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

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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 bio-accumulated 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.

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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 bio-accumulated 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 under-lined 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 micro-nuclei 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 in-crease 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

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′-tetra-bromodiphenyl 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.)

supplemented with Proteinase Inhibitor Cocktail (1 µL of Proteinase Inhibitor Cocktail for 100 µL of CelLytic MT reagent; Sigma–Aldrich Co.). Samples were centrifuged for 10 min at 15 000 × g, 4 °C and the resulting supernatants were 10-fold diluted in homogenization buffer before being used for the assay.

As three different substrates were necessary to measure chitinolytic enzymes activities (i.e. 4-methylumbelliferyl N,N′-diacetyl-β-D-chitobioside, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide and 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotriose used to assess chitobiosidase, β-N-acetylglucosaminidase and endochitinase activity, respectively), we used one 96-well microplate per substrate. Briefly, each 96-well microplate contained a blank (i.e. appropriated substrate solution), a sample blank (i.e. 95 µL of assay buffer and 5 µL of diluted sample), a positive control (i.e. 95 µL of appropriated substrate solution and 5 µL of control chitinase solution) and a five point 4-methylumbelliferone (MUF) standard curve. Each sample was measured in triplicate and the average was calculated and used in all subsequent analyses. Chitinolytic enzymes activities were expressed in µg min^{-1} mg protein^{-1}.

Protein concentrations were measured in 96-well microplate in the diluted supernatants by using the "Protein Quantification Kit-Rapid" from Sigma–Aldrich Co. based on the Bradford method (Bradford, 1976).

2.5. Statistical analysis

All data met normality and homogeneity of variance assumptions (Shapiro and Bartlett tests, $p > 0.05$). Our data were analyzed by using a univariate analysis of variance (ANOVA) to test the effect of PBDE congeners and concentration, gammarid gender, time of exposure and their interactions. Then, TukeyHSD post-hoc tests were used to describe significant differences. All tests were performed with a 5% type-I error risk, using R 2.9.0 Software.

3. Results

3.1. BDE-47 and BDE-99 accumulation

After 96 h of exposure, there was a dose-dependent accumulation of PBDE in *G. pulex*, whatever the gender and the PBDE congeners (Fig. 1). Indeed, the BDE-47 concentrations in *G. pulex* were 7 times higher in individuals exposed to 1 µg L^{-1} than in individuals exposed to 0.1 µg L^{-1}. Similarly, BDE-99 concentrations in *G. pulex* were an average 11-fold higher in gammarids exposed to 1 µg L^{-1} than in gammarids exposed to 0.1 µg L^{-1}. No differences were observed between BDE concentrations in males and females exposed to 0.1 µg L^{-1}, whatever the BDE congener. However, at the highest exposure concentration, the BDE concentrations in *G. pulex* were 1.5-fold higher in females than in males, whatever the BDE congener. Results revealed that, when exposed to the highest concentration, BDE-99 concentrations were 3.5 and 2.7 times higher than BDE-47 in males and females, respectively.

3.2. Chitobiase activity

Chitobiase activity was influenced by BDE congener, *G. pulex* gender and the time of exposure (Table 2, Fig. 2). Indeed, whatever the gender, chitobiase activity was different depending on whether gammarids had been exposed to BDE-47 or BDE-99. When exposed
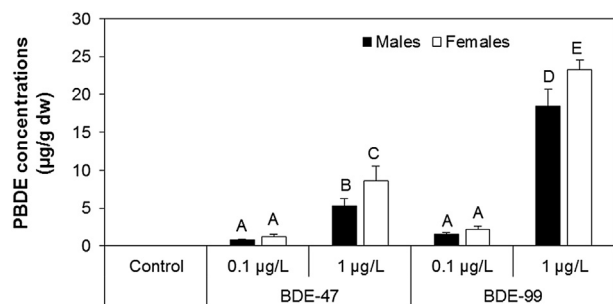
Table 2
Univariate analyses of variance (ANOVA) investigating variations in chitobiase activity in water of exposure according to PBDE concentrations and congeners (PBDE), gender and time of exposure.

