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Fate and effects of sediment-associated polycyclic musk HHCB in subtropical freshwater microcosms

Feng-Jiao Peng[a,*], Fionne Kiggen[a], Chang-Gui Pan[b], Sally A. Bracewell[a], Guang-Guo Ying[c], Daniel Salvito[d], Henriette Selck[e], Paul J. Van den Brink[a,f]

[a] Aquatic Ecology and Water Quality Management Group, Wageningen University, P.O. Box 47, 6700 AA Wageningen, the Netherlands
[b] School of Marine Sciences, Guangxi University, Nanning 530004, China
[c] The Environmental Research Institute, MOE Key Laboratory of Environmental Theoretical Chemistry, South China Normal University, Guangzhou 510006, China
[d] Research Institute for Fragrance Materials, 50 Tice Boulevard, Woodcliff Lake, NJ 07677, USA
[e] Department of Environmental, Social and Spatial Change, Roskilde University, Universitetsvej 1, Denmark
[f] Wageningen Environmental Research, P.O. Box 47, 6700 AA Wageningen, the Netherlands

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ABSTRACT

Galaxolide (HHCB) is used as a fragrance ingredient in household and personal care products, and has been ubiquitously detected in the environment. Here we investigated the fate of HHCB in subtropical freshwater microcosms, and evaluated effects of sediment-associated HHCB on a biological community consisting of algae, Daphnia, benthic macroinvertebrates and bacteria. The concentrations of sediment-associated HHCB did not change significantly during a 28 days exposure period, but HHCB accumulated in worms with biota-sediment accumulation-factor (BSAF) values in the range of 0.29–0.66 for *Branchiura sowerbyi* and 0.94–2.11 for *Limnodrilus hoffmeisteri*. There was no significant effects of HHCB (30 μg/g dry weight (dw) sediment) on chlorophyll-a content, sediment bacterial community composition, and survival and growth of benthic macroinvertebrates. However, the presence of benthic macroinvertebrates altered the sediment bacterial community structure relative to microcosms without introduced organisms. The findings of this study suggest that a single high-dose of HHCB, over 28 days, at environmentally relevant concentrations would not impose direct toxicological risks to aquatic organisms such as benthic macroinvertebrates.

1. Introduction

Polycyclic musks (PCMs) are widely used as fragrances in household and personal care products, such as detergents, perfumes, body lotions and cosmetics (Reiner and Kannan, 2006). Due to their high lipophilicity, PCMs that end up in the waterways can bio-accumulate in aquatic organisms, such as fish and mussels, especially at low trophic levels (Reiner and Kannan, 2011; Díaz-Cruz and Barceló, 2015). Given the current high usage, there are concerns regarding environmental exposure and toxic effects of PCMs (Sun et al., 2014).

Galaxolide (HHCB; 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hex-amethylcyclopenta (g)-2-benzopyran) is a High Production Volume (HPV) chemical substance and one of the most widely used PCMs in a range of consumer and commercial products including perfumes, cosmetics, shampoos, lotions, detergents, fabric softeners, and household cleaners (USEPA, 2014). Due to its widespread use and low rates of degradation, HHCB has been detected throughout the environment (e.g., air, water and sediment) (Osenbrück et al., 2007; McDonough et al., 2016; Peng et al., 2017). For example, HHCB concentrations in the surface water and sediment were in the range of ng/L–low μg/L and ng/g dry weight (dw)–low μg/g dw, respectively (Table S1). In standard laboratory tests, HHCB generally has low acute toxicity to aquatic organisms (e.g. algae, crustaceans, and fish) (Table S2; Breitholtz et al., 2003; Gooding et al., 2006). However, with a log Kow value of 5.9 and log Koc value of 4.86 (Balk and Ford, 1999a), HHCB will bind strongly to suspended and benthic sediment (USEPA, 2014). Indeed, it has been reported that HHCB is only partially biodegraded in the wastewater treatment plants (WWTPs), with removal occurring mainly via sorption onto sludge particles (Federle et al., 2014). Therefore, sediment-associated HHCB may be available for accumulation and subsequent biotransformation in deposit-feeding worms, which might affect the fate of HHCB. For example, HHCB can accumulate in oligochaete worm *Limnodrilus hoffmeisteri* and the presence of worms significantly accelerated HHCB dissipation in a 14-d test (Peng et al., 2018a). Despite the crucial

* Corresponding author.
E-mail address: fengjiaopeng@gmail.com (F.-J. Peng).

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importance in evaluating the environmental risk of sediment-associated HHCB, little is known about the interaction between deposit-feeding worms and HHCB under more ecologically realistic conditions compared to single species studies.

Oligochaete worms, such as *L. hoffmeisteri* and *Branchiura sowerbyi*, are burrowing deposit feeders that are broadly distributed in the sediments of freshwater bodies, such as lakes and rivers (Thorp and Covich, 2009). They ingest large amounts of sediment particles (Wang and Matisoff, 1997), and are therefore likely to be exposed to sediment-associated organic contaminants given their capacity to bioaccumulate such compounds (e.g., Peng et al., 2018a, 2018b). Because oligochaete worms play a key role in nutrient cycling and form important links in detritus food chains (Wang and Matisoff, 1997), understanding the effects of sediment-associated organic chemicals on these organisms is a priority. The effects of sediment-associated HHCB on individual benthic organisms have been well determined in laboratory toxicity experiments, such as snails and worms (Ramskov et al., 2009; USEPA, 2014). For example, HHCB at concentrations of 26 μg/g dry weight (dw) significantly reduced the number of eggs produced per polychaete worm (*Capitella* sp. I) and increased the time between broods, but these individual-level effects were not translated to population growth rate, which was unaffected by HHCB up to 168 μg/g dry weight (Ramskov et al., 2009). Nevertheless, these experiments are often restricted to single species responses. We still do not know how HHCB impacts multi-species communities (e.g., growth and survival) in a setting that mimics more natural conditions (e.g., including water, sediment, and a multispecies community).

Microorganisms, such as bacteria, are also a major component of natural sediments and aquatic ecosystems, playing a key role in the processing of organic matter and nutrient cycling (Grünheid et al., 2005). Contaminated sediments are known to affect bacterial community composition and structure (Staley et al., 2015). For example, municipal wastewater discharging to streams resulted in an increase in the relative abundance of genes associated with restriction-modification systems, nitrogen, sulphur, purine and pyrimidine metabolisms (Li et al., 2016). However, we do not know the impact of sediment-associated HHCB on bacterial communities.

