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1 **Bioaccumulation and biotransformation of triclosan and galaxolide in the freshwater**
2 **oligochaete *Limnodrilus hoffmeisteri* in a water/sediment microcosm**

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19 **ABSTRACT:** Personal care products are widely used in our daily life in considerable
20 quantities and discharged through the down-the-drain route to the aquatic environments,
21 resulting in potential risks to aquatic organisms. We investigated bioaccumulation and
22 biotransformation of two widely used personal care products, triclosan (TCS) and galaxolide
23 (HHCB) spiked to sediment, in the oligochaete worm *Limnodrilus hoffmeisteri* in
24 water/sediment microcosms. After 7 days of sediment exposure to 3.1 μg TCS or HHCB /g
25 dry weight (dw) sediment, the accumulation of TCS and HHCB in *L. hoffmeisteri* reached
26 equilibrium, at which point the biota-sediment accumulation factors (BSAFs) were 2.07 and
27 2.50 for TCS and HHCB, respectively. The presence of *L. hoffmeisteri* significantly
28 accelerated the dissipation of TCS and HHCB in the microcosms, with approximately 9.03%
29 and 2.90% of TCS and HHCB eliminated from the water-sediment systems after 14 d
30 exposure in presence of worms, respectively. Two biotransformation products, methyl
31 triclosan and triclosan-O-sulfate, were identified for TCS in the worm tissue, whereas only
32 methyl triclosan was identified in the sediment. Unlike TCS, no evidence of
33 biotransformation products was found for HHCB in either worm tissue or sediment. These
34 experiments demonstrate that *L. hoffmeisteri* biotransformed TCS through methylation and
35 sulfation, whereas HHCB biotransformation was undetectable.

36

37 **INTRODUCTION**

38 Personal care products (PCPs) are widely used in our daily life and can be a potential risk to
39 the aquatic environment due to their incomplete removal in wastewater treatment plants
40 (WWTPs) and negative effects on aquatic ecosystems.[1] Sediments may act as 'sinks' and
41 long-term reservoirs for hydrophobic PCPs released into the aquatic environment.[2] Those
42 hydrophobic PCPs can accumulate in aquatic organisms and may cause bio-magnification
43 through dietary transfer in the food web,[3] or may potentially be biotransformed as observed in

44 annelid worm *Capitella teleta*, important for sediment biogeochemistry and sediment-
45 associated contaminant turnover,[4] exposed to sediment-associated acetyl cedrene[5], thereby
46 reducing the body burden. Oligochaete worms prevail in aquatic environments worldwide and
47 are exposed to sediment-associated hydrophobic PCPs. However, little is known about their
48 potential to biotransform these chemicals.

49 Triclosan (TCS) and galaxolide (HHCB) are two ingredients widely used in personal care
50 products and are ubiquitous in a variety of aquatic environments.[6] For example, our chemical
51 monitoring results show that TCS and HHCB were the most frequently detected hydrophobic
52 chemicals used in personal care products in the subtropical urban rivers, with concentrations
53 up to 1 μg/g dw.[7] With their hydrophobic nature, these two chemicals may sorb to settling
54 particles and bio-accumulate in deposit-feeding macroinvertebrates.[8, 9] To date, laboratory
55 degradation studies of TCS and HHCB have been limited to soil bacterial cultures,[10]
56 wastewater microorganisms,[11] fungi,[12, 13] diatom,[14] algae,[15] activated sludge[16-19] and iron and
57 manganese oxides.[20] For example, TCS can be transformed into methyl triclosan (Me-TCS) in
58 activated sludge under aerobic conditions[16] and in biosolid-amended agricultural soil by
59 microorganisms[21] or earthworms.[22] Similarly, the biological oxidation of HHCB into HHCB-
60 lactone has been reported in wastewater treatment processes[23] and fish samples.[24] However,
61 little to no research has been performed to investigate their degradation under more
62 ecologically realistic conditions, such as water/sediment systems with the presence of
63 oligochaete worms that may efficiently biotransform organic contaminants. For example,
64 *Lumbriculus variegatus* (Oligochaeta) was reported to biotransform pyrene into 1-
65 hydroxypyrene.[25]

66 Oligochaete worms are an important group of freshwater benthic macroinvertebrates,
67 ubiquitous and abundant in sediments of freshwater ecosystems, such as rivers, ponds and
68 lakes.[26] They are thus widely used to evaluate the toxicity and accumulation of sediment-

69 associated hydrophobic organic contaminants.[27-29] *Limnodrilus hoffmeisteri* (Naididae,
70 Oligochaeta) is the dominant taxon within oligochaete worms in the Pearl River (South
71 China), and it can achieve a density of up to 50.000 ind./m^2.[30] Our recent biological
72 monitoring also demonstrated that *L. hoffmeisteri* was the predominant benthic
73 macroinvertebrates identified in six urban rivers of Guangzhou City, South China. As
74 conveyor-belt feeder, *L. hoffmeisteri* ingests small particles in sediments and egests them as
75 faecal pellets on the sediment surface.[31, 32] Sediment-associated hydrophobic organic
76 contaminants may go through bioaccumulation and biotransformation in the body of *L.*
77 *hoffmeisteri*,[33] thereby influencing the fate of chemicals in environment. During the
78 biotransformation process, both more water-soluble and more hydrophobic products can be
79 produced. For example, 2,4-dichlorophenol, a metabolite of TCS, is more water-soluble but
80 less toxic than its parent compound.[11, 34] However, Macherius et al.[22] reported that *Eisenia*
81 *fetida* can biotransform TCS into Me-TCS that is more environmentally persistent,
82 lipophilic[35, 36] and toxic to *Vibrio fischeri* than TCS.[37] Although both TCS and HHCB are
83 hydrophobic chemicals, they have different physicochemical properties. TCS is an ionizable
84 compound with water solubility of 10 mg/L and a log octanol-water partition coefficient
85 (Kow) of 4.8, whereas HHCB is a non-ionizable compound with water solubility of 1.75
86 mg/L and a log Kow of 5.9. [38] Besides, they have different steric configuration and molecular
87 size. As such, TCS and HHCB are likely to show different bioaccumulation and
88 biotransformation in oligochaete *L. hoffmeisteri*.[39, 40] This, however, has not been studied thus
89 far, despite the importance of understanding the metabolic pathway of TCS and HHCB for
90 evaluation of their persistence and risk in the environment.

