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Toxic effects of two brominated flame retardants BDE-47 and BDE-183 on the survival and protein expression of the tubificid *Monopylephorus limosus*

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ABSTRACT

The toxic effects of two brominated diphenyl ethers (BDE), BDE-47, and BDE-183, on a benthic oligochaete tubificid, *Monopylephorus limosus* were studied under laboratory conditions. Investigated responses included survival, growth, and protein expression profiles, at BDE concentrations of 1, 10, 100, and 700 ng/g on a dry soil weight basis, with isooctane as the carrier solvent. Body weight losses among treatments were insignificant after 8 weeks of exposure. The 8-wk LC_{50} of BDE-47 and -183 were 2311 and 169 ng/g, respectively. By applying multivariate analysis techniques, protein expression patterns were compared and correlated with stressful sources of long-term culture, carrier solvent, BDE-47 and -183. The treatment of 8-wk 100 ng/g BDE-47 was most closely clustered to the 10 ng/g BDE-183 treatment, based on the 40 examined protein spots. This indicated that BDE-183 was more potent to *M. limosus,* than was BDE-47. The 2-wk and 8-wk controls clustered into different groups indicating the occurrence of physiological changes due to long-term laboratory culture. Additionally, solvent effect was shown by grouping the isooctane carrier to different clusters. With further characterization by principle component analysis, it was found that the separation was mainly contributed by the 2nd principal-component. And, the primarily inhibitory variation was at spots 2 (UMP-CMP kinase) and 40 (plasma retinol-binding protein precursor) in the 8-wk groups. On the contrary, protein spots 16 (cell division control protein 2 homolog) and 24 (mitochondrial DNA mismatch repair protein) showed stimulatory variation. In all, the observed proteomic responses suggest that BDEs disrupted metabolic function in *M. limosus* and multivariate analysis tool offers significant potential for the assessment of various stress sources at biochemical level.

1. Introduction

Polybrominated diphenyl ethers are brominated flame retardants widely used to prevent fires involving plastics, textiles, paints, and electronic appliances, including computers, televisions, and other electric household equipment (Alaee and Wenning, 2002; Watanabe et al., 1992). From these various sources, brominated diphenyl ethers (BDEs) enter aquatic systems through surface water or atmospheric pathways. Because of their lipophilic characteristics, they adsorb easily onto particles and bioaccumulate in aquatic food chains through incorporation by aquatic organisms (Boon et al., 2002; Burreau et al., 2004; Johnson-Restrepo et al., 2005). As a result, these compounds have been detected in all compartments including sediments, mussels, fish, and marine mammals (Law et al., 2003; Liu et al., 2005). In a global survey on skipjack tuna (*Katsuwonus pelamis*), the percent contribution of lower brominated congeners (BDE-15, -28, and -47) increased with increasing latitude (Ueno et al., 2004). In contrast, the distribution of higher brominated congeners (i.e., BDE-153, -154, and -183) showed a reverse trend, decreasing on moving to higher latitudes. These patterns suggested that lower brominated congeners were preferentially transported from pollution sources to high-latitude colder regions through the atmosphere (Darnerud et al., 2001). In addition, it has been raised concerns that BDEs and their metabolites may induce toxicity through binding to AhR with subsequent induction of CYP1A and the rest of the CYP1A gene family (Darnerud et al., 2001).

In Taiwan, BDEs have been detected in river and estuary fish (25–490 ng/g lipid) (Chen, 2005; Peng et al., 2007), farmland sediments (0.05–0.31 ng/g dry wt) (Peng, 2002), river sediments (0.8–92 ng/g dry wt) (Chen, 2005; Jiang, 2006) and coastal sediments (0.1–1.8 ng/g dry wt) (Jiang, 2006). BDE-209 was a

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dominant congener in most samples. In some cases, BDE-47, -154, -153, and -183 congeners were predominant in fish samples (Peng et al., 2007). In East Asia, the detection of BDE-153, -154 and -183 in sediments and in dolphins was also well-documented (Wang et al., 2007). However, current knowledge about the effects of higher brominated congeners on aquatic organisms is far from comprehensive.

