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Uptake, metabolism and sub-lethal effects of BDE-47 in two estuarine invertebrates with different trophic positions☆

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ABSTRACT

Two microcosm types -sediment-biota and biota-biota- were constructed to simulate different pathways of BDE-47 uptake, metabolism and oxidative stress effects in two key estuarine invertebrates (polychaete *Laeonereis acuta* and crab *Cyrtograpsus angulatus*). In the sediment-biota experiment, both species were exposed to spiked sediments; an environmentally reported and a high concentration of BDE-47 for 2 weeks. In the biota-biota experiment, crabs were fed with polychaetes pre-exposed to BDE-47 in the sediment-biota experiment. The sediment-biota experiment first revealed that polychaetes significantly accumulated BDE-47 (biota-sediment accumulation factor >2; $p < 0.05$) to a much greater extent than the crab organs (muscle, hepatopancreas, gills) at both sediment concentrations. For oxidative stress responses, polychaete and crab tissues exposed to spiked sediment showed a significant increase ($p < 0.05$) of only glutathione S-transferase (GST) activity with respect to controls in both BDE-47 concentrations. No lipid peroxidation (TBARS) or total antioxidant capacity (ACAP) changes were evident in the species or organs exposed to either BDE-47 sediment concentration. The biota-biota experiment showed that feeding crabs with pre-exposed polychaetes caused BDE-47 accumulation in organs as well as significant amounts of BDE-47 eliminated through feces ($p < 0.05$). Unlike the sediment-biota exposure, crabs fed with pre-exposed BDE-47 polychaetes showed the most conspicuous oxidative stress responses. Significant changes in GST and ACAP in both hepatopancreas and gills, in addition to enhanced TBARS levels in the hepatopancreas with respect to controls ($p < 0.05$), revealed that BDE-47 assimilated by invertebrates represents a potential source of toxicity to their predators. No methoxylated metabolites (MeO-PBDEs) were detected during BDE-47 metabolism in the invertebrates in either of the two different exposure types. In contrast, hydroxylated metabolites (OH-PBDEs) were detected in polychaetes and crab organs/feces in both experiments. Our results demonstrate that PBDE hydroxylation is one of the main biotransformation routes of BDE-47 in estuarine animals, which could be associated with the oxidative stress responses found.

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1. Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of flame-retardant additives that have been widely applied in domestic and industrial materials to reduce their flammability (Gaylor et al., 2013; Tian et al., 2012). Due to the persistent and increasing levels in the environment (Yogui and Sericano, 2009), bioaccumulation potential (Luo et al., 2007; Klosterhaus et al., 2011) and effects on human and wildlife (Eljarrat and Barceló, 2011), some PBDEs were added in 2009 to the list of persistent organic pollutants (POPs) under the UNEP Stockholm Convention. Tetra-BDE congeners, such as 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), are the most predominant PBDEs in aquatic environments worldwide (Darnerud et al., 2001; Eljarrat and Barceló, 2011; Oros et al., 2005; Yogui and Sericano, 2009).

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Moreover, due to its high hydrophobicity (log $K_{ow} > 6$), BDE-47 tends to bind to particulate matter and sediments (Eljarrat and Barceló, 2011). Thus, estuarine sediments can act as important sinks of the PBDEs originated by several anthropogenic activities developed across watersheds (Oros et al., 2005; Xiang et al., 2007), and the PBDEs in sediments are potentially bioavailable to benthic invertebrates due to their ingestion and assimilation of sediment-associated particles (Ciparis and Hale, 2005). In this way, BDE-47, as well as other PBDE congeners, could also be bioavailable to higher trophic levels through deposit feeder invertebrates by primary/secondary carnivores or scavengers. Several studies have been conducted to investigate trophic transfer and accumulation of sediment-associated PBDEs from benthic invertebrates to higher trophic levels (Barón et al., 2013; Ma et al., 2013; Yu et al., 2009). However, little is known about the underlying mechanisms of PBDE bioaccumulation, metabolism/elimination in sediment-associated species, transfer to higher-trophic levels and potential for sub-lethal effects (Klosterhaus et al., 2011; Ma et al., 2013; Vidal-Liñán et al., 2015; Wan et al., 2009; Wiseman et al., 2011). Moreover studies in PBDEs exposure on benthic invertebrates with biological relevant sediments (sandy mud) typically found in estuarine tidal flats are scarce or only assessed with other types of sediments (Klosterhaus, 2007; Klosterhaus et al., 2011). Fecal BDE-47 residues/metabolites after PBDEs exposure were observed in vertebrates (Darnerud et al., 2001; Staskal et al., 2005), however the use of invertebrate feces for the assessment of BDE-47 and its metabolites is not properly investigated.

In terms of adverse effects on biota, BDE-47 and their hydroxylated (OH-PBDEs) and methoxylated (MeO-PBDEs) analogues/metabolites were reported as neurotoxic, genotoxic, endocrine disruptors and reactive oxygen species (ROS) inducers. On this basis, an oxidative stress scenario caused by BDE-47 and its analogues is possible and could be a potential toxicological pathway to promote deleterious effects. Some of the oxidative stress responses usually evaluated include glutathione S-transferase (GST) as a biotransformation enzyme biomarker, total antioxidant capacity against peroxyl radicals (ACAP) as the sum of enzymatic and non-enzymatic defenses, and thiobarbituric reactive substances (TBARS) as biomarker of lipid oxidative damage. The assessment of the above-mentioned responses provides insight regarding the exposure, general redox status and potential cellular damage by chemicals (Monserrat et al., 2007) and valuable data related to sub-lethal effects in biota. The assessment of oxidative stress responses induced by BDE-47 and it's metabolites on invertebrates is scarce and limited to some terrestrial and marine species (Vidal-Liñán et al., 2015; Xu et al., 2015) without studies in estuarine invertebrates.

The deposit feeder polychaete *Laeonereis acuta* (Polychaeta: Nereididae) and the scavenger crab *Cyrtograpsus angulatus* (Crustacea: Varunidae) are dominant benthic invertebrates from the temperate estuaries of the South America Atlantic coast (Palomo and Iribarne, 2000; Spivak et al., 1994). Both species are considered key estuarine species due to their influence on sediment properties, local diversity and tendency to become prey to other species (Botto et al., 1998; Palomo and Iribarne, 2000). Additionally, *L. acuta* as well as *C. angulatus* have been used as effective biomonitors of POPs and oxidative stress responses in the assessment of estuarine sediment pollution (Menone et al., 2001; Monserrat et al., 2007).

