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Identification of the origin of faecal contamination in estuarine oysters using *Bacteroidales* and F-specific RNA bacteriophage markers

S. Mieszkin[1], M.P. Caprais[1], C. Le Mennec[1], M. Le Goff[1], T.A. Edge[2], M. Gourmelon[1,*]

[1] Laboratoire Santé Environnement et Microbiologie, Unité SG2M, Département RBE, IFREMER, Plouzané, France
[2] Water Science & Technology Directorate, National Water Research Institute, Environment Canada, Burlington, ON, Canada

*: Corresponding author : Michèle Gourmelon, email address : Michele.Gourmelon@ifremer.fr

Abstract :

Aims

The aim of this study was to identify the origin of faecal pollution impacting the Elorn estuary (Brittany, France) by applying microbial source tracking (MST) markers in both oysters and estuarine waters.

Methods and Results

The MST markers used were as follows: (i) human-, ruminant- and pig-associated Bacteroidales markers by real-time PCR and (ii) human genogroup II and animal genogroup I of F-specific RNA bacteriophages (FRNAPH) by culture/genotyping and by direct real-time reverse-transcriptase PCR. The higher occurrence of the human genogroup II of F-specific RNA bacteriophages using a culture/genotyping method, and human-associated Bacteroidales marker by real-time PCR, allowed the identification of human faecal contamination as the predominant source of contamination in oysters (total of 18 oyster batches tested) and waters (total of 24 water samples tested). The importance of using the intravalvular liquids instead of digestive tissues, when applying host-associated Bacteroidales markers in oysters, was also revealed.

Conclusions

This study has shown that the application of a MST toolbox of diverse bacterial and viral methods can provide multiple lines of evidence to identify the predominant source of faecal contamination in shellfish from an estuarine environment.
Significance and Impact of the Study

Application of this MST toolbox is a useful approach to understand the origin of faecal contamination in shellfish harvesting areas in an estuarine setting.

Keywords : *Escherichia coli* ; Estuarine and river waters ; F-specific RNA bacteriophages ; host-associated *Bacteroidales* markers ; microbial source tracking ; shellfish

1. Introduction

The microbiological quality of coastal environments can be impacted by urban and agricultural fecal wastes, and shellfish in these areas can accumulate and concentrate pathogenic microorganisms present in surrounding waters by their filter-feeding activities (Potasman *et al.* 2002). This can lead to closures or downgrading of shellfish-harvesting areas, and to outbreaks of food-poisoning through consumption of contaminated shellfish (Rabinovici *et al.* 2004, Iwamoto *et al.* 2010). In Europe, fecal contamination in shellfish-harvesting areas is currently evaluated by enumeration of the fecal indicator bacteria *Escherichia coli* (*E. coli*) in shellfish (Anonymous 2004). Consequently, shellfish harvesting areas are classified in three categories: A, B and C with shellfish having less than 230, 4,600 (for 90% of the samples) and 46,000 *E. coli* per 100g total flesh, respectively.

However, as *E. coli* can be found in diverse human and animal fecal wastes, it is frequently difficult to determine its source which is needed to guide pollution remediation actions. To overcome this problem, Microbial Source Tracking (MST) methods have been developed to discriminate between human and non-human fecal sources, and between different animal fecal sources contaminating the environment (USEPA 2005). Although a variety of different applications of MST methods have been successfully described in waters (Vogel *et al.* 2007, Reischer *et al.* 2008, Gourmelon *et al.* 2010b), few studies have been performed on naturally contaminated shellfish in marine or estuarine environments and were mainly focused on viruses (Vantarakis *et al.* 2006, Wolf *et al.* 2008, 2010). Detection of viral nucleic acid from the shellfish matrix can be difficult and cannot rely on an enrichment step as often used to enhance the detection of bacterial MST markers (Vantarakis *et al.* 2000, Roslev *et al.* 2009, 2010). As microorganisms are mainly concentrated in digestive tissues (DT), their direct detection in shellfish, especially for pathogenic enteric viruses or viral MST markers, has been mainly performed from these tissues (Dore and Lees 1995, Le Guyader *et al.* 2009, Wang *et al.* 2010, Wolf *et al.* 2010). To our knowledge, diverse bacterial and viral library-independent methods in a MST toolbox have yet to be applied in the same study to investigate the sources of fecal contamination in shellfish from an estuarine environment.

In the present study, we selected two types of MST targets i.e. host-associated *Bacteroidales* and genogroups of F-specific RNA bacteriophages (FRNAPH) for investigating shellfish and associated harvesting waters. Firstly, the human-(Hum-1-Bac), ruminant-(Rum-2-Bac) and porcine-(Pig-2-Bac) associated *Bacteroidales* markers by real-time PCR were selected due to their high sensitivity (92-100%) and specificity (93-99%) on target and non-target feces and effluent samples, respectively (Mieszkin *et al.* 2009, 2010a, 2010b). Secondly, viral markers represented by the human FRNAPH genogroup II (FRNAPH II) and the animal FRNAPH genogroup I (FRNAPH I) were also used to identify the origin of the fecal contamination in shellfish areas. The viruses were investigated by culture-dependent methods using genotyping by real-time Reverse-Transcriptase PCR (real-time RT-PCR) on isolates, and by culture-independent method using direct genotyping by real-time RT-PCR on nucleic acid extracts from digestive tissues or water samples (Ogorzaly and Gantzer 2006, Vantarakis *et al.* 2006).

These two types of MST targets were combined in a MST toolbox approach for identifying the origin of fecal contamination in shellfish in our study due to the potential limitations of applying only single MST marker. Host-associated *Bacteroidales* markers have been found to be only weakly detected or tested with the DT of a variety of naturally contaminated shellfish (Gourmelon *et al.* 2010a, Cornelisen *et al.* 2011), and the animal FRNAPH genogroup I has not been useful for tracing bovine fecal contamination in France (Mauffret *et al.* 2012).

The aim of this study was to evaluate the occurrence of both bacterial and viral MST markers and to discriminate the origin of fecal contamination in oyster batches and water samples from a French estuary with contrasting environmental conditions. Both FRNAPH and host-associated *Bacteroidales* makers were investigated in oyster DT. In addition, exploratory analyses also investigated host-associated *Bacteroidales* markers in oyster intravalvular liquids (IL). The IL, a liquid compartment, was selected to investigate its potential for better bacterial DNA recovery and less PCR inhibitors than other compartments and tissues of shellfish (Kaufman *et al.* 2004).

2. Materials and Methods

2.1. Study area

The catchment of the Elorn estuary (Brittany, France) covers 385 km^2, and it is located in the North-East of the bay of Brest. It is mainly characterized by intensive livestock farming (cows [26,680], pigs [308,460], and poultry [889,200]), and the total number of human inhabitants is estimated at approximately 63,500 (Fig. 1).

