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Development of microbial and chemical MST tools to identify the origin of the faecal pollution in bathing and shellfish harvesting waters in France

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Abstract:

The microbiological quality of coastal or river waters can be affected by faecal pollution from human or animal sources. An efficient MST (Microbial Source Tracking) toolbox consisting of several host-specific markers would therefore be valuable for identifying the origin of the faecal pollution in the environment and thus for effective resource management and remediation. In this multidisciplinary study, after having tested some MST markers on faecal samples, we compared a selection of 17 parameters corresponding to chemical (steroid ratios, caffeine, and synthetic compounds), bacterial (host-specific *Bacteroidales*, *Lactobacillus amylovorus* and *Bifidobacterium adolescentis*) and viral (genotypes I–IV of F-specific bacteriophages, FRNAPH) markers on environmental water samples (n = 33; wastewater, runoff and river waters) with variable *Escherichia coli* concentrations. Eleven microbial and chemical parameters were finally chosen for our MST toolbox, based on their specificity for particular pollution sources represented by our samples and their detection in river waters impacted by human or animal pollution; these were: the human-specific chemical compounds caffeine, TCEP (tri(2-chloroethyl)phosphate) and benzophenone; the ratios of sitostanol/coprostanol and coprostanol/(coprostanol+24-ethylcopstanol); real-time PCR (Polymerase Chain Reaction) human-specific (HF183 and *B. adolescentis*), pig-specific (Pig-2-Bac and *L. amylovorus*) and ruminant-specific (Rum-2-Bac) markers; and human FRNAPH genogroup II.

Keywords: Microbial source tracking; Faecal pollution; *Bacteroidales*; *Bifidobacterium adolescentis*; *Lactobacillus*; FRNA bacteriophages; Steroids; Caffeine

1. Introduction

Human and animal faecal pollution may affect inland and coastal water with negative effects on recreational uses, public safety or shellfish sanitary status (Feldhusen 2000; Dorfman and Sinclair Rosselot 2008) and can lead to economic losses due to shellfish bed closures or bathing prohibition (Rabinovici et al., 2004).

In order to improve water quality, management and remediation plans need methods of identifying faecal pollution sources. Indeed, the introduction of the Water Framework Directive (WFD, 2000/60/EC) in the EU has provided a framework for integrated water management by river basin, and this requires the consideration of point- or diffuse-pollution sources. More especially, one of its daughter directives, the revised Bathing Water European Directive (2006/7/EC), sets more stringent microbiological quality targets and requires that Member States establish bathing water profiles that assess pollution risks. These profiles are an inventory and study of the pollution sources likely to affect water quality, and are drawn up for each bathing water zone. In addition, the revised EU Shellfish Directive (Hygiene 3 Regulation N°854/2004) goes through the best means of assessment for potential faecal pollution sources upstream of shellfish farming areas.

A major shortcoming of such evaluations at present is that the faecal microbiological indicators used as standards in these regulations *i.e. Escherichia coli* and enterococci cannot distinguish between human and animal faecal pollution. As identification of nonpoint-source or multiple pollutions is difficult, Microbial Source Tracking (MST) methods have been developed to discriminate between human and nonhuman sources of faecal pollution, and between different animal species. Microbial or chemical targets associated with human and animal faeces, or their related effluents, have been proposed (Glassmeyer et al., 2005; US EPA 2005; Field and Samadpour 2007). For example, chemical markers such as faecal steroids (Leeming et al., 1996; Shah et al., 2007; Tyagy et al., 2007), caffeine and synthetic compounds released into urban wastewaters (Glassmeyer et al., 2005) have been used to distinguish human and animal pollution. Microbial markers including bacteria, *Archaebacteria*, viruses and protozoa have also been tested (Bernhard and Field 2000; Jimenez-Clavero et al., 2003; Ufnar et al., 2007).

Until now, no single source tracking microbial or chemical method appears sufficiently discriminatory to identify the origin of faecal pollution in water (Griffith et al., 2003; Blanch et al., 2006; Field and Samadpour, 2007). Although a MST toolbox combining several methods could potentially improve discrimination, only a few studies have been done to develop and compare the combination of several MST methods (Griffith et al., 2003; Blanch et al., 2006; Ahmed et al., 2007; McQuaig et al., 2009).

Given the few data available on combined chemical and microbial source tracking methods, a multidisciplinary study was conducted by six French research Institutes in order to propose an efficient MST toolbox that could differentiate human, pig and bovine pollution in surface waters. In fact, even though pollution from pets, horses and wild animals such as waterfowl birds could contribute to faecal pollution of waters (Savichtcheva et al., 2007; Lu et al., 2008), human wastes (WWTP discharges, autonomous sanitation systems effluents, or sewage sludge spreading), and agricultural runoff containing faecal pollution from pig and bovine manure spreading and pasture are more often responsible for pollution in catchments in France, which is the top cattle producer and the third largest pig producer in the EU.

In the first phase of this project, each laboratory independently developed and validated its chemical or microbial methods on faeces, manure and wastewater treatment plant (WWTP) effluents.

Chemical markers investigated in this study included i) caffeine and seven synthetic compounds selected from the 35 compounds proven useful as indicators of anthropogenic pollution in the largest study performed to date (Glassmeyer et al. 2005), and ii) faecal sterol and stanol fingerprints, which have been widely used to monitor faecal pollution in water and to distinguish human and animal sources (Leeming et al., 1996; Shah et al., 2007).

The microbiological approach, which included viral and bacterial markers, was based on library-independent methods. The viral markers tested were F-specific RNA bacteriophages (FRNAPH); these have been classified into four genogroups, two of which (II, and III) predominate in WWTP effluents, and two of which (I and IV) are mainly associated with animal faeces and effluents (Schaper et al., 2002; Ogorzaly et al., 2006). Bacterial markers were selected among *Bacteroidales* and *Bifidobacterium*, two bacterial groups that have been reported to contain host-specific bacterial species (Bernhard and Field, 2000; Delcenserie et al., 2004; Dick et al., 2005; Lamendella et al., 2008). The human-specific *Bacteroidales* HF183, first described by Bernhard and Field (2000) and modified for real-time PCR assay by Seurinck et al. (2005), was selected due to its high specificity (Mieszkin et al., 2010). Three other *Bacteroidales* (two pig-specific and one ruminant-specific) markers were selected according to the results of a previous analysis of 16S rRNA *Bacteroidales* gene sequences of animal faeces and effluents (Mieszkin et al., 2009). A third pig-specific marker, *Lactobacillus amylovorus*, which has been successfully tested by Marti et al. (2010), was also selected. Finally, a phylotype related to *Bifidobacterium adolescentis* was chosen that had been identified as a potential human marker after analyzing the diversity of bifidobacteria in treated WWTP effluents, although its specificity had not been tested (Wéry et al., 2010).
Our aims were therefore (i) to select and test chemical markers, FRNAPH and *B. adolescentis* on faeces and effluent samples; (ii) to evaluate the efficiency of a new toolbox composed of these markers, *Bacteroidales* markers and *L. amylovorus*, for differentiation of human and livestock faecal pollution, by analyzing wastewaters, runoff waters and rivers; and (iii) to select the most effective set of markers for identifying the origin of faecal pollution.