91 This study aims to evaluate the importance of *L. hoffmeisteri* in the dissipation of
92 sediment-associated TCS and HHCB in microcosms simulating static water systems. The
93 microcosms were divided into two treatment groups: with and without addition of *L.*

94 *hoffmeisteri*. The exposures lasted for 14 d, and worms were sampled on day 1, 3, 7, 10 and
95 14 to investigate the bioaccumulation kinetics of TCS and HHCB. Our results will improve
96 the understanding of the dissipation kinetics of TCS and HHCB in a water/sediment system,
97 and the accumulation and biotransformation of sediment-associated TCS and HHCB in *L.*
98 *hoffmeisteri*.
99

100 **MATERIALS AND METHODS**

101 **Standards and Reagents.** Standards of triclosan (TCS), methyl triclosan, 2,4-
102 dichlorophenol, 4-chlorocatechol, and d_3-Tonalide (d_3-AHTN) were purchased from Dr.
103 Ehrensorfer (Germany), while triclosan-O-β-D-glucuronide sodium salt (TCSG), triclosan-O-
104 sulfate sodium salt and galaxolidone (HHCB-lactone) were obtained from TRC (Canada). The
105 standard galaxolide (HHCB; 1,3,4,6,7,8-hexahydro-4,6,6, 7, 8, 8-hexamethyl cyclopenta (g)-
106 2-benzopyran) was kindly provided by International Flavors & Fragrances (USA), containing
107 about 10% HHCB-lactone, a technical product.[23] The internal standards $^{13}C_{12}$-triclosan and ^{13}
108 C_{12}-methyl triclosan were obtained from Cambridge Isotope Laboratories (Andover, USA).
109 Sylon BTZ containing trimethylchlorosilane, N,O-bis(trimethylsilyl) acetamide, and N-
110 trimethylsilylimidazole was obtained from Supelco. Further details are provided in the
111 Supporting Information (Text S1).

112 **Test sediment and spiking.** The experimental sediment was collected from an
113 uncontaminated reservoir (113˚47'42"N, 23˚46'01"E) [7, 41], a drinking water source of
114 Guangzhou city (South China). The natural sediment was wet-sieved (300 μm) with deionized
115 water, and then allowed to settle overnight. After removing the overlying water, the resultant
116 sediment was kept frozen at -20 °C until use. The sediments used in the microcosms consisted
117 of 0.49% sand, 40.82% silt, and 58.69% clay, and they had a water content of 57% (24 h at
118 105 °C; n = 4), an organic matter (OM) content of 20.6‰, a total nitrogen (TN) content of

119 1.45‰, a total phosphorus (TP) content of 0.45‰ and an ammonia (NH_4^+) content of
120 0.11‰.[42] The background TCS and HHCB concentrations in the sediment were around 0.002
121 µg/g dry weight (dw), and considered negligible for the purposes of this study. Before
122 chemical application, sediment was thawed at 27 ± 1 °C in the dark and rinsed with Milli-Q
123 water.

124 To spike each test compound into sediment, 15 g wet sediment was weighed into a
125 centrifuge tube (50 mL), producing a sediment height of approximately 2.5 cm, and amended
126 with 10 µL of TCS or HHCB stock solution to achieve a final concentration of 3.1 µg/g dw
127 sed. It should be noted that the presence of HHCB-lactone in the HHCB stock solution
128 resulted in a spiked HHCB-lactone concentration of 0.34 µg/g dw in the spiked sediment.
129 Two controls were used in the experiments: a water control and an acetone control, which
130 were created by replacing the chemical solution with the same volume of Milli-Q water and
131 acetone, respectively. Tubes were wrapped with aluminium foil to minimize photolysis of
132 TCS and HHCB. After 15 min of solvent evaporation in the fume hood under darkness, each
133 tube was vortexed for 5 min and then shaken on a horizontal shaker for 12 h in the dark at
134 16 °C to achieve homogeneity.

135 **Test Organisms.** The *L. hoffmeisteri* was obtained from an aquarium market (Guangzhou,
136 South China). It was acclimatized in a 18-L glass tank containing aerated deionized water and
137 thawed sediment (27 ± 1 °C, dark). The acclimatization phase lasted three weeks before the
138 start of exposure. The culturing water and sediment were renewed once during the
139 acclimatization. The total lipids were extracted with acetone/hexane (1/1, v/v) and quantified
140 gravimetrically.[43] TCS, Me-TCS, HHCB and HHCB-lactone concentrations were below the
141 method quantification limits (MQLs) in the unexposed worm tissue. Therefore, worms used
142 here were suitable for the purposes of this study.

143 **Experimental Design.** TCS and HHCB biotransformation experiments were performed
144 separately in water/sediment microcosms. After sediment spiking, 30 mL of aerated Milli-Q
145 water was gently pipetted into each glass vial along the wall, and stored at 4 °C in the dark for
146 2 days to enable potential suspended particles to settle down. Then 30 *L. hoffmeisteri* (length:
147 20.48±3.17 mm; width: 0.375±0.032 mm; wet weight: 0.0021±0.0006 g) at larval stage were
148 introduced into each tube belonging to system with worms. A parallel set of vials without
149 worms were also included to assess microbial degradation. Constant gentle aeration was
150 provided through a glass Pasteur pipette in each tube of both systems at the water surface.
151 Microcosms were incubated statically at 27±1 °C in the dark. The experiment ran for 14 d.
152 During the exposure period, no food was added into the microcosms as worms live on the
153 organic matter associated with the sediment particles. As such, the exposure used here cannot
154 last for a long period. Nevertheless, 14-d exposure is enough for the purpose of studying the
155 bioaccumulation and biotransformation of chemicals in the worm tissue, as demonstrated by
156 the degradation of acetyl cedrene by *C. teleta*.[5] Water evaporation was minimized by covering
157 the tubes with parafilm during the exposure period. According to our previous experience, the
158 evaporation was negligible after 14 d culturing. To measure the abiotic loss of TCS and
159 HHCB during the exposure period, blanks were prepared by adding 30 mL of aerated Milli-Q
160 water containing TCS or HHCB at concentration of 2 μg/L into microcosms. All experiments
161 were performed in four replicates, thus there were 72 tubes in total for each experiment. To
162 analyse bioaccumulation and dissipation kinetics of TCS or HHCB, 8 tubes were sacrificed on
163 days 0, 1, 3, 7, 10 and 14, respectively. Blank and control vessels were sacrificed only at the
164 start and end of exposure. The TCS and HHCB concentrations in the water phase were
165 determined only at the start of exposure. As Me-TCS and HHCB-lactone have been reported
166 as the main product of TCS[22] and HHCB[12], their concentrations were also measured on each
167 sampling date in the worms and water-sediment phases. The pH value in the overlying water

168 was measured at the start and end of exposure. The biotransformation products were
169 determined on days 0, 7 and 14 in worm tissue and in water-sediment phases.

170 **Sample Pre-treatment.** Tubes from system without worms were directly frozen (-20 °C)
171 until lyophilization. Tubes from system with worms were gently vortexed, the resultant water-
172 sediment mixture was then sieved (300 µm). Worms were transferred to glass beakers with
173 400 mL of aerated tap water, left to depurate overnight, weighted into a 50-mL polypropylene
174 centrifuge tube for chemical extraction. Water and sediment were separated by centrifugation
175 at 4000 rpm. The resultant water phase was immediately filtered through 0.7-µm glass fibre
176 filters, combined with the above tap water, diluted to 1000 mL and extracted using solid-
177 phase extraction (SPE) as previously described.[44] The collected sediment and filters were
178 frozen (-20 °C), lyophilized and stored at 4 °C in the dark until extraction. The detailed
179 explanation for SPE is given in the supporting information (Text S2).

