Discrimination between human, pig and ruminant faecal contaminations in a river catchment by real-time PCR using host-specific markers

Mieszkin Sophie [1], Furet Jean-Pierre [2], Corthie Gérard [2], Pommepuy Monique [1], Le Saux Jean Claude [1], Bougeard Morgane [1], Hervio-Heath Dominique [1] and Gourmelon Michèle [1*]

[1]Ifremer EMP, Laboratoire de Microbiologie, 29280 Plouzané, France,

[2]Unité d'écologie et de physiologie du système digestif (UEPSD), INRA, 78352 Jouy-en-Josas cedex, France

****corresponding author: Michele.Gourmelon@ifremer.fr***

The microbiological quality of coastal waters and shellfish harvesting areas in Brittany (France) can be affected by faecal pollutions from human activities and animal breeding (especially pigs and cattle). To discriminate among faecal pollution of human and animal origin, a library-independent microbial source tracking method was selected: *Bacteroidales* host-specific 16S rRNA gene markers by real-time Polymerase Chain Reaction (PCR). A human-specific *Bacteroidales* marker (Hum-1-Bac) was designed. Tested on faecal samples, the Hum-1-Bac marker showed 95 % sensitivity and 95 % specificity (*n= 80*). Average values (± STD) of the Hum-1-Bac marker were found to be 7.3 ± 1.4 16S rRNA gene copies per g wet faeces in human faeces samples (*n=10*) and 5.7 ± 1.3 $\log_{10}$ copies per 100 ml water in Sewage Treatment Plant (STP) water samples (*n=10*). These results highlight that the human-specific marker present in individual faeces was still present in the effluents.

The human-specific marker developed in this study (Hum-1-Bac) and the human- (HF183), pig- (Pig-2-Bac) and ruminant- (Rum-2-Bac) specific *Bacteroidales* markers previously described by Seurinck *et al.* (2005) and Mieszkin *et al.* (2009, 2010) were then applied to river water samples (*n=33*) collected in 2008-2009 from the catchment of the Daoulas river estuary (Brittany, France), upstream of shellfish harvesting areas. For all sites, at least two host-specific markers were detected. The ruminant-specific *Bacteroidales* marker was more often quantified (60.6 %) than the human- or pig-specific *Bacteroidales* markers (Hum-1-Bac, 45.5 %; HF183, 48.5 % and Pig-2-Bac, 30.3 %) in river water samples. These results show that faecal pollution came generally from multiple origins and that host-specific *Bacteroidales* markers are promising as a quantitative microbial source tracking method to determine sources of faecal pollution in environmental water.

Key words: Microbial Source Tracking / Host-Specific Bacteroidales Marker / 16S rRNA Gene / Real-Time PCR / Faecal Contamination

Introduction

Coastal and river waters are an important resource for both shellfish farming and bathing areas. However faecal contamination from point source discharges, such as effluents from sewage treatment plants (STP), or from non point source discharges, such as effluents from animal rearing can affect the microbial quality of waters and shellfish. Furthermore, human enteric bacteria and viruses, such as *Salmonella,* norovirus and hepatitis A virus, have been isolated from coastal waters and shellfish in recent incidents where they were implicated in shellfish-borne disease outbreaks following oyster consumption (Le Guyader and Atmar, 2007). Management techniques have been proposed to correctly identify the origin of faecal pollution and distinguish between animal and human sources. For example, in response to health risks in recreational waters, the revised European Bathing Water Directive (2006/7/EC) changed the bathing water classification system and introduced more stringent standards. One important change was the requirement to produce bathing water profiles, meaning that the origin of the faecal contamination now has to be identified. For shellfish farming areas, the revision of the EU directive (Hygiene 3 regulation N°854/2004) also requires that assessment be made of the potential faecal sources upstream of shellfish farming areas. However, current standard techniques, which enumerate culturable *Escherichia coli* and enterococci, are not sufficient to determine the origin of contamination, as these bacteria are found in the faeces of both humans and warm-blooded animals. Alternative microbial and chemical (Microbial Source Tracking: MST) methods have been developed to distinguish between human and animal faecal pollution, targeting microorganisms, such as bacteria, viruses or protozoa, and chemical compounds, such as caffeine, sterols and stanols (Bernhard and Field 2000; Glassmeyer *et al.* 2005). Several approaches exist to identify the origin of faecal pollution in a complex catchment: (1) microbial source apportionment studies, (2) catchment modelling or (3) application of MST methods to water samples.