2. Materials and methods

2.1 Adaptation and testing of existing methods on faecal samples

2.1.1 Steroids

Fifteen steroids (cholesterol, coprostanol, epicoprostanol, cholestanol, α-cholestanol, campesterol, campestanol, stigmasterol, stigmastanol, 5β-stigmastanol, 5β-epistigmastanol, sitosterol, sitostanol, 24-ethylcoprostanol and 24-ethylepicoprostanol) were quantified in 9 bovine and 4 porcine manures and 6 WWTP effluents. These samples were frozen for 2 days at -10 °C and freeze-dried for 3 days before analyses. Concentrations were determined as in Jardé et al. (2009). Steroid compounds were separated by liquid chromatography. After derivatization using N,O-bis(trimethylsilyl)trifuoroacetamide - trimethylchlorosilane (99/1, v/v), quantifications were performed on a Shimadzu QP2010+ gas chromatograph mass spectrometer. The chromatographic separation was performed on a SLBTM-5ms column (Supelco, 60 m, 0.25mm, 0.25 µm) with the program: 70°C (held at 1 min) to 130°C at 15°C/min, then 130°C to 300°C (held at 15 min) at 3°C/min. Helium flow was maintained at 1 mL/min. Quantification of steroid compounds was performed by adding of $^{2}H_{6}$-cholestane as an internal standard.

2.1.2 Human faecal chemical pollution markers

Eight compounds (caffeine, diphenhydramine tri(2-chloroethyl)phosphate (TCEP), tri(dichloroisopropyl)phosphate (TDCP), triclosan, ethyl citrate, galaxolide and tonalide) were selected among the 35 compounds that had been shown to be useful as indicators of anthropogenic pollution in the study by Glassmeyer *et al.* (2005). Except caffeine, which was chosen because of its wide use as chemical tracer, all the other compounds were selected due to their 100 % frequency in wastewater effluents and their potential to be detected using a simple multiresidue method.

Water samples (250 mL) were vacuum filtered through a 1.2 µm GF/C filter (Whatman) and extracted with Oasis HLB® cartridges (500 mg, 6 cm^3) as in Zaugg et al. (2002). Phenanthrene-d10 was used as surrogate standard.
Sample extracts (1 mL in ethyl acetate) were analysed using a GC/MS/MS system: Varian GC 3800 equipped with a Varian 8400 autosampler, and coupled to a Varian 4000 ion trap mass spectrometer operating in electron impact ionization mode at 70 eV. Separation was performed using a FactorFour VF-5 ms column.
The GC conditions were: splitless injection (290 °C, 2 min, 2 µL injection volume, 1 mL/min He) and a temperature program of 60 °C for 3 min, 20 °C/min to 200 °C, 5 min isothermal, 10 °C/min to 280 °C and 15 °C/min to 320 °C. The ion trap was maintained at 200 °C, the manifold at 80 °C and the line transfer at 260 °C. The mass detection parameters were individually optimized for each analysis, as collision energy was required to produce the daughter ions.
The most intense peak(s) of daughter ions were used for quantification.

2.1.3 F-specific RNA bacteriophages

FRNAPH were enumerated following the ISO 10705 method (Anonymous 2001). FRNAPH genotyping was done on bacteriophage isolates by plaque hybridization with labelled oligonucleotide probes designed by Beekwilder et al. (1996). Faeces of humans, pigs, cows, sheep and birds (*n=125*), and effluent samples (*n=100*; WWTP effluent, porcine and bovine manure) were analysed to evaluate sensitivity and specificity of this method. However, as the plaque hybridization method is time-consuming, a new genotyping method was also carried out based on real-time RT-PCR (Reverse Transcriptase-PCR) with a One-Step RT-PCR kit (QuantiTech Probe RT-PCR, Qiagen; Ogorzaly et al., 2009) using primers and probes described in Ogorzaly and Gantzer (2006, Table 1).
A comparison was made between the two methods on 60 representative or environmental bacteriophage isolates.
FRNAPH concentrations were expressed in plaque-forming units (PFU) per g faeces or per 100 mL water. The quantification limit is 1 PFU per g faeces and 10 PFU par 100 mL water. Only results with at least 20 successfully-genotyped plaques were considered as valid FRNAPH source tracking results. Indeed, due to the statistical variation of results from FRNAPH tests, those of <20 PFU were considered to be inconclusive (Stapleton et al., 2007). Results are expressed in percentage (%) human (II or III) or animal (I or IV) genogroups/total FRNAPH isolated.

Table 1. Primer and probe sequences for real-time PCR for detection of bacterial markers and of four F-specific RNA phage genogroups

Primers and probe	Sequences (5'→ 3')	Amplicon size (bp)	Annealing temperature (°C)	Final concentration (nmol)	Target	References
AllBac						
AllBac296F	GAGAGGAAGGTCCCCCAC	106	60	200	All-*Bacteroidales* général	Layton *et al.,* (2006)
AllBac467R	CGCTACTTGGCTGGTTCAG			200		
AllBac375Bhqr	**(FAM)CCATTGACCAATATTCCTCACTGCTGCT (BHQ-1)**			100		
HF183						
HF183f	ATCATGAGTTCACATGTCCG	83	60	200	Human - specifc *Bacteroidales*	Seurinck *et al.*, (2005)
HF183r	TACCCCGCCTACTATCTAATG			200		
Rum-2-Bac						
BacB2-590F	ACAGCCCGCGATTGATACTGGTAA	99	60	200	Ruminant-specific *Bacteroidales*	Mieszkin *et al.,* (2010)
Bac708Rm	CAATCGGAGTTCTTCGTGAT			200		
BacB2-626P	**(FAM)ATGAGGTGGATGGAATTCGTGGTGT(BHQ-1)**			200		
Pig-1-Bac						
Pig-1-Bac32Fm	AACGCTAGCTACAGGCTTAAC	129	60	200	Pig-specific *Bacteroidales*	Mieszkin *et al.,* (2009)
Pig-1-Bac108R	CGGGCTATTCCTGACTATGGG			200		
Pig-1-Bac44P	**(FAM)ATCGAAGCTTGCTTTGATAGATGGCG(BHQ-1)**			200		
Pig-2-Bac						
Pig-2-Bac41F	GCATGAATTTAGCTTGCTAAATTTGAT	116	60	300	Pig-specific *Bacteroidales*	Mieszkin *et al.,* (2009)
Pig-2-Bac163Rm	ACCTCATACGGTATTAATCCGC			300		
Pig-2Bac113MGB	**(VIC)TCCACGGGATAGCC(NFQ-MGB)**			200		
L. amylovorus	TTCTGCCTTTTTGGGATCAA	175	60	200	*L. amylovorus*	Konstantinov *et al.,* (2006)
	CCTTGTTTATTCAAGTGGGTGA			200		
Bifid. adolescentis					Human-specific *Bifidobacteria* Phylotype	This study
W257F	GGGTGGTAATGCCGGATG	325	60	300		
W255R	GGTGCTTATTCGAAAGGTACACTCA			300		
W256P	**(FAM)ATGTCCTTCTGGGAAAGATTCATCGGTATG (TAMRA)**			100		
FRNAPH I						
GIF	TCGATGGTCCATACCTTAGATGC	176	60	400	Animal genogroups	
GIR	ACCCCGTTAGCGAAGTTGCT			400		
GIP	**(FAM)CTCGTCGACAATGG(MGBNFQ)**			150		
FRNAPH II						
GIIF	TGCAAACCTAACTCGGAATGG	72	60	400	Human genogroups	Ogorzaly et Gantzer, (2006)
GIIR	AGGAGAGAACGCAGGCCTCTA			400		
GIIP	**(FAM)TCCCTCTATTTCCTC (MGBNFQ)**			150		
FRNAPH III						
GIIIF	CCGCGTGGGGTAAATCC	115	60	400	Human genogroups	
GIIIR	TTCTTACGATTGCGAGAAGGCT			400		
GIIIP	**(FAM) AAGCGGGTGCAGTT(MGBNFQ)**			150		
FRNAPH IV						
GIVF	GCTACTAGCCTTCGTCGCAAGA	62	60	400	Animal genogroups	
GIVR	GAAGGCACTGTCCTGAATCCA			400		
GIVP	**(FAM)AGGTCGGTACAAAGTG (MGBNFQ)**			150		

