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# Bioaccumulation of BDE-47 and effects on molecular biomarkers acetylcholinesterase, glutathione-*S*-transferase and glutathione peroxidase in *Mytilus galloprovincialis* mussels

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**Abstract** Mussels, *Mytilus galloprovincialis*, showed a high bioaccumulation ability when exposed to waterborne tetrabromodiphenyl ether (BDE-47), with a bioconcentration factor of 10,900 L $Kg^{-1}$ wet weight, and slow depuration rates in clean seawater. Kinetic and concentration–response experiments were performed measuring in the exposed mussel the activities of three molecular biomarkers: glutathione *S*-transferase (GST), glutathione peroxidase (GPx) and acetylcholinesterase (AChE). The long term (30 days) exposure of mussels to all concentrations (2–15 μg $L^{-1}$) of BDE-47 significantly inhibited the AChE and GST activities, a result that supports the suitability of these biomarkers in marine pollution monitoring programs. However, GPx activity showed a less consistent pattern of response depending on the concentration and the duration of exposure.

**Keywords** *Mytilus galloprovincialis* · Biomarkers · PBDE-47 exposure · Bioaccumulation · Acetylcholinesterase · Antioxidant enzymes

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## Introduction

Brominated flame retardants (BFRs) are a heterogeneous group of chemical substances whose common points are that they all contain bromine and are used to retard the combustibility and reduce flammability in electronic equipment, plastics, textiles, and building materials (Alaee and Wenning 2002; Prevedouros et al. 2004). About 80 different types of BFRs are used commercially, but since the 1970s the most common are the polybrominated diphenyl ethers (PBDEs), which have drawn much attention because of its persistence in the environment (De Wit 2002). Similarly to polychlorinated biphenyls (PCBs), PBDEs comprise 209 structurally related congeners, but only a few of them: penta-, octa-, and deca-BDEs, are used in commercial mixtures (Lema et al. 2007; Parolini and Binelli 2012).

Due to their high persistence, high potential for bioaccumulation, and toxicity to both humans and wildlife, PBDE release was recently regulated in many countries (Wolkers et al. 2004; Parolini et al. 2012). In fact, in 2009 the Stockholm Convention (Stockholm Convention 2014) on Persistent Organic Pollutants recognised several PBDEs (tetraBDE, pentaBDE, hexaBDE, heptaBDE, octaBDE) as posing serious risk to human health and to the environment, and were targeted for elimination under this global treaty (http://chm.pops.int). In this context, nine PBDE congeners (BDE-28, BDE-47, BDE-66, BDE-85, BDE-99, BDE-100, BDE-153, BDE-154 and BDE-183) were selected, on the basis of their toxicity and occurrence in the environment, to be determined routinely as part of the OSPAR Commission (2014) Coordinated Environmental Monitoring Program (CEMP) (http://www.ospar.org). Despite these regulations, PBDEs are nowadays commonly found in marine organisms at concentrations ranging from a few ng $g^{-1}$ up to

tens of μg $g^{-1}$, depending on the species and sampling site (Parolini and Binelli 2012). For instance, PBDE concentrations in *Mytilus galloprovincialis* mussels collected along the North Atlantic Spanish coast, represented as the sum of nine individual congeners, ranged between 0.052 and 0.741 μg $kg^{-1}$ wet weight (ww), with a mean value of 0.229 μg $kg^{-1}$ ww, and with the highest PBDE levels generally found in proximities of cities (Bellas et al. 2013). Other studies conducted with different species of marine mussels reported concentrations within the same range (e.g. Christensen and Platz 2001; Johansson et al. 2006; Gama et al. 2006; De Wit et al. 2006). The BDE-47 followed by BDE-99 are the dominant congeners in most profiles for different environmental matrices, including marine samples (Bellas et al. 2013; Gama et al. 2006; Johansson et al. 2006). However, despite the widespread distribution of PBDEs in the marine environment, there is still insufficient information about the risk they pose in those ecosystems.

Mussels are widely distributed, sessile filter-feeding organisms, which may be exposed to large amounts of chemical pollutants, and are capable of accumulating and tolerating high concentrations of many organic and inorganic pollutants in their tissues (Livingstone 1991). However, not many works have been published about the effects of PBDEs on these organisms. Aarab et al. (2006) demonstrated that BDE-47 induced serious perturbations in mussel (*Mytilus edulis*) gonad development. Other study by Barsiene et al. (2006) has shown the induction of micronuclei in blue mussel gills and elevated levels of other nuclear abnormalities after exposure to BDE-47.

