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Unsuitability of Quantitative *Bacteroidales* 16S rRNA Gene Assays for Discerning Fecal Contamination of Drinking Water▽

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Bacteroidales **species were detected in (tap) water samples from treatment plants with three different PCR assays. 16S rRNA gene sequence analysis indicated that the sequences had an environmental rather than fecal origin. We conclude that assays for *Bacteroidales* 16S rRNA genes are not specific enough to discern fecal contamination of drinking water in the Netherlands.**

Drinking water in many countries is routinely monitored for recent fecal contamination by testing for fecal indicator organisms *Escherichia coli*, thermotolerant coliforms, and/or intestinal enterococci to demonstrate microbial safety (13, 21, 42). Although these indicator organisms have been used for many decades, they have some limitations: the number of *E. coli*/coliform/enterococcus bacteria in feces is relatively low (18, 38), and they sometimes might be able to grow in the environment (10, 11, 14, 27). Consequently, scientists have been searching for alternative indicator organisms to determine fecal contamination of water. In 1967, bacteria belonging to the genus *Bacteroides* were suggested as alternative indicator organisms (26). *Bacteroides* spp. might have some advantages over the traditional indicator organisms. The numbers of *Bacteroides* spp. in the intestinal tract of humans and animals are 10 to 100 times higher than the numbers of *E. coli* or intestinal enterococci (1, 2, 12, 26). However, the use of *Bacteroides* spp. as indicator organisms was hampered by the complex cultivation conditions required (1, 2). The introduction of molecular methods made it possible to detect bacterial species that belong to the order *Bacteroidales*, an order that includes the genus *Bacteroides*, without cultivation. As a result, real-time PCR methods were developed for the quantitative detection of *Bacteroidales* in surface and recreation water and the potential of *Bacteroidales* species as an indication of fecal contamination of recreational waters was demonstrated (6, 12, 16, 19, 20, 29). *Bacteroidales* species might be useful indicator organisms for fecal contamination of drinking water as well. However, methods to detect fecal contamination in drinking water should be more sensitive, because people ingest more drinking water and the quality assessments and standards for fecal contamination are stricter than for bathing water. Studies exploring real-time PCR for the detection of *Bacteroidales* genes in drinking water have not been published to our knowledge. The objective of our study was, therefore, to determine if assays for the detection of *Bacteroidales* 16S rRNA genes can be used to detect fecal contamination in drinking water.

Unchlorinated tap water samples were obtained in November 2007 and February 2010 from one or more locations in the distribution systems of nine different drinking water treatment plants (plants A to I; Table 1) that produced unchlorinated drinking water from confined (plants B, C, E, F, and G) and unconfined (plants A, D, H, and I) groundwater. The treatment plants are located in the central part of the Netherlands within 90 km of each other. In addition, untreated groundwater from extraction wells and/or untreated raw groundwater (mixture of groundwater from different extraction wells) was sampled in March 2008 (Table 1). Water samples (100 ml) were filtered over a 25-mm polycarbonate filter (0.22-μm pore size, type GTTP; Millipore, Netherlands) and a DNA fragment was added as internal control to determine the recovery efficiency of DNA isolation and PCR analysis (2a, 40). DNA was isolated using a FastDNA spin kit for soil (Qbiogene, United States) according to the supplier's protocol. Primer sets AllBac 296f and AllBac 412r, resulting in a PCR product of 108 bp, were used in combination with TaqMan probe AllBac375Bhqr to quantitatively determine the number of *Bacteroidales* 16S rRNA gene copies in the water samples using a real-time PCR instrument (20). The PCR cycle after which the fluorescence signal of the amplified DNA was detected (threshold cycle [C_T]) was used to quantify the concentration of 16S rRNA gene copies. Quantification was based on comparison of the sample C_T value with the C_T values of a calibration curve graphed using known copy numbers of the *Bacteroidales* 16S rRNA gene, as previously described (12, 20). The correlation coefficient of the calibration curve was 0.99, and the efficiency of the PCR 95 to 105%. Finally, the *Bacteroidales* cell number was calculated by using the recovery rate of the internal standard and assuming five 16S rRNA gene copy numbers per cell (5). The detection limit of this gene assay was 50 *Bacteroidales* cells 100 ml^{-1} (corresponding to 10 16S rRNA gene copies per reaction mixture). Furthermore, the 16S rRNA genes that were obtained from several water samples from treatment plant C with the AllBac and TotBac (12) primer sets were sequenced, and the nearest relatives were obtained from the GenBank database using BLAST searches.