To understand the interaction between sediment-associated HHCB and benthic macroinvertebrates under more environmentally realistic conditions, we spiked HHCB into the sediment and introduced a model freshwater community (algae, *Daphnia*, clams, snails, midges, and benthic worms) that was representative of communities found in urban rivers of Guangzhou (subtropical South China) to half of microcosms. We chose the subtropical region based on the results of our chemical monitoring which showed that HHCB was one of the dominant chemicals used in personal care products in both water and sediment in six urban rivers in Guangzhou (South China) (Peng et al., 2017). Because of the importance of microbes for degradation of organic contaminants, we also evaluated the effects of sediment-associated HHCB on bacterial community in the sediment. The objective of this study was to (i) investigate the fate of HHCB in the microcosms with and without introduced organisms, (ii) evaluate the effect of HHCB on a model freshwater community, and to (iii) assess the effect of HHCB and the presence of macroinvertebrates on sediment bacterial community.

2. Material and methods

2.1. Test sediment and HHCB spiking

The test chemical was galaxolide (HHCB; 1,3,4,6,7,8-hexahydro-4,6,6, 7, 8, 8-hex- amethylcyclopenta (g)-2-benzopyran). Further details on standards and reagents are provided in Supporting information Text S1. Sediments used in the microcosms were collected from an uncontaminated reservoir (113°47'42"N, 23°46'01"E, Guangzhou, South China) in January 2016 (Peng et al., 2017). Sediment collection and pre-treatment followed the same procedures as our previous study (Peng et al., 2018b). The sieved sediment contained 60% water (24 h at 105 °C; n = 4), 20.6‰ organic matter (OM), 0.11‰ ammonia nitrogen (NH_4^+), 1.45‰ total nitrogen (TN), and 0.45‰ total phosphorus (TP). It was composed primarily of clay (58.7%), silt (40.8%), and a small amount of sand (0.49%). The background HHCB concentration in the sediment was around 0.002 μg/g dw sed, which was negligible relative to spiked concentrations.

Sediment spiking followed procedures described in Peng et al. (2018b). Briefly, fresh sediment was wet sieved (300 μm), homogenized, and spiked with HHCB to obtain actual target concentrations of 30, 100, 200 and 300 μg/g dw sed using acetone as carrier (2.2‰). Because HHCB has been proved to have low sediment toxicity to sediment-dwelling organisms in the single species test (Ramskov et al., 2009; USEPA, 2014), here we used environmentally elevated concentrations. After spiking, sediments were further pre-equilibrated for 3 days by manually mixing using a spade. Two controls were used in the study: a water control and an acetone control, that were created by replacing the HHCB solution with the same volume of Mill-Q water and acetone, respectively.

2.2. Microcosm operation

Experimental exposures were identical to those reported by Peng et al. (2018b). Briefly, we used 40 microcosms that consisted of glass aquaria (length and width 30 cm; depth 20 cm) and were placed in a climate controlled room (27 ± 1 °C; white cool fluorescent light with a light intensity of approximately 2200 lx; photoperiod 12 h) for the duration of the experiment, to test the effects of HHCB on subtropical shallow freshwater communities. Of these 40 microcosms, 28 were used to test the effects of different concentrations of HHCB (30, 100, 200 and 300 μg/g dw sed). Because one microcosm per HHCB treatment was sacrificed at the start of exposure to measure HHCB concentrations in the overlying water and sediment, there were six replicates per HHCB treatment (Fig. S1). The remaining 12 microcosms were used as controls (six for the water control and six for the acetone control.

Two different test systems were used for each treatment and control: A and B. Each microcosm was first filled with 4-cm spiked sediment and then 14-cm aerated tap water, and allowed to settle for 3 days. The aerated tap water had the following properties: 0.032% NH_4^+, 0.175% nitrate (NO_3^-), 0.0002% nitrite (NO_2^-), 0.002% TP, 0.067% total organic carbon (TOC). At the beginning of the exposure (day 0), 12 *Daphnia magna*, 6 *Corbicula fluminea*, 6 *Viviparidae bellamya*, 50 *Orthocladiinae*, 240 *Limnodrilus hoffmeisteri*, 30 *Branchiura sowerbyi*, and algae (i.e., *Chlorella pyrenoidosa*, *Scenedesmus obliquus*, *Scenedesmus quadricauda*, *Scenedesmus acuminatus, Selenastrum capricornutum* and *Chlamydomonas reinhardtii*; approximately 10^4 cells/mL in the system) were added to the microcosms of system A, to mimic shallow subtropical freshwater systems. Benthic organisms used here were key benthic macroinvertebrates identified from the biological monitoring of rivers in Guangzhou city (South China) in 2015. Pelagic species, algae and *Daphnia*, cultured in the laboratory were used to create a more realistic system. Clams were collected from a small uncontaminated headwater stream in Huizhou, Guangdong province (South China). The remaining benthic organisms were purchased from an aquatic market in Guangzhou (South China) as insufficient numbers could be collected from the field. Benthic macroinvertebrates were, therefore, of an unknown age. Their culturing followed methods described in Peng et al. (2018b). System B was identical to A, but did not contain any introduced organisms. As periphyton can develop in the microcosms (Rico et al., 2014; Peng et al., 2018b), the effect of sediment-associated HHCB on periphyton communities was also examined. In each microcosm, five microscopic glass slides (7.5 × 2.5 cm) were hung at a depth of 10 cm using nylon sewing thread and left for the duration of exposure (28 days). During the experiment, the microcosms were aerated using a glass pipette connected to an aeration system and any evaporated water was replenished with aerated tap water at weekly intervals. Nitrogen

(0.7 mg/L as urea) and phosphorus (0.09 mg/L as triple super phosphate) were added biweekly to the systems to provide nutrients for algal growth (Rico et al., 2014). No food was added to microcosms, as all introduced invertebrates can feed on algae, microorganisms, and/or sediment (Table S3). The initial body wet weight of the midge larvae (4.5–6.2 mg) and worms (1.9–2.4 mg for *L. hoffmeisteri* and 16.8–18.5 mg for *B. sowerbyi*) were estimated using 100 randomly chosen individuals. The total lipids in *L. hoffmeisteri* (1.62 ± 0.22% wet weight) and *B. sowerbyi* (9.16 ± 0.35% wet weight) were also measured following previously reported method (Bligh and Dyer, 1959). Furthermore, 30 individuals of each species were weighed to estimate the initial wet weight of clams (326–471 mg) and snails (598–766 mg) (Table S3). Other species traits, such as feeding habits and food preferences, are also available in Table S3.