In the present work, we investigated the effects of 2,2′,4,4′- tetrabromodiphenyl ether (BDE-47) on a suite of molecular biomarkers in *M. galloprovincialis*, including an antioxidant enzyme: glutathione peroxidase (GPx), a Phase II detoxification enzyme: glutathione *S*-transferase (GST), and the neurotransmitter catabolism enzyme acetylcholinesterase (AChE). GPx protects cells against the deleterious effects of oxyradical generation by maintaining endogenous reactive oxygen species at relatively low levels, and attenuating the damages related to their high reactivity (Livingstone 1990). GPx catalyzes the reduction of $H_2O_2$ originated from the dismutation of superoxide radical by the superoxide dismutase (SOD) activity into water or the reduction of organic peroxides to their corresponding stable alcohols by oxidizing the reduced glutathione (GSH) into its oxidized form (GSSG). An increased GPx activity has been reported in marine invertebrate species in response to low intracellular $H_2O_2$ concentrations (Orbea and Cajaraville 2006). Parolini et al. (2012) demonstrated that antioxidant enzymes such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) were significantly induced, while catalase (CAT) and glutathione *S*-transferase (GST) were depressed with respect to the baseline levels in zebra mussel (*Dreissena polymorpha*) exposed to BDE-154. The present study tests the effects of BDE-47 exposure on GPx activity of the marine mussel *M. galloprovincialis*.

GST are Phase II detoxification enzymes involved in the conjugation and detoxification of organic compounds, which also play a protective role against oxidative stress by catalysing a selenium-independent glutathione peroxidase activity (Prohaska 1980; reviewed by Sheehan et al. 2001). GST activity has been widely used as a biomarker of exposure to trace metals, PAHs, PCBs and dioxins both in fish and invertebrates (e.g. Fitzpatrick et al. 1997; Van der Oost et al. 2003; Funes et al. 2006), and has been recently identified as a suitable biomarker for monitoring chemical pollution in highly productive marine coastal ecosystems (Vidal-Liñán et al. 2010). We investigate here whether BDE-47 also induces the activity of the biotransformation enzyme GST or it is not metabolised and accumulates in mussel tissues.

AChE is an essential enzyme in the transmission of the nerve impulse frequently used in marine pollution monitoring (e.g. Bocquené and Galgani 1990; Tsangaris et al. 2010) because it has long been considered as a specific biomarker for organophosphate and carbamate insecticides (Galgani and Bocquené 1989; Escartín and Porte 1997), However, recent studies have shown that other types of pollutants, such as heavy metals, surfactants, PAHs and microplastics, may also unselectively inhibit AChE activity (Guilhermino et al. 1998; Akcha et al. 2000; Regoli and Principato 1995; Grintzalis et al. 2012; Oliveira et al. 2013). Therefore, we have investigated the effect of BDE-47 on AChE in order to test whether it is also affected by this chemical.

Another aim of the present work was to determinate the potential bioaccumulation and its kinetic parameters of waterborne BDE-47 in exposed mussels.

## Materials and methods

### Experimental procedure

Mussels between 40 and 45 mm long were collected from a pristine area from the outer part of Ria de Vigo (NW Iberian coast), and acclimated to incubation conditions in the laboratory for 1 week prior to experiments. Exposure was made in 30 L glass tanks with 20 mussels per tank, at constant temperature (15 °C), in darkness, using 1 μm filtered seawater with oceanic characteristics. Exposure tanks were continuously aerated with 0.22 μm filtered air, and were allowed to equilibrate for 1 h, before introducing the mussels. Water was renewed three times per week after feeding mussels for 1 h with a mixed diet of *Isochrysis*

*galbana*, *Tetraselmis suecica* and *Chaetoceros gracilis*. Every day with no water renewal toxicant was added to maintain nominal concentrations assuming an exponential decay.

A primary stock solution of 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), 98 % purity from Chem Service (West Chester, Pensilvania), was prepared by dissolving the product in dimethylsulphoxide (DMSO) to a nominal concentration of 80 mg $L^{-1}$. Fresh stock solutions were prepared every 7 days, and stored at 5 °C in the dark when not in use. Exposure BDE-47 concentrations in all experiments were always selected below the water solubility limit for this chemical.

Water samples intended for chemical analysis were taken at 0 h to check the initial concentration and at 24 and 96 h before water renewal to monitor the concentration of BDE-47 in the exposure media.

### Kinetics experiment

To evaluate the kinetics of accumulation and effects of BDE-47 on the biomarkers after different exposure times, over 450 mussels were exposed for 30 d to experimental solutions containing 8 μg $L^{-1}$ of BDE-47, followed by a 10 d depuration period. Samples of mussels were taken for biochemical and chemical analyses after 0, 2, 5, 9, 15, 20 and 30 d exposure, and after 10 d of depuration.