2.1.4 Bifidobacterium adolescentis marker

Sample collection and DNA extraction

Individual human faecal samples were obtained from 10 healthy adult and child volunteers from Brittany (France). Individual animal faecal samples were collected immediately after excretion from apparently healthy animals (14 pigs, 14 cows, 10 sheep and 10 horses).
Porcine faecal samples were collected from adult sows and boars, young pigs, and piglets mainly housed in stalls on farms in Brittany. Cow faecal samples were collected from animals kept on pasture or housed in stalls, and included samples from adults and heifers on 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. Poultry droppings (*n=4*) were collected from a henhouse in Brittany. Wild bird faeces (*n=15*) were collected on two beaches in Brittany.
Eight WWTP water samples were collected at the outlets of eight independent municipal WWTP (not connected to slaughterhouses) in Pays de la Loire.
Six porcine manure samples were collected from six separate farms in Brittany.

For faeces samples, DNA was extracted from 250 mg wet weight of samples using the Fast DNA Spin Kit for Soil (MP Biomedical, Illkirsh, France) according to Dick et al. (2005). Porcine manure and WWTP samples were centrifuged at 9000 g for 15 min and DNA extracted from 200 mg of the pellet using the DNA stool mini kit.

Real-time PCR

A real-time PCR system was designed to specifically amplify the phylotype SFA 41 related to *Bifid. adolescentis* identified in Wéry et al. (2010) (Table 1). The forward *Bifidobacterium* primer (W257F) was previously published by Germond et al. (2002). The probe (W256P) and reverse primer (W255R) were designed using ARB, BLASTN, Primer BLAST (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Primer Express® (Applied Biosystems).
Real-time PCR were done in 25 µL, using 96-well optical reaction plates. PCR of *Bifid. adolescentis* used 12.5 µL 2X qPCR Mastermix Plus for probes (Eurogentec, France), 5 µL diluted DNA, primers (300 nM) and TaqMan probe (100 nM). Amplifications were run on ABI Prism 7000 SDS equipment (Applied Biosystems). The PCR program was: 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C.
The presence/absence of PCR inhibitors was verified using an Internal Positive Control (IPC) (AppliedBiosystem, France). Samples were diluted if inhibitors were present. DNA samples were tested with the IPC at no-dilution (ND), 1/10 and 1/100 dilution. The DNA samples without inhibition were used for the PCR reactions. Negative controls (no template DNA) were performed for each run.
Host-specific PCR assays were tested on faeces and effluent samples. Sensitivity and specificity were calculated according to Gawler et al. (2007).
All samples were tested in triplicate. Plasmid DNA containing partial 16S rRNA gene sequence insert was used as a standard at 10-fold dilutions ranging from 5×10^7 to 5×10^0 gene copies per real-time PCR, with a quantification limit of 5 target copies per reaction.
Consequently, the lower limit for quantification of *Bifid. adolescentis* marker was 3×10^4 copies per g in faeces and 1×10^3 copies per 100 ml water.

2.2 *In situ* application of host-specific microbial and chemical markers

2.2.1 Runoff, WWTP effluent and river water samples
Independent samples of field runoff were collected after six rainfall simulations on an experimental agricultural plot (Brittany, France) spread with either bovine (samples B1 to B3) or porcine manure (samples P1 to P3).
Four WWTP effluents (WW1 to WW4) from Pays de la Loire (France) were sampled after the secondary sedimentation tank.

Three samples were collected from a river (Brittany, France) flowing through an agricultural area associated with intensive cattle grazing (C1 to C3).
Twenty water samples (R1 to R20) were collected from two rivers in Pays de la Loire, both flowing though agricultural areas and receiving WWTP discharges.
All water samples were collected in plastic bottles and transferred at 4 °C to each laboratory. Samples for chemical analyses were stored at -20°C, whereas samples for microbial analysis were analysed within 24 hours of collection.
E. coli was enumerated using TBX medium (Oxoid, France) and concentrations were expressed in colony-forming units (CFU) per 100 mL water. Some blue colonies were transferred into Peptone Water (Oxoid, France) and incubated for 24 h at 44 °C for the confirmation of *E. coli*. After incubation, 0.5 mL Kovac's Indole reagent (Labogros, France) was added. The detection limit is 1 CFU per 100 mL water.
The Table 2 present all MST markers and general indicators tested in environmental waters.

Table 2. Description of 17 microbial and chemical parameters tested during the *in situ* application of MST markers

MST Markers / general indicators	Abbreviations	Type of marker(target)	Origin of pollution	Methods	References
Host-specific *Bacteroidales* markers		Microbial		Real-time PCR	Mieszkin *et al.*, (2009)
- HF183	HF183	(anaerobic bacteria – 16S rRNA gene)	Human		
- Rum-2-Bac	Rum-2		Ruminant		Mieszkin *et al.*, (2010)
- Pig-1-Bac	Pig-1		Porcine		
- Pig-2-Bac	Pig-2		Porcine		
Pig-specific bacterial marker		Microbial		Real-time PCR	Marti *et al.*, (2010)
- *Lactobacillus amylovorus*	*L. amy*	(anaerobic bacteria – genomic RDA fragment)	Porcine		
Human-specific bacterial marker		Microbial		Real-time PCR	This study
- *Bifidobacterium adolescentis*	*B. ado*	(anaerobic bacteria – 16S rRNA gene)	Human		
F-specific RNA bacteriophages		Microbial		Culture/ genotyping by real -time RT-PCR	This study
- FRNAPH I	FR I	(virus of enteric bacteria)	Animal		Ogorzaly *et al.*, (2009)
- FRNAPH II	FR II		Human		
- FRNAPH III	FR III		Human		
- FRNAPH IV	FR IV		Animal		
Faecal chemical pollution markers		Chemical		Multi-residue procedure and GC/MS/MS	This study
- Caffeine	Caff		Human		Glassmeyer *et al.*, (2005)
- Diphenylhydramine	Diph		Human		
- Benzophenone	Benzo		Human		
- TCEP – tri(2-chloroethyl)phosphate	TCEP		Human		
- TDCP – tri(dichloroisopropyl)phosphate	TDCP		Human		
Steroids		Chemical	Human/porcine-bovine	***Liquid solid extraction*** and capillary column *GC*/MS	This study
- R1: coprostanol/24-ethylcoprostanol	R1				Jardé *et al.*, (2009)
- R2: sitostanol/coprostanol	R2		Bovine/porcine-human		
Escherichia coli	*E. coli*	Microbial		***Culture***	
All-*Bacteroidales*	AllBac	Microbial		***Real-time PCR***	
F-specific RNA bacteriophages	FRNAPH	Microbial		***Culture***	
Total Steroids	T. Steroids	Chemical		***Liquid solid extraction*** and	

capillary column
GC/MS

2.2.2 Chemical markers

For steroid compounds, one litre of each water sample was frozen for 2 days at -10 °C after filtration at 0.45 µm, and freeze-dried for 3 days before analyses. Then, 0.01 to 0.2 g of freeze-dried samples were extracted and analysed as described in section 2.1.1
The other chemical markers were quantified on 250 mL-water samples as described in section 2.1.2.