To determine the abiotic degradation (i.e., volatilization, photolysis and hydrolysis) during the exposure period, we performed two parallel fate experiments under similar conditions as the microcosm experiment. The first experiment was performed in six 1-L Erlenmeyer flasks with 0.5-L de-chlorinated tap water containing 1.2 mg/L HHCB to mimic abiotic degradation in low-turbidity water (the worst scenario). The second experiment was performed in six 0.5-L Erlenmeyer flasks containing 0.25-L overlying water collected from the sacrificed microcosm with HHCB at concentration of 30 μg/g dw sed to mimic abiotic degradation in high-turbidity water. Flasks were divided into 2 groups in each experiment: with and without covering in aluminium foil. As such, potential HHCB hydrolysis and volatilization were determined in flasks covered in aluminium foil, whereas potential HHCB photolysis was determined in those without by comparison with those covered in aluminium foil. The specific procedure of these tests is provided in Text S2.

2.3. Sampling, community, water and sediment quality parameter analysis

To detect whether the overlying water in microcosms maintained a good water quality or not, dissolved oxygen (DO), temperature (T), pH and conductivity were measured on days 0, 1, 3, 7, 10, 14, 21 and 28 in all microcosms. Turbidity was measured on days 1, 7, 14, 21 and 28. At experimental end (day 28), water, sediment and community sampling and analysis followed methods described in Peng et al. (2018b). *D. magna* and benthic macroinvertebrates were recruited from each microcosm of system A. Phytoplankton and periphyton chlorophyll-a concentration were analysed in both system A and B microcosms. *D. magna* and phytoplankton were sampled through collecting overlying water using a Teflon siphon, whereas benthic macroinvertebrates were sampled through gently sieving sediment. Additionally, periphyton were sampled via scraping introduced microscopic glass slides (in 0.5-L de-chlorinated tap water) (Rico et al., 2014). See our previous study for more details on community sampling and analysis (Peng et al., 2018b).

2.4. HHCB analysis

We determined HHCB concentrations in the overlying water, sediment and both species (*L. hoffmeisteri* and *B. sowerbyi*) of worms at the beginning (day 0) and end of the exposure (day 28). We also determined HHCB concentrations in the spiked sediment and original aerated tap water. However, we did not measure HHCB concentrations in snails or clams as they are filter-feeder while we spiked sediment in the current study. Analytical methods used for the analysis of HHCB in water, sediment and worm tissue have been previously described (Chen et al., 2010, 2014; Yao et al., 2016). Briefly, HHCB in water samples were extracted by solid phase extraction using a HLB cartridges (6 mL, 200 mg) (Chen et al., 2010); HHCB in sediment samples were extracted using an accelerated solvent extraction system (ASE) (Dionex 350, Dionex Corporation) (Chen et al., 2014); and HHCB in worm samples were extracted using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method (Yao et al., 2016). HHCB in extracts was analysed using a gas chromatograph (Agilent 6890) coupled to a mass spectrometer (Agilent 5975B) with an electron impact ionization source (EI) according to Chen et al. (2014). Details on extraction procedures and instrumental analysis are provided in the Supporting information (Text S3 and S4). The limits of detection (LODs), limits of quantification (LOQ) and method recoveries of HHCB are listed in Table S4.

2.5. Bacterial analysis

The effect of HHCB and benthic macroinvertebrates on the sediment bacterial community structure and composition was evaluated using deep16S rDNA sequencing. Because there is no information available on the effects of sediment-associated HHCB on bacterial communities, bacterial analysis was performed only on water controls, acetone controls, and the highest HHCB treatment (i.e., 300 μg/g dw sed), to ensure a detectable HHCB effect. Procedures for DNA extraction and amplicon sequencing are detailed in the Supporting information (Text S5). Briefly, DNA was isolated from sediment samples using PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. The bacterial 16S rDNA genes were amplified at V4 and V5 regions with the primers 515F (5′-GTGCCAGCMGCCGC GGTAA-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′). Sequencing libraries were constructed using TruSeq® DNA PCR-Free Sample Preparation Kit according to manufacturer's recommendations and added with index codes and sequenced using an Illumina HiSeq. 2500.

2.6. Data analysis

Methods used for Biota-Sediment-Accumulation-Factor (BSAF) calculation, derivation of No-Observed-Effect-Concentrations (NOECs) of HHCB on benthic macroinvertebrates, and testing significance of differences in HHCB concentrations, HHCB dissipation, water and sediment quality parameters, and chlorophyll-a concentrations between systems or treatments have been described in Peng et al. (2018b). While Williams test (ANOVA, Williams, 1972) was used to derive NOECs with the Community Analysis computer program (Hommen et al., 1994), two-way ANOVA (factors: HHCB and presence of benthic macroinvertebrates), one-way ANOVA, Kruskal-Wallis test, paired *t*-test or Wilcoxon matched-pairs test was used to test the significance of the above mentioned differences using SPSS version 23.0 (IBM, NY). When a significant main effect was detected by the ANOVA, post hoc comparisons were performed with Tukey's test. 5% significance level was defined for all of the statistical tests.

For the bacterial analysis, QIIME V1.7.0 and R software (Version 2.15.3) were used to analyse Shannon index, Chao1 index, and observed number of species. The relative abundances of the bacterial 16S rDNA gene at the phylum level were examined to determine the bacterial community composition. Differences in alpha diversity indices and relative abundance of the ten most abundant phyla between system A and B microcosms or among groups were tested using two-way ANOVA (factors: HHCB treatment and the presence of benthic macroinvertebrates) or one-way ANOVA (factor: treatment).

To test the significance of the effect of benthic macroinvertebrates on the bacterial community structure, Monte Carlo permutation test under redundancy analysis (RDA) was performed on the OTU table using microcosm systems as explanatory variables and groups as covariates and constraining the permutation to the covariates. If significant, both systems were further analysed separately. For each system, a Monte Carlo permutation test was performed to test the significance of the effect of sediment-associated HHCB on the bacterial community structure. If significant, a further Monte Carlo permutation test was performed via placing the water control and acetone control on the one side and the 300 μg/g dw sed treatment on the other side. Also, when differences were significant, a PCA was performed with groups as passive variables to show the placement of the groups and their samples with respect to the OTU's. Unfortunately, due to the death of sediment-

dwelling worms in the highest HHCB treatment, we cannot test whether there is an direct interactive effect of sediment-associated HHCB and the presence of benthic macroinvertebrates on the sediment bacterial community structure. The analyses were performed using the CANOCO Software package, version 5 (Ter Braak and Smilauer, 2012).

