Applied and Environmental Microbiology, Aug. 2007, p. 4857–4866 Vol. 73, No. 15
0099-2240/07/$08.00+0 doi:10.1128/AEM.03003-06

Evaluation of Two Library-Independent Microbial Source Tracking Methods To Identify Sources of Fecal Contamination in French Estuaries[▽]

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Received 29 December 2006/Accepted 23 May 2007

In order to identify the origin of the fecal contamination observed in French estuaries, two library-independent microbial source tracking (MST) methods were selected: (i) *Bacteroidales* host-specific 16S rRNA gene markers and (ii) F-specific RNA bacteriophage genotyping. The specificity of the *Bacteroidales* markers was evaluated on human and animal (bovine, pig, sheep, and bird) feces. Two human-specific markers (HF183 and HF134), one ruminant-specific marker (CF193′), and one pig-specific marker (PF163) showed a high level of specificity (>90%). However, the data suggest that the proposed ruminant-specific CF128 marker would be better described as an animal marker, as it was observed in all bovine and sheep feces and 96% of pig feces. F RNA bacteriophages were detected in only 21% of individual fecal samples tested, in 60% of pig slurries, but in all sewage samples. Most detected F RNA bacteriophages were from genotypes II and III in sewage samples and from genotypes I and IV in bovine, pig, and bird feces and from pig slurries. Both MST methods were applied to 28 water samples collected from three watersheds at different times. Classification of water samples as subject to human, animal, or mixed fecal contamination was more frequent when using *Bacteroidales* markers (82.1% of water samples) than by bacteriophage genotyping (50%). The ability to classify a water sample increased with increasing *Escherichia coli* or enterococcus concentration. For the samples that could be classified by bacteriophage genotyping, 78% agreed with the classification obtained from *Bacteroidales* markers.

Fecal pollution of coastal environments affects shellfish and recreational water quality and safety, in addition to causing economic losses from closure of shellfish harvesting areas and bathing restrictions (22, 30, 42, 62).

Due to difficulties in the identification of nonpoint sources and association of urban and agricultural activities in some watersheds, microbial source tracking (MST) methods are crucial for effective resource management and remediation. Many MST techniques have been developed to discriminate between human and nonhuman sources of fecal contamination and to distinguish contamination from different animal species (55, 58, 60). These methods can be divided into library-dependent (20, 34, 51, 61) or library-independent methods. Many library-dependent techniques require culture of a large number of bacterial strains and are time-consuming and labor-intensive. Furthermore, geographical differences (33, 55) and false positives (59) may be an issue. This study has thus focused on two library-independent methods. Comparisons of MST results using blind water samples demonstrated that no single technique can currently predict a source of fecal contamination (24, 32, 35, 47, 48, 49, 59).

Other published data sets generally have investigated only a limited number of locations and potential fecal sources (32). Using different MST methods at the same time was reported to be the best solution to properly identify the microbial sources in a specified area (9, 29).

Detection of host-specific 16S rRNA gene markers from *Bacteroidales* and genotyping of F-specific RNA bacteriophages were selected for this study. *Bacteroidales* markers, developed by Bernhard and Field (6, 7), require direct PCR amplification of 16S ribosomal gene fragments of the *Bacteroides-Prevotella* group. PCR primers targeting general *Bacteroidales* and host-specific *Bacteroidales* (human-, ruminant-, and pig-associated species) have been designed (7, 18). Detection of these markers has been proposed as an alternative and possible improved water quality indicator (25), as the group represents the main component of human and animal flora.

An alternative method is to detect F-specific RNA bacteriophages by plaque formation using an appropriate host followed by genotyping using specific probes (5, 37). Two probes are specific for bacteriophages associated with human fecal contamination (genotypes II and III represented by GA and Qβ, respectively), while genotype I (represented by MS2) is mostly associated with animal contamination and genotype IV (SP) is characteristic of animal contamination (16). F-specific RNA bacteriophages were originally proposed as indicators of enteric viruses in environmental samples (15, 21, 38) but are not routinely used, as the presence of the two viral groups does not always correlate (31, 41, 46).

For MST, both microorganisms could represent reliable fecal contamination markers because high concentrations of human-specific *Bacteroidales* markers and F-specific RNA bacteriophages can be found in domestic raw or treated sewage: 5.9×10^8 to 3.1×10^9 per 100 ml (56) and 1×10^2 to 1×10^6 PFU/100 ml (14, 16), respectively. However, the two groups of

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▽ Published ahead of print on 8 June 2007.

