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Persistence of microbial and chemical pig manure markers as compared to faecal indicator bacteria survival in freshwater and seawater microcosms

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Abstract :

Natural seawater and freshwater microcosms inoculated with pig manure were set up to determine the persistence of pig faecal microbial and chemical markers in these two types of surface water. The concentrations of *Lactobacillus amylovorus*, the *Bacteroidales* Pig-2-Bac 16S rRNA genetic marker, five stanols and the evolution of two ratios of stanols, R_1 (coprostanol to the sum of coprostanol and 24-ethylcoprostanol) and R_2 (sitostanol to coprostanol) were analyzed during two months along with the concentration of Faecal Indicator Bacteria (FIB). Pig manure was inoculated to unfiltered water microcosms incubated aerobically at 18 °C in the dark. The faecal contamination load represented by the concentrations of culturable *Escherichia coli* and/or enterococci remained for two months in the freshwater and seawater microcosms water column. These concentrations followed a biphasic decay pattern with a 97% reduction of the initial amount during a first rapid phase (<6 days) and a remaining proportion undergoing a slower or null second decline. The *L. amylovorus* marker and five stanols persisted as long as the indicators in both treatments. The Pig-2-Bac marker persisted 20 and 27 days in seawater and freshwater, respectively. The ratios R_1 and R_2 were in the range specific to pig manure until day 6 in both types of water. These results indicate that Pig-2-Bac, *L. amylovorus* and stanol ratios might be used in combination to complement FIB testing to determine the pig source of fecal pollution. However, stanol ratios are to be used when the time point of the discharge is known.

Highlights

► *Escherichia coli* and enterococci from pig manure followed a biphasic decay in water microcosms. ► The marker *Lactobacillus amylovorus* followed the same trend as enterococci in fresh and seawater. ► The marker Pig-2-Bac followed the same trend as *E. coli* in seawater. ► Pig-specific stanols and *L. amylovorus* persisted for two months. ► Stanols and *L. amylovorus* detection and persistence was not affected by salinity.

Keywords : Faecal source tracking; Decay rates; Faecal stanols; Biphasic kinetic; *Lactobacillus amylovorus*; Bacteroidales marker

1. Introduction

Each year, the microbial analyses of the 540 freshwater and seawater bathing areas located in Brittany, North West of France, reveal that these areas might be sporadically contaminated by high faecal loads. Among the three main sources of faecal contamination (human, pig and cow) which can lead to the downgrading of these areas (Soller et al., 2010), pig manure spreading is considered as a potential factor of water pollution (Thurston-Enriquez et al., 2005). Brittany supports more than half of the national pigs' livestock while representing only 5% of the French territory and this high concentration generates from 8 to 10 million tons of pig manure each year. To protect public health whilst bathing and to improve management practices at bathing waters, the revised Bathing Water European Directive (2006/7/EC) requires to establish bathing water profiles to identify the source(s) of a faecal pollution upstream a targeted water body.

To track pollution to livestock facilities or diffuse non point sources, animal-specific markers have been proposed. They include DNA molecules from intestinal bacteria (Dick et al., 2005; Ufnar et al., 2007), endogenous eukaryotic cells (Balleste et al., 2010) and faecal stanols (Tyagi et al., 2009). Among pig genetic markers, studies have focused essentially on bacteria from the *Bacteroidales* order as potential markers preferred for their abundance in the gastrointestinal tract and host specificity occurrence (Dick et al., 2005; Mieszkin et al., 2009). Among chemical markers, faecal stanols are considered as direct markers because they occur in the faeces (Leeming et al., 1996). The distribution of those compounds in animal faeces depends on three host factors: (i) the animal's diet, (ii) the ability to biosynthesize

endogenous sterols and (iii) the occurrence of anaerobic bacteria able to biohydrogenate sterols to stanols of various isomeric configurations. The combination of those three factors determines the “sterol fingerprint” that is characteristic of each animal faeces (Leeming et al., 1996).

In a previous study from our teams, several microbial and chemical markers detected in river waters impacted by faecal pollutions were proposed to be used in a toolbox as Faecal Source Tracking methods (FST) (Gourmelon et al., 2010). Two of which were microbial pig-specific markers previously validated for their host specificity, the genetic *Bacteroidales* marker Pig-2-Bac and the bacterial species *Lactobacillus amylovorus* (Marti et al., 2010; Mieszkin et al., 2009). Five faecal stanols found in pig faeces and two steroids ratios were also selected to be part of the toolbox. Specific markers should exhibit high host-specificity and represent the load of faecal pollution (Field and Samadpour, 2007). In this scope, the evaluation of markers to be used as FST must consider whether the degradation and transport characteristics of the markers are similar to that of one or several pathogens or to the traditional faecal indicator bacteria (FIB) such as *Escherichia coli* (*E. coli*) and enterococci.

The present study examines the detection of Pig-2-Bac marker, *L. amylovorus* and faecal stanols as compared to the detection of FIB in natural surface water microcosm. The faecal stanols selected comprised coprostanol (5β-cholestan-3β-ol) and 24-ethylcoprostanol (24-ethyl-5β-cholestan-3β-ol) dominant in fresh pig manure (Leeming et al., 1996; Shah et al., 2007). We also included epicoprostanol (5β-cholestan-3α-ol) and campestanol (24-methyl-5α-cholestan-3β-ol) since the land spreading of pig manure as a soil fertilizer results in high concentration of those compounds (Jarde et al., 2009). Two ratios of concentrations of steroids were calculated along the experiment: coprostanol to the sum of coprostanol and 24-ethylcoprostanol (R_1) and sitostanol to coprostanol (R_2). In the previous study to this work, pig manure was characterized by a R_1 ratio of 0.57 ± 0.02 and a R_2 ratio of 0.3 ± 0.1 (Gourmelon et al., 2010).

The objective of this study was to estimate the persistence and decay rates of pig genetic markers, faecal stanols, and FIB in fresh and marine water during two months. The evaluation to which extent the detection of tested markers and ratios correlates with that of FIB and hence, represents faecal load contamination should allow to validate their usefulness as markers of pig faecal pollution in these two types of surface water.