3. Results

3.1. Water and sediment quality parameters

Dissolved oxygen (DO) concentration remained stable in the overlying water (~ 7 mg/L) in both systems during the experiment. pH levels were more stable in system B (pH: 6.7–7) without introduced organisms than in system A (decreased from ~ 7 to ~ 6) (Table S5). Conductivity remained at similar levels (90–110 µS/cm) in all microcosms during the 28 days exposure period (Table S6). Turbidity decreased continuously from day 0 (150–180 NTU) to 28 (< 10 NTU) in system B (Table S7), and increased between day 7 (50–60 NTU) and day 14 (110–140 NTU) in all HHCB treatments in system A (Table S7).

After 28 days, the overlying water from the microcosms was compared with the original aerated tap water to determine changes in nutrient concentrations (Table S8). By the end of the experiment, in both system A and B microcosms TOC concentrations increased (from 0.67 mg/L to 4.71–5.24 mg/L in system A or 3.51–4.67 mg/L in system B) while NO_3^- concentrations decreased (from 1.75 mg/L to 1.17–1.23 mg/L in system A or 0.44–0.77 mg/L in system B). In addition, NH_4^+ concentrations decreased in system B microcosms (from 0.32 mg/L to 0.17–0.25 mg/L) after 28 days of exposure. Comparing systems A and B, while significantly higher NH_4^+ and TOC concentrations were found in system A (two-way ANOVA, $p < 0.05$), significantly lower NO_3^- concentrations were found in system A (Wilcoxon matched-pairs test, $p < 0.05$). Comparing treatments and controls, in system B NO_3^- concentrations were significantly lower in all HHCB treatments relative to controls (one-way ANOVA, $p < 0.05$). In both system A and B, TOC concentrations were significantly higher in all HHCB treatments than controls, except for the lowest treatment (two-way ANOVA, $p < 0.05$).

After 28 days, there was no significant difference in any nutrients concentrations between the sediment from the microcosms and the original sediment (Table S9). Comparing systems A (~ 0.06 g/kg dw) and B (~ 0.04–0.06 g/kg dw), NH_4^+ showed significantly higher levels in system A (paired *t*-test, $p < 0.05$). Comparing treatments and controls, in system B NH_4^+ concentrations were significantly lower in the two highest treatments (~ 0.04–0.05 g/kg dw) compared to controls and lower treatments (~ 0.06 g/kg dw) (one-way ANOVA, $p < 0.05$) (Table S9).

3.2. HHCB concentrations in water, sediment and worms

The recoveries of extraction methods were 100–110% and the method limit of quantitation was below 0.002 µg/L or µg/g for overlying water, sediment and worms (Table S4). At the end of the experiment, HHCB concentrations in the overlying water were significantly higher in system A (1.36 ± 0.16–4.73 ± 0.25 µg/L) compared to system B (0.74 ± 0.09–4.01 ± 0.15 µg/L) (two-way ANOVA, $p < 0.05$) (Fig. 1a and Table S10). In both systems A and B, HHCB concentrations in the overlying water of the two highest HHCB treatments (200 µg/g dw and 300 µg/g dw) dropped dramatically over the 28 days of exposure from over a hundred µg/L to only few µg/L (Fig. 1a and b).

The sediment HHCB concentrations did not decrease significantly over time (Table S10; Fig. 2). Mass balance was assessed for HHCB by comparing the HHCB mass in the system on day 28 with the original added HHCB mass (Table S11 and Fig. 3). After 28 days of exposure, the overall amount of HHCB decreased by 0.36–1.54% in system A and 0.36–1.49% in system B (Table S11). However, there was no significant

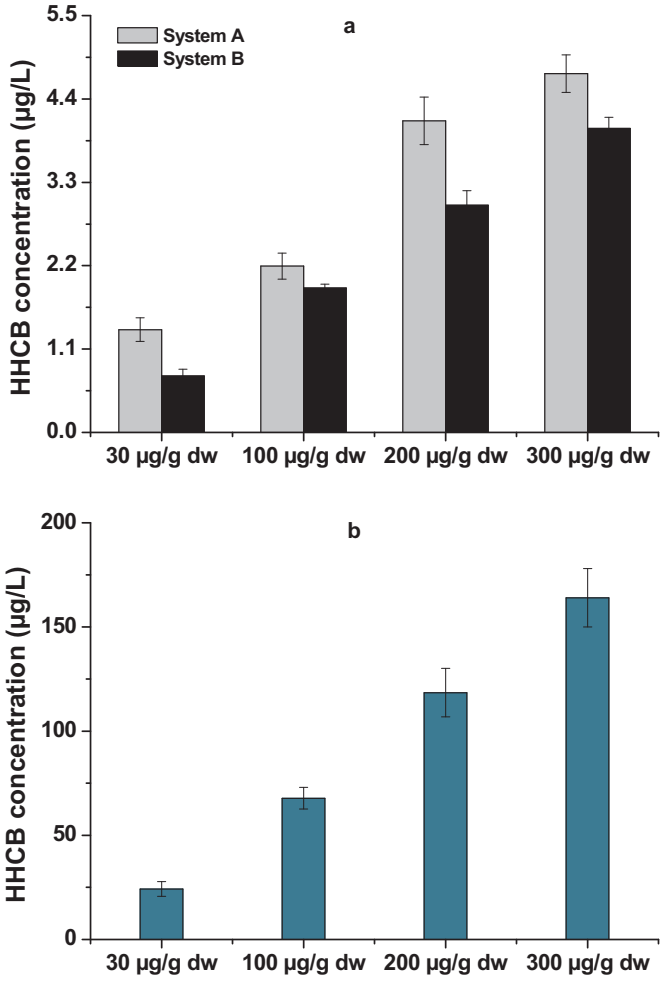

Fig. 1. HHCB concentrations in the overlying water (µg/L) of the microcosms at the end (a; day 28) and start (b; day 0) of exposure. Error bars represent standard errors of means (n = 3). In graph a, grey and black bars represent treatments from systems with (System A) and without (System B) introduced organisms, respectively.

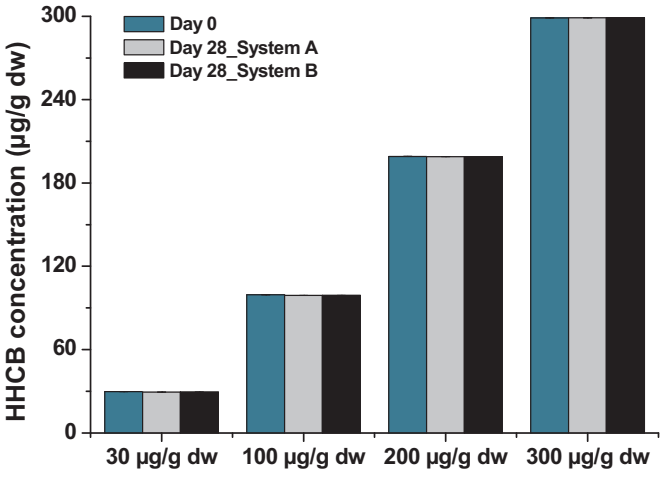

Fig. 2. HHCB concentrations in the sediment (µg/g dw) of the microcosms at the start (day 0) and end (day 28) of exposure. Error bars represent standard errors of means (n = 3). While green bars represent treatments on day 0, grey and black bars represent treatments on day 28 from systems with (System A) and without (System B) introduced organisms, respectively.