180 Sediment samples were extracted by ultrasonic extraction combined with purification by
181 SPE cartridges. Briefly, 15 mL of methanol (for TCS extraction) or acetone/dichloromethane
182 (1:1, v/v) (for HHCB extraction) was added into each tube with dry sediment, vortexed for 5
183 min and further shaken on a horizontal shaker for 2.5 h at 16 °C to thoroughly mix the
184 sediment and solvent. Samples were then extracted in an ultrasonic bath for 0.5 h, and
185 centrifuged at 3000 rpm for 10 min. The clear supernatant was transferred to a 300-mL flat-
186 bottomed flask using a glass pipette. The extraction procedure was repeated three times. For
187 the fourth extraction of TCS, 15 mL of methanol containing 0.1 % (v/v) formic acid was used
188 as extraction solvent. Extraction procedures for particles on the filters were the same as the
189 sediment samples. The supernatants of the sediment and filter from the same microcosm were
190 combined, allowed to evaporate at 37 °C to about 20 mL for TCS whereas to almost dry and
191 reconstituted in 20-mL methanol for HHCB, and diluted with Milli-Q water to a volume of

192 300 mL. Each diluted extract was then purified and enriched on an Oasis HLB cartridge (200
193 mg, 6 mL) using the same procedures for the extraction of water samples.

194 Worms in the tubes were first spiked with 100 ng of d_3-AHTN in case of HHCB samples
195 and 100 ng of $^{13}C_{12}$-triclosan and $^{13}C_{12}$-methyl triclosan in case of TCS samples, vortexed
196 for 30 s, and equilibrated at 4 °C for 30 min. Worm tissue was then homogenized in 4 mL of
197 acetonitrile with two ceramic homogenizers. The homogenates were ultra-sonicated (30 min,
198 20 °C) and centrifuged (10 min, 4000 rpm). The clear supernatants were transferred to 15 mL
199 d-SPE tubes containing 900 mg anhydrous $MgSO_4$, 150 mg PSA, and 150 mg C_{18} to remove
200 lipids. The extraction procedure was repeated twice for each sample. In the third extraction,
201 acetonitrile was replaced by acetonitrile containing 0.2 % acetic acid. The d-SPE tubes
202 containing supernatants were shaken for 2 min and centrifuged (15 min, 4000 rpm). The final
203 supernatants were transferred to 15-mL glass tubes, dried under gentle nitrogen stream, re-
204 dissolved in 1 mL of methanol, filtered through 0.22-μm membrane filters into 2-mL amber
205 glass vials and stored at -20 °C until instrumental analysis.

206 To identify biotransformation products of HHCB, the extracts were derivatized following
207 the procedure described by Martin et al.[12] The details of derivatization are given in the
208 supporting information (Text S3).

209 **Instrumental Analysis.** TCS in the extracts was quantified using an Agilent 1200 high
210 performance liquid chromatograph (Agilent, USA) coupled to an Agilent 6460 triple
211 quadrupole mass spectrometer with electrospray ionization under negative ionization modes
212 (HPLC-MS/MS, ESI-). Me-TCS, HHCB and HHCB-lactone in the extracts were determined
213 by an Agilent 6890N gas chromatograph (Agilent, USA) connected to an Agilent 5975B
214 MSD mass spectrometer (GC-MS), equipped with a DB-5MS column (30 m × 0.25 mm i.d.,
215 0.25 μm film thickness, J&W Scientific Co., USA), in the selected-ion-monitoring (SIM)
216 mode under electron-impact ionization (EI). Qualification of TCS biotransformation products

217 in worm tissue and sediment was performed using Waters ACQUITY UPLC-I Class with
218 Xevo G2-XS QTOF, whereas Agilent 7250 GC/Q-TOF was used to analyse
219 biotransformation products of HHCB. The detailed procedures used for the quantitative and
220 qualitative analysis are provided in the supporting information (Text S4).

221 **Quality Assurance, Quality Control, and Data Analysis.** Solvent blanks and procedural
222 blanks were determined successively for each batch of samples to check background
223 contamination and ensure the performance of the analytical procedure. The MQLs were
224 defined as 10 times the ratio of the signal to instrument noise (Table S1). The recoveries of
225 TCS, Me-TCS, HHCB and HHCB-lactone in each compartment were separately assessed by
226 spiking a standard solution at three levels (0.1, 0.5, and 2) in clean Milli-Q water (μg/L),
227 sediment (μg/g dw), sediment particles on the filters (μg/g dw) and worm tissue (μg/g ww),
228 respectively. All recoveries were in the range of 60% to 110% (Table S2). Concentration data
229 below MQLs were treated as not detected (ND). TCS, Me-TCS, HHCB and HHCB-lactone
230 concentrations were below the MQLs in the clean Milli-Q water and worm tissue in the
231 controls at the end of experiment.

232 The dissipation kinetics of TCS and HHCB in the water/sediment systems were described
233 using both zero-order and first-order kinetic models. For zero-order kinetic model, $C_{(t)} = C$
234 $_{(t=0)}$ - kt and half-life $t_{1/2} = C_{(t=0)} / 2k$; for first-order kinetic model, $C_{(t)} = C_{(t=0)} \times \exp^{(-kt)}$
235 and half-life $t_{1/2} = \ln(2) / k$, where $C_{(t)}$ (μg/g dw) is the TCS or HHCB concentration in the
236 sediment at sampling time t (days) and k is the elimination rate constant.

237 The biota-sediment-accumulation-factor (BSAF) was calculated at each sampling point
238 using the following equation:[45] $\mathrm{BSAF} = (C_o / f_l) / (C_s / f_{OM})$, where C_o is the chemical
239 concentration in the organism (μg/g wet weight (ww)) at each sampling point, f_l is the lipid
240 fraction of the organism (g lipid/g ww) at the start of exposure, C_s is the chemical

241 concentration in the sediment (μg/g dw) at the corresponding sampling point, and f_{OM} is the
242 organic matter fraction of the sediment (g organic matter/g dw) at the start of exposure.
243 Statistical analyses were performed with the software SPSS Statistics (Ver 23.0.0). Two-
244 way ANOVA (factors: presence of *L. hoffmeisteri* and sampling time) with Tukey's multiple
245 comparison tests was used to determine the statistical differences in the chemicals
246 concentrations between systems with and without worms or among sampling dates. Data were
247 checked for normality and variance homogeneity with Shapiro-Wilk test and Levene's test,
248 respectively. Statistical significance was accepted at $p < 0.05$ level.
249

250 **RESULTS**

251 **Concentrations and Dissipation Kinetics of TCS and HHCB in the Microcosms.** The
252 concentrations of TCS and HHCB in the sediment were measured on days 0, 1, 3, 7, 10 and
253 14 and are shown in Table S3 and Figure 1. TCS and HHCB concentrations in the blank
254 samples remained at 2 μg/L during the 14 d incubation period. At the start of exposure, TCS
255 and HHCB concentrations in the water phase were 0.59 μg/L and 0.48 μg/L, respectively.
256 Over the course of experiment, both TCS and HHCB gradually disappeared from the
257 microcosms. However, TCS dissipated faster than HHCB, as demonstrated by greater
258 negative slopes of TCS relative to HHCB in the zero-order model (Figure 1A and B). After 14
259 d exposure, the TCS concentrations decreased from 3.1 μg/g dw to 2.8 μg/g dw (9.03%) and
260 3.0 μg/g dw (3.23%) in systems with and without worms, respectively (Table S3). The HHCB
261 concentrations declined slightly from 3.10 μg/g dw to 3.02 μg/g dw (2.90%) and 3.08 μg/g
262 dw (0.65%) in systems with and without worms, respectively (Table S3). Furthermore, there
263 was a significant difference in TCS and HHCB concentrations between systems with and
264 without worms (two-way ANOVA, $p < 0.05$). Compared to the original spiked concentration,

265 TCS and HHCB concentrations significantly decreased from day 3 and 10 onwards,
266 respectively (two-way ANOVA, $p < 0.05$).

