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Estimation of Pig Fecal Contamination in a River Catchment by Real-Time PCR Using Two Pig-Specific *Bacteroidales* 16S rRNA Genetic Markers

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Abstract:

The microbiological quality of coastal or river water can be affected by fecal contamination from human or animal sources. To discriminate pig fecal pollution from other pollution, a library-independent microbial source tracking method targeting *Bacteroidales* host-specific 16S rRNA gene markers by real-time PCR was designed. Two pig-specific *Bacteroidales* markers (Pig-1-Bac and Pig-2-Bac) were designed using 16S rRNA gene *Bacteroidales* clone libraries from pig feces and slurry. For these two pig markers, 98 to 100% sensitivity and 100% specificity were obtained when tested by TaqMan real-time PCR. A decrease in the concentrations of Pig-1-Bac and Pig-2-Bac markers was observed throughout the slurry treatment chain. The two newly designed pig-specific *Bacteroidales* markers, plus the human-specific (HF183) and ruminant-specific (BacR) *Bacteroidales* markers, were then applied to river water samples (n = 24) representing 14 different sites from the French Daoulas River catchment (Brittany, France). Pig-1-Bac and Pig-2-Bac were quantified in 25% and 62.5%, respectively, of samples collected around pig farms, with concentrations ranging from 3.6 to 4.1 $\log_{10}$ copies per 100 ml of water. They were detected in water samples collected downstream from pig farms but never detected near cattle farms. HF183 was quantified in 90% of water samples collected downstream near Daoulas town, with concentrations ranging between 3.6 and 4.4 $\log_{10}$ copies per 100 ml of water, and BacR in all water samples collected around cattle farms, with concentrations ranging between 4.6 and 6.0 $\log_{10}$ copies per 100 ml of water. The results of this study highlight that pig fecal contamination was not as frequent as human or bovine fecal contamination and that fecal pollution generally came from multiple origins. The two pig-specific *Bacteroidales* markers can be applied to environmental water samples to detect pig fecal pollution.

1. Introduction

Human and animal fecal pollution of coastal environments affects shellfish and recreational water quality and safety, in addition to causing economic losses from the closure of shellfish harvesting areas and from bathing restrictions (13, 19, 33). Human feces are known to contain human-specific enteric pathogens (3, 18, 28), but animals can also be reservoirs for numerous enteric human pathogens such as *Escherichia coli* O157:H17, *Salmonella spp, Mycobacterium spp* or *Listeria spp* that may persist in the soil or surface waters (6, 8, 22, 24). Among animals, pigs are known to carry human pathogens that are excreted with fecal wastes. There are approximately 125 million pigs in the European Union (EU) and 114 million in North America (12, 36, 48), generating an estimated 100 and 91 million tons of pig slurry per year, respectively (4). France, the third largest pig producer in the EU, with about 23,000 farms, generates 8 to 10 million tons of pig slurry per year. Brittany accounts for 56.1% of the total national pig production on only 6% (27,200 km^2) of the French territory though it has 40% (2,700 km) of the coast line. This production could contaminate the environment when tanks on farms overflow, when slurry or compost is spread onto soils or, to a lesser extent, when lagoon surface waters are used for irrigation (38, 47, 52).

Fecal contamination in shellfish-harvesting and bathing areas is currently evaluated by the detection and enumeration of culturable facultative-anaerobic bacteria, such as *E. coli*, enterococci or fecal coliforms (11) in shellfish and bathing waters (European Directives 2006/113/CE; 2006/7/CE). Pigs are among the potential sources of *E. coli* inputs to the environment; a pig produces approximately 1×10^7 *E. coli* per gram of feces which corresponds to an *E. coli* flow rate per day that is 28 times higher than that of one human (16, 34, 55).

E. coli is not a good indicator of fecal sources of pollution in water because of its presence in both human and animal feces, therefore alternative fecal indicators must be used. Microbial Source Tracking methods (43) are being developed to discriminate between human and non-human sources of fecal contamination and to distinguish contamination from different animal species (17, 46, 54). Many of these methods are library-dependent, requiring a large number of isolates to be cultured and tested, which is time-consuming and labor intensive. For these reasons, library-independent methods are preferred for the detection of host-specific markers.

The detection of host-specific *Bacteroidales* markers is a promising library-independent method and has been used for identifying contaminations from human and bovine origins (25, 29, 39, 40, 44). In this study, we selected *Bacteroidales* 16S rRNA gene markers and real time PCR to focus on fecal contamination from pigs. To date, only one pig-specific *Bacteroidales* 16S rRNA gene marker has been developed and used on water samples for the identification of pig fecal contamination by real-time PCR assay (SyberGreen®; 37). When this pig-specific *Bacteroidales* marker was tested on a small number of fecal samples (*n=16*), it showed some cross-reaction with human and cow feces.

The present study investigated pig fecal contamination in a French catchment, the Daoulas estuary (Brittany), which has commercial and recreational shellfish harvesting areas and which is potentially subject to fecal contamination. The aims of the present study were: (i) to design new primers for detection and quantification of pig-specific *Bacteroidales* 16S rRNA genes by TaqMan® analysis; (ii) to validate the sensitivity and specificity of the new primers and TaqMan® assay using target (feces, slurry, compost and lagoon water samples) and non- target (human and other animal sources: include which animals) DNA, respectively; and (iii) to evaluate the TaqMan® assay for proper detection and quantitative estimation of pig-associated fecal pollution. It represents the first application of pig-specific *Bacteroidales* markers using a TaqMan® assay in Europe and includes a monitoring study of marker levels throughout the various stages of slurry treatment.

2. Materials and methods

Sample collection

Fecal samples

Individual human and animal fecal samples were collected from April 2004 to March 2008. Human fecal samples were obtained from 24 healthy adult and child volunteers from Brittany (France). Animal fecal samples were collected immediately after excretion from apparently healthy animals (25 pigs, 10 cows, 10 sheep, and 10 horses). Pig fecal samples were collected from sows and male adults, young pigs and piglets, mainly housed in stalls on 15 farms in Brittany. Cow fecal samples were collected from animals kept on pasture or housed in stalls, and included samples from adults and heifers on six independent beef and dairy farms in Brittany and Normandy. Samples from sheep were collected from one farm in Brittany and from salt meadows on two farms in Normandy. Samples from horses were collected from stud farms in Brittany and Pays de la Loire.

Pig effluent samples

Twenty-three slurry and 14 lagoon surface water samples were collected from 14 and 9 independent farms, respectively, in Brittany during April and December 2007 and March 2008. Seven compost samples obtained by slurry centrifugation were also collected on one of these farms.

Case study of the pig-specific markers through a slurry treatment process on a pig farm
A pig farm that performs slurry treatment was also selected to evaluate the new pig markers during a slurry treatment process. This case illustrates the most frequent slurry treatment process used in Brittany. Indeed, 71% of farms in Brittany use this biologically activated sludge method to treat slurry effluents, while 17% use composting (32). In this case, the main farm collects and processes its own slurry, along with slurries from 4 other surrounding farms: corresponding to the wastes of approximately 800 sows in total. Mixed slurries are centrifuged as part of compost production (60 m^3 per day) and the resulting water is biologically treated through an activated sludge process. After decantation, surface water is stored in a lagoon basin (13,000 m^3). A total of 5 pig fecal and 5 slurry samples (from the main farm and 2 surrounding farms), and 5 compost and 5 lagoon waters samples (from the main farm) were collected in March 2008.

