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Title: Are fecal stanols suitable to record and identify a pulse of human fecal contamination in short-term exposed shellfish? A microcosm study.

Article Type: Research Paper

Keywords: Oyster, Fecal contamination, Seawater, Fecal stanols, Persistence, Microcosms

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Abstract: In this study, the capacity of oysters to bioaccumulate fecal stanols and to record a source-specific fingerprint was investigated by the short-term contamination of seawater microcosms containing oysters with a human effluent. Contaminated oysters bioaccumulated the typical fecal stanols coprostanol and 24-ethylcoprostanol and their bioaccumulation kinetics were similar to that of the Fecal Indicator Bacteria *Escherichia coli* used in European legislation. Although stanol fingerprints of contaminated water allowed the identification of the human specific fingerprint, this was not the case for oysters. This discrepancy is attributed to i) high concentrations of endogenous cholestanol and sitostanol, responsible for "unbalanced" stanol fingerprints, ii) different accumulation/depuration kinetics of fecal coprostanol and 24-ethylcoprostanol and iii) the limits of the analytical pathway used. These results show that fecal stanols bioaccumulated by oysters are useful to record fecal contamination but the usefulness of stanol fingerprints to identify specific sources of contamination in shellfish currently seems limited.

Highlights:

- Oysters were fecally-contaminated with human wastewater in seawater microcosms.
- Stanol concentrations and fingerprints were analyzed in both the water and oysters.
- Fecal stanols coprostanol and 24-ethylcoprostanol were bioaccumulated by the oysters.
- The human-specific stanol fingerprint of water was not transferred to the oysters.

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24 *Keywords:* Oyster, fecal contamination, seawater, fecal stanols, persistence, microcosms.
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Abstract

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4 In this study, the capacity of oysters to bioaccumulate fecal stanols and to record a
5 source-specific fingerprint was investigated by the short-term contamination of seawater
6 microcosms containing oysters with a human effluent. Contaminated oysters bioaccumulated
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9 legislation. Although stanol fingerprints of contaminated water allowed the identification of
10 the human specific fingerprint, this was not the case for oysters. This discrepancy is attributed
11 to i) high concentrations of endogenous cholestanol and sitostanol, responsible for
12 “unbalanced” stanol fingerprints, ii) different accumulation/depuration kinetics of fecal
13 coprostanol and 24-ethylcoprostanol and iii) the limits of the analytical pathway used. These
14 results show that fecal stanols bioaccumulated by oysters are useful to record fecal
15 contamination but the usefulness of stanol fingerprints to identify specific sources of
16 contamination in shellfish currently seems limited.
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2 **1. Introduction**
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7 In coastal environments, shellfish can bioaccumulate pathogenic micro-organisms
8 associated with human and animal fecal contamination originating from the watershed (Fong
9 and Lipp, 2005; Hundesa et al, 2005; Riou et al., 2007; Soller et al., 2010). This type of fecal
10 contamination can result in sanitary risks due to the consumption of contaminated shellfish
11 and has led European authorities to impose a shellfish classification based on the fecal
12 indicator bacteria (FIB) *Escherichia coli* (*E. coli*, European Shellfish Directive 91/492/CEE).
13 In addition, the European Shellfish Directive on shellfish harvesting (854/2004/EC) requires
14 the identification of potential sources of fecal contamination in these environments.
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26 Since *E. coli* is not source-specific, microbial source tracking methods were developed
27 combining microbial and chemical markers to identify the sources of fecal pollution (Simpson
28 et al., 2002; Glassmeyer et al., 2005; Blanch et al., 2006; Vogel et al., 2007; Gourmelon et al.,
29 2010). These methods have been successfully used in several environmental matrices (e.g.
30 water, soil, and sediment), but they remained poorly applied to shellfish and in these latter
31 cases, they only involved microbial markers (Vantarakis et al., 2006; Wolf et al., 2010;
32 Mieszkin et al., 2013). Consequently, chemical markers have not yet been applied to directly
33 identify the sources of fecal contamination in shellfish.
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46 Fecal stanols are direct chemical markers from animal feces. Their distribution in feces
47 depends on i) the animal's diet, ii) the ability of animals to biosynthesize endogenous sterols
48 and iii) the composition of the intestinal flora responsible for sterol biohydrogenation into
49 stanols (Leeming et al., 1996). This species-specific distribution, called the "stanol
50 fingerprint" has been successfully used, via the analysis of stanol ratios or by multivariate
51 analyses, to distinguish between human and animal fecal contamination in water, soil, and
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1 sediment (Bull et al., 2002; Jardé et al., 2007; Shah et al., 2007; Jardé et al., 2009; Tyagi et al.,
2 2009; Gourmelon et al., 2010, Derrien et al., 2011, 2012; Biache and Philp, 2013). Moreover,
3 stanols are sufficiently persistent in the environment to be transferred from the watershed to
4 seawater where the shellfish are living (Solecki et al., 2011; Jeanneau et al., 2012).
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9 In shellfish, studies that investigated the use of fecal stanols to track human
10 contamination focused on the human-associated coprostanol (Sherwin et al., 1993; Cathum
11 and Sabik, 2001; Gagné et al., 2001, 2002; Hellou et al., 2003; Yeats et al., 2008), but the
12 usefulness of the stanol fingerprint to identify human-specific contamination remains
13 unknown. Recently, Harrault et al. (2014) applied the principal component analysis (PCA)
14 model developed by Derrien et al. (2011) to oysters collected in Brittany, which can be used
15 to distinguish between human, porcine and bovine fecal contamination in water in Brittany
16 (Derrien et al., 2012). Harrault et al. (2014) found that oysters fecally contaminated by the
17 FIB *E. coli* presented a specific bovine fingerprint while non-contaminated oysters have no
18 specific fingerprint. However, the transfer of a specific stanol fingerprint from the
19 surrounding water to oyster tissue, in controlled conditions, has not been yet investigated and
20 their persistence has not been compared to that of *E. coli* which are requirements for using
21 them as reliable markers to identify sources of fecal contamination (Blanch et al., 2006).
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41 The present study was conducted to evaluate these issues. In an experimental design
42 conducted in microcosms, the persistence of selected stanols was compared to that of the FIB
43 *E. coli* in seawater initially contaminated with wastewater treatment plant (WWTP) influent
44 and in oysters. In addition, under these experimental conditions, the efficiency of the stanol
45 ratios and Derrien et al.'s (2011) PCA model were investigated to track the human-specific
46 contamination of water and oysters exposed to a short-term contamination.
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58 **2. Material and methods**