Live oysters (*Crassostrea gigas*) from a shellfish-farming area (Logonna Daoulas, Brittany, France) intended for human consumption were directly set on six sampling sites (oysters were emerged at mid-tide when the tide coefficient was superior to 70) throughout the estuary (sites Landerneau, Vervian, Le Pouldu, Pen An Trein, Le Passage and Camfrout) at least one month before the sampling periods. According to the European classification of shellfish-harvesting areas based on *E. coli* concentrations (Anonymous 2004), the sampling sites of Landerneau and Vervian, located in the upstream part of the estuary, are classified as category C (*E. coli*<46,000 CFU per 100 g total flesh), the sampling sites Pen An Trein and Le Pouldu are classified as category B (*E. coli*< 4,600 CFU per 100 g total flesh for 90% of the samples) and Le Passage and Camfrout, located in the downstream part of the estuary, as category B and non-classified corresponding to a prohibited area, respectively. The sampling sites Camfrout and Le Pouldu are also close to water bathing areas classified as categories A (*E. coli*<100 CFU per 100 ml water for 80% of the samples and *E. coli*<2,000 CFU per 100 ml water for 95% of the samples) and B (*E. coli*<2,000 CFU per 100 ml water for 95% of the samples), respectively (CEC 1976) (Figure 1).

2.2. Collection of oyster batches and water samples

Three sampling periods were carried out from March to May 2010 at the six sampling sites previously described. For each sampling period and site, one oyster batch (33 oysters) and a two litre sample of estuarine water surrounding the oysters, were collected at low tide. A total of 18 oyster batches and 18 estuarine water samples were collected during this study. In addition, at the sites Le Pouldu and Camfrout, a two litre sample of tributary water from the main river located upstream to these sites was also collected resulting in a total of six river water samples.

All the samples were placed in sterile containers and transported in insulated coolers. Upon arrival at the laboratory, water samples were directly analysed by membrane filtration and oysters were scrubbed under running tap water to remove debris and attached algae, then opened and dissected aseptically for analyses.

2.3. Bacteroidales markers

2.3.1. Sample preparation and DNA extraction

For *Bacteroidales* markers, filtration of water samples (500 mL) and bacterial DNA extractions were processed as described in Mauffret *et al.* (2012). Oysters were dissected to analyse the digestive tissues (DT) and the intravalvular liquids (IL) and then conserved at -80°C before bacterial DNA extraction. Briefly, around 4-10 g of DT (from nine oysters) were chopped up with a sterile scalpel, and 200 mg (wet weight) of DT were placed into Lysing E Matrix tubes with 1 ml of DNAzol. This was followed by a physical lysis using FastPrep FP120 (speed 5.5; 30 s; MP Biomedical, France). After centrifugation, DNA extractions were performed on supernatants according to the supplier's instructions (DNAzol, Invitrogen, France). For the IL, 15 ml-aliquots were centrifuged at 2,000 g for 15 min, and DNA extractions were performed on pellets using the Fast DNA spin kit for soil according to the supplier's instructions (MP Biomedical, France).

2.3.2. Real-time PCR

General (AllBac), human- (Hum-1-Bac), ruminant- (Rum-2-Bac) and pig- (Pig-2-Bac) associated *Bacteroidales* MST markers were quantified by real-time PCR following Mauffret *et al.* (2012) and using primers and probes described by Layton *et al.* (2006) and Mieszkin *et al.* (2009, 2010a, 2010b), respectively. Standard curves were generated using serial 10-fold dilutions of a plasmid preparation containing the target sequence for *Bacteroidales* markers as described in Mieszkin *et al.* (2009, 2010a, 2010b). Linear DNA plasmids containing partial 16S rRNA gene sequence inserts were used as standards at 10-fold dilutions ranging from 1.6×10^7 to 1.6×10^0 copies per PCR, with a limit of quantification (LOQ) of five target gene copies/reaction 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. Consequently, the lower LOQ of the MST markers was 2.7 Log_{10} gene copies 100 ml^{-1} water, 1.2 Log_{10} gene copies ml^{-1} IL and 4.1 Log_{10} gene copies g^{-1} DT.

2.4. F-specific RNA bacteriophages and FRNAPH genogroups

2.4.1. Sample preparation and FRNAPH enumeration

Water samples (500 ml) were processed as described by Mauffret *et al.* (2012) but were concentrated using a Centricon Plus-70 centrifugal filtration device (Millipore, France). For each sample, the pellet was recovered by inverse centrifugation in 5 ml-elute (NaCl 0.5 M, beef extract 1%, Tween 80 3%). Then, three milliliters of the undiluted elute was assayed for the enumeration of total FRNAPHs by using three 1-ml portions, 90-mm petri dishes, and a standard double agar overlay method with *Salmonella typhimurium* strain WG49 (ISO 10705-1, 1995; Havelaar *et al.* 1986). Briefly, 1-ml portions of undiluted elute were added to 2.5-ml portions of molten 1% tryptone-yeast extract glucose agar (TYGA) with nalidixic acid (100 mg l^{-1}), and to 1-ml portions of culture of the host *Salmonella typhimurium* WG49 in its early log phase. Then, the mixture was poured onto 1% TYGA base in a 90-mm petri dish. Finally, plaques were counted after overnight incubation at 37°C. The host strain used ensures a highly selective count of total FRNAPHs (Havelaar *et al.* 1986). Results were expressed in Plaque Forming Units (PFU) 100 ml^{-1} water.

For oysters, 4-10 g of DT were prepared as described for *Bacteroidales* markers, and then homogenized in two volumes of 1% peptone saline water with a Polytron (Kinematica AG, Switzerland) using three bursts of 20 s. The supernatant was then centrifuged at 2,000 g for

20 min and total FRNAPHs were enumerated as previously described using three 1 ml-portions of supernatant. Results were then expressed in PFU g^{-1} DT.

2.4.2. FRNAPH genotyping

By culture/genotyping

More than 20 plaques were picked up and these isolates were spotted on two agar plates containing *Salmonella typhimurium* WG 49 host, one with 100 µl ml^{-1} of RNase A (Roche Diagnostics, France) and the other one without RNase to distinguish between DNA and RNA phages. Isolates growing on agar containing RNase and corresponding to F-DNA bacteriophages were not considered.