The *Bacteroidales* 16S rRNA gene, quantified with the AllBac primer set, was detected in all tap water samples in November 2007 and February 2010. The number of cells varied between 154 and 7,862 *Bacteroidales* cells 100 ml^{-1}, and the numbers in tap water of each plant were similar in 2007 and

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TABLE 1. Numbers of *Bacteroidales* cells in extraction wells, raw groundwater, and unchlorinated tap water of nine different groundwater plants in the Netherlands[a]

Plant	Source of sample	No. (100 ml^{-1}) of *Bacteroidales* cells in:		
		2007	2008	2010
A	Tap water 1[b]	5,948 ± 950		
	Tap water 2	2,682 ± 1,459		1,254 ± 216
	Tap water 3	4,362 ± 947		439 ± 136
	Raw water		96 ± 15	
B	Tap water 1	3,553 ± 981		5,302 ± 2,952
	Tap water 2	4,487 ± 391		2,119 ± 1,367
	Tap water 3	7,862 ± 4,588		3,896 ± 3,003
	Raw water		3,209 ± 833	
C	Tap water 1	661 ± 75		386 ± 199
	Tap water 2			1,051 ± 626
	Tap water 3			831 ± 584
	Tap water 4			1,254 ± 216
	Extraction well 1		1,126 ± 262	
	Extraction well 2		2,666 ± 51	
	Extraction well 3		<50	
	Raw water		90 ± 44	
D	Tap water	1,103 ± 29		1,254 ± 216
	Raw water		48 ± 16	
E	Tap water	1,302 ± 222		1,254 ± 216
	Extraction well 1		671 ± 97	
F	Tap water	1,317 ± 198		
	Raw water		<50	
G	Tap water 1	675 ± 92		439 ± 300
	Tap water 2	216 ± 65		249 ± 98
	Tap water 3	154 ± 6		322 ± 137
	Raw water		<50	
H	Tap water	7,073 ± 845		
	Raw water		511 ± 254	
I	Tap water	1,577 ± 176		
	Raw water		420 ± 66	

[a] Values are the average results and standard deviations from replicate PCRs on the same water sample using the AllBac primer set (20). In November 2007, the distribution systems (tap water) of plants A, B, and G were sampled at three different locations, whereas for the other plants, one location in the distribution system was sampled. In March 2008, raw water of plants A to G was sampled, as well as one (plant E) or three (plant C) different extraction wells. Finally, in February 2010, the distribution systems of plants A, B, C, D, E, and G were sampled again.

[b] More than one tap water sample from a treatment plant means that samples were taken at different locations in the distribution system.

2010 (Table 1). The *Bacteroidales* counts were high compared to the number of *E. coli* that are occasionally observed in fecally contaminated drinking water (17a) but low compared to numbers observed in surface water (4, 20, 22). Water from the extraction wells and raw water used for unchlorinated drinking water production were analyzed, and *Bacteroidales* species were detected in 10 out of 15 samples (Table 1). These results would imply that the extracted groundwater, raw water, and tap water were fecally contaminated. According to the Dutch drinking water decree (2b), both raw and tap water from the nine different treatment plants are regularly analyzed for fecal contamination by monitoring for *E. coli*, F-specific RNA phages, and somatic coliphages. For at least the last 10 years, these indicator organisms have not been detected in these waters.

