BDE-47 exposure modulates cellular responses, oxidative stress and biotransformation related-genes in Mytilus galloprovincialis

BDE-47 exposure on *Mytilus galloprovincialis* modulates cellular effects and gene expression related to oxidative stress and biotransformation.

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Abstract

Polybrominated diphenyl ethers (PBDEs) are persistent pollutants characterized by elevated stability, and easily accumulated by marine organisms, where they induce a wide panel of negative effects. In this study, bioaccumulation, detoxification mechanisms and the toxic effects of BDE-47 were studied in *Mytilus galloprovincialis* fed with microalgae previously treated with increasing concentrations of PBDEs (maximum dose 100ng L-1 of BDE-47 per day). After 15 days of treatment, mussels were fed with the same diet without BDE-47 for additional 15 days. Gills and digestive glands were analyzed after 0, 15 and 30 days of exposure. Histopathological lesions were assessed in digestive glands, while expression of genes related to cell cycle, multidrug resistance, oxidative stress and detoxification was evaluated on both gills and digestive glands. After 15 days, BDE-47 exposure significantly affected the cell activity in digestive gland while, at 30 days, only mussels exposed to the lower doses showed a certain recovery. Regarding the gene expression, both gills and digestive glands showed a significant reduction of the target genes at 15 days, although most of them were up-regulated at 30 days in digestive gland. The results on BDE-47 accumulation in mussels revealed a dose-dependent increase of tissue concentrations, which remained elevated after further 15 days of depuration. This trend supports the responses of the biomarkers, indicating that exposure at environmentally realistic concentrations of BDE-47, strongly modulates oxidative stress and patterns of gene expression .

1 Introduction

Polybrominated diphenyl ethers (PBDEs) are used as flame retardants, being widely added to building materials, electronic products, textiles and foams. These compounds were introduced in the 70s, becoming the most popular class of flame retardants in the last decades (Covaci et al., 2011). PBDEs are characterized by high stability and environmental persistence (Eljarrat and Barceló, 2011), and they are known to accumulate in sediments, marine and terrestrial animals, including humans (Darnerud et al., 2001; Horri et al., 2018). Among PBDEs, commonly found compounds include the 2, 2′, 4, 4′-tetrabromodiphenyl ether (BDE-47), and the 2, 2′, 4, 4′, 5-pentabromodiphenyl ether (BDE-99) (Bi et al., 2007; Leung et al., 2006). The effects of PBDEs have been studied for many years in several biological models (Chao et al., 2017; Espinosa et al., 2019a, 2019b; Manuguerra et al., 2019; Mercado-Feliciano and Bigsby, 2008; Tang et al., 2018; Zhang et al., 2017),

and their toxicity has been demonstrated in terms of disruption on immune, reproductive, endocrine and neurological systems, being also suggested to be involved in cancer progression (Legler, 2008; McDonald T.A., 2002; Yu et al., 2015).

Marine mussels are frequently used as bioindicators for monitoring programs due to their capacity of accumulate and tolerate high levels of environmental pollutants, allowing to identify both temporal and geographical trends (Booij et al., 2002). The effects of PBDEs on mussels have been previously reported on hemocytes and gills damage (Ji et al., 2013; Riva et al., 2007; Vidal-Liñán et al., 2016), or immune responses (Jiang et al., 2017a, 2017b). The different effects of the pollutants in the organisms not only determine direct effects in their immunity, reproductive or endocrine systems, but could entail several alterations in bioenergetics and tissues/specimens development (Canesi et al., 2003; Luster et al., 1988).

In vertebrates, PBDEs can be metabolized to more polar compounds, for being eliminated through the bile or urine. It has been reported that PBDEs in mice or rats are transformed by phase I and phase II enzymes to hydroxylated, methoxylated and/or conjugated metabolites (OH-, Meo- and/or GS-PBDEs) (Malmberg et al., 2005; Qiu et al., 2007). In this respect, the phase I, II and III xenobiotic metabolizing enzymes have a key role in PBDEs metabolism and elimination. Some specific isoforms of cytochrome P450 enzymes (CYPs), such as CYP1A2, CYP3A4 and CYP2B, have been suggested as crucial in the biotransformation of these compounds (Stapleton et al., 2009; Szabo et al., 2009).