After lysis step at 95°C for 5 min, genotyping of the FRNAPH I and II from isolates was performed by real-time RT-PCR with a One-Step RT-PCR kit (QuantiTech Probe RT-PCR, Qiagen, France) (Ogorzaly *et al.* 2009) using the appropriate final concentrations of primers and probes previously described by Ogorzaly and Gantzer (2006) with a final volume of 25 µl. Positive controls: FRNAPH I/MS2 and FRNAPH II/GA, and negative controls (no template RNA) were performed for each run.

Only results with at least 20 FRNAPH plaques successfully genotyped per sample were considered. Results were expressed in term of presence/absence. A FRNAPH genogroup was considered to be present in a sample when at least five of the 20 FRNAPH plaques genotyped were associated to this genogroup (Mauffret *et al.* 2012).

By direct real-time RT-PCR

The two remaining millilitres of 5 ml-elutes from concentrated waters were used for RNA extraction to perform direct real-time RT-PCR genotyping. Briefly, two volumes of NucliSens lysis buffer (BioMerieux, France) were added to these concentrated water samples, followed by 30 min of incubation at 56°C. RNAs were then extracted with a NucliSens Magnetic extraction kit (BioMérieux, Lyon, France) as previously described (Zakhour *et al.* 2010).

Concerning oysters, RNA extraction for direct real-time RT-PCR was performed from an additional 6 g of DT using the NucliSens magnetic extraction kit according to Le Guyader *et al.* (2009) and Zakhour *et al.* (2010).

Real-time RT-PCR assays of FRNAPH I and FRNAPH II were performed on RNAs extracted from water and oyster samples using the primers and probes designed by Ogorzaly and Gantzer (2006) and the RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen, France). Amplifications were performed using a Stratagene MX3000P with software version 4. Standard curves were generated using serial 10-fold dilutions of plasmid preparations containing the target sequence for FRNAPH I and II. Linear plasmids containing partial genome sequence inserts were used as standards at 10-fold dilutions ranging from 2.5×10^6 to 2.5×10^0 and from 2.1×10^6 to 2.1×10^0 genome copies/real-time RT-PCR, with a LOQ of 25 and 20 genome copies/reaction per PCR well in the triplicate PCR assays, respectively, which was the lowest quantity of linear plasmid DNA of the standard curve made for each PCR assay. Consequently, the lower LOQ for the FRNAPH I and II was 2.4 and 2.3 Log_{10} genes copies 100 ml^{-1} water, and 1.8 and 1.9 Log_{10} genes copies g^{-1} DT, respectively.

2.5. Enumeration of E. coli bacteria

E. coli concentrations in oysters were estimated by analysing six oysters per batch using the AFNOR standardized five-tube MPN method (Anonymous 2000) with a detection limit of <15 MPN (Most Probable Number) 100 g^{-1} total flesh. In water samples, *E. coli* concentrations were obtained by the microplate method (Anonymous 1999) with a detection limit of <38 MPN 100 ml^{-1} water.

2.6. Chemophysical analysis

Measurements of physical and chemical parameters were performed on all waters sampled. Temperature (T°C), dissolved oxygen (mg l^{-1}) and salinity (practical salinity unit (psu)) measurements were taken *in situ* using oxygen and salinity probes (HACH, HQ30d flexi and WTW, Cond 340i). Turbidity (Nephelometric Turbidity Units; NTU) measurements were performed at the laboratory using a turbidity meter (Neotek-Ponsel, Odeon). Rainfall data were obtained from Météo-France station of Guipavas (Brittany, France).

2.7. Statistical analyses

Statistical analyses were performed using XLSTAT Version 2011.2.02 to assess the correlations between *E. coli,* MST markers and chemophysical parameters using Spearman coefficients. Quantified values for *E. coli* and the general markers were used with values below the LOQ set at 0. For the MST markers, quantified values were analysed in terms of presence/absence (values were set at 1 when values > LOQ and values were set at 0 when values < LOQ, respectively). Thresholds were considered significant when *p-value < 0.05.*

Results

3.1. Rainfall intensity and chemophysical parameters in estuarine water samples

Sampling was carried out under contrasting meteorological conditions. The 1st, 2nd and 3rd collections were characterized respectively by a rainfall intensity of 29.6, 6.8 and 6.0 mm/48h before the sampling date.

Chemophysical parameters of the estuarine water samples collected are presented in the supplemental material (Table S1). Values or concentrations of temperature, dissolved oxygen and pH remained stable between the water samples for each collection, while salinity and turbidity varied between the sites. Low salinities were mainly obtained for the sites located upstream: Landerneau, Vervian Pen An Trein and Pouldu (mean = 11.5 ± 13.5 psu, *n = 12*) and higher salinities were obtained for sites located downstream: Le Passage and Camfrout (mean = 25.4 ± 8.8 psu, *n = 6*). High values of turbidity were mainly obtained for the sites Vervian and Camfrout (mean = 105.9 ± 56.2 NTU, *n = 6*).

3.2. E. coli concentrations in oyster batches

Concentrations of the fecal indicator *E. coli* in the 18 oyster batches analysed were found to vary according to the sites and the sampling date. *E. coli* concentrations were < 2.4 Log_{10} MPN 100 g^{-1} total flesh in three of the nine oyster batches collected in the sites classified in B category (Pen An Trein, Le Pouldu and Le passage). Intermediate *E. coli* concentrations, between 2.4 – 3 Log_{10} MPN 100 g^{-1} total flesh, were obtained in two of the nine oyster batches collected from the sites classified in B category, and in three of the six oyster

batches collected from the sites classified in C category (Vervian and Landerneau). Finally, *E. coli* concentrations > 3 Log_{10} MPN 100 g^{-1} total flesh were found in four of the nine oyster batches collected from the sites classified in B category, three of the six oyster batches collected from the sites classified in C category and in all of the three oyster batches collected in the non-classified site (Camfrout, located in a prohibited area for shellfish collection) (Table S2).

3.3. Detection of MST markers in oyster batches

3.3.1. FRNAPH genogroups I and II

The human FRNAPH II marker was the most commonly detected in oyster batches with detection in 83% and 94% of samples analysed (n=18) by culture/genotyping and by direct real-time RT-PCR, respectively. The animal FRNAPH I was always detected in association with the FRNAPH II, and it was detected in 51% and 61% of samples by culture/genotyping and by direct real-time RT-PCR, respectively (Table 1). Concentrations of the FRNAPH II were 1.3 to 2.2 Log_{10} higher than concentrations of the FRNAPH I, highlighting even more the predominance of human fecal contamination in these samples (Table S2).