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3 *2.1. Reagent and chemicals*
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8 Organic solvents were of high performance liquid chromatography (HPLC) grade.
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10 Dichloromethane (DCM) was purchased from Carlo-Erba SDS (Val de Reuil, France),
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12 methanol (MeOH), isopropanol, hydrochloric acid 37% and cyclohexane were purchased
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14 from VWR (West Chester, PA). N,O-bis- (trimethylsilyl)trifluoroacetamide and
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16 trimethylchlorosilane (99/1, v/v) (BSTFA + TMCS) and SPE disks (Supelco ENVI-18DISK,
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18 47 mm in diameter) were purchased from Supelco (St. Quentin Fallavier, France).
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20 Coprostanol (5 β -cholestan-3 β -ol), cholestanol (5 α -cholestan-3 β -ol), 5 α -cholestane and
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22 anhydrous magnesium sulfate (MgSO₄) were purchased from Sigma (St. Quentin Fallavier,
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24 France). Sitostanol (24-ethyl-5 α -cholestan-3 β -ol) was purchased from Steraloids (Newport,
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26 United States). 24-Ethylcoprostanol (24-ethyl-5 β -cholestan-3 β -ol) and 24-ethylepicoprostanol
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28 (24-ethyl-5 β -cholestan-3 α -ol) were purchased from BCP Instruments (Irigny, France). Silica
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30 gel (40-63 μ m) was purchased from Merck (Darmstadt, Germany). Cholesterol d₆
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32 ([2,2,3,4,4,6-²H₆]-cholest-5-en-3 β -ol) was purchased from CDN Isotopes (Pointe-Claire,
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34 Canada).
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44 *2.2. Incubations and sampling*
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50 Five-hundred and sixteen oysters (*Crassostrea gigas*) were purchased in Cancale
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52 (France), 200 L of seawater was sampled at Dinard (France) and 30 L of raw WWTP sewage
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54 was sampled at Acigné (France) in January 2013. In the laboratory, oysters were placed in 43
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56 11-liter plastic boxes (39.5 \times 26.5 \times 15.6 cm, L \times l \times h) pre-washed with distilled water and
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58 ethanol to remove any plasticizers. Twelve oysters were submerged in each box filled with 4
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1 L of unfiltered seawater. This volume corresponds to more than 300 mL of seawater per
2 oyster, which is necessary for their survival in controlled conditions (Mauffret et al., 2013).
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4 Batches were continuously oxygenated using aquarium pumps and kept in the dark to avoid
5 phytoplankton development and photodegradation of the organic compounds. At the
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7 beginning of the experiment, oysters were allowed to acclimate to laboratory conditions for
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9 72 h (Charles et al., 1992).
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14 The experiment began by diluting the raw WWTP sewage to 10% (“10%” treatment
15 thereafter) in 18 batches and 20% (“20%” treatment thereafter) in 18 other batches. The
16 remaining batches without WWTP sewage addition were used as blanks. Except at the
17 beginning of the experiment (0h), 1 “blank” batch and 3 batches (triplicates) for both the
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19 “10%” and “20%” treatments were sampled at each sampling time. At 0h, oysters sampled
20 from the “blank” batch were considered as 0h samples for the “blank”, “10%” and “20%”
21 treatments. The water and oysters were sampled at 1h, 6h, 24h, 48h, 196h (8 days) and 332h
22 (14 days) after the start of the experiment. At each sampling time, the 12 oysters from the
23 sampled batches were collected for *E. coli* and stanol analyses and 2.5 L of the water was
24 sampled for *E. coli* and chemical analyses including dissolved organic carbon analysis,
25 suspended particulate matter concentrations, and dissolved stanol analyses. Results for the
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27 “10%” and “20%” treatments are the means of the three replicates.
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46 2.3. Physico-chemical parameters 47 48 49 50

51 Suspended particulate matter concentrations were determined by filtrating the water
52 samples through pre-weighted glass-fiber filters at 0.7 μm . Filters were freeze-dried and
53 weighed to determine the particle concentrations. Dissolved organic carbon concentrations
54 were determined on filtered (0.7 μm) water samples using a Shimadzu TOC 5050 total carbon
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1 analyzer (Noisiel, France). Dissolved oxygen, pH, salinity and temperature were measured
2 throughout the experiment using a multisensor probe (WTW, Ales, France).
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4 *2.4. Escherichia coli analysis*

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9 The concentration of the FIB *E. coli* was determined by the CVPA laboratory (Saint-
10 Malo, France) using culture methods (NF EN ISO 9308-3 for the water and NF V08 600 for
11 the oysters). These methods required 500 mL of the water samples and six living oysters. Due
12 to technical constraints, *E. coli* counts were limited to an upper limit of 4.5 log₁₀ colony
13 forming units (log CFU) per 100 mL for the water and 4.2 log CFU per 100 g of flesh for the
14 oysters.
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23 *2.5. Stanol extraction and analysis*

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28 *2.5.1. Water*

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36 Solid phase extractions (SPE) were performed to extract fecal stanols from filtered (0.7
37 μm glass-fiber) water samples as described by Jeanneau et al. (2011). Briefly, known amounts
38 of the recovery standard cholesterol d₆ were added to 1 L of filtered water samples before
39 adjusting its pH to 1 with a 1 M hydrochloric acid solution and the addition of 100 mL of
40 isopropanol. Then, the mixture was passed through pre-washed (DCM) and activated (MeOH)
41 SPE disks. Stanols bound to the disk were eluted with DCM and analyzed by gas
42 chromatography-mass spectrometry (GC-MS).
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53 *2.5.2. Oysters*

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2 Stanols from oyster tissue were extracted and analyzed as described by Harrault et al.
3 (2014). Briefly, the oyster flesh (about 4 g dry weight, DW) was freeze-dried and ground and
4 the total lipids were extracted using an Accelerated Solvent Extractor (ASE 200, Dionex,
5 Courtaboeuf, France) with DCM. Then, the total lipids were fractionated on silica gel
6 columns into an apolar fraction eluted with a mixture of cyclohexane/DCM (2/1, v/v) and a
7 stanol-containing polar fraction eluted with a mixture of DCM/MeOH (1/1, v/v).
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10 11 12 13 14 15 16 17 2.5.3. *Stanol analysis using gas chromatography-mass spectrometry (GC-MS)* 18 19

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23 Stanols extracted from the water and oyster samples were derivatized using a mixture of
24 BSTFA + TMCS (99/1, v/v) at 60°C for 20 minutes to convert hydroxyl groups into
25 trimethylsilyl (TMS) ether groups. Derivatized stanols were then analyzed by GC-MS with
26 a Shimadzu QP2010 + MS gas chromatograph/mass spectrometer (Shimadzu, Tokyo, Japan)
27 with electron ionization at 70 eV. Separation was achieved using a fused silica column coated
28 with SLB-5 MS (Supelco, 60 m, i.d. 0.25 mm, film thickness 0.25 µm) with helium as a
29 carrier gas at a flow of 1 ml min⁻¹. The GC oven temperature was maintained at 70°C for 1
30 min, then increased to 130°C at 15 °C min⁻¹, then to 300°C at 3°C min⁻¹ and held at this
31 temperature for 15 min. Analyses were performed in SIM (selective ion monitoring) mode.
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33 Quantification was based on the internal standard (5α-cholestane) method and a five-point
34 calibration curve was used (standards: coprostanol, epicholestanol, epicoprostanol,
35 cholestanol and sitostanol). The limits for the quantifications were 0.1 µg/L for the water and
36 5 µg/g DW for the oysters.
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58 2.6. Stanol ratios 59 60 61 62 63 64 65

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3 In order to investigate the usefulness of fecal stanols to record a specific human
4 contamination in water and shellfish, three ratios for the fecal stanol concentrations were
5 used:
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11 - $R1 = \frac{[Coprostanol]}{[Cholestanol]}$. With R1 considered as specific from human contamination when >
12 0.50 (Shah et al., 2007; Furtula et al., 2012 and references therein).
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15 - $R2 = \frac{[Coprostanol]}{([Coprostanol]+[24-Ethylcoprostanol])}$. With R2 considered as specific from human
16 contamination when > 0.73 (Leeming et al., 1997, Furtula et al., 2012 and references
17 therein).
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20 - $R3 = \frac{[Sitostanol]}{[Coprostanol]}$. With R3 considered as specific from human contamination when
21 < 0.20 (Gourmelon et al., 2010).
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35 2.7. Statistical analyses 36 37 38 39 40