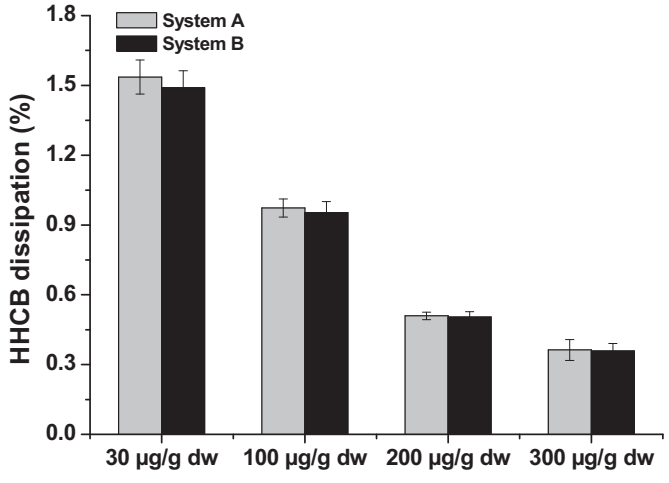

Fig. 3. The HHCB dissipation (%) in the microcosms after 28 days of exposure. Error bars represent standard errors of means (n = 3). Grey and black bars represent treatments from systems with (System A) and without (System B) introduced organisms, respectively.

difference in HHCB mass between values on day 28 and on day 0 or between system A and B on day 28 (Fig. 3). Among the four HHCB treatments, the rates of HHCB dissipation in the microcosms decreased with increasing concentrations of HHCB (two-way ANOVA, $p < 0.05$).

HHCB was detected in the tissue of both worms tested, *B. sowerbyi* and *L. hoffmeisteri*. In *B. sowerbyi*, HHCB concentrations were 98.2, 241, 350 and 431 μg/g ww in treatments of 30, 100, 200 and 300 μg/g dw sed, respectively. The corresponding HHCB concentrations in *L. hoffmeisteri* were 56.4, 135, 217 and 279 μg/g ww, respectively (Fig. 4 and Table S10). In contrast to HHCB concentrations in worm tissues, BSAF values decreased with increasing spiked HHCB concentrations, with values of 0.29–0.66 and 0.94–2.11 for *B. sowerbyi* and *L. hoffmeisteri*, respectively (Table S10). For the same treatment, *B. sowerbyi* showed higher HHCB body residues (two-way ANOVA, $p < 0.05$) but smaller BSAF values (two-way ANOVA, $p < 0.05$) relative to *L. hoffmeisteri* (Table S10). In contrast, *B. sowerbyi* had larger body weight and lipid content than *L. hoffmeisteri* (Table S3).

The results of abiotic degradation experiments showed that there was a significant decrease in HHCB concentrations from day 14 onwards under both light and dark conditions in the aerated tap water

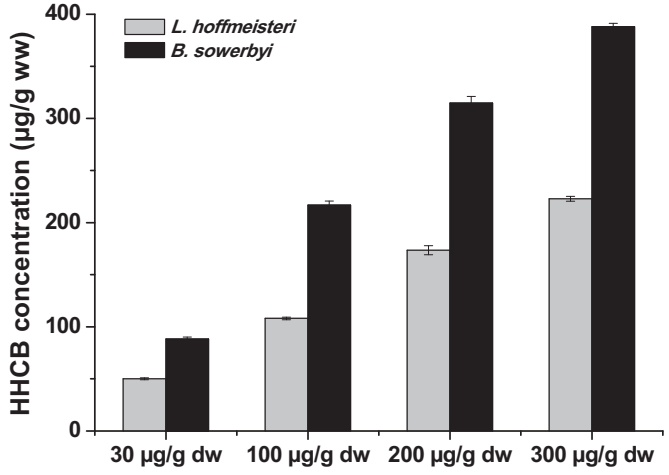

Fig. 4. HHCB concentrations (μg/g ww) in the *L. hoffmeisteri* and *B. sowerbyi* tissue after 28 days of exposure to different concentrations of HHCB spiked sediment. Error bars represent standard errors of means (n = 3). Grey and black bars represent treatments corresponding to *L. hoffmeisteri* and *B. sowerbyi*, respectively.

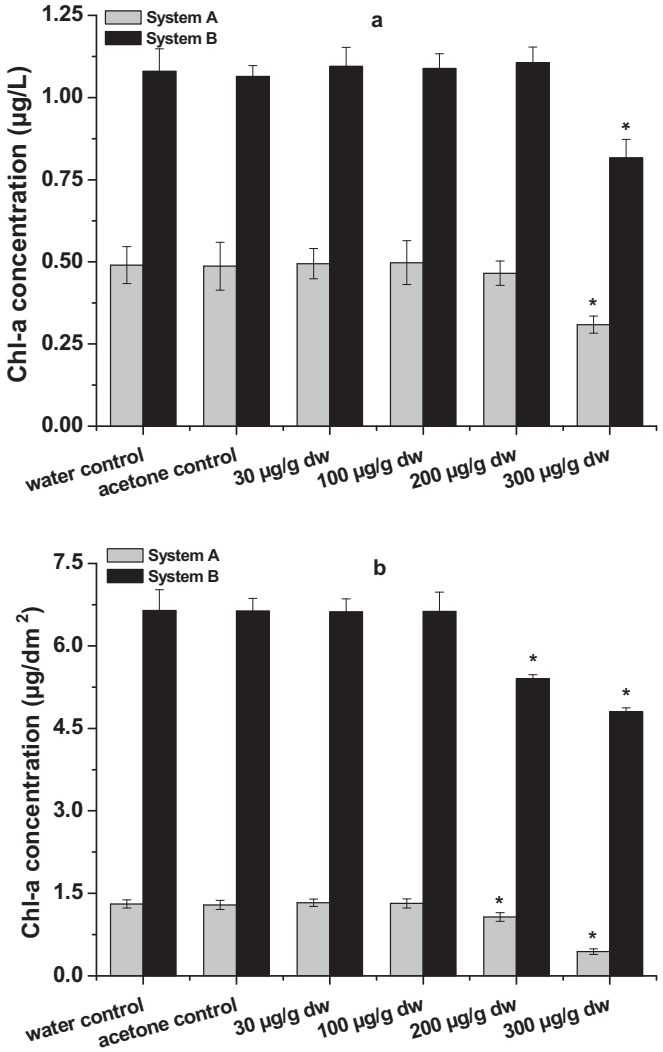

Fig. 5. Chlorophyll a content in the overlying water (a) and on the glass slides (b) at the end of exposure (day 28). Error bars represent standard errors of means (n = 3). Grey and black bars represent treatments from systems with (System A) and without (System B) introduced organisms, respectively.