The specific pattern of BDE-47 metabolism, as well as the different effects that such compound can exert on mussels defense mechanism and cellular integrity, are not well understood. With the aim to clarify some of these aspects, the effects of PBDEs were investigated in *Mytilus galloprovincialis* fed with microalgae containing PBDE for 15 days, followed by a depuration period of additional 15 days with microalgae without contaminant. Histopathological analyses were carried out in digestive glands, while the expression of genes related to detoxification, oxidative stress, cell proliferation and multidrug resistance were measured both in digestive gland and gills.

2 Material and Methods

2.1 Mussels maintenance

Specimens of *Mytilus galloprovincialis* were adults (shell length: 6.5 ± 0.46 cm), obtained in May 2018 from a mussel farm of Messina (Italy), and acclimated to laboratory conditions for a month. Organisms were maintained in 10 L tanks containing filtered and aerated sea water under semi static conditions. The pH and salinity were adjusted to 7.95± 0.05 and 37.82 ± 0.24 %, respectively, temperature was kept at 22.6 ± 0.5°C with a 12:12 dark-light cycle, according to local environmental factors and the optimal condition for mussel's culture (Barón et al., 2016; Ji et al., 2013; Parrino et al., 2019). Mussels were fed with *Nannochloropsis sp*. at an approximate concentration of 1.2 x 10^6 cells mL^{-1}. The seawater was renewed periodically to maintain constant conditions.

2.2 BDE-47 inclusion into microalgae

BDE-47 standard was provided by SPECTRA (Rome, Italy). A stock solution (20 mg mL-1) was prepared by dissolving the powder compound in acetone. The feeding administration was done according to an exposure procedure for filter-feeding organisms and organic contaminants with high Log K_{ow} (Barón et al., 2016). Serial dilutions of the stock solution were done to prepare four doses of the contaminant in 4 mL of acetone. The different solutions were mixed in hermetically closed glass

vials with 1g of lyophilized microalgae (*Nannochloropsis sp.*), maintained in agitation for 24h using a programmable rotator-mixer Multi RS-60 (Biosan Ltd. Riga, Latvia), to support the BDE-47 inclusion in the microalgae. The vials were then incubated at 37°C overnight until all the acetone was evaporated. As a result, different vials were obtained with the microalgae containing 0, 0.333, 3.33 and 33.3 μg g^{-1} of BDE-47, as nominal concentrations, respectively. The microalgae with the different concentrations of BDE-47 were resuspended in 100 mL of filtered seawater, aliquoted and stored at -20°C until mussel treatment.

2.3 Experimental design

Acclimated mussels were randomly divided into five experimental groups, each containing three aquaria (12 mussels per aquarium). Mussels from each aquarium were daily placed in separate beakers containing 1L of seawater with aeration and acclimated for 30 min, before being fed with 3 ml of seawater containing 3 mg of *Nannochloropsis* sp. (approx. 1.2 x 10^6 cell mL^{-1}) treated as follow: control, acetone (vehicle control), and contaminated microalgae (0.333, 3.33 e 33.3 μg of BDE-47 per g of microalgae). This procedure corresponded to an exposure concentration of 1, 10 and 100 ng L^{-1} of BDE-47 each day (D1, D2 and D3, respectively), in the same magnitude previously reported by others authors (Ji et al., 2013; Jiang et al., 2017a; Vidal-Liñán et al., 2016; Parolini and Binelli, 2012; Vidal-Liñán et al., 2016).