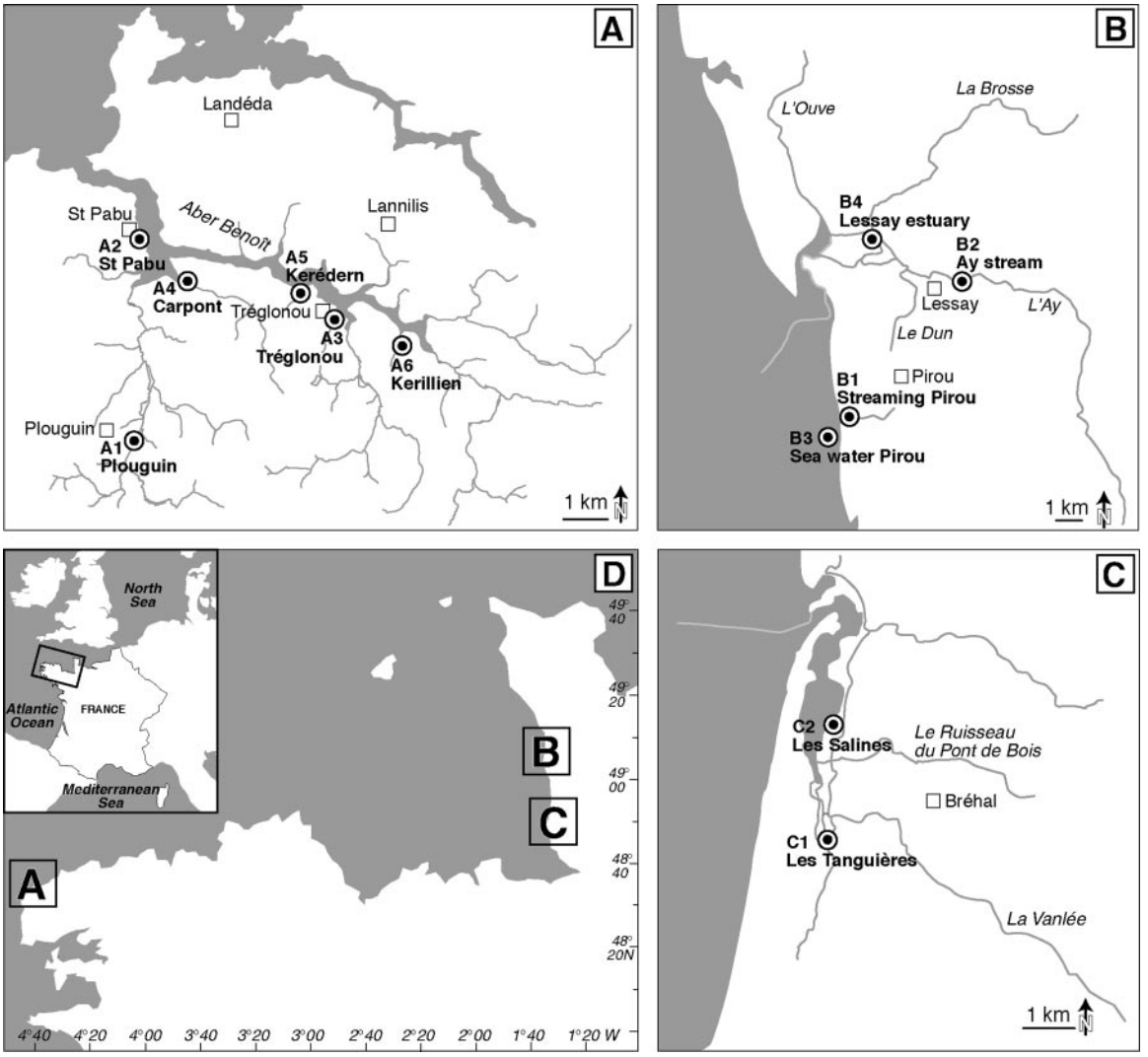

FIG. 1. Location of water sampling sites. (A) Aber Benoît estuary (Britanny) with sampling points A1 to A6; (B) Havre de l'Ay (Normandy) with sampling points B1 to B4; (C) Havre de la Vanlée (Normandy) with sampling points C1 and C2. Small squares, locations of towns; circles, sampling points. (D) Location of studied sites on a map of France.

microorganisms do not necessarily behave in the same way in the environment. Members of the *Bacteroidales* have high concentrations in human and animal feces (e.g., about 10^9 to 10^{11} per gram of human feces) and do not maintain culturability for long once released into fresh or marine waters due to their low oxygen tolerance (1, 17, 25). The fate of these bacteria in the environment is still poorly understood though, as with other enteric bacteria, cells will be affected by both biotic and abiotic factors (52). The persistence is longer for *Bacteroidales* 16S rRNA genetic markers than for culturable *Bacteroidales*, as the former considers dead and living bacteria. Cell fate also depends on environmental factors, such as water temperature or predation (40, 56). Furthermore, *Bacteroidales* PCR markers have been shown to persist in a similar way to an *Escherichia coli* PCR marker and for longer than the culturable *E. coli* in Lake Michigan (United States) waters (12). F-specific RNA bacteriophages are not frequently found in human and animal feces (14, 41), but they are detected more often in wastewater samples and slurry lagoon samples (10, 15, 41, 44). As enteric viruses, F-specific bacteriophages are sensitive to various environmental factors, such as sunlight or temperature (50). Moreover, genotypes I and II are the most frequently detected in the environment (13, 16) and show the highest persistence (13, 43, 54).

Three French estuaries, with commercial and recreational shellfish harvesting and bathing areas and subject to potential fecal contamination, were investigated in this study (Fig. 1). The Aber Benoît estuary, located on the northern coast of Brittany (English Channel, France [Fig. 1A]) has a watershed of 224 km^2 containing urban areas and intensive livestock farming (dairy cows [26,700], pigs [225,500], and poultry [803,900]). The total number of inhabitants in the watershed is about 25,000. The two other sites were located on the western coast of Normandy (English Channel, France [Fig. 1B and C]). The watershed from Havre de l'Ay (Fig. 1B) encompasses 211 km^2 with approximately 7,000 permanent inhabitants and ap-

proximately twice this in summer. Near the sea, about 300 ewes (and their lambs in spring) graze on salt meadows. Sheep and cattle (about 3,000) farming also occurs upstream. About 5,800 permanent inhabitants (10,000 to 15,000 in summertime) live in the third watershed of Havre de la Vanlée (60 km^2) (Fig. 1C). Agricultural activity includes sheep farming, with approximately 3,500 cows and 1,400 ewes on salt meadows.

These sites contain shellfish-farming areas which have been classified as B category by European legislation (European Directive 91/492/EEC [2]). The bathing areas are of good or sufficient quality (European Directive 2006/7/EEC). This means that the shellfish have been found to contain between 230 and 4,600 *E. coli* per 100 g of total flesh and must be depurated for ≥48 h in good quality water prior to sale. To be classified as of sufficient quality, the bathing waters have been assessed as having 90% values for microbiological numbers of ≤500 CFU per 100 ml.

The aim of this study was to optimize and validate the two MST techniques on human and animal feces, sewage treatment plant (STP) sludge, wastewater samples, and pig liquid manure (PLM; pig slurry) collected in Brittany and Normandy (France). Both techniques were then applied to water samples collected at different times from each estuary.

MATERIALS AND METHODS

Water sampling sites. Water samples were collected as shown in Fig. 1. In the Aber Benoît area, six water points were selected (Fig. 1A). A stormwater drain (Saint-Pabu [Fig. 1A, A2]) and two streams (Plouguin [A1] and Tréglonou [A3]), all downstream from different small towns were sampled. Sampling point A1 was in a river which crossed pasture land grazed by cows and was also close to an STP outlet. Subsequently, one river (Carpont [A4]) downstream of a watershed with mainly agricultural activities with scattered houses and two streams (Keredern [A5] and Kerilien [A6]) in rural areas were selected. Average flow rates were approximately 5 liters/s (Keredern [A5] and Kerilien [A6]) or approximately 100 liters/s (Carpont [A4]). Samples were taken three times: 13 April, 25 June, and 13 October 2005.