Oligochaete tubificids are mostly found in fresh and brackish waters containing decomposing organic materials. These sediment-ingesting endobenthic animals are exposed to sediment-bound substances by all potential routes, e.g., overlying water, interstitial water, and ingestion of sediment. The animals absorb and accumulate pollutants in their bodies and may develop cumulative effects. Because of this unique feature, we selected a large freshwater tubificid *Monopylephorus limosus*, with adult size ranging from 15 to 70 mm (Erseus and Hsieh, 1997), to evaluate the effects of BDE congeners in this study.

When aquatic oligochaete *Lumbriculus variegates* (family Lumbriculidae) was treated with 1500 ng/g dry wt commercial DE-71 for 4 weeks, the bioaccumulation factors (BAFs) of BDE-47, -100, -99 were 8.1, 9.9, 4.0 in artificial sediments and 1.7, 1.2, and 0.8 in sewage sludge, respectively (Ciparis and Hale, 2005). In natural sediments with 181 and 221 ng/g dry wt BDE-47 and -99, the BAFs of *L. variegates* were 3.1 and 3.0 (Leppanen, Kukkonen, 2004). Because sediment characteristics can vary the sorptive strength of the contaminant to sediments, BDE congeners are bioavailable to aquatic oligochaetes to different degrees.

We herein compare the lethal and sublethal effects of BDE-47 and -183 on the oligochaete tubificid *M. limosus* under laboratory conditions. The examined toxicophysiological responses included survival, growth, and protein expression profiles derived from the two-dimensional gel electrophoresis (2-DE) in combination with identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Apraiz et al., 2006). Proteomic approaches are designed to study the protein profiles associated with specific treatments. A typical proteomic approach combines 2-DE and mass spectrometry can directly identify and quantify a large number of proteins. Our toxicophysiological studies compared and correlated protein expression patterns with stresses of long-term culture, carrier solvent, BDE-47, and -183.

2. Materials and methods

2.1. Animals

Adult *M. lomsus* were sampled from domestic wastewater drainage in Kaohsiung, Taiwan (120° 20′24.3″E and 22° 36′12.9″N) by sieving sediment through a 1-mm mesh, during the period of December 2004 to January 2005. Collected worms were cultured in an aquarium ($27 \times 7.5 \times 6.5$ cm) containing agar-substratum 2.5 cm in height and aerated deionized water 70 ml. Worms were acclimated in the laboratory for 4 weeks before experimental use. Culture density was 40 worms per aquarium at 25 ± 2 °C and 12*D*:12*L*. Water lost due to evaporation was adjusted daily and dead worms were removed. The worms were fed weekly with fine rice bran (5% of worm biomass) and the substratum was replaced once a week.

2.2. Exposure experiments

Test sediment was collected from the field which was mainly composited with grain size < 3.9 μm (i.e., 67% of the substrate). It was sterilized at 105 °C for 30 min then dehydrated at the same temperature for 24 h. Then, 20 g of dry sediment were spread into each exposure container (4 cm in diameter and 9 cm in height) and isooctane stock solution of BDE-47 or -183 (AccuStandard, USA) was added and thoroughly mixed. The dry soil weight-based concentrations were 1, 10, 100, and 700 ng/g for both congeners. Isooctane 4 μl/g was used to make stock solutions of 1, 10, and 100 ng/g concentrations, and 14 μl/g was for 700 ng/g concentration. The two solvent controls were isooctane 4 μl/g and 14 μl/g. So, the 100 ng/g BDE groups and 4 μl/g solvent control contained isooctane 80 μl in total. And, the 700 ng/g BDE groups and 14 μl/g solvent control contained isooctane 280 μl in total. After adding deionized water (20 ml), 20 adult worms (> 2 cm) were put into each container. Each treatment setup had three replicates and the exposing period was 8 weeks. During the exposure period, worms were fed weekly and water level was checked daily.

To determine the survival rate and growth (expressed as wet weight changes), alive worms were expelled to crawl out from the substrate by heating the exposure container in a 33 °C hot water bath on a weekly basis. The number of expelled worms were counted then transferred to tared weight pans, blotted dry and weighed. The survival rates and growth for each treatment were determined and compared using analysis of variance (ANOVA) test. If there was a significant difference at the 5% level, the results were compared by Duncan's test. The LC_{50}, LT_{50}, and their corresponding 95% confidence intervals (C.I.) were determined using probit analysis.