### Concentration–response experiment

To study the concentration–response relationship between BDE-47 exposure and the biomarker responses, over 240 mussels were exposed for 30 d to experimental solutions containing 2, 4, 8 and 15 μg $L^{-1}$ of BDE-47. After 0, 7 and 30 d exposure, samples were taken for biochemical and chemical analyses.

In both experiments seawater control, DMSO control and BDE-47 treatment groups were processed at the same time. DMSO control and BDE-47 treatment tanks contained <0.1 % DMSO (v/v) which was constant in all treatments for a given experiment.

Thirty individuals from each experimental group were taken at every sampling time. Gills from 24 mussels were dissected out and processed for subsequent biomarker analysis. Studied end-points included AChE, GST and GPx activities in the gills. The remaining 6 individuals from each treatment were used for chemical analyses.

### Chemical analyses in water and mussels

Water samples intended for BDE-47 analysis were extracted with hexane, dried over sodium sulphate, and concentrated in a vacuum evaporator (Büchi Rotavapor R-200).

Mussels intended for chemical analysis were placed in clean artificial seawater for 5 min and then washed with ultrapure water to remove the exposure media and the weakly adsorbed toxicant from the mussel cavity. Subsequently, one pool tissues from 6 mussels were kept at −20 °C until analysis. The analytical method followed for the analysis of mussels (Covaci et al. 2003) is based on a Soxhlet extraction using a solvent mixture of n-hexane:dichloromethane (1:1) for 8 h. For each extraction, homogenised tissue (1 g) was mixed with sodium sulphate and spiked with appropriate recovery standard (BDE-77). Mussel samples were stored overnight in a desiccator before extraction. Lipids were removed from the extract using a chromatography column on 6 % deactivated alumina, n-hexane being the eluent, and fractionated on activated silica gel column with iso-octane as the eluent.

The concentration and composition of the congeners of PBDEs were determined by GC–MS using an Agilent gas chromatograph 6890 N equipped with an electronically controlled split/splitless injection port, coupled to an Agilent 5973 N mass selective detector operated in negative chemical ionization (NCI) mode. A concentrated extract (2 μL) was injected onto a CPSil8 CB (30 m × 250 μm i.d. × 0.25 μm film thickness) capillary column. The carrier gas was helium set at a constant flow 1 mL $min^{-1}$. The injection was made in pulsed splitless mode (pulse 58 psi, purge 1.5 min). The injector temperature was 275 °C and the oven temperature program was as follows: 90 °C held for 3 min, ramped to 210 °C at 30 °C $min^{-1}$, held for 20 min, ramped to 290 °C at 5 °C $min^{-1}$, held for 17 min and to 310 °C at 35 °C $min^{-1}$ and held for 5 min.

The monitored ions (m/z) were 79, 81, 159 and 161 for all congeners. For the internal standard (octachloronaphtalene) the ion (m/z) was 403.80. Methane was used as reagent gas. The MS transfer line temperature was held at 290 °C, the MS source and quadrupole temperatures were 200 °C and 106 °C respectively.

Quality assurance and quality control was performed through the analysis of procedural blanks and a duplicate sample for each batch of 10 samples. All the steps were performed under a quality assurance system that was checked periodically by participation in interlaboratory exercises (Wells and Cofino 1997). In addition, all the analyses were standardised and followed a strict quality control system, which guaranteed accuracy. The quality of the chemical analyses was controlled internally by analysing Certified Reference Materials [BDE-COC (Mixture of Commonly Occurring PBDE Congeners for Precision and Recovery) and BDE-AAP-A (Mixture of PBDEs Standard Solution for Accuracy & Precision from Accu-Standard)] along with each batch of samples, and externally, by participation in intercalibration exercises promoted by international institutions as the QUASIMEME Laboratory

Performance Studies from 1996 to date (QUASIMEME 2004). In QUASIMEME, a Z-score is calculated for each participant's data for each matrix/determinand combination which is given an assigned value. This assessment method is being used as an ISO/IUPAC standard. The |Z| values reported by the QUASIMEME Project Office were between −2 and 2, indicating satisfactory results.

### Bioaccumulation model

Bioaccumulation was modelled assuming first-order kinetics and constant BDE-47 concentration in water according to the expression (Landrum et al. 1992):

$$C_a(t) = \frac{C_w K_u}{K_d}\left(1 - e^{-K_d t}\right) \quad (1)$$

where $C_a$ $(t)$ is the concentration (μg $Kg^{-1}$) accumulated in mussels at time $t$, $C_w$ is the water concentration of BDE-47 (μg $L^{-1}$), $K_u$ is the uptake rate coefficient (L $Kg^{-1}$ $day^{-1}$), $K_d$ is the depuration rate coefficient ($day^{-1}$) and t is the time (days). $K_u$ and $K_d$ were estimated by least square fits of the accumulation data to Eq. (1) model.