2. MATERIALS AND METHODS

2.1. Microcosms design

Microcosms consisted of 6 one hundred-litres inert glass aquariums placed in a dark room, protected from sunlight and fluctuating temperature. Three of which were filled with seawater and the remaining aquariums with freshwater. Both waters were not filtered to study the persistence of FIB and specific markers in presence of protozoa. Waters were seeded with pig liquid manure. Constant mixing was achieved with the aid of a helix agitator and oxygen saturation with air pumped in throughout the experiment. A plastic film cover limited evaporation of water. The type of surface water was the changing parameter. Ambient temperature (around 18 $^{\circ}$C) corresponded with surface water temperature during the warmer months in Brittany (France). Nine hundred mL of untreated liquid pig manure was added to 90 L of water (1:100 dilution). This ratio was chosen to represent a high faecal load contamination likely to remain for two months. However, the turbidity resulting from this parameter hindered light treatment.

Sampling took place on the starting day, then on day 2, 6, 13, 20, 27, 34, 41, 48 and 55. Both unseeded initial types of water were kept in the same conditions during the whole experiment to use as controls, sampling took place on the starting day and on day 55. Culturable *E. coli* (cEC), culturable enterococci (cENT), bacterial genetic markers and stanol concentrations were measured at each sampling point. Dissolved O_2 concentration and temperature were measured every 3 to 4 days. Although constant mixing was achieved in the middle of the

aquarium, sedimentation occurred during the course of the experiment and biofilms formation occurred on the walls. Samples were drawn from the water column.

2.2. Water and pig manure samples

The seawater was collected in the end of January 2010 in the Atlantic Ocean from Landunvez, in the NW Brittany region of France (lat × long: 48.540819 × -4.751587). Salinity was 33 g/L, total dissolved organic carbon measurement was 0.4 mg C/L, total dissolved nitrogen was 4.0 mg N/L. The freshwater was sampled from a lake in Commana in Brittany (lat × long: 48.3887488 × -4.0177564) on the same day, the total dissolved organic carbon was 2.7 mg C/L, the total dissolved nitrogen was 9.3 mg N/L. Pig manure was collected from a farm located in Brittany and samples were taken from a storage tank after homogenisation with propeller agitator for 20 minutes, the total dissolved carbon and total dissolved nitrogen were 6.5 g C/L, and 2.9 g N/L, respectively.

2.3. Enumeration of FIB

Depending on sample turbidity, FIB counts were achieved either by serial dilution in buffered peptoned water (Oxoid, Basingstoke, England) or by filtration of 100 mL of sample on a 0.45 µm cellulose membrane (Whatman, Dassel, Germany). Filters or 0.1 mL of the dilution was plated on TBX agar (Oxoid) and on Slanetz and Bartley agar (Biokar Diagnostics, Beauvais, France). TBX plates were incubated for 24 h at 44 °C. Blue colonies (glucuronidase positive) were counted to determine the concentration of *E. coli*. After incubation at 37 °C for 48 h, membranes on Slanetz and Bartley agar were transferred onto Bile-Esculin-Azide agar (BEA) (Biokar Diagnostics) and incubated for 2 h at 44 °C. Black colonies on BEA were counted as enterococci. The detection limit of both methods was 1 colony forming units (CFU) per 100 mL.

2.4. Microbial markers analyses

2.4.1. Samples preparation and DNA extraction

Two hundreds mL of samples were either centrifuged (9000 g for 15 min) or filtered onto 0.2 µm polycarbonate membrane (Sartorius, Goettingen, Germany) depending on suspended matter density. Filtration was the preferred method to recover DNA. However, filtration was not possible until day 13. On that day, samples from one microcosm of both types of water were treated by both methods. Since qPCR results were similar, it was decided to carry on with filtration. From 0.30 to 250 mg of solid matter were recovered. DNA extraction was performed on sample solid matter with the aid of the FastDNA® SPIN for Soil kit (MP Biomedicals, Illkirch, France), following manufacturer's instructions. The elution volume was 100 µL.

2.4.2. Real-time PCR

2.4.2.1. Oligonucleotide primers and probes

The pig-specific *Bacteroidales* 16S rRNA gene marker (Pig-2-Bac) and the *L. amylovorus* marker were quantified with the primers and probe described by Mieszkin et al. (2009) and Konstantinov et al. (2005), respectively.

2.4.2.2. DNA standard curves

For the quantification of the *Bacteroidales* marker Pig-2-Bac, standard curves were generated from serial dilutions of known concentration of plasmid DNA ranging from 5×10^7 to 5×10^0 copies per reaction. Linear plasmids were extracted with the QIAquick Miniprep Extraction Kit (Qiagen), following the manufacturer's instructions. The linear form of plasmid was obtained with *Not*I enzyme (Roche Diagnostics, Meylan, France) in a final volume of 50 µL for 3 h at 37 °C. The PCR standard curve for the *L. amylovorus* markers was prepared by 10-fold dilution of bacterial genomic DNA extracted from one mL of a pure culture of *L. amylovorus* DSM16698, with the Wizard genomic DNA purification Kit (Promega, Madison,

158 USA) according to the manufacturer's instructions. Dilutions ranged from 4.5×10^6 to $4.5 \times$
159 10^0 CFU equivalent from direct plating count considering that 100 % of the DNA from the
160 culture was recovered. Standard curves were generated by plotting threshold cycles (Ct)
161 against 16S rRNA genes or CFU equivalent, depending on the marker. Standard curves were
162 obtained by means of 3 replicates per point.

163 *2.4.2.3. Real-time PCR assays*

164 For Pig-2-Bac marker, amplification was performed using the Chromo4 real-time detection
165 system associated with Bio-Rad Opticon Manager software version 3.1 (Bio-Rad, Hercules,
166 CA). Real-time PCR was performed using the TaqMan® Brilliant II QPCR Master Mix kit
167 (Agilent technologies, Massy, France). Each reaction was run in triplicate. The cycle
168 conditions were 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60
169 °C for 1 min. Reactions were carried out in a final volume of 25 µL with primers and probe
170 final concentration being 300 nM and 200 nM respectively. Quantification limit was 1250
171 16S rRNA gene copies per 100 mL. The presence/absence of PCR inhibitors was verified
172 using an Internal Positive Control (IPC; AppliedBiosystem, France). Samples were diluted if
173 inhibitors were present.