2.3. Proteomic study

2.3.1. Two-dimensional gel electrophoresis (2DE)

The protein profiles of worms exposed to 2-wk BDE-47, 8-wk BDE-47 and 8-wk BDE-183 were determined. Live worms were washed with deionized water and homogenized for proteomic analysis (Chiu et al., 2007). Homogenates were centrifuged at 13,000 *g* for 10 min at 4 °C. The homogenous supernatant was collected and the protein concentration was determined by Bradford assay, using bovine serum albumin as standard.

For the first dimension, immobiline Dry-Strip (13 cm, pH3 −10NL) was incubated with 200 ml rehydration buffer at 20 °C and 50 V 12 h (Chiu et al., 2007; Lin et al., 2011; Westermeier and Naven, 2002). After rehydration, 650 μl of total protein mixed with 1% DTT and 2% IPG buffer was placed in sample cups, and isoelectric focusing (IEF) was performed on an electrophoretic apparatus (IPGphorTM, GE Healthcare). The voltage program was 50 V for 12 h, 100 V for 5 h, 1,000 V for 1 h, and finally 8000 V for 8 h at 20 °C. The focused strips were equilibrated for 20 min in SDS equilibration buffer solution (6 M urea, 30% glycerol, 2% SDS, 0.01% bromophenol blue, and 50 mM Tris–HCl at pH 8.8) with 30 mM DTT. A second equilibration step was then performed using an equilibration solution with 135 mM iodoacetamide equilibration buffer for 20 min to replace the DTT. The second dimension separation was performed with 12% acrylamide gels at 30 mA constant current on a Hoefer SE600 Ruby vertical electrophoretic apparatus until the dye front was approximately 1 mm from the bottom of the gel. The gels were stained using a sensitive colloidal Coomassie G-250 protocol (Candiano et al., 2004).

2.4. Image acquisition

The stained gels were scanned and transformed into digitalized 2-DE images using an imaging system (LAS-3000; Fujifilm). In this experiment, each treatment had triplicate gels analyzed. The protein spots were assigned a spot number and their intensity levels were calculated as their relative volume to the total protein volume on the gel. Then the spots were processed with Melanie software to yield the isoelectric point (pI) and molecular weight (Mw). In addition, the intensity of each protein spot was compared by one-way ANOVA and Duncan's multiple-comparisons test (StatView, 1996; SAS Institute). A cluster analysis of the Bray–Curtis similarity (BCs) Indices (Primer 6.0) was employed to compare the expression of overall protein patterns among treatments (Clarke and Warwick, 2001). The contribution of each protein spot was further estimated by Principal component analysis (PCA). Afterward, the best gel from each treatment was selected for further protein identification.

2.5. Protein identification

Protein spots were excised from the Coomassie blue-stained gels. Then, they were washed, destained, dehydrated, and subjected to digestion buffer (Promega, Madison, WI, USA) at 37 °C overnight. The tryptic peptides were re-extracted twice in 10 μl of a 5% formic acid and 50% acetonitrile solution. The extracted peptide solution was concentrated by vacuum centrifuge and dissolved in 2 μl of 5% formic acid. The peptides were mixed with matrix solution (50% acetonitrile, 0.5% trifluoroacetic acid saturated with α-cyano-4-hydroxycinnamic acid), then sat until dry on a sample plate. Peptide examination was conducted by a MALDI-TOF MS (AutoFlex; Bruker Daltonik GmbH, Bremen, Germany) in linear reflection mode. Spectra was calibrated externally using a ProteoMass peptide and protein MALDI-MS calibration kit (Sigma–Aldrich) containing bradykinin fragment 1–7 (*m/z* 756.3997), angiotensin II (*m/z* 1045.5423), ACTH fragment 18–39 (*m/z* 2464.1989), and insulin chain B oxidized (*m/z* 3493.6513) standards. The MS data and peak lists were generated using flex Analysis (Version 2.0, Bruker).

Proteins were identified using peptide mass fingerprinting (PMF) with the search programs MS-FIT (Version 4.27.1) of the Protein Prospector program (prospector.ucsf.edu, 28.06.2010). The SWISS–PROT (10.09.2007) and NCBInr (10.09.2007) databases were used for comparison with the following parameters: a mass tolerance of 1Da, one missed cleavage, at least four matched peptides, and Pfactor set as 0.4. Fixed carboxyamidomethylation at cysteine and possible oxidation at methionine were also considered for the queries. Calculations of experimental pI and Mw on gels were performed using PDQuest (Bio-Rad), based