In the present study, a library-independent MST method was selected using host-specific *Bacteroidales* 16S rRNA gene markers (human-, ruminant- and pig-specific markers already published and a human-specific marker developed in this study) by real-time Polymerase Chain Reaction (PCR) to discriminate the origin of faecal contamination from the catchment of the Daoulas river estuary in France. This selected catchment is mainly characterised by intensive livestock farming (dairy cows, pigs and poultry) with the total number of inhabitants estimated at 15 000 (Pommepuy *et al.*, 2008). The coastal shellfish harvesting areas are classified in the B category according to European legislation (European Directive 91/492/EEC). This means that the shellfish have been found to contain between 230 and 4600 *E. coli* per 100 g of total flesh and intravalvular fluid and must be depurated for ≥ 48 h in good quality water prior to sale.

The objective of this study was to use data obtained from a microbial source apportionment study on the Daoulas river catchment (Brittany, France) to test the relevance of the host-specific *Bacteroidales* markers developed or selected in our laboratory.

1. Materials and methods

1.1. Development of a human-specific *Bacteroidales* marker

1.1.1. Faecal sample collection and DNA extraction

Twenty samples of human origin (faeces and sewage treatment plant (STP) water) and 60 samples of animal origin (pig faeces and liquid manure, bovine faeces and manure, ovine and equine faeces) were collected. DNA was extracted from faecal samples using the Fast DNA Spin Kit for Soil (MP Biomedical, Illkirsh, France). For STP water samples, 25-200 ml were 0.22 µm-filtered and DNA was extracted using the DNeasy Tissue kit (Qiagen, Courtaboeuf, France) as described in Mieszkin *et al.* (2009).

1.1.2. Oligonucleotide primers and probe

Human-specific *Bacteroidales* primers (Hum-1-Bac) were designed from multiple alignments of partial *Bacteroidales* 16S rRNA gene sequences obtained by Mieszkin *et al.* (2010) from human faeces and STP water samples. The HF183 set of primers was used to amplify human-specific *Bacteroidales* 16S rRNA genes (Seurinck *et al.,* 2005). The primers and probe AllBac, Pig-2-Bac and Rum-2-Bac were used to amplify all *Bacteroidales*, pig- and ruminant-specific *Bacteroidales* 16S rRNA genes, respectively (Layton *et al.* 2006; Mieszkin *et al.* 2009, 2010).

Oligonucleotide specificity for human-associated *Bacteroidales* 16S rRNA genes was verified using the BLAST (NCBI) and Probe Match (Ribosomal Database Project: RDP II) programs.

1.2. Application of host-specific *Bacteroidales* markers to river water

1.2.1. River water sample collection and DNA extraction

River water samples were collected on the catchment of the Daoulas river estuary. Thirty-three river water samples were collected at 8 different sites. Three sites (N°s 2, 4 and 5) were selected for their proximity to pig farming activities, 4 sites (N°s 1, 6, 7 and 8) for their proximity to cattle farming and 1 site (N° 3) downstream, close to an urban area (Daoulas) (Fig. 1). For river water samples, 200 ml were 0.22 µm-filtered and DNA was extracted using the DNeasy Tissue kit (Qiagen, Courtaboeuf, France).