2.2.3 FRNAPH

FRNAPH were enumerated following the ISO method, directly or after concentration by ultrafiltration with a Centricon Plus-70 (Millipore) for the less contaminated water samples. Bacteriophage isolates were then genotyped by real-time RT-PCR, with the One-Step RT-PCR kit, as in Ogorzaly et al. (2009).

2.2.4 Bacterial markers

For river water and WWTP effluent samples, 25-200 mL water were filtered on 0.22 µm polycarbonate membrane (Nuclepore) for *Bacteroidales* and *L. amylovorus* marker analyses, and on 0.22 µm polyethersulfone filter (Supor 200; Pall Corporation, Ann Arbor, MI) for *Bifid. adolescentis*. Filters were stored at -20°C. DNA was extracted with a modified DNA mini kit (Qiagen) protocol as in Mieszkin et al. (2009) or by using the DNA stool mini kit (Qiagen) as in Marti et al. (2010). Results from these two protocols were similar (data not shown).
For runoff, approximately 50 mL water were centrifuged at 9000 g for 15 min, and 250 mg of the pellet were transferred into microtubes and stored at -20 °C. DNA was extracted using the Fast DNA Spin Kit for Soil (MP Biomedicals, Illkirsh, France), according to Dick et al. (2005).
Previously described primers-and-probe and primer sets for all-*Bacteroidales* (AllBac), HF183, Pig-1-Bac, Pig-2-Bac, Rum-2-Bac and *L. amylovorus* markers (Seurinck et al., 2005; Layton et al., 2006; Mieszkin et al., 2009, 2010; Marti et al. 2010; Table 1) were used to amplify total *Bacteroidales*; human-, pig-, and ruminant-specific *Bacteroidales* and 16S rRNA genes; and the *L. amylovorus* RDA gene. PCR amplifications of general and host-specific *Bacteroidales* markers, *L. amylovorus* and *Bifid. adolescentis* were performed as in Mieszkin et al. (2009, 2010), Marti et al. (2010) and section 2.1.4.
A standard curve was generated using serial 10-fold dilutions of a plasmid preparation containing the target sequence for *B*acteroidales and Bifid. adolescentis markers or bacterial genomic DNA extracted from a pure-strain culture for the L. amylovorus marker. The presence/absence of PCR inhibitors was verified using an Internal Positive Control (IPC) (AppliedBiosystem, France). All samples were tested in triplicate.
The lower limit of quantification of the all-*Bacteroidales*, host-specific *Bacteroidales* and *Bifid. adolescentis* markers was 1×10^3 16S RNA gene copies per 100 ml water, and the lower limit of quantification of *L. amylovorus* marker was 1×10^3 cells per 100 ml.

2.3. Statistical analysis

To take into account values below the quantification limit, quantitative data were transformed into categorical data (0 = not quantified; 1-3 = relative to concentrations or ratios). Relations between samples and presence of each marker were studied by Multiple Correspondence Analysis (MCA) using FactoMineR and Hierarchical Classification on Principle Components (HCPC) by Ward's method. Euclidean distance was calculated between individuals. All calculations were performed and graphics drawn using R version v.2.9.2.

3. Results

3.1 Development and testing of methods for the selection of chemical and microbial markers on faecal samples

3.1.1 Steroids

The 19 samples of animal faeces and WWTP effluents were characterized by the presence of C_{27}, C_{28} and C_{29} sterol and stanol compounds. The major compounds in the samples of bovine manure were the C_{28} and C_{29} steroids, which represented between 65 % and 75 % of the steroids quantified. Among C_{28} and C_{29} steroids, sitostanol (12 to 14 %) and 24-ethylepicoprostanol (14 % to 18 %) were the main compounds present in bovine manure. In porcine manure samples, the distribution of steroids was roughly the same in C_{27} compounds (45 to 53 %) as in C_{28} and C_{29} compounds (46 to 55 %). The major compounds were coprostanol (20 to 27 %), 24-ethylcoprostanol (17 to 19 %), and sitosterol (7 to 8 %). These results are consistent with the previous studies of Leeming et al. (1996) and Jardé et al. (2007). In WWTP effluent samples, C_{27} steroids, which are the main compounds, represent between 64 and 73 % of the quantified steroids. They were represented essentially by coprostanol (30 to 41 %), cholesterol (21 to 29 %), 24-ethylcoprostanol (10 to 16 %) and sitostrol (6 to 12 %). According to the distribution of the steroid in the animal manure and WWTP effluent samples, two ratios were calculated to differentiate the samples:

- R1 or (cop/cop+24-ethylcop) $\times$ 100, *i.e.* coprostanol concentration/sum of coprostanol and 24-ethylcoprostanol, expressed as a percentage.
- R2 or sitostanol/coprostanol, *i.e.* sitostanol concentration/coprostanol concentration. This ratio illustrates the proportion of stanol produced by the degradation of cholesterol over the proportion of stanol derived from phytosterol.

Table 3 displays the mean values of R1 and R2 ratios for bovine and porcine manures and WWTP effluents.

R1 showed different discrete ranges and means for bovine, porcine and human (WWTP) sources. R1 ratio therefore distinguishes animal manures (R1 < 60 %) from WWTP effluents (R1 > 60 %). R2 ratio provides complementary information for sample discrimination. R2 ratios were >1 for bovine manures, and <1 for porcine manures and WWTP effluents.

Table 3. Steroid ratios for animal manures and WWTP effluents

Origin	No. samples	(cop/(24ethyl+cop))×100 [a] (R1, in %)	sitostanol/coprostanol (R2)
		Mean ±S.E [b]	Mean ±S.E
Bovine manure	9	41±1	2.8±0.1
Porcine manure	4	57±2	0.3±0.1
WWTP effluent	6	71±2	0.1±0.01

[a]coprostanol/coprostanol+24-ethylcoprostanol; [b]standard error

3.1.2 Human faecal chemical pollution markers

The instrumental limits of quantification were determined below 10 µg/L according to the AFNOR XP T90-210 standard method, leading us to set an analytical quantification limit of 0.04 µg/L for all compounds in water.

The procedure was validated in tap and surface water according to the SANCO/825/00 rev.7 standard. The majority of compounds gave 70-110 % acceptable recoveries with < 20 % RSD (Relative Standard Deviation); concentrations ranged from 0.04 µg/L to 0.4 µg/L. Matrix effects were observed for ethyl citrate and TDCP, giving unacceptable recoveries, and

diphenhydramine was not recovered at all. These three molecules were excluded from further analyses.