In the present study, two experiments were performed in order to determine sediment-biota and biota-biota transference of BDE-47 in simple models and their effects on two estuarine invertebrates with different feeding behaviors. The scarcity of data related to BDE-47 uptake/accumulation, metabolism in estuarine invertebrates and utilization of different uptake methods led us to analyze the importance of sediment transfer and predator-prey relationships in assessing sub-lethal effects of PBDEs. Thus, the objectives of this study were to investigate the following: (1) the accumulation and metabolism of BDE-47 from sediment or food sources in estuarine benthic invertebrates; (2) the effects of BDE-47 on pro-oxidant/antioxidant balance/status in estuarine benthic invertebrates, (3) the influence of different uptake mechanisms (sediment and pre-exposed food) of BDE-47 on sub-lethal effects in crab tissues and (4) the assessment of crab feces as a reliable biological matrix to evaluate BDE-47 uptake/metabolism.

2. Materials and methods

2.1. Test sediment

Estuarine sediment was collected from the Mar Chiquita coastal lagoon (37° 44′ 27″ S; 57° 25′ 30 W) and used for control and spiked sediments in the experiments. Sediment (12 kg) was air-dried until a constant weight was maintained to homogenize it and evenly reduce agglomerates. Once dried, the sediment was sieved through a 400-μm mesh to exclude gross particles. This sandy-mud sediment contained 0.4 ± 0.05% organic carbon (OC), 85.4 ± 0.2% sand, 6.4 ± 0.1% clay and 8.3 ± 0.1% silt. Sediments were spiked with the BDE-47 standard (labels provided in supplementary material) following the procedures reported in previous studies, with modifications (Tian et al., 2012). Briefly, the stock solution of BDE-47 (550 μL/ml in acetone) was transferred to the sediment to obtain the desired BDE-47 concentration. Then, after the solvent was volatilized, control and spiked dry sediments were rotated (end over end) for 20 days and stored in the dark for 4 days to equilibrate. Sediments were additionally equilibrated with filtered seawater (salinity: 20 PSU) for 24 h prior to the addition of invertebrates. The BDE-47 concentrations were determined in the control (non-spiked) and spiked sediments using the analytical procedures described below. The results are listed in Table 1.

2.2. Experimental design

The assays were divided into sediment-biota (Experiment I) and biota-biota (Experiment II) exposures. In experiment I, the polychaetes and crabs were individually exposed for 14 d to sediment spiked with environmentally reported levels (Luo et al., 2007; Oros et al., 2005) and high levels of BDE-47. Experiment II used polychaetes exposed to BDE-47-spiked sediment from experiment I as food for non-exposed crabs for 14 d. Experiments were conducted in 10 L rectangular glass aquariums for crabs and 1 L rectangular glass trays for polychaetes. Both crabs and polychaetes were

Table 1
BDE-47 concentrations in sediment (ng g^{-1} dw) and water (ng L^{-1}) from sediment-biota and biota-biota experiments at different exposure times.

Matrix/compartment	Treatment	BDE-47
Sediment (Day 0)	Control	0.17 ± 0.04
	Environmental	41.4 ± 14.7
	High	401 ± 66.2
Sediment (Day 14)	Polychaeta Environmental	34.1 ± 4.39
	Polychaeta High	393 ± 102
	Crab Environmental	32.6 ± 11.4
	Crab High	361 ± 2.24
Water (Day 14)	Control	bdl
	Polychaeta Environmental	0.83 ± 0.50
	Polychaeta High	17.5 ± 1.00
	Crab Environmental	0.67 ± 0.50
	Crab High	61.6 ± 25.7

bdl: Below detection limits.

collected from the Mar Chiquita coastal lagoon at the same site of sediment collection. Invertebrates were acclimatized in the laboratory for 2 days in filtered seawater (20 PSU, pH 8.5 and 20 °C) prior to the exposure experiments. Assays were run in a temperature-controlled room (18 °C), with the water gently aerated to maintain oxygen saturation, and exposed to a photoperiod of 14:10 h light:dark. Experimental conditions of pH, temperature and salinity of seawater were 8.4 (±0.3), 18.3 (±0.4 °C) and 20 (±2 PSU), respectively.

2.2.1. Sediment-biota exposure (Exp. I)

In this experiment, both crabs and polychaetes were exposed to sediments spiked with two different BDE-47 concentrations or control sediments to assess BDE-47 uptake and effects after exposure to (1) environmentally reported BDE-47 levels and (2) high BDE-47 levels (Table 1) that could allow proper quantification and/or induce the generation of MeO-BDE-47 and OH-BDE47 metabolites in target species. For the *C. angulatus* exposure, 48 male crabs of approximately 3.12 ± 0.04 cm carapace width were kept in 12 aquariums (n = 4 per aquarium) with 1 kg of spiked/unspiked sediments plus 5 L of filtered seawater each. The same treatment setup was used for the *L. acuta* exposure using 132 (0.19 ± 0.01 g w.w) adult polychaetes in 6 glass trays (n = 22 per tray). Additionally, for their later use in the Experiment II (Exp. II), 140 adult polychaetes (0.15 ± 0.02 g w.w) were added in 4 glass trays with unspiked sediments and sediments spiked at the highest BDE-47 levels. Eight crabs and 22 polychaetes were sampled from each treatment on days 0, 7 and 14, while polychaetes for Exp. II were only sampled at the end of the experiment (14 d). Together with invertebrates, sediments were sampled on days 0 and 14. Water samples obtained at 14 d from both BDE-47 concentrations were filtered through a 0.45 μm cellulose nitrate membrane filter (Sartorius Stedim Biotech) under vacuum.

2.2.2. Biota-biota exposure (Exp. II)

Thirty-two male crabs of approximately 3.34 ± 0.04 cm carapace width were added individually to aquariums with 5 L of filtered seawater after a starving and acclimation period of 2 days. The crabs were fed every 2 days with frozen polychaetes from Exp. I (ensuring that the crabs ate the polychaetes quickly and completely) in an amount of 0.5 g of exposed/unexposed polychaetes per week, equivalent to 110 ± 10 ng/g wet weight (w.w) of BDE-47 per day for the exposed crabs. At days 0, 7 and 14, eight crabs were sampled from each treatment; simultaneously, water containing crab feces from the bottom was pumped and filtered through a 0.45 μm cellulose nitrate membrane filter (Sartorius Stedim Biotech) under vacuum to obtain fecal material.

