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Relevance of *Bacteroidales* and F-Specific RNA Bacteriophages for Efficient Fecal Contamination Tracking at the Level of a Catchment in France

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The relevance of three host-associated *Bacteroidales* markers (HF183, Rum2Bac, and Pig2Bac) and four F-specific RNA bacteriophage genogroups (FRNAPH I to IV) as microbial source tracking markers was assessed at the level of a catchment (Daoulas, France). They were monitored together with fecal indicators (*Escherichia coli* and enterococci) and chemophysical parameters (rainfall, temperature, salinity, pH, and turbidity) by monthly sampling over 2 years ($n = 240$ water samples) and one specific sampling following an accidental pig manure spillage ($n = 5$ samples). During the 2-year regular monitoring, levels of *E. coli*, enterococci, total F-specific RNA bacteriophages, and the general *Bacteroidales* marker AllBac were strongly correlated with one another and with Rum2Bac ($r = 0.37$ to 0.50, $P < 0.0001$). Their correlations with HF183 and FRNAPH I and II were lower ($r = 0.21$ to 0.29, $P < 0.001$ to $P < 0.0001$), and HF183 and enterococci were associated rather than correlated (Fisher's exact test, $P < 0.01$). Rum2Bac and HF183 enabled 73% of water samples that had ≥2.7 $\log_{10}$ most probably number (MPN) of *E. coli*/100 ml to be classified. FRNAPH I and II enabled 33% of samples at this contamination level to be classified. FRNAPH I and II complemented the water sample classification obtained with the two *Bacteroidales* markers by an additional 8%. Pig2Bac and FRNAPH III and IV were observed in a small number of samples ($n = 0$ to 4 of 245). The present study validates Rum2Bac and HF183 as relevant tools to trace fecal contamination originating from ruminant or human waste, respectively, at the level of a whole catchment.

Human, livestock, pet, or wildlife fecal wastes alter river and coastal water quality due to the organic matter, odors, and enteric pathogens they bring, which cause environmental and human health issues (e.g., reference 27). Two fecal indicators, *Escherichia coli* and enterococci, are in standard use to assess the level of fecal microorganisms in water and the associated risk. From 2011, the revised European Bathing Water Directive (7) additionally requires bathing water profiles to be established and the identification of any pollution sources, to ensure efficient water quality management. Over the last decade, a panel of host-associated markers, known as microbial source tracking (MST) markers, has been derived from enteric bacteria or viruses or from chemical products (3, 10, 35). Criteria required for an MST marker to be useful and reliable, and thus relevant for inclusion in an operational toolbox, include (i) high sensitivity and specificity to the targeted species, independent of geographic region, and (ii) the potential to offer a credible evidence-based description of the fecal loading in environmental waters that do not comply with quality standards (13). Accordingly, for stakeholders who need an easy-to-use toolbox, an MST marker should have a dynamic, including persistence, similar to that of the standard criteria for fecal loadings (*E. coli* or enterococci) in environmental waters. In addition, an MST toolbox should target several host species, including human, livestock, pets, and wildlife, ideally with two or more types of markers for each host, to confirm the pollution source.

Real-time PCR markers designed from *Bacteroidales* 16S RNA, such as the human (HF183)-, ruminant (Rum2Bac)-, or pig (Pig2Bac)-associated ones, have been found to offer high sensitivity and specificity, ranging from 78 to 100% (20) (Table 1). At the catchment level, strong correlations between rainfall, water turbidity, *E. coli*, and levels of host-associated *Bacteroidales* markers have been observed in Austria (28) and the United States (31). However, in other instances, low correlations have been found with fecal indicators, e.g., following UV disinfection processes (33) or following high rainfall in summer, when there is an increase in *E. coli* but a decrease in ruminant-associated *Bacteroidales* markers (31). Such results have cast doubt on the usefulness of *Bacteroidales* markers in an operational MST toolbox.

Total F-specific RNA bacteriophages (FRNAPH) were initially proposed as indicators of viral pathogens (6), but the absence of a clear correlation between FRNAPH and these pathogens has limited their standardization (23). F-specific RNA bacteriophages do, however, contain four genogroups that could potentially be useful, since genogroups I and IV are typically associated with animal contamination and genogroups II and III with human contamination (5). The four genogroups, and more frequently FRNAPH I and II, have also been observed in environmental waters (5, 11, 12). However, the reading methodology for culture and genotyping outcomes (e.g., the criteria for one specific genogroup to be considered present) needs to be standardized before assessing the sensitivity and specificity rates for each genogroup and the on-site applicability of these markers.

To establish the potential relevance of these markers as MST tools and their usefulness for the development of effective water-quality management, their long-term monitoring has been per-

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TABLE 1 Specificity and sensitivity of host-associated *Bacteroidales* markers and FRNAPH genogroups[a]

Indicator	Marker or genogroup	Host	No. of positive samples/no. of total samples									% sensitivity (*n*)	% specificity (*n*)
			Feces						Effluent				
			Human	Pig	Bovine	Ovine	Equine	Avian	WWTP outlet	Pig manure	Bovine manure		
Bacteroidales	HF183	Human	15/24	0/25	1/15	0/10	0/10	NT	17/17	0/23	0/15	78 (41)	99 (98)
	Rum2Bac	Bovine/ovine	1/24	0/25	15/15	10/10	0/10	NT	5/15	0/23	14/15	98 (40)	94 (97)
	Pig2Bac	Pig	0/24	25/25	0/15	0/10	0/10	NT	1/17	23/23	0/15	100 (48)	99 (91)
F-specific RNA bacteriophage	FRNAPH I	Animal	NT	15/40	0/16	1/8	0/3	7/34	7/47	12/47	0/13	22 (161)	85 (47)
	FRNAPH II	Human	NT	0/40	0/16	0/8	0/3	1/34	45/47	0/47	0/13	85 (47)	99 (161)
	FRNAPH III	Human	NT	0/40	0/16	0/8	0/3	0/34	14/47	0/47	0/13	26 (47)	100 (161)
	FRNAPH IV	Animal	NT	5/40	0/16	0/8	0/3	2/34	0/47	1/47	0/13	4 (161)	100 (47)

[a] Markers were designed by Seurinck et al. (30) (HF183), Mieszkin et al. (22) (Rum2Bac), Mieszkin et al. (21) (Pig2Bac), and Ogorzaly et al. (25) (FRNAPH I to IV). Specificity and sensitivity outcomes reported in Mieszkin (20) (*Bacteroidales*) and the present study (F-specific RNA bacteriophage). A *Bacteroidales* marker result was positive when the real-time PCR amplification of the DNA sample resulted in 5 or more copies per PCR well in the triplicate PCR assays (LOQ). An FRNAPH genogroup result was positive when 20 plaques were successfully genotyped for one sample, including ≥5 plaques identified as belonging to a particular genogroup (LOD). NT, not tested.

formed at the catchment level, combined with parallel measurements of fecal indicators and chemophysical parameters. In addition, we assessed the sensitivity and specificity rates for each FRNAPH genogroup and proposed standards to define their detection limit.