(Kruskal-Wallis test, $p < 0.05$). HHCB decreased from 1.2 mg/L on day 0 to 1.10 mg/L (with light) and 1.15 mg/L (without light) on day 28 in the aerated tap water (Table S12). Also, there was a significant difference in HHCB concentrations between the light and dark conditions in the aerated tap water (Wilcoxon matched-pairs test, $p < 0.05$). Although HHCB also showed a slight decrease in the overlying water during the 28 days period (from 24.2 μg/L to ~ 23.7 μg/L), there was no significant difference in HHCB concentration between day 0 and day 28 or the light and dark conditions (Table S12).

3.3. Phytoplankton and periphyton chlorophyll-a

Chlorophyll-a levels in the overlying water were significantly higher in system B (0.82–1.10 μg/L) than A (0.31–0.50 μg/L) (two-way ANOVA, $p < 0.05$) (Table S13 and Fig. 5a). In both systems A and B, microcosms with the highest sediment HHCB concentration had significantly lower chlorophyll-a concentrations compared to other treatments and controls (two-way ANOVA, $p < 0.05$) (Table S13). Similarly, chlorophyll-a levels of periphyton measured on the glass slides introduced to the microcosms were also higher in system B (4.99–6.64 μg/dm^2) than A (0.44–1.30 μg/dm^2) (two-way ANOVA, $p < 0.05$) and were significantly lower at the two highest HHCB

treatment in both systems (two-way ANOVA, $p < 0.05$) (Table S13 and Fig. 5b).

3.4. Daphnia and benthic macroinvertebrates

By the end of experiment, there were no *Daphnia* or midge individuals in any microcosms of system A. Emergence of midge larvae was observed during the exposure period. However, as here we used open systems, midge emergence could not be recorded. The survival rates for snails and clams were close to 100% in all treatments and controls (Table S14). There was no evidence of reproductive activity, such as clam or snail larvae, during the experiment. At low concentrations of HHCB (30 μg/g), there was no observable effect of HHCB on the survival of *B. sowerbyi* and *L. hoffmeisteri* (Table S14). However, at higher concentrations, HHCB significantly reduced the survival of *B. sowerbyi* (at 100 μg/g dw) by 15.5% and *L. hoffmeisteri* (at 200 μg/g dw) by 21%. The NOECs based on the survival were 30 μg/g dw for *B. sowerbyi* and 100 μg/g dw for *L. hoffmeisteri* (Table S15). Similar to snails and clams, both worms showed no reproductive activity during the exposure period. There were no significant differences in the body wet weight of snails, clams and worms between treatments and controls or between the beginning and end of the experiment.

3.5. Bacterial community

The richness, evaluated using OTUs (4237–4301) and Chao 1 parameter (5753–5956), was similar between system A and B (Table S16). Likewise, there was no significant difference in mean Shannon indices between system A (10.25–10.29) and B (10.19–10.23) (Table S16). Moreover, there was no significant differences in the values of these indices between controls and the highest HHCB treatment.

A total of 61 phyla were observed in all sediment samples, and the top ten dominant phyla are presented in Table S17 and Fig. 6. Among all the identified phyla, *Proteobacteria* (26–29%), *Actinobacteria* (16–20%), *Chloroflexi* (11–16%), *Firmicutes* (9–15%), and *Acidobacteria* (5–6%) were the five most dominant bacterial phyla in both controls and the HHCB treatment (Table S17 and Fig. 6). Among the top ten phyla, system A had a higher relative abundance of *Chloroflexi* (two-way ANOVA, $p < 0.05$) and *Nitrospirae* (two-way ANOVA, $p < 0.05$), whereas system B had a higher relative abundance of *Firmicutes* (two-way ANOVA, $p < 0.05$). There was no significant difference in the abundances of the top ten phyla between HHCB treatment and controls, except that the abundance of *Nitrospirae* was significantly higher in the water controls of system A compared to acetone control and HHCB treatment (one-way ANOVA, $p < 0.05$).

The result of the Monte Carlo permutation test indicates that the bacterial community structure was significantly different between system A and B ($p = 0.039$; Table S18). While the bacterial community structure was significantly different between the 300 μg/g dw HHCB treatment and controls in system A microcosms ($p = 0.015$; Table S18), it was similar between the HHCB treatment and controls in system B microcosms ($p = 0.457$; Table S18). Likewise, the distance among groups or samples were larger in the PCA biplot of system A than system B (Fig. S2).

4. Discussion

Overall, the fate data of HHCB obtained in the present study are in agreement with data reported by other studies performed with HHCB (EC European Commission, 2008; Chen et al., 2014; USEPA, 2014; ECHA, 2015), all indicating that HHCB is not readily degradable. Similar to other aquatic organisms such as freshwater fish (Hu et al., 2011) and marine organisms (Nakata et al., 2007), oligochaete worms (*L. hoffmeisteri* and *B. sowerbyi*) can accumulate HHCB. The presence of oligochaete worms, however, did not significantly influence the dissipation of sediment-associated HHCB (Table S10). This can be attributed to the low biomass of worms (~ 0.96–1.13 g ww) compared to the amount of sediment (~ 4.35 kg ww) present in each microcosm of the system A. Sediment-associated HHCB significantly influenced the survival of *B. sowerbyi* and *L. hoffmeisteri* at concentrations of 100 μg/g dw and 200 μg/g dw, respectively (Table S14). While sediment-associated HHCB had no effect on the bacterial community structure, richness and diversity at concentration up till 300 μg/g dw sed, the presence of benthic macroinvertebrates significantly altered the bacterial community structure in the sediment at the density used in the present study (e.g., 2667 and 333 ind./m^2 for *L. hoffmeisteri* and *B. sowerbyi*, respectively) that were similar to those we found in the urban rivers of Guangzhou (South China).