174 Concerning the *L. amylovorus* marker, PCR was performed on the CFX96 real time system
175 (Bio-Rad), with the software Opticon Monitor version 3.1.32 and CFX manager version 1.1
176 (Bio-Rad), using the IQ SYBR-Green Supermix (Bio-Rad). The cycle conditions were 1 cycle
177 at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. Reactions were
178 carried out in a final volume of 25 µL with primers final concentration being 200 nM.
179 Quantification limit was 112.5 CFU equivalents per 100 mL.

180 No filtration, extraction and template positive controls from a known concentration of a
181 control DNA fragment were included in the method to evaluate the yield of DNA recovery.

182

183 **2.5. Faecal stanols analysis**

Five faecal stanols, namely coprostanol, epicoprostanol, 24-ethylcoprostanol, campestanol and sitostanol were investigated in this study. Analyses were performed on 1 L of non seeded initial waters and 500 mL of microcosm waters. Faecal stanols were extracted from the dissolved phase (< 0.7 µm) by solid phase extraction and quantified by gas chromatography – mass spectrometry (GC-MS) according to the protocol developed by Jeanneau et al. (2011). Cholesterol d6 (2,2,3,4,4,6-$^{2}H_6$-5-cholesten-3β-ol) was used as a recovery standard and was added to water samples prior to the extraction step. 5α-cholestane was used as an internal standard and was added prior to the GC-MS analysis. Faecal stanols were quantified by the internal standard method using a five-point calibration curve. The resulting calibration ranged from 0.01 to 40 µg/L for stanols in water.

2.6. Decay rate calculations

The decay rates of faecal stanols were calculated based on a first order decay model (Chick model):

$C_{(t)} = C_0 \times e^{-kt}$ or $\ln(C_{(t)}/C_0) = -kt$

Where C_0 is the average initial concentration of the target in µg/L, $C_{(t)}$ is the target average concentration at time t in µg/L, t is the time in days and *k* the decay constant or rate in days^{-1}. The model describes a linear regression, *k* is the slope of the regression line and R^2 the regression coefficient.

The biphasic model described by Lee et al. (2001) was used to calculate decay rates for every microbial target in both treatments. Two constants were calculated from a biphasic first order decay model (Cerf model):

$C_{(t)} = C_0 \times (f \times e^{-k1t} + (1-f) \times e^{-k2t})$ or $\ln(C_{(t)}/C_0) = \ln(f \times e^{-k1t} + (1-f) \times e^{-k2t})$

Where f is the proportion of C_0 that declined during the first phase, k_1 is the decay constant of the first phase and k_2 the decay constant of the second phase. $C_{(t)}$ and C_0 are expressed in CFU/100 mL for FIB, in CFU equivalent/100 mL for *L. amylovorus* and in DNA copies/100

mL for Pig-2-Bac. The biphasic model and associated parameters were obtained with the aid of XLSTAT 2010.4 using the nonlinear regression modelling.

Decay rates were calculated until the day concentrations were below quantification limit or until day 55 when the detection limit was not reached.

The length of time (expressed in days) needed to obtain a 90% reduction in initial concentration of stanols or bacteria was calculated as follows: $T_{90} = -\ln(0.1)/k$ (Chick model) or, if f was > 90%, $T_{90} = -\ln(0.1)/k1$ (Cerf Model)

2.7. Regression tests

In order to compare occurrence and concentration of FIB and stanols with time, regressions were performed by plotting average concentrations of each stanol against average concentrations of FIB. Regression lines were drawn between four or several time points. The regression coefficient R^2 values illustrate the relationship between the two variables compared.

2.8. Statistical analysis

2.8.1. Decay rates distribution

The validity of parametric tests is limited to samples following a normal distribution. When the distribution is unknown non parametric tests should be preferred. To infer on normality, a large number of samples is required, therefore to check whether the decay rates obtained in this study were normally distributed, we performed an additional experiment where 20 microcosms were investigated. These latter consisted of 2 L-polypropylene bottles placed in the same conditions as in the study, filled with the same freshwater and the same pig manure added to 1:100. For practical and economical reasons, only, culturable *E. coli* concentrations were measured. The concentrations of cEC were followed every week for 6 weeks. Decay constants *k1* and *k2* were found to be normally distributed with the Normality tests from

XLSTAT 2010.4 (n=20) (data not shown). As a result parametric tests were performed to compare decay constants of FIB and microbial markers. However, regression coefficients R^2 were found not to follow a normal distribution, hence all R^2 were compared with the Mann and Whitney test.

2.8.2. Tests of significance

Two null hypotheses were posed (i) no difference exists between decay rates of one target in either treatments, (ii) no difference exists between decay rates of either indicator (cEC or cENT) and marker in a particular treatment (seawater or freshwater). The risk α to reject the null hypothesis while it might be true was set at 0.05.

All statistical analyses were calculated from the regression coefficients (R^2) and decay rates (k) of three independent experimental replicates. Concerning microbial markers, an F-test was performed to determine variance equality between set of samples prior to a two tailed Student's *t*-test assuming equal or unequal variance depending on the F-test results. Analyses were achieved by Microsoft Office Excel 2003.

It was not possible to compare decay rates from FIB and faecal stanols because the former decay was described with a non linear regression model whilst the latter with a linear model. Only the first null hypothesis could be considered. The distribution of stanols decay rates could not be determined. As a consequence, to compare decay rates of stanols in freshwater and seawater microcosms; a non parametric test for small samples of unknown distribution (Mann-Whitney) was performed on XLSTAT 2010.4.

Decay rates were compared only if the R^2 of the linear regressions they originated from were not statistically different. Otherwise, the test is stated to be non applicable (NA).

