95%. However, such data should be interpreted with care because these studies evaluated the performance of these markers by testing a small number of samples, predominantly raw sewage and pooled human fecal samples, which provides less information about the distribution of the marker in fecal samples from individuals. Further host sensitivity evaluation of these markers may be useful prior to their field application. Nonetheless, the overall host sensitivity values indicate that human-specific *Bacteroides* 16S rRNA and non-16S rRNA markers are highly prevalent in sewage and meet one of the essential performance characteristics of an ideal MST marker.

3.2. Host Specificity

Host specificity is one of the most important performance indicators for a marker. Host specificity also reaches a maximum of 100% [53,64]. It is expressed by the formula $\frac{d}{b+d} \times 100$, where b is a false positive (fecal and wastewater samples from non-target hosts are positive for the marker) and where d is a true negative result (fecal and wastewater samples from non-target hosts are negative for the marker).

The host specificity of a human marker is determined by testing non-human fecal samples in the designated study area [26]. However, there is no consensus on the number of samples needed to determine the host specificity of a marker. The more, the better, however: the U.S. EPA MST guide document [60] recommends that at least 10 animals per host type should be screened for specificity. Currently, there is no benchmark for a host specificity measure of a marker, but a specificity value >80% has been used as a benchmark and >90% is excellent [48,60].

In this review, we calculated the overall host specificity values for the *Bacteroides* markers by compiling specificity values reported in the relevant research articles (see Supplementary Tables S11–S20). Figure 1 shows the overall host specificity values for each human-specific *Bacteroides* 16S rRNA and non-16S rRNA marker. For the determination of the host specificity value for a particular

marker, studies have generally tested a panel of individual non-human fecal samples. Several studies also tested composite DNA or non-human fecal samples [46,63,65]. The advantage of this approach is that many samples can be screened in a PCR reaction, which is cost-effective. However, the disadvantage is that composite fecal or DNA samples do not provide information on the frequency of the marker in the population, as it generates a common signal for the composite sample. Studies that analyzed composite non-human DNA or fecal samples, as opposed to individual fecal or DNA samples, were excluded from the overall host specificity calculation for consistency.

Among the human-specific *Bacteroides* markers, the host specificity values of the HF183 marker have been more rigorously evaluated than others. Since its development, 2966 individual non-human fecal samples have been screened for the presence of the HF183 marker, of which 2807 were PCR negative, yielding an overall host specificity value of 94.6% (Supplementary Table S11). Several studies have reported 100% host specificity of the HF183 marker for non-human fecal samples [51,66–70]. In contrast, the occasional presence of the HF183 markers in non-human fecal samples has also been reported [52,57,61,71,72], particularly in dog, deer, and chicken feces.

The host specificity values of BacH and BacHum-UCD markers were also evaluated by several studies [51,73,74]. The overall host specificity value (88.7%) of BacH was lower than that of HF183 (Supplementary Table S15). However, the specificity value of the BacHum-UCD was much lower (78.1%), suggesting that this marker is not useful in areas where the feces of non-targeted hosts contain this marker (Supplementary Table S14). The overall host specificity values of the HuBac (64.6%) and Human-Bac1 (50%) markers were quite low, suggesting that these markers are not suitable for MST field studies (Supplementary Tables S13 and S16). The overall host specificity values of the non-16S rRNA markers (HumM2, HumM3, *B. thetaiotaomicron*, and *gyrB*) were higher than most of the 16S rRNA markers and may potentially provide accurate results (Supplementary Tables S17–S20). However, none of the non-16S markers showed 100% host specificity. Caution should also be exercised when using these non-16S rRNA markers because only a few studies have determined the host specificity values of these markers [38,39]. The overall host specificity values may decrease if more validation is performed.

In summary, from the available data, it can be concluded that human *Bacteroides* 16S rRNA markers such as HF183, HF134, and BacH and non-16S rRNA markers such as HumM2, HumM3, *B. thetaiotaomicron*, and *gyrB* are highly host-specific, although they could occasionally be present in non-human fecal samples. The non-specific markers are unreliable for field studies due to the possibility of yielding false positive detection, which may result in a wasted capital venture for mitigation activities. Obtaining additional information on the concentrations of non-specific markers in non-human fecal samples may provide more insight into the marker's usefulness. If the concentration of a marker is high in a human host, it is unlikely that it being detected at a low concentration in the non-human host(s) would be a limitation to MST results interpretation [75].

4. Concentrations of Human-Specific *Bacteroides* Markers in Human Feces, Wastewater and Water Environment

4.1. Human Feces and Wastewater

Host sensitivity validation provides information on the occurrence of a marker in its host in the presence/absence form, but not the concentrations. The concentration of a marker in its host is also an important factor because it is likely that a marker whose level is high will be consistently and more easily detected in polluted water samples. Markers whose concentrations are highly variable or low can be difficult to detect in the environment due to dilution. The concentrations of a marker in published studies are generally estimated by analyzing human fecal and wastewater samples using qPCR. The marker concentrations are expressed as gene copies per *g* or mg wet weight feces or per mL, 100 mL, or L of wastewater. To estimate the overall mean concentration and ease of data visualization, we standardized the concentrations of each human-specific *Bacteroides* marker to per *g* of feces or L of

wastewater. Figure 2 shows the overall mean concentrations and range of the *Bacteroides* 16S rRNA and non-16S rRNA markers in human feces and wastewater samples around the globe.