1.2.2. Real-time PCR assays

All amplifications were performed using the Chromo4 real-time detection system associated with Bio-Rad Opticon Manager software version 3.1 (Bio-Rad, Hercules, CA). Real-time PCR for the Hum-1-Bac and HF183 markers were performed using the Brillant® SYBR® Green QPCR Master Mix (Stratagene, Massy, France). Real-time PCR for the AllBac, Pig-2-Bac and Rum-2-Bac markers were performed using the TaqMan® Brilliant II QPCR Master Mix kit (Stratagene, Massy, France). Each reaction was run in duplicate with cycle conditions as described in Mieszkin *et al.* (2009, 2010). For the Hum-1-Bac marker, the PCR mixture contained 200 nM of each primer and a dissociation step was added to control amplification specificity

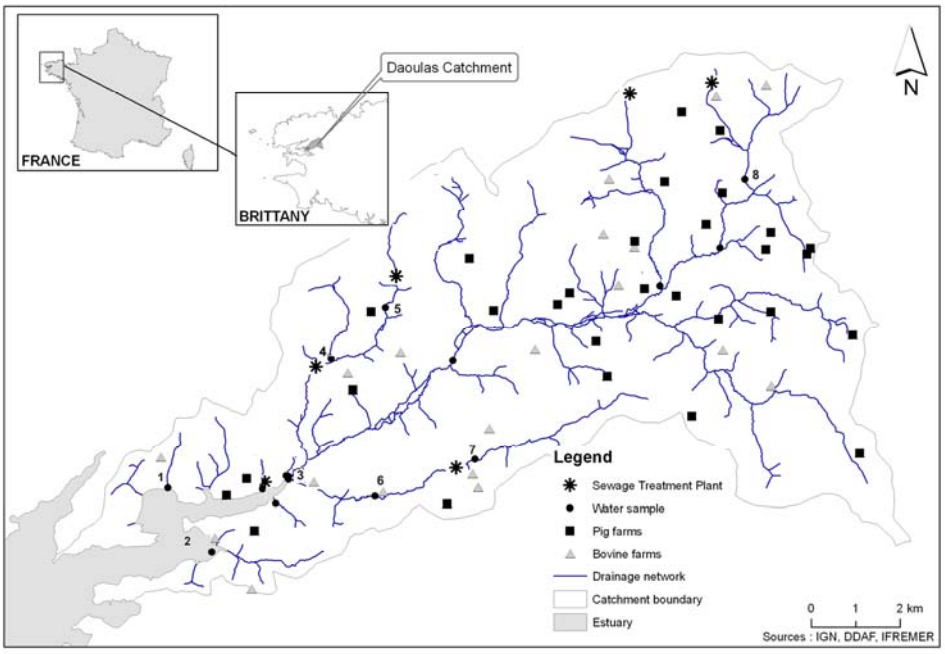

Figure 1. Location of water sampling sites, pig and cattle farms and sewage treatment plants on the catchment of the Daoulas river estuary, Brittany, France

. The presence/absence of PCR inhibitors was verified using an Internal Positive Control (IPC; Applied Biosystems, Villebon sur Yvette, France). Samples were diluted if inhibitors were present. Negative controls (no template DNA) were performed in triplicate for each run. The sensitivity and the specificity of the human-specific *Bacteroidales* markers (Hum-1-Bac and HF183) were tested on target (*n=20*) and non target DNA (*n=60*).

1.2.3. DNA standard curves and quantification

Linear plasmid DNA used to generate standard curves was obtained as described in Mieszkin *et al.* (2009). 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 gene copy numbers.

1.2.4. Enumeration of E. coli

E. coli enumerations were performed by the microplate method (EN ISO 9308-3. 1999) with a detection limit of < 15 MPN (Most Probable Number) per 100 ml water.

1.2.5. Data treatment

The results of general and host-specific *Bacteroidales* marker quantifications were expressed in numbers of 16S rRNA gene copies per g faeces and per 100 ml water.