The bioconcentration factor (BCF) is usually calculated as the ratio of the uptake rate coefficient to the depuration rate coefficient (BCF = $K_u/K_d$) (L $Kg^{-1}$). The Eq. (1) can be reparametrised to obtain directly the confidence intervals of BCF:

$$C_a(t) = C_w BCF\left(1 - e^{-K_d t}\right) \quad (2)$$

### Biomarker analyses

GST and GPx were analysed in the gills, which were homogenized with an Ultra-Turrax, at 1:2 (w/v) ratio, in 0.05 M potassium-phosphate buffer at pH 7.5 containing 2 mM EDTA. Samples were centrifuged at 15.000×g for 15 min at 4–7 °C. For the analysis of AChE activity, the gills were weighed and homogenized with a Potter glass homogenizer at a 1:2 (w/v) ratio, in 0.02 M phosphate buffer at pH 7.0 with 0.1 % Triton X-100 and centrifuged at 10.000×g for 10 min at 4 °C. Aliquots of the supernatant were utilized for the spectrophotometric determination of activity of the enzymes GST, GPx and AChE with an absorbance microplate reader (Biotek ELx 808) at a constant temperature of 20 °C.

GST activities were evaluated, according to Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The measurement was carried out at 340 nm (extinction coefficient, ε = 9.6 $mM^{-1}cm^{-1}$) in 0.1 M potassium-phosphate buffer at pH 6.5, 60 mM CDNB and 10 mM GSH.

**Table 1** Nominal and measured tetrabromodiphenyl ether (BDE-47) concentrations in the exposure water

| Nominal concentration (μg $L^{-1}$) | Measured concentration (μg $L^{-1}$) | | |
|---|---|---|---|
| | $t_0$ | $t_{24}$ | $t_{48}$ |
| 2 | 1.57 | 0.15 | 0.13 |
| 4 | 2.94 | 0.29 | 0.22 |
| 8 | 3.83 | 0.31 | 0.14 |
| 15 | 14.58 | 1.02 | 0.85 |

After 24 h exposure, samples were taken for analysis and water was spiked again with the nominal BDE-47 concentrations

GPx activity was measured in a coupled enzyme system where NADPH is consumed by glutathione reductase to convert the formed oxidized glutathione form (GSSG) to its reduced form (GSH), according to Halliwell and Gutteridge (1999). The decrease of absorbance was monitored at 340 nm (ε = 5.598 $mM^{-1}cm^{-1}$) in 0.1 M potassium-phosphate buffer at pH 6.5, 10 mM sodium azide ($NaN_3$), 40 mM GSH, 20 unit $mL^{-1}$ glutathione reductase, 2.4 mM NADPH and 2 mM hydrogen peroxide as substrate.

AChE activity was spectrophotometrically determined, as described in Bocquené and Galgani (1998), by measuring the increase in absorbance of the sample at 412 nm in the presence of 2.6 mM acetylthiocholine as substrate and 0.5 mM 5,5′- dithiobis-2-dinitrobenzoic acid.

Protein concentrations in the supernatants were measured according to Lowry et al. (1951) by using bovine serum albumin as standard. All the enzymatic activities are expressed as nmol $min^{-1}$ $mg^{-1}$ of protein.

### Statistical analyses

Data normality and homoscedasticity were verified using Shapiro–Wilk and Levene's tests, respectively. Differences among the means of the enzymatic activities in seawater control and DMSO control were analyzed by the Student's $t$ test. Once it was verified that there were no significant differences, DMSO control was used as reference. Two-way analysis of variance (ANOVA) was performed to investigate possible time-effect and concentration-effect relationships using time and BDE-47 concentrations as variables. Bonferroni post hoc test was used to evaluate significant differences ($p < 0.05$) between treated samples and related controls (time to time), as well as among exposure levels. The tests were performed using the SPSS statistical package version 15.0 and GraphPad Prism software version 4.01.