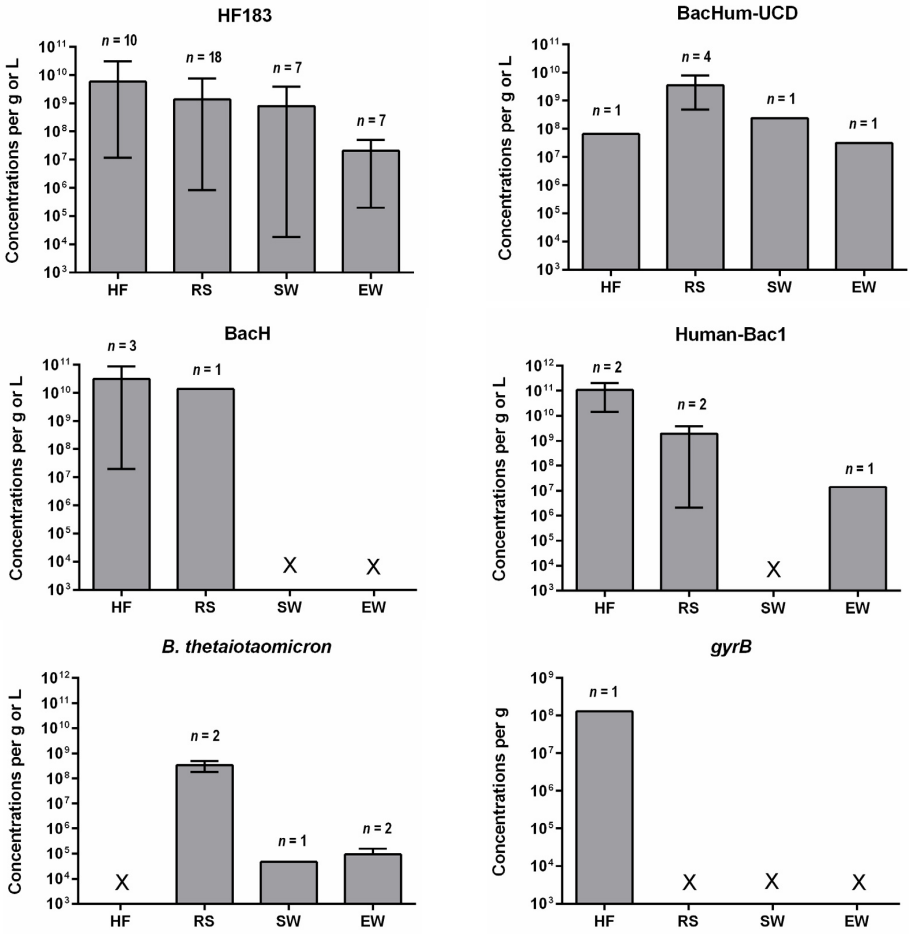

Figure 2. Concentrations (g per L) of human *Bacteroides* 16S rRNA and non-16S rRNA markers in human feces (HF), raw sewage (RS), septic wastewater (SW), and effluent wastewater (EW) samples. X denotes data not available. *n* represents the total number of studies reported the concentration of the marker in human feces and wastewater.

Among the *Bacteroides* markers, the HF183 has been the most thoroughly studied. The mean concentrations of the HF183 markers for pooled human feces, raw sewage, septic wastewater, and treated effluent were 5.8×10^9 gene copies per *g*, 1.7×10^9 gene copies per L, 8.0×10^8 gene copies per L, and 2.1×10^7 gene copies per L, respectively. The mean concentrations were higher in human feces followed by raw sewage, septic, and treated wastewater. Similar trends were also observed for the Human-Bac1 marker but not for the BacHum-UCD. The mean concentrations of the BacHum-UCD in human feces were approximately two orders of magnitude lower than raw wastewater. Two to four orders of magnitude variations were reported on the concentrations of the HF183 and BacH markers [35,44,74]. In general, the concentration of the HF183, BacHum-UCD, Human-Bac1, and *B. thetaiotaomicron* α-1-6-mannanase in secondary wastewater was one to two orders of magnitude lower than raw sewage, which is expected in sewage treatment processes.

The concentrations of the HF183, BacHum-UCD, BacH, and Human-Bac1 were $>1.0 \times 10^9$ gene copies per L of raw sewage, suggesting that these markers have the potential to detect diluted sewage pollution in environmental waters. A recent study determined the temporal variability of the HF183 markers in raw sewage samples from three climatic zones in Australia [76]. The concentrations of the HF183 markers in raw sewage samples showed little or no temporal variation over the course of

the study. Considering the elevated and consistent levels of HF183 markers in raw sewage samples from three climatic zones, the authors concluded that HF183 is useful for detecting sewage pollution across Australia.

