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

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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.

1. Introduction

In coastal environments, shellfish can bioaccumulate pathogenic micro-organisms associated with human and animal fecal contamination originating from the watershed (Fong and Lipp, 2005; Hundesa et al., 2006; Riou et al., 2007; Soller et al., 2010). This type of fecal contamination can result in sanitary risks due to the consumption of contaminated shellfish and has led European authorities to impose a shellfish classification based on the fecal indicator bacteria (FIB) *Escherichia coli* (*E. coli*, European Shellfish Directive 91/492/CEE). In addition, the European Shellfish Directive on shellfish harvesting (854/2004/EC) requires the identification of potential sources of fecal contamination in these environments.

Since *E. coli* is not source-specific, microbial source tracking methods were developed combining microbial and chemical markers to identify the sources of fecal pollution (Simpson et al., 2002; Glassmeyer et al., 2005; Blanch et al., 2006; Vogel et al., 2007; Gourmelon et al., 2010). These methods have been successfully used in several environmental matrices (e.g. water, soil and sediment), but they remained poorly applied to shellfish and in these latter cases, they only involved microbial markers (Vantarakis et al., 2006; Wolf et al., 2010; Mieszkin et al., 2013). Consequently, chemical markers have not yet been applied to directly identify the sources of fecal contamination in shellfish.

Fecal stanols are direct chemical markers from animal feces. Their distribution in feces depends on (i) the animal's diet, (ii) the ability of animals to biosynthesize endogenous sterols and (iii) the composition of the intestinal flora responsible for sterol biohydrogenation into stanols (Leeming et al., 1996). This species-specific distribution, called the "stanol fingerprint" has been successfully used, *via* the analysis of stanol ratios or by multivariate analyses, to distinguish between human and animal fecal contamination in water, soil and sediment (Bull et al., 2002; Jardé et al., 2006, 2009; Shah et al., 2007; Tyagi et al., 2009; Gourmelon et al., 2010; Derrien et al., 2011, 2012; Biache and Philp, 2013). Moreover, stanols are sufficiently persistent in the environment to be transferred from the watershed to seawater where the shellfish are living (Solecki et al., 2011; Jeanneau et al., 2012).

In shellfish, studies that investigated the use of fecal stanols to track human contamination focused on the human-associated coprostanol (Sherwin et al., 1993; Cathum and Sabik, 2001; Gagné et al., 2001, 2002; Hellou et al., 2003; Yeats et al., 2008), but the usefulness of the stanol fingerprint to identify human-specific contamination remains unknown. Recently, Harrault et al. (2014) applied the principal component analysis (PCA) model developed by Derrien et al. (2011) to oysters collected in Brittany, which can be used to distinguish between human, porcine and bovine fecal contamination in water in Brittany (Derrien et al.,

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2012). Harrault et al. (2014) found that oysters fecally contaminated by the FIB *E. coli* presented a specific bovine fingerprint while non-contaminated oysters have no specific fingerprint. However, the transfer of a specific stanol fingerprint from the surrounding water to oyster tissue, in controlled conditions, has not been yet investigated and their persistence has not been compared to that of *E. coli* which are requirements for using them as reliable markers to identify sources of fecal contamination (Blanch et al., 2006).

The present study was conducted to evaluate these issues. In an experimental design conducted in microcosms, the persistence of selected stanols was compared to that of the FIB *E. coli* in seawater initially contaminated with wastewater treatment plant (WWTP) influent and in oysters. In addition, under these experimental conditions, the efficiency of the stanol ratios and Derrien et al.'s (2011) PCA model were investigated to track the human-specific contamination of water and oysters exposed to a short-term contamination.

2. Materials and methods

2.1. Reagent and chemicals

Organic solvents were of high performance liquid chromatography (HPLC) grade. Dichloromethane (DCM) was purchased from Carlo-Erba SDS (Val de Reuil, France), methanol (MeOH), isopropanol, hydrochloric acid 37% and cyclohexane were purchased from VWR (West Chester, PA). N,O-bis-(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99/1, v/v) (BSTFA + TMCS) and SPE disks (Supelco ENVI-18DISK, 47 mm in diameter) were purchased from Supelco (St. Quentin Fallavier, France). Coprostanol (5β-cholestan-3β-ol), cholestanol (5α-cholestan-3β-ol), 5α-cholestane and anhydrous magnesium sulfate ($MgSO_4$) were purchased from Sigma (St. Quentin Fallavier, France). Sitostanol (24-ethyl-5α-cholestan-3β-ol) was purchased from Steraloids (Newport, United States). 24-Ethylcoprostanol (24-ethyl-5β-cholestan-3β-ol) and 24-ethylepicoprostanol (24-ethyl-5β-cholestan-3α-ol) were purchased from BCP Instruments (Irigny, France). Silica gel (40–63 μm) was purchased from Merck (Darmstadt, Germany). Cholesterol d_6 ([2,2,3,4,4,6-$^2H_6$]-cholest-5-en-3β-ol) was purchased from CDN Isotopes (Pointe-Claire, Canada).