To evaluate the performance of the MST methods on faecal samples, sensitivity (r) and specificity (s) were defined as r = a/(a+c) and s = d/(b+d), where a, corresponded to faecal samples positive for the marker of its own species (true positive); b, corresponded to

faecal samples positive for a marker of another species (false positive); c, corresponded to faecal samples negative for a marker of its own species (false negative); and d, corresponded to faecal samples negative for a marker of another species (true negative) (Fisher and Van Bell 1993).

2. Results

2.1. Design and validation of a new human-specific *Bacteroidales* marker

Human-specific *Bacteroidales* sequences were identified from *Bacteroidales* 16S rRNA gene sequences from human faeces and STP water samples obtained by Mieszkin *et al.* (2010). They were used to design PCR primers (Hum-1-Bac marker: Hum-1-Bac32F-AACGCTAGCTACAGGCTTAAC and Hum-1-Bac169R-CATGCGGACATGTGAACTCATG) to identify a human source of faecal pollution in water.

For the Hum-1-Bac marker, plasmid DNA containing a 16S rRNA gene sequence insert was run as a standard, using 10-fold dilutions ranging from 1.6×10^7 to 1.6×10^0 copies per PCR mixture with a quantification limit of 1.6 target copies per reaction. Consequently, the lower limits of quantification were 4.5 $\log_{10}$ copies per g faeces and manure and 2.6 $\log_{10}$ copies per 100 ml water.

The primers of the Hum-1-Bac marker were found to be both sensitive (95 %) and specific (95 %). Indeed, on 20 samples of human origin, only one DNA sample extracted from human faeces was not amplified. Hum-1-Bac marker concentrations were 7.3 ± 1.4 $\log_{10}$ copies per g in human faeces and 5.7 ± 1.3 $\log_{10}$ copies per 100 ml in STP water samples (Table 1). Concerning non target DNA (*n=60*), three pig faeces samples were amplified but at weak concentrations (from 4.7 to 5.2 $\log_{10}$ copies per g) (Table 1).

Table 1. Percentage of sensitivity and specificity of host-specific Bacteroidales markers and their quantification in faecal and effluent samples; percentage of positive results and concentrations

	Host-specific *Bacteroidales* markers							
	Human marker: Hum-1-Bac		Human marker: HF183[1]		Ruminant marker: Rum-2-Bac[1]		Pig marker: Pig-2-Bac[1]	
Source of sample (*n = 10*)	**Positive samples (%)**	**Concentrations[2]**	**Positive samples (%)**	**Concentrations**	**Positive Samples (%)**	**Concentrations**	**Positive Samples (%)**	**Concentrations**
Faeces								
Human	90	7.3 ± 1.4	70	8.4 ± 1	0	<4.5[4]	0	<4.5
Pig	30	4.7 to 5.2	0	<4.5	0	<4.5	100	8.6 ± 0.8
Bovine	0	<4.5	0	<4.5	100	8.2 ± 0.5	0	<4.5
Ovine	0	<4.5	10	4.8	100	8.4 ± 1.3	0	<4.5
Equine	0	<4.5	0	<4.5	0	<4.5	0	<4.5
Effluent								
STP[3] water	100	5.7 ± 1.4	100	7.7 ± 1.1	20	3.1 ; 4	0	<2.6
Pig liquid manure	0	<4.5	0	<4.5	0	<4.5	100	7.2 ± 0.7
Bovine manure	0	<4.5	0	<4.5	100	7 ± 0.5	0	<4.5
Sensitivity (%)	**95**		**85**		**100**		**100**	
Specificity (%)	**95**		**98**		**96**		**100**	

[1]Results published in Mieszkin *et al.* (2009, 2010), [2]Concentrations are expressed in $\log_{10}$ copies per gram faeces or manure and in $\log_{10}$ copies per 100 ml pig liquid manure and STP water samples, [3]STP: sewage treatment plant, [4]Quantification limit of *Bacteroidales* markers