Several studies have presented the concentration of these markers per ng DNA rather than volume of wastewater or wet weight [46,50,63,65]. This approach has some advantages such as avoiding the need to measure and correct for DNA extraction efficiencies and error introduced by sample variability (solid or liquid phases). The standardization process may also relieve PCR inhibition [77]. The mean concentrations of HF183 and HuBac in human feces were approximately two orders of magnitude higher than BacHum-UCD and BacH markers. For raw sewage samples, HumM2 markers had the highest concentration (Figure 3). More studies are warranted to determine the concentrations of these markers in new geographical locations to identify their potential for global application. Also, it is important to reach a consensus on the units of measurement used to express markers' concentrations.

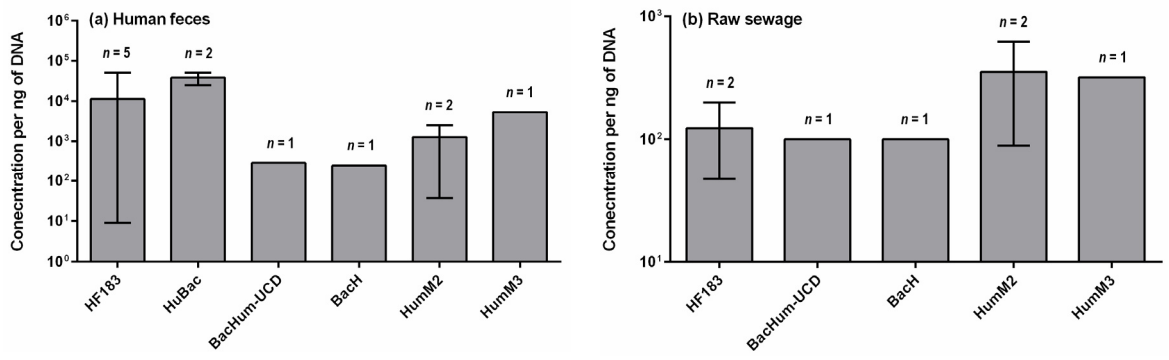

Figure 3. Concentrations (per ng of DNA) of human *Bacteroides* 16S rRNA and non-16S rRNA markers in (**a**) human feces and (**b**) raw sewage. *n* represents the total number of studies that reported the concentration of the marker.

4.2. Water Environment

While human-specific *Bacteroides* markers have been assessed by end-point PCR in a wide range of environmental waters, qualitative data are difficult to interpret regarding pollution magnitude, prioritizing remediation efforts, and human health risk. Qualitative data are therefore not included in this review.

Table 2 summarizes the prevalence and concentration of human *Bacteroides* markers in various types of environmental water samples. More quantitative data are available for HF183 than for other markers; samples were collected from river water, storm drains, and beach water in USA, Canada, Singapore, France, Bangladesh, Kenya, and Belgium. A few studies also provided quantitative data on the BacHum-UCD and HuBac markers in surface water, storm, and river water in the USA and Kenya. In the published articles, the concentrations of the HF183 marker per L of water ranged from as low as 1.6×10^1 to as high as 1.9×10^8 per L of water. Sercu and colleagues [78] reported the concentrations of the HF183 markers were as high as 1.9×10^8 per L of stormwater, suggesting a dominant wastewater component in the urban stormwater. High occurrence and concentrations of HF183 markers in storm drains, creeks, lakes, and coastal water samples have been reported by several studies, indicating a high proportion of human fecal pollution and potential public health risk [41,79,80]. Eichmiller and colleagues [81] quantified HF183 markers in water, sand, and sediment samples from a Lake Superior harbor site that received continuous contributions from wastewater effluent. Water samples from all sites were positive for HF183 with an average concentration of 1.3×10^5 per L, slightly below that of wastewater effluent. The concentrations of the HF183 markers in the sand and sediment samples were 1.3×10^2 per *g*. The authors concluded that sand and sediment may act as reservoirs for human-associated markers of fecal pollution at some sites.

Table 2. Prevalence and concentrations of human *Bacteroides* markers in environmental waters.