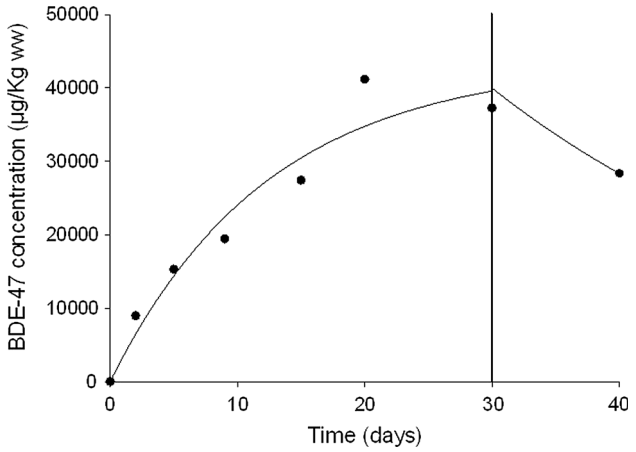


**Fig. 1** Concentration of tetrabromo diphenyl ether in mussels (*Mytilus galloprovincialis*) exposed for 30 days to 8 µg $L^{-1}$ and placed in clean seawater for 10 further days (adjusted $R^2 = 0.946$)

## Results

### Mussel bioaccumulation

Analysis of water samples revealed that initial BDE-47 concentrations in the dose–response experiments were 73–97 % of the nominal concentrations, but a rapid decrease was observed after 24 h. The same decrease took place again from 24 to 48 h after spiking nominal concentration at 24 h (Table 1). Therefore, actual measured BDE-47 concentrations in the water were used for calculating the BCF.

BDE-47 concentrations in mussel tissues measured before exposure were 0.018 µg $Kg^{-1}$ ww. Those concentrations are equivalent to the background levels found in the NW Iberian coast (Bellas et al. 2013). After only 48 h of exposure, BDE-47 concentration was 8,960 µg $Kg^{-1}$ ww in mussels exposed to 8 µg $L^{-1}$. BDE-47 uptake was approximately linear with time up to 20 days of exposure, but saturation was observed further on up to day 30 (Fig. 1). Kinetics of bioaccumulation was accurately described by Eq. (1), and parameters were statistically significant. High adjusted $R^2$ (0.946) and consistency of the model ($p < 0.001$) were obtained. A high bioconcentration factor (BCF) was observed (10,900 ± 4,100 L $Kg^{-1}$), with an uptake rate coefficient ($K_u$) of 875 ± 437 L $Kg^{-1}$ $day^{-1}$. The model predicted a low excretion rate constant (0.081 ± 0.067 $day^{-1}$), and this was confirmed by the depuration experiment. After 10 d of exposure in clean seawater the reduction in BDE-47 in the mussels was only 23.89 %.

The dose response experiments also showed very rapid BDE-47 uptake (Table 2), and at the end of the 30 d exposure BDE-47 concentrations were 52.6, 48.7, 37.2 and 144.3 mg $kg^{-1}$ ww, in mussels exposed to 2, 4, 8 and 15 µg $L^{-1}$, respectively. However, the strong decrease in dissolved BDE-47 that took place after 24 h caused actual BDE-47 concentrations very similar in the three lower treatments (Table 1). This explains the lack of dose–response pattern in the BDE-47 accumulated in mussels.

**Table 2** Nominal tetrabromodiphenyl ether (BDE-47) concentration in water and BDE-47 concentrations measured in mussels exposed for 7 and 30 days

| Nominal concentration in water (µg $L^{-1}$) | Measured concentration in mussel (µg $Kg^{-1}$ ww) | |
|---|---|---|
| | $t_7$ | $t_{30}$ |
| 2 | 6,958.18 | 52,607.99 |
| 4 | 17,460.21 | 48,744.57 |
| 8 | nm | 37,248.19 |
| 15 | 75,688.79 | 144,267.72 |

*nm* not measured

### Biomarker analyses

Figure 2 shows AChE, GST and GPx activities in gills of mussels exposed to a nominal concentration of 8 µg $L^{-1}$ of BDE-47 for 30 days. AChE baseline levels are comparable with those obtained in Vidal-Liñán and Bellas (2013). Significant inhibition of AChE activity was observed at 2, 9, 20 and 30 days (Fig. 2). After 10 days of depuration, the enzyme activity increased 2 times its value, observing a recovery to pre-exposure levels.

GST baseline levels are comparable with those obtained in wild mussels (*Mytilus galloprovincialis*) located in highly productive ecosystems (Vidal-Liñán et al. 2010). Exposed animals showed significantly lower GST activity than the corresponding DMSO controls after 20 and 30 days (Fig. 2). In this case, after 10 days of depuration, GST activity levels remained low, but the same pattern was observed in the control and no significant differences were observed between exposed and unexposed animals after recovery.

In the case of the GPx activity in gills, background values obtained were similar with those obtained in Vidal-Liñán et al. (2010). GPx activity in BDE-exposed mussels was significantly lower than DMSO controls after 20 and 30 days exposure, but recovered up to values not significantly different to control after the 10 days depuration period (Fig. 2).