3.3.2. Host-associated Bacteroidales markers

None of the host-associated *Bacteroidales* markers were detected in the DT of oysters (data not shown). The detection of host-associated *Bacteroidales* markers was greatly improved by investigating another shellfish part, the intravalvular liquids (IL). The Hum-1-Bac marker was detected in 56% of the oyster batches analysed while the Rum-2-Bac (22%) and the Pig-2-Bac (0%) MST markers were rarely detected in these batches. Furthermore, the Rum-2-Bac marker was detected each time in association with the Hum-1-Bac marker, as was the FRNAPH I with the FRNAPH II (Table 1).

3.3.3. Spearman correlation analyses

Correlation analyses for oyster batches showed that the Hum-1-Bac marker was most highly correlated with *E. coli* and the general markers (Tot FRNAPHs and AllBac) (p<0.05) (Table 2). Indeed, the Hum-1-Bac marker was not detected in oyster batches associated with a concentrations of *E. coli* <2.4 Log_{10} MPN 100 g^{-1} total flesh while it was detected in up to 80% of the oyster batches analysed with a concentration of *E. coli* > 3 Log_{10} MPN 100 g^{-1} total flesh (Fig. 2). Therefore, the Hum-1-Bac marker was found to increase in frequency of occurrence in oyster batches as *E. coli* concentrations increased (Fig. 2). The FRNAPH II by culture/genotyping and direct real-time RT-PCR were correlated with each other (p<0.05), but not with other variables for oysters. Interestingly, the Hum-1-Bac marker, *E. coli* and the general markers were correlated (p<0.05) with two-day rainfall (Table 2).

3.4. Detection of viral and bacterial MST markers in waters

3.4.1. In estuarine waters surrounding oysters

The human FRNAPH II was detected in 67% of estuarine water samples (n=18) by culture/genotyping but in only 6% of estuarine water samples by direct real-time RT-PCR. In addition, the Hum-1-Bac marker was detected in 33% of estuarine water samples. Animal MST markers were not common in these waters; only the Rum-2-Bac marker and the

FRNAPH I by culture/genotyping were detected in 11% and 22% of the estuarine waters, respectively (Table 1).

3.4.2. In river tributaries

The human FRNAPH II was detected in 67% of river water samples by culture/genotyping, although in only 17% of river samples by direct real-time RT-PCR. The Hum-1-Bac marker was detected in all river samples at high concentrations (from 3.3 to 6.5 Log_{10} copies 100 ml^{-1}). None of the animal MST markers were detected in these river water samples (Table 3S).

3.4.3. Spearman correlation analyses for estuarine waters

Correlation analyses for estuarine waters showed that the Hum-1-Bac marker was also significantly correlated with *E. coli* and the general AllBac marker (p<0.001), and to a lesser extent with the Tot FRNAPHs and the FRNAPH II by culture/genotyping (p<0.05) (Table 3). Indeed, Hum-1-Bac was not detected in estuarine water samples associated with a concentrations of *E. coli* <2.7 Log_{10} MPN/100 ml water while it was detected in up to 83% of estuarine water samples with concentrations of *E. coli* >3 Log_{10} MPN 100 ml^{-1} water (Fig. 2). As previously mentioned for oyster batches, the Hum-1-Bac marker, and to a lesser extent the FRNAPH II by culture/genotyping, were found to increase in frequency of occurrence in estuarine water samples as *E. coli* concentrations increased (Fig. 2). The human FRNAPH II by culture/genotyping was also weakly correlated with *E. coli* and the general markers (p<0.05). In addition, *E. coli*, the general markers, and the host-associated markers, with the exception of the FRNAPH II by direct real-time PCR, were all highly negatively correlated with salinity (p<0.01) (Table 3).

4. Discussion

Application of both bacterial and viral MST methods found that oyster batches and estuarine waters collected throughout the Elorn estuary were predominantly impacted by human fecal pollution and to a lesser extent by animal fecal pollution.

The human FRNAPH genogroup II was the most common marker detected in digestive tissues (DT) of oysters using both culture/genotyping (83% of samples) and direct real-time RT-PCR (94% of samples) methods. These results confirmed the great interest in using FRNAPH genogroups to identify the origin of fecal contamination in shellfish (Vantarakis *et al.* 2006, Wolf *et al.* 2008, 2010). Detection of host-*Bacteroidales* markers by real-time PCR in DT of oyster batches led to poor results and confirmed that this method was not efficient for identifying the origin of fecal contamination in shellfish. These results are similar to results obtained by Gourmelon *et al.* (2010a), where ruminant-(BacR) and human-(HF183) associated *Bacteroidales* markers were detected in only one clam and two mussel samples of DT from a naturally contaminated area (Guerande-Atlantique peninsula, n=80). Several hypotheses may explain why host-associated *Bacteroidales* markers were not detected in these tissues: (i) the low quantity of DT used to extract bacterial DNA using commercial kits (200 mg) leads to a high limit of quantification, (ii) there would have some difficulties for separating bacteria from oyster tissues and then concentrating them, (iii) the presence of PCR inhibitors in DT requires greater dilution of bacterial DNA extracts (10-fold in this study) and, (iv) there would be a low level of fecal contamination in the environment leading to a low presence of *Bacteroidales* cells in the DT and consequently of 16S rRNA gene sequences in these tissues. These results also confirm the difficulties involved in quantifying bacteria in shellfish tissues using PCR techniques without a prior enrichment step (Brands *et al.* 2005, Gourmelon *et al.* 2006, Roslev *et al.* 2009, 2010). In contrast, the detection of host-

associated *Bacteroidales* markers in oyster batches was greatly improved by investigating another shellfish part, the intravalvular liquids (IL). The predominance of human fecal contamination was confirmed by the more frequent occurrence of the human-associated *Bacteroidales* marker (Hum-1-Bac) in oyster batches (56% of IL samples). Consequently, even though the IL fraction of shellfish may contain a lower concentration of bacteria than in total flesh or DT (Plusquellec *et al.* 1990), the ease of recovery of genomic bacterial DNA from the liquid (using the commercial kit "Fast DNA spin kit for soil"), is a useful and promising alternative method for applying host-associated *Bacteroidales* markers in oysters.

Correlation analyses for oysters showed some interesting results. The Hum-1-Bac marker was found to increase in frequency of occurrence in oyster batches as *E. coli* concentrations increased and it was also correlated with the general markers (Tot FRNAPHs and AllBac). Rainfall events were found to have a major role for increasing bacterial contamination in oysters at the scale of an entire estuary as the Hum-1-Bac marker, the general markers (Tot FRNAPHs and AllBac) and *E. coli* were significantly correlated with two-day rainfall. These results are in agreement with the study of Riou *et al.* (2007) where it was demonstrated that *E. coli* concentrations in estuarine waters close to shellfish harvesting areas were 50-fold higher when rainfall was greater than 10 mm per day.