River water samples

River water samples were collected on the catchment of the Daoulas estuary (Brittany) from January 2006 to January 2008 (Fig. 1). This catchment is located about 20 km south-east of Brest and covers 113 km^2, with 90 km of river system. It is mainly characterised by intensive livestock farming (dairy cows [5,300], pigs [151,000] and poultry [782,000]) with the total number of human inhabitants in the catchment estimated at 15,000. The coastal shellfish harvesting areas are classified as B-category according to European legislation (European Directive 91/492/EEC). This means that the shellfish have been found to contain between 230 and 4,600 *E. coli* per 100 g of total flesh and must be depurated for $\geq$ 48h in good quality water prior to sale. Twenty-four water samples were collected at 14 different sites. Six sites (2, 10, 11, 12, 13 and 14) were selected for their proximity to pig farming activities, 3 sites (1, 8, 9) for their proximity to cattle farming and 5 sites (3, 4, 5, 6, 7) downstream and near an urban area (Daoulas). Sites 1, 3, 8, 9, 11 and 13 were sampled twice while sites 5 and 6 were sampled three times. All samples were placed in sterile containers and transported in insulated coolers. Cells were captured on filters upon arrival to the laboratory and stored at -20°C.

Sample preparation and DNA extraction

For water samples, approximately 200 ml were filtered through 0.22 µm Nuclepore membrane filters (Whatman, Scleicher and Schuell, Germany). Filters were then placed in 0.5 ml of GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH = 8.0], 0.5% Sarkosyl) (8) and frozen at -20°C until extraction. DNA was extracted using the DNeasy Tissue kit (Qiagen, Courtaboeuf, France) with two modifications: the proteinase K step was omitted and 700 µl of Qiagen Buffer AL were added to the filters after the GITC buffer (9).
For fecal and compost samples, DNA was extracted from 250 mg wet weight using the Fast DNA Spin Kit for Soil (MP Biomedical, Illkirsh, France), according to the supplier's instructions, with an additional wash using the SEWS-M reagent as suggested by Dick and Field (9). Pig slurry samples (50 ml) were centrifuged at 9,000 × g for 15 minutes. DNA was then extracted from 250 mg of the pellet using the Fast DNA Spin Kit for Soil.

PCR and 16S rRNA gene library construction

The primers Bac32F and Bac708R (Table 1) were used to selectively amplify Bacteroidales 16S rRNA genes from DNA extracts from 10 pig feces and 10 pig slurry samples. Reactions were performed in a Peltier Thermal Cycler (PTC 200; MJ Research, Waltham, MA, USA) for 30 cycles of 94°C for 5 min, 94°C for 30 s, 61°C for 30 s and 72°C for 30 s. Final extension was carried out at 72°C for 7 min. PCR products from each library were pooled to obtain 2 different clone libraries (from pig feces and pig slurry). Pooled PCR products were gel purified (Nusieve GTG agarose 2%; BMA, Rockland, USA) using the QiaQuick gel purification kit (Qiagen, France). They were cloned into the pCR2.1 vector using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. The vectors were transformed into chemically competent *E. coli* cells (One Shot TOP10F'; Invitrogen). Ninety-six transformants were randomly picked on Luria-Bertani (LB) agar plates from each host-specific library and used to inoculate 96-well culture plates (Deep-Well; Millipore) containing 1 ml LB 2X broth with ampicillin (50 µg ml^{-1}). Culture plates were incubated at 37°C for 24 h with shaking (130 rpm). After centrifugation, they were stored at -20°C prior to sequencing the inserts.

Sequencing and phylogenetic analysis

Sequencing was performed on an ABI PRISM 9700 capillary sequencer using the ABI Prism Big Dye Terminator Cycle sequencing kit with M13-f and M13-r primers, as described by the manufacturer (Perkin–Elmer Applied Biosystems).
Sequences were edited using BioEdit (21). DNA sequences (approximately 690 bp) were processed using the MALLARD software (2) to eliminate chimeric sequences. Sequences were aligned using MAFFT (version 5) and the distance matrix calculated using the software DNADIST (version 3.5c). The distance matrix was used with DOTUR software (45) to assign sequences to operational taxonomic units (OTUs). OTUs were defined by assigning 16S rRNA gene sequences of >98% similarity to the same species. Representative OTU sequences were retrieved rationally with the pipeline RapidOTU (30) and compared with the GenBank database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST). Multiple alignments were performed using CLUSTAL W (51). A phylogenetic tree was constructed using the PHYLO-WIN program (15). The root was determined using the 16S rRNA gene sequence of *Cytophaga fermentans* (accession number M58766) as an out-group. Distance trees were constructed using Neighbor-Joining algorithms (41) with the Kimura two-parameter correction (26). The statistical significance of tree branches was evaluated by bootstrap analysis using 500 resampling (values <70 omitted from figure).

Oligonucleotide primers and probes

The primers and probe All-*Bacteria* (Bact2, modified from reference 50) and All-*Bacteroidales* (AllBac) from Layton *et al.* (29), were used to amplify total bacterial, and total *Bacteroidales* 16S rRNA genes. Detection of human and ruminant-specific *Bacteroidales* 16S rRNA gene markers (HF183 and BacR) was performed with the primers and probe described by Seurinck *et al.* (44) and Reischer *et al.* (39), respectively (Table 1). Two pig-specific *Bacteroidales* primers and probe (Pig-1-Bac and Pig-2-Bac) were designed from multiple alignments of partial *Bacteroidales* 16S rRNA genes obtained in this study (Table 1). Oligonucleotide specificity for pig-associated *Bacteroidales* 16S rRNA genes was verified using the BLAST (NCBI) and the Probe Match (Ribosomal Database Project: RDP II) programs. The pig-specific *Bacteroidales* marker (Pig-Bac2) described by Okabe *et al.* (37) was also tested on target and non target DNA preparations and compared with the two pig-specific *Bacteroidales* designed in this study.

Real-Time PCR assays

All real-time PCR were performed using the TaqMan® Brilliant QPCR Core reagent kit (Stratagene), except for the human-specific and the pig-specific markers defined by Okabe *et al.* (36) that used the Brillant SYBR-Green® QPCR Master Mix (Stratagene).
Amplifications were performed using a Stratagene MX 3000 P with software version 4. Each reaction was run in duplicate with the following cycle conditions: 1 cycle at 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. For SYBR-Green® amplifications, a dissociation step was added to improve amplification specificity.
TaqMan and SYBR-Green® reactions were carried out in a final volume of 25 µl with appropriate final concentrations of primers and probe (Table 1).
The presence/absence of PCR inhibitors was verified using an Internal Positive Control (IPC; AppliedBiosystem, France). Samples were diluted if inhibitors were present. Negative controls (no template DNA) were performed in triplicate for each run.
Host-specific *Bacteroidales*, All-*Bacteroidales* and All-*Bacteria* markers were tested on all feces, pig waste effluent and river water samples.

DNA standard curves and quantification

Linear plasmid DNA used to generate standard curves were extracted with the QIAquick Miniprep Extraction Kit (Qiagen), following the manufacturer's instructions. The linear forms of pig, bovine and human plasmids were obtained with NotI enzyme (Roche Diagnostics) in a final volume of 50 µl for 3 h at 37°C. For the quantification of *Bacteroidales* markers, standard curves were generated from serial dilutions of a known concentration of plasmid DNA. Standard curves were generated by plotting threshold cycles (Ct) against 16S rRNA copy numbers.
A PCR standard for the Bact2 marker was prepared using a 10-fold dilution of bacterial genomic DNA extracted from pure culture of *E. coli* with the Wizard genomic DNA purification Kit (Promega) according to the manufacturer's instructions. The copy number of 16S rRNA /genome was considered to be 7 copies for the *E. coli* strain (27).

Enumeration of E. coli

E. coli was enumerated in a sub-set of the pig fecal samples, effluent samples and in all water samples from the Daoulas catchment by the microplate method (standard NF IN ISO 9308-3 (1)) with a detection limit of <10 Most Probable Number (MPN) per g for feces and compost and <15 MPN per 100 ml of water.

Nucleotide sequence accession numbers

Sequence data used in this study has been submitted to the GenBank database under numbers EU797125 to EU797175.