Water samples from Normandy were collected from two sites on the western coast of Normandy (Fig. 1B and C). For the first area (Havre de l'Ay [Fig. 1B]), samples were collected in four places. The stream at Pirou, B1, represented an urban area and the stream l'Ay (B2) an agricultural area, while the two other points (seawater from Pirou [B3] and Lessay estuary [B4]) both had agricultural and urban influences. For the second area (Havre de la Vanlée [Fig. 1C]), samples were collected at high and low tides in two places in the estuary (Les Tanguières [C1] and Les Salines [C2]). Both were under both agricultural (cow and sheep breeding) and urban influences. Water samples were taken on 19 September and 16 November 2005.

Collection of samples of known origin. Individual human and animal fecal samples were collected from April 2004 to September 2005. Human feces were obtained from 44 healthy adult and child volunteers from Brittany and Normandy (France). Fresh feces were collected immediately after excretion from apparently healthy animals (43 cows, 12 sheep, 26 pigs, and 10 chickens) using sterile containers. Bovine feces from animals kept at pasture or housed in stalls, including beef and dairy cattle, adults and heifers from more than five independent farms in Brittany and Normandy, were sampled. Pig feces were collected from sows and male adults, young pigs, and piglets mainly housed in stalls from seven farms in Brittany. All chicken samples were from the same farm and collected in Brittany. Samples from sheep were collected from one farm in Brittany and from salt meadows from two farms in Normandy. Three or more different animals were usually analyzed per farm. Fresh feces from seagulls and from other seabirds were collected from two beaches in Brittany and in Normandy. Wild duck feces were collected from Brittany. PLM samples were collected from five outside storage tanks, on independent farms, and sludge was sampled from a domestic sewage treatment plant in the Aber Benoît watershed (Brittany). Water samples (28 environmental and 5 STP water samples) were collected in sterile 1-liter containers and transported in insulated coolers to the laboratory. Sewage treatment plant waters were from four different plants from the two Normandy sites.

All fecal, STP sludge, and PLM samples were placed in sterile containers and analyzed upon arrival at the laboratory for bacteriophages. Material for *Bacteroidales* 16S rRNA gene marker detection was stored at −20°C.

Detection of general and host-specific *Bacteroidales* 16S rRNA gene markers. DNA was extracted from 250 mg (wet weight) of feces using the Fast DNA spin kit for soil (MP Biomedical, Illkirsh, France), according to the supplier's instructions, except that an additional wash using the SEWS-M reagent was performed as suggested by Dick and Field (18). For water samples, duplicate samples of 100 ml were filtered through 0.45-μm membrane filters (HAWP04700; Millipore, St. Quentin en Yvelines, France). Filters were then placed in 0.5 ml of GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH 8.0], 0.5% Sarkosyl) (18) and frozen at −20°C in this lysis buffer until extraction. Afterwards, DNA was extracted using the DNeasy tissue kit (QIAGEN, Courtaboeuf, France) with two modifications: the proteinase K step was omitted, and 700 μl of QIAGEN AL buffer was added to the filters placed in GITC buffer.

Detection of 16S rRNA gene markers by PCR was performed with the primers previously described (6, 8, 19). Forward primers Bac32F, HF183F, CF128F, CF193′F, and PF163F were paired with the *Bacteroidales* reverse primer Bac708R, and the forward HF134F primer was paired with HF654R. The sequence of the CF193′F primer was different from that of the CF193F primer given by Bernhard and Field (6), as the last nucleotide was changed from a C to a G, according to the nucleotide sequences deposited by these authors in the GenBank database. Two microliters of template DNA was added to a reaction mixture containing 4 μl of 10× PCR buffer (Roche Diagnostics, Meylan, France), 200 μM of each deoxynucleotide triphosphate, from 0.2 to 0.8 μM of each primer (depending on the primer set used; Bac32F/Bac708R, HF183F/Bac708R, HF134F/HF654R, and CF128F/Bac708R at 0.5 μM; CF193′F/Bac708R at 0.2 μM; PF163F/Bac708R at 0.8 μM), 0.5 mM additional $MgCl_2$, and 1 U *Taq* DNA polymerase (Roche Diagnostics) for a final volume of 40 μl. The amplifications were performed in a Peltier thermal cycler (PTC 200; MJ Research, Waltham, MA) with the following cycle conditions: initial denaturation at 94°C for 5 min and 40 cycles of denaturation at 94°C for 30 s, primer annealing at 61°C (or at 63°C for Bac32F/Bac708R and PF163F/Bac708R primer sets) for 30 s, and elongation at 72°C for 1 min, followed by a 5-min extension at 72°C and holding at 4°C. Positive (DNA from feces from the target source previously found positive) and negative (DNA template is replaced by water) controls were included in each experiment. The presence/absence of PCR inhibitors was controlled for each sample by adding DNA from a positive control to the tested sample. An amplification resulted in the absence of inhibitors. If inhibitors were present, the sample was diluted (10-fold dilutions). Each PCR assay was carried out in triplicate. Amplified products (15 μl) were visualized after electrophoresis through 1.5% (wt/vol) agarose gels stained with ethidium bromide.

Detection of F-specific RNA bacteriophages. For sample preparation, 5 g of sludge samples was eluted in 45 ml of phosphate buffer (145 mM NaCl, 7.7 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, 15% glycerol, and 0.2% Tween). The whole suspension was mixed for 1 h 30 min at 4°C. For feces, 5 g was homogenized in 45 ml of phosphate buffer using a Polytron (Kinematica AG, Luzern, Switzerland; high speed, 15 s, twice). The homogenate was then centrifuged at 5,000 × *g* for 20 min at 4°C. The supernatant was recovered for analysis.

For water and liquid manure samples, F-specific RNA bacteriophages were counted according to the ISO 10705-1 method (39). For less contaminated samples, a concentration step was necessary and was performed according to the methods of Mendez et al. (45). In this case, a 300-ml water sample was shaken for 15 min in the presence of 0.05 M of $MgCl_2$ at room temperature and filtered through 47-mm 0.22-μm-diameter membrane filters (GSWP047S0; Millipore, St. Quentin en Yvelines, France). The filter was cut in small pieces and then placed in 5 ml of eluent (1% beef extract, 3% [vol/vol] Tween 80, 0.5 M NaCl solution, pH 9). After vortexing and sonicating for 4 min, the filter pieces were removed and the eluate analyzed. Plaques were transferred onto nylon membranes, and denaturation and neutralization steps were performed as described by Schaper and Jofre (53). For samples with low bacteriophage concentrations, single plaques were transferred individually using the protocol described by Long and Sobsey (43) and studied individually. Hybridizations were performed using oligonucleotide probes labeled using the Dig oligonucleotide tailing kit (Roche Diagnostics) to determine plaque numbers for each genotype (I, II, III, and IV) of F-specific RNA bacteriophages (5). Probe-target hybrids were detected using the chemiluminescence assay with CDP-Star as enzyme substrate (Roche Diagnostics). For a consistent interpretation, only results with at least 20 plaques that were successfully genotyped were considered.