2.2. Incubations and sampling

Five-hundred and sixteen oysters (*Crassostrea gigas*) were purchased in Cancale (France), 200 L of seawater was sampled at Dinard (France) and 30 L of raw WWTP sewage was sampled at Acigné (France) in January 2013. In the laboratory, oysters were placed in 43 11-L plastic boxes (39.5 × 26.5 × 15.6 cm, $L \times l \times h$) pre-washed with distilled water and ethanol to remove any plasticizers. Twelve oysters were submerged in each box filled with 4 L of unfiltered seawater. This volume corresponds to more than 300 mL of seawater per oyster, which is necessary for their survival in controlled conditions (Mauffret et al., 2013). Batches were continuously oxygenated using aquarium pumps and kept in the dark to avoid phytoplankton development and photodegradation of the organic compounds. At the beginning of the experiment, oysters were allowed to acclimate to laboratory conditions for 72 h (Charles et al., 1992).

The experiment began by diluting the raw WWTP sewage to 10% ("10%" treatment thereafter) in 18 batches and 20% ("20%" treatment thereafter) in 18 other batches. The remaining batches without WWTP sewage addition were used as blanks. Except at the beginning of the experiment (0 h), 1 "blank" batch and 3 batches (triplicates) for both the "10%" and "20%" treatments were sampled at each sampling time. At 0 h, oysters sampled from the "blank" batch were considered as 0 h samples for the "blank", "10%" and "20%" treatments. The water and oysters were sampled at 1 h, 6 h, 24 h, 48 h, 196 h (8 days) and 332 h (14 days) after the start of the experiment. At each sampling time, the 12 oysters from the sampled batches were collected for *E. coli* and stanol analyses and 2.5 L of the water was sampled for *E. coli* and chemical analyses including dissolved organic carbon analysis, suspended particulate matter concentrations, and dissolved stanol analyses. Results for the "10%" and "20%" treatments are the means of the three replicates.

2.3. Physico-chemical parameters

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

2.4. Escherichia coli analysis

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

2.5. Stanol extraction and analysis

2.5.1. Water

Solid phase extractions (SPE) were performed to extract fecal stanols from filtered (0.7 μm glass-fiber) water samples as described by Jeanneau et al. (2011). Briefly, known amounts of the recovery standard cholesterol d_6 were added to 1 L of filtered water samples before adjusting its pH to 1 with a 1 M hydrochloric acid solution and the addition of 100 mL of isopropanol. Then, the mixture was passed through pre-washed (DCM) and activated (MeOH) SPE disks. Stanols bound to the disk were eluted with DCM and analyzed by gas chromatography-mass spectrometry (GC–MS).

2.5.2. Oysters

Stanols from oyster tissue were extracted and analyzed as described by Harrault et al. (2014). Briefly, the oyster flesh (about 4 g dry weight, DW) was freeze-dried and ground and the total lipids were extracted using an Accelerated Solvent Extractor (ASE 200, Dionex, Courtaboeuf, France) with DCM. Then, the total lipids were fractionated on silica gel columns into an apolar fraction eluted with a mixture of cyclohexane/DCM (2/1, v/v) and a stanol-containing polar fraction eluted with a mixture of DCM/MeOH (1/1, v/v).

2.5.3. Stanol analysis using gas chromatography-mass spectrometry (GC–MS)

Stanols extracted from the water and oyster samples were derivatizated using a mixture of BSTFA + TMCS (99/1, v/v) at 60 °C for 20 min to convert hydroxyl groups into trimethylsilyl (TMS) ether groups. Derivatizated stanols were then analyzed by GC–MS with a Shimadzu QP2010 + MS gas chromatograph/mass spectrometer (Shimadzu, Tokyo, Japan) with electron ionization