Data treatment and statistical analyses

The results of the general and host-specific marker quantifications were expressed in 16S rRNA gene copies per g or ml of feces, compost, slurry or water.
All statistical analyses were performed using STATISTICA version 6.1 (StatSoft; France). To evaluate the performance of the MST methods on fecal samples, sensitivity (r) and specificity (s) were defined as r = a/(a+c) and s = d/(b+d), where a, when a fecal sample was positive for the marker of its own species (true positive); b, when a fecal sample was positive for a marker of another species (false positive); c, when a fecal sample was negative for a marker of its own species (false negative); d, when a fecal sample was negative for a marker of another species (true negative) (14). Fisher's exact test was used to verify if the observed differences in the frequency of detection of the various markers in their target or non target fecal samples were significantly different. Analysis of variance (ANOVA), followed by comparisons of means using Fisher's LSD test, was used to test for significant differences between the concentrations of the different markers at the same stage of slurry treatment and between the different stages of the slurry treatment ($P<0.05$). Linear regression analysis (analysis of the completed model) was used to verify if differences in concentration between *Bacteroidales* markers and *E. coli* concentrations were significant.

3. Results

Phylogenetic analysis of Bacteroidales 16S rRNA genes from pig feces and pig slurry samples
Of the 96 clones obtained from *Bacteroidales* 16S rRNA gene libraries derived from pig feces and pig slurry samples, 94 and 86 clones yielded unambiguous sequence data, respectively. Twenty-seven and 24 different OTUs were obtained for pig feces and slurry, respectively, with 16 OTUs showing common clone sequences from both feces and slurry. Forty-five and 60% of the sequences from pig feces and pig slurry libraries had more than 98% similarity to bacterial 16S rRNA gene sequences published in GenBank (NCBI).
The 16S rRNA pig *Bacteroidales* sequences were predominantly *Prevotella*-like, 85% from feces and 55% from slurry. From pig feces, 60% of the sequences were closely related to isolates from pig feces and 26% were related to sequences derived from human tissues and stools. From pig slurry samples, 55% of the sequences showed >98% similarity with bacterial 16S rRNA gene sequences isolated from pig fecal samples, 21% with sequences associated with human tissues or stools and 13% corresponded to environmental clones.
To design *Bacteroidales* pig-specific primers, clusters of pig-specific sequences were investigated using: (i) the partial *Bacteroidales* 16S rRNA gene sequences obtained from the slurry and fecal samples; and (ii) partial *Bacteroidales* 16S rRNA gene sequences of human and bovine fecal origin in the GenBank database. Phylogenetic analysis of the 51 OTUs showed two distinct clusters of pig-specific sequences (Fig. 2). Sequences from clusters I and II were selected to design *Bacteroidales* pig-specific primers to detect the pig-specific *Bacteroidales* markers. Cluster I contained only one OTU (Fpc59) which represented 8 *Bacteroidales* 16S rRNA gene sequences with 5 sequences isolated from pig feces and 3 from pig slurry. The 'Fpc59' OTU was closely related (99% similarity) to PigA4 uncultured *Bacteroidales* sequences from a pig fecal sample (10) and to a lesser extent, to *Prevotella brevis (*AJ011682; 90% similarity). This cluster was used to design the first pig-associated real-time PCR assay, Pig-1-Bac. Cluster II contained 12 OTUs: 8 from pig feces OTUs and 4 from pig slurry. In cluster II, the OTUs 'Fpc8', 'Lpc61' and 'Fpc37' were closely related to the pig marker PigC1 described by Dick *et al.* (10) with 99%, 96% and 94% similarity, respectively. Clone sequence 'Fpc35' was 96% similar to clone sequence 'P93' (AB237869) obtained by Okabe *et al.* (37). Clone sequence 'Fpc3' showed 94% similarity to clone

sequences 'P80' (AB237867; 37). This cluster was used to design the second pig-associated real-time PCR assay named Pig-2-Bac.

Real-Time PCR assays and limits of quantification

For the two pig-specific *Bacteroidales* markers designed in this study and for AllBac, HF183 and BacR, plasmid DNA containing partial 16S rRNA gene sequence insert were run as standards using 10-fold dilutions ranging from 1.6×10^7 to 1.6×10^0 copies per PCR with a quantification limit of 1.6 target copies per reaction.
Genomic DNA from a pure culture of *E. coli,* ranging from 7×10^6 to 7×10^1 rRNA 16S rRNA gene copies per PCR, were run as standards for the Bact2 assay, with a quantification limit of 70 target copies per reaction.
Consequently, the lower limits for quantification of the All-B*acteroidales* and host-specific *Bacteroidales* markers were 4.5 $\log_{10}$ copies per g in feces and composts and 3.5 $\log_{10}$ copies per 100 ml of water.

Testing general and host-specific Bacteroidales markers in feces and effluent samples

The sensitivity and specificity of the general and host-specific *Bacteroidales* primers and probes developed in this present study or described previously (29, 39, 44) were tested on target and non target fecal DNA samples.
The two pig-specific *Bacteroidales* primers and probe sets were both applied to 69 samples of pig origin (Table 2). For Pig-1-Bac and Pig-2-Bac, average concentrations were estimated to be 8.6 ± 0.8 and 8.5 ± 0.6 $\log_{10}$ copies per g wet weight feces, 4.8 ± 0.9 and 4.9 ± 0.7 $\log_{10}$ copies per ml of slurry, 2.4 ± 0.4 and 2.6 ± 0.4 $\log_{10}$ copies per ml of lagoon water and 5.3 ± 0.5 and 5.3 ± 0.6 $\log_{10}$ copies per g of compost samples, respectively. No amplification was observed with non target DNA. The two pig-specific *Bacteroidales* markers showed 98-100% sensitivity and 100% specificity. The pig-specific *Bacteroidales* primers (Pig-Bac2) described by Okabe *et al.* (37) demonstrated 100% sensitivity *(n=10;* pig feces and effluent samples). However, positive results were also observed on non-target DNA (*n=30*; human, bovine, sheep and horse feces samples), with an overall score of 54% specificity.
All fecal and pig waste samples were positive for both the All-*Bacteria* and the All-*Bacteroidales* markers (Table 2). For samples of pig origin, the All-*Bacteroidales* marker concentrations were 10.1 ± 0.7 $\log_{10}$ copies per g of pig feces, 6.9 ± 1.1 $\log_{10}$ copies per ml of slurry, 4.7 ± 0.6 $\log_{10}$ copies per ml of lagoon water and 9.5 ± 0.4 $\log_{10}$ copies per g of compost samples. The human-specific *Bacteroidales* marker HF183 was present in 13 of 24 human stool samples with average concentrations of 7.8 ± 2.1 $\log_{10}$ copies per g of wet feces, implying 54% of sensitivity. Negative results with HF183 were obtained for all non target DNA, showing 100% specificity. The ruminant-specific *Bacteroidales* marker BacR gave positive results on all bovine and sheep feces with average concentrations estimated at 10 ± 0.3 $\log_{10}$ copies per g of wet feces corresponding to 100% sensitivity. Amplifications were observed with pig effluent samples and human feces samples showing 89% specificity. The observed differences in the frequency of detection of host-specific *Bacteroidales* markers in their target and non target samples were significantly different ($P<0.05$).

Case study of the pig-specific markers through a slurry treatment process on a pig farm
All markers and especially the pig-specific markers and *E. coli* enumerated by cultural technique were detected throughout the pig waste treatment chain, and their concentrations were seen to decrease throughout the slurry treatment process (Fig. 3). Indeed, Pig-1-Bac concentrations were 8.5 ± 0.7 $\log_{10}$ copies per g in feces, 4.8 ± 0.7 $\log_{10}$ copies per ml in slurry, 2.1 ± 0.3 $\log_{10}$ copies per ml in lagoon water and 5.3 ± 0.5 $\log_{10}$ copies per g in compost samples. Pig-2-Bac concentrations were 8.6 ± 0.5 $\log_{10}$ copies per g in feces, 4.9 ± 0.7 $\log_{10}$ copies per ml in slurry, 2.4 ± 0.2 $\log_{10}$ copies per ml in lagoon water and 5.1 ± 0.5 $\log_{10}$ copies per g in compost samples. ANOVA confirmed that the concentrations of the two markers were not significantly different in any of the stages of slurry treatment ($P>0.05$).