Application of the MST toolbox in estuarine waters surrounding oysters also found that both viral and bacterial human MST markers were mainly detected, confirming the presence of human fecal pollution impacting this estuary.

The human FRNAPH genogroup II (detected by culture/genotyping) was the most common marker detected in estuarine water samples (67% of samples) followed by the Hum-1-Bac marker (33% of samples). A weak detection of the human FRNAPH genogroup II using the direct real-time RT-PCR method was obtained (6% of samples) highlighting the need for protocol improvement to detect such viruses in water. The FRNAPH II and FRNAPH I were previously quantified by real-time RT-PCR in water samples collected from a river located in an urbanised watershed characterised by high *E. coli* concentrations (average of 3.99 Log_{10} MPN 100 ml^{-1} water (n=23)) (Ogorzaly *et al.* 2009). Therefore, the low frequency of detection in the present study could be due to (i) PCR inhibition, (ii) the lower level of fecal contamination and *E. coli* concentrations (average of 2.8 Log_{10} MPN 100 ml^{-1} (n=18)) or, (iii) due to different RT-PCR procedures (reverse transcription and PCR in only one step were used in this study while reverse transcription and PCR performed with two different steps were used in Ogorzaly *et al.* (2009)).

Correlation analyses for estuarine waters demonstrated that the Hum-1-Bac marker and the human FRNAPH II by culture/genotyping were also significantly correlated with *E. coli* and the general markers. These results are in accordance with those obtained in oysters and suggest that MST markers could be useful to identify the origin of *E. coli* present in an estuarine environment. In addition, *E. coli*, the general markers, and the human-associated markers, with the exception of the FRNAPH II by direct real-time PCR, were all significantly highly negatively correlated with salinity showing a trend with sites located upstream associated with a lower salinity (Landerneau, Vervian, Pen an Trein and Pouldu; mean salinity 11.5 psu) and sites located downstream associated with a higher salinity (Le Passage and Camfrout; mean salinity 25.4 psu).

It was also suggested that fecal contamination impacting the estuary could be also likely due to the inputs of its various tributaries. Indeed, the Hum-1-Bac marker and the human FRNAPH II by culture/genotyping were detected respectively in 100% and 67% of river water samples collected from two river tributaries impacting the shellfish sites Camfrout and Le Pouldu. The frequent detection of the human MST markers in river water samples highlighted the importance of these tributaries for influencing the microbial quality of the estuary. Furthermore, these results were in accordance with the study of Pommepuy *et al.* (1987)

which demonstrated that the Elorn estuary could be mainly contaminated through inputs from human activities *via* river tributaries, WWTP effluents and sediment resuspension during spring tide.

This study has also demonstrated that the occurrence of MST markers in oyster batches was higher than in their surrounding estuarine waters. While it is well known that MST markers can be applied in water to successfully identify the origin of fecal contamination (US EPA 2005), these results show that they can also be applied on naturally contaminated oysters from coastal and shellfish-harvesting areas. Other MST markers at other locations have also been detected more commonly in shellfish than surrounding waters, likely due to the capacity of oysters to accumulate microorganisms through their filter feeding activities (Love *et al.* 2008, Roslev *et al.* 2009, 2010). Studies on MST bacterial markers in Denmark have shown that after the use of an enrichment step, human-associated *E. coli* and *Enterococcus* markers were more often detected by PCR in mussels than in seawater samples, despite direct contamination of the seawater by a nearby wastewater treatment plant (Roslev *et al.* 2009, 2010). A higher prevalence of FRNAPH I to IV, detected by real-time RT-PCR reverse line blot hybridization, was also observed in clams and mussels rather than in overlaying brackish or saline waters in the United States (Love *et al.* 2008). In a general manner, considering the results obtained in this study all together (oyster batches and water samples) for each site, the occurrence of the human MST markers (Hum-1-Bac and FRNAPH II) was more frequent at sites classified as C category (according to the European classification of shellfish-harvesting areas based on *E. coli* concentrations (Anonymous 2004)) such as Landerneau and Vervian, than for the site Le Passage classified as B category. This indicated shellfish sites categorized by higher *E. coli* concentrations were associated with more frequent occurrence of human MST markers indicative of sewage contamination.

In conclusion, this study has demonstrated that a MST toolbox of bacterial and viral methods, along with *E. coli* enumeration, can provide multiple lines of evidence to better understand the origin of fecal contamination in shellfish harvesting areas in an estuarine setting. The results identified the importance of human fecal pollution as a frequent source of contamination in oysters and their surrounding estuarine waters in this French estuary. Among the MST markers used, the detection of FRNAPH genogroups using a culture/genotyping method was the most efficient method to trace the origin of fecal contamination in both oyster batches and estuarine waters. Host-associated *Bacteroidales* markers were also successfully quantified in oyster batches when the intravalvular liquids were analysed instead of the digestive tissues.

Even though these results are promising, it would be important to evaluate the efficiency of these methods on other shellfish species and locations for validation at a larger scale. Furthermore, the comparison of these MST methods with others, such as mitochondrial DNA, chemical products, or other viruses such as noroviruses on the same shellfish batches, could also be of interest to obtain the most applicable MST toolbox for shellfish.

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Tables

Table 1 Discrimination of the origin of the fecal contamination in oyster batches and in estuarine and river water samples using bacterial and viral MST markers (% detection)

	Oysters (*n=18*)	Estuarine waters (*n=18*)	River waters (*n=6*)
Human-associated MST markers			
Hum-1-Bac	10 (56) *	6 (33)	6 (100)
FRNAPH II by culture/genotyping	15 (83) †	12 (67)	4 (67)
FRNAPH II by direct real-time RT-PCR	17 (94) †	1 (6)	1 (17)
Any human marker	17 (94) *,†	12 (67)	6 (100)
Animal-associated MST markers			
Rum-2-Bac	4 (22) *	2 (11)	0 (0)
Pig-2-Bac	0 (0) *	0 (0)	0 (0)
FRNAPH I by culture/genotyping	9 (50) †	4 (22)	0 (0)
FRNAPH I by direct real-time RT-PCR	11 (61) †	0 (0)	0 (0)
Any animal marker	11 (61) *,†	6 (33)	0 (0)

* Host-associated *Bacteroidales* markers were detected using the intravalvular liquids of oysters (none of them was detected in the digestive tissues of oysters); † FRNAPH genogroups were detected using the digestive tissues of oysters.

Table 2 Spearman correlations between *E. coli*, general and human-associated MST markers in oyster batches and rainfall at six sites on the Elorn estuary (18 observations per variable). Animal-associated markers were not taken into account for the analyses as there were too few detections.