Source of variation	Sum of square	d.f	F	p-Values
PBDE	1274.4	4	188.65	< 0.001
Gender	214.2	1	126.81	< 0.001
Time	2137.6	3	421.91	< 0.001
PBDE × Gender	153.6	4	22.75	< 0.001
PBDE × Time	3187.7	12	157.29	< 0.001
Gender × Time	501.0	3	98.88	< 0.001
PBDE × Gender × Time	819.1	12	40.42	< 0.001
Residuals	608.0	360		

to BDE-99, the major effect was observed in gammarids exposed for 48 h compared to respective controls. In females exposed for 48 h, whatever the BDE-99 concentration, the chitobiase activity was inhibited as compared to 48 h-females controls. In males exposed for 48 h to BDE-99 at 0.1 µg L^{-1}, the chitobiase activity was inhibited, while no effect was observed after a 48 h-exposure to BDE-99 at 1 µg L^{-1} as compared to 48 h-males controls.

When exposed to BDE-47 at 0.1 µg L^{-1}, only *G. pulex* males exposed for 48 h displayed a 1.5-fold increase of the chitobiase activity as compared to control. No differences were observed at other times of exposure in comparison to respective controls. After 24 and 48 h of exposure to BDE-47 at 1 µg L^{-1}, chitobiase activity was 3.5- and 1.5-fold higher than respective controls while after 96 h of exposure, the chitobiase activity was 3.3-fold lower than respective control. In females exposed to BDE-47 at 0.1 µg L^{-1}, chitobiase activity was 3.8-fold lower after 48 h of exposure and 1.5-fold higher after 96 h than respective controls. On the contrary, when females were exposed to BDE-47 at 1 µg L^{-1}, the chitobiase activity was 9-fold higher after 24 h of exposure, and was 1.6 to about 4.5 times lower after 48 h, 72 h and 96 h of exposure, as compared to respective controls.
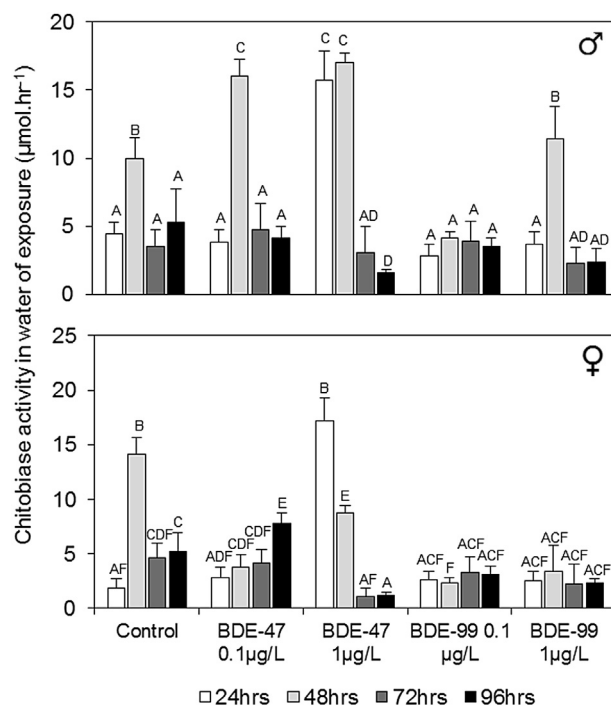

Fig. 2. Chitobiase activity in media from *G. pulex* males (♂) and females (♀) exposed to BDE-47 and BDE-99 at 0.1 µg L^{-1} and 1 µg L^{-1} (mean ± s.d., N = 5). Different letters above the bars indicate significantly different values (Tukey's HSD test, p-values < 0.05).