Additional qualitative PCR analyses using TotBac and BacUni primer sets (12, 19) targeting other parts of the *Bacteroidales* 16S rRNA gene were performed to confirm the presence of *Bacteroidales* species in the water samples of November 2007 and March 2008. Nine or 10 of the 11 samples that were positive with the AllBac primer set were also positive with the TotBac and BacUni primer sets (data not shown). The BacUni primer set has a higher detection limit (30 gene copies per PCR; 19), which could explain the difference from the results with the AllBac primer set. The TotBac primer set has the same detection limit as the AllBac primer set (12), but small differences in PCR efficiencies might have resulted in different results, since some water samples showed *Bacteroidales* 16S rRNA gene copy numbers around the detection limit (Table 1). Nevertheless, the additional PCR analyses demonstrated that the detection of *Bacteroidales* species in tap, raw, and extracted well water with the AllBac primer set was not an artifact. The primer sets used were developed in three different studies (12, 19, 20) but have been applied in a number of

TABLE 2. Nearest relatives in GenBank to the *Bacteroidales* 16S rRNA gene sequences obtained from groundwater and unchlorinated tap water from plant C using different primer sets[a]

Primer set used, source of sample, and OTUs[b]	GenBank sequence accession no.	Source of sequence (GenBank sequence accession no.)	Similarity[c]	Nearest cultivated bacterium in GenBank (sequence accession no.)	Similarity
AllBac					
Extraction well 1 (3/6)	GQ169588	Rhizosphere (EF605968)	108/108	*Prevotella oralis* (AY323522)	105/108
Extraction well 1 (3/6)	GQ169589	Water from watershed (DQ886209)	108/108	*Tannerella forsythia* (AB035460)	107/108
Extraction well 2 (1/6)	GQ169590	Phyllosphere Brazilian forest (DQ221468)	108/108	*Tannerella forsythia* (AB035460)	106/108
Extraction well 2 (5/6)	GQ169591	Bovine rumen (EU348207)	108/108	*Tannerella forsythia* (AB035460)	106/108
Extraction well 3 (1/6)	GQ169592	Phyllosphere Brazilian forest (DQ221468)	108/108	*Prevotella oralis* (AY323522)	104/108
Extraction well 3 (5/6)	GQ169593	*Prevotella corporis* (L16465)	108/108	*Prevotella corporis* (L16465)	108/108
Raw water (3/6)	GQ169594	Spitsbergen permafrost (EF034756)	108/108	*Tannerella forsythia* (AB035460)	106/108
Raw water (3/6)	GQ169595	Hindgut beetle larvae (FJ374179)	108/108	*Tannerella forsythia* (AB035460)	107/108
Tap water (6/6)	GQ169596	*Prevotella timonensis* (DQ518919) *Prevotella buccalis* (L16476) *Prevotella ruminicola* (AF218617) *Bacteroides vulgatus* (NC_009614)	108/108	*Prevotella timonensis* (DQ518919) *Prevotella buccalis* (L16476) *Prevotella ruminicola* (AF218617) *Bacteroides vulgatus* (NC_009614)	108/108
TotBac					
Extraction well 1 (1/10)	GQ169597	Deep subsurface groundwater (AB237705)	339/369	*Salinimicrobium terrae* (EU135614)	315/370
Extraction well 1 (1/10)	GQ169598	Songhuajiang River sediment (DQ444125)	363/377	*Paludibacter propionicigenes* (AB078842)	357/376
Extraction well 1 (4/10)	GQ169599	Freshwater pond sediment (DQ676447)	352/360	*Paludibacter propionicigenes* (AB078842)	313/372
Extraction well 1 (4/10)	GQ169600	Pine River sediment (DQ833352)	364/371	*Bacteroides oleiciplenus* (AB490803)	334/375
Extraction well 2 (4/10)	GQ169601	Groundwater (AF273319)	364/371	*Xanthobacillum maris* (AB362815)	338/375
Extraction well 2 (6/10)	GQ169602	Human saliva (AB028385)	381/382	*Prevotella intermedia* (AY689226)	380/382
Extraction well 3 (1/10)	GQ169603	Pig manure (AY816766)	354/377	*Bacteroides thetaiotaomicron* (AE015928)	311/380
Extraction well 3 (3/10)	GQ169604	Pig manure (AY816867)	371/376	*Butyricimonas virosa* (AB443949)	307/379
Extraction well 3 (6/10)	GQ169605	Swedish lake (AY509350)	343/362	*Parabacteroides distasonis* (AB238927)	320/374
Raw water (10/10)	GQ169606	*Prevotella timonensis* (AF218617)	378/379	*Prevotella timonensis* (AF218617)	378/379
Tap water (1/10)	GQ169607	Deep subsurface groundwater (AB237705)	338/369	*Salinimicrobium terrae* (EU135614)	312/370
Tap water (2/10)	GQ169608	Yukon River, AK(FJ694652)	367/372	*Psychroserpens burtonensis* (U62913)	312/375
Tap water (7/10)	GQ169609	Deep subsurface groundwater (AB237705)	341/369	*Salinimicrobium terrae* (EU135614)	315/370