267 Dissipation data of TCS and HHCB fitted well to both zero-order and first-order reaction
268 kinetic models in both systems with and without worms (Figure 1). Under zero-order model
269 (Figure 1A and B), estimated $t_{1/2}$ values for TCS were 79 d and 218 d, and for HHCB were
270 320 d and 1105 d in systems with and without worms, respectively. However, under first-
271 order model (Figure 1C and D), estimated $t_{1/2}$ values for TCS were 103 d and 301 d, and for
272 HHCB were 433 d and 1386 d in systems with and without worms, respectively.

273 **Identification of Biotransformation Products in the Sediment.** The concentrations of
274 Me-TCS increased in both systems during the exposure period, with significantly higher
275 concentrations in systems with than without worm presence (two-way ANOVA, $p < 0.05$)
276 (Table S3 and Figure 2). HHCB-lactone concentration remained at similar levels throughout
277 the exposure period in both systems (two-way ANOVA, $p > 0.05$), with values around the
278 initial spiked concentration, i.e. 0.34 µg/g dw (Figure 2). However, after 14 d exposure, the
279 final HHCB-lactone concentration was slightly lower in the system with (~0.33 µg/g dw) than
280 without (~0.34 µg/g dw) worms (Table S3). Me-TCS concentrations significantly increased
281 from day 7 onwards (two-way ANOVA, $p < 0.05$), whereas there was no significant
282 difference in HHCB-lactone between sampling dates (two-way ANOVA, $p > 0.05$). No other
283 products were found for TCS or HHCB in the sediment by UPLC- QTOF and GC-QTOF,
284 respectively.

285 **Bioaccumulation and Biotransformation Products of TCS and HHCB in the Worm**
286 **Tissue.** The lipid content of *L. hoffmeisteri* was 2.26% ww. During the 14 d exposure period,
287 there was no mortality of *L. hoffmeisteri* in any treatments. The pH was around 6.6 in the
288 overlying water at the start and end of exposure. TCS and HHCB concentrations showed
289 similar change trends in the worm tissue, i.e. increasing from day 0 to day 3 and remaining

290 stable from day 7 onwards (Figure 3). After 1 d exposure, the TCS and HHCB concentrations
291 were 2.4 µg/g ww and 6.5 µg/g ww, respectively. After 3 d exposure, the TCS and HHCB
292 concentrations reached 6.5 and 8.4 µg/g ww, respectively (Table S3 and Figure 3). The BSAF
293 values of TCS and HHCB were in the range of 0.70 to 2.07 and 1.84 to 2.50 during the
294 exposure period, respectively (Table S3).

295 As was observed in the sediment, Me-TCS and HHCB-lactone were also detected in the
296 worm tissue, with concentrations in the range of 0.06-0.15 µg/g ww and 0.70-0.81 µg/g ww,
297 respectively (Table S3 and Figure 3). Moreover, the results of mass balance show that HHCB-
298 lactone accumulation in worms was responsible for the loss of HHCB-lactone in systems with
299 worm presence. Me-TCS and HHCB-lactone concentrations reached the steady state in *L.*
300 *hoffmeisteri* on day 7 and day 10, respectively.

301 In addition to Me-TCS, triclosan-O-sulfate (TCS-O-sulfate) was detected by LC-Q-TOF
302 (Figure 4 and Figure S1). The identification of TCS-O-sulfate was further confirmed by its
303 authentic standard. However, no biotransformation products were identified for HHCB in the
304 worm tissue by GC-QTOF, except for HHCB-lactone.

305

306 **DISCUSSION**

307 This study showed that the TCS and HHCB dissipation in the microcosms fitted well to
308 both zero-order and first-order reaction kinetics models. Likewise, fitting to both models has
309 been reported for TCS[16, 19, 46] and HHCB[12] dissipation by biosolids-amended soil
310 microorganisms and fungi, respectively. TCS dissipated slowly in systems without worm
311 presence with a $t_{1/2}$ value of 218 d (zero-order model) or 301 d (first-order model). While
312 these values are larger than the $t_{1/2}$ value of 58 d detected in the pond water-silty clay loam
313 sediment system under aerobic conditions[47], they are comparable to the $t_{1/2}$ value of 239 d in
314 the lake water-silty clay sediment system with dissolved oxygen levels above 3 mg/L.[48] These

315 differences are likely related to different microbial communities and sediment properties
316 including organic matter and clay content between studies.[21, 49, 50] In addition, although in this
317 study the oxygen was supplied in the overlying water during the incubation period, the
318 sediment in systems without worms was likely under reducing condition due to the microbial
319 respiration[51] and lack of bioturbation, which might hamper the dissipation of TCS because
320 TCS dissipated faster under aerobic than anaerobic conditions.[9, 10] The estimated $t_{1/2}$ for
321 sediment-associated HHCB were > 300 d in both systems under both kinetics models,
322 suggesting that HHCB was persistent in the water/sediment system under the conditions in the
323 present study. However, in the EU Risk Assessment Report (EU RAR) for HHCB, $t_{1/2}$ of 79 d
324 in the sediment was deemed most relevant for modelling the fate of HHCB in sediment using
325 the European Union System for the Evaluation of Substances (EUSES) model.[52] These
326 differences could be attributed to differences in sediment properties, microbial communities
327 and exposure scenarios. Under both kinetics models, the estimated $t_{1/2}$ values of HHCB were
328 ~3 and ~4 times longer than those of TCS in systems with and without the presence of
329 worms, respectively, indicating that HHCB was more persistent than TCS in the water-
330 sediment system. Likewise, a longer $t_{1/2}$ value of HHCB (900 d[53]) relative to TCS (258 d[53]
331 and 107 d[21]) has been reported in biosolid-amended soils in field. However, a faster
332 dissipation of HHCB in biosolid-amended soils has been described by DiFrancesco et al.[54]
333 The corresponding $t_{1/2}$ values were 141 and 144 d in the spiked and unspiked biosolids-
334 amended soils, respectively.[52] The dissipation of TCS and HHCB were faster in systems with
335 than without worms, suggesting that *L. hoffmeisteri* stimulated the dissipation of the two
336 hydrophobic compounds in the water/sediment systems in this study. This is likely to be
337 associated with the bioaccumulation and biotransformation in *L. hoffmeisteri* and enhanced
338 microbial degradation due to the sediment reworking by worms.[55, 56]