Fig. 1. Bioaccumulation of BDE-47 and BDE-99 in *Gammarus pulex* males and females exposed at two concentrations, 0.1 µg L^{-1} and 1 µg L^{-1} for 96 h (mean ± s.d., N = 3). Different letters above the bars indicate significantly different values (Tukey's HSD test, p-values < 0.05).

3.3. Chitinolytic enzymes activities

All the three chitinolytic enzymes were influenced by the BDE congener and concentrations, but only the β-N-acetylglucosaminidase and endochitinase activities were also influenced by the gammarid gender (Table 3, Figs. 3 and 4). However, only the β-N-acetylglucosaminidase activity was impacted by the interaction between these three factors. Whatever the gender, the total activity of enzymes involved in the chitin degradation (i.e. the sum of the three enzyme activities) is mainly represented by the activity of the β-N-acetylglucosaminidase.

In *G. pulex* males exposed to BDE-47 (Fig. 3), chitinolytic enzymes activities were decreased whatever the concentration compared to respective controls. At 0.1 μg L^{-1}, the activities of the β-N-acetylglucosaminidase, chitobiosidase and endochitinase enzymes were 1.4, 1.9 and 1.7-fold lower than control, respectively. At 1 μg L^{-1}, the three enzymes activities were 2.2, 1.8 and 1.5-fold lower than control. In addition, comparing the enzymes activities between the low and high BDE-47 concentration, only the β-N-acetylglucosaminidase activity was lower in males exposed to BDE-47 at 1 μg L^{-1} than in males exposed to BDE-47 at 0.1 μg L^{-1} (i.e. 1.5-fold lower). In contrast, no significant differences were observed when *G. pulex* males were exposed to BDE-99 at 0.1 μg L^{-1}. However, when exposed at 1 μg L^{-1}, the activities of β-N-acetylglucosaminidase and chitobiosidase were 2.1 and 1.5-fold lower than control, respectively.

In females, chitinolytic enzymes activities of females were weakly or not impacted by the BDE exposure (Fig. 4). Indeed, when exposed to BDE-47 at 0.1 μg L^{-1}, only the β-N-acetylglucosaminidase activity was 1.2-fold higher than control, while in females exposed to BDE-47 at 1 μg L^{-1}, only the endochitinase activity was 1.5-fold higher than control. After the BDE-99 exposure, whatever the concentration, no significant differences were observed in the β-N-acetylglucosaminidase activity, while the endochitinase activity was 1.7-fold higher than control. Chitobiosidase activity was significantly 1.4-fold higher and tended to be higher than control at 0.1 μg L^{-1} and 1 μg L^{-1}, respectively.

4. Discussion

4.1. Dose- and congener-dependent impact of PBDEs

Results of PBDEs bioaccumulation in *G. pulex* have highlighted that accumulation depends on the PBDE congener and concentration. Indeed, although at the lowest concentration of exposure and whatever the gender, no differences were observed between BDE-47 and BDE-99 bioaccumulated concentrations in *G. pulex*, at the highest concentration of exposure, BDE-99 was more accumulated in both genders than BDE-47. To our knowledge, no study was devoted to compare the bioaccumulation of BDE-47 and BDE-99 in freshwater invertebrates in laboratory conditions. However, this result is surprising as several field studies have highlight a greater bioaccumulation of BDE-47 as compared to BDE-99 in various aquatic species as flounder, blue mussel, aquatic oligochaete or amphipods (Christensen et al., 2002; Wu et al., 2008; Ciparis and Hale, 2009; Tlili et al., 2012; Waszak et al., 2012). In our study case, the higher BDE-99 accumulation in *G. pulex* as compared to BDE-47 accumulation could be explained by the fact that the BDE-99 concentration in the water of exposure was higher than the BDE-47 one, especially at 1 μg L^{-1} (see Table 1). It could be interesting to renew this bioaccumulation experiment over a range of BDE concentrations in order to confirm this result.