MATERIALS AND METHODS

Sites and water sampling strategy. (i) Two-year monitoring on Daoulas catchment. Daoulas catchment is located in Brittany, on the west coast of France (Fig. 1). It covers 113 km^2, including 90 cumulative kilometers of streams, and is an area of intensive livestock rearing, with 170 farms (4).

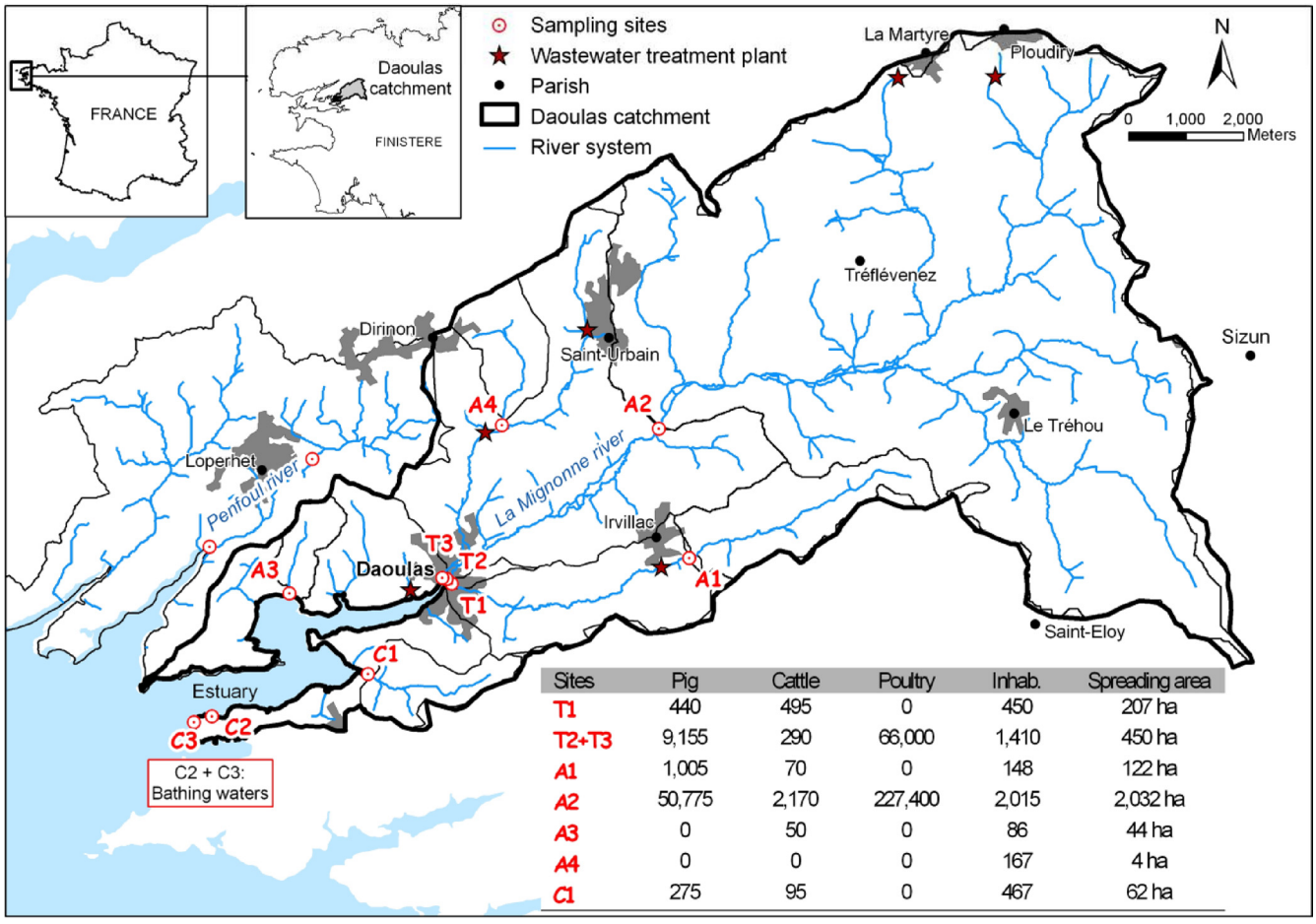

FIG 1 The Daoulas catchment with locations and descriptions of 10 sites sampled monthly over 2 years. The Loperhet subcatchment, where an accidental pig manure spillage occurred, is also indicated. (Printed with permission of Ifremer and IDHESA Bretagne Océane.)

Water samples were collected monthly over 2 years, from July 2009 to June 2011, at 10 sites on the Daoulas catchment ($n = 240$ samples) (Fig. 1). Three sites, T1, T2, and T3, were located in the town of Daoulas, which is located in the downstream area of the catchment. Four sites, A1, A2, A3, and A4, were situated on upstream agricultural subcatchments. Finally, three sites, C1, C2, and C3, were situated on the coast. At sites T1, T2, T3, A3, and C1, water samples were collected at low tide to avoid their dilution by seawater. At sites A1, A2, and A4, water level was not affected by the tide, so tidal fluctuations were not taken into account when planning sampling times. At C2 and C3, both of which are bathing sites, water samples were collected at high tide, in typical bathing conditions. Water samples were collected from the top 10-cm surface layer using sterilized 1-liter bottles and immediately placed in an ice chest, where they were kept until analysis. All water filtrations were performed within 24 h of sampling.

(ii) Monitoring of an accidental pig manure tank spillage. Water samples were collected downstream of an accidental spillage from a pig manure tank, which happened due to a pipe fracture, in a nearby catchment (Loperhet; 17 March 2011; $n = 5$ samples) (Fig. 1). For 3 h 30 min, from 7 a.m. to 10:30 a.m., pig manure poured into a pond and then into a watercourse that flows into the Glanvez River, 50 m downstream; this river reaches the sea at Penfoul, 3 km downstream of the farm. After 2 h 30 min of spillage (9:30 a.m., day 1), one sample was collected at the point where the watercourse leading from the tank joins the Glanvez River, and a second sample was collected at the Penfoul site. The day after the spillage (8 a.m., day 2), two samples were collected from the watercourse connected to the pond, 40 m downstream of the farm, and one sample was collected from the point at which the watercourse from the tank joins the Glanvez River.

Water sample analysis. (i) Enumeration of *E. coli* cells, enterococci, and total F-specific bacteriophage. Most probable numbers (MPN) of fecal indicators, *E. coli* cells, and enterococci were assessed on microplates at the Idhesa laboratory (Brest, France), with 1.6 $\log_{10}$ MPN/100 ml as the limit of quantification (LOQ) ($n = 1$ analysis per sample) (16, 17). Total F-specific bacteriophage (total FPH) concentrations were determined by plaque assays, according to the ISO 10705 recommendations (15), with 1 $\log_{10}$ PFU/100 ml water as the LOQ ($n = 1$ analysis per sample).