Concentrations of the All-*Bacteroidales* marker were 10.5 ± 0.1 $\log_{10}$ copies per g in feces, 7.8 ± 0.4 $\log_{10}$ copies per ml in slurry, 2.1 ± 0.3 $\log_{10}$ copies per ml in lagoon water and 5.3 ± 0.3 $\log_{10}$ copies per g in compost samples (Fig. 3). Concentrations of *E. coli* by culture were 6.9 ± 0.7 $\log_{10}$ MPN per g in feces, 6.6 ± 0.8 $\log_{10}$ MPN per ml in slurry, 2.4 ± 0.1 $\log_{10}$ MPN per ml in lagoon water and 4.3 ± 0.4 $\log_{10}$ MPN per g in compost samples (Fig. 3). ANOVA indicated that concentrations of Pig-1-Bac and Pig-2-Bac markers were not significantly different to *E. coli* concentrations in slurry and they were not either significantly different to *E. coli* concentrations in lagoon water samples whereas concentrations of the All-*Bacteroidales* marker were significantly different to *E. coli* concentrations between at all the stages. A high level of correlation was found between the concentrations of the two pig-specific *Bacteroidales* markers and *E. coli* obtained by culture in pig wastes (R^2 = 0.77 for Pig-1-Bac and R^2 = 0.88 for Pig-2-Bac) (Fig. 4).

Marker concentrations in environmental river water samples

Escherichia coli was found in all samples at concentrations that varied, with sampling point and date, from 2.5 to 4.3 $\log_{10}$ MPN per 100 ml (Table 3). The All-*Bacteroidales* marker was quantified in all samples, at concentrations that ranged between 4.6 and 7.8 $\log_{10}$ copies per 100 ml of water. At least one host-specific marker was quantified in all sites, with most cases having multiple markers. The Pig-1-Bac marker was quantified in 25% of water samples collected around pig farms corresponding to site 10 with 4 $\log_{10}$ copies per 100 ml and to site 11 with 3.9 $\log_{10}$ copies per 100 ml and it was detected in 37.5% of these samples. It was detected in 30% of water samples collected downstream corresponding to site 6 and never detected in samples collected near cattle farms. The Pig-2-Bac marker was quantified in 62.5% of water samples collected around pig farms (site 2, 4.1 $\log_{10}$ copies per 100 ml; site 10, 4 $\log_{10}$ copies per 100 ml; site 11, 3.6 and 3.8 $\log_{10}$ copies per 100 ml and site 14, 3.6 $\log_{10}$ copies per 100 ml) and detected in 87.5% of these samples. It was detected in 20% of water samples collected downstream corresponding to sites 6 and 7 and never detected in samples collected near cattle farms.
The HF 183 marker was quantified in 90% of water samples collected downstream, near Daoulas town corresponding to sites 3, 4, 5, 6 and 7. The concentrations of the marker in these sites ranged between 3.5 and 4.4 $\log_{10}$ copies per 100 ml. It was quantified in 50% of water samples collected around pig farms corresponding to sites 11, 12 and 13. The concentrations in these sites ranged between 3.9 and 5.1 $\log_{10}$ copies per 100 ml. It was also quantified in 17% of water samples collected around cattle farms corresponding to site 9 with 4.3 $\log_{10}$ copies per 100 ml. The BacR marker was quantified in all water samples collected around cattle farms corresponding to sites 1, 7 and 8. The concentrations of the marker in these sites ranged between 4.6 and 6.0 $\log_{10}$ copies per 100 ml. It was quantified in 62.5% of water samples collected around pig farms corresponding to sites 2, 10, 12 and 13 and concentrations ranged between 4.5 to 6 $\log_{10}$ copies per 100 ml. It was also quantified in
60% of water samples collected downstream corresponding to sites 5, 6 and 7 and concentrations ranged between 5.7 and 6 $\log_{10}$ copies per 100 ml.
No significant correlation was observed between *E. coli* concentration and the concentrations of the two pig-specific *Bacteroidales* markers (R^2 = 0.12 and 0.11), the human and ruminant-specific *Bacteroidales* marker (R^2 = 0.11 and 0.10), or the All-*Bacteroidales* marker (R^2 = 0.15) in the river water samples.

4. Discussion

In this study, *Bacteroidales* 16S rRNA gene sequences were obtained from pig feces and slurries, and host-specific TaqMan® real-time PCR primers and probes were designed to identify pig fecal contamination in natural water samples. Most of the *Bacteroidales* sequences identified in the present study were related to uncultured *Prevotella* bacteria, thus

indicating a high level of (as yet uncultured) diversity similar to that observed by Dick *et al.* (10) and Okabe *et al.* (37). Sequence analyses showed that clusters with only pig-specific sequences (from both feces and slurry samples) were represented in the genus *Prevotella.* In the phylogenetic tree, among a total of 51 OTUs, 16 OTUs represented sequences from pig feces and slurry; underlining that the *Bacteroidales* present in pig feces can also be found in pig slurry.
Two clusters (I and II) were identified and used for designing primers and probes for two pig-specific *Bacteroidales* markers with OTUs from both feces and slurry samples. The first cluster (cluster I) has not been previously described in the literature whereas the second cluster (cluster II) contained sequences closely related to the clone sequence "PigC1" obtained by Dick *et al.* (10) and to the clones "P80" and "P93" described by Okabe *et al.* (37). These studies highlight that pig-specific sequences can be obtained from different geographical areas (USA, Japan and France) and that a cosmopolitan distribution of the *Bacteroidales* can be observed. Thus, bacteria belonging to the *Bacteroidales* order seem to be promising fecal indicators to identify pig fecal pollution sources (9, 10) and could be used to design pig-specific PCR primer and probe sets for real-time PCR assays. The Pig-1-Bac and Pig-2-Bac markers were designed from clusters I and II, respectively. These pig-specific markers succeeded in identifying pig fecal pollution in target samples, and their concentrations were correlated with culturable *E. coli* concentrations throughout a pig waste treatment chain (from feces to compost or lagoon waters). However, concentrations of these pig-specific markers were low in comparison to All-*Bacteroidales* marker concentrations. One explanation for these lower concentrations could be that the total *Bacteroidales* 16S rRNA gene sequences from pig samples are not all pig-specific. Indeed, 30% of the *Bacteroidales* 16S rRNA gene sequences from pig samples obtained in this study showed 98%-similarity with *Bacteroidales* 16S rRNA gene sequences isolated from human samples published in GenBank. The similarity between *Bacteroidales* from pig and human *Bacteroidales* sequences was also found previously (29, 49) and was explained by their common omnivorous diet and similar digestive tract (10). The proportion of pig-specific markers relative to the All-*Bacteroidales* marker and proportion of the All-*Bacteroidales* marker relative to the All-*Bacteria* marker decreased along the waste treatment chain. Explanations could include (i) a loss of *Bacteroidales* during slurry storage, slurry treatment under aerobic conditions or by activated sludge or (ii) dilution of *Bacteroidales* in bacteria community during pig slurry storage. Indeed, Peu *et al.* (38) observed changes in the dominant microbial population between feces and slurry, and between a slurry storage tank and a pond by performing PCR-SSCP (PCR-Single Strand Conformation Polymorphism) profiles. Furthermore, aerobic bacteria such as *Bacillus thuringiensis*, *Sphingobacterium mizutae* or *Paenibacillus* sp have been shown to appear during slurry storage (31).
Previously, pig-specific *Bacteroidales* primers were described and found to be specific to pig feces from geographically distant sources in the USA (10) and to pig feces and pig waste effluent samples from France (20). However, these studies used conventional PCR assays, and as such only provided qualitative data (presence/absence) of the pig-specific marker. In 2007, pig-specific *Bacteroidales* primers (Pig-Bac2) were designed by Okabe *et al.* (37) for real-time PCR with the SYBR-Green® assay. However, when tested in the laboratory, this marker was found to amplify non specific DNA extracted from human, bovine, sheep and horse feces. These results are similar to those obtained by Okabe *et al.* (37) where a few human and bovine fecal samples showed non specific amplifications.