2.3. Depuration and sample processing

At the end of each sampling time, polychaetes for chemical analysis were removed and placed into aquariums with filtered seawater for 8 h to purge recently ingested particles. Polychaetes were washed and weighed, whereas crabs were immediately dissected to extract and weigh the organs (muscle, hepatopancreas and gills). For chemical analysis, each sample contained 3 composites of 2 polychaetes and 3 composites of 2 crab organs made from animals of similar weight/length and subsequently frozen at −20 °C. For biochemical analysis, both polychaetes and crab organs were immediately dissected and frozen individually at −80 °C.

2.4. Chemical analysis

2.4.1. Sample preparation

The sample preparation method applied was performed following the procedures described by Gonzalez et al. (2013) and Metcalfe and Metcalfe (1997) with the modifications of Miglioranza et al. (2003) for water, sediments and invertebrate tissues. For water samples, 1 L of water was spiked with 10 ng of PCB #103 as an internal standard, and 500 mL of spiked water was shaken with 300 mL of hexane:dichloromethane (2:1) for 2 h in an amber glass bottle with a Teflon-lined cap. Cleanup was performed by chromatography on activated (200 °C, 24 h) silica gel, and elution was carried out with hexane and hexane:dichloromethane (1:1). Then, the extracts were evaporated with N_2 to incipient dryness and reconstituted in 50 μL of hexane prior to analysis. For sediments and invertebrate tissues, ~5 and 0.5 g, respectively, were homogenized with sodium sulfate and spiked with the surrogate standards (10 ng of BDE-181 and 5 ng ^{13}C-6-OH-BDE-47, labels provided in supplementary material). Then, the sediments, tissues and filters containing feces were Soxhlet extracted (8 h) with a 50:50 mixture of hexane:dichloromethane. Extracts were concentrated under a vacuum and nitrogen flow to 2 mL followed by a gravimetrically determined lipid percentage calculation. Sulfurs compounds were eliminated from sediment extracts by reaction with pre-activated copper particles. Before cleanup, samples were divided into two extracts to determine PBDEs, MeO-PBDEs and OH-PBDEs separately using different cleanup methods. For PBDEs and MeO-PBDEs, lipids were removed by H_2SO_4 treatment prior to cleanup. The cleanup of the organic phase for PBDEs and MeO-PBDEs was carried out using solid phase extraction (SPE) with alumina cartridges (AL-N 5 g, ISOLUTE®, Biotage AB). Cartridges were conditioned with 10 mL of hexane and eluted with 10 mL of a mixture of hexane:DCM (1:2). Extracts were then evaporated with N_2 to incipient dryness and reconstituted in 50 μL of toluene prior to the analysis. For OH-PBDEs, the cleanup was carried out following the procedure of Sun et al., 2013, with modifications. Briefly, a glass micro column with 1.5 g of acid silica (44% of H_2SO_4) was coupled with 5 g silica cartridges (Si-N). The column with acid silica was conditioned with 5 mL of DCM and eluted with 10 mL of DCM. Silica cartridges were conditioned with 10 mL of DCM and eluted with 20 mL of DCM. Then, the extracts were evaporated with N_2 to incipient dryness and reconstituted in 50 μL of acetonitrile (ACN) prior to analysis.

2.4.2. Analysis of PBDEs and MeO-PBDEs

Analysis of PBDEs and MeO-PBDEs in sediments and tissues was performed following the procedure described by Barón et al., 2013. Briefly, PBDEs and MeO-PBDEs were analyzed using an Agilent 7890C gas chromatograph connected to an Agilent 5975A Network mass spectrometer working in negative chemical ionization mode (NCI) using $NH4^+$ as reagent gas. PBDEs in water samples were analyzed using a gas chromatograph with an electron capture detector (GC-ECD, Shimadzu 17-A) using a SPB-5 (Supelco, Bellefonte, PA, USA) capillary column. PBDEs and MeO-PBDEs recoveries, detection limits and quantification limits for invertebrate tissues are provided in the supplementary material (Table S1).

2.4.3. Analysis of OH-PBDEs

Analysis of OH-PBDEs was performed following the procedure described by Feo et al. (2013a). Briefly, OH-PBDEs were analyzed by liquid chromatography—electrospray chemical ionization tandem mass spectrometry. The chromatographic separation of OH-PBDEs was carried out with a LC system Symbiosis Pico (Spark Holland, Emmen, The Netherlands) using a Purospher® STAR RP-18 endcapped column (125 mm × 2 mm × 5 μm particle size), preceded by a C18 guard column (2.1 mm × 10 mm) supplied by Waters. The mobile phases were water at pH 10.0 (or pH 8.0) and methanol (3:2, v/v) (solvent A) and ACN (solvent B). Acetic acid/ammonium acetate buffer was used to maintain the pH during the working conditions. Mass spectrometric analysis was performed with a hybrid

QqLIT Applied Biosystems MSD Sciex 4000QTRAP (Applied Biosystems, Foster City, CA) in electrospray ionization (ESI) mode. Experiments were carried out in negative ionization (NCI) mode. OH-PBDE recoveries, detection limits and quantification limits for invertebrate tissues are depicted in the supplementary material (Table S1).

2.4.4. QA/QC

The MS-MS identification of analytes was based on the following criteria: (i) simultaneous responses for the two monitored transitions (SRM1 and SRM2) must be obtained at the same retention time than those of available standards; (ii) signal-to-noise ratios must be >3; and (iii) relative peak intensity ratio must be within ±20% of the theoretical values obtained with standard solutions. Quality parameters of the method had been evaluated. For this purpose, recovery tests were carried out by addition of each analyte to an invertebrate tissue. These samples were previously analyzed in order to determine analyte presence before spiking (blank). Three replicates were prepared for the evaluation of the reproducibility of the method. The limits of detection of the method (mLOD), defined as 3 times the noise level, and the limit of quantification of the method (mLOQ), defined as 10 times the noise level, were calculated. Quality parameters were summarized in Table S1. Recoveries ranged between 45 and 89%, with relative standard deviation (RSD) values lower than 15%. mLODs ranged between 0.4 and 22 pg/g ww, and mLOQs ranged between 1.2 and 75 pg/g ww.