Assays	Country	Sample Types	No. of Samples Tested (% of Sample Positive)	Concentrations of Gene Copies	References
HF183 [a]	USA	Surface water	10 (30)	3.2×10^3–1.4×10^4 per L	[82]
HF183 [a]	USA	Creek and storm drain	90 (44)	Approx. 1.0×10^3–1.9×10^8 per L	[78]
HF183 [a]	Canada	Beach water, stormwater, creek water	203 (79)	3.2×10^2–4.0×10^3 per L	[83]
HF183 [a]	USA	Storm drains, creek water	26 (27)	4.1×10^2–1.5×10^7 per L	[80]
HF183 [a]	Singapore	Reservoir and catchment water	54 (93)	2.5×10^4–6.0×10^5 per L	[84]
HF183 [a]	USA	Coastal water	230 (29)	1.2×10^4–1.5×10^4 per L	[85]
HF183 [a]	USA	Stormwater	6 (100)	1.0×10^4–3.0×10^6 per L	[86]
HF183 [a]	USA	Harbor water	10 (100)	1.3×10^5 per L	[81]
HF183 [a]	France	River water	14 (7)	7.3×10^6 per L	[87]
HF183 [a]	France	Catchment water	240 (17)	1.6×10^1–2.5×10^2 per L	[88]
HF183 [a]	France	Surface water	63 (43)	1.6×10^4–1.0×10^6 per L	[89]
HF183 [a]	France	River water	23 (43)	2.3×10^4–9.6×10^5 per L	[90]
HF183 [a]	France	River water	24 (58)	3.2×10^4–1.3×10^6 per L	[70]
HF183 [a]	Canada	River water	1095 (10)	7.1×10^4 per L	[91]
HF183 [b]	USA	Surface water	189 (NM)	5.6×10^3 per µL of extract	[92]
HF183 [b]	USA	Surface water	29 (76)	5.1×10^2–2.9×10^4 per L	[93]
HF183 [a]	USA	Stormwater	14 (43)	3.9×10^3–6.3×10^6 per L	[94]
HF183 [c]	USA	Stormwater, surface water	32 (100)	3.5×103–1.4×10^6 per L	[95]
HF183 [a]	Bangladesh	Lake water	20 (70)	3.9×10^5–6.3×10^8 per L	[79]
HF183 [a]	Kenya	River water	18 (11)	9.5×10^3–4.5×10^4 per L	[67]
HF183 [a]	Canada	Surface water	374 (10)	6.3×10^4–6.3×10^6 per L	[72]
HF183 [a]	Canada	Surface water	184 (72)	1.0×10^5 per L	[96]
HF183 [a]	USA	River water	35 (34)	1.3×10^3–2.4×10^4 per L	[5]
HF183 [a]	Belgium	Coastal water	80 (76)	Approx. 1.0×10^5–1.0×10^7 per L	[41]
HuBac	USA	Surface water and stormwater	249 (98)	4.3×10^4–7.8×10^6 per L	[97]
HuBac	USA	Creek water	7 (100)	0.6×10^0–2.4×10^2 mg of feces per L	[45]
BacHum-UCD	USA	Stormwater	828 (57)	3.0×10^3–4.1×10^6 per L	[98]
BacHum-UCD	USA	Surface water and stormwater	73 (88)	3.0×10^5–5.0×10^5 per L	[99]
BacHum-UCD	Kenya	River water	18 (11)	1.3×10^5–1.6×10^5 per L	[67]
BacH	Canada	River water	130 (88)	8.0×10^1–1.0×10^3 per L	[74]
BacH	Austria	Spring water, river water, watering brook	6 (50)	6.5×10^2–1.1×10^6 per L	[35]
HuBac	Bangladesh	Pond water	43 (84)	1.3×10^5–6.8×10^8 per L	[100]
BacH	Israel	Spring water	46 (100)	1.1×10^4–3.2×10^5 per L	[101]
Human-Bac1	Japan	River water	30 (100)	2.7×10^3–6.5×10^4 per L	[102]
HumM2	USA	Surface water	29 (76)	7.6×10^2–2.1×10^3 per L	[91]
HumM3	Israel	Spring water	46 (65)	1.1×10^3–9.6×10^3 per L	[101]
B. thetaiotaomicron	USA	Surface water	29 (76)	6.7×10^2–1.2×10^6 per L	[58]

Notes: [a]: [44]; [b]: [46]; [c]: [50]; NM: Not mentioned.

Less information is available on the concentrations of other *Bacteroides* markers such as BacHum-UCD, BacH, Human-Bac1, HumM3, and *B. thetaiotaomicron*, α-1.6-mannanase in the environment compared to HF183. Like HF183, the concentrations of these markers ranged widely, from 8.0×10^1 to 6.8×10^8 per L of water. Sauer and colleagues [98] determined the concentrations of the BacHum-UCD marker in 828 water samples from 45 stormwater outfalls over a four-year period. Fifty-seven percent of samples were positive for the BacHum-UCD marker, with concentrations ranging from 3.0×10^3 to 4.1×10^6 gene copies per L in positive samples, suggesting widespread human fecal pollution in that urban environment. Ohad and colleagues [101] measured the concentrations of the BacH markers in 46 water samples from three Karst Springs in Israel by monitoring for a year and including rainfall events. All samples were positive for the BacH marker and concentrations ranged between 1.1×10^4 and 6.5×10^4 gene copies per L of water. The presence of human fecal pollution during the dry season indicated that septic systems in the study area were a contributing factor.

Knappett and colleagues [100] investigated the impacts of latrines on fecal pollution of ponds used for bathing and washing clothes and utensils in a community in Bangladesh. The ponds were polluted by latrines and were believed to a contributing factor to diarrheal disease. Forty-three pond water samples were analyzed for the HuBac marker, which was detected in 84% of samples at concentrations ranging from 1.3×10^5 to 6.8×10^8 gene copies per L of water. The authors concluded that continued use of unsafe pond water would contribute to diarrheal diseases in the community.