4.1. Fate of HHCB in the microcosms

HHCB concentrations decreased in the overlying water of both systems after 28 days of exposure (Fig. 1a and b), likely due to the deposition of suspended particles in the water column (Table S7) and HHCB partition between the water phase and sediment phase considering its hydrophobic property (log Koc value of 4.86) (Balk and

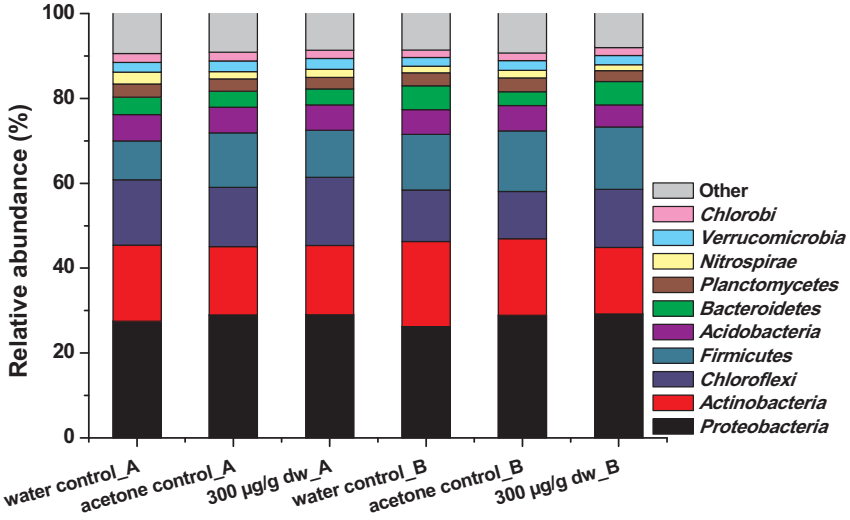

Fig. 6. The relative abundance (%) of the top ten phyla in the sediment at the end of exposure (day 28). A and B represent systems with (System A) and without (System B) introduced organisms, respectively.

Ford, 1999a). Moreover, in the overlying water higher HHCB concentrations were found in the system A than system B. This is likely due to the worm (i.e., *L. hoffmeisteri* and *B. sowerbyi*) bioturbation creating a flux of HHCB from sediment to the overlying water (Karickhoff and Morris, 1985).

Because of the lipophilic properties, HHCB accumulated in *B. sowerbyi* and *L. hoffmeisteri* with BSAF values of 0.29–0.66 and 0.94–2.11, respectively (Table S10). This is in agreement with findings of Hu et al. (2011), who found that HHCB accumulated in the carps from the Haihe River (China) with similar BSAF values of 1.6–2.5. Additionally, HHCB accumulation in other wildlife, such as marine benthic organisms, has also been reported in earlier studies (Table S1). For example, Nakata et al. (2007) found that HHCB accumulated in lugworm (unknown scientific name) to concentrations of 0.003 μg/g ww tissue in the Ariake Sea (Japan). Unfortunately, these authors did not report corresponding HHCB concentrations in the sediment. In our previous tests with sediment-associated HHCB and *L. hoffmeisteri* in a water/sediment system, we found that the HHCB bioaccumulation in *L. hoffmeisteri* reached the steady state after 7 days of exposure (Peng et al., 2018a). Therefore, we can infer that the bioaccumulation of HHCB also reached the steady state in both *B. sowerbyi* and *L. hoffmeisteri* by the end of exposure (day 28). For the same treatment, the BSAF value of *B. sowerbyi* was smaller than *L. hoffmeisteri*, which is likely related to its lager body weight and lipid content relative to *L. hoffmeisteri*. BSAF values measured here decreased with increasing exposure concentration for both *B. sowerbyi* and *L. hoffmeisteri*. This corresponds well to the sediment-associated triclosan (TCS, an antibacterial agent used in personal care products) bioaccumulation in *L. hoffmeisteri* (Peng et al., 2018b). In that study, the results of bioaccumulation modelling showed that the decrease in ingestion rate with increasing TCS exposure concentration was likely responsible for the BSAF values variation among TCS treatments (Peng et al., 2018b). Likewise, the changes of BSAF values found in the present study could also be attributed to the HHCB effects on the ingestion rate of worms. Indeed, a previous study reported that sediment-associated HHCB significantly reduced the average weekly feeding rates of the adult gastropod *Potamopyrgus antipodarum* at 30 and 100 μg HHCB/g dw sed compared to the control group during a 12 weeks of incubation (Pedersen et al., 2009).

There was a significant reduction in HHCB concentrations in the aerated tap water for abiotic degradation test under both light and dark conditions during the 28 days period. Since HHCB showed negligible photochemical degradation in the surface water of lake Zurich (Switzerland) in summer (Buerge et al., 2003), HHCB volatilization that played an important role in the removal of HHCB in the WWTPs (Federle et al., 2014) is likely to be responsible for the HHCB reduction observed here. Indeed, it has been reported that HHCB can volatilize into the atmosphere where it quickly reacts with OH radicals, resulting in short atmospheric lifetimes (Aschmann et al., 2001). In contrast, in the overlying water test there was no significant decrease in HHCB concentrations under the same conditions as the aerated tap water. This is likely due to the high turbidity (~ 130 NTU) of the overlying water. As such, HHCB will strongly bind to the suspended particles in the overlying water, reducing potential volatilization of HHCB. These results indicate that volatilization, photolysis and hydrolysis processes of HHCB are negligible in the overlying water under the conditions in the present study. Therefore, any decrease in HHCB in microcosms during the experiment could be attributed to bioaccumulation and biodegradation by either microorganisms or the introduced organisms. However, here we only found a minimal loss of HHCB from the microcosms (0.36–1.54% in system A and 0.36–1.49% in system B) (Table S11), indicating that HHCB was not readily degradable under the experimental conditions of the present study. Similar results have been reported in other studies (e.g., Buerge et al., 2003; EC European Commission, 2008; USEPA, 2014; ECHA, 2015). For example, in a CO_2 evolution test for ready biodegradability, only 2% of the HHCB was degraded during a 28 days period (European Chemicals Agency ECHA, 2015). Actually, in the European Union Risk Assessment Report (EC European Commission, 2008), a half-life of 79 days in the sediment was deemed most relevant for modelling the fate of HHCB in sediment using the European Union System for the Evaluation of Substances (EUSES) model. However, the rate of HHCB dissipation was slower in the current study relative to the half-life of 79 days. This could be associated with differences in exposure scenario, content of organic material, microbial activity, etc between the current study and that study. In fact, adsorption onto activated sludge particles has been demonstrated to be the major removal mechanism of HHCB in WWTPs, as HHCB is poorly biodegradable or susceptible to chemical degradation (e.g., USEPA, 2012, 2014; Federle et al., 2014).