2.5. Biochemical analysis

GST, ACAP and TBARS were measured using 5–8 organisms/organs per treatment following the procedure described in Díaz-Jaramillo et al. (2013). For total protein, GST and ACAP, organs were homogenized (1:3 w/v) in ice-cold buffer (20 mM Tris-base, 1 mM EDTA, 1 mM DL-dithiothreitol, 500 mM sucrose and 150 mM KCl) with pH adjusted to 7.6. Homogenates were centrifuged at 9000 × g for 45 min (4 °C), and the supernatants were collected and stored at −80 °C for further analysis. The GST activity was expressed as nmol/min/mg proteins. ACAP results were expressed as the relative difference between ROS area with and without the pro-oxidant 2,2′-azobis (2-methylpropionamidine) dihydrochloride (ABAP). In this method, high values of relative area reflect low antioxidant capacity to neutralize peroxyl radicals, and low values reflect high antioxidant capacity (Amado et al., 2009). For TBARS measurements, tissues were homogenized in 1.15% KCl (1:5 w/v) containing 35 mM butylated hydroxytoluene (BHT) (Oakes and Van Der Kraak, 2003). The measurement of lipid peroxidation through TBARS determination was performed using fluorometric assays and expressed as nmol TBARS/mg of wet tissue, using tetramethoxypropane (TMP) as the external standard (Díaz-Jaramillo et al., 2013).

2.6. Data analysis

The biota-sediment accumulation factor (BSAF) was assessed to establish differences in the BDE-47 bioaccumulation rates between species and among tissues. Sediment data were normalized to organic carbon, and organ data were normalized to lipids before analysis (g_{lipid}/g_{TOC}). A two-compartment (sediment and invertebrate/tissue) first-order kinetic model was used to describe the movement of contaminants into the invertebrates. For this model, uptake rates were derived by the differential equation described by Klosterhaus et al. (2011).

$$C_i = C_s(K_1/K_2)\left(1 - e^{-K2t}\right)$$

where Ci is the concentration of contaminant in the invertebrate tissue (ng/g lipid), Cs is the concentration of contaminant in the sediment (ng/g carbon), K_1 is the uptake rate constant (g (carbon/g lipid·h), K_2 is the depuration rate constant (1/h), and t = time (h). The uptake rate constant was estimated to fit a linear regression of BDE-47 concentrations (assuming K_2 as negligible) from day 0–14. Uptake data were normalized to the mean day 0 sediment concentration, versus time. Changes in total BDE-47 tissue concentrations, BSAF and biochemical responses were evaluated by analysis of variance (ANOVA) using the Neumann–Keuls post-hoc comparisons test ($\alpha = 0.05$). Data were checked to meet the assumptions of normality and homogeneity of variances prior to analysis. Data without normal distribution were analyzed using the Kruskal–Wallis non-parametric test.

3. Results

3.1. Sediment-biota experiment

BDE-47 concentrations in sediment samples were 0.17 ± 0.04, 41.4 ± 14.7 and 401 ± 66.2 ng/g dw for control, environmental and high level sediment exposure, respectively (Table 1), whereas the levels in water from polychaetes and crabs at environmental and high sediment exposure ranged from 0.83 ± 0.50 to 61.6 ± 25.7 ng/L (Table 1). Field collected invertebrates for sediment-biota and biota-biota exposures showed BDE-47 levels of 1.08 ng/g ww for *L. acuta* and 0.95–5.24 ng/g ww in *C. angulatus* tissues, respectively (Supplementary material, Table S2).

3.1.1. BDE-47 sediment-biota accumulation

BDE-47 concentrations in polychaetes and crabs exposed to spiked sediments showed similar accumulation patterns between environmental and high BDE-47 concentrations with little interspecies variability (Fig. 1 a,b). At both concentrations, polychaetes exhibited significantly higher BDE-47 accumulation ($p < 0.05$) compared to crab organs, reaching a total BDE-47 concentration of 146 ± 18.1 and 2233 ± 1264 ng/g ww at day 14, for environmental and high BDE levels, respectively (Fig. 1a,b). In general, crab organs showed significantly higher BDE-47 accumulation in their gills ($p < 0.05$) compared to hepatopancreas and muscle at both concentrations, reaching a total BDE-47 concentration in gills of 23.4 ± 6.2 and 378 ± 82 ng/g ww after 14 of exposure, respectively (Fig. 1a,b). Wet-weight-based BDE-47 concentrations in polychaetes and crab tissues did not show significant increases over time (7 d–14 d) at either sediment concentration (Fig. 1a,b). The BSAF was determined for both species/tissues and BDE-47 concentrations to compare the relative bioavailability after 7 and 14 d. The BSAF at 14 d ($BSAF_{14}$) for polychaetes and crab tissues exposed to both spiked sediment concentrations ranged from 0.03 to 2.56 (Fig. 1c). Polychaetes exposed to the environmental BDE-47 concentration had significantly higher $BSAF_{14}$ compared to all crab organs ($p < 0.05$; Fig. 1c). The $BSAF_{14}$ from both invertebrates exposed to the highest BDE-47 concentration showed different bioaccumulation patterns, whereas only crab hepatopancreas had significantly low $BSAF_{14}$ compared to polychaetes and the other crab organs ($p < 0.05$; Fig. 1c). The $BSAF_{14}$ in each invertebrate/tissue showed no differences between sediment concentrations ($p < 0.05$; Fig. 1c). However, polychaetes and crabs hepatopancreas exposed to the environmental BDE-47 concentration showed a significant BSAF increase over time between 7 and 14 d ($p < 0.05$, Fig. 1c). At the highest BDE-47 sediment concentration, only crab