Enumeration of fecal indicator organisms. *E. coli* and enterococci were enumerated by microplate methods (EN ISO 9308-3 [3] and EN ISO 7899-1 [4]) with a detection limit of <15 most probable number (MPN) per 100-ml water sample.

TABLE 1. Detection of general *Bacteroidales* and host-specific markers in individual feces of known human or animal origin

Source of individual fecal samples	No. of samples	No. of samples with indicated result[a] with the following primers:					
		General Bac32F/ Bac708R	Human HF183F/ Bac708R	Human HF134F/ HF654R	Ruminant CF128F/ Bac708R	Ruminant CF193′F/Bac708R	Pig PF163F/Bac708R
Human	44	44 +	38 +, 5 +/−, 1 −	34 +, 3 +/−, 7 −	41 −, 2 +/−, 1 +	44 −	44 −
Pig	25	25 +	25 −	25 −	17 +, 7 +/−, 1 −	25 −	25 +
Cow	32	32 +	28 −, 4 +/−	32 −	32 +	30 +, 2 +/−	32 −
Sheep	12	12 +	12 −	12 −	12 +	8 +, 2 +/−, 2 −	12 −
Chicken	10	10 +	1 +, 9 −	1 +/−, 9 −	0 +, 10 −	0 +, 10 −	1 +, 1 +/−, 8 −
Wild bird	13	4 +, 3 +/−, 6 −	7 −	7 −	7 −	7 −	7 −

[a] +, positive signal; −, negative signal; +/−, weak positive result or both positive and negative results were obtained in three replicate analyses.

Statistical analyses. All statistical analyses were performed using STATISTICA version 6.1 (StatSoft; France) and XLSTAT (Addinsoft; France). To evaluate the performance of MST methods on fecal samples, sensitivity (r) and specificity (s) were defined as follows: $r = a/(a + c)$ and $s = d/(b + d)$, where a is the frequency that a fecal sample was positive for the marker of its own species (true positive), b is the frequency that a fecal sample was positive for a marker of another species (false positive), c is the frequency that a fecal sample was negative for a marker of its own species (false negative), and d is the frequency that a fecal sample was negative for a marker of another species (true negative) (26). Fisher's exact test was used to verify if the observed differences in the frequency of detection of the various markers in their target and nontarget fecal samples were significantly different. The significance level was set at 0.05. Binary logistic regressions were also performed to test whether the presence/absence of the marker in water samples correlated with indicator bacteria concentrations (expressed as $\log_{10}$ *E. coli*/100 ml). The significance level was again set at 0.05.

RESULTS

Detection of general and host-specific *Bacteroidales* in feces. In total, 136 fresh feces from 44 humans, 25 pigs, 32 cows, 12 sheep, 10 chickens, and 13 seabirds and ducks were collected and analyzed with the different primer sets (Table 1). Ninety-five percent of individual feces tested were positive with the general *Bacteroidales* Bac32F/Bac708R primer set (only six seabird feces were found negative) (Table 1). Human-specific *Bacteroidales* markers were present in 43 and 37 human feces when using the two human marker sets HF183F/Bac708R and HF134F/HF654R, respectively. Ruminant-specific *Bacteroidales* markers were present in 44 and 42 ruminant (cow and sheep) feces when using CF128F/Bac708R and CF193′F/Bac708R primer sets, respectively. The pig-specific *Bacteroidales* marker was detected in all 25 pig feces tested (Table 1).

Detection and genotyping of F-specific RNA bacteriophages in feces. F-specific bacteriophages were researched in 76 animal feces and detected in only 3 cow, 11 pig, and 2 bird feces with highly variable concentrations (1 to 5.5×10^5 PFU per g of feces) (Table 2). Genotypes I and IV were detected in pig feces with sufficient bacteriophage concentrations, and only genotype I was detected in cow and duck feces.

MST application on STP sludge, wastewater, and PLM samples. All 10 PLM, 6 STP sludge, and 5 STP water samples were found positive for general *Bacteroidales* markers (Table 3). F-specific RNA bacteriophages were isolated from all STP sludge and water samples and from 60% of the PLM samples (Table 3).

Positive results were found for all PLM samples with the *Bacteroidales* pig-specific primer set, and negative results were obtained with HF183F/Bac708R, HF134F/Bac708R, and CF193′F/Bac708R *Bacteroidales* species primer sets (Table 3). A weak positive result was obtained for 1 of the 10 PLM samples with the CF128F/Bac708R primer set (Table 3). Only 4 of the 10 PLM samples contained enough phage plaques for typing, and their genotyping showed the presence of genotype I or IV (Table 3).

For the STP sludge samples (Table 3), positive results were obtained with both human-specific *Bacteroidales* primer sets in all samples, whereas all other host-specific *Bacteroidales* primer sets were negative. F-specific RNA bacteriophages were detected in high numbers in all sludge samples ($>3 \times 10^4$ PFU per 100 g) coming from genotype II for five of six samples (data not shown) and genotypes II and III for the last sample (STP-S5).

In the STP water samples, the two human-specific primer sets and the CF128F/Bac708R ruminant-specific *Bacteroidales* primer set gave positive results with all samples, whereas the pig-specific *Bacteroidales* primer and ruminant-specific CF193′F/Bac708R *Bacteroidales* primer sets were negative (Table 3). F-specific RNA bacteriophages were also detected in high numbers (2.4×10^5 to 5.9×10^6 PFU per 100 ml) and belonged to genotypes II and III in all samples.