[a] Primer sets AllBac (20) and TotBac (12) were used in PCRs of samples, and GenBank was searched for relatives using BLAST.
[b] OTUs are indicated by the values in parentheses (number of sequences belonging to the OTU/total number of sequences analyzed).
[c] Number of base pairs identical in both sequences/total number of base pairs in sequences.

recent studies to detect fecal contamination of surface water (3, 4, 16, 22, 33, 34). The results from most of these studies showed that 16S rRNA genes of *Bacteroidales* were present in all surface water samples tested. Only Sinigalliano et al. (34) observed that 2 out of 4 water samples were negative with the TotBac primer set. However, the detection limit of the assay was not specified in that study.

The nine different treatment plants tested in our study produce unchlorinated drinking water from groundwater, which is considered to be of high hygienic quality. In addition, the extraction wells are protected from fecal contamination by a protection zone where no activities related to human waste or animal manure are allowed. In the Netherlands, this protection zone is based on a 60-day residence time of the water. Previous studies have demonstrated that a residence time of 60 days is highly effective in removing fecal bacteria and viruses (30, 31, 39). Moreover, the *Bacteroidales* numbers in tap water in November 2007 were significantly higher than the numbers in raw groundwater in March 2008 (Mann-Whitney U test; $P < 0.01$). Because the recovery efficiency of the internal control was the same between raw water and tap water samples, this result demonstrates that *Bacteroidales* cell numbers increased during treatment and/or drinking water distribution. This result could suggest that the water was fecally contaminated during drinking water treatment and/or distribution. However, it is unlikely that the integrity of nine different treatment trains and/or supply systems was affected in the sampling period. The statutory monitoring did not show the presence of *E. coli* at these sites. Another hypothesis is that the increase of *Bacteroidales* cell numbers in tap water was caused by the growth of *Bacteroidales* species in (drinking) water systems. In summary, it is unexpected that the majority of the tap water, raw water, and extracted groundwater samples were fecally contaminated. These unexpected observations raise the question of whether the PCR methods detect only fecal *Bacteroidales* species and, thus, if the gene assays are suitable to discern fecal contamination in drinking water in the Netherlands.