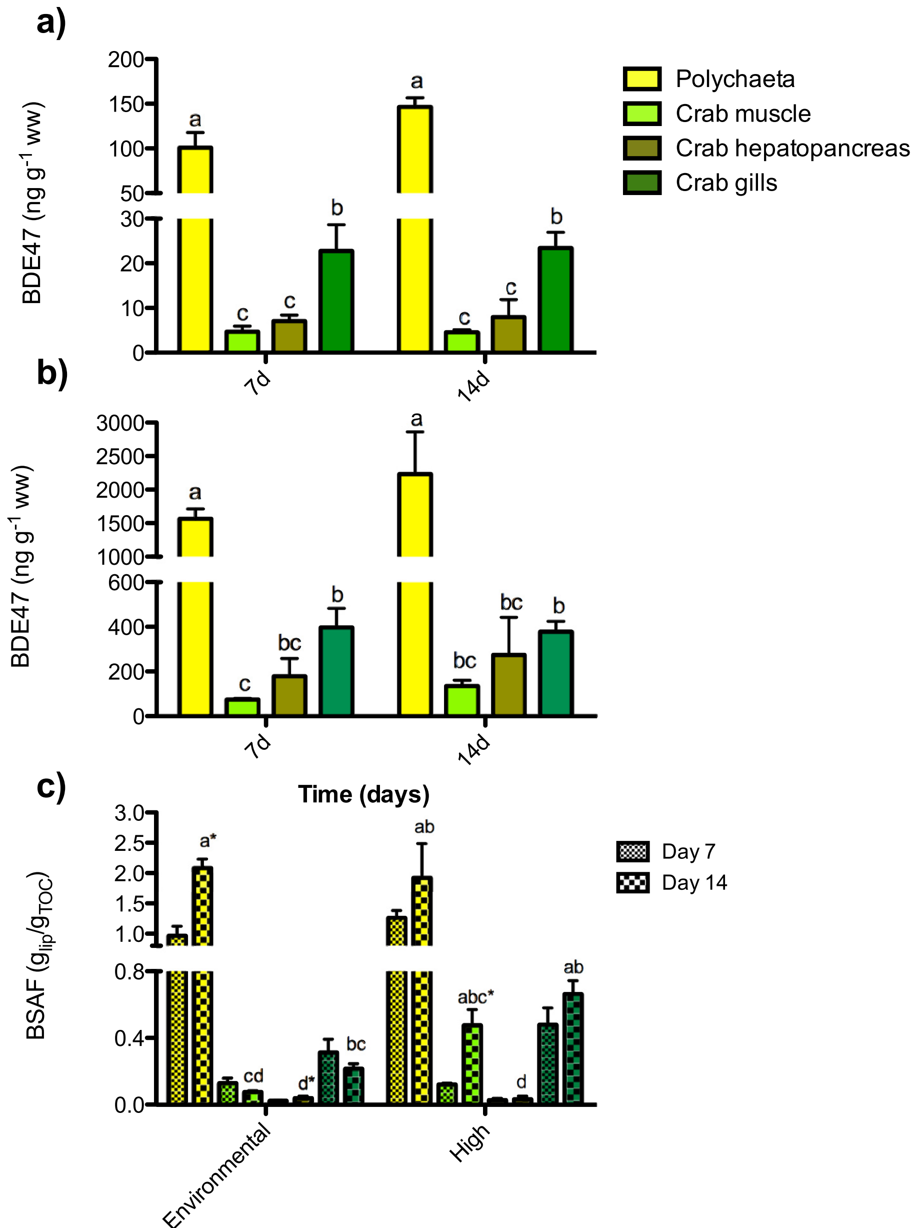

Fig. 1. Sediment-biota experiment. BDE-47 concentrations (ng g^{-1} ww) in invertebrate/tissues at a) environmental and b) highest BDE-47 level-spiked sediments. c) BSAF values in invertebrate/organs from sediment-biota experiment at different exposure times. Measurements represent the mean ± SE. Different letters indicate significant differences ($p < 0.05$). Asterisk in BSAF graphs indicate significant differences with respect to day 7.

muscles showed significant BSAF increases over time ($p < 0.05$; Fig. 1c). Uptake rates (K_1) for BDE-47 from both spiked sediment concentrations into polychaetes were similar and ranged from 6.4×10^{-3} to 7.0×10^{-3} g carbon/g lipid/h for environmental and high BDE-47 concentrations, respectively. The uptake rate for crab hepatopancreas was 1.0×10^{-4} for both BDE-47 sediment concentrations.

3.1.2. Sediment-biota oxidative stress responses

In general, oxidative stress responses showed similar patterns between the invertebrates/tissues exposed to BDE-47 sediment concentrations. The polychaetes only showed a significant increase in GST activity relative to the control when exposed to environmental concentrations at 14 d ($p < 0.05$; Fig. 2a). No significant differences were observed in ACAP and TBARS in the polychaetes exposed to both BDE-47 concentrations at all exposure times ($p < 0.05$). No significant differences were observed in control polychaetes with respect to the initial condition (day 0) ($p < 0.05$; Fig. 2). Crabs showed a significant increase in GST activity in all organs at the end of the sediment exposure ($p < 0.05$; Fig. 2b–d). Crab muscle only showed significant differences at exposure to environmental levels, while crab hepatopancreas and gills showed a significant increase in GST activity relative to the control at the highest sediment BDE-47 concentration ($p < 0.05$; Fig. 2b–d). No significant differences were observed in ACAP and TBARS crab organs exposed to both BDE-47 concentrations at all exposure times ($p < 0.05$; Supplementary material, Fig. S1). Significant differences were only observed in control TBARS muscle at 14 d with respect to the initial conditions ($p < 0.05$; Supplementary material, Fig. S1).

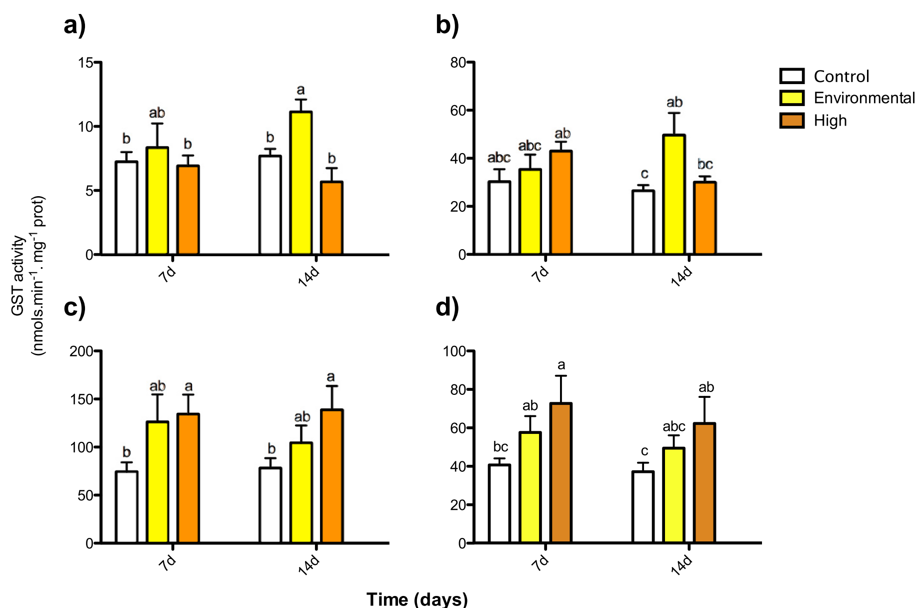

Fig. 2. Sediment-biota experiment. Values for glutathione S-transferase (GST) activity of the a) polychaete *Laeonereis acuta* and crab *Cyrtograpsus angulatus* b) muscle, c) hepatopancreas and d) gills exposed to environmental and high levels of BDE-47 spiked sediments. Measurements represent the mean ± SE. Different letters indicate significant differences ($p < 0.05$). Asterisk indicates significant increment (+) or decrease (−) of biochemical responses relative to initial conditions (day 0).