41 Statistical analyses were performed using the R software (2.15.3, [http://www.r-](http://www.r-project.org/)
42 [project.org/](http://www.r-project.org/)). Two-way ANOVAs, with treatment and time as explanatory variables, were
43 performed to compare the evolution of the stanol concentrations in water and oysters between
44 treatments. When the “10%” and/or “20%” treatments had a significant effect on the
45 concentrations compared to the blank, post-hoc Tukey’s tests were performed to compare one
46 treatment to another. Data were log-transformed when necessary to normalize distributions
47 and homogenize variances. A significant threshold of $p < 0.05$ was chosen for all analyses.
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1 Stanol fingerprints of the water and oysters were investigated using the PCA model set up
2 by Derrien et al. (2011) with XLSTAT 2013 (Addinsoft, Paris, France). Briefly, this model is
3 based on the distribution of six main fecal stanols (i.e., coprostanol, epicoprostanol, 24-
4 ethylcoprostanol, 24 ethylepicoprostanol, campestanol, and sitostanol) from 88 various
5 samples of bovine, porcine (feces, manures, slurries, etc.) and human origin (raw and diluted
6 waste water treatment plant effluent, sewage sludge). With 78.3% of the total variance
7 explained by the first two components, this model distinguishes stanol fingerprints from the
8 three previous origins into three distinctive clusters, allowing the identification of the origin
9 of fecal contamination in the environmental matrix between bovine, porcine and human
10 contamination. Each source sample was used as an individual and the water and oyster
11 samples were used as supplementary individuals.

30 **3. Results**

35 *3.1. Physico-chemical parameters of water*

41 The mean values of the physico-chemical parameters of water throughout the experiment
42 are presented in Table 1. The dissolved oxygen, pH, temperature and suspended particulate
43 matter concentration remained similar between all treatments over time. The different dilution
44 factors between seawater and WWTP sewage in the “10%” and “20%” batches resulted in
45 decreasing salinity and increasing dissolved organic carbon concentrations in water from the
46 “blank” up to the “20%” batches.

59 *3.2. Escherichia coli concentrations*

3.2.1. Water

The concentration of *E. coli* in the seawater and raw WWTP effluent was respectively 1.7 and 7.3 log CFU/100 mL (Supplementary Table 1). Except at 1h after the start of the experiment (2.8 log CFU/100 mL), the concentration of *E. coli* in the non-contaminated water sample remained lower than the European threshold indicative of fecal contamination (2.7 log CFU/100 mL, Figure 1a). Until 48h after the start of the artificial contamination, the concentration of *E. coli* in the “10%” and “20%” water samples (4.5 ± 0.0 log CFU/100 mL) was higher than 2.7 log CFU/100 mL. Then their concentrations decreased below 2.7 log CFU/100 mL to reach low levels of contamination with 2.3 ± 0.2 log CFU/100 mL at 196h and 1.2 ± 0.0 log CFU/100 mL at 332h for the “10%” water samples and 1.8 ± 0.3 log CFU/100 mL at 196h and 1.3 ± 0.3 log CFU/100 mL at 332h for the “20%” water samples (Figure 1a).

3.2.2. Oysters

The “blank” oysters were not fecally contaminated since their concentrations of *E. coli* were below the limit of quantification for the method used, except at 48h with a concentration of 2.5 log CFU/100 g, which remained lower than the European threshold indicative of fecal contamination of shellfish (3.7 log CFU/100 g, Figure 1b). At the start of the experiment, the “10%” and “20%” oysters were not fecally contaminated (below the limit of quantification for both, Figure 1b) but from 1h to 48h, they were both highly contaminated since their concentration of *E. coli* was higher than 3.7 log CFU/100 g (4.2 ± 0.0 log CFU/100 g for both

1 treatments, Figure 1b). Then, the concentration of *E. coli* in the “10%” oysters decreased
2 below the contamination threshold at 196h (3.0 ± 0.4 log CFU/100 g) until 332h (2.8 ± 0.4 log
3 CFU/100 g). The concentration of *E. coli* in the “20%” oysters decreased below the
4 contamination threshold at 196h (3.3 ± 0.3 log CFU/100 g) and increased at the end of the
5 experiment to reach 3.5 ± 0.1 log CFU/100 g at 332h.
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14 3.3. Stanol concentrations 15 16 17 18 19

20 This section presents the temporal concentration variations in water and oysters of four
21 particularly interesting stanols (Figure 2): coprostanol, 24-ethylcoprostanol, cholestanol and
22 sitostanol. Coprostanol and 24-ethylcoprostanol were chosen because they are the two main
23 fecal stanols of WWTP sewage (71% and 15% of the total stanols, respectively,
24 Supplementary Table 1) and thus are used as qualitative biomarkers of human fecal
25 contamination. Cholestanol and sitostanol were chosen because they are the main stanols in
26 the oysters quantified in this study (Supplementary Table 3). Individual concentrations of all
27 stanols quantified in the water and oysters are presented in Supplementary Tables 2 and 3,
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46 3.3.1. Water 47 48 49 50 51

52 On average throughout the experiment, the addition of WWTP sewage in seawater had a
53 significant effect on the concentration of coprostanol, 24-ethylcoprostanol, cholestanol and
54 sitostanol in water (treatment effect: $p < 0.0001$ for the four compounds, Figure 2a, b, c, d):
55 “blank” concentrations were lower than the “10%” ones (Tukey tests: $p < 0.0001$ for the four
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compounds), which were lower than the “20%” ones (Tukey tests: $p < 0.0001$, $p < 0.001$, $p < 0.01$ and $p < 0.0005$, respectively).

In non-contaminated water, the concentration of coprostanol, 24-ethylcoprostanol, cholestanol and sitostanol slightly varied over time and remained below 0.14, 0.45, 1.83 and 0.12 $\mu\text{g/L}$, respectively (Figure 2a, b, c, d).

On the contrary, the concentration of these four compounds in the “10%” and “20%” water batches had high temporal variations which occurred at three different rates (time effect: $p < 0.0001$ for the four compounds, Figure 2a, b, c, d): on average, their concentrations first increased by 951% until 48h, then decreased by 95% until 196h and finally stabilized until 332h (Supplementary Table 2).

3.3.2. Oysters

Throughout the whole experiment, the concentration of both coprostanol and 24-ethylcoprostanol in the “10%” and “20%” oysters were on average higher than those of the “blank” oysters (treatment effect: $p = 0.002$ and $p = 0.025$, respectively) but there was no significant dose effect between the “10%” and “20%” treatments (Tukey: $p = 0.49$ and $p = 0.61$, respectively; Table 2e, f; Supplementary Table 3). In addition, the kinetics of these two compounds were quite similar (time effect: $p < 0.0001$ for the four modalities): their mean concentration increased by 154% from 0h to 24h or 48h and then decreased by 38% until 332h.