(ii) Quantification of AllBac, HF183, Rum2Bac, and Pig2Bac levels. Water samples collected in the first year (500 ml, except at C2 and C3 [1 liter]) were filtered under sterile conditions through 0.22-μm Nuclepore membranes (Whatman, Millipore, France). For the samples collected in the second year, these volumes were doubled (1 liter, except at C2 and C3 [2 liters]), and the samples were filtered through 0.45-μm cellulose membranes (Pall Gelman GN-6 Metricel; Pall Corporation). For the samples collected after the accidental manure spillage, turbidity was high, and it was possible to filter only 10 and 20 ml of the samples collected on day 1 at Glanvez and Penfoul, respectively, and 50 ml and 500 ml of the samples collected on day 2 from the watercourse connected to the pond and Glanvez River, respectively. The filters were then mixed with 500 μl GITC (5 M guanidine, 100 mM EDTA [pH = 8.0], 0.5% Sarkosyl) and stored at −80°C until DNA extraction.

DNA was extracted from the filters using the QIAamp DNA minikit (Qiagen, GmbH) following the manufacturer's recommendations, except for two modifications: proteinase k treatment was omitted and AL buffer (700 μl) was added to the filter with the GITC buffer ($n = 1$ extraction per sample). DNA extracts were eluted in 100 μl AE buffer and stored at −80°C until PCR analysis.

Primers, probes, and amplification conditions are summarized by Mieszkin et al. for AllBac, HF183, and Pig2Bac (21) and for Rum2Bac (22). The TaqMan exogenous internal positive control (IPC) reagent kit (EXO IPC; Applied Biosystems, France) was added to the AllBac assays to distinguish true target negatives from PCR inhibition. Concentration of the IPC primers in the PCR assay is limiting, and competition between AllBac and the IPC reaction was not observed. When PCR inhibitors were present, DNA extracts were diluted 10- or 100-fold to prevent the inhibitors from influencing the subsequent host-associated marker PCRs. Controls included nontemplate controls, as well as filtration and DNA extraction blanks, each performed in triplicate. LOQ was set at 5 copies per PCR well in the triplicate PCR assays, which was the lowest quantity of linear plasmid DNA of the standard curve made for each PCR assay. Correlation coefficients (r^2) for all the standard curves were >0.97, and PCR efficiency ranged between 95 and 105%. The equivalent LOQ concentrations ranged from 2.4 to 3.7 $\log_{10}$ copies/100 ml water over the 2-year monitoring period and up to 4.4 $\log_{10}$ copies/100 ml water during the accidental pig manure contamination study, depending on the volume of sample filtered and the dilution of the DNA extract for the PCR assay.

Detection of FRNAPH. During the first year of monitoring, FRNAPH were concentrated with a Vivaflow 50 tangential flow system equipped with a polyethersulfone (PES) membrane with a 50,000 molecular weight cutoff (Vivascience, Sartorius, France). Cassettes were rinsed with 500 ml milliQ water (Millipore, France) prior to filtration, and the water samples (1 liter, except at C2 and C3 [2 liters]) were then concentrated to 35 ml. The cassettes were finally rinsed with 10 ml glycine buffer (0.05 M, pH 9.5), which was added to the concentrate. During the second year of monitoring, FRNAPH genogroups were concentrated from 500 ml water from each of the 10 sites by flocculation with $MgCl_2$ (0.05 M) followed by filtration. The viruses on the filter were then recovered in 5 ml eluent according to Gourmelon et al. (11). Concentrated samples (5 ml) were cultivated according to ISO 10705 recommendations (15). Lysis plaques on petri dishes were individually picked out and stored in 15% phosphate-buffered saline (PBS)-glycerol at −20°C until genotyping. Isolates were cultivated on petri dishes with or without RNase. Isolates that were not sensitive to this treatment corresponded to F-DNA bacteriophages and were removed from the analysis. FRNAPH were genotyped using a QuantiTech probe reverse transcription (RT)-PCR kit (Qiagen, France) according to Ogorzaly et al. (25). Limit of detection (LOD) was defined as 20 plaques successfully genotyped for one sample, arbitrarily set as the minimum number of isolates needed to represent FRNAPH diversity of a sample. The presence of a genogroup among the isolates of a sample was considered confirmed when there were ≥5 plaques identified as belonging to that genogroup.

Chemophysical analysis. Rainfall data were provided by the meteorological station at Sizun (Meteo France) (Fig. 1). On-site water temperature (°C) and salinity (practical salinity units [psu]) were measured using a Cond 340i (WTW); oxygen level (mg/liter) and saturation (%) were measured using an HQ 30d equipped with an LDO 101 electrode (HACH). Turbidity (NTU) and pH were measured at the laboratory within 24 h, using an HACH 2100N turbidimeter and a metrohm pH meter, respectively.

FRNAPH genogroup sensitivity and specificity. Individual human and animal fecal ($n = 101$) and effluent ($n = 115$) samples, collected from 2007 to 2011, were analyzed according to Gourmelon et al. (11), except that genotyping was performed using the QuantiTech probe RT-PCR kit (Qiagen, France), as detailed above.

Statistics. Sensitivity is the proportion of true positive outcomes (%) in the analyzed fecal or effluent samples of a species traced by a marker. Specificity is the proportion of true negative outcomes (%) in the analyzed fecal and effluent samples of a species not traced by a marker (8).

Statistical analyses were performed using XLSTAT version 2011.2.02. Real-time PCR outcomes obtained with the *Bacteroidales* markers, bacterial culture outcomes obtained with *E. coli* and enterococci, and chemophysical variables were used as quantitative data. Values below the LOQ were assigned a value of 0. Culture and genotyping outcomes obtained with the FRNAPH genogroups were used as semiquantitative data, and a value of 0 or 1 was attributed when the genogroup was absent or present, respectively. Enterococcus levels were not assessed for the two first months of the study, and the missing values were estimated by the nearest-neighbor method. Microbial concentrations and turbidity were log transformed to bring their distributions closer to normal.

To assess correlations between pairs of variables, Pearson coefficients

(linear correlation) were calculated. When Pearson correlations were not significant, variables were divided into up to four groups to build contingency tables, which were then used to assess Fisher's exact probability to look for types of associations other than linear ones. To account for the effect of the sample size on the meaning of the significance tests, a P value of <0.05 was used as the significance level with the data set used to compare the sites ($n = 24$), and a P value of <0.01 was used for larger data sets, e.g., with the pooled data set from the 10 sites collected over 2 years ($n = 240$).

To look for significant differences in levels of microbial and chemophysical variables between sites, one-way analyses of variance (ANOVA) were performed with Newman-Keuls *post hoc* tests. Significance was inferred at P values of <0.01 ($n = 72$ observations per variable for urban and coastal sites [3 sites, 24 monthly samples each], 96 observations per variable for agricultural sites [4 sites, 24 monthly samples each]).