3.2. Biota-biota experiment

3.2.1. BDE-47 accumulation and excretion

BDE-47 concentrations in crabs fed with pre-exposed polychaetes showed non-significant differences between crab organs at the same exposure time ($p > 0.05$; Fig. 3). In contrast, crab feces showed significant differences compared to crab organs, reaching a total BDE-47 concentration of 418.0 ± 238.0 and 1412.0 ± 311.0 ng/g ww after 7 and 14 days of exposure, respectively ($p < 0.05$; Fig. 3). BDE-47 concentrations in crabs showed significant increases over time in gills and feces, respectively ($p < 0.05$; Fig. 3).

3.2.2. Biota-biota oxidative stress responses

Oxidative stress responses in crabs fed with polychaetes pre-exposed to BDE-47 showed differences between crabs fed with non-exposed (control) polychaetes (Fig. 4). Unlike muscle, which showed non-significant differences in all evaluated responses ($p < 0.05$; Fig. 4a), crab hepatopancreas and gills showed significant oxidative stress responses ($p < 0.05$; Fig. 4b, c). GST activity showed a significant increase in the hepatopancreas at 7 d compared to the control group ($p < 0.05$; Fig. 4b). The crab hepatopancreas showed a

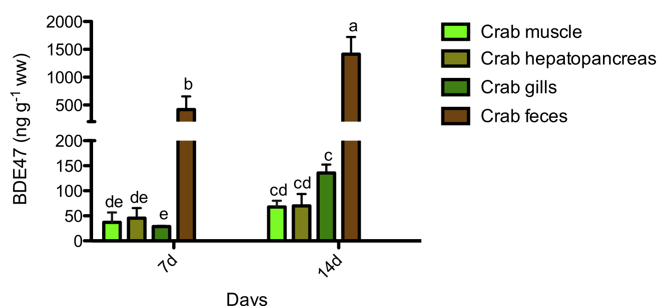

Fig. 3. Biota-biota experiment. BDE-47 concentrations (ng g^{-1} ww) in crab *Cyrtograpsus angulatus* organs/feces fed polychaetes pre-exposed to BDE-47 at different exposure times. Measurements represent the mean ± SE. Different letters indicate significant differences ($p < 0.05$).

significantly higher antioxidant capacity at 7 d and a subsequent decrease after 14 d measured by ACAP ($p < 0.05$; Fig. 4b). Lipid peroxidation in the crab hepatopancreas showed significant differences in TBARS levels compared to the control at 14 d ($p < 0.05$; Fig. 4b). Crab gills showed a significant increase in GST activity relative to the control in both exposure times ($p < 0.05$; Fig. 4c). The gill ACAP exhibited a significant decrease relative to the control at 14 d ($p < 0.05$; Fig. 4c). No TBARS differences were observed in the gills of pre-exposed crabs relative to the control ($p < 0.05$; Fig. 4c). Significant differences were observed in control GST and ACAP gills at both exposure times relative to the initial conditions (day 0) ($p < 0.05$; Fig. 4c).

3.3. MeO-PBDE and OH-PBDE metabolites

No methoxylated metabolites (MeO-PBDEs) were detected in target invertebrates from different exposure types. In contrast, the hydroxylated metabolites (OH-PBDEs) were detected in the polychaetes and crab organs/feces in both experiments. Analysis of the OH-BDE-47 metabolites in polychaete tissues exposed to spiked sediment showed that 3-OH-BDE-47 and 5-OH-BDE-47 were the most abundant congeners, ranging from nd to 18.4 pg/g ww (Table 2). 3-OH-BDE-47, 5-OH-BDE-47 and 6-OH-BDE-47 were detected in the crab organs exposed to spiked sediment, and the hepatopancreas and gills consistently showed the highest values, ranging from nd to 138 pg/g ww (Table 2). Crabs feed with pre-exposed polychaetes (biota-biota experiment) showed the presence of 3-OH-BDE-47, 5-OH-BDE-47 and 6-OH-BDE-47 in the hepatopancreas, gills and feces (Table 2). The highest levels of OH-BDE-47 were found in crab feces, ranging from nd to 2781 pg/g ww (Table 2). Percentages of Σ OH-BDE-47 (sum of the three major concentrations of each OH-BDE47 congener) relative to the parent BDE-47 compound was in general below or similar to 1% in both experiments (Table 2). 4-OH-BDE-17 and 3-OH-BDE-28, two OH-tri-BDE congeners, were also detected in all invertebrate/organs and experiments at values ranging from nd to 430 pg/g ww (Table 2).