5. Decay of Human-Specific *Bacteroides* Markers in Environmental Matrices

Ideally, a human-associated *Bacteroides* marker should have a decay profile similar to those of human pathogens. Quantitative detection of *Bacteroides* markers involves quantifying DNA or RNA from viable, viable but non-culturable (VBNC) cells, and also from cells with compromised structural integrity. Since DNA from live or dead cells can be detected by PCR, it is important to understand how fast these cells and markers decay in environmental waters [103]. Little has been documented on the decay of *Bacteroides* cells and DNA in the aquatic environments. Table 3 shows the T_{90} decay (1 log reduction or 90% loss) of *Bacteroides* DNA, RNA, and cells in environmental matrices. However, caution should be exercised when comparing the results of T_{90} inactivation times of the *Bacteroides* markers among studies because decay rates and corresponding inactivation times can be influenced by factors such as predation, higher temperature, and sunlight, types of seeding materials, sample matrices, and decay model used [103–106]. Most of the decay studies listed in Table 3 have been conducted in laboratory microcosms designed to imitate at least some aspects of ambient environmental conditions, including varying sunlight, temperature, and salinity. Microcosms were seeded with raw sewage or cells.

Bacteroidales culturable cells tend to persist a relatively short time in environmental waters compared to DNA signal [107,108]. For example, Okabe and Shizuma [107] monitored the decay of *Bacteroides fragilis* cells in filtered and non-filtered river and seawater. The results indicated a 90%–99% reduction of *B. fragilis* cells within the first two days. Similarly, Bae and Wuertz [108] also reported the short survival (T_{90} < 2 days) of *Bacteroidales* cells in seawater and freshwater exposed to sunlight and dark conditions. In contrast, T_{90} decay of *Bacteroidales* DNA and RNA range from three to 12 days [44,106]. Microcosm studies also suggested that *Bacteroidales* markers persist longer at low temperatures than at high temperatures [44,105,109]. A strong effect of temperature was also noted for the HF183 marker, which persisted for up to 24 days at 4 °C and eight days at 28 °C in river water when measured by qPCR [44]. Several studies also reported faster decay rates for *Bacteroides* markers exposed to dark compared to sunlight [103,109–111]. In contrast, slower decay rates of *Bacteroidales* markers exposed to sunlight have been observed by others [105,108,110]. Studies also reported that the decay rates of the several *Bacteroides* markers including human-specific *Bacteroides* (BsteriF1, BuniF2, GenBac3, HF183, HF134, and HumM2) in freshwater were somewhat greater than the corresponding decay rate in seawater [106,112].

Table 3. Decay of human *Bacteroides* markers in environmental matrices.

Assays	Matrices	Temperature (°C)	Salinity (ppt)	Conditions	T_{90} (Days)	Reference
HF183	Freshwater	4			>24.0 [a]	[44]
		12			10.0 [a]	
		28			3.00 [a]	
HF183	Canal water	6–26		Sunlight	2.72 [a]	[113]
					2.08 [b]	
HF183	Seawater	20	33	Dark	2.30 [a]	[112]
	Freshwater				1.70 [a]	
HF183	Beach sand (14% moisture)	25		Dark	1.50 [a]	[114]
	Beach sand (28% moisture)				4.90 [a]	
HF183	Freshwater	13		Light	0.72 [a]	[106]
				Dark	0.75 [a]	
	Marine water			Light	3.98 [a]	
				Dark	0.49 [a]	
HF183	Freshwater	13		Light	0.41 [b]	[106]
				Dark	1.03 [b]	
	Marine water			Light	5.50 [b]	
				Dark	1.40 [b]	
HF183	Freshwater	14–21		Sunlight	3.44 [a]	[115]
	Seawater				2.70 [a]	
HF183	River water	25		Artificial sunlight	0.85 [a]	[105]
		15			1.26 [a]	
	Sediment	25			1.44 [a]	
HF183	Freshwater	14		Sunlight	1.37 [a]	[110]
				Dark	2.59 [a]	
	Seawater			Sunlight	1.45 [a]	
				Dark	1.65 [a]	
HF183	River water	13		Sunlight	0.59 [a]	[116]
					0.71 [b]	
				Dark	0.83 [a]	
					0.91 [b]	
HF134	River water	13		Sunlight	0.71 [a]	[116]
					0.66 [b]	
				Dark	0.83 [a]	
					0.91 [b]	
BacHum-UCD	Watered Beach sand	22		Dark	14.5 [a]	[117]
	Control beach sand				36.2 [a]	
BacHum-UCD	Seawater	17	34.2	Sunlight	1.79 [a]	[116]
				Dark	8.72 [a]	
BacHum-UCD	Seawater	14	31.5	Sunlight	3.75 [a]	[108]
				Dark	3.58 [a]	
				Sunlight	0.56 [c]	
				Dark	0.71 [c]	
BacHum-UCD	Freshwater	22		Sunlight	0.52 [c]	[118]
				Dark	1.29 [c]	
				Sunlight	2.14 [a]	
				Sunlight	1.29 [a]	

