Ecotoxicology and Environmental Safety 79 (2012) 247–255

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Cyto-genotoxic effects induced by three brominated diphenyl ether congeners on the freshwater mussel *Dreissena polymorpha*

Marco Parolini *, Andrea Binelli

Department of Biology, University of Milan, Via Celoria 26, 20133 Milan, Italy

ARTICLE INFO

Article history:
Received 26 September 2011
Received in revised form
9 January 2012
Accepted 9 January 2012
Available online 26 January 2012

Keywords:
Polybrominated diphenyl ethers (PBDEs)
Dreissena polymorpha
Biomarkers
Cytogenotoxicity

ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are a group of highly hydrophobic and persistent chemicals that has been used as flame retardants in several industrial applications. They have been detected in various environmental matrices worldwide and an increasing number of studies have recently been carried out to investigate their potential toxicity on ecosystem communities. Although a variety of biological damage has been documented in vertebrates, the effects on invertebrates are largely unknown. The objective of the present study was to determine the cyto-genotoxic effects induced by single exposure to three concentrations of 2,4,2′,4′-tetra BDE (BDE 47), 2,2′,4,4′,6-penta BDE (BDE-100) and 2,2′,4,4′,5,6′-hexa BDE (BDE-154) on the freshwater mussel *Dreissena polymorpha* by a multi-biomarker approach. We performed on bivalve hemocytes the Single Cell Gel Electrophoresis (SCGE) assay, the DNA Diffusion assay and the Micronucleus test (MN test) to assess genotoxicity, while the Neutral Red Retention Assay (NRRA) was used to evaluate cytotoxic effects. Results showed that BDE-47 did not produce any genetic damage at the tested concentrations (0.1 μg/L, 0.5 μg/L and 1 μg/L), while BDE-100 and BDE-154 can be considered moderately genotoxic, since both primary and fixed DNA injuries were induced. The NRRA indicated a moderate increase in cellular stress in BDEs-treated bivalves. Thus, our data seems to suggest that investigated BDEs may pose a low risk to freshwater mussels at environmental concentrations.

1. Introduction

Since the 1970s, polybrominated diphenyl ethers (PBDEs) have been used as flame retardants in products such as plastics, textiles, polyurethane foam and electrical equipment (Lema et al., 2007). Three technical PBDE products have been extensively used as non-covalent additives in several products: Penta-BDE, Octa-BDE and Deca-BDE formulation. Penta-BDE contains primarily tetra- (BDE-47), penta- (BDE-99, -100) and hexa-BDE (BDE-153, -154) congeners. Octa-BDE contains primarily a hepta-BDE (BDE-183) plus hexa- (BDE-153, -154) and octa-BDEs, while Deca-BDE consists mainly of the fully brominated BDE-209 (La Guardia et al., 2006).

Since these molecules are not chemically bound to the products, PBDEs may be released into the environment via manufacturing processes, handling of treated products and disposal or recycling of PBDE-containing materials (Hale et al., 2003). These chemicals share a similar molecular structure, physicochemical and toxicological features with other persistent organic pollutants (POPs; Watanabe and Sakai, 2003), mainly polychlorinated biphenyls (PCBs). They are toxic, bioaccumulative and can undergo long-range atmospheric transport. Therefore, they are also on the proposed list of new chemicals to be included in the Stockholm Convention and in the POPs Protocol to the UN ECE Convention on Long-range Transboundary Air Pollution. Due to growing environmental and human health concerns, Penta- and Octa-formulations were banned in the European Union in 2004 (Restriction of Hazardous Substances Directive; EU 2002/95/CE), while their manufacturing was voluntarily ended in the US. Lastly, the use of Deca-BDE in the EU has been banned since 1 July 2008 (European Court of Justice, 2008). Unfortunately, these restrictions have not eliminated PBDE release from products currently in-service or new products manufactured using recycled PBDE-containing material (La Guardia et al., 2006). To date, investigations have reported more than 40 specific PBDEs, ranging from mono- to deca-BDEs in environmental matrices. Among these and in contrast to the production capacity, the less brominated BDEs (in particular BDE-47,-99 and -100) are the predominant congeners detected in biological samples, with BDE-47 generally accounting for 70 percent of the total PBDE content (Wollenberger et al., 2005). PBDEs are commonly found in the tissues of invertebrates, fish, birds and mammals from both marine (Bayen et al., 2003; Hites, 2004; de Wit et al., 2010) and freshwater (Covaci et al., 2002; Vives et al., 2004; de Boer et al.,

* Corresponding author. Fax: +39 02 50314713.
E-mail address: marco.parolini@unimi.it (M. Parolini).