TABLE 2. Enumeration and genotyping of F-specific RNA bacteriophages from animal (cow, sheep, pig, and wild bird) feces

Origin of fecal samples	Total no. of samples	No. of positive samples	Range of F-specific RNA bacteriophage concn (PFU/g of feces)[a]	Identification (%) of genotype[b]			
				I (MS2)	II (GA)	III (Qβ)	IV (SP)
Cow	29	3	<1–1.6×10^5	+ (46–57)	ND	ND	ND
Sheep	7	0	<1	NT	NT	NT	NT
Pig	27	11	<1–5.5×10^5	+ (25–100)	ND	ND	+ (25–100)
Seabird	12	1	<1–10	NT	NT	NT	NT
Duck	1	1	1×10^3	+ (100)	− (0)	− (0)	− (0)

[a] Detection limit, 1 PFU/g of feces.
[b] ND, not detected; NT, not tested because of the insufficient number of phage isolated.

TABLE 3. Results of *Bacteroidales* and F-specific RNA bacteriophage source tracking methods on PLM and STP sludge and water samples

Sample[a]	Result with *Bacteroidales*-specific marker[b]						Result with F-specific RNA bacteriophages[c]				
	General Bac32F/ Bac708R	Human HF183F/ Bac708R	Human HF134F/ HF654R	Ruminant CF128F/ Bac708R	Ruminant CF193F′/ Bac708R	Pig PF163F/ Bac708R	PFU/100 g or PFU/100 ml	% with animal genotype		% with human genotype II + III, GA + Qβ	No. of plaques genotyped
								I, MS2	IV, SP		
PLM 1	+	−	−	−	−	+	1.1×10^3	0	100	0	110
PLM 2	+	−	−	−	−	+	450	0	80	0	45
PLM 3	+	−	−	−	−	+/−	<10	NT	NT	NT	0
PLM 4	+	−	−	−	−	+/−	<10	NT	NT	NT	0
PLM 5	+	−	−	+/−	−	+	<10	NT	NT	NT	0
PLM 6	+	−	−	−	−	+/−	<10	NT	NT	NT	0
PLM 7	+	−	−	−	−	+	9.8×10^3	97	0	0	98
PLM 8	+	−	−	−	−	+	8.4×10^3	99	0	0	84
PLM 9	+	−	−	−	−	+	50	NT	NT	NT	0
PLM 10	+	−	−	−	−	+	50	NT	NT	NT	0
STP-S1	+	+	+	−	−	−	9.4×10^4	0	0	28	137
STP-S2	+	+	+	−	−	−	7.7×10^4	0	0	41	110
STP-S3	+	+/−	+	−	−	−	3.6×10^4	0	0	81	81
STP-S4	+	+	+	−	−	−	3.3×10^4	0	0	70	95
STP-S5	+	+	+	−	−	−	1.3×10^5	0	0	70	84
STP-S6	+	+	+	−	−	−	4.3×10^4	0	0	26	82
STP-W1	+	+	+	+	−	−	5.7×10^5	0	0	77	137
STP-W2	+	+	+	+/−	−	−	2.5×10^5	0	0	61	110
STP-W3	+	+/−	+	+/−	−	−	8.5×10^5	0	0	53	81
STP-W4	+	+	+	+	−	−	2.4×10^5	0	0	53	95
STP-W5	+	+	+	+/−	−	−	5.9×10^6	0	0	70	82

[a] STP-S, STP sludge sample; STP-W, STP water sample.
[b] +, positive signal; −, negative signal; +/−, weak positive result or both positive and negative results were obtained in three repeated experiments.
[c] NT, not tested because insufficient phage was isolated.

Performance of the *Bacteroidales* 16S rRNA gene and F-specific bacteriophages as markers for human or animal feces. The sensitivities of the human-specific *Bacteroidales* markers were 98% and 84% in human feces and the specificities were 94% and 99% when using HF183F/Bac708R and HF134F/HF654R, respectively (Table 4). The sensitivities of ruminant-specific *Bacteroidales* markers were 100% in bovine fecal samples and 100% and 83% in sheep feces using CF128F/Bac708R and CF193′F/Bac708R primer sets, respectively. Although the average specificity of the CF128F/Bac708R marker was correct (69%), its performance in terms of specificity differed significantly between nonruminant fecal samples (human, 93%; pig, 4%; birds, 100%). A specificity of 100% was obtained with the second ruminant primer set (CF193′F/Bac708R). The sensitiv-

TABLE 4. Performance statistics for tests in which detection of *Bacteroidales* host-specific markers and genotyping of F-specific RNA bacteriophages were tested against individual fecal samples and waste effluents for ability or failure to detect the origin of fecal contamination

Test description[a]	Target	Host category	Sample type[b]	% Sensitivity (no. of samples)	% Specificity (no. of samples)
Bacteroidales (PCR)	HF183F/Bac708R	Human	Individual feces	98 (44)	94 (86)
Bacteroidales (PCR)	HF183F/Bac708R	Human	Waste effluents	100 (11)	100 (10)
Bacteroidales (PCR)	HF134F/HF654R	Human	Individual feces	84 (44)	99 (86)
Bacteroidales (PCR)	HF134F/HF654R	Human	Waste effluents	100 (11)	100 (10)
Bacteroidales (PCR)	CF128F/Bac708R	Ruminants	Individual feces	100 (44)	69 (86)
Bacteroidales (PCR)	CF128F/Bac708R	Ruminants	Waste effluents	NT[c]	71 (21)
Bacteroidales (PCR)	CF193′F/Bac708R	Ruminants	Individual feces	95 (44)	100 (86)
Bacteroidales (PCR)	CF193′F/Bac708R	Ruminants	Waste effluents	NT	100 (21)
Bacteroidales (PCR)	PF163F/Bac708R	Pigs	Individual feces	100 (25)	98 (105)
Bacteroidales (PCR)	PF163F/Bac708R	Pigs	Waste effluents	100 (10)	100 (11)
F-specific RNA bacteriophage (genotyping)	Genotypes II + III	Human	Individual feces	NT	100 (76)
F-specific RNA bacteriophage (genotyping)	Genotypes II + III	Human	Waste effluents	100 (11)	100 (10)
F-specific RNA bacteriophage (genotyping)	Genotypes I + IV	Animal	Individual feces	21 (76)	NT
F-specific RNA bacteriophage (genotyping)	Genotypes I + IV	Animal	Waste effluents	40 (10)	100 (11)

[a] All samples tested by host-specific *Bacteroidales* were previously found positive with the general *Bacteroidales* Bac32F/Bac708R primer set.
[b] Waste effluents included sewage treatment plant waters and sludges and pig slurries.
[c] NT, not tested.

ity and specificity of the pig-specific *Bacteroidales* marker were 100% and 98%, respectively (Table 4). The observed differences in the frequency of detection of all host-specific *Bacteroidales* markers in their target and their nontarget fecal samples were significantly different ($P < 0.001$). The sensitivity of detection of F-specific RNA bacteriophages in animal feces was 21% (Table 4).