339 TCS, Me-TCS, HHCB and HHCB-lactone were detected in the worm tissue, with
340 concentrations increasing from exposure day 1 to 7 and reaching the steady state from then
341 onwards (Table S3), which indicates that *L. hoffmeisteri* can accumulate these hydrophobic
342 compounds. Similar time to reach steady state has previously been observed for sediment-
343 associated polybrominated diphenyl ether (PBDE) accumulation in the oligochaete
344 *Lumbriculus variegatus* (a similar species to *L. hoffmeisteri*).[57, 58] The stabilized BSAF values
345 of TCS (~2.07) in *L. hoffmeisteri* were larger than the 28-day BSAF value (1.4) reported by
346 Dang et al,[59] who studied the bioaccumulation of TCS in *L. variegatus*. However, another
347 study has reported a greater BSAF (9.04) of TCS in *L. variegatus* than the present study.[60]
348 These differences are most likely related to differences in sediment characteristics and species
349 traits between the studies.[60, 61] The stabilized BSAFs of HHCB were around 2.50 in *L.*
350 *hoffmeisteri*, similar to the values (1.5-2.5) reported in carps from the Haihe River (China).[62]
351 HHCB showed higher BSAF values than TCS in *L. hoffmeisteri*, which is likely associated
352 with the lower metabolism and water solubility but higher log K_{ow} value of HHCB than
353 TCS.[39, 40]

354 Me-TCS was detected in both the sediment and worm tissue whereas TCS-O-sulfate was
355 only detected in the worm tissue. These two metabolites were products from phase II reaction,
356 i.e., methylation and sulfation. However, no phase I (e.g., oxidation, reduction and hydrolysis
357 reactions) products were observed in this study. This may be related to the fast transformation
358 of phase I to phase II products, as described by Malmquist et al.[63] who investigated the
359 biotransformation of pyrene by the benthic invertebrate *Nereis diversicolor*. Also, analyses of
360 the overlying water would have provided more information on the fate of phase I products. In
361 the future work, we therefore recommend to analyse metabolites in the overlying water. Yet,
362 the formation of Me-TCS via biological methylation has been reported for different stages of
363 wastewater treatment plants.[16, 64] Besides, Macherius et al.[22] found that TCS was transformed

364 into Me-TCS by earthworms in biosolid-amended agricultural field. However, compared to
365 TCS, Me-TCS is more persistent and also more prone to bio-accumulate in aquatic
366 organisms.[36] The formation of TCS-O-sulfate has been reported in activated sludge,[17] plants[65],
367 rats[66] and human urine[67].

368 Unlike TCS, no products were identified for HHCB. Although HHCB-lactone was
369 detected in both sediment and worm tissue, the results of mass balance show that the presence
370 of HHCB-lactone was due to the spiking rather than HHCB degradation by microorganisms
371 or worms. HHCB-lactone has been reported as a HHCB degradation metabolite for activated
372 sludge[8] and cultures of fungi such as *Myrioconium* sp.[12] and *Phanerochaete chrysosporium*.[8]
373 However, our results demonstrate that *L. hoffmeisteri* and microorganisms in the sediment did
374 not degrade HHCB or HHCB-lactone to a measurable degree. Unlike HHCB, Dai et al.[5] found
375 that after 14 days of exposure sediment-associated acetyl cedrene (another fragrance material)
376 was reduced by 88-99% and 13-31% in the sediment with and without *C. teleta*, respectively.
377 However, another study reported that acetyl cedrene in the sediment decreased 72% in both
378 treatments with and without *C. teleta* after 16 days.[68] One explanation for these findings is
379 that the microbial activity was very low initially in the present study due to the freezing of the
380 sediment, which would potentially decrease microbial degradation compared to a full-active
381 microbial community in previous studies. In addition, it seems that macrofaunal
382 biotransformation is both species- and chemical specific (e.g., Malmquist et al.[63]).

383 In conclusion, our results demonstrate that oligochaete worm presence significantly
384 accelerated HHCB and TCS dissipation in water-sediment systems. *L. hoffmeisteri* either
385 cannot or has a very low ability to biodegrade HHCB but can biotransform TCS through
386 methylation and sulfation. However, currently little information is available for TCS-O-
387 sulfate. Further work is therefore needed to evaluate the (eco)toxicity and persistence of TCS-
388 O-sulfate.

ASSOCIATED CONTENT

Supporting Information

Additional information about sample preparation, instrumental analysis, measured chemical concentrations and predicted biotransformation pathways.

Notes

The authors declare no competing financial interest.

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615

616 List of Figure Captions

617 **Figure 1.** Time courses of TCS (A and C) and HHCB (B and D) concentrations in the

618 sediment from microcosms with and without *Limnodrilus hoffmeisteri*.

619 **Figure 2.** Time courses of Me-TCS (A) and HHCB-lactone (B) concentrations in the

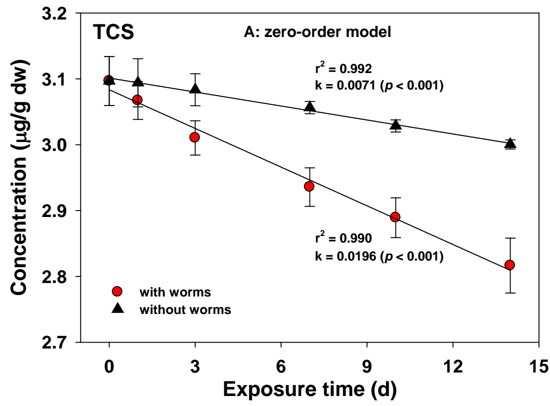
620 sediment from microcosms with and without *Limnodrilus hoffmeisteri*.

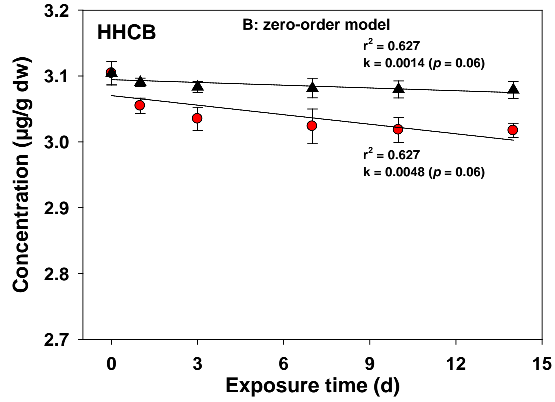
621 **Figure 3.** Time courses of TCS, HHCB (A), Me-TCS and HHCB-lactone (B) concentrations

622 (µg/g ww) in the tissue of *Limnodrilus hoffmeisteri*.

623 **Figure 4.** UPLC-Q-TOF product ion spectra and chromatogram of sulfonated metabolite of

624 TCS in worm tissue.





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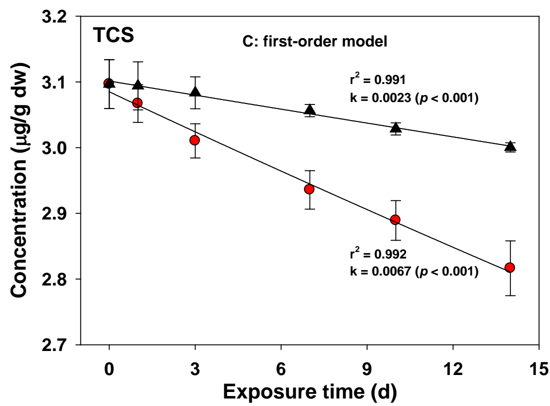

