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Effects of two PBDE congeners on the moulting enzymes of the freshwater amphipod *Gammarus pulex*

Eric Gismondi*, Jean-Pierre Thomé

Laboratoire d'Ecologie Animale et d'Ecotoxicologie (LEAE), Institue de Chimie, Bât. B6C, 15 allée du 6 Août, Sart-Tilman, Université de Liège, B-4000 Liège, Belgium

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ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are abundant in aquatic environment. However, only few studies have investigated their impacts on freshwater invertebrates. This work aimed to study the effects of BDE-47 and BDE-99 congeners on the chitobiase and chitinolytic enzymes activities of the freshwater amphipod *Gammarus pulex*, according to gender, PBDE concentration and time of exposure. In addition, the bioaccumulation of BDE-47 and BDE-99 were measured. Results revealed that females have bioaccumulated more PBDE than males, and BDE-99 was more accumulated than BDE-47. PBDE exposures for 96 h have caused chitobiase and chitinolytic enzymes inhibition. This study not only indicate the importance of taking into account various confounding factors (gender, congeners, concentration) to understand PBDE effects, but underline also disruptions of molting enzymes activities. These disturbances suggest effects on the gammarid development and reproduction, and consequently effects on the gammarid population, and on a larger scale, a dysfunction of the ecosystem.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) constitute a class of chemical compounds included to the composition of usual products such as plastics, textiles or electrical equipment, due to their flame retardants properties (Alaee et al., 2003). Since 2004, PBDEs are banned in Europe and listed as Priority Substances within the European Union Water Frame Work Directive (Coquery et al., 2005). Unfortunately, the release of PBDEs in ecosystems continues due to their presence in products currently in use and new products manufactured using recycled PBDE-containing material (La Guardia et al., 2006). As PBDEs are environmental persistent xenobiotics and bioaccumulated in biota (de Wit et al., 2010), the need to determine their effects on biota is important. Indeed, recent studies have underlined that PBDEs could be bioaccumulated in tissues of some aquatic organisms such as fish (Tomy et al., 2004; Isosaari et al., 2005; Lema et al., 2007), bivalves (Gustafsson et al., 1999; Parolini and Binelli, 2012) or amphipods (Tlili et al., 2012). Despite these observations and the fact that PBDEs have a widespread distribution in environment, most of the studies which have investigated their potential toxic effects, have been conducted on mammalian models (e.g. rats, mice – Darnerud et al., 2001). However, investigations of PBDEs effects on aquatic organisms are increasing, and particularly the effects of the BDE-47 and BDE-99 congeners. Indeed, Breitholtz and Wollenberger (2003) have demonstrated that the BDE-47 congener could induce a decrease of the larval development and population growth rates of the copepod *Nitocra spinipes* exposed for full–life cycle. In addition, Baršienė et al. (2006) have underlined the induction of nuclear injuries in the gill tissues of the blue mussel (*Mytilus edulis*) exposed to 5 μg L^{-1} of BDE-47 for three weeks. These authors have also described an increase of micronuclei frequency as well as inductions of bi-nucleated and fragmented-apoptotic cells and nuclear buds. An increase of the micronuclei proportion was also observed in the bivalve *Dreissena polymorpha* exposed to BDE-47 (Parolini and Binelli, 2012). PBDEs can also affect the reproduction of organisms. For example, Muirhead et al. (2006) have observed, in the fish *Pimephales promelas*, a reduction of sperm maturation in males (>50%) and an egg production stopped in females exposed for 10 days to BDE-47. In this same organism, Lema et al. (2008) have highlighted that PBDEs could be an endocrine disruptor as BDE-47 caused an increase of the thyroid hormone (TH) receptor α in females and a decrease of the TH receptor β in both genders. The endocrine disruptor potential of PBDEs was also underlined by Wollenberger et al. (2005) in the copepod *Acartia tonsa* in which a BDE-99

* Corresponding author.
E-mail address: gismondi.eric@gmail.com (E. Gismondi).

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exposure induced an antagonistic effect of the ecdysteroid hormones (steroid hormones regulating development and reproduction in arthropods). More recently, Zhang et al. (2012) have demonstrated that BDE-47 could increase estrogen receptor in the scallop *Chlamys farreri*. In *Daphnia magna* neonates, Davies and Zou (2012) have shown that PBDEs disrupt the moult, which is controlled by ecdysteroid hormones.