Measurements of chitobiase activity in the water of exposure revealed also that PBDEs effects are congener- and concentration-dependent. Indeed, whatever the gender, BDE-99 induces only few alterations of the chitobiase activity, while BDE-47 impacts the activity of this enzyme in most of the exposure conditions. No studies were found in the literature on the effect of PBDE on chitobiase activity. However, our results are consistent with those of Mhadhbi et al. (2012) who have demonstrated, in embryos and larvae of turbot *Psetta maxima*, that the toxic potency of BDE-47 was higher than the toxic potency of BDE-99. These authors have shown that LC$_{50}$ for embryos and larvae were 27.35 and 14.13 μg L^{-1} for BDE-47, and 38.28 and 29.64 μg L^{-1} for BDE-99, respectively. Similarly, Breitholtz and Wollenberger (2003), studying the larval

Table 3
Univariate analyses of variance (ANOVA) investigating variations in chitinolytic enzymes activities in *Gammarus pulex* according to PBDE congener and concentrations and gammarid gender (*p*-values in bold indicate significant effects of the considered parameter).

Parameters	Source of variation	Sum of square	d.f	F	p-Values
β-N-acetylglucosaminidase	Concentration	43.99	2	186.5	**< 0.001**
	Congener	0.57	1	4.89	**0.032**
	Gender	5.07	1	42.98	**< 0.001**
	Concentration × Congener	0.66	2	2.82	0.069
	Concentration × Gender	27.13	2	115.02	**< 0.001**
	Congener × Gender	3.04	1	25.75	**< 0.001**
	Concentration × Congener × Gender	5.37	2	22.79	**< 0.001**
	Residuals	5.66	48		
Chitobiosidase	Concentration	0.36	2	13.22	**< 0.001**
	Congener	0.71	1	50.84	**< 0.001**
	Gender	0.01	1	0.99	0.324
	Concentration × Congener	0.47	2	17.02	**< 0.001**
	Concentration × Gender	0.52	2	18.79	**< 0.001**
	Congener × Gender	0.001	1	0.10	0.752
	Concentration × Congener × Gender	0.04	2	1.39	0.259
	Residuals	0.67	48		
Endochitinase	Concentration	0.23	2	5.94	**0.005**
	Congener	0.68	1	34.50	**< 0.001**
	Gender	0.66	1	33.85	**< 0.001**
	Concentration × Congener	0.53	2	13.59	**< 0.001**
	Concentration × Gender	0.93	2	23.83	**< 0.001**
	Congener × Gender	0.01	1	0.64	0.426
	Concentration × Congener × Gender	0.006	2	0.16	0.849
	Residuals	0.94	48		

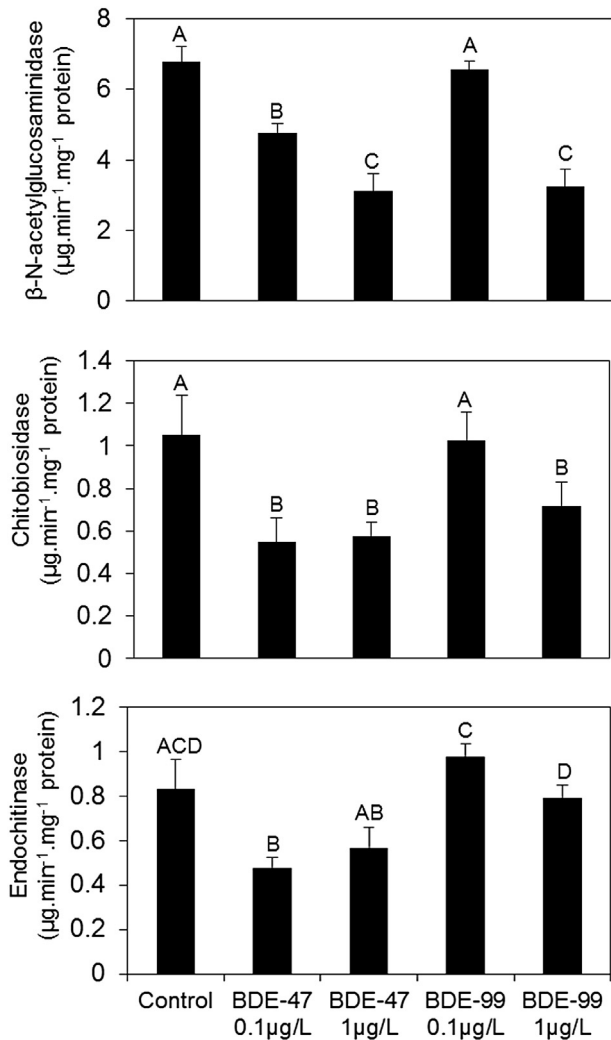**Fig. 3.** Activities of the three chitinolytic enzymes of *G. pulex* males exposed for 96 h to BDE-47 and BDE-99 at 0.1 μg L^{-1} and 1 μg L^{-1} (mean ± s.d., N = 5). Different letters above the bars indicate significantly different values (Tukey's HSD test, *p*-values < 0.05).