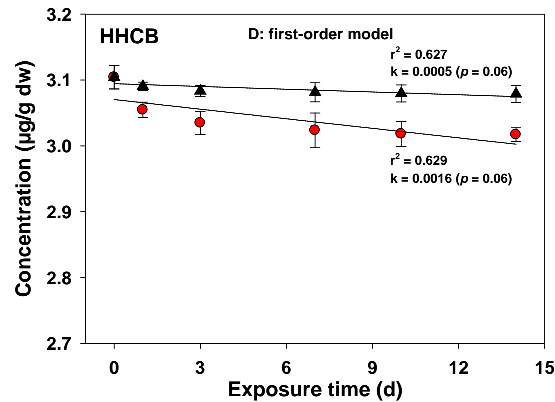

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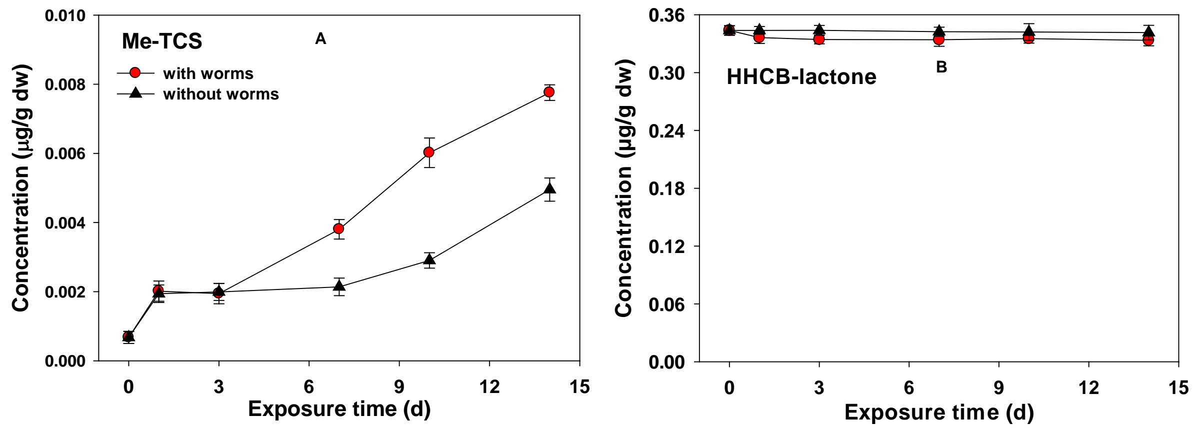
628 Figure 1. Time courses of TCS (A and C) and HHCB (B and D) concentrations in the
629 sediment from microcosms with and without *Limnodrilus hoffmeisteri*. Red circle symbols
630 and black up triangle symbols represent averages of chemical concentrations in systems with
631 and without worms, respectively.

632

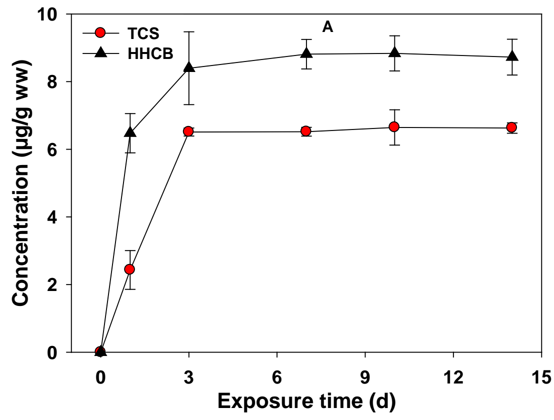


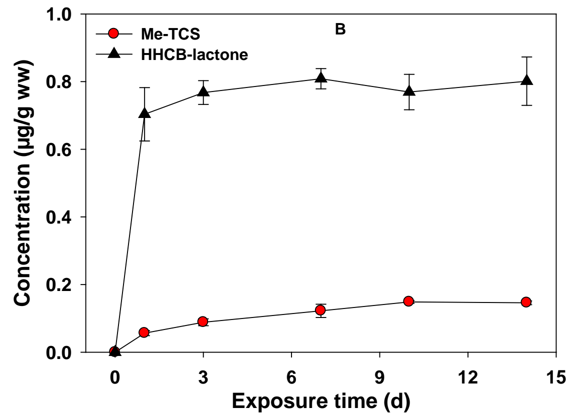
633 Figure 2. Time courses of Me-TCS (A) and HHCB-lactone (B) concentrations in the sediment

634 from microcosms with and without *Limnodrilus hoffmeisteri*. Red circle symbols and black up

635 triangle symbols represent averages of chemicals concentration in systems with and without

636 worms, respectively.





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638 Figure 3. Time courses of TCS, HHCB (A), Me-TCS and HHCB-lactone (B) concentrations

639 (μg/g ww) in the tissue of *Limnodrilus hoffmeisteri*.

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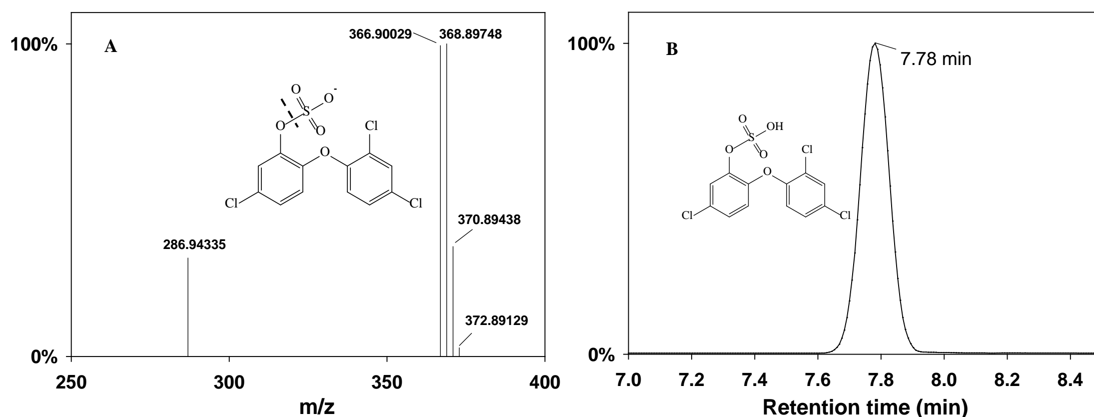

649 Figure 4. UPLC-Q-TOF product ion spectra and chromatogram of sulfonated metabolite of
650 TCS in worm tissue. (A) Product ion spectra of the m/z 368.89748 peak (7.78 min), the
651 product was identified as TCS-O-sulfate. (B) Extracted ion chromatogram of TCS-O-sulfate
652 in the worm tissue.

Supporting Information

Bioaccumulation and biotransformation of triclosan and galaxolide in the freshwater oligochaete *Limnodrilus hoffmeisteri* in a water/sediment microcosm

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Figure S1 Predicted biotransformation pathways of TCS in *Limnodrilus hoffmeisteri*.