3. RESULTS

In this study, we examined the persistence of pig faecal markers in three independent controlled unfiltered water microcosms under aerobic condition. Temperature oscillated between 16 and 20 °C and dissolved O_2 ranged from 8.3 to 9.7 mg/L. The waters used to constitute microcosms were free or slightly contaminated with FIB and stanols. In the seawater, FIB were not detected. Among stanols, only coprostanol and sitostanol were quantifiable with a concentration of 0.02 and 0.05 µg/L, respectively. In the freshwater, cEC concentration was 60 CFU/100 mL and cENT were not detected. The concentrations of coprostanol, epicoprostanol, 24-ethylcoprostanol and sitostanol were 0.04, 0.02, 0.02 and 0.07 µg/L, respectively whereas campestanol was not detected. Pig-specific DNA markers Pig-2-Bac and *L. amylovorus* were not detected in both types of water. At the end of the experiment FIB were not detected in both types of water controls in 100 mL. We thus inferred that microflora and stanols present in the microcosms at day 0 arose from the pig manure.

3.1. Decay curves

3.1.1 FIB and microbial markers

FIB and microbial markers followed a biphasic first order decay kinetic (Fig. 1). We, therefore, used the Cerf biphasic decay model to determine the decay rates of the first and second phase. An example of this model, illustrated by the behaviour of cENT in seawater is shown on Fig. A.2. The model shows a sharper slope for the regression of the first phase than for the second phase. Hence *k1* is always higher than *k2* in this model (Table 1). All decay curves fitted to the model (R^2 > 0.90). As indicated by the value of the f parameter – proportion of the initial concentration of the target that declined during the first phase-, more than 97% of the initial inoculum was lost during this phase. The decimal reduction time occurred thus during the first phase soon after the beginning of the experiment and was reached in less than 6 days independently of the bacteria or the microcosm conditions. However, in many instances a small remaining proportion persisted until the end of the

287 experiment. Culturable *E. coli* were recovered until day 20 in seawater whilst it was still
288 detected on day 55 in freshwater microcosms. Culturable enterococci and *L. amylovorus*
289 concentrations were over the limit of quantification in both types of water on the last day of
290 the experiment. In seawater, Pig-2-Bac marker followed the same trend as *E. coli*, it reached
291 the quantification limit by day 13. On day 20, it was detected just below quantification limit
292 in the three microcosms. It was decided to take this point into account for calculations (Fig.
293 A.1). The week after Pig-2-Bac was still detected in two microcosms. In freshwater, it was
294 found over quantification limit until day 27 in the three microcosms (Fig. B.1), but remained
295 detectable until day 34 in two microcosms.
296
297 **3.1.2. Faecal stanols**
298 Faecal stanols followed a monophasic first order decay kinetic as illustrated by Fig. B.2. In
299 seawater microcosms, initial concentrations of coprostanol, episcoprostanol, 24-
300 ethylcoprostanol, campestanol and sitostanol were 16.7, 3.1, 12.1, 2.6 and 4.7 µg/L,
301 respectively. Their final concentrations reached 0.15, 0.05, 0.18, 0.03 and 0.10 µg/L,
302 respectively (Fig A.3). The concentrations observed after 55 days represented a degradation
303 of 98 to 99% of the initial amounts of the five stanols. Nevertheless, they remained higher
304 than the initial concentrations in seawater before the addition of pig manure. In freshwater
305 microcosms, initial concentrations of coprostanol, epicoprostanol, 24-ethylcoprostanol,
306 campestanol and sitostanol were higher than in seawater microcosms and were 33.4, 9.3, 25.5,
307 6.9 and 11.8 µg/L, respectively whereas their final concentrations were 0.49, 0.15, 0.53, 0.10
308 and 0.20 µg/L, respectively. At the end of the experiment, they were in average 13 ± 6 times
309 higher than the initial concentrations in freshwater before the addition of pig manure.
310 Furthermore, as observed in the seawater microcosms, they represented a degradation of 98 to
311 99% of the initial amount of the five stanols (Fig. B.3).
312

3.2. Progression of stanol ratios

At the beginning of the experiment, the initial values of R_1 (coprostanol/ (coprostanol + 24-ethylcoprostanol)) and R_2 (sitostanol/coprostanol) ratios ranged between 0.57 and 0.58 and between 0.20 and 0.23, respectively. Regardless the microcosms, the ratio R_1 progressively decreased to reach a value of 0.47 (Fig. A.4), due to the difference of decay rate between coprostanol and 24-ethylcoprostanol (Table 1). The changes in the values of R_2 were also similar in freshwater and in seawater. They were most marked than those of R_1 ratio as R_2 ranged mainly from 0.20 to 0.91 with however a maximum value of 1.55 on day 20 (Fig. B.4).

3.3. Decay rates statistical analysis

3.3.1. Decay rates comparisons from seawater and freshwater microcosms

The regression coefficients from non linear regressions were not statistically different. As a consequence every *k1* and *k2* drawn from microbial markers kinetic models could be compared. The results concerning the first null hypothesis are shown in Table 1. *L. amylovorus* decay first constants (*k1*) were statistically different whilst their seconds *(k2*) were not. The opposite was noted for Pig-2-Bac, its first decay constant was not different in seawater or freshwater microcosms whilst they were significantly different during the second phase.

For stanols, the regression coefficients from linear regressions were also not statistically different. Thus, every *k* drawn from faecal stanols kinetics could be compared. According to the Mann and Whitney non parametric test, stanols decay rates were not significantly different in freshwater or seawater microcosms (Table 1).

3.3.2. Decay rates comparisons from FIB and microbial markers

In seawater, the first phase constant from Pig-2-Bac decay did not significantly differed from that of FIB. Contrary to this pig-specific marker, the decrease of *L. amylovorus* was slower than the ones of cEC ($p < 0.0001$) and of cENT ($p = 0.027$) during the first phase. During the second phase, the decays of both genetic markers were not significantly different from that of cENT whilst they were lower than that of cEC ($p < 0.001$). In freshwater, Pig-2-Bac and *L. amylovorus* decays did not significantly differed from that of cENT in the first phase while they were faster than cEC ($p = 0.001$). During the second phase, decay rates of both markers were not statistically different from those of FIB.