Variables	*E.coli*	Tot FRNAPHs	AllBac	Hum-1-Bac	FRNAPH II rRT-PCR	FRNAPH II Cult/Gen
E.coli						
Tot FRNAPHs	0.39					
AllBac	0.46	0.17				
Hum-1-Bac	**0.57***	**0.56***	**0.66****			
FRNAPH II rRT-PCR	0.35	0.40	0.02	0.27		
FRNAPH II Cult/Gen	-0.17	0.21	-0.22	-0.10	**0.54****	
2-d rain	**0.52***	**0.63****	**0.51****	**0.69****	0.30	0.18

Quantified values for *E. coli* and the general markers (Tot FRNAPHs and AllBac) were used with values below the LOQ set at 0. Quantified values for MST markers were analyzed in terms of presence/absence (values were set at 1 when values ≥ LOQ and values were set at 0 when values< LOQ, respectively). Significance thresholds were rated as significant (*, $P < 0.05$) or highly significant (**, $P < 0.01$); *E. coli*, *Escherichia coli*; Tot FRNAPHs, total F-specific bacteriophages; AllBac, general *Bacteroidales* marker; Hum-1-Bac, human-associated *Bacteroidales* marker; FRNAPH II rRT-PCR, human genogroup II of FRNAPH by direct real-time RT-PCR; FRNAPH II Cult/Gen, human genogroup II of FRNAPH by culture/genotyping; 2-d rain, two-days rainfall.

Table 3 Spearman correlations between *E. coli*, general and human-associated MST markers and chemophysical variables in estuarine waters at six sites on the Elorn estuary (18 observations per variable). Animal-associated markers were not taken into account for the analyses as there were too few detections.

Variables	*E. coli*	Tot FRNAPHs	AllBac	Hum-1-Bac	FRNAPH II rRT-PCR%	FRNAPH II Cult/Gen	2-d rain	Salinity	Temp
E. coli									
Tot FRNAPHs	0.40								
AllBac	**0.62****	0.31							
Hum-1-Bac	**0.73*****	**0.53***	**0.82*****						
FRNAPH II rRT-PCR	-0.38	0.15	-0.26	-0.17					
FRNAPH II Cult/Gen	**0.57***	**0.54***	**0.53***	**0.50***	0.17				
2-d rain	**0.59***	-0.01	**0.62****	0.43	-0.30	0.43			
Salinity	**-0.66****	**-0.60****	**-0.61****	**-0.66****	0.07	**-0.73*****	-0.33		
Temp	**-0.59***	0.05	**-0.78*****	**-0.53***	0.30	0.46	**-0.95*****	0.46	
Turbidity	0.22	0.07	**-0.49***	-0.14	-0.12	0.02	-0.17	0.16	0.31

Quantified values for *E. coli* and the general markers (Tot FRNAPHs and AllBac) were used with values below the LOQ set at 0. Quantified values for the MST markers were analyzed in terms of presence/absence (values were set at 1 when values $\geq$ LOQ and values were set at 0 when values < LOQ, respectively). Significance thresholds were rated as significant (*, $P < 0.05$), highly significant (**, $P < 0.01$), or very highly significant (***, $P < 0.001$); *E. coli*, *Escherichia coli*; tot FRNAPHs, total F-specific bacteriophages; AllBac, general *Bacteroidales* marker; Hum-1-Bac, human-associated *Bacteroidales* marker; FRNAPH II rRT-PCR, human genogroup II of FRNAPH by direct real-time RT-PCR; FRNAPH II Cult/Gen, human genogroup II of FRNAPH by culture/genotyping; 2-d rain, 2-days rainfall; Temp, temperature.

Figures

Figure 1 Location of water and oyster sampling sites, waste water treatment plants (WWTP) and pig, bovine and poultry farms in the Elorn estuary, Brittany, France. Sources: IDHESA Bretagne Océane, BMO, DDAE, INSEE.