Mussels were fed with the microalgae alone (control) or the vehicle alone (acetone) and the different concentrations (D1, D2 and D3) for 1-2h until the water was clear. Clearance rate was calculated for each treatment during the experiment, to ensure the mussels fed the microalgae. After treatment, mussels were maintained in renewed sea water for 30 min, before being placed again into their respective aquaria. Mussels were daily fed with the different diets for 15 days and with control diet (microalgae alone) for the following 15 days (depuration period). Three mussels from each aquarium (9 from each treatment) were randomly chosen at the beginning, the middle and the end of experiment (on day 0, 15 and 30). Samples of digestive gland and gills were stored in PUREzol Reagent (Bio-Rad, Hercules, CA, USA) at -80°C for gene expression analysis, while digestive glands were also processed for routine light microscopy.

2.4 Histopathological and morphometric study in hepatopancreas

Tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich, Saint Louis, USA) for 24 h, then dehydrated using increasing concentrations of ethanol and embedded in paraplast (Leica Biosystem, Richmond, IL, USA). Sections were cut at 3 μm, dewaxed, rehydrated, and stained with haematoxylin-eosin (HE). Slides were studied in a light microscope (Olympus BX60), images obtained with a Nikon DS-L3 digital camera (Nikon Corporation, Japan) and DS-L3 Digital Camera Controller acquisition software and used for the morphometric study by ImageJ 1.46r analysis software (National Institutes of Health, USA). The characterization of the digestive tubules was performed at different phases (absorptive phase, digestive phase, disintegrating phase, reconstructing phase, and holding phase, supplementary figure 1) as previously described (Tunali and Erkan, 2008). Loss of epithelial integrity edema, epithelial inflammation, nuclei diameter and presence of cell debris in the lumen of digestive tubules were monitored. Measurements included secondary digestive tubule height (μm, measured from the tip to the base of secondary tubule epithelium) and nuclei diameter (μm) from cells of secondary digestive tubules. The presence of cells in the lumen of digestive tubules was monitored, as well as signs of inflammation and edema per area of epithelium layer. Results from 3 independent specimens were obtained and analyzed (at least four slides from each specimen and six images from each slide).

2.5 Gene expression

Total cellular RNA was isolated from the samples of digestive glands and gills in PUREzol using Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA), and the concentration was assessed spectrophotometrically at 260 nm. The absorbance ratios A260/A280 and A260/A230 were evaluated as indicators of RNA purity. Then, 1µg of RNA were reverse-transcribed for each sample, in a volume of 20µL, by the 5X iScript Reaction Mix Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. The amplification was performed in a total volume of 20µL, which contained: 0.4µmol L-1 of each primer, cDNA diluted 1:10 of the final reaction volume, 1X IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and nuclease-free water. Conditions for real-time PCRs were optimized in a gradient cycler (C1000 Touch Thermal Cycler, Bio-Rad, USA) using the following run protocol: an initial activation step at 95°C for 3min, followed by 39 cycles of 95°C for 10s and 60°C for 30s, with a single fluorescence measurement. Melting curve program was achieved at 65-95°C with heating rate of 0.5°C/cycle and a continuous fluorescence measurement. All reactions were performed in triplicate. For each PCR, we checked linear range of a standard curve of serial dilutions. The relative quantification of [*catalase (cat*), *superoxide dismutase (sod), ras, multidrug resistance P-glycoprotein (pgp), cytochrome p450-4Y1 (cyp4Y1), glutathione-S-transferase (gst), glutathione-S-transferase subunits σ1* and *σ2 (gst-σ1* and *gst-σ2*, respectively)] gene expression was evaluated after normalization with the reference genes. Data processing and statistical analysis were performed using CFX Manager Software (Bio-Rad, Hercules, CA, USA). The primers used are shown in Table 1. The relative expression of all genes was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), using *Mytilus galloprovicialis* β-actin and 18S as the endogenous reference.