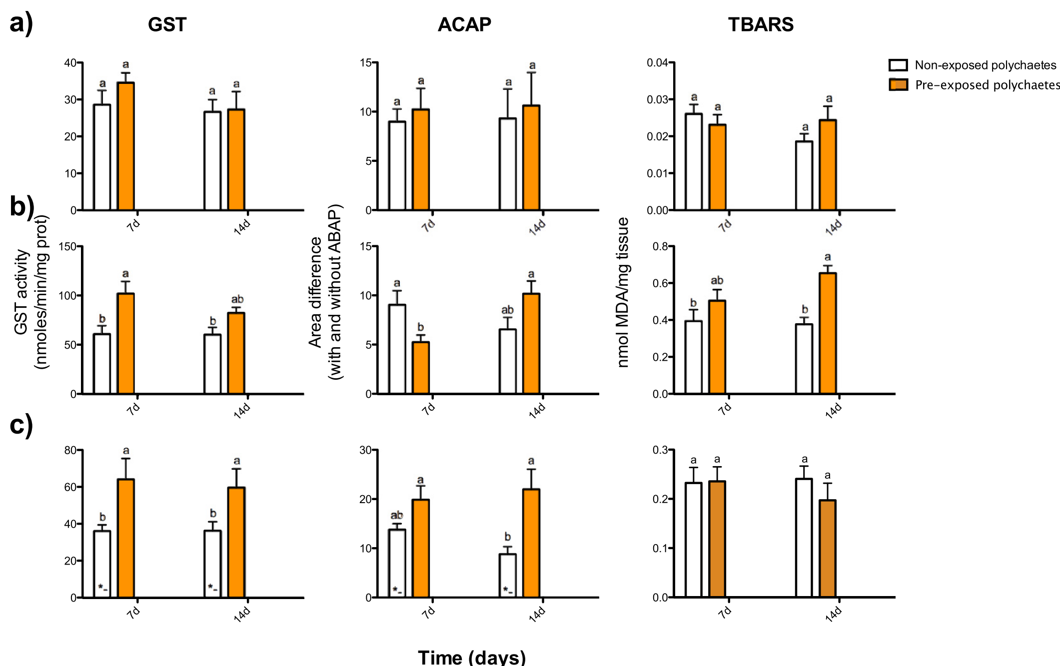

Fig. 4. Biota-biota experiment. Values for glutathione S-transferase (GST) activity, total antioxidant capacity (ACAP) and thiobarbituric acids reactive substances (TBARS) levels in a) muscle, b) hepatopancreas and c) gills from crabs *Cyrtograpsus angulatus* fed non-exposed and BDE-47-pre-exposed polychaetes. Measurements represent the mean ± SE. Different letters indicate significant differences ($p < 0.05$). An asterisk indicates a significant increase (+) or decrease (−) of biochemical responses relative to initial conditions (day 0).

Table 2
Range of concentrations (expressed in pg g^{-1}wet weight (ww)) for the OH-PBDEs congeners and percentage respect parent BDE-47 compounds at different BDE-47 exposures/experiments.

Experiment	Specie/tissue	Time (day)	4-OH-BDE-17	3-OH-BDE-28	3-OH-BDE-47	5-OH-BDE-47	6-OH-BDE-47	% Respect parent BDE-47
Sediment-Biota[a]	Polychaeta	7	bdl-bql	bdl-10.7	bdl-43.6	bql-18.4	bdl	3.98×10^{-4}
		14	bql-3.1	bdl-bql	bdl	bql-2.0	bdl	7.16×10^{-5}
	Crab muscle	7	bdl-23.4	bql-7.1	bdl	bdl	bdl	na
		14	bdl	bdl-24.5	bdl-92.0	bdl	bdl	0.07
	Crab hepatopancreas	7	bdl-8.1	bdl-30.2	3.2-113	2.9-113	4.8-63.4	0.16
		14	7.0-23.9	16.8-85.0	bdl-27.3	bdl-41.0	bdl-35.9	0.04
	Crab gills	7	bdl-16.6	bdl-13.7	29.0-138	bdl-20.5	bdl-38.1	0.04
		14	21.2-30.4	17.5-25.1	39.0-55.7	26.2-37.5	48.5-69.5	0.04
Biota-Biota	Crab muscle	7	bdl-4.7	bdl-6.5	bdl	bdl	bdl	na
		14	bdl-14.8	bdl-24.2	bdl	bdl	bdl	na
	Crab hepatopancreas	7	bdl-27.1	bdl-24.7	bdl	bdl-12.1	bdl	0.02
		14	5.5-43.1	4.3-66.0	44.5-62.3	42.6-52.5	bql-59.9	0.25
	Crab gills	7	16.1-20.8	13.3-17.1	29.5-38.1	19.7-25.6	36.8-47.4	0.39
		14	18.4-22.2	bdl-17.5	bdl-38.8	bdl-26.2	bdl-48.4	0.08
	Crab feces	7	bdl	bdl-222	bdl-2780	bdl-2012	bdl-222	1.19
		14	bdl-429	bdl-358	980-2030	228-755	bdl-286	0.61

[a] Only high BDE-47 sediment exposure. bdl: Below detection limits. bql: Below quantification limits. na: Data not available n = 3 per tissue/species.

4. Discussion

The differences observed between the polychaetes and the organs of crabs exposed to both BDE-47 concentrations demonstrate the ability of these species to accumulate hydrophobic organic contaminants ($K_{ow} > 6$) from sediments. Infaunal deposit feeders, such as polychaetes and some mollusks, accumulation of organic contaminants at concentrations even higher that those reported in benthic scavengers and some fishes could occur (Tracey and Hansen, 1996; Magnusson et al., 2003). The BSAF values observed in *L. acuta* exposed at both spiked concentrations are similar to those observed in benthic annelids exposed to field- and spiked BDE-47 and similar organic compounds (Klosterhaus, 2007; Magnusson et al., 2003; Mehler et al., 2011; Tracey and Hansen, 1996). However, experiments with exposed nereid polychaetes to field- and spiked PBDE sediments reported lower BSAF values for BDE-47, although the exposure was longer (28 d) than that in the present work (Klosterhaus et al., 2011). The lower BASF values observed in nereid worms at 28 d could be related to differences in the sediment characteristics compared to our work. Sediments with low sand percentage (<30% of sand) and high OC content are not environmentally realistic sediments for sand flat species, reducing the desorption and bioavailability of PBDEs and similar organic compounds (Millward et al., 2005). However, Mehler et al. (2011) reported that fine sediments (1.73% OC) diminish sediment uptake rates of BDE-47 in annelids compared to coarse sediments (1.53% OC) without significant changes in the BSAF values. Sediment characteristics such as particle size distribution and OC differences are important matrix constituents to take into consideration when comparing sediment-bioaccumulation