References

Text S1 Standards and Reagents

All solvents used for chemical analysis, including methanol, ethyl acetate, n-hexane, acetone, dichloromethane, and acetonitrile were of high-performance liquid chromatography (HPLC) grade and purchased from CNW Technologies (Shanghai, China) or Merck (Germany). Acetic acid and ammonium acetic were bought from Sigma-aldrich (St.Louis, USA), while formic acid was obtained from Tedia (USA). Oasis HLB cartridges (60 mg, 3 mL) and Oasis HLB cartridges (200 mg, 6 mL) were supplied by Waters Corporation (Milford, MA, USA). Glass fiber filters (GF/F, pore size 0.7 μm) were obtained from Whatman (Maidstone, UK). Ceramic homogenizer, Z-Sep tube, anhydrous magnesium sulfate ($MgSO_4$), primary-secondary amine (PSA) and C_{18} bulk sorbent were purchased from Agilent (Santa Clara, USA). Ultrapure water was provided by a Milli-Q system from Millipore (Watford, UK). TCS and HHCB were dissolved in acetone to make a stock solution of 2 g/L. The resultant stock solutions with concentration of 100 mg/L in methanol were individually prepared, and stored at -18 ˚C until use. It should be noted that HHCB stock solution contains about 10% HHCB-lactone.

Text S2 Details of solid phase extraction

Oasis HLB SPE cartridges were preconditioned with 10 mL of methanol and 10 mL of Milli-Q water. Samples were passed through cartridges at a flow rate of 5-10 mL/min. Each sample bottle was rinsed twice with 50 mL of Milli-Q water containing 5 % methanol (v/v) and passed through the SPE cartridge. The cartridges were then dried under vacuum for 3 h. The cartridges were eluted with 3×3 mL of methanol followed by 3×3 mL of ethyl acetate, 3×3 mL of dichloromethane and 3×3 n-hexane. The eluates were dried under a gentle nitrogen gas, re-dissolved in 1 mL of methanol, transferred to a 2 mL amber glass vial with filtering through a 0.22 μm nylon membrane filter, and finally stored at -18 °C until analysis.

Text S3 Derivatization of HHCB and its biotransformation products

The derivatization method for HHCB and its biotransformation products reported by Martin et al.[1] was used in this study. Specifically, 100 μL of an extract in methanol was transferred to a 2 mL amber glass vial with polytetrafluoroethylene (PTFE) screw cap and dried under a gentle nitrogen stream, added with 100 μL of Sylon BTZ and derivatized at 60 ˚C for 1 hour. Then, 200 μL of Milli-Q water at pH 3.0 was added to remove the excess derivatization reagent. The derivatization products were extracted with 500 μL of n-hexane, dried over anhydrous sodium sulfate, concentrated to a final volume of 100 μL, and analysed by GC-Q-TOF.

Text S4 Details of instrumental analysis

LC-MS/MS for TCS quantification: TCS was analysed by an Agilent 1200 rapid resolution liquid chromatograph coupled to Agilent G6460A triple quadrupole mass spectrometer under electrospray negative ionization (ESI) mode[2]. A 10 µL aliquot of each extract was injected into an Agilent SB-C18 column (3.0 mm × 100 mm ID, 1.8 µm particle size) at temperature of 40 ˚C with an RRLC in-line pre-column filter (4.6 mm, 0.2 µm filter), with Milli-Q water containing 0.01% acetic acid (v/v) (solvent A) and acetonitrile : methanol (1:1, v/v) (solvent B) as the mobile phase at a flow rate of 0.3 mL/min. The gradient program was given as follows: 60% B at 0 min, then increased to 90% B at 3 min and kept at 90% B for 4 min, then returned to the initial 60% B at 9 min and let column re-equilibrate for 6 min. The capillary was maintained at 3500 V. Dry and sheath gas flows were kept at 8 and 12 mL/min, respectively. Both dry and sheath temperatures were kept at 350 ˚C.

GC-MS for Me-TCS quantification: Me-TCS was analysed using an Agilent 6890N GC interfaced to a 5975B MSD (GC-MS), equipped with a DB-5MS column (30 m × 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific Co., USA), under electron-impact ionization (EI) mode. Helium (purity > 99.999%) was used as the carrier gas at a constant flowrate of 1.0 mL/min. Splitless mode was applied for injection, with injection volume of 1 µL for each samples. The temperatures for the GC-MS interface, ion source, quadrupole and injector were kept at 300 ˚C, 230 ˚C, 150 ˚C and 250 ˚C, respectively. The column temperature was programmed as follows: from 100 ˚C (2 min) to 180 ˚C at 5 ˚C/min (2 min), from 180 ˚C to 300 ˚C at 10 ˚C/min (2 min), and then to the temperature 310 ˚C at 10 ˚C/min (10 min). The characteristic ions were 314, 264 and 243.9 for $^{13}C_{12}$-Me-TCS, 301.9, 251.9 and 232 for Me-TCS.

ACQUITY UPLC-I Class with Xevo G2-XS QTOF: the qualification of TCS biotransformation products were analysed by an Waters ACQUITY UPLC-I Class with Xevo G2-XS QTOF under a negative ion mode. For chromatographic conditions, a 2 μL aliquot of each extract was injected into a HSS T3 column (2.1 × 100 mm ID, 1.8 μm particle size) at temperature of 40 ˚C, with Milli-Q water containing 10 mM ammonium acetate (solvent A) and methanol (solvent B) as the mobile phase at a flow rate of 0.4 mL/min. The gradient program was given as follows: kept 2% B from 0 to 0.25 min, increased to 98% B at 12 min and kept at 98% B for 3 min, then returned to the initial 2% B at 18 min and let column re-equilibrate for 6 min. For mass spectrometry conditions, the capillary and cone voltage were maintained at 2500 V and 20 V, respectively. The cone gas flow was kept at 50 L/h, and the source temperature was 120 ˚C. The desolvation temperature and desolvation gas flow were set as 500 ˚C and 800 L/h. The samples were scanned using MSE scan mode at a range of 50-1000 m/z with scan time of 0.2 s. Leucine-enkephalin was used as reference for mass correction. The data were processed using the UNIFI Scientific Information System to identify the putative compounds present in the extracts.

GC-MS for HHCB and HHCB-lactone quantification: HHCB and HHCB-lactone in the extracts were measured using an Agilent 6890 N GC interfaced to a 5975B MSD (GC-MS), equipped with a DB-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific Co., USA), in selected ion monitoring (SIM) mode under electron-impact ionization (EI). Helium (purity > 99.999%) was used as carrier gas at a constant flow of 1.0 mL/min. Splitless mode was used for injection, with injection volume of 2 μL for each samples. The temperatures for the GC-MS interface, ion source, quadrupole and injector were 280 ˚C, 250 ˚C, 150 ˚C and 280 ˚C, respectively. The GC oven temperature was programmed as follows:

80 ˚C for 0 min, increased to 170 ˚C at 15 ˚C/min, from 170 ˚C to 185 ˚C at 1 ˚C /min, then to 300 ˚C at a rate of 20 ˚C /min for 5 min.

GC/Q-TOF for HHCB biotransformation qualification: HHCB biotransformation products qualification was analysed using an Agilent 7890B GC interfaced to a 7250 QTOF, equipped with a HP-5MS UI column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific Co., USA) under electron-impact ionization (EI). Helium (purity > 99.999%) was used as carrier gas at a constant flow of 1.0 mL/min. Splitless mode was applied for injection, with injection volume of 2 μL for each samples. The temperatures for the GC/Q-TOF interface, ion source, quadrupole and injector were 300 ˚C, 200 ˚C, 150 ˚C and 280 ˚C respectively. The GC oven temperature was programmed as follows: 80 ˚C for 0 min, increased to 170 ˚C at 15 ˚C/min, from 170 ˚C to 185 ˚C at 1 ˚C /min, then to 300 ˚C at a rate of 20 ˚C /min for 5 min. The samples were scanned using full scan TOF mode at a range of 50-550 m/z with scan time of 0.20 s.