Throughout the whole experiment, cholestanol was by and large the main stanol in the oyster tissue (ca. 450 $\mu\text{g/g}$ DW and ca. 80% of the total stanols, Supplementary Table 3) and its concentrations exhibited high standard deviations (i.e. 17% of the mean relative standard

1 deviation). Moreover, the concentration of cholestanol in oysters for the three treatments did
2 not follow a specific trend (time effect: $p = 0.84$, Figure 2g) and was similar among the
3 treatments (treatment effect: $p = 0.42$).
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7 The sitostanol concentration in oysters tended to increase from the start until the end of
8 the experiment (time effect: $p < 0.0001$, 36.05 $\mu\text{g/g DW}$ at 0h to ca. 80 $\mu\text{g/g DW}$ at 332h,
9 Figure 2h) but did not differ between non-contaminated and contaminated microcosms
10 (treatment effect: $p = 0.26$).
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21 3.4. Stanol fingerprints 22

23 3.4.1. Water 24 25

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27 The maximum values for the R1 and R2 ratios in “blank” water were respectively 0.16
28 and 0.28 at 6h, which remains below the thresholds indicative of a major human
29 contamination (0.50 for R1, 0.73 for R2, respectively; Figures 3a, b). Similarly, the minimum
30 value for the R3 ratio in “blank” water was 0.50 at 332h, which remains above the 0.20
31 threshold indicative of a major human contamination (Figure 3c).
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45 The R1 ratio value for “10%” and “20%” water decreased from 0h to 6h (from 16.53 to
46 2.35 ± 0.83 and 9.53 to 4.96 ± 1.40 , respectively), then respectively increased to 4.04 ± 0.29
47 until 48h and to 6.13 ± 0.38 until 24h, greatly decreased to 0.34 ± 0.07 and 0.37 ± 0.07 until
48 196h and finally decreased to 0.09 ± 0.04 and 0.04 ± 0.00 until 332h.
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56 The R2 ratio value for “10%” water decreased from 0h (0.90) to 6h (0.76 ± 0.03), then
57 increased to 0.83 ± 0.01 until 48h and finally decreased to 0.21 ± 0.08 until 332h. The R2
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1 ratio value for “20%” water decreased from 0h (0.87) to 6h (0.82 ± 0.00), then remained
2 constant until 48h and finally decreased to 0.10 ± 0.02 until 332h.
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5 For “10%” and “20%” water, the R3 ratio values increased from 0h (0.02 and 0.01,
6 respectively) to 1h (0.10 ± 0.03 and 0.05 ± 0.01 , respectively), then both slightly decreased to
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8 0.03 ± 0.00 until 48h, slightly increased to respectively 0.26 ± 0.02 and 0.22 ± 0.02 until 196h
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10 and finally greatly increased to 1.71 ± 0.50 and 3.07 ± 0.54 , respectively, until 332h.
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15 The projections of the stanol fingerprints for the “10%” and “20%” water onto the 2D
16 plane of the PCA developed by Derrien et al. (2011) were located in the human cluster during
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18 the whole course of the experiment (Figure 4).
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27 3.4.2. Oysters

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33 The values of the R1 ratio for the “blank”, “10%” and “20%” oysters were lower than
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35 0.03 ± 0.02 and therefore remained under the threshold of 0.50 indicative of human
36 contamination (Figure 3d). Moreover, they did not greatly vary over time and, on average, did
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38 not differ (mean values for the three treatments: 0.02 ± 0.00).
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43 The values of the R2 ratio for the “blank”, “10%” and “20%” oysters were lower than
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45 0.52 ± 0.03 and remained below the threshold of 0.73 indicative of human contamination
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47 (Figure 3e). In addition, they remained stable over time and were not different on average
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49 (mean values: 0.49 ± 0.03 for “blank” oysters, 0.51 ± 0.02 for “10%” oysters and 0.51 ± 0.02
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51 for “20%” oysters).
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56 Similarly, the values of the R3 ratio for the “blank”, “10%” and “20%” oysters remained
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58 largely above the threshold of 0.20 (minimum value: 1.78) and were consequently not
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indicative of human contamination (Figure 3f). They did not differ between treatments and increased by 243% from the start to the end of the experiment.

In the PCA model developed by Derrien et al. (2011), stanol fingerprints of oysters from the “blank”, “10%” and “20%” treatments were not specifically located in the human cluster, but between the human and bovine clusters (Figure 4).

4. Discussion

4.1. Comparison between *E. coli* and fecal stanol kinetics

4.1.1. Water

According to the European legislation on recreational water based on the *E. coli* concentration, the level of fecal contamination for “10%” and “20%” water decreased from highly contaminated levels until 48 hours to non-contaminated levels from 8 to 14 days. This pattern is in agreement with the patterns presented by Jeanneau et al. (2012), who observed the highest *E. coli* decay rate between 2 and 6 days of incubation followed by a lower decay rate until 13 days in seawater microcosms contaminated with a WWTP effluent, but without oysters. This reduction could be due to the sedimentation of *E. coli* associated with particles and/or predation by bacteriophage protozoans (Marty et al., 1995; Solecki et al., 2011).

The initial increase observed in the fecal stanol concentrations might be the result of i) the release of hydrophobic dissolved stanols from particles to water or ii) the exo-enzymatic hydrolysis of ester-bonded stanols by bacteria (Thoumelin et al., 1990; Marty et al., 1995). Similarly to the *E. coli* concentrations, there was a high decrease for fecal stanols in water

1 between 2 and 8 days, like in the study by Jeanneau et al. (2012), who attributed this decrease
2 to a combination of processes such as biodegradation and sedimentation.
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5 These results shows that in these experimental conditions mimicking a short-term
6 exposure to a human effluent, FIB *E. coli* and stanol markers in water are mainly degraded
7 and/or removed during the course of the experiment (14 days).
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12 4.1.2. Oysters 13 14 15 16 17 18

19 As previously observed in studies that investigated the kinetics of *E. coli* bioaccumulation
20 by shellfish (Selegan et al., 2001; Jozic et al., 2012), oysters contaminated with WWTP
21 effluent bioaccumulated *E. coli* within 1 hour in the present study. *Escherichia coli*
22 concentrations in oysters remained higher than the European regulatory value until 2 days and
23 then decreased below this value until 8 days. *Escherichia coli* concentrations remained
24 relatively high in the contaminated oysters compared to the blanks until the end of the
25 experiment while the contaminated water finally decreased to the blank values. These results
26 are in agreement with those of Selegan et al. (2001) who conducted a similar experiment but
27 on freshwater mussels contaminated by a WWTP effluent. Moreover, these results suggest
28 that under these experimental conditions, the depuration process leading to the elimination of
29 *E. coli* by defecation and lysozomal digestion of microorganisms (McHenery et al., 1979;
30 Power and Collins, 1989; Love et al., 2010) from oyster tissues is not yet finished after 2
31 weeks. This depuration time is longer than those observed in depuration experiments in which
32 fecally-contaminated oysters were allowed to depurate from *E. coli* in non-contaminated
33 seawater (Buisson et al., 1981; Doré and Lees, 1995; Selegan et al., 2001; Pommepeuy et al.,
34 2003; Love et al., 2010), while in our experiment, oysters remained in fecally-contaminated
35 water until ca. 6 days (Figure 1a). However, a comparison of the depuration kinetics observed
36 here and in the previous studies should be considered cautiously since the depuration kinetics
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1 of shellfish are highly dependent on the species studied and on the experimental parameters
2 such as water temperature, salinity, dissolved oxygen and particle concentrations and
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4 phytoplankton uptake (Richards, 1988; Pommepeuy et al., 2003).
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8 Among the four stanols focused upon in this study, two groups can be identified. The first
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10 comprises cholestanol and sitostanol: their concentration, and especially that of cholestanol,
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12 was naturally higher than that of the other stanols and remained high until the end of the
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14 experiment. Their respective reduction did not follow the pattern of *E. coli*. The second group
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16 comprises coprostanol and 24-ethylcoprostanol: their concentrations in oysters were lower
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18 than those of cholestanol and sitostanol and exhibited larger temporal variations. Moreover,
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20 their concentration in contaminated oysters followed quite similar patterns to that of *E. coli*: a
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22 first increase step several hours after contamination until 2 days followed by a decay step until
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24 2 weeks. Although different in amplitude, the similarities between the kinetics of these two
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26 stanols and *E. coli* in oysters suggest that these compounds are indicative of the fecal
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28 contamination of oysters. Moreover, contrary to the *E. coli* concentration that did not reach its
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30 initial value after 2 weeks, the coprostanol and 24-ethylcoprostanol concentrations reached
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32 their respective initial values at the same time. This could be the result of a higher depuration
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34 efficiency of these compounds compared to that of *E. coli*.
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45 4.2. Implications for the use of fecal stanols as markers of human fecal contamination