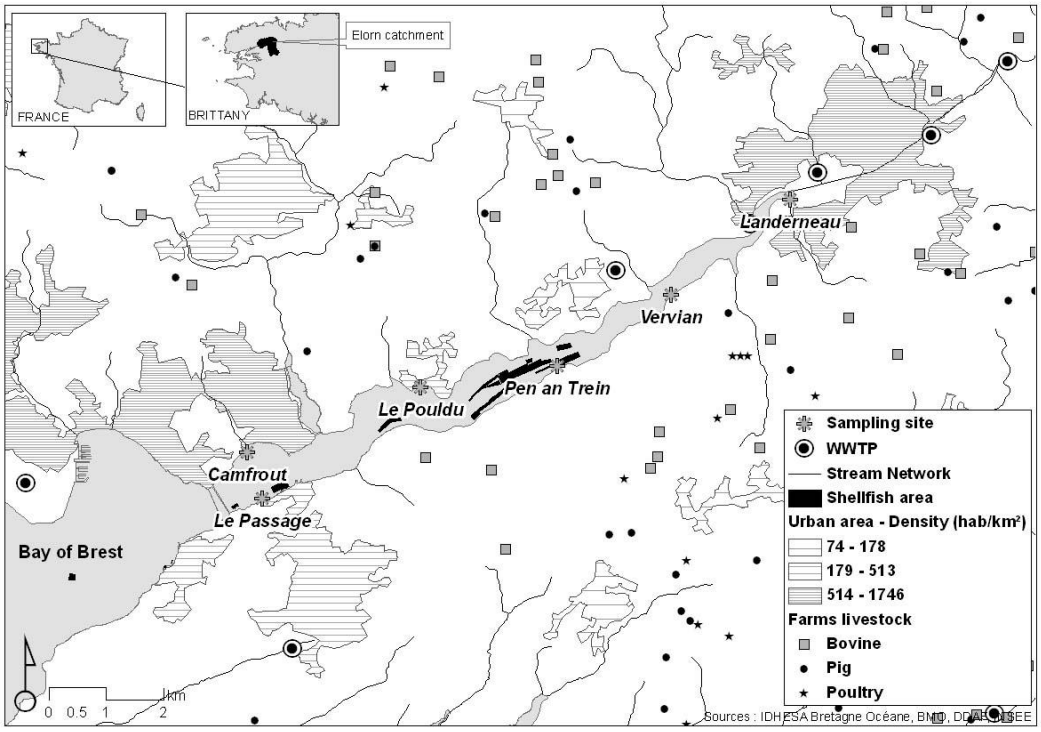


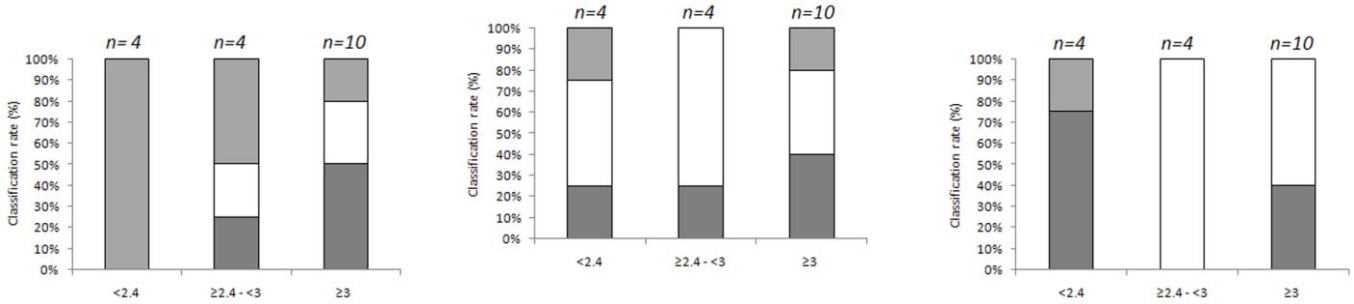
Figure 2 Fecal pollution source occurrence rate (%), at three levels of *E. coli* concentration in (a) oyster batches and, (b) estuarine water samples using host-associated *Bacteroidales* markers by real-time PCR, FRNAPH I and II by culture/genotyping and direct real-time RT-PCR.

a – Oyster batches

Host-associated *Bacteroidales* markers

FRNAPH I-II by culture/genotyping

FRNAPH I-II by direct real- time RT-PCR



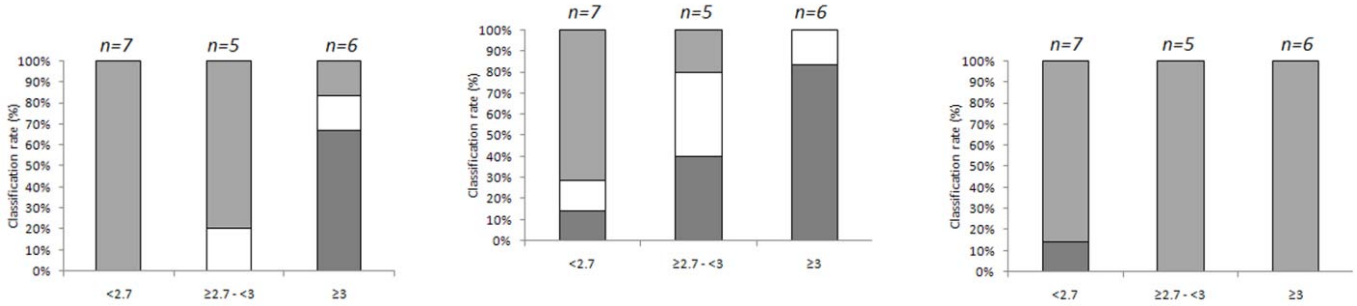
E. coli rank (Log_{10} MPN/100 g total flesh)

b – Estuarine waters

Host-associated *Bacteroidales* markers

FRNAPH I-II by culture/genotyping

FRNAPH I-II by direct real-timeRT-PCR



E. coli rank (Log_{10} MPN/100 ml water)

Human Mixed (Human + Animal) No assigned

Supporting Information

Table S1 Range of values or concentrations of chemophysical parameters in estuarine water samples collected throughout the Elorn estuary.

Estuarine waters	Rainfall (mm/48 h)	Tidal coefficient	Temp; (T°C)	Dissolved oxygen (mgl^{-1})	Salinity (psu)	Turbidity (NTU)
			Range (Min – Max)	Range (Min – Max)	Range (Min – Max)	Range (Min – Max)
1st sampling period - 03/01/2010 (*n=6*)	29.6	113	7.5 – 9.8	8.5 – 11.4	0.0 – 25.5	19 – 86.1
2nd sampling period - 03/29/2010 (*n=6*)	6.8	101	10.3 – 11.5	7.5 – 11	0.0 – 28.8	3.4 – 44.1
3rd sampling period - 05/16/2010 (*n=6*)	6.0	87	12.8 – 14.8	9.3 – 11.6	0.0 – 32.2	12.1 – 67.7

Table S2 Concentrations of the fecal indicator *E. coli*, the general markers (AllBac and Tot FRNAPHs), the host-associated *Bacteroidales* markers by real-time PCR and the FRNAPH I and II by direct real-time RT-PCR and presence of the FRNAPH I and II by culture/genotyping in oyster batchessampled from the six sites of the Elorn estuary.

Sites	Sampling date (mo/day/yr)	*E. coli* conc. (Log_{10} MPN/100 g total flesh)	*Bacteroidales* marker conc. (Log_{10} copies/ml IL)				FRNAPH genogroups by culture/genotyping				FRNAPH genogroup conc. by direct real-time RT-PCR (Log_{10} genome copies/g DT)	
			General AllBac	Human Hum-1-Bac	Ruminant Rum-2-Bac	Porcine Pig-2-Bac	Log_{10} PFU/g DT	Human genogroup isolates	Animal genogroup isolates	No. of plaques genotyped	Human FRNAPH II	Animal FRNAPH I
								II (GA)	I (MS2)			
Landerneau	03/01/2010	3.1	3.0	1.5	<1.2	<1.2	1.7	21**	1	22¶	4.2	2.1
	03/29/2010	2.8	3.6	2.0	1.5	<1.2	2.1	14	10	24	4.1	2.2
	05/16/2010	3.5	2.3	<1.2	<1.2	<1.2	1.6	17	5	22	3.0	<1.8§
Vervian	03/01/2010	3.1	2.8	1.8	<1.2	<1.2	2.0	23	1	24	4.6	2.9
	03/29/2010	2.8	3.3	1.4	<1.2	<1.2	1.9	10	14	24	4.1	2.3
	05/16/2010	2.4	<1.2*	<1.2	<1.2	<1.2	1.2	19	5	24	2.6	<1.8
Pen An Trein	03/01/2010	3.4	3.8	2.2	1.9	<1.2	1.7	19	4	23	2.4	2.5
	03/29/2010	3.1	3.8	1.8	<1.2	<1.2	1.5	18	6	24	3.5	<1.8
	05/16/2010	2.1	2.6	<1.2	<1.2	<1.2	1.9	24	0	24	2.6	<1.8
Le Pouldu	03/01/2010	3.5	3.5	2.3	1.9	<1.2	2.3	10	7	17	4.4	2.6
	03/29/2010	2.9	2.9	<1.2	<1.2	<1.2	1.3	10	13	23	3.9	2.5
	05/16/2010	1.7	2.7	<1.2	<1.2	<1.2	1.2	12	10	23	2.2	<1.8
Camfrout	03/01/2010	3.2	3.8	2.6	1.6	<1.2	2.2	19	6	25	4.1	1.9
	03/29/2010	3.0	2.4	<1.2	<1.2	<1.2	1.5	20	3	23	2.5	<1.8
	05/16/2010	3.4	3.7	3.2	<1.2	<1.2	0.7	2	0	6	2.2	<1.8
Le passage	03/01/2010	3.2	3.6	2.2	<1.2	<1.2	1.9	10	13	23	4.2	2.9
	03/29/2010	2.7	2.9	<1.2	<1.2	<1.2	1.1	20	1	21	3.3	2.2
	05/16/2010	1.7	2.9	<1.2	<1.2	<1.2	0.1	0	NT	0	<1.9‡	<1.8