development inhibition of the copepod *Nitocra spinipes*, have observed that the Lowest Observed Effect Concentration (LOEC) of BDE-47 was lower than the LOEC of BDE-99 (i.e. 13 and 30 μg L^{-1}, respectively). Results of these studies as well as our results are in line with the general hypothesis which is based on the fact that the higher-numbered brominated congeners are less toxic than lower-numbered ones. The weak effect we have observed in gammarids exposed to BDE-99 could be explained by the possible biotransformation of the BDE-99 congener in BDE-47 by a debromination process, as shown by Noyes et al. (2010), and not to the presence of BDE-99 itself. This hypothesis is supported by the fact that we have measured low concentrations of BDE-47 congener in *G. pulex* males and females exposed to BDE-99 at 1 μg L^{-1} (i.e. 1616.1 ± 82.8 and 1286 ± 73 μg g^{-1} dw, respectively).

Another factor that could play a role in BDE impact on chitobiase activity is the congener configuration as it has been observed by Zou and Fingerman (1999a, 1999b) with 4-octylphenol and 4-tert-octylphenol in the crab *Uca pugilator*. Indeed, authors have shown that the first molecule inhibited the hepatopancreas chitobiase activity but not the epidermis one, whereas the opposite effect was observed for the second molecule.

4.2. PBDEs toxicity and gammarid gender

Except the chitobiosidase activity, all results revealed that BDE-47 and BDE-99 impacts in *G. pulex* were gender-dependent. The accumulation of these two congeners in *G. pulex* is not surprising due to the fact that Tlili et al. (2012) have previously demonstrated, in a field study, a PBDEs bioaccumulation in *G. pulex*. However, our results go further, since we have demonstrated a higher PBDE accumulation in females compared to males. This observation could be explained by the fact that PBDEs are highly lipophilic compounds (K$_{ow}$ ranges from 6.5 to 10; European Union (EU), 2001) and that Gammarid females have higher lipids content than males (Gismondi et al., 2012b). This hypothesis is supported by Viganò et al. (2009) who have shown that the accumulation of lower PBDEs is directly related