3.4. Regression tests

In seawater microcosms, the changes in concentrations of cEC from day 0 to day 13 were well correlated with those of coprostanol ($R^2 = 0.92$) and 24-ethylcoprostanol ($R^2 = 0.91$). However the decrease in concentrations of cEC was not correlated to concentrations of epicoprostanol, campestanol and sitostanol ($R^2 < 0.40$) due to their increases between day 6 and day 13. The correlation between the decrease of concentrations of cENT and stanols showed a same trend with $R^2 > 0.95$ for coprostanol and 24-ethylcoprostanol and $R^2 < 0.60$ for epicoprostanol, campestanol and sitostanol. In freshwater microcosms, the five stanols were better correlated to the change in concentrations of cEC ($R^2 > 0.85$) than to cENT with R^2 ranging from 0.73 (sitostanol) to 0.78 (epicoprostanol).

4. DISCUSSION

The objective of this research was to evaluate the persistence of bacterial and chemical markers as compared to the survival of indicator organisms that are measured currently to assess water microbial quality. It is expected that a microbial load added to a water body by a faecal pollution for instance would decline with time due to the effects of several parameters including sunlight, sedimentation, dilution, transport or grazing by biological agents (Barcina

et al., 1997; Easton et al., 1999). Although here, conditions of natural water bodies were not fulfilled, since the experiment was performed in a closed environment, a decline was observed for every target: molecular and living organisms. This observation is consistent with other recent microcosm studies in freshwater and seawater (Dick et al., 2010; Walters et al., 2009).

4.1. Decay curves

The kinetics of the FIB and microbial markers followed a biphasic curve (Fig. 1). The first phase occurred within 6 days on average. During this phase, a high proportion of the starting inoculum decayed. The remaining proportion persisted in the water column and seemed to be more resistant to decline, since the second phase decline was slow or even null. This was reported before by Easton et al. (1999) who showed, using in-situ chambers that faecal microorganisms did not die-off at a constant rate, and this was only true for the initial decline. Their experiment demonstrated that the die-off rate slowed down as the organism level approached equilibrium with the environment. They found that the initial rapid die-off occurred, generally during the first seven days of the experiment which is consistent with our findings. They proposed two hypotheses to explain this observation (1) the microorganism die-off at a rapid rate until the carrying capacity of the environment is reached, (2) microorganisms would use quorum sensing to regulate their numbers and adapt to their new environment. Although here other parameters than the genetic programming of organisms might have triggered the decline such as sedimentation (Hartz et al., 2008), grazing by protozoa (Bell et al., 2009) or loss of culturability (Barcina et al., 1997), it seems that bacteria can regulate their numbers in a microcosm. Thus, Hellweger et al. (2009) established the biphasic decay kinetic of a pure strain of culturable *E. coli* inoculated in sterile phosphate buffered saline. They observed a decline of the initial inoculum during two days followed by a slight increase of *E. coli* densities. Their experiment established that the resistant fraction

was not a population or strain dependent parameter since they used a pure strain. They proposed as possible explanation that this resistant fraction was made of mutants growing on nutrients released by dead cells.

In agreement with our results, Bae and Wuertz (2009) demonstrated the biphasic persistence of *Bacteroidales* gene markers and *Enterococcus* 23S rRNA gene from human, cattle and dog faecal samples in seawater. Furthermore, Dick et al. (2010) also observed a decay with a biphasic pattern for cEC and *Bacteroidales* genetic markers from human wastewater in freshwater. They also noted that 99% of the initial inoculum was inactivated during the first phase regardless the microcosm conditions. It is noteworthy that in the mentioned studies, although the type of water, the polluting matrix and the physical conditions differed, the biphasic pattern of every different microbial target was observed. This is consistent with our results as each bacterial target in both treatments followed a biphasic decay trend. Furthermore, it has been reported that a high level of prey would be reduced by predators to an equilibrium density that would ensure the survival of the predators (Marino and Gannon, 1991; Menon et al., 2003). It appears that numerous factors are involved in the biphasic decay and the mechanisms responsible for this trend. Results from our study would probably depend on both predation, as the waters were not filtered, and on intrinsic characteristic of the studied bacteria.

The persistence pattern of the DNA markers tended to follow the survival pattern of the living organisms. We could then speculate that the DNA we quantified arose from living cells. This is somehow illustrated by the positive second decay rate from the *L. amylovorus* marker (Table 1). This very low rate could be due to sampling or measurement variations or to a multiplication of the marker . However, it was not possible to verify this hypothesis because no medium enables the isolation of *L. amylovorus* from a complex matrix.

Stanol decay results were concordant with the evolution of coprostanol in seawater during microcosm experiment performed in darkness at 19 °C (Thoumelin et al., 1990). The increase

of the concentration of sitostanol, campestanol and epicoprostanol between day 6 and day 13 could be due to the death of living organisms inherited from the pig manure. As a consequence the sterols that constitute those organisms were liberated in the dissolved phase (Marty et al., 1996) and further hydrogenated into sitostanol, campestanol and epicoprostanol (Pratt et al., 2008). This explanation would agree with the observed microbial decay.