MST application to environmental water samples. The general *Bacteroidales* primer set Bac32F/Bac708R allowed amplification of PCR products from all water samples, except one sample from Ay stream (September 2005) (Table 5). This sample showed only a low level of fecal contamination (<500 MPN of indicator organisms per 100 ml).

At least one host-specific *Bacteroidales* marker was detected in 82% of water samples (Table 6). Host-specific markers were found in 91% of the 23 samples for which one of the fecal indicator concentrations (*E. coli* or enterococci) was above 500 per 100 ml. Also, markers were found in all samples contaminated with more than 3×10^3 fecal indicators per 100 ml (Table 5). Human *Bacteroidales* markers HF183F/Bac708R and HF134F/Bac708R were detected in 42.9% of water samples and in 80% of samples from areas mainly downstream of urban areas.

For the animal-specific *Bacteroidales* markers, ruminant-specific primer sets CF128F/Bac708R and CF193′F/Bac708R gave positive results in 60.7% of water samples: 72.7% of samples preclassified as from rural areas and 71.4% of samples preclassified as mixed (under rural and urban influences) areas. With the pig-specific primer set PF163F/Bac708R, only two water samples were positive with weak PCR signals. Human- and animal-specific markers were both found in 21.4% of water samples (Table 5).

F-specific RNA bacteriophages were detected in 71% of water samples, and genotyping was performed in 50% of samples (Table 5). Genotypes II and III were detected in 64% of these water samples, mainly in areas downstream of urban activities: Plouguin (2/3), Saint Pabu (3/3), and Tréglonou (2/3). Genotype I was detected only in three sampling sites with sufficient phage concentrations: Plouguin in a high proportion (73.5%) at the last sampling date (October 2005) and Les Tanguières and Les Salines (>65%). No bacteriophage from genotype IV was detected in any of the water samples (Table 5).

When present, genotype I represented a high proportion of the total bacteriophages (50 to 100% of hybridized bacteriophages) except in the case of the sample from Lessay estuary (22%), where high levels of genotypes II and III (78%) suggested a predominant human impact in this area.

Fecal indicator (*E. coli* and enterococci) concentrations varied with sampling point and date (Table 5). Some sample sites contained low numbers of indicator bacteria ($<2 \times 10^3$ MPN/100 ml for *E. coli* from the Kerilien, Keredern, and Ay streams and seawater from Pirou), whereas others were more heavily polluted (mainly from 2×10^3 to 1.3×10^5 MPN/100 ml for *E. coli*, at Plouguin, St. Pabu, Carpont, and Tréglonou, Lessay estuary, Les Tanguières, and Les Salines). The detection of host-specific *Bacteroidales* markers and human or animal bacteriophages increased with the *E. coli* concentration (Table 6). Significant correlations were obtained between *E. coli* concentrations and the presence/absence of at least one host-specific marker *Bacteroidales* ($P = 0.002$), one human host-specific *Bacteroidales* ($P < 0.001$), F RNA bacteriophages ($P = 0.01$), and human F RNA bacteriophages ($P = 0.015$), whereas no significant correlations between *E. coli* concentrations and the presence/absence of ruminant host-specific *Bacteroidales* (CF128, $P = 0.85$; CF193, $P = 0.75$) or animal F RNA bacteriophages ($P = 0.15$) were found. While host-specific *Bacteroidales* markers were detected in some water samples with an *E. coli* concentration of <500 MPN per 100 ml (40%; $n = 5$), no F-specific RNA bacteriophages were detected in water samples with an *E. coli* concentration of $<2 \times 10^3$ MPN per 100 ml (Table 6).

DISCUSSION

This study aimed to select reliable fecal indicators to identify origins of fecal contamination in France. Two methods were used: detection of 16S rRNA gene markers from *Bacteroides-Prevotella* and F-specific RNA bacteriophage genotyping. The methods were initially evaluated on defined samples (feces, STP, and agricultural effluents) and then further tested on environmental samples.

The first step in investigating the 16S rRNA gene markers was to test for the general *Bacteroidales* marker. This marker was found to be a reliable indicator of fecal contamination and was observed in 95% of fecal samples, in all STP and PLM samples, and in 96.4% of environmental water samples (with *E. coli* concentrations from 80 to 1×10^5 MPN per 100 ml). When this marker was absent, or gave a weak signal only (3/28 of environmental water samples), no host-specific *Bacteroidales* were detected. Absence or weak signals from *Bacteroidales* markers in wild bird samples have been observed previously (27) and could be explained by the variability of the fecal flora of these animals.

Data for the host-specific *Bacteroidales* markers revealed that human-specific markers could be identified correctly by both primer pairs tested. However, HF183 was found to be more sensitive than HF134. This was also noted by Bernhard and Field (6).

The pig-specific marker, identified by PF163, was detected in all pig feces and liquid manure tested. A ruminant pollution source could be identified by the CF128 and/or CF193′ markers. The CF193′ marker was present more frequently in bovine feces than in ovine feces, and the CF128 marker was found in all bovine feces, all sheep feces, and in 96% of pig feces. Positive results on pig feces from sows and male adults, young pigs, and piglets mainly housed in stalls from different farms by using the CF128F/Bac708R primers were reported in a recent study of other European countries (28). The detection of a signal with both CF128 and CF193′ markers or with CF128 alone could thus indicate contamination from bovine, sheep, and/or pigs, while the presence of CF193′ would suggest pollution of sheep or bovine origin. However, in the case of contamination from pigs, both CF128 and PF163 markers should be detected. These data support the identification of CF128 as an animal marker rather than as a ruminant marker, as previously suggested by Bernhard and Field (6). The primer set CF128F/Bac70R also amplified a fragment of the expected size in STP samples (45%) and in a few human fecal samples. It should be noted that these wastewater samples were col-

TABLE 5. *Bacteroidales* markers and F-specific RNA bacteriophage genotyping results from water samples taken from three sites in France