To illustrate microbial variability on the Daoulas catchment, principal component analysis (PCA) based on Pearson correlations was applied to the pooled microbial data set, with chemophysical parameters as supplemental variables. Pig2Bac and FRNAPH III and IV markers were not used in the PCA, as there was insufficient data.

RESULTS

Description of the pooled data set covering 10 sites over 2 years. *E. coli* and AllBac marker levels were quantified in 93% (222 of 240 samples) and nearly 100% of the samples (239 of 240 samples), respectively. *E. coli* levels exceeded the threshold for the "excellent" classification category of inland waters according to EC directive 2006/7/EC (7) ($\geq$2.7 $\log_{10}$ MPN/100 ml) in 38% of the samples (90 of 240 samples). Levels of enterococci, total FPH, Rum2Bac, HF183, and Pig2Bac were higher than the LOQ in 60%, 33%, 36%, 17%, and 2% of the samples, respectively (132 of 220 samples and 79, 86, 41, and 4 of 240 samples, respectively). Enterococcus levels were higher than the threshold for the excellent classification of inland waters ($\geq$2.3 $\log_{10}$ MPN/100 ml [7]) in 24% of the samples (53 of 220 samples). FRNAPH I and II were present in 15 and 17% of the samples, respectively (36 and 40 of 240 samples, respectively). FRNAPH III and IV were observed in 0 and 1 of the 240 samples, respectively. The levels of the standard indicators and *Bacteroidales* marker or FRNAPH presence rate were mainly higher at urban sites than at agricultural ones and much higher than at coastal ones (Table 2).

Turbidity was significantly higher at urban sites than at the other sites. It ranged from 0.1 to 40 NTU in the pooled 2-year data set ($n = 240$), with two additional higher values, 149 and 167 NTU at C1 and T1, respectively. The pH levels increased significantly in the downstream direction. Oxygen levels were significantly lower at coastal sites than at sites upstream. Inversely, water temperatures were higher at coastal sites than at sites upstream. Salinity at the seven urban and agricultural sites was below 3 psu. Salinity at C2 and C3 ranged mainly from 27 to 35 psu. At C1, salinity ranged from 0 to 30 psu with no significant correlation with the tide amplitude or time elapsed between sampling and low tide ($P > 0.05$). Rainfall data were obtained at one upstream meteorological station (Meteo France, Sizun) for the whole catchment. One-day rainfall was <10 mm on 19 occasions and $\geq$10 mm on five.

Correlations between variables in the pooled data set covering 10 sites over 2 years. (i) Correlations between microbial variables. Rum2Bac, *E. coli*, enterococcus, AllBac, and total FPH levels in water samples were all strongly correlated with one another (Table 3), explaining 41% of the microbial variability observed across the Daoulas catchment during the 2-year monitoring period (see Fig. S1 in the supplemental material). To a lesser extent, the HF183 level was correlated with *E. coli*, AllBac, and total FPH (Table 3). HF183 was not correlated with enterococcus levels (Table 3), but HF183 and Enterococcus levels were associated with each other (Fisher's exact test, $P < 0.01$). FRNAPH I and II presences were correlated with *E. coli*, enterococcus, and total FPH levels. The two human-associated markers, HF183 and FRNAPH II, were correlated, but the two animal-associated markers, FRNAPH I and Rum2Bac, were not (Table 3).

(ii) Correlations between chemophysical variables. Rainfall was correlated with turbidity in log form (Table 3). Consequently, specific effects of rainfall or turbidity on the microbial variables cannot be separated. Rainfall was negatively correlated with oxygen level, but rainfall, temperature, and pH were not correlated (Table 3).

(iii) Correlations between chemophysical and microbial variables. One-day rainfall and turbidity were positively correlated with levels of Rum2Bac, *E. coli*, enterococci, and total FPH (Table 3; see also Fig. S1 in the supplemental material). However, 1-day rainfall and turbidity were neither correlated nor associated with HF183 levels or the presence of FRNAPH I and II markers (Pearson and Fisher's exact tests, $P > 0.04$). Salinity was negatively correlated with levels of HF183, Rum2Bac, FRNAPH II, *E. coli*, AllBac, and total FPH (Table 3; see also Fig. S1). Similar trends were observed between salinity and FRNAPH I or enterococci, although they were not significant. Salinity was higher at the coastal sites downstream, which are the sites the furthest away from the pollution sources, situated 2 to 14 km upstream (Fig. 1). Temperature, oxygen level, and pH were weakly correlated with the microbial variables (Table 3; see also Fig. S1).

Classification of water samples according to the contamination origin. Classification rate increased with *E. coli* level in the host-associated *Bacteroidales* markers and, to a much lower extent, in the FRNAPH genogroups (Fig. 2).

Water samples with $\geq$2.7 $\log_{10}$ MPN *E. coli*/100 ml. In water samples which exceeded the threshold for excellent quality of inland waters ($\geq$2.7 $\log_{10}$ MPN *E. coli*/100 ml [7]), the combined use of HF183 and Rum2Bac enabled 73% of samples to be classified ($n = 90$ samples) (Table 4). Fecal contamination at the three urban sites was identified as being mainly of mixed human-ruminant origin (33% of the samples) (Fig. 2). Contamination at the four agricultural sites was identified as being mainly of ruminant origin (60% of the samples) (Fig. 2). At A1 and A2, Rum2Bac was detected in 95% of the samples $\geq$2.7 $\log_{10}$ MPN *E. coli*/100 ml (21 of 22 samples), including 90% (19 samples) collected between May and December. At sites A3 and A4, HF183 and Rum2Bac were quantified in 16 and 26% of the samples (3 and 5 of 19 samples), respectively. At the three coastal sites, the excellent classification threshold of inland waters (7) was exceeded in only 11% of the samples collected over the 2 years (8 of 72 samples). HF183 was quantified in 13% (1 of 8 samples) and Rum2Bac in 50% of these samples (4 of 8 samples). According to the *Bacteroidales* markers, ruminant contamination was twice as frequent as human contamination on the scale of the study catchment (Table 4).