4.2. Effects of HHCB on algae

Although no algae were initially introduced into system B, higher levels of phytoplankton and periphyton chlorophyll-a were observed in system B than system A (Table S13), suggesting the growth of microalgae. Compared to system B, in system A the negative effects of HHCB on the periphyton chlorophyll-a occurred at higher HHCB treatments, indicating that the toxic effects of HHCB on periphyton was mediated in system A. This is likely to be associated with the presence of snails and higher turbidity values in system A than B, as the turbidity and grazing activities of snail can impose an additional stress on the periphyton relative to HHCB. For the phytoplankton chlorophyll-a, the toxic effect of HHCB was only observed at the 300 μg/g dw treatment in both systems, with the initial HHCB concentration of 164 μg/L in the overlying water. However, this level is slightly lower than the reported NOEC value (72 h algal growth inhibition) of 201 μg/L (Table S2) based on *Pseudokirchneriella subcapitata* exposure to HHCB (Balk and Ford, 1999b). This could be attributed to the different sensitivity of different algae, the longer exposure duration (28 days) and more complicated growth conditions in our study (e.g., diverse organisms and water-sediment systems).

4.3. Sensitivity of Daphnia and benthic macroinvertebrates to HHCB stress

No *Daphnia* were found in any of the microcosms by the experimental end (Table S14), which is likely related to food deficiency rather than HHCB stress, as *Daphnia* were also absent in the controls and the overlying water maintained a good water quality during the test. Indeed, while the reported EC_{50}-48 h (immobilization) and NOEC-21 d (reproduction) of HHCB for *Daphnia* were 282 μg/L and 111 μg/L, respectively, in the overlying water of the highest treatment (300 μg/g dw) the HHCB concentration was 164 μg/L and 4.73 μg/L at the beginning and end of exposure, respectively. Therefore, *Daphnia* was unlikely to suffer stress from HHCB. As *Daphnia* were absent in the controls, in the future work, we therefore recommend to include a lower density of *Daphnia* in such systems or a higher nutritional state for algal growth. For midges, as the reported NOEC-28 d (emergence) of HHCB for *Chironomus riparius* was 200 μg/g dw, emergence was likely to be responsible for the absence of midge in the lower treatments (≤ 200 μg/g dw) at the end of experiment. However, effects of HHCB on midges cannot be evaluated in the 300 μg HHCB/g dw treatment, as here we used fourth-instar larvae and open systems. It is therefore recommended to use first-instar larvae and emergent trap for effects evaluation on midges. No productive activities were observed for clams, snails or worms in any microcosms at experimental end (day 28), which is probably associated with their age (juvenile). The lack of effect of HHCB on survival and growth of clams and snails, even at quite high concentration (300 μg/g dw), corresponds well with previous studies which found limited effects of HHCB on freshwater gastropods at 100 μg/g dw (Pedersen et al., 2009) and terrestrial gastropods at 289 μg/g dw (Wang et al., 2015). In contrast to the snails, HHCB significantly reduced the survival of *B. sowerbyi* and *L. hoffmeisteri* at 100 and 200 μg/g dw and higher, respectively (Table S14). This is likely

related to differences in food preference and feeding habits between these organisms. For example, while *B. sowerbyi* and *L. hoffmeisteri* are deposit feeder that can ingest silt and clay particles, clams and snails are filter-feeder that feed on detritus and living microphytes (Table S3). However, these concentrations are higher than those occurring in the aquatic environment (Table S1). Therefore, our results suggest that both oligochaete worms are highly tolerant to HHCB, which is in line with earlier findings (Milbrink, 1973; Sang, 1987). Likewise, sediment-associated HHCB (168 μg/g dw sed) had no detectable effects on a polychaete (*Capitella* sp. I) in terms of adult survival and growth (Ramskov et al., 2009). Based on these findings, it seems that *B. sowerbyi* was more sensitive to sediment-associated HHCB than *L. hoffmeisteri* and *Capitella* sp. I, indicating the species-specific toxicity of HHCB.

4.4. Effects of HHCB and benthic macroinvertebrates on sediment bacterial community

HHCB at 300 μg/g dw had no effect on the sediment bacterial community structure, richness and diversity in system B under the conditions in this study. This is likely due to the strong binding of HHCB to the sediment and its physicochemical properties (USEPA, 2014). Our results indicate that HHCB alone would probably not affect the bacterial community richness, diversity and structure at environmentally relevant concentrations. Likewise, HHCB had no effect on soil bacterial richness and carbon sources utilization at concentration of 100 μg/g dw soil (Lv et al., 2017).

In addition, we found that the presence of benthic macroinvertebrates significantly altered the bacterial community structure and relative abundance of some dominant bacteria in the sediment. This is most likely due to their reworking, irrigating, and feeding activities (Lohrer et al., 2004; Selck et al., 2005; Bertics and Ziebis, 2009). Similar results have been reported for bacterial community composition in sediments inhabited by benthic macroinvertebrates (i.e., *C. fluminea*, *Chironomidae* larvae and tubificid worms) (Cuny et al., 2007; Zeng et al., 2014). The relative abundance of *Chloroflexi*, a common phylum in the field sediment which is involved in carbon cycling (Hug et al., 2013), was significantly higher in the sediments of system A than system B. Thus, the presence of benthic macroinvertebrates may indirectly influence the degradation of organic contaminants via altering bacterial communities. In contrast, the phylum *Firmicutes* showed statistically lower relative abundance in the system A than system B, which is likely associated with the relatively aerobic conditions in the sediment of system A compared to system B, due to the bioturbation of introduced benthic organisms in system A. In line with this, a previous study has demonstrated that aerobic treatment caused approximately 8-fold decrease in the relative abundance of *Firmicutes* (McGarvey et al., 2007).

4.5. Implications for environmental risk assessment

Our results demonstrate that HHCB at 30 μg/g dw had no significant effects on chlorophyll-a content, sediment bacterial community composition, and survival and growth of benthic macroinvertebrates under the conditions used in the present study. Oligochaete worms at the environmentally relevant density (2667 and 333 ind./m^2 for *L. hoffmeisteri* and *B. sowerbyi*, respectively) made insignificant contribution to the loss of HHCB in the microcosms, but significantly changed the bacterial community composition. As the HHCB concentration employed here was higher than what have been reported in field sediments (i.e., 0–1.48 μg/g dw; Table S1), HHCB at environmentally relevant levels is unlikely to pose direct toxicological risks to algae, benthic macroinvertebrates and sediment bacterial communities in the short term.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.11.092.

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