Table 3. *Cont.*

Assays	Matrices	Temperature (°C)	Salinity (ppt)	Conditions	T_{90} (Days)	Reference
BacHum-UCD	Freshwater	22		Sunlight Dark	2.44 [a] 2.50 [a]	[111]
				Sunlight Dark	0.79 [c] 1.98 [c]	
BacHum-UCD	River water	25 15		Artificial sunlight	0.77 [a] 1.17 [a]	[105]
	Sediment	25			1.04 [a]	
BacH	Creek water	20–25			0.75 [a]	[74]
					3.85 [a]	
Human-Bac1	River water	4 10 20 30 10	 0 10	Dark	14.3 [a] 3.57 [a] 1.96 [a] 1.66 [a] 3.33 [a] 3.33 [a]	[107]
	Seawater		20 20		3.70 [a] 3.70 [a]	
HumM2	Freshwater	13		Light Dark	1.10 [a] 0.18 [a]	[106]
	Marine water			Light Dark	4.23 [a] 6.95 [a]	
B. thetaiotaomicron, α-1.6-mannanase	River water (solid matrix) River water	4		Dark	27.0 [a] 9.60 [a]	[109]
	River water (solid matrix) River water	27			18.0 [a] 1.80 [a]	
	River water (solid matrix) River water	37			3.20 [a] 1.00 [a]	

Notes: [a]: DNA; [b]: RNA; [c]: cells.

Bae and Wuertz [108] used propidium monoazide (PMA)-qPCR to differentiate between live and dead cells or extracellular DNA. The authors concluded that this approach might be explored to estimate the age (recent *vs.* old) of fecal pollution in water. Green and colleagues [106] determined the decay rates of several *Bacteroides* DNA and RNA markers in marine and freshwater microcosms seeded with raw sewage and exposed to either sunlight or dark. While the DNA and RNA-based markers decayed at the same rate, some markers decayed significantly faster than others. It is not clear whether such variable decay of *Bacteroides* markers will affect their application in field studies. Caution should be exercised when choosing a marker for data interpretation because differential decay affects the quantitative estimation of the contribution from each source [11,116].

It is desirable that the decay of MST markers in environmental waters should have similar decay patterns to those of FIB and pathogens. This is particularly important for water quality regulation and health risk context.Several studies reported that the decay of the *Bacteroidales* DNA markers in water is comparable to culturable FIB [103,105,108,115,116]. These findings emphasize the potential for using *Bacteroidales marker* for MST field studies. Bae and Wuertz [118] reported similar decay of *Bacteroidales* and *Campylobacter* cells exposed to sunlight. DNA from *Bacteroides* and pathogens such as *Campylobacter* spp., *Salmonella* spp., and adenoviruses decayed at comparable rates. The authors concluded that the application of PMA-qPCR and qPCR might yield more realistic information about recent sources of fecal pollution and waterborne pathogens.

In summary, understanding the decay patterns of *Bacteroides* markers in relation to FIB and pathogens in environmental waters is important in order to develop fate and transport models. The

decay of HF183 and BacHum-UCD markers has been well studied in laboratory freshwater and seawater microcosms with variable temperatures and conditions. Several studies have consistently shown that higher temperature, higher salinity, and predation accelerate the decay of *Bacteroides* markers. More studies should be undertaken to compare the decay profiles of various *Bacteroides* markers with those pathogens, which would aid in the better interpretation of fecal pollution and associated health risks in water.

6. Correlation of Human-Specific *Bacteroides* Markers with Enteric Pathogens in Water

An ideal MST marker should be able to predict human health outcomes, such as the probability of gastroenteritis from exposure to recreational waters. Because the expense and logistical difficulty of epidemiology studies limit the number that is conducted, most studies attempt to correlate MST markers with FIB levels (regulatory standards) and/or with pathogens. From a public health perspective, the relationship between human *Bacteroides* markers and pathogens is more critical than that of the markers to FIB. Several studies reported the significant positive correlations between *Bacteroides* markers such as HF183, HF134, HuBac, Human-Bac1, and BacHum-UCD with fecal culturable fecal coliform, *E. coli*, and *Enterococcus* spp. in environmental waters [5,98,119–121] (Table 4, 2nd column). In contrast, negative correlations between human-specific *Bacteroides* and FIB have been reported by others [37,42,86,120] (Table 4, 3rd column). Contradictory results have also been reported on the correlations between human-specific *Bacteroides* markers and pathogens (see Table 4). These results suggest that, while some of these markers may be useful indicators of sewage pollution, they may fail to indicate human health risks from non-human fecal sources [82].

Table 4. Studies reporting significant positive and negative correlation between human *Bacteroides* and fecal indicator bacteria or pathogens in environmental waters.