Among freshwater species, *Gammarus* sp. is a suitable organism for ecotoxicological assessment of environmental pollutants at a large scale, because gammarids are present in most (if not all) rivers in Europe (Jażdżewski, 1980). Gammarids are known to be sensitive to pollutants and can easily be used in laboratory and field studies (Kunz et al., 2010). Hence, many ecotoxicological studies have been carried out using gammarids to evaluate impacts of xenobiotics (Leroy et al., 2010; Sornom et al., 2010, 2012; Gismondi et al., 2012a, 2013; Vellinger et al., 2012). However, to our knowledge, no study has been devoted to PBDEs effects on *Gammarus* sp., although this specie offers advantages (e.g. easy identification, widespread distribution) and plays important roles in the trophic chain (i.e. food resource for fish, amphibians and birds), but also in the organic matter recycling (i.e. leaf degradation).

In the present study, we investigated the effects of PBDEs on the physiological responses of the freshwater amphipod *Gammarus pulex*. According to the field study of Tlili et al. (2012), we have chosen to work particularly with the two major congeners bioaccumulated in *Gammarus pulex*, i.e. 2,2′,4,4'-tetrabromodiphenyl ether (BDE-47) and 2,2′,4,4′,5-penta-bromodiphenyl ether (BDE-99). In a first experiment, we studied the bioaccumulation of each PBDE in *Gammarus pulex* whole body. Then, as PBDEs are suspected to be endocrine disruptors (Wollenberger et al., 2005; Zhang et al., 2012) and that crustacean moult is hormonally controlled (Hyne, 2011), we investigated the effects of BDE-47 and BDE-99 on the activity of the three chitinolytic enzymes: β-N-acetylglucosaminidase, chitobiosidase and endochitinase. This measure ensures to assess the moulting disruption called the invisible endocrine disruption since the disruption of crustacean moulting is not readily seen in the field (Zou, 2005). In parallel, we measured the chitobioase activity (enzyme released into the medium during the moult) in the water of exposure, according to the time of exposure. To ensure a comprehensive view of the toxic effects of a PBDEs exposure on a population of Gammarid, all measurements were carried out in males and females separately.

2. Material and methods

2.1. Sample collection

Male and female *Gammarus pulex* were collected using a pont net in the Blanc-Gravier brook (50°34′60″ N and 5°34′60″ E, Colonster, Belgium) in autumn 2012. This site was chosen as a reference site due to its good physico-chemical quality (see Leroy et al., 2010 for details). Individuals were sorted out on the spot by observing gnathopods (smaller in females than in males) and transferred to the laboratory (~10 km) where they were maintained at 15 °C in large aerated aquaria and fed ad libitum with alder leaves, until PBDEs exposures.

2.2. Experimental solutions

Solutions of BDE-47 and BDE-99 were purchased from Sigma–Aldrich Co. BDE-47 and BDE-99 stock solutions of 1000 μg L^{-1}, diluted in acetone, were store in amber glass vials at −20 °C. The experimental concentrations of each PBDE were obtained by diluting the respective stock solution in mineral water (Volvic, France). To give a marked ecological relevance to our research, the exposure concentrations of BDE were chosen according to BDE concentrations measured in invertebrates collected in the field in a preliminary study (Thomé and Leroy, 2012). In addition, to compare the toxicity of the two studied congeners, we decided to test the same concentrations for each BDE, i.e. 0.1 μg L^{-1} and 1 μg L^{-1} (Table 1). Acetone controls were carried out in parallel to Volvic controls. As no significant differences were observed between these two controls, only the Volvic controls were included in results to avoid overloading results.

Table 1
Nominal and measured concentrations of BDE-47 and BDE-99 (μg.L^{-1}) in water of exposure.