2.2 Application of *E. coli* and *Bacteroidales* markers to river waters

Escherichia coli was enumerated in all river water samples at concentrations that varied with sampling point from <1.6 to 4.3 $\log_{10}$ MPN per 100 ml river water. The AllBac

marker was also found in all samples at concentrations varying from 5.1 to 6.9 $\log_{10}$ copies per 100 ml water between samples (Table 2).
For all sites, at least two host-specific markers were detected with concentrations ranging from 2.7 to 5.7 $\log_{10}$ copies per 100 ml water.
In river water samples collected downstream and near the urban area of Daoulas (site 3), both human-specific markers were quantified in 62.5 % of samples, with similar concentrations ranging from 2.7 to 4.1 $\log_{10}$ copies per 100 ml water, respectively. The Rum-2-Bac and Pig-2-Bac markers were weakly quantified (50 and 37.5 % of water samples, respectively), with concentrations ranging from 3.4 to 5 and from 3.4 to 3.5 $\log_{10}$ copies per 100 ml water, respectively (Table 2).
In river water samples collected near cattle farms (sites 1, 6, 7 and 8), the Rum-2-Bac marker was quantified in all samples, with concentrations ranging from 3.5 to 5.7 $\log_{10}$ copies per 100 ml water. Both human-specific markers were quantified in 42-50 % of water samples, with similar concentrations, ranging from 3.2 to 5.4 $\log_{10}$ copies per 100 ml water. The Pig-2-Bac marker was only quantified twice (at concentrations of 3.5 and 3.9 $\log_{10}$ copies per 100 ml water).

Table 2. Concentration medians and ranges of general and host-specific Bacteroidales markers and E. coli *in river waters samples taken from the catchment of the Daoulas river estuary*

Site	Faecal contamination expected		*E. coli*[1]	General *Bacteroidales* (AllBac)[1]	Human-specific *Bacteroidales* (Hum-1-Bac)[1]	Human-specific *Bacteroidales* (HF183)[1]	Ruminant-specific *Bacteroidales* (Rum-2-Bac)[1]	Pig-specific *Bacteroidales* (Pig-2-Bac)[1]
1	Urban and rural (bovine, ovine and poultry)	Median	3.2	6	<2.6[2]	<2.6	4.8	<2.6
		Range	1.9 - 4.3	5.5 - 6.7	<2.6 - 3.7	<2.6 - 3.9	4.4 - 5.4	<2.6
n = 4		N[3]	4	4	1	1	4	0
2	Urban and rural (bovine and pig)	Median	3	5.8	<2.6	<2.6	4.3	3.2
		Range	2.3 - 3.5	5.6 - 6.8	<2.6	<2.6	<2.6 - 4.9	<2.6 - 4.1
n = 3		N	3	3	0	0	2	2
3	Urban and rural (pig, bovine and poultry)	Median	2.8	5.9	3	3.1 <2.6 – 4.1	3	<2.6
		Range	1.9 - 3.3	5.1 - 6.5	<2.6 - 3.9		<2.6 - 5	<2.6 - 3.5
n = 8		N	8	8	5	5	4	3
4	Rural (pig)	Median	2.9	5.6	3.2	3.7 <2.6 – 4.4	<2.6	<2.6
		Range	2.2 - 3.1	5.1 - 5.8	D - 4.2		<2.6 - 3.9	<2.6 - 3.9
n = 3		N	3	3	2	2	1	1
5	Rural (pig and poultry)	Median	3.7	6.6	4.5	4.7 <2.6 – 5.1	<2.6	3.5
		Range	2.9 - 4.3	6.1 - 6.6	<2.6 - 4.9		<2.6	<2.6 - 4.4
n = 4		N	4	4	3	3	0	2
6	Urban and rural (pig and bovine)	Median	3.8	6.2	4.1	4.3 3.2 - 5.4	5	<2.6
		Range	2.9 - 4.1	5.2 - 6.9	3.2 - 5.2		4.5 - 5.7	<2.6 - 3.9
n = 4		N	4	4	4	4	4	1
7	Rural (bovine)	Median	2.6	6	<2.6	<2.6 <2.6 - 4.3	4.5	<2.6
		Range	2.1 - 3	5.6 - 6.4	<2.6		3.5 - 4.9	<2.6
n = 4		N	4	4	0	1	4	0
8	Rural (pig and bovine)	Median	2.3	5.6	<2.6	<2.6	<2.6	<2.6
		Range	<1.6 - 3.5	5.5 - 5.8	<2.6	<2.6	<2.6 - 4.4	<2.6 - 3.5
n = 3		N	3	3	0	0	1	1