Analytes	Studies	
	Weak to Strong Positive Correlation	**Negative to No Correlation**
HF183 *vs.* Fecal coliform	[96]	[91,121]
HF183 *vs. E. coli*	[5,84,88,98,119,122]	[42,70,86,91,123]
HF183 *vs. Enterococcus* spp.	[81,98,119,120,122]	[86,88,91,121,123]
HF183 *vs. C. perfringens*	-	[91]
HF183 *vs.* Adenoviruses	[124]	-
HF183 *vs. Campylobacter* spp.	[125,126]	[69,72]
HF183 *vs.* Enteroviruses	[127,128]	[129]
HF183 *vs. Salmonella* spp.	[72,91]	[69,129]
HF183 *vs. Cryptosporidium* spp.	-	[91,129]
HF183 *vs. Giardia* spp.	-	[91,129]
HF183 *vs. E. coli* O157:H7	-	[69,72,91]
HF183 *vs.* Shiga-toxin producing *E. coli*	-	[69]
HF134 *vs. E. coli*	[119]	-
HF134 *vs. Enterococcus* spp.	[119]	-
HuBac *vs. E. coli*	[100]	-
BacHum-UCD *vs. Campylobacter* spp.	[126,130]	-
BacHum-UCD *vs. Cryptosporidium* spp.	[130]	-
BacHum-UCD *vs. E. coli* O157:H7	[130]	-
BacHum-UCD *vs. Leptospira* spp.	[126,130]	-
BacHum-UCD *vs. Salmonella* spp.	[130]	-
BacHum-UCD *vs.* Enteroviruses	[131]	-
BacHum-UCD *vs.* Adenoviruses	-	[132]
BacHum-UCD *vs.* noroviruses	-	[132]
Human-Bac1 *vs.* Fecal coliform	-	[37]
Human-Bac1 *vs. C. perfringens*	-	[102]

Many factors such as types of water, dilution effect, turbidity, differences in analytical methods (culture-based *vs.* qPCR), sources of fecal inputs, differential decay, and the magnitude of fecal pollution may explain the lack of correlations observed. Poor correlations between FIB and pathogens and human-specific *Bacteroides* markers may not necessarily hinder their application as MST tools if the objective of the study is to determine the sources of fecal pollution for mitigation purpose. Because some pathogens and all FIB have non-human sources, a comprehensive "toolbox" approach that targets as many host types as possible is likely to produce more correlations with pathogens and FIB than a study that assesses only human sources of pollution.

7. Future Directions

The currently used *Bacteroides* PCR/qPCR assays rely on detecting or quantifying a single marker in a water sample. A negative result based on the detection of a single marker does not effectively rule out the presence of fecal pollution since various markers have differential decay rates and host-sensitivity. This is a limitation for the management of water quality and protection of public health risk. Microbial community analysis may ultimately allow for a more comprehensive assessment of source contributions by identifying multiple sources of fecal pollution in a single sample [133]. Microarray technology, where hundreds of probes targeting multiple markers including *Bacteroides* and pathogens can be tested simultaneously by hybridization, is a powerful tool for MST studies. Li and colleagues [134] developed a custom microarray targeting pathogens, MST markers, and antibiotic resistance genes. In a subsequent study, the microarray was used to detect multiple pathogenic genes and MST markers in fresh and marine water and as well as sewage and treated effluent seeded environmental water [135]. One drawback of this approach is that it does not provide quantitative data. However, it can serve as a screening tool, and based on the frequency of detection qPCR assays can be used to obtain quantitative data (if required).

The recent advances in metagenomics using next-generation sequencing allow accurate identification of candidates for novel genetic markers [133]. By far for MST studies, the most explored taxonomic group is the order *Bacteroidales*. However, human and other animal gut microbiota contain an array of other taxonomic groups that may serve as novel indicators of fecal pollution along with *Bacteroidales*. Unno and colleagues [136] reported the development of a new library-dependent method using pyrosequencing-derived shared operational taxonomic units (OTUs) to identify the sources of fecal pollution in waterways in South Korea. Their results indicated that the majority of bacteria in the feces of humans and domesticated animals belonged to the phyla *Bacteroidetes* and *Firmicutes*, whereas the predominant bacteria in the feces of geese and in freshwater samples belonged to *Proteobacteria*. Using this method, the authors were able to determine that human and swine feces were the sources of fecal pollution in the river.

A recent study compared the V6 region of the 16S rRNA gene in fecal samples from six animals to sewage [137]. The authors focused on 10 abundant genera and used oligotyping, which can distinguish ecologically distinct organisms whose rRNA sequences differ from each other by few nucleotides [138]. The results indicated that 99 human oligotypes were specific to human wastewater. The advantage of this approach is that specific oligotypes can be used to develop molecular assays, or all oligotypes can be used to indicate the presence of sewage signature in water. One of the most important aspects of metagenomic analysis is that it has the potential to detect multiple human and animal markers from different taxonomic groups. However, further improvements in bioinformatics will be required for routine use of these methods to identify sources of fecal pollution in the environment.

8. Conclusions

- Application of human-associated *Bacteroides* for MST field studies is promising due to their high host specificity and abundance in human wastewater. PCR methods avoid the issue of culture bias but introduce methodological issues such as efficiency of DNA extraction and inhibition of amplification. PCR/qPCR-based methods can be less expensive and are more rapid than

culturing bacteria—they can yield results in as little as 4–6 h. However, standardization of assays, units, and the markers' performance characteristics will be substantial challenges from a regulatory perspective.