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48 4.2.1. Water

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56 As suggested by Furtula et al. (2012), the identification of human fecal contamination
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58 from wastewater by the use of fecal stanol ratios is more effective when several ratios are
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1 used. In the present study, the three ratios and the PCA model used on water contaminated by
2 WWTP sewage confirmed their human fingerprint. This reinforces the usefulness of these
3 ratios and/or the model to identify human fecal contamination in water (Derrien et al., 2012;
4 Jeanneau et al., 2012; Furtula et al., 2012 and references therein). However, the sensibility of
5 these ratios and the PCA model appears to differ. The three ratio values remained
6 characteristic of human fecal contamination up to 2 to 8 days, while the projection of the
7 stanol fingerprint onto the 2D plane of the PCA model remained in the human cluster up to
8 the end of the experiment. These differences are probably due to the higher sensibility of the
9 three stanol ratios to coprostanol variations than the PCA model. Indeed, the ratios are each
10 based on the variations of two fecal stanols comprising the major stanol coprostanol, while the
11 PCA model is based on the distribution of six stanols and is consequently less sensitive to
12 coprostanol variations (Derrien et al., 2011).

32 4.2.2. Oysters

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39 In oysters, the concentrations of coprostanol and 24-ethylcoprostanol, which are typical
40 of feces and dominated the distribution of the raw WWTP effluent (Supplementary Table 1),
41 were higher in contaminated oysters compared to non-contaminated ones (Figures 2e, f). The
42 R1, R2 and R3 ratios and the PCA model applied to oysters could not be used to record a
43 specific human fingerprint. Several assumptions can be put forward to explain these
44 discrepancies. Firstly, the natural occurrence of cholestanol in oysters at high concentrations
45 compared to that of coprostanol strongly decreased the relative importance of the latter with
46 regards to the R1 ratio. As a consequence, the R1 values were much lower than the “human
47 threshold”, which makes this ratio inappropriate to identify a specific human fingerprint in
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1 oysters. Secondly, in terms of the R1 ratio, the natural occurrence of sitostanol in oysters at
2 high concentrations compared to that of coprostanol meant that the R3 ratio was not able to
3 identify a human fingerprint in oysters. In addition, the naturally high concentration of
4 sitostanol in oysters compared to those of the other stanols used to build the PCA model (i.e.
5 coprostanol, epicoprostanol, 24-ethylcoprostanol, 24-ethylepicoprostanol and campestanol)
6 could produce PCA score plots for oysters that are primarily calculated from the sitostanol
7 proportions, which makes the PCA model inadequate to identify a specific human fingerprint
8 in oysters. Thirdly, the similar concentrations of both coprostanol and 24-ethylcoprostanol in
9 oysters led to low values for the R2 ratio, which makes this ratio inappropriate to identify a
10 specific human fingerprint in oysters. The similarity between the concentrations of these two
11 compounds in oysters raises issues because in the surrounding contaminated water the
12 concentration of coprostanol was at least three times higher than that of 24-ethylcoprostanol.
13 A first hypothesis could be that the assimilation and/or depuration processes of these two
14 compounds by oysters do not follow similar kinetics, resulting in large shifts in the R2 values
15 from the surrounding water to oyster tissue. A second hypothesis could be that the
16 concentrations of 24-ethylcoprostanol in oysters might have been overestimated because of its
17 coelution with several compounds during GC-MS analysis (see the Experimental section in
18 Harrault et al., 2014); in which case, the analytical pathway developed by Harrault et al.
19 (2014), and especially the fractionation step, has to be improved.

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47 In contrast to the present study, Harrault et al. (2014) found that oysters (*C. gigas*)
48 sampled in a French bay presented a bovine fingerprint according to the currently used PCA
49 model, which was in agreement with the activities of the surrounding watershed. The fact that
50 oysters sampled in the field (Harrault et al., 2014) had a specific stanol fingerprint and that
51 oysters artificially contaminated in the present batch experiment did not could be due to
52 different exposure times to fecal contamination. Indeed, the present study was designed to
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1 represent a short-term exposure to a pulse of pollution by a WWTP effluent and the fecal
2 coprostanol concentration decreased after 2 days in contaminated water. This decrease might
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4 not have occurred if the batches were continuously filled with WWTP sewage in order to
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6 represent constant pollution and the ratio values for the oysters used here might have reached
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8 a “human” value if the oysters were continuously supplied with the fecal stanol coprostanol
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10 from the surrounding water. This assumption could be investigated in a further study where
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12 oysters are exposed to constant concentrations of fecal stanols. On this basis, the theoretical
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14 kinetics of R1 for the “20%” oysters was calculated by considering that the highest
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16 coprostanol accumulation rates (between 6h and 24h) remained constant over time if the
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18 WWTP sewage discharge was continuous in our experiment (Figure 2e). As the cholestanol
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20 concentration did not vary over time in the “20%” oysters (Figure 2g), its concentration was
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22 considered as being constant for the calculation of the R1 equation (mean of the 6h and 24h
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24 samples = 430.8 $\mu\text{g/g DW}$). The result presented in Figure 5 can be used to determine, under
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26 the previous assumptions, the theoretical exposure time of oysters to a continuous WWTP
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28 sewage discharge needed to bioaccumulate enough coprostanol to reach a human fingerprint
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30 with regards to their R1 ratios. The theoretical exposure time of R1 was reached at 38 days
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32 with a coprostanol concentration of 217.3 $\mu\text{g/g DW}$, which is very high compared to the
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34 concentrations observed in field studies (Sherwin et al., 1993; Cathum and Sabik, 2001;
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36 Gagné et al., 2001, 2002; Hellou et al., 2003; Yeats et al., 2008; Harrault et al., 2014) and
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38 seems practically unreachable in the laboratory. As a consequence, the stanol fingerprint does
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40 not seem to be suitable for the determination of human fecal contamination.
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55 **5. Conclusion**

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In this study, the ability of oysters to record specific human contamination through the use of fecal stanols was investigated by means of punctual artificial contamination of the surrounding seawater with a human WWTP effluent. The typical fecal stanols coprostanol and 24-ethylcoprostanol exhibited higher bioaccumulation in the contaminated oysters than in the non-contaminated ones, and their bioaccumulation kinetics were quite similar to that of the FIB *E. coli*, suggesting that these two compounds could be used as markers for the human fecal contamination of oysters. On the contrary, the naturally high concentrations of cholestanol and sitostanol in oysters and their different kinetics compared to that of *E. coli* make them unreliable to record fecal contamination.