Target compound concentrations were then measured in triplicate in five WWTP effluents from two sampling campaigns to verify that the selected molecules were present in the effluents. Extraction was satisfactory for all samples in the first campaign, since recoveries of the surrogate standard (phenanthrene-d10) were 101-111 %, and the results are reported in table 4. Apart from tonalide, the five molecules were detected in all samples at concentrations above the quantification limit. The value obtained for tonalide is, however, acceptable (individual LQ=0.02 µg/L). Mean levels varied between 0.03 µg/L (tonalide) and 0.51 µg/L (TCEP) and concentrations of the individual compounds were similar among WWTP, as indicated by the RSD. In the second sampling campaign, recovery rate of phenanthrene-d10 was good for WWT2 (96 %) but much lower (52 to 62 %) for the other samples. A correction factor, taking into account the recovery rate of phenanthrene-d10, was therefore applied to results from the 4 other WWTP. Some levels (in italics in Table 4) were out of the range of the calibration curve; the values for this second campaign are therefore estimates. Despite these approximations, it is clear that the selected molecules are present in WWTP effluents and are good candidates for tracing human faecal pollution.

Table 4. Concentrations (µg/L) of human faecal chemical pollution markers in WWTP effluents during the two sampling campaigns

WWTP Effluent	Benzophenone	TCEP	Galaxolide	Tonalide	Caffeine
	1st – 2nd [a]	1st – 2nd	1st – 2nd	1st – 2nd	1st – 2nd
WWTP1	0.2- 1.7	0.85- 0.6	0.4- *12.6*	0.07- 0.6	0.5- *30.1*
WWTP2	0.3- 0.3	0.45- 0.6	0.5-1.9	0.03- 0.07	0.4- 0.2
WWTP3	0.15- 1.2	0.35- 0.2	0.4- *3.6*	0.03- 0.4	0.2 – *26.9*
WWTP4	0.2- *3.2*	0.6- 0.2	0.4- *8.6*	0.02- 0.4	0.15- *21.5*
WWTP5	0.3- 1.9	0.35- 0.2	0.6- *11.2*	0.03 – 0.4	0.2- *32.1*
Mean	0.2- 1.65	0.5- 0.3	0.5- 7.6	0.03- 0.4	0.3- 22.2
RSD (%) [b]	29 - 54.7	41 - 59.5	20 - 62.1	63 - 34.5	53 - 58.3

[a] First and second sampling campaigns; [b] Related Standard Deviation expressed in percentage Data in bold: out of calibration curve range

3.1. FRNAPH

Among the 121 animal faeces analysed, FRNAPH were only detected in 26.4 % of the faeces: 2 bovine, 18 porcine, 1 ovine and 11 avian faeces samples (at 1 to 5.5×10^5 PFU per g of faeces) (Table 5). FRNAPH genotypes identified were mainly animal ones *i.e*. FRNAPH I and, to a lesser extent, FRNAPH IV. Human-specific FRNAPH II and FRNAPH III were also detected in avian and porcine faecal samples, respectively. Nevertheless, FRNAPH were more frequently detected in WWTP effluent samples (94.3 %) and porcine manure (40 %) than in faeces samples. FRNAPH I and IV represented 81.6 % of FRNAPH isolated in porcine manure samples whereas FRNAPH II and III represented 75.3 % of those isolated in WWTP samples. FRNAPH I and IV appeared particularly inefficient for tracing bovine pollution, as they were detected in less than 7 % of bovine (faeces and manure) samples.

To improve genotyping, plaque hybridization and real-time RT-PCR were compared in a total of 60 FRNAPH strains for the four genogroups. Results were 97 % concordant (only two

FRNAPH isolates differed), showing real-time RT-PCR genotyping to be equivalent to plaque hybridization.

Table 5 Percentages of the four genogroups of FRNAPH in human and animal faeces and effluents

Samples	No. samples	No. positive samples	Total no. of isolates	% of positive samples	FRNAPH I [a] (%)	FRNAPH II[a] (%)	FRNAPH III [a] (%)	FRNAPH IV [a] (%)	Genotypes not identified (%)
Human faeces	4	0	0	0	_	_	_	_	_
Porcine faeces	48	18	425	37.5	71.5	0	0	28.5	0
Bovine faeces	32	2	24	6.2	52.1	0	0	0	47.9
Ovine faeces	12	1	24	8.3	100	0	0	0	0
Avian faeces	29	11	259	38	72.6	10.4	0	8.2	8.8
WWTP effluent	35	33	810	94.3	6.9	57.9	17.4	1.9	15.9
Porcine manure	35	14	364	40	65.9	0	13.7	15.7	4.7
Bovine manure	30	2	26	6.7	12	30.7	0	0	57.7

[a]Percentages of FRNAPH I, II, III or IV / total FRNAPH isolates

3.1.4 B. adolescentis marker

A new real-time PCR system was developed to quantify the phylotype related to *B. adolescentis*, previously identified as a potential human marker (Wéry et al., 2010). Phylotype concentrations in different sample types are given in Table 6. The marker was found in all WWTP effluents and in 9/10 human faeces tested, implying 92 % sensitivity. Concentrations in human faeces ranged from 5×10^5 to 1×10^9 gene copies/g. As this marker was not found in porcine, avian, equine or ovine faeces but was present in two bovine faeces and two avian faeces, specificity was 94.5 %. Although this new marker was not strictly specific to humans, its presence and concentrations were lower in bovine and avian faeces than in human faeces, and it was not recovered in porcine faeces or manure. It therefore represents an interesting marker for tracing human pollution, but only when combined with other markers.

Table 6. Specificity of the phylotype 'SFA41' related to *Bifidobacterium adolescentis*

Origin	No. samples	No. positive samples	Concentrations min-max $\log_{10}$ copies per g
Human faeces	10	9	5×10^5-1×10^9
Porcine faeces	14	0	
Bovine faeces	14	2	4×10^5-1.3×10^6
Ovine faeces	10	0	
Equine faeces	10	0	
Avian faeces	19	2	6.3×10^4-7.9×10^6
WWTP effluent[a]	8	8	1×10^4-7.9×10^6
Porcine manure	6	0	

[a] expressed by 100 mL

3.2 *In situ* application of host-specific microbial and chemical markers

Table 7 gives average concentrations for faecal indicators, MST markers and ratios of steroids in 33 water samples including WWTP effluent, runoff and river samples. FRNAPH, and total *Bacteroidales* were significantly correlated ($p < 0.001$) with *E. coli* levels.

Highly contaminated samples (WWTP effluent and runoff waters, with *E. coli* concentrations ranging between 6.6×10^3 and 1.1×10^6 CFU/ 100 mL), could be differentiated with most of the human and animal MST markers. None of the 5 chemical markers of human pollution selected in the first phase of the study (*i.e.* caffeine, benzophenone, TCEP, tonalide and galaxolide) were detected in runoff waters impacted by livestock manures. However, tonalide was only detected in raw WWTP effluents and galaxolide was never detected in any of the rivers analysed (data not shown). The steroid ratios (R1 (cop/cop+24-ethylcop)$\times$100) and R2 (sistostanol/ coprostanol) clearly differed between waters contaminated by human and animal pollution. Microbial markers Rum-2-Bac, Pig-2-Bac and *L. amylovorus* were only detected in water impacted by the respective manures. The human *Bacteroidales* marker HF183 and *Bifid. adolescentis* were quantified in all the WWTP effluent samples, although HF183 appeared more discriminating since *Bifid. adolescentis* was also quantified in 1/3 of the bovine-contaminated runoff samples. FRNAPH II and III were always present in variable proportions in urban sewage, but were also detected in bovine-impacted runoff waters. Moreover, animal FRNAPH I were not found only in animal effluents but also in 2/4 WWTP effluents.