* The quantification limit of the *Bacteroidales* markers is 1.2 Log_{10} copies ml^{-1} IL, † Not Tested because insufficient phage was isolated, ‡ The LOQ of the human FRNAPH II is 1.9 Log_{10} genome copies g^{-1} DT, § The LOQ of the animal FRNAPH I is 1.8 Log_{10} genome copies g^{-1} DT, ¶ For the number of FRNAPH plaques genotyped, only results with at least 20 FRNAPH plaques successfully genotyped per sample were considered (highlighted in grey), ** A FRNAPH genogroup was considered to be present in a sample when at least five of the 20 FRNAPH plaques genotyped were associated to this genogroup (highlighted in grey).

Table S3 Concentrations of the fecal indicator *E. coli*, the general markers (AllBac and Tot FRNAPHs), the host-associated *Bacteroidales* markers by real-time PCR and the FRNAPH I and II by direct real-time RT-PCR and presence of the FRNAPH I and II by culture/genotyping in estuarine and river waters sampled from the six sites in the Elorn estuary.

Sites	Sampling date (mo/day/yr)	*E. coli* conc. (Log_{10} MPN/100 ml water)	*Bacteroidales* marker conc. (Log_{10} copies/100 ml water)				FRNAPH genogroups by culture/genotyping				FRNAPH genogroups conc. by direct real-time RT-PCR (Log_{10} genome copies/100 ml water)	
			General AllBac	Human Hum-1-Bac	Ruminant Rum-2-Bac	Porcine Pig-2-Bac	Log (PFU/100 ml)	Human genogroup isolates II (GA)	Animal genogroup isolates I (MS2)	No. of plaques genotyped	Human FRNAPH II	Animal FRNAPH I
Estuarine waters												
Landerneau	03/01/2010	3.3	5.8	4.4	<2.7	<2.7	2.1	23‡‡	1	24††	<2.3¶	<2.4**
	03/29/2010	3.3	5.4	4.1	3.0	<2.7	2.3	21	3	24	<2.3	<2.4
	05/16/2010	3.4	4.6	3.8	<2.7	<2.7	2.3	18	6	25	<2.3	<2.4
Vervian	03/01/2010	5.0	4.8	3.1	<2.7	<2.7	1.3	21	3	24	<2.3	<2.4
	03/29/2010	2.8	3.8	<2.7	<2.7	<2.7	<1.0‡	8	11	20	<2.3	<2.4
	05/16/2010	2.9	3.8	<2.7	<2.7	<2.7	2.5	17	6	23	<2.3	<2.4
Pen An Trein	03/01/2010	3.0	4.8	3.3	3.0	<2.7	1.6	21	2	23	<2.3	<2.4
	03/29/2010	1.9	4.4	<2.7	<2.7	<2.7	<1.0	15	3	18	<2.3	<2.4
	05/16/2010	<1.6*	2.8	<2.7	<2.7	<2.7	2.1	34	1	35	3.6	<2.4
Le Pouldu	03/01/2010	3.4	4.4	<2.7	<2.7	<2.7	1.6	22	2	24	<2.3	<2.4
	03/29/2010	1.9	4.5	<2.7	<2.7	<2.7	<1.0	19	5	24	<2.3	<2.4
	05/16/2010	<1.6	<2.7†	<2.7	<2.7	<2.7	2.2	14	1	15	<2.3	<2.4
Camfrout	03/01/2010	3.8	4.8	3.6	<2.7	<2.7	2.3	24	0	24	<2.3	<2.4
	03/29/2010	3.0	<2.7	<2.7	<2.7	<2.7	<1.0	10	1	11	<2.3	<2.4
	05/16/2010	1.9	<2.7	<2.7	<2.7	<2.7	1.0	NT§	NT	0	<2.3	<2.4

Le passage	03/01/2010	2.7	4.2	<2.7	<2.7	<2.7	1.3	20	3	23	<2.3	<2.4
	03/29/2010	1.9	4.5	<2.7	<2.7	<2.7	<1.0	3	2	5	<2.3	<2.4
	05/16/2010	2.4	4.1	<2.7	<2.7	<2.7	<1.0	NT	NT	0	<2.3	<2.4
River waters												
Le Pouldu	03/01/2010	3.7	6.5	3.3	<2.7	<2.7	2.7	24	0	24	<2.3	<2.4
	03/29/2010	2.4	6.2	4.2	<2.7	<2.7	<1.0	10	0	10	<2.3	<2.4
	05/16/2010	3.5	6.5	4.4	<2.7	<2.7	2.2	13	2	18	<2.3	<2.4
Camfrout	03/01/2010	3.8	7	5.3	<2.7	<2.7	1.7	23	0	23	<2.3	<2.4
	03/29/2010	4.6	7.3	5.9	<2.7	<2.7	2.9	22	1	21	<2.3	<2.4
	05/16/2010	4.8	7.4	6.5	<2.7	<2.7	3.3	17	0	24	3.1	<2.4

* The detection limit of *E. coli* is 1.6 U. Log_{10} MPN 100 ml^{-1} water, † The quantification limit of *Bacteroidales* markers is 2.7 Log_{10} copies 100 ml^{-1} water, ‡ The limit of quantification (LOQ) of total F-specific bacteriophages is 1 Log_{10} PFU 100 ml^{-1} water, § Not Tested because insufficient phage was isolated, ¶ The LOQ of the FRNAPH II is 2.3 Log_{10} genomes 100 ml^{-1} water, ** The LOQ of the FRNAPH I is 2.4 Log_{10} genomes 100 ml^{-1} water, †† For the number of FRNAPH plaques genotyped, only results with at least 20 FRNAPH plaques successfully genotyped per sample were considered (highlighted in grey), ‡‡ A FRNAPH genogroup was considered to be present in a sample when at least five of the 20 FRNAPH plaques genotyped were associated to this genogroup (highlighted in grey).