FRNAPH I and II together enabled 33% of samples with $\geq$2.7 $\log_{10}$ MPN *E. coli*/100 ml to be classified (Table 4). At urban sites, FRNAPH genogroups detected mainly a mixed human-animal contamination (28% of the samples), and at the four agricultural sites, they detected animal and mixed contamination in 14 and 5% of samples, respectively (Fig. 2). FRNAPH I and II were observed

TABLE 2 Chemophysical and microbial data assessed over 2 years at three urban sites, four agricultural sites, and three coastal sites on the Daoulas catchment[a]

Variable	Town sites T1, T2, T3 ($n = 72$ samples)						Agricultural sites A1, A2, A3, A4 ($n = 96$ samples)						Coastal sites C1, C2, C3 ($n = 72$ samples)					
	Mean	Min	Max	95% CI low	95% CI high	Subg.	Mean	Min	Max	95% CI low	95% CI high	Subg.	Mean	Min	Max	95% CI low	95% CI high	Subg.
Microbial																		
Fecal indicators																		
E. coli ($\log_{10}$ MPN/100 ml)	2.9	2.1	4.4	2.8	3.0	a	2.7	0.0	4.3	2.5	2.8	a	1.7	0.0	4.7	1.5	2.0	b
Enterococci ($\log_{10}$ MPN/100 ml)	1.7	0.0	4.5	1.4	2.0	a	1.2	0.0	3.9	1.0	1.5	b	1.1	0.0	3.9	0.8	1.4	b
Bacteroidales																		
AllBac ($\log_{10}$ copies/100 ml)	5.5	3.1	6.6	5.3	5.6	a	5.3	3.8	6.4	5.2	5.4	a	4.8	0.0	6.2	4.6	5.0	b
HF183 ($\log_{10}$ copies/100 ml)	1.4	0.0	5.2	1.0	1.8	a	0.2	0.0	4.5	0.0	0.4	b	0.2	0.0	3.7	0.0	0.4	b
Rum2Bac ($\log_{10}$ copies/100 ml)	1.9	0.0	5.9	1.5	2.4	a	1.5	0.0	6.1	1.1	1.9	a	0.4	0.0	4.4	0.1	0.7	b
Pig2Bac ($\log_{10}$ copies/100 ml)	0.1	0.0	3.5	0.0	0.3	a	0.0	0.0	0.0	0.0	0.0	a	0.1	0.0	4.0	−0.1	0.2	a
Bacteriophage																		
Total FPH ($\log_{10}$ PFU/100 ml)	1.3	0.0	3.2	1.0	1.5	a	0.6	0.0	2.7	0.4	0.8	b	0.3	0.0	2.6	0.2	0.4	c
FRNAPH I (0/1)	0.3	0	1	0.2	0.4	a	0.1	0	1	0.0	0.2	b	0.0	0	1	0.0	0.1	b
FRNAPH II (0/1)	0.4	0	1	0.3	0.5	a	0.1	0	1	0.0	0.1	b	0.1	0	1	0.0	0.1	b
FRNAPH III (0/1)	0.0	0	0	0.0	0.0	a	0.0	0	0	0.0	0.0	a	0.0	0	0	0.0	0.0	a
FRNAPH IV (0/1)	<0.0	0	1	<0.0	<0.0	a	0.0	0	0	0.0	0.0	a	0.0	0	0	0.0	0.0	a
Chemophysical																		
1-day rainfall at Sizun (mm/day)	4.3	0.0	21.0	2.9	5.7	a	4.3	0.0	21.0	2.9	5.7	a	4.3	0.0	21.0	2.9	5.7	a
Turbidity (NTU)	10	2	167	5	14	a	6	1	40	5	7	b	9	0	149	4	13	b
Turbidity ($\log_{10}$ NTU)	0.8	0.3	2.2	0.7	0.9	a	0.6	−0.1	1.6	0.6	0.7	b	0.6	−1.0	2.2	0.5	0.8	b
Temperature (°C)	11.1	4.8	16.8	10.2	12.0	b	11.0	4.8	18.2	10.3	11.7	b	13.6	4.3	25.0	12.4	14.8	a
Oxygen (%)	98	91	111	97	99	a	98	88	122	96	99	a	104	82	162	100	109	a
Oxygen (mg/liter)	10.6	7.8	12.3	10.4	10.9	a	10.5	2.0	12.3	10.2	10.7	a	9.5	6.7	14.9	9.0	10.0	b
pH	7.7	6.7	8.6	7.6	7.8	b	7.5	6.7	8.2	7.4	7.6	c	7.9	7.2	8.4	7.8	7.9	a
Salinity (psu)	0.2	0.0	10.5	−0.1	0.5	b	0.4	0.0	9.4	0.1	0.6	b	25.2	0.0	34.6	22.6	27.8	a

[a] Data below the limit of quantification were set at 0 for the statistical analyses. a, b, and c are homogeneous subgroups according to ANOVA with Newman-Keuls *post hoc* tests ($P < 0.01$). Subg., subgroup.

together in 13% (1 of 8 samples) of the samples collected at the coastal sites (13%) (Fig. 2).

The FRNAPH genogroups more frequently classified mixed animal-human contamination in proportion to the total classification (16/33 = 49%) than did host-associated *Bacteroidales* markers (17/73 = 23%) (Table 4). Both *Bacteroidales* and FRNAPH markers suggested the same contamination source in 23% of the samples (Table 4). They failed to give a unanimous result in 58% of the samples. If the classifications defined by the four markers, HF183, Rum2Bac, and FRNAPH I and II, were merged, as they would be in the case of an MST toolbox, FRNAPH genogroups complemented the classifications obtained with the host-associated *Bacteroidales* markers by an additional 13% (42% − 29%) and 8% (69% − 61%) of the water samples classified as human and animal contaminated, respectively (Table 4). FRNAPH genogroups thus complemented the total classification obtained with both *Bacteroidales* markers by an additional 8% (81 to 73%).

Water samples with a low *E. coli* level (<2.7 $\log_{10}$ MPN *E. coli*/100 ml). In the data set with <2.7 $\log_{10}$ MPN *E. coli*/100 ml ($n = 150$), host-associated *Bacteroidales* markers were quantified in 47, 26, and 11% of the samples at the urban, agricultural, and coastal sites, respectively. FRNAPH genogroups were detected in 44, 15, and 6% of these samples at the urban, agricultural, and coastal sites, respectively (Fig. 2). For instance, at A1 and A2, Rum2Bac was detected in 46% of the samples <2.7 $\log_{10}$ MPN *E. coli*/100 ml (12 of 26 samples), including 75% (9 samples) collected between May and December. At the urban sites, *E. coli* was never <2 $\log_{10}$ MPN *E. coli*/100 ml. At agricultural and coastal sites, Rum2Bac and HF183 were quantified in 12% of the samples with <2 $\log_{10}$ MPN *E. coli*/100 ml (7 of 57 samples) and FRNAPH were detected in 6% (4 of 57 samples).