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Stanol fingerprints, such as the stanol ratios and PCA model that were successfully used to identify the human fingerprint in water, could not be used for this identification in oysters. This lack of specificity for the identification of a human fingerprint is the result of i) the high levels of cholestanol and sitostanol and ii) a large shift in the coprostanol concentration compared to that of 24-ethylcoprostanol in oysters compared to the surrounding water, probably due to either differences in the accumulation/depuration processes of these compounds or the limits of the analytical pathway used.

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Thus this study shows that oysters are able to record a pulse of specific human contamination using specific fecal stanols. However, contrary to constant contamination, the stanol fingerprints of the oysters (ratios and PCA model) appear unreliable to identify a specific source of fecal contamination.

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Last, the stanol fingerprint is a useful tool that can be used as a microbial source tracking toolbox to identify specific sources of fecally-contaminated water; however, the current analytical method of stanols in oysters might not allow the use of the stanol fingerprint as an applicable microbial source tracking tool in this type of matrix. This underlines the need to further investigate the analysis of stanols in a biological matrix. This limitation needs to be

1 taken into account in further investigations of the application of stanol fingerprints to identify
2 fecal contamination sources in shellfish harvesting areas.
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8 **Acknowledgments:**
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17 [mullin.html](http://www.trad8.eu/us/sara-mullin.html)).
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Table1 : Mean values of physico-chemical parameters over the whole experiment. Uncertainties are standard deviations.

Treatment	Blank	10%	20%
<i>pH</i>	7.98 ± 0.05	8.03 ± 0.03	8.10 ± 0.06
<i>O₂ (%)</i>	94.1 ± 7.4	92.2 ± 8.9	90.3 ± 10.1
<i>Salinity (g/kg)</i>	34.5 ± 0.7	31.8 ± 1.2	29.0 ± 2.2
<i>T (°C)</i>	13.1 ± 1.4	12.9 ± 1.4	12.6 ± 1.4
<i>DOC (mg/L)</i>	2.51 ± 1.31	3.75 ± 2.05	4.12 ± 1.07
<i>SPM (mg/L)</i>	20.1 ± 10.5	22.3 ± 11.1	27.2 ± 14.6

Figure 1 : Temporal variations of the concentration of *Escherichia coli* in waters and oysters expressed as colony forming unit (CFU) \log_{10} units/100 mL and \log_{10} units/100 g of flesh, respectively. Open triangles are blanks, grey squares '10%' treatment and black circles '20%' treatment. Errors are standard deviations ($n = 3$). Horizontal dotted lines represent European fecal contamination thresholds for water and oyster of sufficient quality (2.7 \log CFU/100 mL and 3.7 \log CFU/100 g, respectively).

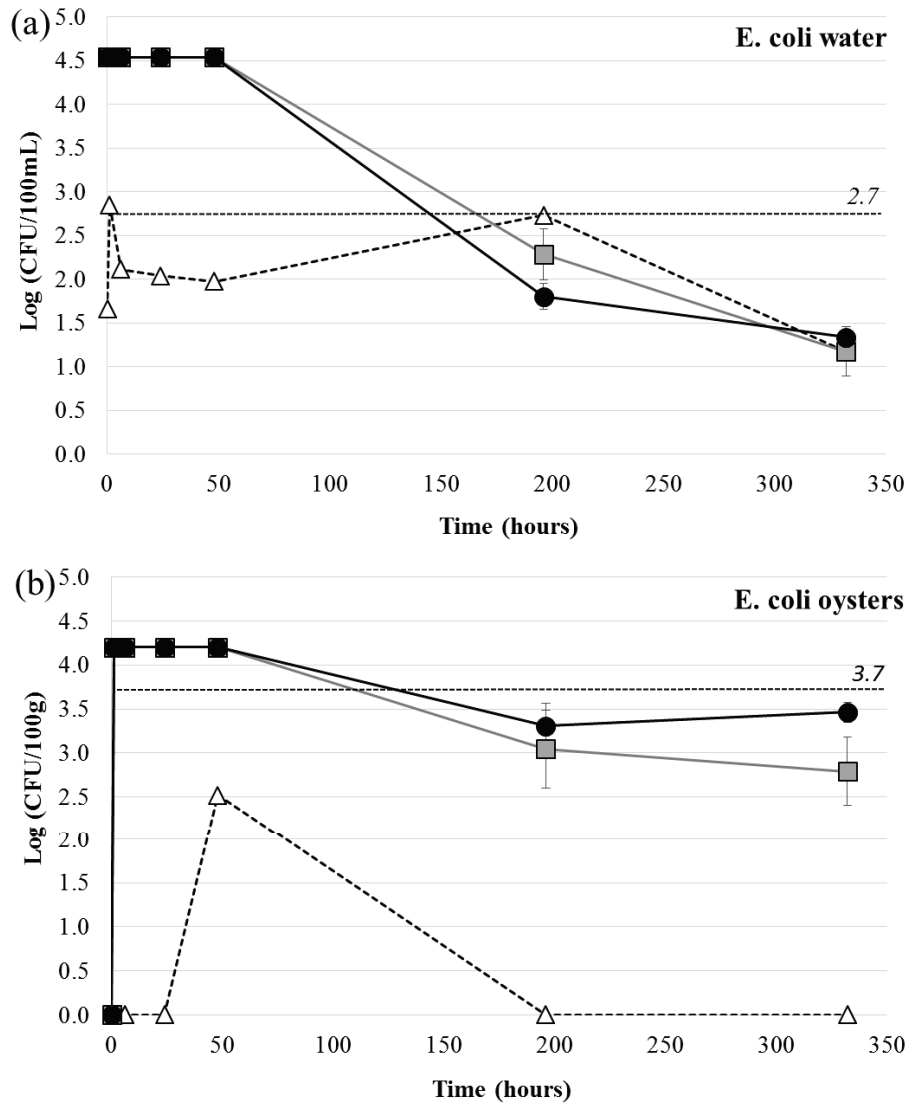


Figure 2: Temporal variations of the concentrations of coprostanol, 24-ethylcoprostanol, cholestanol and sitostanol of water ($\mu\text{g/L}$) and oysters ($\mu\text{g/g}$). Open triangles are blanks, grey squares '10%' treatment and black circles '20%' treatment. Errors are standard deviations ($n = 3$).