patterns to prevent under or overestimating BDE-47 sediment uptake by deposit feeders. Other authors have observed differences in some *C. angulatus* organs, showing dose-dependent BASF values and increasing BSAF values with time and dose (Gaylor et al., 2013). The lower BDE-47 concentrations and BASF values in the hepatopancreas compared to the other crab organs may be due to a higher metabolic activity in the digestive organs (Van den Steen et al., 2007). Moreover, the high BDE-47 levels and BASF values in the crab gills and the presence of BDE-47 in the dissolved water fraction from both experiments suggest that suspended particulate matter could also play an important role because the sediment resuspension observed in Experiment I could be a complementary pathway for BDE-47 uptake in epifaunal benthic species (Tian et al., 2012). Comparing the $BASF_{14}$ values between species also showed that nereid polychaetes such as *L. acuta* tend to accumulate high levels of tetra-PBDEs (Klosterhaus et al., 2011). Significant differences between 14 and 7 d suggest that *L. acuta* could not have reached a steady state at 14 d Klosterhaus et al. (2011) also observed a lack of steady state in nereid polychaetes after 28 d of PBDEs exposure. Therefore, *L. acuta* and other nereid polychaetes might tend to hyperaccumulate BDE-47 and could represent a pathway for transfer to high trophic levels. BDE-47 uptake in *C. angulatus* using polychaetes pre-exposed to BDE-47 as food represents an environmentally realistic scenario to predict trophic transferences, establishing food as another important PBDE uptake pathway (Staskal et al., 2005). Scavenger crabs accumulate BDE-47 in different organs based on food source and reflects their ability to excrete/eliminate BDE-47 by feces. As in other animals exposed to PBDEs or POPs by food, the amount excreted in feces is significant and increases with time (Darnerud et al., 2001; Staskal et al., 2005). The novelty of studying crab feces, which showed high BDE-47 levels, highlights the use of this matrix in future biomonitoring/uptake studies. Similar to feces, crab gills presented significant accumulation at 14 d relative to 7 d, suggesting that fecal BDE-47 residues at the bottom of the aquarium could be accumulated again through the gills. This is a factor to take in consideration in future experiments.

The absence of significant changes in total antioxidant and oxidative damage in both invertebrate species/tissues exposed to spiked sediments are in accordance with Ji et al. (2011). This author suggested that a high level of PBDEs by itself does not trigger high ROS production to induce oxidative stress. However, the significant increases in GST activity observed in both invertebrates exposed to spiked BDE-47 sediments suggests the involvement of GSTs in PBDE metabolic processes in aquatic animals (Roberts et al., 2011). GSTs are responsible for phase II conjugation of many xenobiotics with reduced glutathione and are capable of catalyzing reductive reactions in halogenated compounds (Tang and Tu, 1994). Particularly, *L. acuta* showed significant changes in GST activity at environmental levels of BDE-47, suggesting the need for further studies to determine the sensitivity of GST at lower BDE-47 levels. BDE-47 uptake in crabs through food induced oxidative stress responses related to enzyme activation and changes in ACAP in both crab hepatopancreas and gills. The observed enzyme activation and high ACAP on the first days of food exposure were not sufficient to prevent lipid oxidative damage, revealing hepatotoxicity by pre-exposed BDE-47 polychaetes as a food source to crabs. Comparing average BDE-47 concentrations expressed as ng/g lipid and ng/g ww in crab tissues at 14 d for both exposure routes revealed that BDE-47 levels from biota-biota exposure were lower than those in crabs exposed to the highest BDE-47 concentrations in sediment, assuming that BDE-47 by itself does not induce the oxidative stress responses observed in feeding crabs. Since many other anthropogenic/natural stressors could influence this non-specific biomarkers (Monserrat et al., 2007), the oxidative stress responses evaluated could not be qualified as specific toxic endpoints related to PBDEs exposure.

The non-detection of MeO-PBDE generation in the tested invertebrates from both experiments agree with results of some authors, who have reported that MeO-BDEs have not been observed to be formed in controlled exposure studies with PBDEs performed either *in vitro* or *in vivo* (Wiseman et al., 2011). No MeO-BDEs were detected in species with different trophic positions exposed to BDE-47 and PBDE mixtures at different cellular levels or with different uptake mechanisms (McKinney et al., 2006; Wan et al., 2010, 2009). Based on these studies and our results, the MeO-PBDE formation in aquatic invertebrates exposed to BDE-47 would be negligible. In contrast, the presence of OH-PBDEs observed in different invertebrates/organs and uptake methods in the present work suggest that OH-PBDEs represent a reliable metabolic route of tetra-PBDEs in animals (Wiseman et al., 2011). Trace concentrations of OH-PBDEs observed in the present work are in accordance with some authors that reported less than 1% of the concentration of the parent PBDEs (Feo et al., 2013b; Malmberg et al., 2005; Stapleton et al., 2009). The low OH-PBDE levels reported represent a challenge to estimating the importance of hydroxylated PBDE metabolism in lower trophic level organisms exposed to environmental PBDE levels. 6-OH-BDE-47 is described as both natural and a metabolite produced by PBDE exposure. In contrast, the 3-OH-BDE-47 and 5-OH-BDE-47 identified in the current work are described as non-natural products and could be reliable markers of PBDE biotransformation products (Wiseman et al., 2011). Hydroxylated-BDE-47 compounds generate oxidative stress in aquatic species (Usenko et al., 2012) and could be related to the oxidative stress responses observed in crabs from biota-biota experiments. However, as was previously mentioned, the observation of similar trace levels of hydroxylated-BDE-47 in crab organs from sediment exposure suggests that BDE-47 hydroxylated metabolism by itself does not cause toxicity different from the ingestion of OH-BDE-47 previously metabolized by invertebrates. Therefore, special attention might be given to nereid polychaetes because of their ability to accumulate PBDEs, biotransform them to hydroxylated compounds and potentially transfer them to higher trophic levels. The high levels of hydroxylated-BDE-47 in crab feces could represent an important source of OH-PBDEs for the estuarine environment. Moreover, the OH-PBDE levels observed in feces and gills could be related to the suspension process of feces during the experiments, which may represent another uptake route that needs to be analyzed in future studies. Finally, the presence of 4-OH-BDE-17 and 3-OH-BDE-28 in both invertebrates in both experiments would indicate that the debromination process could also play an important role in BDE-47 metabolism in aquatic organisms (Mhadhbi et al., 2014). This hypothesis deserves further study in terms of low bromination generation during long-term exposures.

4.1. Conclusions

Our results showed differences in uptake and BSAF patterns between *L. acuta* and *C. angulatus* tissues exposed to BDE-47-spiked sandy mud sediments. *L. acuta* whole individuals as well as *C. angulatus* gills were good indicators of low brominated PBDE exposure. Pre-exposure to BDE-47 for low trophic organisms as a food source for predators revealed the importance of predator-prey relationships in BDE-47 uptake and metabolism. The identification of OH-BDE-47 and the absence of MeO-BDE-47 products in the tested organisms revealed the importance of hydroxylation processes in tetra-PBDE metabolism by key estuarine invertebrates. Finally, crab feces emerged as a good biological matrix to evaluate low exposure to brominated compounds and their hydroxylated products in benthic animals.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.03.009.

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