Sequence analyses of the *Bacteroidales* 16S rRNA genes were performed to determine the relatedness of sequences from the different sampling sites to sequences from the nearest relatives in the GenBank database. All sequences contained the primer regions, indicating that nonspecific amplification had not occurred in the PCRs. Because the PCR product from the AllBac primer set was small (108 bp), many 16S rRNA gene sequences (100 to 5,000) in the GenBank database were identical to the *Bacteroidales* 16S rRNA gene sequences obtained from groundwater and unchlorinated tap water samples from plant C. These identical 16S rRNA gene sequences were in general obtained from fecal sources, but some of them came from environmental rather than fecal sources (Table 2). The AllBac 16S rRNA gene sequences from tap water and groundwater had relative high similarities (96.3 to 100%) to sequences from bacterial species of the genera *Bacteroides*, *Prevotella*, and *Tannerella* (Table 2), which all belong to the order *Bacteroidales*.

16S rRNA gene sequences obtained with the TotBac primer set were longer (~370 bp) and did not show 100% similarity with the nearest relatives in the GenBank database (Table 2).

Sequences from the GenBank database that showed the highest similarity (91.6% to 99.7%) with the 16S rRNA gene sequences from tap water and groundwater from plant C were in general isolated from environmental sources (Table 2). The 16S rRNA gene sequences from cultivated bacterial species that showed the highest similarity to the 16S rRNA gene sequences obtained in our study belonged to different genera (Table 2). Some of these genera (*Salinimicrobium*, *Xanthobacillum*, and *Psychroserpens*) did not belong to the order *Bacteroidales*. However, the 16S rRNA gene sequences from bacterial species of these genera showed low similarities with the sequences obtained in this study (83.2% to 90.1%) and six mismatches to the TotBac primers. Thus, it is unlikely that DNA from bacterial species belonging to *Salinimicrobium*, *Xanthobacillum*, and *Psychroserpens* was amplified in the gene assay. More importantly, the majority of the nearest environmental clone sequences retrieved from the GenBank database showed no or a single mismatch with the AllBac and TotBac primer and probe sequences. Thus, these primer sets are capable of amplifying 16S rRNA genes from bacteria that have been observed in ecosystems outside the intestinal tract of humans and animals.

16S rRNA gene sequences related to *Prevotella* species were commonly observed in extracted groundwater, raw water, and tap water (Table 2). The isolation of *Prevotella paludivivens* from rice roots in a rice field soil (35) demonstrated the environmental nature of some *Prevotella* species. In addition, primer sequences developed for the detection of fecal *Bacteroidales* species (8, 12, 19, 20, 25, 29) showed no or a single mismatch with 16S rRNA gene sequences from *P. paludivivens*, *Xylanibacterium oryzae*, *Paludibacter propionicigenes*, *Proteiniphilum acetatigenes*, and *Petrimonas sulfuriphila* that are present in the GenBank database. These five *Bacteroidales* species have all been isolated from ecosystems other than the gastrointestinal tract. Consequently, primer sets for 16S rRNA genes of *Bacteroidales* species cannot always be used to discern fecal contamination in water.

A number of 16S rRNA gene sequences observed in groundwater and tap water fell in the genus *Bacteroides*. The presence of *Bacteroides* 16S rRNA gene sequences in groundwater and tap water might also suggest that some *Bacteroides* species are capable of growth in the environment. However, until now, type strains of *Bacteroides* species growing outside the animal intestinal tract have not been published. Another possible explanation is that the observed 16S rRNA gene sequences originate from *Bacteroides* species that inhabit the anoxic intestinal tract of insects. Previous studies have shown that bacterial species belonging to the genus *Bacteroides* are common inhabitants of the hindguts of insects (15, 23, 24, 28, 32). Some of the 16S rRNA gene sequences obtained with the AllBac primer set in our study showed 100% similarity to 16S rRNA gene sequences from the hindgut of insects. Moreover, a number of 16S rRNA gene sequences isolated from the hindguts of insects (15, 23, 24, 32) showed no or a single mismatch with the TotBac and AllBac primer and probe sequences. In conclusion, these primer sets are capable of detecting *Bacteroides* species from the hindgut of insects as well. Water insects are normal inhabitants of groundwater and drinking water distribution systems (7, 41) and might be a source of *Bacteroides* species in water. *Bacteroides* species from insect feces do not indicate fecal pollution by warm-blooded animals, and insects do not normally shed human fecal pathogenic microorganisms. *Bacteroides* species from insect feces, therefore, can hamper *Bacteroides* gene assays developed for the detection of water fecally contaminated by warm-blooded animals. Additional cultivation techniques in combination with molecular tools are required to demonstrate the persistence or growth of *Bacteroides* bacteria in groundwater and drinking water or whether *Bacteroides* bacteria are present in water insects. However, these experiments were beyond the scope of our study.