4.2. FIB survival and markers persistence

In seawater, Pig-2-Bac presented a rapid decay rate close to that of cEC (1.1 and 1.3 d^{-1}, respectively) during the first phase, twice faster than that of cENT. It has been described that protozoa eliminate gram positive bacteria at lower rates than gram negative bacteria (Barcina et al., 1997; Davies et al., 1995). Furthermore, in a recent study Balleste and Blanch (2010) proved that *Bacteroides fragilis* survival was highly hindered by grazing predators in warm conditions in a river. Additionally, Jin et al. (2005) established in a natural slightly salted water storm event experiment that the percentage of *E. coli* attached to suspended particles was 21.8 to 30.4 % compared to 8.3 to 11.5 % for enterococci. However, in salty water, Gram positive bacteria would try to protect themselves from the osmotic pressure by attaching to organic matter (Hartz et al., 2008). In this experiment, turbidity was not measured, but it was clear since we could filtered the water from day 13, that suspended organic matter had settled and thus the water column was poorer in organic matter from that day. As a consequence, the concentration of organic matter might have accounted for in the survival of *E. coli* and Pig-2-Bac marker in seawater. Predation and sedimentation might explain the more rapid decay of Gram negative compared to Gram positive markers in seawater. Another factor that might explain the sensibility of the *Bacteroidales* marker compared to the three other organisms in freshwater, is their sensibility to oxygen. It is well established that *Bacteroidales* cells are negatively affected by increased dissolved oxygen in water (Bae and Wuertz, 2009; Balleste and Blanch, 2010). In freshwater, the *Bacteroidales* marker was the only target not detected

442 until the end of the experiment although, Pig-2-Bac decay constant was not different from
443 cENT decay constants (Table 1), thus the shorter relative persistence time period might also
444 be explained by the higher detection limit of the method. This could be improved by filtering
445 higher volumes of water especially, this would be possible in natural environment normally
446 less concentrated in suspended organic matter, or improving DNA extraction yield.
447 No microcosm studies have yet reported on the persistence of *L. amylovorus*. It is thus
448 interesting to note that the pig-specific *L. amylovorus* marker followed the same trend as
449 cENT until day 55 in seawater and in freshwater, although the detection limit was elevated
450 (112 CFU equivalent/100 mL) compared to that of FIB (1 CFU/100 mL). However, the
451 marker follows cEC evolution only in freshwater as it was inactivated more rapidly in
452 seawater. This is not surprising as it has been demonstrated that unlike *Enterococcus faecalis*
453 *or Lactobacillus casei*, *E. coli* does not harbour resistance mechanisms to high osmotic
454 pressure (Lee et al., 1977). However, as stated before, attachment to organic matter aids the
455 bacteria to resist to this pressure.
456 The progressions of the amount of FIB and of the concentration of stanols followed the same
457 trends except for cEC in seawater. Coprostanol and ethylcoprostanol were correlated with
458 cEC until it was no more detected in seawater. Regression coefficients were higher between
459 the five stanols and cENT than with cEC in seawater. On the other hand in freshwater
460 microcosms the five stanols concentrations were more closely related to the amount of cEC
461 than of cENT. However the regression coefficients were still high *i.e.* $R^2 > 0.70$ and defined a
462 close relationship between the occurrence and concentrations of stanols and FIB.
463
464 **4.3. Effect of the type of water**
465 To determine whether the type of water influenced the persistence of markers in the
466 conditions of the experiment, we compared decay rates observed from both microcosms for
467 each target. Pig-2-Bac was not significantly influenced by the type of water during the first

phase maybe due to the presence of suspended particles but salinity or other factors from the seawater accelerated the decay during the second phase (Table 1) when the particles had settled. As a consequence, the marker was detected one more week in freshwater microcosms. These results are somehow different from those from Okabe and Shimazu (2007) who exposed their *Bacteroidales* pig marker Pig-Bac2 to different salinities and observed no real difference in the persistence or decay of the marker to 0, 10, 20 and 30 g/L under dark conditions; however their experiments were performed at 10°C which decreases the decay. The lactobacillus marker was affected by the type of water at the beginning of the experiment, but the remaining proportion of the population was not affected and persisted for the same length of time in freshwater and seawater microcosms. It has been shown that *L. amylovorus* can multiply in NaCl concentration of 5-10 g/L and can survive in concentrations from 20 to 40 g/L (Neysens et al., 2003). This new genetic marker, which belongs to a genus phylogenetically close to the *Enterococcus* genus, might be useful to monitor marine waters.

The concentrations of stanols after the addition of pig manure were twice to three times higher in the dissolved phase (< 0.7µm) of freshwater than of seawater. This observation was probably due to the increased salinity in seawater that induced an aggregation of dissolved macromolecules instead of allowing them to suspend in the dissolved phase as colloids. In spite of slight differences observed on decay curves (Fig. 3), the decay rates calculated for stanols in seawater and freshwater microcosms did not exhibit significant differences (Table 1).

4.4. MST toolbox validation

Pig-2-Bac and *L. amylovorus* markers were selected to be part of a toolbox to identify sources of faecal pollution in water in our previous study (Gourmelon et al., 2010), we wanted to estimate how their detection is representative of a faecal load. In seawater and freshwater, in the conditions of the study, according to the concentrations in culturable enterococci, the

faecal contamination was present until the end of the experiment. *L. amylovorus* followed globally the same trend as cENT and its detection was thus representative of the faecal load during two months. Pig-2-Bac followed also the same trend as cENT but only until day 20 and day 27 in seawater and freshwater, respectively. Thus it was no longer detected whilst the faecal contamination was still present. However, although Pig-2-Bac appeared less persistent than *L. amylovorus*, both markers were detected for a long period of time (at least 20 days) that would allow water managers to take necessary actions in cases of important discharges. The development of the MST toolbox in our previous study has highlighted two ratios allowing the discrimination between human, pig and cow faeces. Thus, coprostanol to the sum of coprostanol and 24-ethylcoprostanol (R_1) allows the differentiation between human (> 0.71), porcine (0.55-0.59) and herbivore (< 0.41) contributions and the second ratio, sitostanol to coprostanol (R_2) exhibits values > 1 for bovine manures and < 0.4 for pig manures and waste water treatment plant effluents (Gourmelon et al., 2010). In seawater and freshwater microcosms, between day 0 and day 13, R_1 exhibited values characteristic of pig manure. However, over the course of the experiment, R_1 remained above the specific values of bovine manure, suggesting that slight changes in R_1 values over time do not lead to misinterpretation of the origin of the contamination. In both microcosms, R_2 was characteristic of pig manure from day 0 to day 6. As a consequence, the combination of stanol ratios R_1 and R_2 can be investigated in order to indicate a faecal contamination from pig manure up to six days from the beginning of the discharge, which allows time to enumerate FIB in order to determine if further analyses are necessary.