Relationships between sites located up- and downstream or between urban sites. *E. coli*, enterococcus, AllBac, and total FPH levels at the urban sites were correlated with the levels at the corresponding agricultural sites upstream ($r = 0.46$ to 80; see Table

TABLE 3 Pearson correlations between microbial and chemophysical variables at 10 sites on the Daoulas catchment over 2 years (240 observations per variable)[a]

	Pearson correlation														
Variable	*E. coli*	Entero	AllBac	HF183	Rum2Bac	Pig2Bac	Tot FPH	PH I	PH II	Salinity	Temp	1-d rain	O_2	pH	$\log_{10}$ NTU
E. coli															
Entero	0.50***														
AllBac	0.49***	0.37***													
HF183	0.25**	0.14	0.29***												
Rum2Bac	0.49***	0.43***	0.45***	0.15											
Pig2Bac	0.01	−0.09	0.05	0.33***	0.04										
Tot FPH	0.43***	0.43***	0.30***	0.27***	0.49***	0.03									
PH I	0.21**	0.23**	0.06	0.12	0.15	0.12	0.36***								
PH II	0.21**	0.25***	0.22**	0.24**	0.16	0.03	0.39***	0.44***							
Salinity	−0.57***	−0.16	−0.45***	−0.17*	−0.33***	−0.06	−0.31***	−0.14	−0.19*						
Temp	−0.06	0.27***	−0.16	−0.22*	0.06	−0.18*	−0.02	0.07	−0.07	0.35***					
1-d rain	0.33***	0.36***	0.19	0.02	0.30***	−0.08	0.43***	0.05	0.11	−0.05	−0.03				
O_2	0.04	−0.09	0.07	0.19*	−0.04	0.15	0.01	−0.04	0.08	−0.40***	−0.39***	−0.27***			
pH	−0.28***	−0.13	−0.20*	−0.03	−0.22**	−0.12	−0.04	−0.07	−0.07	0.40***	0.29***	−0.16	−0.04		
$\log_{10}$NTU	0.38***	0.34***	0.10	0.08	0.21**	0.05	0.30***	0.07	0.11	−0.23**	−0.02	0.42***	0.06	−0.27***	

[a] Significance thresholds were rated as very weakly significant (*, $P < 0.01$), weakly significant (**, $P < 0.001$), or significant (***, $P < 0.0001$). Entero, enterococci; Tot FPH, total F-specific bacteriophage; PH I, FRNAPH I; PH II, FRNAPH II; Temp, temperature; 1-d rain, 1-day rainfall; O_2, oxygen concentration (mg/liter); $\log_{10}$ NTU, turbidity in log form.

S1 in the supplemental material), except in 3 of the 20 correlations considered. Inversely, for the host-associated markers, up- and downstream correlation was observed in only two cases: for Rum2Bac levels between A1 and T1 ($r = 0.59$) and A2 and T3 ($r = 0.48$).

Bacterial levels (except enterococci) and FRNAPH I and II presence at T2 were correlated with their corresponding values at T3 ($r = 0.44$ to 0.80, see Table S1 in the supplemental material). Both T2 and T3 are located on the La Mignonne subcatchment downstream of the urban area (Fig. 1). Lower correlations than the ones reported between T2 and T3 were observed between T2 and T1 or between T3 and T1; T1 is situated downstream of a separate subcatchment (Irvillac subcatchment).

Case study of an accidental pig manure spillage. In water samples collected 2 h 30 min after the spillage, the turbidity and *E. coli* level were higher 3 km downstream at Penfoul (650 NTU, 4.0 $\log_{10}$ MPN *E. coli*/100 ml) than 50 m downstream of the farm (381 NTU, 3.5 $\log_{10}$ MPN *E. coli*/100 ml). The day after the spillage, 50 m downstream from the farm, the water turbidity and *E. coli* level decreased to 4 NTU and 2.5 $\log_{10}$ MPN/100 ml. In the watercourse connected to the pond, 40 m downstream from the farm, the turbidity and *E. coli* level remained at about 130 NTU and 3.2 $\log_{10}$ MPN/100 ml, respectively ($n = 2$). For the 4 samples where turbidity was >100 NTU, 10 to 50 ml water was filtered and the LOQ for *Bacteroidales* markers was therefore high, ranging from 3.7 to 4.4 $\log_{10}$ copies/100 ml. Pig2Bac was detected in the 5 samples, but real-time PCR signals were below the quantification limit. Total FPH and FRNAPH genogroups I to IV were not observed in these samples.

Inhibitors in the *Bacteroidales* marker quantification procedure. PCR inhibitors were detected in all the DNA extracts. The extracts were consequently diluted 10-fold (128 samples) or 100-fold (117 samples) for the subsequent host-associated *Bacteroidales* marker PCRs ($n = 245$). Samples collected at A4 and at the bathing sites (C2 and C3) were mostly diluted 100-fold (in 17 to 20 of 24 samples). In contrast, samples collected at T1, T2, A1, and A2 (16 to 21 of 24 samples) and the 5 samples collected after the pig manure spillage, and having a high turbidity, were diluted 10-fold. At the other sites (T3, A3, and C1), no clear dilution trend was observed.

FRNAPH genogroup specificity, sensitivity, and detection. Specificity of the four FRNAPH genogroups ranged from 85 to 100% (Table 1). FRNAPH I was detected in 15% of wastewater treatment plant (WWTP) samples. Sensitivity of the human-associated genogroup FRNAPH II was high (85%). Inversely, FRNAPH I, III, and IV sensitivities ranged from 26 to 4%. FRNAPH I was detected in 31% of pig feces and manure samples and 21% of avian fecal samples. Neither FRNAPH animal marker (I or IV) traced feces or manure of bovine or ovine origins (0 of 29 samples).

FRNAPH I was observed in 11 and 34% of the samples with ≥2.7 MPN *E. coli*/100 ml in the first year and second year, respectively, and FRNAPH II was observed in 14 and 37% in the first year and second year, respectively ($n = 90$ samples, $P < 0.01$). Sample concentration by flocculation thus appeared more sensitive than in the Vivaflow system. In contrast, host-associated *Bacteroidales* markers, *E. coli*, and enterococcus levels were similar between the first and the second years ($n = 240$, $P \geq 0.12$), suggesting that the modification of the volume filtered and pore size of the filter between the 2 years of monitoring did not affect Rum2Bac or HF183 quantification rates.

DISCUSSION

The frequent quantification of Rum2Bac among our samples was consistent with the livestock on the Daoulas catchment (Fig. 1). Rum2Bac, *E. coli*, and enterococci showed (i) a strong correlation during the 2-year monitoring period and (ii) a pattern of decreasing concentration from upstream to downstream. HF183 was quantified in the majority of water samples with ≥2.7 $\log_{10}$ MPN *E. coli*/100 ml collected at urban sites and occasionally at upstream agricultural sites located away from densely inhabited areas. The HF183 pattern was consistent with the assumed profile of human contamination (Fig. 1). HF183 was correlated with *E. coli* and associated with enterococci. These results showed Rum2Bac and