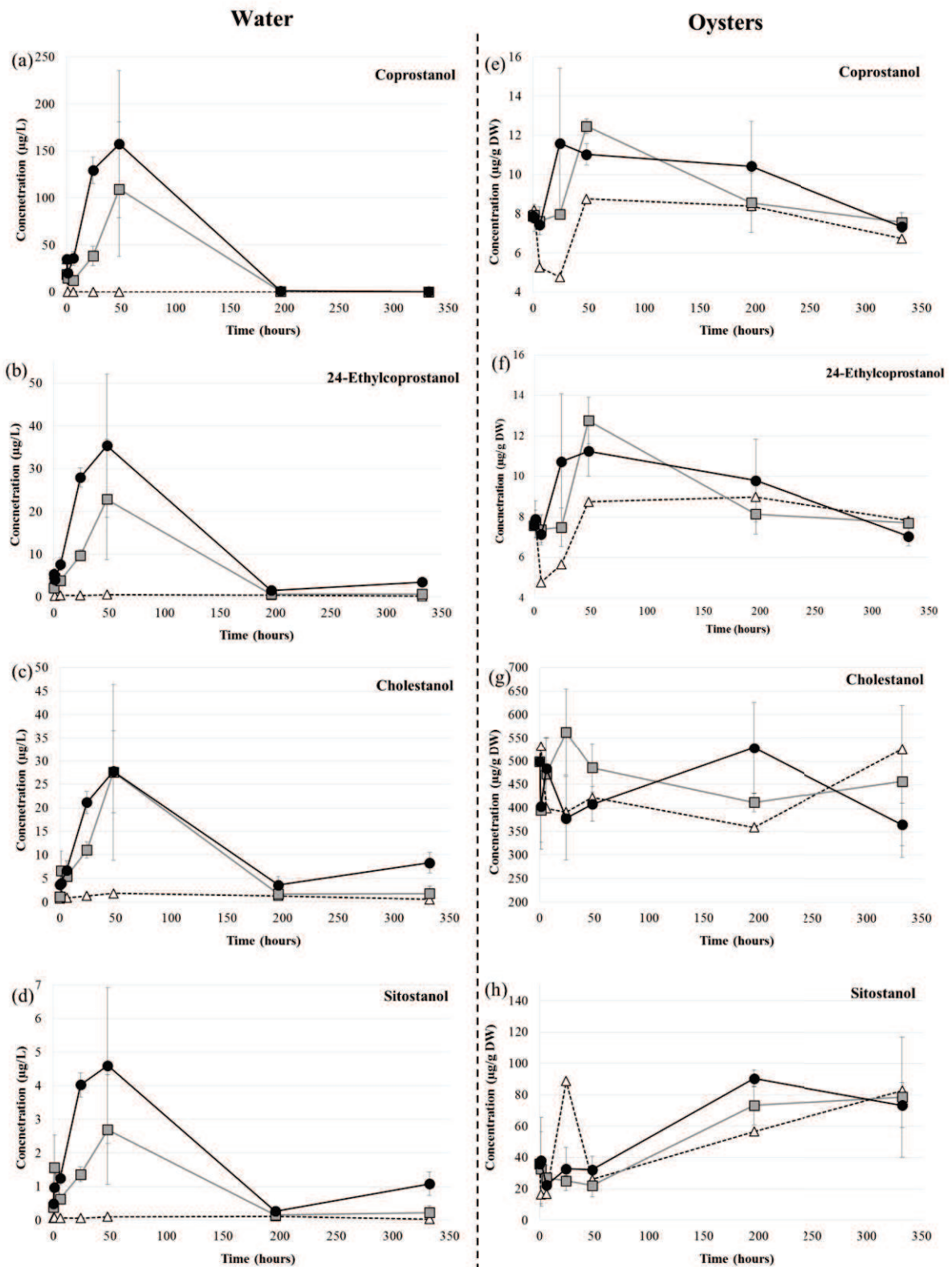


Figure 3 : Temporal variations of stanol ratios in water and oysters over time : (a, d) R1 = coprostanol/ cholestanol, (b, e) R2 = coprostanol/(coprostanol + 24-ethylcoprostanol) and (c, f) R3 = sitostanol/ coprostanol. Open triangles are blanks, grey squares ‘10%’ treatment and black circles ‘20%’ treatment. Horizontal dashed lines represent the upper limits above which each ratio is indicative of a major human contamination. Vertical dotted lines represent the theoretical time at which ratios become non-indicative of a human contamination. Errors are standard deviations (n = 3).

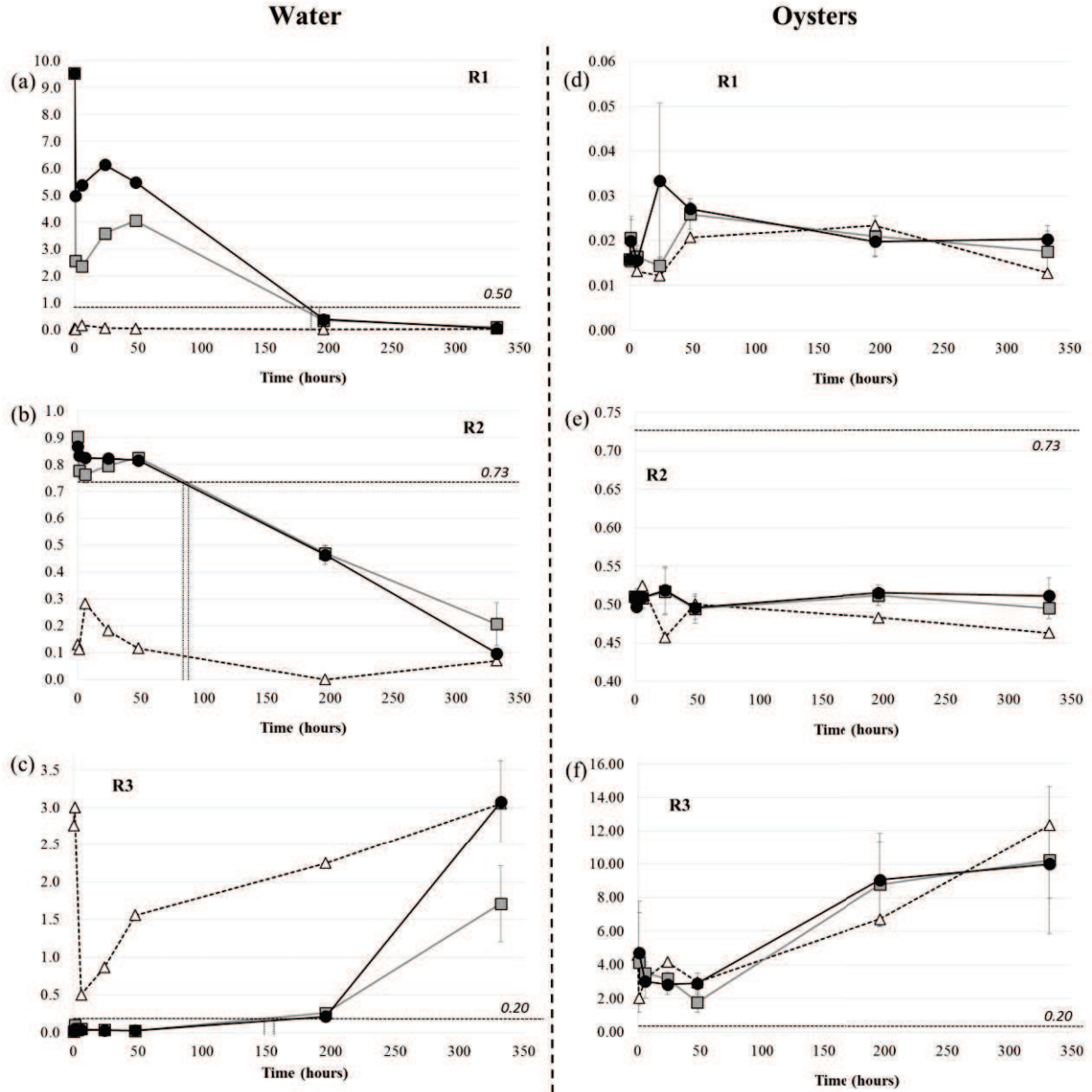


Figure 4 : Plot of the principal component analysis comparing the 88 source samples and water and oyster samples using the 6 most discriminant stanol compounds proposed by Derrien et al. (2012). Sample fingerprints were not graphically distinguished between treatments and time. F1 axis: principal component 1; F2 axis: principal component 2.