2.6 Extraction and analysis of BDE-47 in tissues

Total lipids were extracted from lyophilized mussels (individuals on the whole) according to Bligh and Dyer method (1957). In order to remove lipid compounds 500 µl of the extract was poured in a SPE Florisil (3 ml/200 mg) and eluted with 2 ml of Hexane:Acetone (80:20). The eluted was collected in a glass test tube and dried in gently current of N2. 1 ml of n-Hexane:Acetone (80:20) (containing internals standard) was spilled inside the test tube and recovered for GC/MS. Analysis of BDE-47 was carried out using a GC/MS Triple Quadrupole (by Thermo Fisher GC Trace 1310 coupled with a TSQ8000 mass spectrometry and Triplus RSH autosampler). The GC was equipped with a TG-5ms capillary column (15m x 0.25mm, 0.25µm) and with a PTV injector set in large volume mode. BDE47 was determined in SRM Mode using m/z 485.7 → 325.8 (CE 30eV) as quantificator and 325.8 → 219 (CE 30eV) as qualificator.

2.7 Statistical analysis

Statistical differences among the groups were assessed by one-way ANOVA analyses, followed by the Bonferroni or Games Howell test, depending on the homogeneity of the variables. The normality of the variables was confirmed by the Shapiro–Wilk test and homogeneity of variance by the Levene test. The significance level was 95% in all cases ($P < 0.05$). All the data were analyzed by the computer application SPSS for Windows® (version 20.0, SPSS Inc., Chicago, USA).

3 Results

3.1 Effect of sub-lethal PBDEs concentrations on survival and feeding

During the experiment, vitality and feeding were monitored. No mortality was observed except in mussels treated with D1 and D3, that showed 4% and 12% mortality at the end of the experiment, respectively. No changes occurred in the feeding, as attested by the clearance rate (data not shown).

3.2 Histology analyses

The exposure to BDE-47 caused various histopathological changes, such as increase in digestive tubule epithelial height, epithelial disruption, cellular and nuclear hypertrophy, hyperplasia, as well as cellular apical portion desquamation along with occurrence of nucleus towards the lumen (fig. 1-4). The percentage of edema in digestive tubules in absorptive phase, the presence of cells in the lumen, as well as the thickness of secondary digestive tubules and the nuclei diameter were quantified by image analysis at 15 and 30 days on mussels exposed to the control, lower and highest dose of BDE-47 (Table 2).

Our results revealed that exposure to BDE-47 significantly affected the percentage of edema in digestive tubules in absorptive phase, increasing from 8.61±0.63% in the control group, to 12.48±1.45% in mussels fed the D1 and 22.97±2.54% in mussels fed the D3 after 15 days. Furthermore, the percentage of edema was recovered after 30 days in D1 exposed mussels (7.91±1.54%), but remained elevated in D3 exposed mussels (15.68±1.31%) ($P<0.001$). The presence of cells in the lumen of digestive tubules significantly increased in mussels fed the D3 both at 15 and 30 days (22.06 ±2.4% and 20.1±2.1%, respectively) compared to the control (9.75±1.47%), while mussels fed the D1 showed lower percentages both at 15 and 30 days (3.85±0.44% and 2.37±1.28, respectively).

A significant increase of digestive tubule thickness was observed in mussels fed the D3 for 15 days (27.58±1.02µm) with respect to the controls (17.35±0.79µm) ($P<0.001$); however, this effect was significantly reduced after 30 days (14.29±0.35µm). No significant changes on digestive tubule thickness appeared in mussels fed the D1 both at 15 and 30 days (20.31±054 and 22.18±0.11µm, respectively). Our results showed a significant increase of nuclei diameter in cells of mussels fed the D3 at 15 days (4.51±0.12µm) compared to the control group (4.31± 0.09µm) ($P<0.001$), while a reduction occurred at 30 days (4.01±0.08µm). A significant reduction of nuclei diameters was also measured in mussels fed the D1, both at 15 and 30 days (3.95± 0.14 and 2.87± 0.06 µm, respectively) ($P<0.001$).