In the present study, *Escherichia coli* concentrations, measured in the river Daoulas catchment area, were in agreement with the level of fecal contamination in shellfish collected downstream. Multiple fecal sources from pig-, human- or ruminant-specific markers were detected at least once at each sampling site. Due to the large-scale pig production (approximately 150,000 pigs) in this catchment, frequent detection of the pig markers was expected in most of the samples collected around pig farm sites. The Pig-1-Bac and Pig-2-Bac markers were quantified in 25% and 62.5% of samples collected around pig farms, respectively. In sub-catchments with farms producing only pigs or pigs and cattle, only pig

markers, or pig and ruminant markers were detected, respectively (sites 2, 10 and 14). Other sampling sites which could be contaminated by pig wastes showed the presence of markers for multiple sources (sites 6, 7 and 13). No pig markers were found in samples from sites without pig farms nearby (sites 1, 3, 4, 5, 8 and 9).
The concentrations of the pig-specific *Bacteroidale*s markers were similar to those observed for the human-specific marker but lower than those for the ruminant-specific marker. This latter marker was found to be from 4.1 to 6 $\log_{10}$ copies per 100 ml of water sample in the Daoulas catchment. These results were in agreement with those obtained in target feces samples in which the pig- and human-specific marker concentrations were 8.6 ± 0.7 and 7.8 ± 2.1 $\log_{10}$ copies per g of feces, respectively and the ruminant marker concentrations were 10 ± 0.3 $\log_{10}$ copies per g of feces. The lower frequency of detection of the pig marker in river water could be explained by the transfer mechanisms of pig fecal contamination such as slurry and compost spreading or by irrigation with lagoon water (38). Spreading pig effluent on soil has been found to significantly reduce the numbers of fecal coliforms or *Salmonella spp* present in such effluent (17). Subsequent transfer of bacteria into surface and groundwater requires a certain level of rainfall after slurry spreading (7, 35). This weak detection of pig fecal pollution in these environmental water samples using pig-specific *Bacteroidales* markers was confirmed by results obtained using another pig-specific marker, the pig-specific archaeal molecular marker developed by Ufnar *et al*. (53). Indeed, no positive results in these water samples were obtained with this marker. However, testing this marker on target fecal samples showed weak PCR signals for half of samples tested (data not shown). For environmental contamination from humans, transfer occurs mainly from point sources such as sewage treatment plants, although diffuse pollution by leaking septic systems may also occur (23). Cattle fecal pollution may occur during grazing, movement or access of cattle to rivers and to a lesser extent from bovine slurry or manure spread on arable land.
Detection of pig-specific *Bacteroidales* markers in river water samples was performed in two previous studies. The pig-specific *Bacteroidales* marker described by Dick *et al.* (10) was previously tested on another French catchment (the Aber Benoît estuary which is also important for pig production with approximately 225,500 pigs). It was rarely detected in river water samples from this area (20). In contrast, the pig-specific *Bacteroidales* marker designed by Okabe *et al.* (37) was detected in all 4 Japanese rivers sampled and at higher levels than the All-*Bacteroidales* marker (42). Savichtcheva *et al.* (42) recommended further validation of this pig-specific marker. Among the 2 pig-specific markers described in the present study, the Pig-2-Bac marker was detected more often in environmental waters than the Pig-1-Bac marker although both were detected in similar concentrations in pig feces and effluents from different farms and geographical areas. Thus, a study on the persistence of both of these markers in the environment could be useful to evaluate the difference in detection in river samples.

In conclusion, this study has provided efficient TaqMan® real-time PCR assays targeting pig-specific *Bacteroidales* 16S rRNA genes to discriminate pig fecal contamination in natural waters. Moreover, the detection of the pig-specific *Bacteroidales* markers over a 48 month period demonstrates their temporal stability. Among the two pig *Bacteroidales* markers designed, the Pig-2-Bac marker appears to be the most suitable, as it was detected more frequently in rivers. This study confirms that fecal pollution in river waters often comes from multiple sources and were mainly of human and bovine origin on the sampling dates investigated in the Daoulas catchment. However, additional sampling should be carried out during high rainfall events within the pig slurry spreading period to determine whether the pig markers and thus pollution from pigs could be more prevalent. These pig-specific *Bacteroidales* markers could represent an efficient tool in a microbial source tracking toolbox, to discriminate fecal pollution from pigs from other fecal sources. This tool will assist in the management of microbial water quality of bathing and shellfish-farming areas.

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Tables

Table 1: Oligonucleotide sequences for conventional and real time PCR assays with the annealing temperature, the final concentration and the expected size for each amplified product

Primers and Probes	Primer and probe sequence (5'→ 3')	Size of amplicon (bp)	Annealing Temp.(°C)	Final concentration (nmol)	Target	Reference
Bac32F	AACGCTAGCTACAGGCTT	690	60	500	All	
Bac708R	CAATCGGAGTTCTTCGTG			500	*Bacteroidales*	(5)
Bact2[a]						
BACT1369F	CGGTGAATACGTTCCCGG	142	60	200	All *Bacteria*	(50)
PROK1492R	TACGGCTACCTTGTTACGACTT			200		
TM1389F	(FAM)CTTGTACACACCGCCCGTC(NFQ-MGB)			250		
AllBac						
AllBac296F	GAGAGGAAGGTCCCCCAC	106	60	200	All	(29)
AllBac467R	CGCTACTTGGCTGGTTCAG			200	*Bacteroidales*	
AllBac375Bhqr	(FAM)CCATTGACCAATATTCCTCACTGCTGCT(BHQ-1)			100		
HF183						
HF183f	ATCATGAGTTCACATGTCCG	83	60	200	Human	(44)
HF183r	TACCCCGCCTACTATCTAATG			200	*Bacteroidales*	
BacR						
BacR_f	GCGTATCCAACCTTCCCG	100	60	100	Ruminant	(39)
BacR_r	CATCCCCATCCGTTACCG			200	*Bacteroidales*	
BacR_p	(FAM)CTTCCGAAAGGGAGATT(NFQ-MGB)			200		
Pig-Bac2						
qBac41F	TACAGGCTTAACACATGCAAGTCG	145	60	300	Pig-specific	(37)
qPS183R	CTCATACGGTATTAATCCGCCTTT			300	*Bacteroidales*	
Pig-1-Bac						
Pig-1-Bac32Fm	AACGCTAGCTACAGGCTTAAC	129	60	200	Pig-specific	This study
Pig-1-Bac108R	cgggctattcctgactatggg			200	*Bacteroidales*	
Pig-1-Bac44P	(FAM)ATCGAAGCTTGCTTTGATAGATGGCG(BHQ-1)			200		
Pig-2-Bac						
Pig-2-Bac41F	gcatgaatttagcttgctaaatttgat	116	60	300	Pig-specific	This study
Pig-2-Bac163Rm	ACCTCATACGGTATTAATCCGC			300	*Bacteroidales*	
Pig-2Bac113MGB	(VIC)TCCACGGGATAGCC(NFQ-MGB)			200		

[a] Modified from reference (50)

Table 2. All-*Bacteria* and *Bacteroidales* markers tested with different fecal and effluent samples from human, pig and other animal sources

% of samples positive with the different PCR assays[a]						
Samples	All-*Bacteria*	All-*Bacteroidales*	Pig-specific *Bacteroidales*		Human-specific *Bacteroidales*	Ruminant-specific *Bacteroidales*
	Bact2	AllBac	Pig-1-Bac	Pig-2-Bac	HF183	BacR
Pig						
Feces (*n=25*)	100	100	100	100	0	0
Slurry (*n=23*)	100	100	100	100	0	17
Lagoon water (*n=14*)	100	100	93	100	0	28
Compost (*n=7*)	100	100	100	100	0	43
Human						
Feces (*n=24*)	100	100	0	0	54	4
Bovine						
Feces (*n=10*)	100	100	0	0	0	100
Ovine						
Feces (*n=10*)	100	100	0	0	0	100
Equine						
Feces (*n=10*)	100	100	0	0	0	0

[a] A result was considered positive when marker concentration was greater than 4.5 $\log_{10}$ copies per g in feces and composts and 3.5 $\log_{10}$ copies per 100 ml of water.