to the lipid content of invertebrates. The higher concentrations of PBDE in females could induce a higher toxic impact in *G. pulex* females than in males. However, in our study, it seems difficult to confirm this hypothesis since PBDE exposure did not have the same effect on the chitobiase activity in females and males, especially when exposed to the BDE-47 congener. Indeed, when gammarids were exposed for 48 h to BDE-47, the chitobiase activity was inhibited in females while an increase was observed in males. At this point, we cannot determine which of these effects has a more negative impact than the other; although both
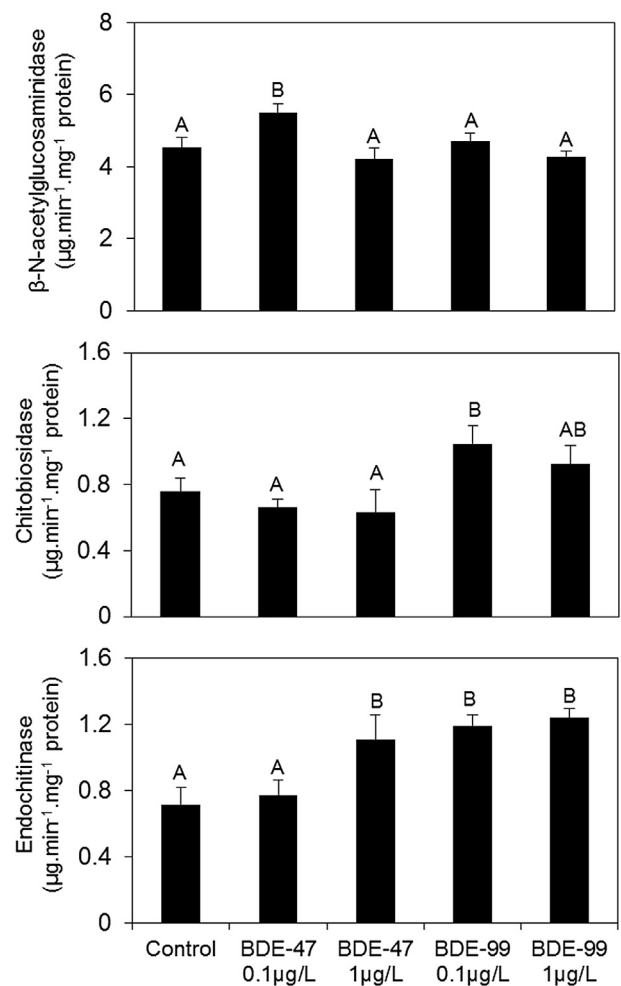

Fig. 4. Activities of the three chitinolytic enzymes of *G. pulex* females exposed for 96 h to BDE-47 and BDE-99 at 0.1 μg L^{-1} and 1 μg L^{-1} (mean ± s.d., N = 5). Different letters above the bars indicate significantly different values (Tukey's HSD test, *p*-values < 0.05).

could have effect on the gammarid moult cycle and thus, on gammarid reproduction and population (see section below).

Results of the activities of chitinolytic enzymes measured in organism after 96 h of exposure emphasize the fact that PBDEs effects are gender-dependent. Indeed, after BDE-47 exposure, we have observed an inhibition of the three chitinolytic enzymes in males, while there is overall a slight increase in females. Although chitobiosidase and endochitinase enzymes were not studied in aquatic invertebrates to our knowledge, the inhibition of the β-N-acetylglucosaminidase activity by xenobiotics was already observed, especially with pollutant having similar properties than PBDEs. In fact, Zou and Fingerman (1999a) have shown that the β-N-acetylglucosaminidase was inhibited in the epidermis and hepatopancreas of the crab *Uca pugilator* exposed for 3 and 7 days to PCB-29 congener. Our results confirm the fact that PBDEs disrupt the degradation of the chitin and thus, the moult cycle of gammarids, and that this disturbance is different in males and females. Therefore, it clearly appears that a PBDEs pollution could affect the gammarid population and thus, at long, the ecosystem functioning.