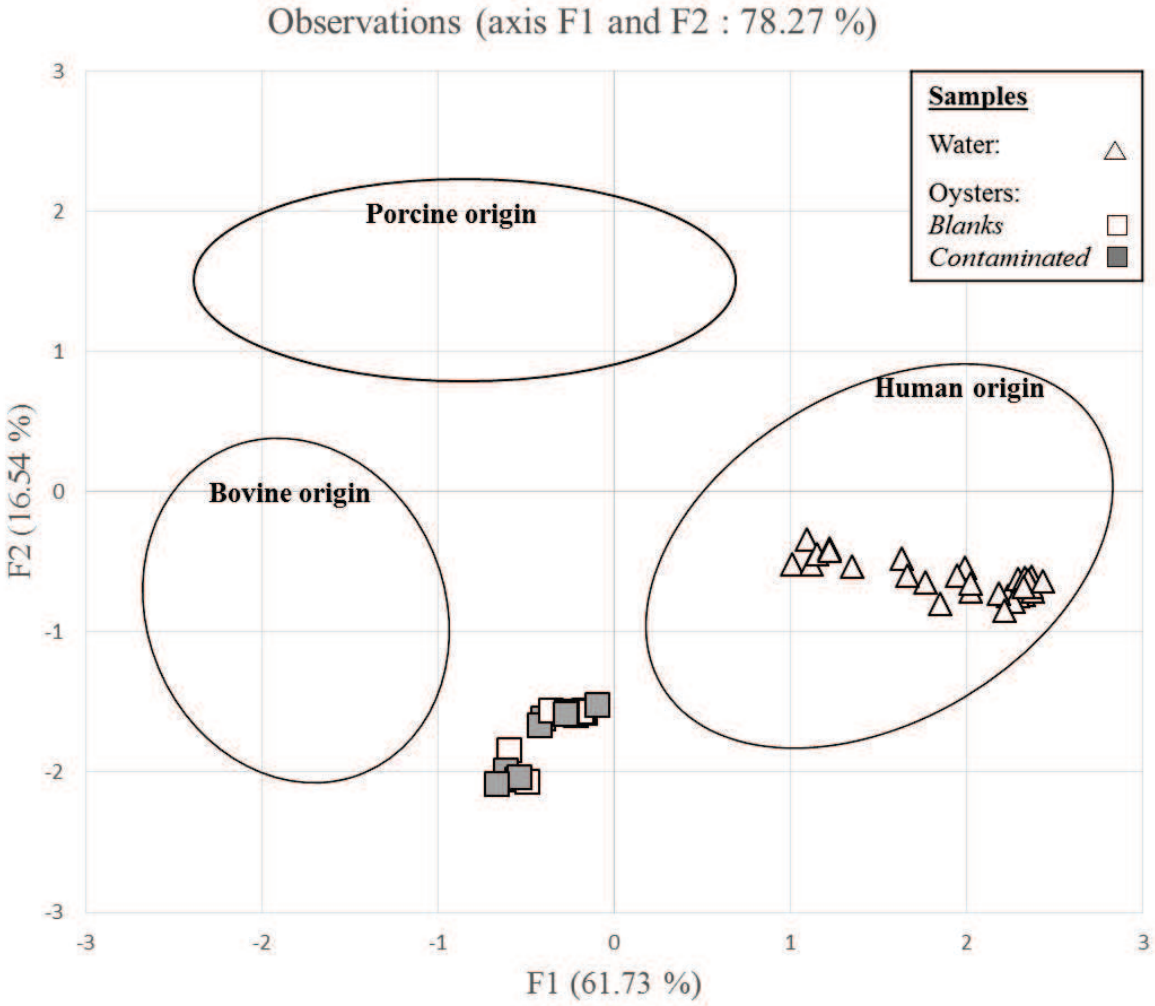
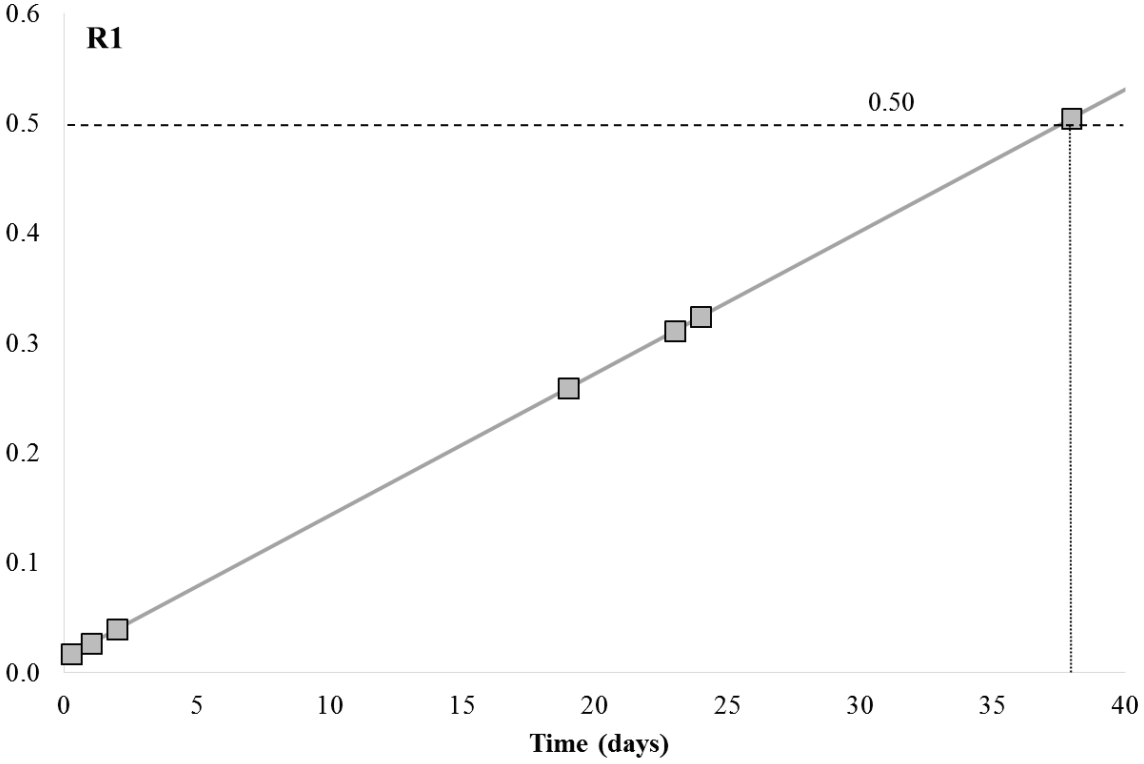


Figure 5 : Theoretical kinetics of stanol ratios R1 (coprostanol/ cholestanol) of oysters from '20%' treatment. Horizontal dashed lines represent the upper limits above which R1 is indicative of a major human contamination. Vertical dotted line represent the theoretical time at which R1 become indicative of a human contamination.



Supplementary Data

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