Table 3: *Bacteroidales* markers results from water samples taken from the estuary of catchment of Daoulas river

River water site no.	Sampling date	No of *E. coli* log(MPN/100ml)	Results with *Bacteroidales* markers (log_{10}copies/100ml)[a]					
			All *Bacteria*	All *Bacteroidales*	Pig-specific *Bacteroidales*		Human-specific *Bacteroidales*	Ruminant-specific *Bacteroidales*
			BACT2	AllBac	Pig-1-Bac	Pig-2-Bac	HF 183	BacR
1	12/06/2006	2.5	7	5.1	<3.5	<3.5	<3.5	4.6
	01/14/2008	4.3	7.6	6.7	<3.5	<3.5	<3.5	6.0
2	01/17/2006	3.3	7.9	6.9	<3.5	4.1	<3.5	4.8
3	01/17/2006	3.9	7.5	6.5	<3.5	<3.5	3.6	<3.5
	03/28/2006	3.9	7.5	5.4	<3.5	<3.5	<3.5	<3.5
4	01/17/2006	3.1	7.9	6.9	<3.5	<3.5	3.7	<3.5
5	01/17/2006	4.0	8.2	7.3	<3.5	<3.5	4.4	5.7
	03/28/2006	3.0	7.8	6.1	<3.5	<3.5	3.6	5.7
	12/06/2006	3.0	8.5	6.5	<3.5	<3.5	3.8	5.9
6	01/17/2006	3.5	8.4	7.8	<3.5 ; D[b]	<3.5	3.9	6.0
	01/14/2008	3.3	7.6	5.9	<3.5 ; D	<3.5 ; D	3.6	<3.5
	01/17/2008	3.1	6.5	5.6	<3.5 ; D	<3.5	3.7	5.7
7	01/17/2006	3.5	9.1	7.7	<3.5	<3.5 ; D	3.6	5.9
8	01/14/2008	3.9	7.7	6.4	<3.5	<3.5	<3.5	5.6
	01/17/2008	3.1	7.7	6.4	<3.5	<3.5	<3.5 ; D	5.3
9	01/14/2008	3.0	7.5	6.2	<3.5	<3.5	<3.5	5.5
	01/17/2008	2.9	6.4	6.2	<3.5	<3.5	4.3	5.3
10	01/17/2008	3.1	6.3	4.7	4	4	0.0	4.1
11	01/14/2008	4.3	7.8	6.9	<3.5	3.6	5.1	<3.5
	01/17/2008	3.9	8.1	6.8	3.9	3.8	5.1	<3.5
12	01/17/2008	3.0	7.2	4.6	<3.5	<3.5	<3.5 ; D	4.5
13	01/14/2008	3.0	7.9	6.2	<3.5	<3.5; D	4	4.8
	01/17/2008	3.3	6.6	5.1	<3.5	<3.5 ; D	3.9	4.8
14	01/17/2008	3.5	6.5	4.9	<3.5 ; D	3.6	<3.5	<3.5

[a] The quantification limit of *Bacteroidales* markers is 3.5 log_{10}copies/100ml of water samples

[b] D: detected; positive results were obtained in two repeated experiments

Figures

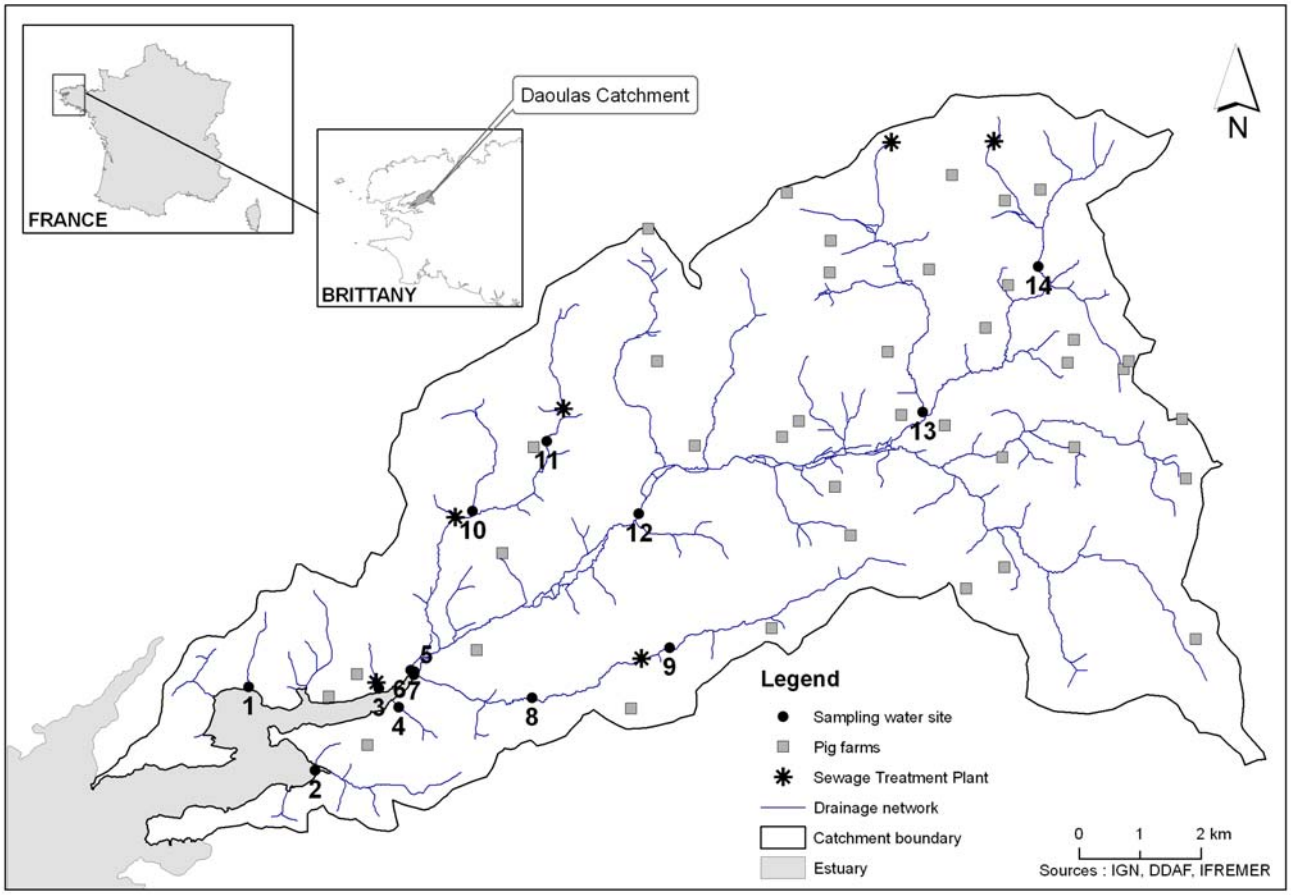


FIG. 1: Location of water sampling sites, pig farms and sewage treatment plants on the catchment and estuary of the Daoulas river, Brittany, France. Cattle farms are not shown.