- Among the human-specific *Bacteroides* markers, the performance characteristics and application of the HF183 markers have been more thoroughly evaluated than others. However, BacH, HumM2, and HumM3 have been shown to be useful quantitative tools for tracking sewage pollution in environmental waters. Rapid decay of these markers in the environment makes them very useful markers for tracking recent fecal pollution. However, differential decay rates and a lack of complete host specificity indicate that these *Bacteroides* markers should be accompanied with more host-specific markers such as human adenoviruses or polyomaviruses in a toolbox format for the accurate and sensitive detection of sewage pollution in environmental waters.
- Little research has been undertaken on the host specificity and sensitivity of the HumM2, HumM3, *B. thetaiotaomicron*, α-1.6-mannanas, and *gyrB* markers. Since some animal fecal samples produced false-positive results with these markers due to the presence of the target (or a very closely related) bacteria, further evaluation of performance characteristics should be undertaken to determine the broader applicability of these markers. Non-specific markers may still be useful for source tracking if information is available on the contributing sources and if possible testing should be accompanied with additional markers in a toolbox format.
- Quantitative data on the occurrence of human-specific *Bacteroides* markers in their hosts is limited other than for HF183. Such data are important to determine the suitability of a marker for detecting fecal pollution in environmental waters. The baseline concentration, which is appropriate to detect fecal pollution and also indicate health risks, needs to be established.
- The high prevalence and levels of human-specific *Bacteroides* markers in environmental waters in the USA, France, Bangladesh, Canada, Belgium, and several other countries indicate chronic sewage pollution in environmental waters. The application of these tools is encouraged in continents such as Asia and Africa (where possible) where wastewater pollution due to improper sanitation and gastrointestinal diseases is a major concern, but only after assessing the performance of the markers in these regions.
- Regulatory and public health concerns mandate that more studies should be undertaken to gain an understanding of how human-specific *Bacteroides* markers correlate with FIB, pathogens, and human health risks. The absence of correlations does not necessarily impede the utility of these markers in identifying fecal pollution and the potential for mitigation. Little is known regarding the concentrations of human-specific *Bacteroides* markers in soils, sediments, and beach sand. These environmental matrices harbor FIB and the limited data indicate that BacHum-UCD markers persist longer in watered beach sand [117]. *Bacteroides* associated with particular matter in suspension or solid matrices may remain viable for a longer time than if they are dispersed in water. These results have implications for the accuracy of MST tools as regulatory standards for the protection of water quality.
- A significant challenge associated with the field application of these markers is effective, quantitative recovery of DNA from complex environmental samples. Little has been documented on the recovery of these markers from environmental matrices. Quantification of these markers in environmental waters can be difficult due to factors such as dilution, sorption to particulate matters, environmental decay, loss due to recovery and DNA extraction, and the fact that only a small volume of a DNA sample is used for PCR analysis. Recent developments in qPCR technology such as droplet digital PCR may provide more sensitive detection of DNA from environmental waters. Incorporation of quantitative microbial risk assessment (QMRA) with MST science will improve our understanding of the relative public health risks associated with various sources of fecal pollution.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4441/8/6/231/s1. Table S1: Detection of *Bacteroides* 16S rRNA HF183 marker in human fecal and wastewater samples reported in the research literature, Table S2: Detection of *Bacteroides* 16S rRNA HF134 marker in human fecal and wastewater samples reported in the research literature, Table S3: Detection of *Bacteroides* 16S rRNA HuBac marker in human fecal and wastewater samples reported in the research literature, Table S4: Detection of *Bacteroides* 16S rRNA BacHum-UCD marker in human fecal and wastewater samples reported in the research literature, Table S5: Detection of *Bacteroides* 16S rRNA BacH marker in human fecal and wastewater samples reported in the research literature, Table S6: Detection of *Bacteroides* 16S rRNA HumanBac1 marker in human fecal and wastewater samples reported in the research literature, Table S7: Detection of *Bacteroides* non-16S rRNA HumM2 marker in human fecal and wastewater samples reported in the research literature, Table S8: Detection of *Bacteroides* non-16S rRNA HumM3 marker in human fecal and wastewater samples reported in the research literature, Table S9: Detection of *Bacteroides* non-16S rRNA *Bacteroides thetaiotaomicron* marker in human fecal and wastewater samples reported in the research literature, Table S10: Detection of *Bacteroides* non-16S rRNA *gyrB* marker in human fecal and wastewater samples reported in the research literature, Table S11: Detection of *Bacteroides* 16S rRNA HF183 marker in non-human fecal samples reported in the research literature, Table S12: Detection of *Bacteroides* 16S rRNA HF134 marker in non-human fecal samples reported in the research literature, Table S13: Detection of *Bacteroides* 16S rRNA HuBac marker in non-human fecal samples reported in the research literature, Table S14: Detection of *Bacteroides* 16S rRNA BacHum-UCD marker in non-human fecal samples reported in the research literature, Table S15: Detection of *Bacteroides* 16S rRNA BacH marker in non-human fecal samples reported in the research literature, Table S16: Detection of *Bacteroides* 16S rRNA HumanBac1 marker in non-human fecal samples reported in the research literature, Table S17: Detection of *Bacteroides* non-16S rRNA HumM2 marker in non-human fecal samples reported in the research literature, Table S18: Detection of *Bacteroides* non-16S rRNA HumM3 marker in non-human fecal samples reported in the research literature, Table S19: Detection of *Bacteroides* non-16S rRNA *Bacteroides thetaiotaomicron* marker in non-human fecal samples reported in the research literature, Table S20: Detection of *Bacteroides* 16S rRNA *gyrB* marker in non-human fecal samples reported in the research literature.

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