3.3 Gene expression

The results on expression of genes related to antioxidant activity, cell proliferation, drug resistance and xenobiotics detoxification in both digestive gland and gills are given in fig. 5-6. In digestive gland, the relative gene expression of *cat* resulted significantly decreased in mussels fed D2 and D3 for 15 days and significantly increased in mussels fed D3 at 30 days in respect to values of control mussels (fig. 5A). Transcription of *sod* decreased in mussels fed the highest dose of BDE-47 at 15 days, while no differences were observed between groups at 30 days (fig. 5B). Genes related to cell proliferation and drug resistance were significantly affected by the BDE-47 exposure: the relative expression of *ras* was significantly up-regulated in mussels fed D2 and D3 at 15 days, while mussels exposed to D3 maintained the up-regulation also at 30 days compared to control values (fig. 5C). The expression of *pgp* decreased in mussels exposed to D2 and D3 at 15 days, while it was still significantly increased at 30 days in mussels exposed to D3 (fig. 5D). The expression of genes related to the detoxification showed a significant decrease of *cyp4Y1* in mussels fed the D2 and D3 diets

after 15 days while, at 30 days, the mussels fed the highest dose of BDE-47 showed an increase of the same marker (fig. 5E). Also *gst* significantly decreased in mussels fed the highest dose of BDE-47 at 15 days, but no differences were found between groups at 30 days (fig. 5F). Finally, the relative expression of *gst-σ1* and *gst-σ2* showed a decrease in mussels fed the D2 and D3 at 15 days, while these genes were significantly up-regulated in mussels fed the D3 at 30 days (fig 5G-H).

In the gills, expression of *cat* significantly decreased in mussels exposed to D1, D2 and D3 at 15 days, and such decrease persisted in D2 and D3 treated mussels after 30 days (fig. 6A). Concomitantly, the expression of *cyp4Y1* significantly decreased in the gills of specimens exposed to D2 and D3 at 15 days, remaining down-regulated after 30 days in mussels exposed to the highest dose of BDE-47 (fig. 6B). Finally, the expression of *gst* showed a significant reduction in the gills of mussels exposed to the highest dose of BDE-47 at 30 days (fig. 6C).

3.2 BDE quantification in tissues

The determination of BDE-47 in mussels tissues highlighted a linear trend of increase in specimens fed from D1 to D3 (figure 7a), even after 15 days of detoxification: only specimens fed with the higher dose showed a slight reduction of the BDE content (as ng/g dry tissue) at 30 days. When data were normalized to the lipid fraction, the increasing trend is visible in all treatments, from D1 to D3 both after 15 and 30 days (figure 7b).

4 Discussion

In the present study we evaluated the molecular and cellular effects of BDE-47 on *M. galloprovincialis* chosen as a marine organism model. To provide environmental relevance to our results, realistic exposure concentrations were used, and administered through the diet (Gu et al., 2017; Ji et al., 2013; Jiang et al., 2017a; Vidal-Liñán et al., 2016). In fact, considering the high affinity of BDE-47 for particulate organic matter (K_{oc}=5.74 x 104L Kg^{-1} estimated by EPI Suite software of Environmental Protection Agency United States), the major route of exposure for this contaminant to mussels is expected to be via food (Gustafsson et al., 18AD; Vidal-Liñán et al., 2016).

In our study, histopathological parameters related to health and welfare of marine organisms revealed that mussels fed in presence of BDE-47 exhibit slight signs of inflammation at 15 days (edema in digestive tubule, increase of tubule thickness, change on nuclei diameters and presence of cells debris in tubule lumen), as previously described in mussels exposed to sub-lethal concentrations of dimethoate (Kumar et al., 2011; Tunali and Erkan, 2008). Both the edema and increase of tubule thickness are markers of inflammation, as cellular and nuclear hypertrophy is considered prognostic of toxicity, while the presence of cells debris in the lumen of the digestive tubule reflects the death of digestive cells (Kumar et al., 2011). These alterations have been described to be produced by increased sensibility, oxidative stress and excessive metabolic detoxification of organic xenobiotics, thus being considered as defense reactions (Kumar et al., 2011). The BDE-47 has been reported to produce oxidative stress and cytotoxicity in mussels (Jiang et al., 2017a; Parolini and Binelli, 2012), in line with our observations on both histopathological effects and accumulation of this compound. After 15 days of depuration (30 days of experiment), most of these alterations were disappeared in mussels exposed to D1, suggesting a certain capacity of recovery but also a limited bioaccumulation. Conversely, in mussels fed the higher dose of BDE-47, all the analyzed histopathological parameters were still affected and supported by data on concentrations of BDE-47 which were elevated after 30