	Control	BDE-47		BDE-99	
Nominal concentration (μg L^{-1})	0	0.1	1	0.1	1
Measured concentration (μg L^{-1})	0	0.17 ± 0.015	0.77 ± 0.036	0.12 ± 0.010	1.3 ± 0.006

2.3. Bioaccumulation assays

2.3.1. Exposure conditions

Contaminations were performed in glass Petri dishes (100 mm diameter and 20 mm high), previously saturated with the corresponding tested solutions for 3 days, to avoid BDE adsorption during the gammarids exposure. For each tested condition, three replicates of 5 *G. pulex* males or females were exposed at 15 °C for 96 h with a photoperiod of 16 h light and 8 h darkness. Experimental media renewed every day in order to guarantee a constant concentration of BDE over the exposure. During exposure, gammarids were not fed. At the end of the experiment, 3 pools of 5 *G. pulex* males or females were constituted for each condition, frozen in liquid nitrogen and stored at −80 °C before bioaccumulation analyses.

2.3.2. BDE concentrations in G. pulex

Each pool of *G. pulex* was lyophilized overnight using a Cryotec Lyophiliser. Then, pressurized liquid extraction (PLE) was performed on freeze-dried samples using an Accelerated Solvent Extractor (ASE™ 200) (Dionex, Sunnyvale, USA) with hexane Pestinorm ®:dichloromethane Pestinorm ® (90:10, v:v) as solvent. Fat extracts were dried under a gentle stream of nitrogen at 37 °C (TurboVap). Samples were resuspended in 3 mL of hexane Pestinorm® and doped with 50 μL of PCB 112 at 100 pg μL^{-1} as a surrogate, to controlled clean-up processes.

For the acid clean-up, samples were mixed with 2 mL of sulphuric acid 98% for 2 min and centrifuged for 3 min at 3000 rpm. Supernatants were transferred in new test tubes and the remaining samples were washed with 3 mL of hexane Pestinorm® and centrifuged for 3 min at 3000 rpm. The second supernatants were pooled with the first, and concentrated to 1 mL under a gentle stream of nitrogen, prior to the second clean-up on Florisil cartridges.

The Florisil clean-up was performed using supelclean Envi-Florisil SPE 6 mL column (Supelco, Bellefonte, USA). The 1 mL supernatants from the acid clean-up were eluted on the Florisil cartridges, which were then washed with 2 mL of hexane Pestinorm®, to ensure full recovery of the sample. The obtained samples were dried under a gentle stream of nitrogen and resuspended in 50 μL of hexane Pestinorm® and 50 μL of Mirex at 100 pg μL^{-1} as internal standard. Samples were analysed in capillary gas chromatography with an electron capture detector (GC-ECD) using a Thermoquest Trace GC 2000 (Milan, Italy).

Commercial solutions of BDE-47 and BDE-99 were used for the standard curve. PBDE concentrations in *G. pulex* were expressed in μg g^{-1} dry weight (dw).

2.4. Chitobiase activity and chitinolytic enzymes activities

2.4.1. Exposure conditions

BDE contamination was performed with the same BDE concentrations and photoperiod conditions as described above (see Bioaccumulation assays). The experiment was conducted using five replicates of one *G. pulex* male or female in similar intermoult C stage. Water of exposure were changed daily and 4 mL of each replicate of the same condition were pooled in a 20 mL-amber glass vial. Chitobiase activity measurements were carried out on the day water collection. At the end of the experiment, gammarids were frozen individually in liquid nitrogen and stored at −80 °C awaiting chitinolytic enzymes assays.

2.4.2. Chitobiase activity in water of exposure

The chitobiase activity assessment was performed according to the method developed by Hanson and Lagadic (2005) in 96-well microplate. The assay is based on the degradation of the 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (MUF-NAG) in 4-methylumbelliferyl acetate (NAG). Before proceeding to the assay, water of exposure was filtered on 0.22 μm membrane to eliminate bacteria that could also display chitobiase activity. Each sample was measured in duplicate; the average was calculated and used in all subsequent analyses. Chitobiase activity was expressed in μmol.hr^{-1}.

2.4.3. Chitinolytic enzymes activities in G. pulex

Chitinolytic enzymes activities were assessed by using the "Chitinase Assay Kit, Fluorescence" from Sigma–Aldrich Co.

Each *G. pulex* male or female was homogenized with a manual Eppendorf pestle in microtubes. The homogenization buffer was adjusted to the *G. pulex* wet weight (ratio 10:1, v:w) and was composed to CelLytic MT reagent (Sigma–Aldrich Co.)