0147-6513/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2012.01.008

2003; Binelli et al., 2008) environments, showing levels for individual congeners ranging from a few ng/g up to tens of μg/g lipid weight, depending on species and sampling site. Even if PBDEs have a widespread distribution, current knowledge of their potential toxic effects is limited. Some studies investigating the toxicity of commercial PBDEs on classical mammalian models have shown a wide range of effects, such as alteration in phase I biotransformation, morphological changes in hepatic and thyroid size and histology, interference with thyroid hormone homeostasis, developmental neurotoxicity and immunological alterations, caused by technical mixtures of PBDEs, single congeners and/or their hydroxylated metabolites (Darnerud et al., 2001). However, there is limited research on non-target aquatic organisms, revealing that PBDEs seem to have low acute toxicity, but a marked capability to induce sub-lethal effects. In fish, PBDEs inhibit liver enzyme activity, affect phase I and phase II biotransformation, produce fatty livers, alter blood levels of glucose and hematocrit, and reduce spawning success (Holm et al., 1993; Tjarnlund et al., 1998). Moreover, morphological abnormalities, as well as the impairment of cardiovascular function and cerebrospinal fluid flow in treated *Danio rerio* specimens, are caused by exposure to PBDEs (Lema et al., 2007). To date, toxicity information on invertebrates is limited to a few studies regarding selected congeners, which have revealed BDE-induced adverse effects on the development, reproduction and growth rate of copepods (Breitholtz and Wollenberger, 2003; Wollenberger et al., 2005) and shrimps (Key et al., 2008). Moreover, most of the studies focus on BDE-209, while data on the toxicity of low-brominated BDEs are absolutely inadequate and limited for several aquatic species (Kallqvist et al., 2006), even if they are more toxic (Birnbaum and Staska, 2004) and have a greater tendency to bioaccumulate with respect to the high-brominated ones (McDonald, 2002).

To expand knowledge on this topic, a suite of biomarkers was used on a classical freshwater biological model, the zebra mussel *Dreissena polymorpha,* to investigate potential cyto-genotoxic effects induced by three low-brominated BDE congeners: BDE-47, BDE-100 and BDE-154. We decided to test these BDEs because they are the main congeners of the Penta-BDE mixture, accounting for 42.8, 7.82 and 2.68 percent of the technical formulation, respectively. Accordingly, they are three of the most frequently found congeners in tissues of several aquatic organisms, including zebra mussel (Covaci et al., 2005; Binelli et al., 2008). End-points of four different biomarkers were measured in mussel hemocytes. The Neutral Red Retention Assay (NRRA) was used to assess cytotoxicity by evaluating lysosome membrane stability, while genotoxicity was evaluated by the Single Cell Gel Electrophoresis (SCGE) assay, DNA Diffusion assay and micronucleus (MN) test. Lastly, we performed simultaneous assessments of the levels of each single congener in zebra mussel soft tissues, investigating the dose-effect relationship.

2. Materials and methods

2.1. Reagent and standards

Standards of 2,2′,4,4′-tetra BDE (BDE-47, CAS number 5436-43-1), 2,2′,4,4′,6-penta BDE (BDE-100; CAS number 189084-64-8) and 2,2′,4,4′,5,6′-hexa BDE (BDE-154; CAS number 207122-15-4) were purchased from AccuStandard (New Haven, CT, USA). All reagents and chemicals used for biomarker determination were purchased from Sigma-Aldrich (Steinheim, Germany). All solvents used in chemical analyses were of pesticide grade. Florisil (100–200 mesh) was obtained from Fluka (Steinheim, Germany), while silica gel for column chromatography (70–230 mesh) was supplied by Sigma-Aldrich (Steinheim, Germany). Mixtures of commonly occurring BDE congeners (PBDE-COC) used for quali-quantitative determination of investigated congeners and two $^{13}C_{12}$-labeled mixtures used as internal surrogate standards (PBDE-MXA and PDE-MXB; composed of $^{13}C_{12}$-labeled BDE-47, 99, 153 and BDE-28, 154 and 183, respectively) were purchased from AccuStandard (New Haven, CT, USA).