Site no. (name)	Sample date (mo/day/yr)	*E. coli* concn (100 ml^{-1})	Enterococcal concn (100 ml^{-1})	Result with *Bacteroidales* marker[b]						Result with F-specific RNA bacteriophages				
				General Bac32F/ Bac708R	Human HF183F/ Bac708R	Human HF134F/ HF654R	Ruminant CF128F/ Bac708R	Ruminant CF193F′/ Bac708R	Pig PF163F/ Bac708R	PFU/100 ml	% with animal genotype		% with human genotypes II + III, GA + Qβ	No. of plaques genotyped
											I, MS2	IV, SP		
A1 (Plouguin)	04/13/05	8.2×10^4	3.8×10^4	+	+	+	−	+/−	−	4.2×10^3	0	0	51	51
	07/25/05	2.4×10^3	5.3×10^2	+/−	−	−	−	−	−	2.3×10^2	0	0	73.5	53
	10/13/05	4.6×10^4	ND[a]	+	+	−	−	−	−	1.6×10^5	73.5	0	0	166
A2 (St. Pabu)	04/13/05	1.3×10^5	9.8×10^3	+	+	−	+/−	+/−	+/−	3.5×10^4	0	0	>92	348
	07/25/05	2.4×10^4	7.8×10^2	+	+	−	−	−	−	4.1×10^3	0	0	27	44
	10/13/05	1.3×10^4	ND	+	+	−	−	−	−	7.1×10^5	0	0	74	110
A3 (Tréglonou)	04/13/05	7.2×10^4	6.9×10^3	+	+	+/−	−	−	−	8.7×10^2	0	0	82	13
	07/25/05	9.6×10^4	4.4×10^3	+	+	+/−	−	−	−	9.3×10^3	0	0	71	98
	10/13/05	1.1×10^5	ND	+	+/-	−	+/−	−	−	3.7×10^2	0	0	86	29
A4 (Carpont)	04/13/05	1.4×10^5	9.3×10^4	+	+/-	−	+/−	+/−	−	10	NT[c]	NT	NT	0
	07/25/05	7.2×10^2	1.5×10^2	+	−	−	−	−	−	10	0	0	100	16
	10/13/05	2.1×10^2	ND	+	+/-	−	+/−	+/−	−	40	0	0	83.5	11
A5 (Keredern)	04/13/05	4.3×10^3	1.7×10^3	+	−	−	−	+/−	+/−	2×10^2	NT	NT	NT	0
	07/25/05	1.1×10^3	3.4×10^2	+	−	−	+	+/−	−	10	100	0	0	1
	10/13/05	8.3×10^3	ND	+	+/-	−	−	−	−	1.1×10^2	0	0	100	1
A6 (Kerilien)	04/13/05	8.9×10^2	3.0×10^2	+	−	−	+/−	+/−	−	<10	NT	NT	NT	0
	07/25/05	1.6×10^3	6.4×10^2	+	−	−	+/−	+/−	−	10	50	0	0	2
	10/13/05	4.6×10^3	ND	+	+	−	+/−	−	−	3.7×10^2	0	0	17	52
B1 (streaming Pirou)	11/16/05	2×10^2	80	+	−	−	+/-	−	−	100	NT	NT	NT	0
B2 (Ay stream)	09/19/05	4.7×10^2	40	−	−	−	−	−	−	<10	NT	NT	NT	0
	11/16/05	1.2×10^3	2.9×10^2	+	−	−	+/−	−	−	40	NT	NT	NT	0
B3 (seawater Pirou)	09/19/05	1.9×10^2	1.9×10^2	+	−	−	-	−	−	<10	NT	NT	NT	0
	11/16/05	80	80	+/−	−	−	−	−	−	10	NT	NT	NT	0
B4 (Lessay estuary)	09/19/05	1.6×10^4	1.7×10^3	+	−	−	+	−	−	1.3×10^2	22	0	78	23
C1 (Les Tanguières)	09/19/05	3.4×10^4	3×10^3	+	−	−	+/−	−	−	3.7×10^3	65	0	0	176
	11/16/05	3.1×10^3	8.3×10^2	+	−	−	+/−	−	−	3.5×10^3	90	0	0	41
C2 (Les Salines)	09/19/05	4×10^3	3×10^3	+	−	−	+/−	−	−	2.3×10^2	72	0	0	102
	11/16/05	2.2×10^3	5.7×10^2	+	−	−	+	−	−	4.6×10^2	100	0	0	86

[a] ND, not done.
[b] +, positive signal; −, negative signal; +/−, weak positive result or both positive and negative results were obtained in three repeated experiments.
[c] NT, not tested because insufficient phage was isolated.

TABLE 6. Detection of host-specific *Bacteroidales* and F-specific bacteriophages in environmental water in conjunction with numbers of *E. coli*

E. coli concn (100 ml)	No. of water samples[a]	No. (%) of samples with host-specific *Bacteroidales* markers		No. (%) of samples with human and animal F-specific RNA bacteriophages	
		Absent[b]	Present[c]	Absent[b]	Present[c]
<500	5	3 (60)	2 (40)	5 (100)	0 (0)
500–2×10^3	5	1 (20)	4 (80)	5 (100)	0 (0)
2×10^3–1.5×10^4	7	1 (14.3)	6 (85.7)	2 (28.5)	5 (71.5)
>1.5×10^4	11	0 (0)	11 (100)	2 (18)	9 (82)

[a] A total of 28 environmental water samples were analyzed.
[b] Absence of host-specific markers, presumed unable to classify by MST.
[c] Presence of host-specific markers, presumed classified as agricultural/urban pollution by MST.

lected from STP in agricultural areas and, consequently, the positive detection of the CF128F marker could be due to (i) the presence of *Bacteroidales* from cow or sheep manure runoff after rain or (ii) the presence of the CF128 marker in human populations (6.8% of human feces were positive with CF128F/Bac708R in this study, and Bower et al. [12] noted a positive signal for an STP studied in an agricultural watershed). These findings clearly demonstrate that it is important to use the three animal-specific *Bacteroidales* primer sets on each sample, rather than only CF128F/Bac708R.