4.3. Gammarid population consequences

The increase or inhibition of the chitobiase activity observed in *G. pulex* suggests an increase or a decrease of the rate of chitobiase released by gammarids into the water and thus, modifications of the moult cycle. This result is in line with the recent study of Davies and Zou (2012) who have reported that PBDE exposure disrupt the moult of *Daphnia magna* neonates. Others studies which investigated the effect of xenobiotics on the chitobiase activity have shown similar results. For example, Richards et al. (2008) have demonstrated that an 24 h-exposure to fluoxetine caused the increase of the chitobiase activity in *Daphnia magna* and authors have suggested that this effect could accelerate the moult. An acceleration of the moult cycle has also been observed in other ecotoxicological studies, as in the crab *Uca pugilator* exposed to *p—p′*-DDT (Weis and Mantel, 1976) or in *Homarus americanus* exposed to emamectine benzoate (Waddy et al., 2002). In this last study, the authors have explained their results by the fact that the xenobiotic could inhibit the release of the moult-inhibition hormone (MIH), hormone which prevents the release of the molting hormone, i.e. hydroxyecdysone hormone (20-HE). Indeed, a MIH inhibition could cause an increase of the release of 20-HE and therefore, an increase of the activity of chitinolytic enzymes. However, it seems unlikely that this mechanism is involved in our study, due to the fact that our results have not shown a continuous increase of chitobiase activity during exposure, but only an increased after 24 h or 48 h, then an inhibition or a return to a similar activity than controls. Here, the principal hypothesis which could explain an increase of the chitobiase activity after 24 h of exposure would be an interaction between PBDEs and ecdysteroid hormone receptors, suggesting that BDE-47 could have ecdysteroid agonistic activity. However, the inhibition effect observed after 72 h of exposure suggests that BDE-47 congener, as well as BDE-99, could have ecdysteroid antagonistic activity, at long. These observations are in accordance with Wollenberger et al. (2005), who have demonstrated ecdysteroid antagonistic activity of BDE-99 in the copepod *Acartia tonsa* exposed for 5days. In addition, the paradoxical effect of BDE-47 observed at 24 h and 72 h are in line with results of Richards et al. (2008) who have shown an activation of the chitobiase activity of *D. magna* exposed for 24 h to fluoxetine, but an inhibition of this same enzyme activity after 72 h of exposure. These results underscore the importance of the response time in the assessment of the PBDEs effects.

The potential endocrine disruption of PBDEs in individual *G. pulex* could have serious consequences on the gammarid population.

Indeed, knowing that the gammarid reproduction is linked to moult cycle (Sutcliffe, 1992), changes in the moult cycle may cause disturbances in gammarids reproduction. First, due to the fact that new juveniles (brood in females marsupium) are released in environment after the moult of the females (Sutcliffe, 1992), we can hypothesis that an inhibition of the moult process in ovigerous females could lead to a late release of juveniles compared to their development. The second hypothesis is that PBDEs exposure could directly impact the juvenile's development in marsupium since this embryo development uses vitellus which is produced from lipids. Hence, a transfer of pollutant from females to eggs could happen, and PBDEs compounds ended up in eggs. This hypothesis is supported by Breitholtz and Wollenberger (2003) who have demonstrated that PBDEs reduced the larval development rate as well as the population growth rate of the copepod *Nitocra spinipes*. However, although our results suggest negative impacts of PBDEs exposures on juvenile's development and gammarid population, further works are required to investigate long-term effects of these pollutants.

5. Conclusions

To our knowledge, this study was the first to highlight physiological effects of PBDE exposure on chitobiase and chitinolytic enzymes activities in an amphipod gammarid, *Gammarus pulex*. Firstly, results underline some confounding factors which could bias the interpretation of the effects of PBDEs exposure. We demonstrated that females have bioaccumulated more PBDEs than males, and that the activities of the chitobiase and chitinolytic enzymes were more impacted by the BDE-47 exposure than BDE-99. Hence, it appeared that BDE-47 seems to be more toxic than BDE-99, particularly at the highest concentration of exposure ($1 \mu g.L^{-1}$). Thought the disturbance of chitobiase and chitinolytic enzymes activities observed in this study, PBDEs could affect the moult cycle of *G. pulex*, probably by affecting the hormonal system linked to the moult. Therefore, PBDEs appear as endocrine disruptors for gammarids, as there was previously observed by Wollenberger et al. (2005) on copepod. As in Gammaridae species, the reproduction and juvenile's release in environment are linked to the moult cycle, PBDE exposures could ultimately affect gammarids population, and therefore the freshwater ecosystem functioning. However, further work is needed to understand more accurately the impact of these emergent xenobiotics on the reproductive metabolism of *Gammarus pulex*.

Acknowledgements

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