2.2. Mussel acclimation and maintenance conditions

Several hundred *D. polymorpha* specimens attached to rocks by their byssi were collected in September 2008 by a scuba diver at a depth of 4–6 m in Lake Lugano (Northern Italy), which is considered a reference site due to its low chemical pollution (Binelli et al., 2005). Mussels were quickly transferred to the laboratory in bags filled with lake water. After rinsing under running tap water, rocks were immersed in 100-L glass holding aquaria filled with tap and lake water (75:25 v/v) in order to avoid a drastic change in water chemistry and to guarantee a food supply to mussels for the first 24 h of acclimation. Specimens were maintained at a natural photoperiod, constant temperature (20 ± 1 °C), pH (7.5) and oxygenation (>90 percent of saturation). Bivalves were fed daily with an algae replacement-substitute-enrichment medium (AlgaMac-2000®, Bio-Marine Inc., Hawthorne, USA) and water was regularly changed for at least two weeks to gradually purify mollusks of possible pollutants previously accumulated in their soft tissues. Several specimens (~2000) with the same shell length (20 ± 2 mm) were chosen for in vivo tests. They were gently cut from the rocks and placed on glass sheets suspended in 15-L aquaria filled with 10 L of de-chlorinated tap water and maintained at the same conditions as described above. Three hundred specimens were put in each aquarium (Binelli et al., 2009; Parolini et al., 2010). Only specimens that were able to re-form their byssi and reattach themselves to the glass sheet were used in the experiments. Mussel viability was checked daily by the Trypan blue exclusion method, while biomarker baseline levels were checked weekly. Mussels were exposed to each single BDE only when target biomarker levels were comparable with the baseline ones obtained in previous studies (Binelli et al., 2009; Parolini et al., 2010).

2.3. Exposure assays

To give a marked ecological relevance to our research, BDE exposure concentrations were as similar as possible to those measured in surface water. To compare the toxicity of different tested congeners, we decided to test the same concentrations for each single BDE (0.1 μg/L, 0.5 μg/L and 1 μg/L). We carried out experiments in semi-static conditions for 96 h, since previous studies demonstrated that this period of time is enough to highlight sub-lethal effects on POP-treated zebra mussel specimens, including PBDEs (Riva et al., 2007; Binelli et al., 2008b). Control, solvent control (0.1 percent of acetone; ACE) and exposure aquaria were processed at the same time. The entire water volume (10 L) was changed daily and each single BDE was added up to the selected concentrations. A working solution (10 mg/L) for each congener was prepared by diluting the BDE standard in acetone. Exact volumes of working solution were added daily to each exposure aquarium, until the selected concentrations were reached. This procedure should guarantee a constant solution concentration of each single BDE congener over each 24-h period and prevent losses of contaminant, as well as the transformation of the parental compound into its metabolites. Specimens were fed daily 2 h before each water and chemical change in order to avoid the chemical from adhering to food particles and prevent changes in chemical bioavailability. Temperature (20 ± 1 °C), pH (7.5) and oxygenation (>90 percent of saturation) were checked daily. Specimens ($n=33$) were collected every 24 h from each aquarium to evaluate cyto-genotoxic effects on the hemocytes. After withdrawal, the soft tissues of each single specimen were pooled, frozen and stored at -20 °C until chemical analyses were performed.

2.4. Biomarkers of cyto-genotoxicity

Since methods and procedures of all the biomarkers applied in this study were described in detail by Parolini et al. (2010), only a brief description of the followed procedures are reported here. The NRRA method followed the protocol proposed by Lowe and Pipe (1994). Slides were examined systematically at 15-min intervals to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. Tests finished when dye loss was evident in at least 50 percent of the hemocytes. The mean retention time was then calculated from five replicates. The alkaline (pH > 13) SCGE assay was performed on hemocytes, according to the method adapted for zebra mussels by Buschini et al. (2003). Fifty cells per slide were analyzed using an image analysis system (Comet Score®), for a total of 500 analyzed cells per specimen ($n=10$). Two common DNA damage endpoints were evaluated: the ratio between migration length and comet head diameter (LDR) and the mean percentage of DNA in the comet tail. The apoptotic cell frequency was evaluated through the protocol described by Singh (2000). Two hundred cells per slide were studied for a total of 1000 cells per sample ($n=5$). The MN test was performed according to the method of Pavlica et al. (2000). Four hundred cells were counted per slide ($n=10$), giving a total of 4000 cells/treatment. Micronuclei were identified by the criteria proposed by Kirsch-Volders et al. (2000) and the MN frequency was calculated (MN‰).