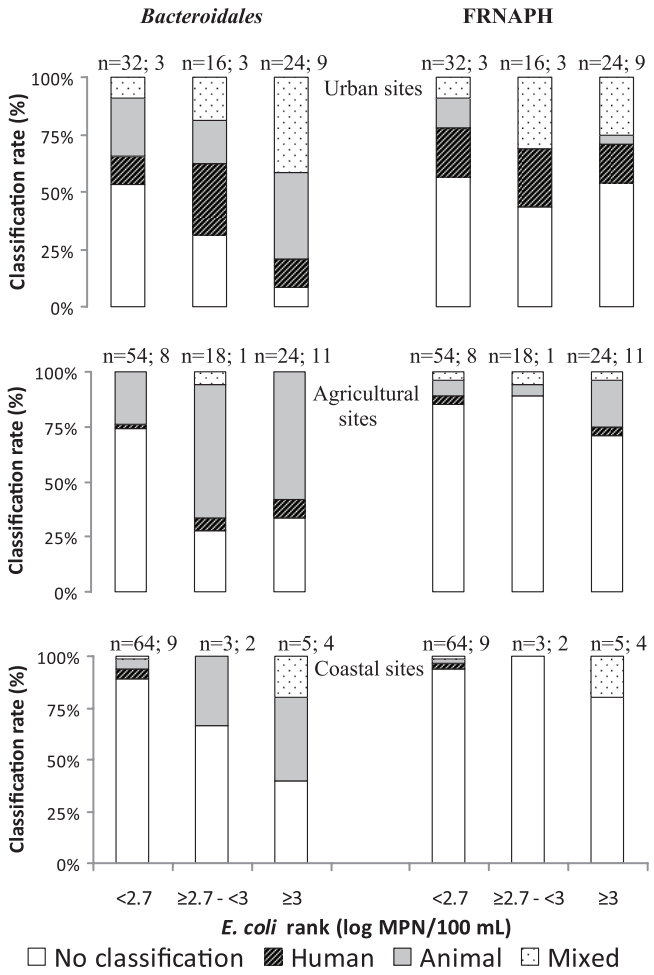

FIG 2 Classification rate (%) of water samples collected at urban, agricultural, and coastal sites according to host-associated *Bacteroidales* (HF183 and Rum2Bac) or FRNAPH (I and II) genogroups, at three *E. coli* levels. Total number of samples and number of samples collected when 1-day rainfall was ≥10 mm are indicated at the tops of the columns for each *E. coli* level considered.

HF183 to be potentially useful MST markers for stakeholders seeking a measurement method that complies with the fecal indicator standards. Higher correlations with fecal indicators for ruminant-associated *Bacteroidales* marker levels than for human-associated ones were previously reported in other catchment systems in the Alps (Europe) and Oregon (United States) (28, 31).

Absence of correlation between HF183 levels and 1-day rainfall indicates that rainfall did not impair the efficiency of the treatment systems on the present catchment to an extent where any contamination increase was detected at the sampling sites situated 2 to 4 km downstream of the WWTP. WWTP at Daoulas (T2 and T3), Irvillac (T1), La Martyre (A2), and Pouldiry (A2) are equipped with lagoon plants, built 15 to 25 years ago. Saint Urbain (T2 and T3) is equipped with an activated sludge plant built in 1975. As there was no correlation between turbidity and HF183 level in the present study, the previously suggested use of turbidity as a cost-effective surrogate for *E. coli* and fecal contamination (24, 26) did not appear reliable where human contamination is concerned. Inversely, ruminant contamination was likely to have been brought in by streaming or associated with high turbidity.

At A1, although cattle numbers are low (70 individuals), the sampling site was downstream from a pasture where cattle had direct access to the riverside and 800 m downstream from the farm. The microbial contamination detected at A3 and A4 is likely to be of an origin other than human or bovine. Spreading of poultry droppings has been observed on the field upstream of A3. However, FRNAPH I was weakly detected at A3.

During the winter, putting animals to pasture depends on the weather forecast and is not usual practice from October to March (H. Goriou, Chambre d'Agriculture, personal communication). Manure spreading on cultured land from July to mid-January is limited due to prohibition by local administration. Farmers do not report the extent of their pasturing or manure spreading practices. However, the period during which Rum2Bac was quantified (from May to December) seemed to be more similar to the pasturing period than to the spreading period. In addition, microbial levels, including MST markers and fecal indicators, are typically lower in manure samples than in fecal ones (20, 21). Consequently, MST markers may have a greater potential for tracing contamination caused by feces than contamination caused by manure. Agricultural practices should be improved so as to avoid streaming, with rules such as (i) excluding the livestock from the riverside and establishing a buffer area around pasture, (ii) spreading during the dry period only, on flat areas excluding water meadows, and (iii) adapting the culture to the land contours and establishing filter strips planted with grass.

Pig2Bac quantification during the 2-year study was less frequent than expected (4 of 240 samples) at the level of Daoulas catchment, which has 63,500 pigs. This classification rate was lower than that during a screening study undertaken in 2008 at other sampling sites on the Daoulas catchment, where Pig2Bac was observed in 21% of water samples (5 of 24 samples) (21). In addition, in a worst-case scenario, with an accidental pig manure spillage causing up to 4 $\log_{10}$ MPN *E. coli*/100 ml downstream of the accident, Pig2Bac was detected but not quantified in river water samples. This last observation suggests that the low Pig2Bac quantification rate at the level of the Daoulas catchment would be due to a methodological issue and probably did not provide an accurate picture of the importance of the pig-associated contamination on this catchment. The Pig2Bac quantification rate could be improved by reducing the quantification limit of the method,

TABLE 4 Classification rate (%) according to *Bacteroidales* and/or FRNAPH markers in the water samples with ≥2.7 $\log_{10}$ MPN *E. coli*/100 ml (n = 90)

Origin of contamination	Classification rate (%)			
	Bacteroidales alone (Rum2Bac or HF183)	FRNAPH alone (I or II)	*Bacteroidales* or FRNAPH (total classification)	*Bacteroidales* and FRNAPH (classification suggested by both marker types together)
Human	29	26	42	12
Animal	61	23	69	16
Including mixed[a]	17	16	31	4
Total classification	73	33	81	23
No classification	27	67	19	19

[a] Mixed total is included in human and animal totals.

which may be achieved by reducing the level of PCR inhibitors in the water samples, for example. In a similar way as with Rum2Bac, Pig2Bac should be more efficient for tracing contamination by pig feces than by pig manure (21). However, pig pasturing has not been reported or observed on the Daoulas catchment. Separately, *Lactobacillus amylovorus* GE39/GE40, another pig-associated marker (19), was quantified at concentrations ranging from 4.6 to 6.5 $\log_{10}$ copies/100 ml in 5 out of the 5 water samples collected downstream of the accidental spillage monitored in the present study (Olivia Solecki, Irstea, Rennes, France, personal communication). This marker has a greater persistence than Pig2Bac in fresh and marine waters (32) and might be considered for pig manure tracking at the catchment level.

Correlation between the general *Bacteroidales* marker AllBac and the fecal indicators *E. coli* and enterococci has been previously reported (11). In the present study, we used AllBac as an indicator of the total *Bacteroidales* level in the samples. Host-associated *Bacteroidales* markers were not quantified in samples with low AllBac concentrations, i.e., between the LOQ and 1.5 log higher ($n = 39$). AllBac was quantified in more samples than either of the traditional fecal indicators and appeared, therefore, as a potential tool to confirm the presence of microbes of fecal origin in cases of low contamination. However, a major limitation to the use of AllBac as a fecal indicator is that *Bacteroidales* found in the environment may not always be of enteric origin (37).