In the 23 river water samples (C1-C3 and R1-R20), the concentration of *E. coli* ranged between 3.2×10^1 and 1.9×10^4 CFU / 100 mL. The number of *E. coli* was less or equal to 5×10^2 CFU/ 100 mL in 9 water samples. In 11 other water samples, the level of *E. coli* exceeded the limit value of the inland water guidelines of the European Directive on the management of bathing water quality.

The origin of pollution in the river flowing through cattle pasture (samples C1 to C3) was clearly identified by the R1 and R2 ratios, Rum-2-Bac and by the absence of human-specific *Bacteroidales* and the three human chemical faecal markers. *Bifid. adolescentis* was, however, quantified in 2/3 of the water samples. In the 11 river water samples with highest *E. coli* concentrations, our results indicated mixed bovine and human pollution. Faecal pollution sources in the other 9 river water samples with low levels of *E. coli* ($\leq 5\times10^2$ CFU / 100 mL) (i.e. samples R12 to R20) were more difficult to identify. Indeed, apart from caffeine and steroid ratios, the marker levels were below the limits of quantification in 7 of the 9 samples.

Analysis of the data obtained with the MCA gave the dendrogram shown in figure 1, which allows us to separate the water samples into six clusters characterized according to their significant modalities ($p<0.05$). Three clusters were associated with one specific pollution source: clusters I, III and VI contained water samples contaminated by only porcine, bovine or human pollution, respectively.

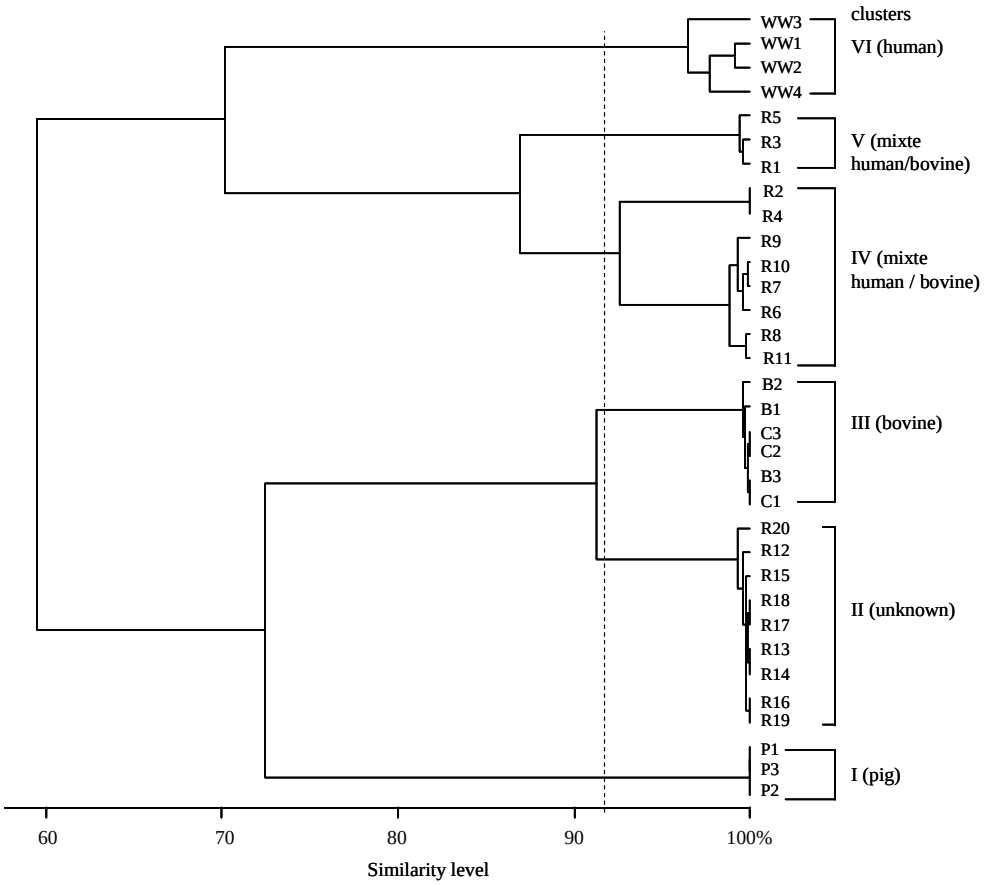

Figure 1. Dendrogram showing clustered water samples contaminated by waste water (WW1 to WW4), bovine manure or bovine pasture (B1 to B3 and C1 to C3), porcine manure (P1 to P3) and by undetermined pollution (R1 to R20)

The three other clusters corresponded to water samples from rivers where we had no specific knowledge about pollution origin, although human and bovine pollution was suspected. No human or animal markers were quantified in cluster II, and caffeine concentration was < 0.16 µg/L. Cluster IV was characterized by intermediate concentrations of human markers (0.17 < caffeine < 0.29 µg/L; 10^3 < *Bifid. adolescentis* < 10^5 copies / 100 mL; 10^3 < HF183 < 10^5 copies / 100 mL). In cluster IV, the presence of human bacteriophages (FRNAPH II) and the ruminant-specific *Bacteroidales* marker Rum-2-Bac suggested mixed pollution. Cluster V results also implied mixed pollution, with a high percentage of FRNAPH I, presence of the Rum-2-Bac marker and of the human chemical faecal markers.

Legend Table 7: [a] correspondence between abbreviations and names of markers is presented in Table 1; [b] no quantified. Limit of quantification were < 0.04µg/ L for caffeine, benzophenone and TCEP, <0.01µg/L for steroids used to calculate ratios R1 and R2, <10 PFU/100 mL for FRNAPH, < 1×10^3 gene copies or cells/100 mL for bacterial markers; [c] percentage were considered not representative when < 20 PFU isolates; [d] not tested

Table 7. Average concentrations of faecal indicators (microorganism /100 mL) and steroid (µ/L), ratios of steroids, percentage of FRNAPH genogroups and average concentrations of chemical markers (µg/ L), of microbial markers (copies or cells/ 100 mL) in WWPT effluent, runoff water and river water samples