5. CONCLUSION

This laboratory microcosm study aimed to compare the decay rates of FIB, *Bacteroidales* Pig-2-Bac and *L. amylovorus* pig genetic markers and stanol ratios in both freshwater and seawater inoculated with pig manure.

520 Regardless the microcosm conditions and the target, the persistence or survival profiles of the
521 two genetic markers and of the FIB followed a biphasic curve whereas the five stanols
522 followed a monophasic first order decay kinetic.
523 The persistence of genetic and chemical markers were similar in freshwater and in seawater.
524 According to the values of the ratio R_1 and R_2, and as the persistence of *L. amylovorus* and
525 Pig-2-Bac *Bacteroidales* marker was relatively close to that of cultivable *E. coli* and
526 enterococci, all these markers can be used to complement *E. coli* and enterococcus detection
527 method to identify a source of pig pollution at least during 6 days when the time point of the
528 contaminating discharge is known. Furthermore, they should prove useful to trace a pig faecal
529 pollution from bathing areas and shellfish farming waters that are sporadically classified as
530 "non satisfactory" in terms of microbial quality.
531

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539

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Figures Captions:

Fig. 1 – Decay curves of pig specific microbial markers in water microcosms seeded with liquid pig manure (1:100 dilution), (A) seawater, (B) freshwater. Conditions: dark, O_2 saturation, around 18 °C. The uncertainties represent standard errors. Limit of quantification was 1 CFU/100 mL for FIB, 1250 gene copies/100 mL for Pig-2-Bac and 112 CFU equivalent/100 mL for *L. amylovorus*.

Fig. 2 - Modelisation of the mean concentration of cENT (A) by Cerf biphasic decay model (R^2 = 0.97) and of 24-ethylcoprostanol (B) by Chick first order decay model (R^2 = 0.98) from the seawater microcosms

Fig. 3 - Decay curves of stanols in seawater (A) and freshwater (B) microcosms. The uncertainties represent standard errors. The scale of Y-axes are different

Fig. 4 - Evolution of stanols ratios R_1 (A; coprostanol/coprostanol+24-ethylcoprostanol) and R_2 (B; sitostanol/coprostanol). Grey areas correspond to the range of values characteristic of pig manure. SWP: seawater, FWP: freshwater.

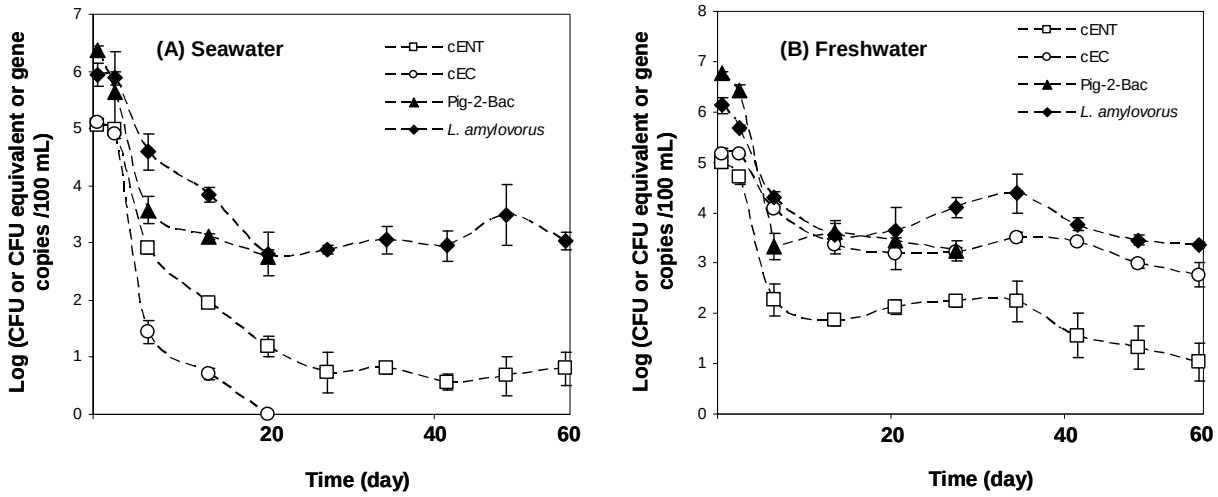
(A) Seawater
(B) Freshwater
cENT
cEC
Pig-2-Bac
L. amylovorus
Log (CFU or CFU equivalent or gene copies /100 mL)
Time (day)