The F-specific RNA bacteriophage data showed low concentrations of phages in animal feces (21%) but more frequent detection in PLM (60%) and good detection in STP (100%). These results confirm that bacteriophages are more suitable to evaluate contamination at the level of the population than individual. These results are in agreement with other studies (14, 32, 36, 54). Calci et al. (14) observed variability in the detection and concentrations of F-specific RNA bacteriophages in 1,081 animal feces, with most of the 11 animal species shedding relatively low numbers. When sufficient phage was isolated, the expected genotypes were found. Genotypes I and IV were detected only in animal fecal samples (individual or PLM) and genotypes II and III only in STP samples. The results for genotypes I, II, and IV were in agreement with those obtained by Blanch et al. (10). In this multilaboratory study, genotypes I and IV were significantly more abundant in animal samples, and genotype II was significantly more abundant in human samples (10). However, genotype III was found to be nonspecific by Blanch et al. (10), as it was present in 33% of animal wastewater effluents and farm slurry samples ($n = 116$). F-specific RNA bacteriophages seem to be more frequent in human-contaminated samples (STP) than in pig-contaminated samples (PLM). These results suggest that these bacteriophages are useful for detecting human contamination from wastewater treatment plants but less efficient for detecting animal contamination, due to their sporadic occurrence. Overall, the application of F-specific RNA bacteriophage genotyping to environmental waters was often limited by low concentrations of phages.

The library-independent markers tested here succeeded in distinguishing human and animal sources. We suggest that, unlike library-dependent markers, they are not spatially constrained. Applying the two markers on environmental water samples collected from sites preclassified as urban, rural, or mixed showed that host-specific *Bacteroidales* were more often detected than genotypes of bacteriophages. For example, host-specific *Bacteroidales* enabled us to classify areas in 60% of water samples with *E. coli* concentrations less than 2×10^3 per 100 ml, while no F-specific bacteriophage genotyping could be performed because of insufficient numbers of bacteriophage in these water samples.

In urban areas of the Aber Benoît site, both methods successfully identified human contamination. The human *Bacteroidales* marker HF183F/Bac708R was frequently detected in water samples from a site preclassified as urban and so seems to be a reliable human indicator. Previous studies have shown this marker to be widely distributed within the United States (11, 23) and in New Zealand (29). Genotyping was successfully performed in all urban water samples of the Aber Benoît site, with 89% of phage predominantly from genotypes II and III. In rural areas, host-specific *Bacteroidales* markers and especially F-specific RNA bacteriophage were detected less often than in urban sites, indicating that it is more difficult to identify the origin of the contamination by runoff from cow pasture and pig slurry spreading than from urban point sources.

Genotyping of *Bacteroidales* revealed that the fecal contamination in these water samples did not come from pigs. Although ruminant-specific markers were present, the pig-specific marker was rarely detected, despite pig farming being important in the Aber Benoît area. Pig-specific *Bacteroidales* markers were found in stockage tanks in these areas. The local practice is for pigs to be housed in stalls in different farms, and their slurries are stored in tanks for at least 1 month before being spread. However, contamination by pig slurry cannot be excluded following heavy rains after pig manure spreading (not encountered during this study). Another explanation could be that pig *Bacteroidales* markers do not persist for a long time in the environment and that the detection level decreases during transfer from the animal to the water. In addition to the detection of animal-specific markers, the human-specific marker HF183 was observed in a few water samples. Scattered houses are present in these areas. For F-specific RNA bacteriophages, genotypes I, II, and III were identified in a small number of samples.

In the samples preclassified as mixed sources of contamination, Les Tanguières and Les Salines, ruminant marker (CF128F/Bac708R) and genotype I (71%) were the only markers detected. This suggests fecal contamination by sheep grazing the salt meadow near sampling sites, rather than urban activities upstream.

In the sites preclassified as rural or mixed (agricultural inputs), only bacteriophage genotype I was found. Similar results have been reported for surface water samples from the United States (13). This result can be explained by the low detection of genotype IV in animal fecal samples (this study and reference 16) and its lower survival in comparison to other genotypes (13, 16, 43).

E. coli and enterococcus concentrations in environmental waters sampled in this study varied according to the date of sampling, weather conditions, and location of sampling site. A significant correlation between *E. coli* concentrations and the presence of human markers (HF183 and genotypes II and III)

was found, whereas no correlation was observed for animal markers (CF128, CF193′, and genotype I). In urban areas, human pollution is mainly from point sources (such as STP outfalls), whereas in rural areas, pollution from ruminant animals will dominate essentially from nonpoint sources, such as rainfall runoff (this study and reference 57). Some inconsistencies between *E. coli* concentrations and some of the host-specific *Bacteroidales* (57) or F-specific bacteriophages (46) in environmental samples have been found previously. Many hypotheses could explain a lack of correlation in some situations: differential fecal inputs, persistence and survival of *E. coli*, bacteriophages, and *Bacteroidales* in the environment (13, 16, 57), and differential detection methods, including molecular biology for host-specific *Bacteroidales* markers and culture for *E. coli*, enterococci, and bacteriophages. To identify the origin of fecal contamination, which is currently identified by *E. coli* or enterococcus counts, more information is needed about the survival of *Bacteroidales* and human and animal genotypes of bacteriophages and their correlation to these fecal indicator bacteria (43, 57).

In conclusion, host-specific *Bacteroidales* markers were found to be more sensitive than F-specific bacteriophage genotyping in identifying the origin of fecal contamination in environmental water samples, especially where low numbers of *E. coli* were found. From the results obtained in this study, we would recommend the use of different animal-specific *Bacteroidales* marker primer sets to increase confidence in the identification of animal (bovine, ovine, or pig) fecal pollution. Among human-specific *Bacteroidales* markers, the use of the HF183 marker alone should be sufficient, as the occurrence of this marker in human and environmental samples was greater than that of HF134. F-specific bacteriophages could be a useful method to confirm human contamination. We are currently developing quantitative PCR for the F-specific bacteriophage genotypes to improve their detection.

ACKNOWLEDGMENTS

This work was in part supported by ICReW (INTERREG 3B Atlantic Area ICReW 2003–2006), and we thank our partners (A. Gawler, M. Walters, B. Masterson, W. Meijer, N. Caroll, L. Falcao, B. Nunes, R. Rodrigues, and J. Brandao) for their cooperation. We thank the Agence de l'Eau Seine Normandie and the Conseil Régional de Basse Normandie for their support for the water and STP sample collection from Normandy.

We thank also D. Hervio-Heath, S. Le Guyader, and J. Porter for critical reading of the manuscript, R. Gnouma for his assistance in statistical analyses, Richard Casseron for his technical assistance, and P. Bodenes for realization of Fig. 1.

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