Figure 3 shows AChE, GST and GPx activities in gills of mussels exposed to 2, 4, 8 and 15 µg $L^{-1}$ of BDE-47 for 7 and 30 days. Both AChE and GST showed a significant inhibition at all concentrations tested only after 30 days exposure (Fig. 3). In contrast, GPx response was more dose-dependent. At low exposure levels (2 µg $L^{-1}$), GPx activity was significantly induced after 7 days exposure, going back to control levels after 30 days. However, animals exposed to medium and high concentrations (4 and 15 µg $L^{-1}$) showed an initial GPx significant inhibition followed by a significant increase of activity in the long term (30 days).

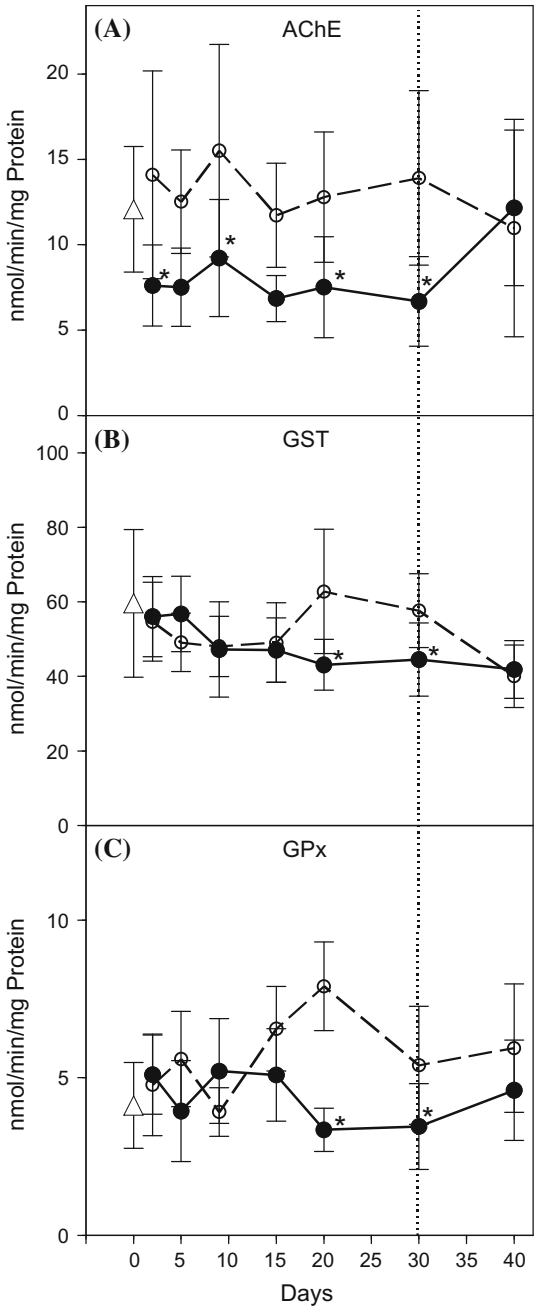


**Fig. 2** AChE (**a**), GST (**b**) and GPx (**c**) activities in gills of mussels exposed to 8 μg $L^{-1}$ of BDE-47 during 30 days and after 10 days depuration in clean seawater. *Open* and *close symbols* represent DMSO control and treatment, respectively. *Triangle symbol* represents baseline activity. Values show mean ± SD ($n = 12$). *Asterisks* indicate significant differences with respect to DMSO control (Bonferroni test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