Fig 1

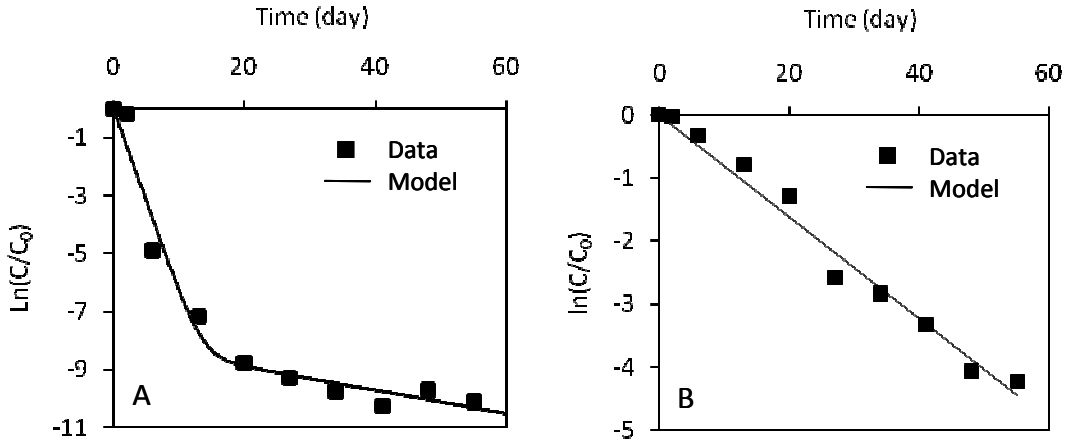
Time (day)
Ln(C/C0)
Data
Model
A
B

Fig 2

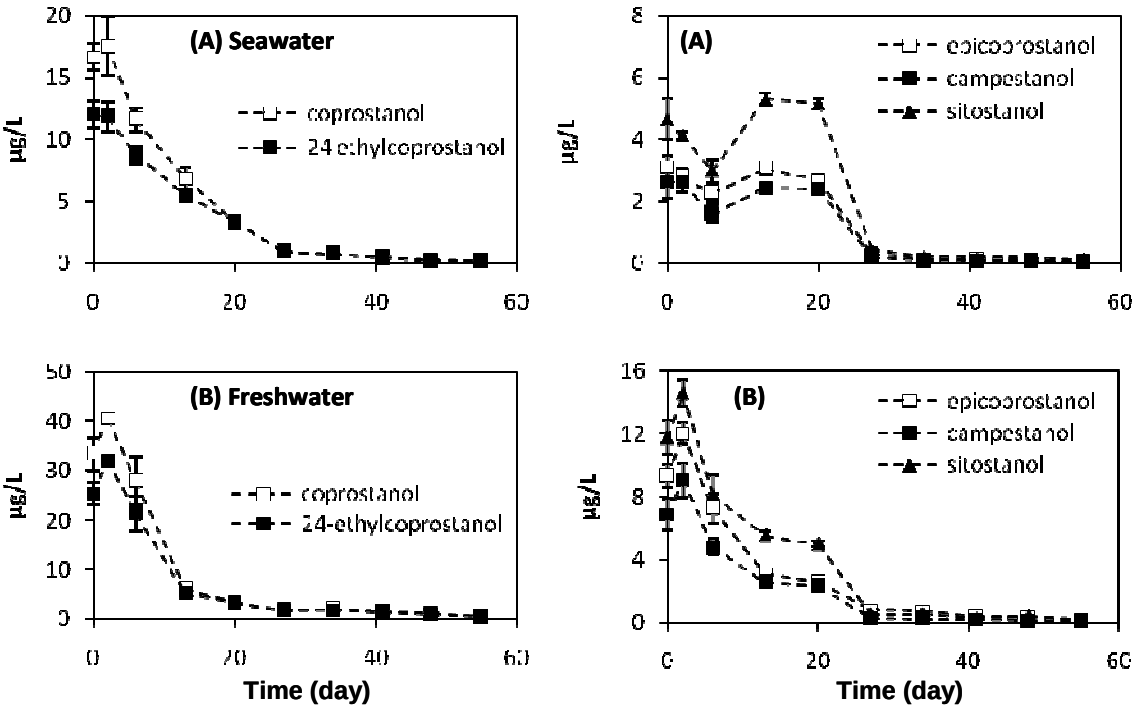
(A) Seawater
coprostanol
24-ethylcoprostanol
μg/L
(A)
epicoprostanol
campestanol
sitostanol
(B) Freshwater
coprostanol
24-ethylcoprostanol
(B)
epicoprostanol
campestanol
sitostanol
Time (day)
Time (day)

Fig 3

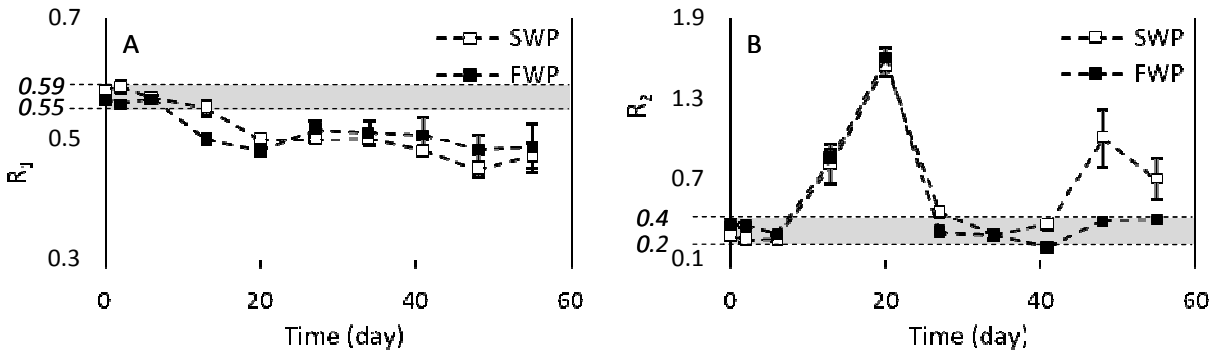
A
SWP
FWP
0.59
0.55
R1
B
R2
Time (day)
Time (day)

Fig4

Table 1 - Decay rates and T90 of FIB and pig specific microbial and chemical markers in water microcosms seeded with liquid pig manure (1:100 dilution). p-values represent comparison between decay rates in seawater and freshwater. Conditions: dark, O_2 saturation, around 18 °C.

	Seawater					Freshwater						
Marker	*k1* (d^{-1})	*k2* (d^{-1})	f (%)	T_{90} (d)	R^2	*k1* (d^{-1})	*k2* (d^{-1})	f (%)	T_{90} (d)	R^2	*p*-value *k1*	*p*-value *k2*
c*E. coli*	1.291	0.388	99.7	1.8	0.96	0.428	-0.021	97.8	5.4	0.94	0.0006	0.0004
cENT	0.605	0.016	99.9	3.8	0.97	1.015	-0.048	99.7	2.3	0.94	0.0377	0.6714
Pig-2-Bac	1.100	0.075	99.9	2.1	0.99	1.247	-0.001	99.9	1.9	0.95	0.7488	0.0412
L. amylovorus	0.406	-0.031	99.9	5.7	0.97	0.737	-0.014	99.4	3.1	0.94	0.0295	0.3089
Coprostanol	0,092			23.7	0.98	0,082			29.5	0.92	0,1000	
Ethylcoprostanol	0,086			26.2	0.98	0,075			32.0	0.93	0,2000	
Epicoprostanol	0,078			25.0	0.91	0,075			29.1	0.98	0,3000	
Campestanol	0,078			25.6	0.88	0,083			25.6	0.93	1,0000	
Sitostanol	0,072			32,0	0,82	0,079			29,1	0,91	0,3000	

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