		General indicators				MST Markers												
Pollution Origin	**Samples**	***E. coli***	**AllBac[a]**	**FRNAPH**	**T. Steroids**	**R1**	**R2**	**Caff**	**Benzo**	**TCEP**	**FR I-IV (%)**	**FR II-III (%)**	**HF183**	**B. ado**	**Pig-1**	**Pig-2**	**L. amy**	**Rum-2**
WWTP effluent	WW1	1.4×10^5	1.1×10^8	1.2×10^3	3213	75.6	0.1	0.83	0.2	0.49	0	100	7.2×10^5	1.9×10^6	-	-	-	6.1×10^3
	WW2	2.3×10^5	1.0×10^8	1.7×10^3	2446	79	0.02	0.3	0.16	0.08	0	100	6.3×10^6	4.1×10^5	9.9×10^4	-	-	-
	WW3	6.3×10^5	3.5×10^7	1.7×10^4	1356	70.5	0.24	1.27	0.2	0.16	21	79	4.4×10^4	7.1×10^6	1.8×10^3	-	-	-
	WW4	6.9×10^4	1.8×10^6	3.4×10^3	1797	61.4	0.52	0.33	0.27	0.3	71	21	3.1×10^6	1.8×10^5	-	-	-	-
Cattle	C1	1.9×10^4	6.1×10^6	-[b]	18	42.9	1.4	-	-	-	nd[c]	nd	-	-	-	-	-	3.6×10^5
	C2	1.5×10^4	3.8×10^6	-	31	38.7	1.76	-	-	-	nd	nd	-	3.2×10^4	-	-	-	3.0×10^5
	C3	1.7×10^4	8.5×10^6	-	30	38.3	1.8	-	-	-	nd	nd	-	4.9×10^4	-	-	-	5.9×10^5
	B1	1.1×10^6	5.9×10^9	4.6×10^2	145	49.8	2.3	-	-	-	nt[d]	nt	-	2.8×10^5	-	-	-	1.9×10^7
	B2	1.610^5	1.2×10^8	1.1×10^3	118	49	2.35	-	-	-	0	22	-	-	-	-	-	8.8×10^6
	B3	2.9×10^4	9.6×10^8	1.6×10^2	104	48.3	2.13	-	-	-	nt	nt	-	-	-	-	-	5.0×10^6
Pig	P1	1.4×10^4	3.8×10^8	-	107	57.4	0.59	-	-	-	nd	nd	-	-	1.3×10^7	1.0×10^6	4.5×10^7	-
	P2	6.6×10^3	6.9×10^8	-	98	56	0.63	-	-	-	nd	nd	-	-	4.4×10^6	7.3×10^5	4.2×10^7	-
	P3	2.0×10^4	2.4×10^8	-	133	56	0.64	-	-	-	nd	nd	-	-	8.1×10^5	3.2×10^5	1.9×10^7	-
Unknown	R1	1.7×10^3	1.7×10^7	20	72	45.4	0.64	0.13	0.2	0.06	93	0	-	-	-	-	-	3.9×10^5
	R2	1.1×10^4	9.0×10^6	20	88	50.5	0.39	0.14	0.17	0.06	44	56	2.0×10^4	4.6×10^4	-	-	-	1.6×10^5
	R3	1.2×10^4	1.1×10^7	66	38	45.3	0.76	0.14	0.08	0.08	63	37	3.0×10^4	2.9×10^4	-	-	-	1.7×10^5
	R4	1.2×10^4	2.2×10^7	50	41	55	0.53	0.17	0.16	0.09	44	56	3.6×10^4	3.3×10^4	-	-	-	1.1×10^5
	R5	5.5×10	6.3×10^6	66	55	42.	0.8	1.99	0.14	0.08	53	47	2.3×10	-	-	-	-	2.9×10^5

	1.5×10^{3}				1	3						1.5×10^{3}					
R6		7.5×10^{6}	6.0×10^{2}	39	43.7	0.56	0.13	0.14	-	0	100	1.5×10^{4}	-	-	-	-	2.3×10^{5}
R7	2.2×10^{3}	1.2×10^{7}	4.8×10^{2}	63	49.7	0.48	0.15	-	-	0	100	1.7×10^{4}	3.3×10^{4}	-	-	-	3.9×10^{5}
R8	2.5×10^{3}	7.6×10^{6}	5.8×10^{2}	60	50.7	0.45	0.1	0.17	-	0	100	5.1×10^{4}	-	-	-	-	2.8×10^{5}
R9	1.7×10^{3}	1.4×10^{7}	5.5×10^{2}	38	47	0.45	0.14	0.12	-	0	100	9.6×10^{4}	7.4×10^{3}	-	-	-	3.2×10^{4}
R10	3.2×10^{3}	2.1×10^{7}	8.3×10^{2}	40	42	0.53	0.16	0.08	-	0	100	9.2×10^{4}	4.4×10^{4}	-	-	-	8.4×10^{5}
R11	3.2×10^{3}	1.0×10^{6}	3.2×10^{2}	13	45.5	1.01	0.11	-	-	0	96	8.1×10^{4}	8.1×10^{4}	-	-	-	-
R12	53	4.1×10^{4}	-	8	56.2	0.76	0.09	-	-	0	0	nd	-	-	-	-	-
R13	5.0×10^{2}	3.3×10^{5}	-	13	51	0.96	0.08	-	-	nd	nd	nd	-	-	-	-	-
R14	3.3×10^{2}	9.0×10^{4}	-	16	53.6	0.86	0.08	-	-	nd	nd	nd	-	-	-	-	-
R15	34	5.1×10^{5}	30	15	50.1	1.03	0.2	-	-	0	0	nd	-	-	-	-	-
R16	32	3.5×10^{3}	-	12	-	-	0.07	-	-	nd	nd	nd	-	-	-	-	-
R17	63	5.3×10^{3}	-	12	50.9	0.99	0.06	-	-	nd	nd	nd	7.1×10^{4}	-	-	-	-
R18	55	3.9×10^{3}	20	18	50.1	0.97	0.08	-	-	0	0	nd	9.3×10^{3}	-	-	-	-
R19	41	5.1×10^{4}	<10	23	50.1	1.03	0.07	-	-	nd	nd	nd	-	-	-	-	-
R20	41	5.9×10^{3}	10	25	50.6	1.01	0.09	-	-	25	16.7	nd	-	-	-	-	-

4. Discussion

An efficient MST toolbox is crucial for identifying the origin of faecal pollution in water and is, therefore, essential for effective resource management and remediation. In this study, we compared a selection of chemical, bacterial and viral markers on water samples characterized by different faecal pollution loads. Some of these markers, such as host-specific *Bacteroidales* and *L. amylovorus*, had previously been described and tested on faecal samples elsewhere (Bernhard and Field, 2000; Seurink et al, 2005; Mieszkin et al, 2009, 20010; Marti et al, 2010), whereas others, such as faecal steroids, chemical synthetic compounds, FRNAPH and *Bifid. adolescentis*, were developed or improved in the present study.

For faecal sterols, rather than considering individual concentrations of the 15 compounds investigated, we found that the use of two derived variables, *i.e.* the R1 and R2 ratios, was more efficient to distinguish human, bovine and porcine pollution. The results obtained with R1 were found similar to those obtained by Shah et al. (2007), who used this ratio to differentiate between animal manure, (R1<45%) dominated by 24-ethylcoprostanol, and human manure (R1>60%), dominated by coprostanol. The difference could be explained by the distributions of steroids: in bovine manure these are dominated by phytosteroids, whereas in WWTP effluents and porcine manures they are dominated by coprostanol (Leeming et al., 1996; Tyagi et al., 2007). The choice of the R2 ratio was based on the distribution of the steroid compounds in our samples of animal manure and WWTP effluents. This ratio allowed us to refine the differentiation between bovine manure (R2>1), porcine manure and WWTP effluent samples (R2<1).