252 days both in the whole tissues and lipid fraction, conforming the significant accumulation and
253 biological reactivity of this chemical in mussels.

254 Regarding the gene expression, BDE-47 exposure significantly modulated key markers related to
255 oxidative stress and phase I & II enzymes. The expression of *cat* was significantly down-regulated
256 after 15 days of BDE-17 exposure both in digestive gland and gills, and a similar trend was observed
257 also for *sod* in digestive gland. In the latter tissue the inhibition of these genes was recovered after 30
258 days (or even increased for *cat*), while the down-regulation was still evident in gills after the
259 depuration period. The induction of antioxidant defenses is interpreted as a counteracting capacity of
260 the organism toward an oxidative challenge, while the inhibition often reflects the overwhelming of
261 such defenses (Regoli and Giuliani, 2014). In this respect, digestive gland would present a greater
262 oxidative recovery compared to gills, in line with the generally higher levels of antioxidant defenses
263 in this tissue. Our results would confirm that BDE-47 exposure modulate oxidative stress *in vivo*,
264 consistent with the increase of ROS, MDA, superoxide anions, H_2O_2 and hydroxyl radicals observed
265 in hemocytes of *Mytilus edulis* exposed to this compound (Jiang et al., 2017a, 2017b). Despite these
266 authors observed a significant increase of catalase and superoxide dismutase activities on hemocytes
267 (Jiang et al., 2017b), antioxidant responsiveness greatly vary in different tissues and also the
268 comparison between antioxidant enzyme activities and gene expression is hampered by several
269 factors including exposure time, contaminant concentration, post-transcriptional, translational and
270 protein degradation regulations, availability of cell machinery for elevated protein synthesis (Vogel
271 and Marcotte, 2012; Giuliani et al., 2014). Further, antioxidant in marine organisms frequently
272 exhibit transitory responses, with both inductions and inhibition difficult to predict and partly related
273 to the complex interactions occurring within the oxidative/antioxidant network (Regoli and Giuliani,
274 2014). In general, our results support that PBDEs, are able to exert their toxicity via oxidative stress
275 (Fonnum et al., 2006; Tseng et al., 2008).

276 The significant induction of *ras* expression in D2 and D3 exposed mussels allow to suggest cell
277 proliferation as a protective mechanism toward oxidative damages and enhanced histological lesions
278 caused by BDE-47. On the other hand, down-regulation of *pgp* might contribute to explain the
279 limited efflux of this chemical and the resulting bioaccumulation.

280 The expression of phase I and II enzymes was significantly affected by BDE-47 exposure both at 15
281 and 30 days. The *cyp4Y1* expression was down-regulated in digestive gland and gills of mussels
282 exposed to the D2 and D3 doses after 15 days; at 30 days gene expression was at control values in
283 tissues of mussels exposed at D2, while upregulated in digestive gland and still inhibited in gills of
284 mussels exposed to the highest dose of BDE-47. Our results suggest that *cyp4Y1* is involved in
285 metabolism of BDE-47, in agreement with reported decrease of CYP4Y1 levels or *cyp4Y1* expression
286 in *M. galloprovincialis* after exposure to various toxicants or mixtures of contaminants (Snyder,
287 1998; Cappello et al., 2013; Gonzalez-Rey et al., 2014). The inhibition of CYP gene expression
288 confirm an oxidative challenge caused by BDE-47 since ROS can down-regulate the
289 biotransformation pathway from pre-transcriptional to catalytic, functional levels (Regoli and
290 Giuliani, 2014). Regarding to the markers of phase II, the expression of *gst*, *gstσ1* and *gstσ2* was
291 significantly down-regulated in mussels exposed to D2 and D3 of BDE-47 and a general recovery
292 was observed after 30 days only in digestive gland. Our results support a general modulation of the
293 phase I and phase II pathways by BDE-47, but also a minor biotransformation and excretion of this
294 chemical, confirming a limited metabolism of organic xenobiotics in mussels.