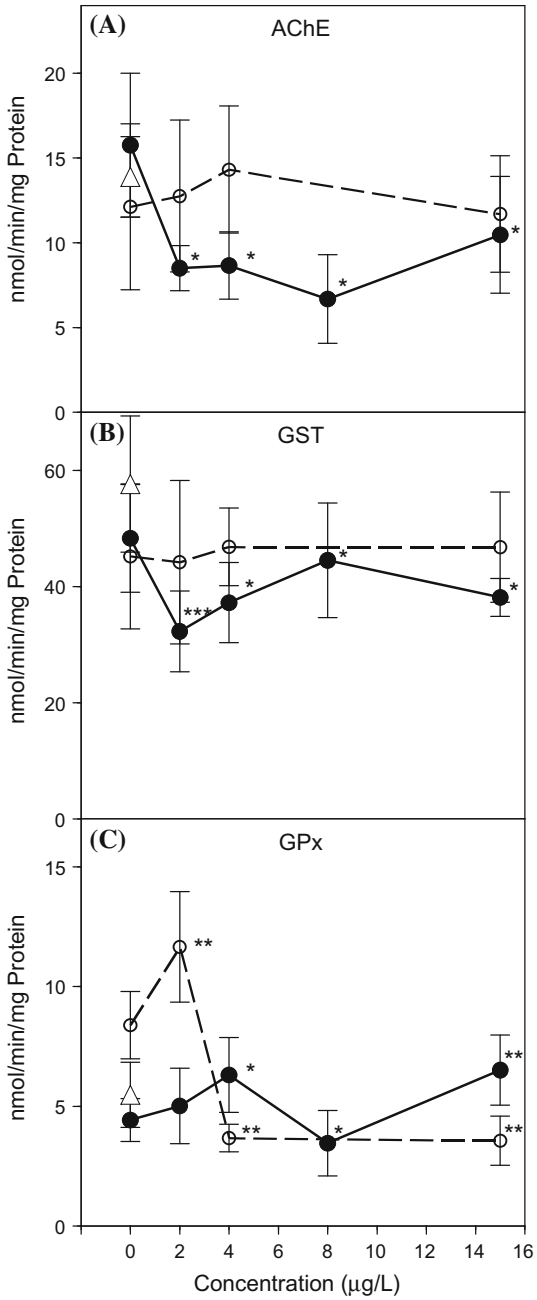


**Fig. 3** AChE (**a**), GST (**b**) and GPx (**c**) activities in gills of mussels exposed to 0 (DMSO control), 2, 4, 8 and 15 μg $L^{-1}$ of BDE-47 during 7 and 30 days. *Open* and *close symbols* represent treatments at 7 and 30 days, respectively. Values show mean ± SD ($n = 12$). *Asterisks* indicate significant differences with respect to DMSO control (Bonferroni test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

## Discussion and conclusions

### Bioaccumulation

European regulations (REACH) classify as Very Persistent and Very Bioaccumulative (vPvB) substances those with a half-life in marine, fresh or estuarine water higher than 60 days, and a bioconcentration factor greater than 5,000 L $Kg^{-1}$ (E.C. 2008).

In our study, the calculated BCF for BDE-47 in *Mytilus galloprovincialis* was 10,900 L $Kg^{-1}$ ww, confirming the high tendency of this compound to accumulate in biota.

Furthermore, a previous study from Gustafsson et al. (1999) using the same species, found a BCF for BDE-47 of 26,000 L $Kg^{-1}$ ww. However, these authors used a different methodology and mussels were fed during the exposure. Since BDE-47 shows a high affinity for particulate organic matter (Koc = 5.74 + E04 L $Kg^{-1}$) (estimated by EPI Suite software of Environmental Protection Agency United States), most BDE-47 could have been taken up by the mussels via food, which would explain the higher BCF values found in that study. Mhadhbi (2012) reported a BDE-47 BCF in turbot of 24,000 L $Kg^{-1}$ ww, in the same order of magnitude that those reported for mussels.

In the exposure phase of the kinetics experiment we have found an almost linear uptake of BDE-47 with time and very limited ability of excretion, as indicated by the low $K_d$ value (0.081 $day^{-1}$). The same feature has been previously found in mussels by Gustafsson et al. (1999) ($K_d$ = 0.09 $day^{-1}$) and in turbot by Mhadhbi (2012) ($K_d$ ranging from 0.006 to 0.02 $day^{-1}$). In fact, Gustafsson et al. (1999) found a better fit of bioaccumulation data to a straight line ($r^2$ = 0.996) than to an asymptotic model ($r^2$ = 0.80), and that was also the case of the turbot experiments from Mhadhbi (2012). According to the first order bioaccumulation model, dCt/dt = Cw*Ku − Ct*Ke, a linear uptake would indicate that dCt/dt = Cw*Ku = constant, which is consistent with $K_d$ values close to zero.

The behaviour of BDE-47 in solution is not stable, as demonstrated by the analysis of water samples (Table 1), which shows a rapid decrease with time. After 24 h the BDE-47 concentration in water falls to approximately 10 % of initial values. This decrease cannot be explained by the uptake of mussels which, considering the mussel biomass per aquarium, aquarium volume, and measured BDE uptake, should account approximately for a 30–40 % reduction in the water concentration in 24 h. Exposures were conducted in glass aquariums in the dark. However analytical measurements detected a rapid decrease in BDE-47 concentrations in the exposure water. Under these conditions one of the assumptions of the asymptotic model normally used for BCF calculations, namely constant water concentration, is not fulfilled. Therefore the BCF values calculated using the asymptotic model are underestimated, and actual values would be higher should water concentrations be actually constant.