The three extraction wells of plant C are located close to each other and extract water from the same aquifer. Subsequently, extracted water from the three wells is mixed and enters the treatment plant as raw water. We hypothesize that if a fecal source in the vicinity of the extraction field of plant C contaminated the groundwater, water from the extraction wells and raw water should (partly) have the same *Bacteroidales* species. Although a relatively limited amount of clones was sequenced per sample (16), the diversity of *Bacteroidales* operational taxonomic units (OTU) within a sample was low (Table 2). In contrast, unique 16S rRNA gene sequences were observed between the different water types (e.g., extracted groundwater, raw water, and tap water) and sequence overlap between water types was low. These results demonstrate that the *Bacteroidales* 16S rRNA gene sequences at the sampling locations were not from the same fecal source and imply once again that *Bacteroidales* species were environmental rather than fecal.

Finally, we hypothesized that if the *Bacteroidales* species observed in tap water were of nonfecal origin, human- and/or bovine-specific *Bacteroidales* strains should not be present in tap water. We tested for the presence of human- or bovine-specific *Bacteroidales* strains by using source-specific 16S rRNA gene assays (5) on tap water samples from February 2010. The results showed that human- and bovine-specific *Bacteroidales* 16S rRNA genes could not be detected in tap water, whereas a PCR product was always detected with the positive control. Again, these results indicate that the *Bacteroidales* species observed in tap water were of nonfecal origin.

Overall, the results from our study indicate that gene assays for *Bacteroidales* detected environmental rather than fecal *Bacteroidales* species in groundwater and tap water from treatment plants in the Netherlands. First, *Bacteroidales* 16S rRNA gene sequences obtained from water samples taken at plant C showed (high) similarity to clone sequences that were isolated from environmental sources. The majority of these clone sequences and several *Bacteroides* clone sequences from the hindguts of insects showed no or a single mismatch with AllBac, TotBac, and BacUni primer and probe sequences. Second, the primer and probe sequences used for the gene assays have no or a single mismatch with 16S rRNA gene sequences of environmental *Bacteroidales* species *P. paludivivens*, *X. oryzae*, *P. propionicigenes*, *P. acetatigenes*, and/or *P. sulfuriphila* (9, 17, 35–37). Third, *Bacteroidales* 16S rRNA gene sequences from raw water and water from extraction wells were unique, and sequence overlap between water types was low. It is expected that in the case of fecal contamination of groundwater, different water types from the same groundwater area have similar *Bacteroidales* species. Fourth, the quantitative assays for *Bacteroidales* 16S rRNA genes commonly used to detect

fecal contamination (3, 4, 12, 16, 19, 20, 22, 33, 34) detected *Bacteroidales* species in deep groundwater and tap water that have no history of fecal contamination. Fifth, *Bacteroidales* gene copy numbers were significantly higher in tap water than in raw groundwater, demonstrating an increase or growth of *Bacteroidales* species during the treatment and/or distribution of drinking water. Finally, human- and bovine-specific *Bacteroidales* strains were not detected in tap water. Consequently, (quantitative) assays for general *Bacteroidales* 16S rRNA genes are not suitable to discern fecal contamination in groundwater and unchlorinated drinking water in the Netherlands.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences obtained in this study were deposited in the GenBank database under accession numbers GQ169588 to GQ169609.

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