[1]Concentrations of *Bacteroidales* markers are expressed in $\log_{10}$ copies per 100 ml and *E. coli* in $\log_{10}$ MPN per 100 ml.[2]Quantification limit of *Bacteroidales* markers. [3]Numbers of results>quantification limit for each *Bacteroidales* marker and *E. coli.* [4]Detected: positive results were obtained in three repeated experiments

In river water samples collected near pig farms (sites 2, 4 and 5), the Pig-2-Bac marker was quantified in 50 % of water samples, with concentrations ranging from 3.2 to 4.4 $\log_{10}$ copies per 100 ml water. The Rum-2-Bac, Hum-1-Bac and HF183 markers were quantified in 30, 40 and 38 % of water samples with concentrations ranging from 3.9 to 4.9, from 3.2 to 4.9 and from 3.7 to 5.1 $\log_{10}$ copies per 100 ml water, respectively (Table 2).

3. Discussion and conclusion

This study illustrates the difficulty of obtaining a human-specific *Bacteroidales* marker that is highly specific and sensitive. Sensitivity (95 %) of the Hum-1-Bac marker was better than the sensitivity of the human-specific marker HF183 (85 %) developed by Seurinck *et al.* (2005), but concentrations were 1 and 2 log units weaker than the concentrations of the HF183 marker in human faeces and STP water samples, respectively. However, for the Hum-1-Bac marker, amplifications were obtained with DNA extracts from pig faeces, although these concentrations were 100 to 1000 fold weaker than those obtained with target DNA. In river water samples, similar concentrations of the two human markers were obtained. Further sampling and analyses on faeces and effluent samples from this catchment could be useful to understand the concentration differences between these two human markers in human faeces and STP waters, and the similar results in river water samples. Comparisons of several human-specific *Bacteroidales* markers with the HF183 marker were performed by Kildare *et al.* (2007) and Ahmed *et al.* (2009). These studies also showed the difficulty of obtaining a human-specific *Bacteroidales* marker that is both specific and sensitive. However, among the different human-specific *Bacteroidales* markers tested, the HF183 marker had the best performance.

In the second part of this study, human, pig and ruminant faecal pollution were discriminated in river water samples from the Daoulas catchment using host-specific *Bacteroidales* markers and real-time PCR. For all sites, at least two host-specific markers were detected, implying that faecal pollution generally came from multiple origins. The ruminant-specific *Bacteroidales* marker was more often quantified (60.6 %) than the human or the pig-specific *Bacteroidales* markers (Hum-1-Bac, 45.5 %; HF185, 48.5 % and