## Molecular biomarkers

As far as we know, this study is the first one to report the effects of BDE-47 on the GST, GPx and AChE activities in marine mussels, *Mytilus galloprovincialis*. In this work, we demonstrate by first time neurotoxic effects of BDE-47 on mussels. AChE activity presented significant inhibition at all concentrations tested after 30 days exposure, and after 2, 9 and 20 days exposure in the kinetics experiment. Nevertheless, this inhibition was reversible, and this enzyme recovered the pre-exposure levels after the 10 d depuration period (Fig. 2). McHenery et al. (1997) also showed that mussel gill AChE activity can recover to almost pre-exposure levels after a first exposure of dichlorvos, though the degree of recovery is not as great after a second exposure. Recent studies have addressed the effects of PBDEs on AChE activity in other aquatic organisms. Chen et al. (2012) observed that this enzyme activity was significantly inhibited in larvae derived from the adult zebrafish (*Danio rerio*) exposed to 0.16, 0.8 and 4 μg $L^{-1}$, compared with controls. In other study with the Artic spider crab (Minier et al. 2008), muscle AChE activity was significantly inhibited in individuals exposed to 5 μg $L^{-1}$ during 3 weeks. On the contrary, Key et al. (2009) observed that AChE activity from the estuarine fish, *Fundulus heteroclitus*, showed values significantly higher than controls in the lowest concentration of 0.0125 mg $L^{-1}$.

In the present work, GST activity exhibited values significantly lower than control at all concentrations tested after 30 days exposure and after 20 days exposure to 8 μg $L^{-1}$. Therefore our results do not support a role of GST in BDE-47 detoxification by mussels. The decrease in GST activity may suggest a failure in detoxification and occurrence of oxidative stress in the cells (Santos et al. 2004). For instance, Zhao et al. (2011) indicate that the inhibition of GST activity in *Carassius auratus* when exposed to BDE-47 possibly results from the combination of reactive oxygen free radical and of electrophilic $Br^-$ with the GST active centre, leading to the disorder of its oxidation resistance and further to the toxic reaction of GST. Ji et al. (2013) observed that one protein of sigma class glutathione *S*-transferase 2 was significantly down-regulated in mussel gills exposed to 10 μg $L^{-1}$ of BDE-47, which was related to the reduction of reactive oxygen species (ROS) production. In accordance with our results, Parolini et al. (2012) observed lower GST activity in freshwater mussels, *D. polymorpha*, exposed to all concentrations of BDE-154 after only 24 h of exposure. Other laboratory studies with fish have proved that GST enzyme is not involved in PBDE debromination (Roberts et al. 2011; Browne et al. 2009). Therefore, we have hypothesized that such inhibition or lack of induction may be partially dependent upon the altered detoxification capabilities of this enzyme in the presence of BDE-47.

Unlike AChE and GST, the pattern of GPx activity in the control mussels was not time independent and showed marked variations at different recording times. Background GPx activity ranged from 4 to 8 nmol $min^{-1}$ $mg^{-1}$ prot, a 100 % variation, much wider than that for the other two biomarkers studied. In addition, when GPx activities in

mussels exposed to nominal concentrations of 8 μg $L^{-1}$ BDE-47 were compared to the corresponding controls at the same exposure times, significantly lower values were found at 20 and 30 days. However this was due to increased GPx activity in the DMSO controls at those exposure times, rather than to a decrease in GPx in the exposed mussels, which showed levels similar to the background recorded in pre-exposed individuals (Fig. 2). In contrast, when exposed to 2 μg $L^{-1}$ BDE-47 for 7 days, or to 4 μg $L^{-1}$ for 30 days GPx activity was significantly induced. This might be explained considering the theoretical bell-shaped pattern of response in inducible biomarkers that are inhibited at high doses, but this is not consistent with the significant induction found at the highest concentration tested after 30 days exposure (Fig. 3). Parolini et al. (2012) also found a time-dependent pattern of GPx response in zebra mussels exposed to low concentrations of BDE-154. This inconsistent and time-dependent pattern of response does not support the use of GPx as a biomarker of PBDE exposure in mussels.

In conclusion, this study confirmed that BDE-47 tend to accumulate in biota due to their low capacity for elimination which suggest that *M. galloprovincialis* mussels have a limited capability for the biotransformation of this compound. This study also validated the use of AChE and GST activities in exposed mussels as biological tools for determine the potential impact of BDE-47. AChE was the biomarker that yielded the highest sensitivity to this compound, proving for first time the existence of neurotoxic effects in mussels due to the exposure to BDE-47. The toxic effect of BDE-47 was also revealed by the inhibition of the GST at long exposure times and by the increased oxidative stress suggested by the induction of GPx at low concentrations.

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