The five selected chemical compounds tested were present in the WWTP effluents at similar levels as those previously reported (Ternes et al., 2001; Weigel et al., 2004; Glassemeyer et al., 2005). Only three chemical markers (caffeine, TCEP and benzophenone) were suitable for identifying human pollution in the environment, as these were detected not only in WWTP effluents but also in river waters impacted by human wastes.

Among the four genogroups of FRNAPH, only human-specific FRNAPH II could be could be used to trace specific pollution. Indeed, this genogroup, which was not detected in porcine, bovine or ovine faeces, was found in all of the 94.3 % of WWTP effluent samples in which FRNAPH were detected, and made up approximately 60 % of the FRNAPH isolated in these WWTP effluent samples. This group was also detected in some avian faeces, possibly because birds that inhabit beaches, picnic areas, and landfills come into contact with human wastes (Dick et al., 2005) and in 2 of the 30 bovine manure samples tested. The other FRNAPH genogroups were found to be less efficient. Thus, FRNAPH III, which represented only 17.4 % of FRNAPH isolated in WWTP effluent samples, was also detected in porcine manure. Our results are in accordance with the data reported by Blanch et al. (2006), who detected this genogroup in 33 % of animal effluent and farm manure samples. Although FRNAPH I was mainly present in bovine, porcine, ovine and avian faeces and in porcine manure, it was also found in WWTP effluents. This lesser specificity led Stapleton et al. (2007) to exclude FRNAPH I from their analyses. FRNAPH IV were found more specific but also less sensitive as they were only present in porcine faeces and manure and avian faeces samples and at a lot weaker percentages than FRNAPH I. Moreover, animal-specific FRNAPH (FRNAPH I and IV) were found particularly inefficient for tracing bovine pollution, as only 20 FRNAPH I were isolated from the 62 cow faeces and manure samples. A weak prevalence of FRNAPH in cow faeces has already been observed in other studies (Calci et al., 1998 ; Long et al., 2005).

No MST bacterial indicator has yet been demonstrated to be strictly human-specific. The human specificity of *B. adolescentis* has been much debated. Some consider it to be human-specific (King et al., 2007), while others have detected it in pig (Dorai-Raj et al., 2009), cattle (Lamendella et al., 2008; Dorai-Raj et al., 2009) and poultry manure (Bonjoch et al., 2004). This study confirmed that, although the specificity of *B. adolescentis* for humans is high (94.5

%), it can also be found in some bovine and avian faeces. The new real-time PCR system we developed, however, seems more human-specific than the previous PCR system used for quantification of *Bifid adolescentis* by Dorai-Raj et al. (2009) and Bonjoch et al. (2004), since our results were negative for porcine samples and poultry manure. It should be underlined that *Bifid. adolescentis* was never detected in porcine manures in our study, and the concentrations in bovine manures were lower than those in treated effluents from WWTP urban sewage.

Microbial markers, Rum-2-Bac, Pig-2-Bac and *L. amylovorus* were only detected in water impacted by the respective manures, confirming host specificity, as observed by Mieszkin et al. (2009, 2010) and Marti et al. (2010). Furthermore, it is interesting to note that the human-specific *Bacteroidales* marker HF183 was also found to be highly specific, in line with the successfully testing of specificity obtained in the EU with this marker (Seunrick et al., 2005; Mieszkin et al., 2009), in the USA by Kildare et al (2007) and in Australia by Ahmed et al. (2009).

MST toolboxes tested in previous studies essentially focused on microbial markers (Griffith et al., 2003, Blanch et al., 2006, Ahmed et al., 2007; MacQuaig et al., 2009). Thus, Griffith et al. (2003) compared the efficiency of phenotypic and genotypic analyses on *E. coli,* genotypic analysis on the *Bacteroides / Prevotella* group, typing of FRNAPH, and identification of human viruses. They concluded that the most specific method was the host-specific PCR. Ahmed et al. (2007) compared the efficiency of a library-dependent method using *E. coli* and enterococci with library-independent methods (host-specific PCR of *Bacteroidales* 16S rRNA gene markers and PCR of a human-specific *Enterococcus* surface protein marker), concluding that the library-independent methods were more sensitive than the library-dependent one. However, these two studies highlight the interest of combining different methods to enhance the identification of the origin of the faecal pollution. In accordance with these data, we selected only library-independent methods and our toolbox combined microbial and chemical markers including steroids. Steroids had been previously tested by Blanch et al. (2006) who found that, while coprostanol and 24-ethylcoprostanol were not sufficiently discriminating, the ratios sitostanol/coprostanol and coprostanol/coprostanol+24-ethylcoprostanol were clearly different between human and animal samples. While Blanch et al. (2006), tested only wastewaters and slurries for their markers, we also evaluated marker efficiency on rivers, without *a priori* knowledge of the *E. coli* level. Our results clearly demonstrate the influence of the faecal pollution loads, as the 11 selected markers were only able to identify the source of pollution in waters when *E. coli* concentration was more than 5×10^2/100 mL. Unlike Blanch et al. (2006), who studied the presence of their markers in several geographic areas in Europe, our sampling was performed only in France. It should be noted, however, that many of our markers have been detected in other countries by other studies: *L. amylovorus* (Konstantinov et al, 2006; Pieper et al., 2008, Su et al., 2008), HF183 (Bernard and Field, 2000; Seurink et al, 2005; Ahmed et al, 2007), *B. adolescentis* (King et al., 2007; Lamendella et al.,2008); FRNAPH II (Blanch et al., 2006), the steroids (Leeming et a, 1996; Shah et al., 2007) and the synthetic compounds (Glassmeyer et al, 2005), pointing to the universal distribution of these markers.

Conclusion

In conclusion, among the 17 MST markers investigated in this study, seven host-specific markers (caffeine, TCEP, benzophenone and bacterial markers HF183, Pig-2-Bac, Rum-2-Bac and *L. amylovorus*) and the two steroid ratios were found to be the most efficient for discriminating the origin of the faecal pollution. *Bifid. adolescentis* and FRNAPH II, which were slightly less discriminating, could make useful complementary markers.

Although knowledge is lacking on the persistence of the different types of markers (chemical, viral and bacterial) in environmental waters, our results show that when the level of faecal pollution is sufficiently high (i.e. > 500 *E. coli* / 100 mL), the origin of the pollution can be determined using the set of markers tested in this study.

More precisely, to trace the origin of the faecal pollution, we recommend:

- For human pollution, the use of caffeine, TCEP and benzophenone, the steroid ratios sitostanol/coprostanol (R1) <60 % and coprostanol/coprostanol+24-ethylcoprostanol (R2) > 1, real-time PCR bacterial markers (HF183 and *Bifid. adolescentis*) and genotype II of FRNAPH,
- For porcine pollution, the use of steroid ratios (R1<60% and R2<1) and real-time PCR bacterial marker Pig-2-Bac and *L. amylovorus*,
- For ruminant pollution, the use of steroid ratios (R1<60% and R2>1) and real-time PCR bacterial marker Rum-2-Bac.

To improve confidence in these MST markers, the next steps should be to compare their persistence in river or marine waters and to apply them at the level of a catchment with a variety of pollution sources.

Finally, when established, these analysis methods will be transferred to water analysis laboratories. Such laboratories are increasingly required to identify the origin of water pollution, particularly to assist with the management of microbial water quality of bathing and shellfish farming areas and to establish bathing water profiles.

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