Specificity of the four FRNAPH genogroups was high (87 to 100%). Presence of FRNAPH I in WWTP effluents in the present study (11%) was similar to that previously reported in South Carolina (11%, $n = 133$) (34) or Korea (28%, $n = 38$) (18). This could have been due either to FRNAPH I presence in human effluents or to the presence of feces from wild animals such as seagull, duck, or dog in the WWTP effluents. In contrast, low sensitivity of FRNAPH genogroups I, III, and IV and their absence from bovine and ovine feces and effluents means that they are not suitable for use as routine MST tools (Table 1). FRNAPH I and IV were not detected at site A2, where Rum2Bac was quantified in 95% of the samples that had ≥2.7 MPN/100 ml, providing further confirmation that these genogroups cannot be used to trace ruminant contamination. The animal FRNAPH I and IV markers appeared to weakly trace avian feces and pig manure in point source samples (Table 1). However, they were not found in the water samples collected following the accidental pig manure spillage and so did not appear efficient for tracing pig manure contamination in environmental waters. Sensitivity of FRNAPH II (86%) was satisfactory for an MST approach. Similarly, Blanch et al. (3) reported that, among the four genogroups, FRNAPH II was the marker that showed the highest difference in abundance between human and animal effluent samples ($n = 185$). On the scale of the present catchment, FRNAPH I and II were weakly correlated with fecal bacterial indicators. FRNAPH III and IV were not present in water samples of the data set covering the 10 sites monitored over 2 years (0 and 1 of 240, respectively), providing another indication that they were not relevant for use as MST tools at the catchment level.

We recommend the use of the host-associated *Bacteroidales* markers HF183 and Rum2Bac for routine MST application on environmental waters. When the *E. coli* level exceeded 2.7 $\log_{10}$ MPN/100 ml, both markers enabled 73% of the water samples to be classified (Table 4). This classification rate is similar to the one obtained on wastewater, feces, and manure point source samples with the combined use of 3 markers (*Bifidobacterium adolescensis* and cattle- and pig-associated mtDNA Bomito and Pomito) (76% correct classification) (1). The classification rate increases with the number of markers used to trace either the same host or additional ones. In the present study, the combined use of four MST markers, both *Bacteroidales* markers and the two main FRNAPH genogroups (I and II), enabled the classification of 81% of the water samples that had ≥2.7 $\log_{10}$ MPN/100 ml (Table 4). The improvement in the classification rate with the addition of FRNAPH genogroups I, III, or IV and their specificities was not sufficient to justify the extra time and cost required for plaque analysis at the level of this catchment. Ballesté et al. (1) reported 90% correct point source sample classification with the combined use of 5 markers (the three mentioned above, plus human-associated *Bacteroidales* HF134 and *Bifidobacterium dentium*). Based on the outcome of our 2 years of monitoring and on their specificity and sensitivity, we do not recommend FRNAPH I, III, or IV by culture/genotyping for routine MST application in water samples at the catchment level. FRNAPH II could be useful and provide additional information to that obtained with HF183.

Since DNA extraction recovery was not assessed in the present study, *Bacteroidales* marker concentrations were probably underestimated, and the variability in the extraction efficiencies between samples may affect the assessment of the microbial variability in the environment. The present results did not reveal any effect of the turbidity on PCR inhibitor content or negative associations between turbidity and the *Bacteroidales* marker concentrations (Table 3). Stoeckel et al. (36) showed that the maturation of microbial source tracking as an environmental monitoring and management tool brings with it the need for enhanced standardization and quality control of DNA extraction.

The various *Bacteroidales* marker concentrations below the LOQ were known to be somewhere between zero and the LOQ and are typically attributed a value in this range. In our case, LOQ varied greatly among the samples (from 2.4 to 3.7 $\log_{10}$ copies/100 ml water). Values below the LOQ were thus arbitrarily assigned a value of 0, as previously used by Nnane et al. (24), to avoid the statistical outcomes of the environmental variability being affected by LOQ variation. When the LOQ did not vary, authors of another study replaced the censored values by the LOQ or excluded them from the analysis altogether, which had the same effect on the results even with the reduced data set (28). Another previous study replaced these nonquantified values with random values between zero and the LOQ to compute sums and test hypotheses (29). However, though these practices are common in the environmental sciences, the strength of statistical analyses would be improved by the use of methods designed specifically for handling censored data without modifying the distribution artificially (14). Such methods are standard procedures in medical studies and should be generalized so that software for censored data methods is available for our specific field (14).

Consequently, one may question our decision to use the parametric statistical approach, though assumptions of normality or equality of variance were violated in several cases, even after log transformation (data not shown) and the *Bacteroidales* markers were used as quantitative variables. The use of parametric or nonparametric approaches gave very similar results, as did the *Bacteroidales* markers as quantitative or semiquantitative (absence/presence) variables (data not shown). Indeed, homogeneous subgroups differed on only 4 occasions out of 19 comparisons between ANOVA (parametric) or Kruskal-Wallis (nonparamet-

ric) tests, including 3 for which an intermediate subgroup (ab) was defined by the Kruskal-Wallis approach when ANOVA defined two significantly different groups (a and b). The same homogeneous subgroups as those shown in Table 2 were generated by using the *Bacteroidales* marker results as qualitative variables. Correlations assessed using the parametric approach (Pearson) with *Bacteroidales* markers as quantitative variables (Table 3) or using the nonparametric approach (Spearman) with *Bacteroidales* markers as semiquantitative variables differed by only 0.001 ± 0.053. Additionally, the numbers of observations used in all the tests were high (from 24 to 240). Parametric statistical testing on quantitative variables was selected for its sensitivity, i.e., this method is more powerful for revealing differences or associations than its nonparametric counterparts. We, nevertheless, used the *Bacteroidales* marker data as semiquantitative to trace the most frequent contamination observed at each group of urban, agricultural, or coastal sites (Fig. 2).

Specificity and sensitivity of host-associated markers should be locally validated in the region or country where they will be used before their inclusion in a routine MST toolbox. HF183 specificity and sensitivity have already been validated in several regions of the world (35). However, to the best of our knowledge, Rum2Bac has been tested only in France (22). Regional variations in specificity and sensitivity may occur due to diet or feeding differences. For instance, the ruminant-associated marker *Bacteroidales* CF128, reported in ≤10% of pig feces samples in Oregon and Ireland, was reported in ≥90% of pig feces samples in France, Portugal, and the United Kingdom (10% specificity) (2, 9). Conclusions on the source of the fecal contamination should not be based on individual water samples. Instead, MST campaigns should be run at regular intervals and combined with classical fecal indicator monitoring and should include samples taken in both low- and high-rainfall periods, so as to obtain an overall picture of fecal input dynamics. Replicate sampling should be planned at regular intervals to ensure the reliability of the procedure. Future studies should focus on (i) DNA extraction recovery assessment and the establishment of standardized quality control, (ii) testing additional host-associated markers to complete the MST toolbox, with at least two host-associated markers for each host and host-associated markers targeting other hosts besides human and ruminant, e.g., markers associated with dog feces, bird feces, or pig manure.

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