Pig-2-Bac, 30.3 %). These results correspond to the large number of cattle (3300 animals) over the catchment sites as a whole. Indeed, bovine grazing or drinking near rivers was observed near the sites. Furthermore, cattle production could lead to faecal pollution entering the river due to bad manure spreading practices on arable land. Human faecal pollution was also high in this catchment and could be due to the presence of 6 STP and of numerous individual houses. For example, the subcatchment with the sites 6 and 7 is mainly characterised by cattle farming (500 animals) and there is a STP located between these two sites. Ruminant and human faecal pollution were detected, with the ruminant-specific markers quantified in all the water samples from the two sites and human-specific marker mainly detected at site 6, downstream of the STP. Furthermore, on site 1, which is near a farm with 50 bovines and also near sheep farms, the ruminant-specific marker could be quantified in all the river water samples collected. Sites 4 and 5 are characterised by high pig production, indeed site 5 is located near a farm with about 7000 pigs and a STP is located upstream. Detection of pig- and human-specific markers at these sites revealed pig and human faecal contamination. Human, ruminant and pig pollution were all

observed at the site 3 located downstream the catchment, in the Daoulas town (1770 inhabitants), which represents a subcatchment with 60 000 pigs and 2400 cattle. The human-, ruminant- and pig-specific markers were detected in five, four and three out of eight samples, respectively, underlining importance of human and animal faecal pollution at this site.

Interestingly in this study, the results we obtained with host-specific *Bacteroidales* markers in river waters were in accordance with the description of the potential sources of faecal contamination in the catchment. However, additional sampling should be carried out to improve identification of faecal pollution origin. For the Daoulas catchment, it could be interesting to develop a domestic bird-specific marker (turkey and chicken) due to the importance of poultry production (383 000 birds) on the catchment.

Acknowledgments

Sophie Mieszkin is the recipient of an Ifremer-Région Bretagne fellowship.

References

Ahmed W., Goonetilleke A., Powell D., Chauhan K., Gardner T., 2009. Comparison of molecular markers to detect fresh sewage in environmental waters. *Water Research*, 43, 4908-4917.

Bernhard A.E., Field K.G., 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA, *Applied and Environmental Microbiology*, 66, 4571-457.

Fisher L. D., Van Belle G., 1993. Biostatistics: a methodology for health sciences. Wiley, New York, NY.

Glassmeyer S.T., Furlong E.T., Kolpin D.W., Cahill J.D., Zaugg S.D., Werner S.L., Meyer M.T., Kryak D.D., 2005. Transport of chemical and microbial compounds from known wastewater discharges: potential for use as indicators of human fecal contamination, *Environmental Science and Technology*, 39, 5157-5169.

Kildare B.J., Leutenegger C.M., McSwain B.S., Bambic D.G., Rajal V.B., Wuertz. S., 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: A Bayesian approach, *Water Research*, 41, 3701-3715.

Layton A., McKay L., Williams D., Garrett V., Gentry R., Sayler G., 2006. Development of *Bacteroides* 16S rRNA gene TaqMan-Based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water, *Applied and Environmental Microbiology*, 72, 4214-4224.

Le Guyader F. S., Atmar R. L., 2007. Viruses in shellfish, *Perspectives in Medical Virology,* 17, 205-226.

Mieszkin S., Furet J.P., Corthier G., Gourmelon M., 2009. Estimation of pig fecal contamination in a river catchment by real-time PCR using two pig-specific *Bacteroidales* 16S rRNA genetic markers, *Applied and Environmental Microbiology*, 75, 3045-3054.

Mieszkin S., Yala J.F., Joubrel R., Gourmelon M., 2010. Phylogenetic analysis of *Bacteroidales* 16S rRNA gene sequences from human and animal effluents and assessment of ruminant faecal pollution by real-time PCR, *Journal of Applied Microbiology*, 108 : 974-984

Pommepuy M., Le Guyader F.S., Le Saux J.C., Guilfoyle F., Doré, B., Kershaw S., Lees D., Lowther J.A., Morgan O.C., Romalde J.L., Furones D., Roque A.**,** 2008. Reducing microbial risk associated with shellfish in European countries. Borresen T., Improving seafood products for the consumer. England, Woodhead publishing in *food science, technology and nutrition*, pp. 212-246.

Seurinck S., Defoirdt T., Verstraete W., Siciliano S.D., 2005. Detection and quantification of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater, *Environmental Microbiology*, 7, 249-259.