Table S1 Method quantification limits of target compounds in surface water, sediment, particles and worms by HPLC-MS/MS or GC-MS.

Compound	Surface water (ng/L)	Sediment (ng/g)	Particles (ng/g)	Worm (ng/g)
Triclosan	0.08	0.10	0.14	0.16
Methyl triclosan	0.94	1.17	1.28	1.55
Galaxolide	1.01	1.36	1.41	1.78
Galaxolidone	1.40	1.72	2.05	2.36

Table S2 Recoveries of target compounds in surface water (µg/L), sediment (µg/g dw), particles (µg/g dw) and worm (µg/g ww).

Compounds	Spiked concentrations in water			Spiked concentrations in sediment			Spiked concentrations in particles			Spiked concentrations in worm		
	0.1	0.5	2	0.1	0.5	2	0.1	0.5	2	0.1	0.5	2
Triclosan	105 ± 5.31	98.1 ± 4.84	94.1 ± 4.66	102 ± 4.15	95.3 ± 5.16	91.9 ± 2.28	102 ± 4.17	97.5 ± 4.82	92.5 ± 3.94	102 ± 5.17	105 ± 4.36	95.7 ± 3.09
Methyl triclosan	97.0 ± 4.19	93.9 ± 4.07	85.9 ± 5.65	101 ± 5.14	90.6± 5.38	82.5 ± 3.16	103 ± 5.43	89.4 ± 5.39	80.7 ± 4.62	105± 4.31	100 ± 4.75	97.9 ± 4.82
Galaxolide	90.4 ± 4.97	82.4 ± 4.11	75.8 ± 3.96	79.6 ± 4.36	70.6 ± 4.47	62.7 ± 2.43	81.3 ± 4.37	74.2 ± 4.19	64.9 ± 4.42	99.3 ± 4.97	103 ± 3.76	106 ± 4.24
Galaxolidone	92.3 ± 5.12	85.7 ± 4.65	77.6 ± 3.77	82.3 ± 4.18	75.4 ± 3.53	68.1 ± 3.16	83.0 ± 5.13	77.9 ± 5.77	70.5 ± 3.46	105 ± 4.83	98.5 ± 3.77	94.7 ± 4.98

Three replicates were used to determine recovery.

Table S3 TCS, Me-TCS, HHCB and HHCB-lactone concentrations in sediment (μg/g dw) and worm tissue (μg/g ww) during the exposure period.

Exposure time (d)	Sediment								Worm				BSAFs	
	TCS		Me-TCS		HHCB		HHCB-lactone		TCS	Me-TCS	HHCB	HHCB-lactone	TCS	HHCB
	with worms	without worms	with worms	without worms	with worms	without worms	with worms	without worms						
0	3.10±0.04	3.10±0.04	< MQL	< MQL	3.10±0.02	3.10±0.02	0.34±0.01	0.34±0.01	< MQL	< MQL	< MQL	< MQL	-	-
1	3.07±0.03	3.09±0.04	0.0020±0.0003	0.0019±0.0003	3.05±0.01	3.09±0.01	0.34±0.01	0.34±0.01	2.43±0.57	0.06±0.01	6.47±0.58	0.70±0.08	0.70±0.17	1.84±0.16
3	3.01±0.03	3.08±0.02	0.0019±0.0003	0.0020±0.0002	3.03±0.02	3.08±0.01	0.33±0.01	0.34±0.01	6.51±0.12	0.09±0.01	8.40±1.08	0.76±0.04	1.90±0.04	2.38±0.31
7	2.94±0.03	3.06±0.01	0.0038±0.0003	0.0021±0.0003	3.02±0.03	3.08±0.01	0.33±0.01	0.34±0.01	6.52±0.13	0.12±0.02	8.81±0.44	0.81±0.03	1.95±0.04	2.50±0.12
10	2.89±0.03	3.03±0.01	0.006±0.0004	0.0029±0.0002	3.02±0.02	3.08±0.01	0.33±0.01	0.34±0.01	6.65±0.52	0.15±0.02	8.84±0.52	0.77±0.05	2.02±0.16	2.50±0.15
14	2.82±0.04	3.00±0.01	0.0078±0.0002	0.0050±0.0003	3.02±0.01	3.08±0.01	0.33±0.01	0.34±0.01	6.63±0.15	0.15±0.01	8.72±0.53	0.80±0.07	2.07±0.04	2.47±0.15

MQL means method limit of quantitation.

with and without worms represent microcosms with and without worm, respectively.

BSAFs: biota-sediment accumulation factor.

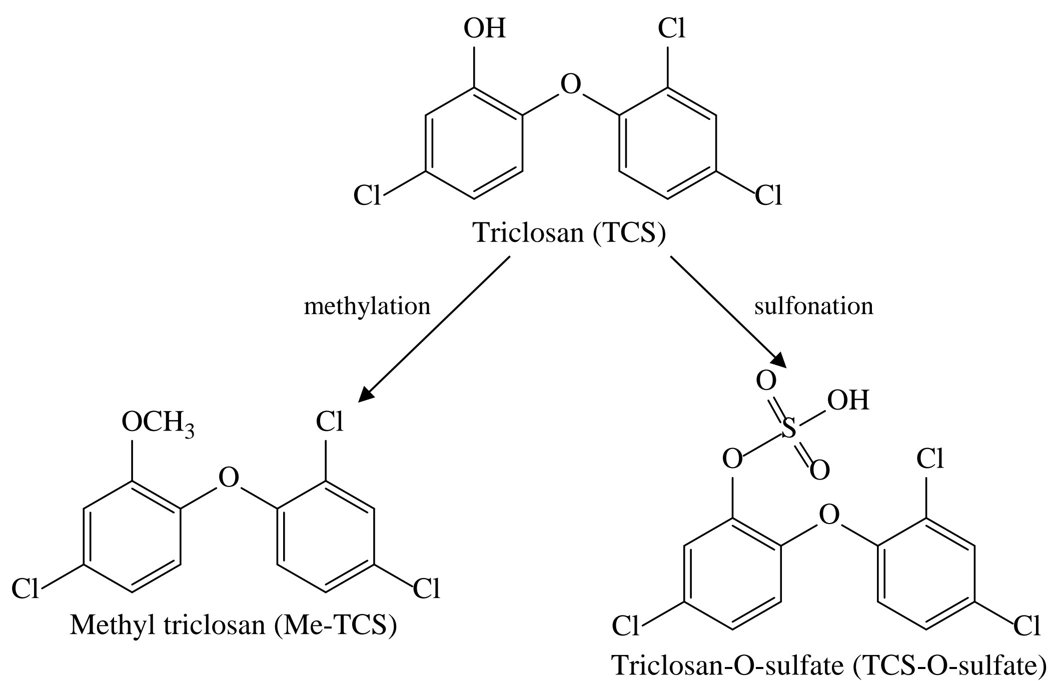

Figure S1 Predicted biotransformation pathways of TCS in *Limnodrilus hoffmeisteri*.

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