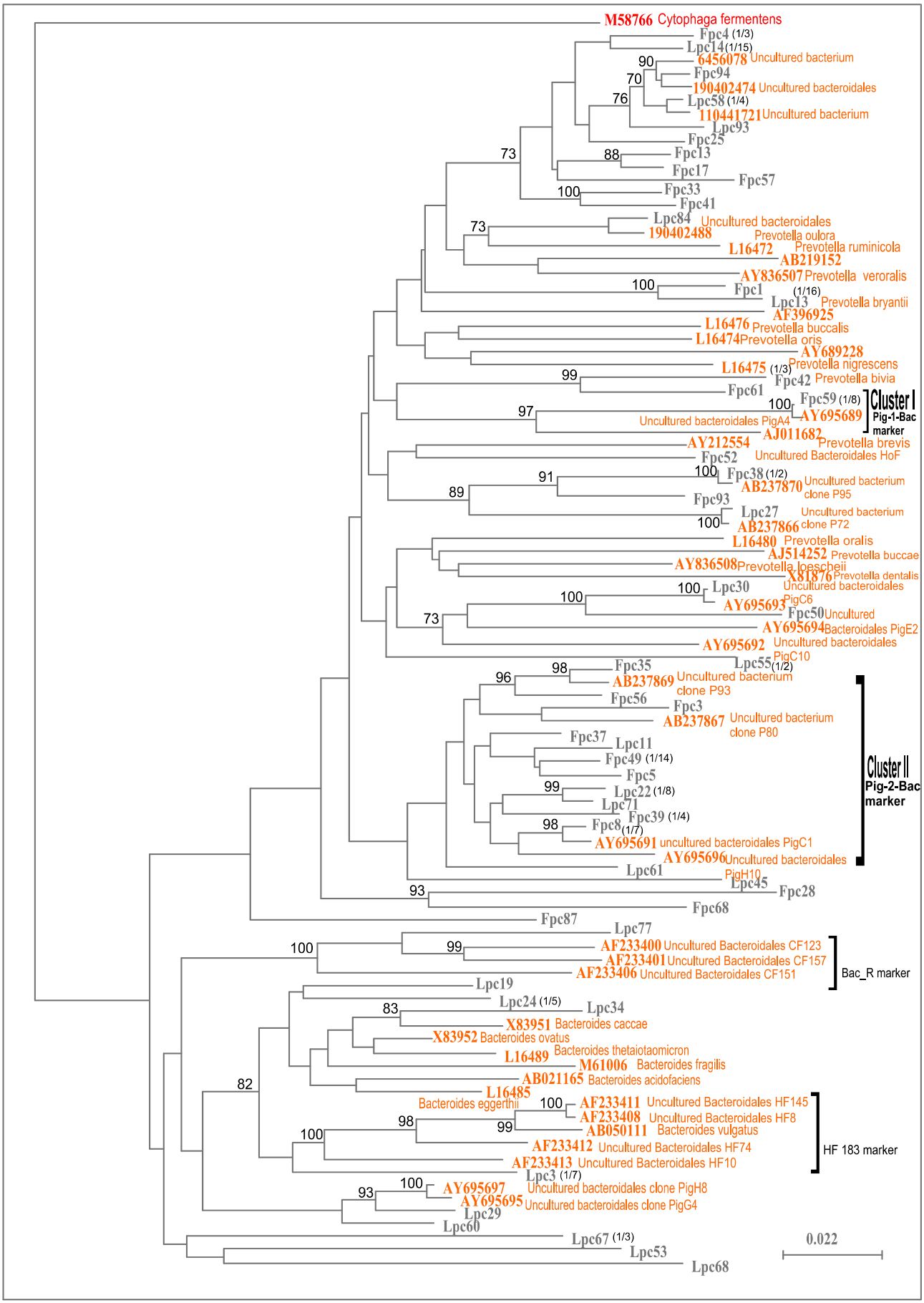

M58766 Cytophaga fermentens
Fpc4 (1/3)
Lpc14 (1/15)
6456078 Uncultured bacterium
Fpc94
190402474 Uncultured bacteroidales
Lpc58 (1/4)
110441721 Uncultured bacterium
Lpc93
Fpc25
Fpc13
Fpc17
Fpc57
Fpc33
Fpc41
Lpc84 Uncultured bacteroidales
190402488 Prevotella oulora
L16472 Prevotella ruminicola
AB219152
AY836507 Prevotella veroralis
Fpc1 (1/16)
Lpc13 Prevotella bryantii
AF396925
L16476 Prevotella buccalis
L16474 Prevotella oris
AY689228
L16475 (1/3) Prevotella nigrescens
Fpc42 Prevotella bivia
Fpc61
Fpc59 (1/8)
AY695689 Uncultured bacteroidales PigA4
Cluster I
Pig-1-Bac marker
AJ011682
AY212554 Prevotella brevis
Fpc52 Uncultured Bacteroidales HoF
Fpc38 (1/2)
AB237870 Uncultured bacterium clone P95
Fpc93
Lpc27 Uncultured bacterium
AB237866 clone P72
L16480 Prevotella oralis
AJ514252 Prevotella buccae
AY836508 Prevotella loescheii
X81870 Prevotella dentalis
Lpc30 Uncultured bacteroidales
AY695693 PigC6
Fpc50 Uncultured
AY695694 Bacteroidales PigE2
AY695692 Uncultured bacteroidales
Lpc55 (1/2) PigC10
Fpc35
AB237869 Uncultured bacterium clone P93
Fpc56
Fpc3
AB237867 Uncultured bacterium clone P80
Fpc37
Lpc11
Fpc49 (1/14)
Fpc5
Lpc22 (1/8)
Lpc71
Fpc39 (1/4)
Fpc8 (1/7)
AY695691 uncultured bacteroidales PigC1
AY695696 Uncultured bacteroidales PigH10
Lpc61
Lpc45
Fpc28
Fpc68
Fpc87
Cluster II
Pig-2-Bac marker
Lpc77
AF233400 Uncultured Bacteroidales CF123
AF233401 Uncultured Bacteroidales CF157
AF233406 Uncultured Bacteroidales CF151
Bac_R marker
Lpc19
Lpc24 (1/5)
Lpc34
X83951 Bacteroides caccae
X83952 Bacteroides ovatus
L16489 Bacteroides thetaiotaomicron
M61006 Bacteroides fragilis
AB021165 Bacteroides acidofaciens
L16485 Bacteroides eggerthii
AF233411 Uncultured Bacteroidales HF145
AF233408 Uncultured Bacteroidales HF8
AB050111 Bacteroides vulgatus
AF233412 Uncultured Bacteroidales HF74
AF233413 Uncultured Bacteroidales HF10
HF 183 marker
Lpc3 (1/7)
AY695697 Uncultured bacteroidales clone PigH8
AY695695 Uncultured bacteroidales clone PigG4
Lpc29
Lpc60
Lpc67 (1/3)
Lpc53
Lpc68
0.022

FIG. 2: Phylogenetic relationships of 51 OTUs obtained from partial 16S rRNA gene sequences from pig fecal (Fpc) and pig slurry samples (Lpc) using Bac32F and Bac708R Bacteroidales specific-primers (5). An OUT is defined by assigning 16S rRNA gene sequences of >98% similarty to the same species. The numbers above the branch points are the percentages of bootstrap replicates that support the branching order. Scale bar represents 2.2% sequence divergence. The numbers in parentheses indicate the frequency of identical clones (example: 1/8 – 1 OTU represents 8 sequences). Known Bacteroides and Prevotella sequences obtained from GenBank are also included.

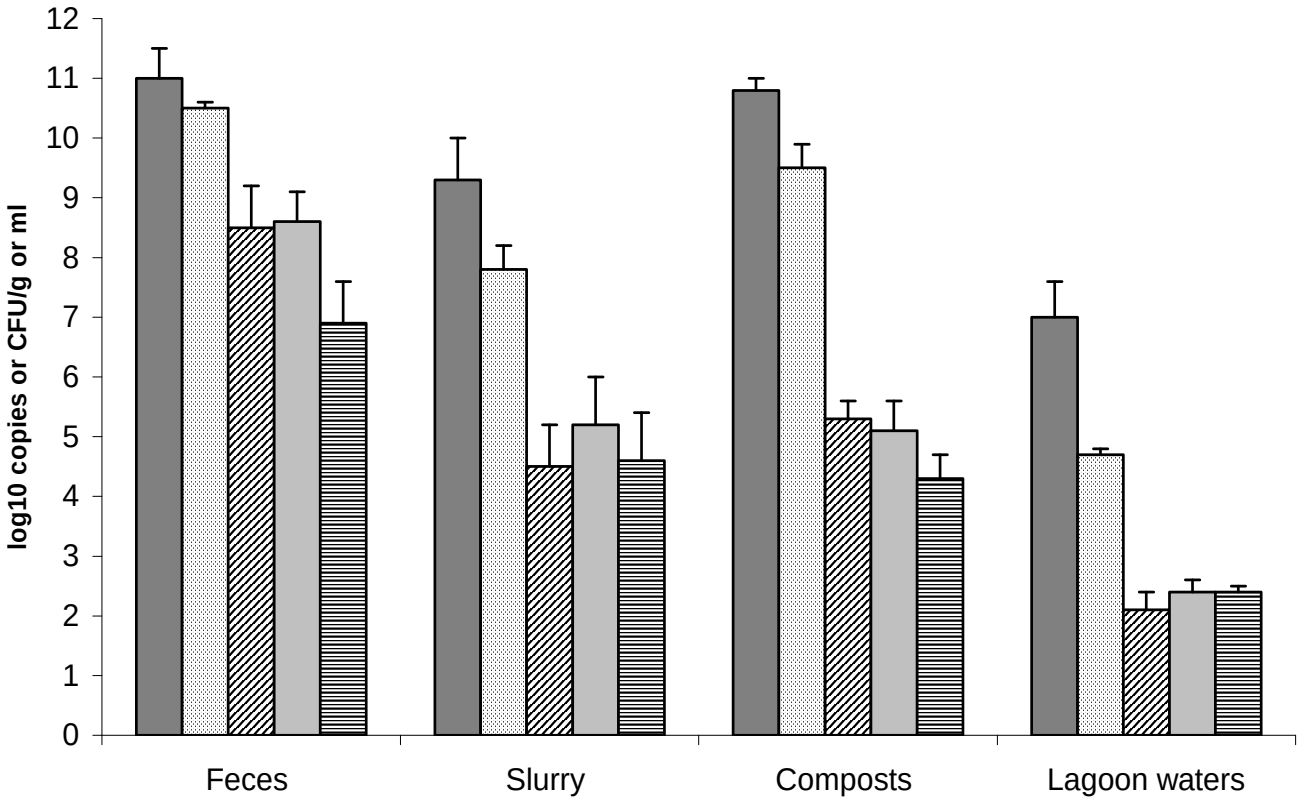
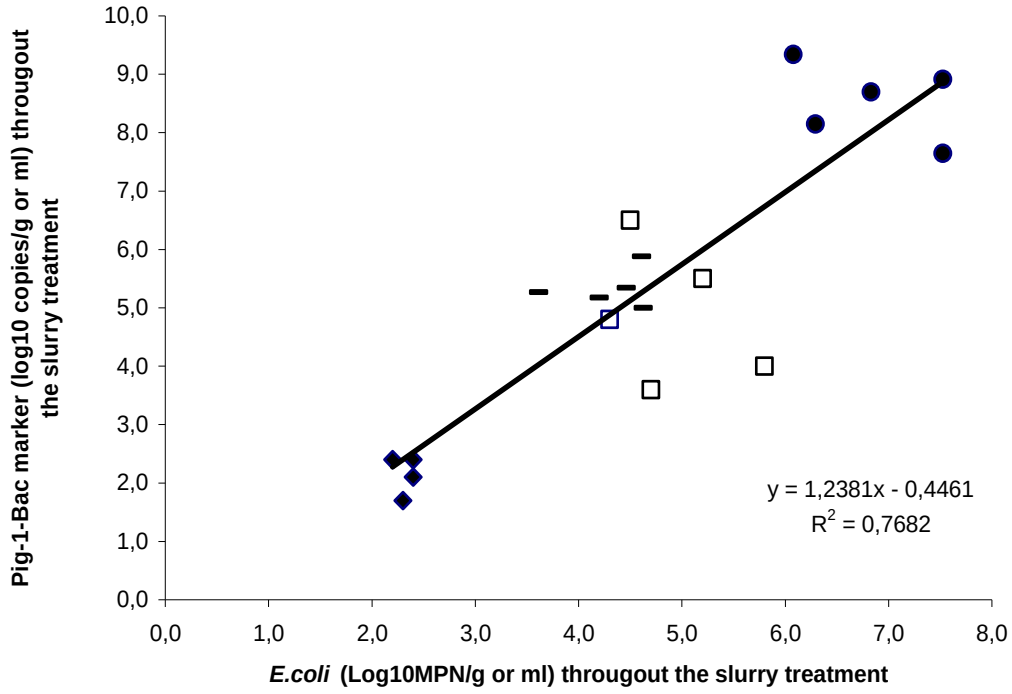


FIG. 3: Concentration of all-*Bacteria* ( ), all-*Bacteroidales* ( ), pig-1-Bac ( ) and Pig-2-Bac ( ) markers (log_{10} copies / g or ml) and *E. coli* bacteria in CFU / g or ml (; MPN per g or ml) in different target samples (feces and waste effluents samples; *n=5*) from three farms, obtained throughout the slurry treatment process. Error bars show standard deviations.

Figure 4



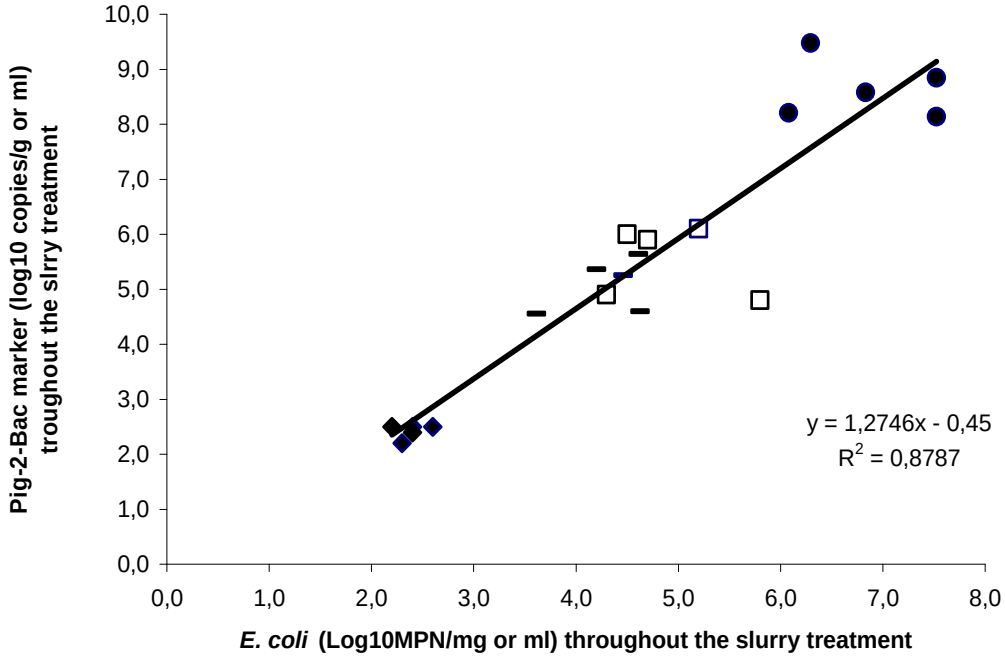


FIG. 4: Relation between concentrations of pig-specific *Bacteroidales* markers (Pig-1-Bac and Pig-2-Bac) and *E. coli* throughout the slurry treatment process (• feces, □ slurry, ▬ compost and ♦ lagoon water samples).