295 Overall, our results showed that cell activity on digestive gland was severely impaired by BDE-47
296 exposure. Concomitantly, gene expression of antioxidant defense and of phase I/II biotransformation

pathway were affected by BDE-47 exposure. A low degree of recovery after 30 days is in accord with data on BDE-47 quantification in tissues, attesting only a limited elimination of the compound via biotransformation. The exposure of BDE-47 at environmentally realistic concentrations, produced oxidative stress and promoted patters of gene expression that could entail cell transformation and/or long-term effects on growth, survival, reproduction of the species.

5 Conflict of interest

The authors declare that they have no conflicts

6 Author Contributions

The Author Contributions section is mandatory for all articles, including articles by sole authors. If an appropriate statement is not provided on submission, a standard one will be inserted during the production process. The Author Contributions statement must describe the contributions of individual authors referred to by their initials and, in doing so, all authors agree to be accountable for the content of the work. Please see here for full authorship criteria.

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8.1 Life Science Identifiers

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Figure 1. Representative micrograph of digestive glands in hepatopancreas from *Mytilus galloprovincialis* fed with a control-vehicle diet. DT: digestive tubule, L: lumen, SP: secretary products, CT: connective tissue. Stain: haematoxylin + eosin.

Figure 2. Representative micrographs of digestive glands in hepatopancreas from *Mytilus galloprovincialis* fed with different diets containing 1ng L^{-1} at 15 (A) and 30 days (B) and 100ng L^{-1} at 15 (C) and 30 days (D) of treatment. L: lumen, SP: secretary products, CT: connective tissue, →Edema, *Cell presence in the lumen and/or secretary products. Stain: haematoxylin + eosin.

Figure 3. Representative micrograph of secondary tubule in hepatopancreas from *Mytilus galloprovincialis* fed with a control-vehicle diet. HT: Hepatopancreatic tubule, CT: connective tissue. Stain: haematoxylin + eosin.

Figure 4. Representative micrographs of secondary tubule in hepatopancreas from *Mytilus galloprovincialis* fed with a control vehicle diet. fed with different diets containing 1ng L^{-1} at 15 (A) and 30 days (B) and 100ng L^{-1} at 15 (C) and 30 days (D) of treatment. HT: Hepatopancreatic tubule, CT: connective tissue, →Edema. Stain: haematoxylin + eosin.

Figure 5. Relative gene expression of some genes related to oxidative stress (*cat, sod*), cell proliferation (*ras*), multidrug resistance (*Pgp*) and xenobiotic detoxification (*cyp4Y1, gst, gstσ1* and *gstσ2*) in hepatopancreas from *Mytilus galloprovincialis* fed with different diets containing 1ng L^{-1} and 100ng L^{-1} at 0, 15 and 30 days of treatment. Values are the mean ± SEM (n=6). Statistical differences ($P < 0.05$) between groups are indicated by different letters.

Figure 6. Relative gene expression of some genes related to oxidative stress (*cat*) and xenobiotic detoxification (*cyp4Y1* and *gst*) in gills from *Mytilus galloprovincialis* fed with different diets containing 1ng L^{-1} and 100ng L^{-1} at 0, 15 and 30 days of treatment. Values are the mean ± SEM (n=6). Statistical differences ($P < 0.05$) between groups are indicated by different letters.

Figure 7. determination of BDE-47 in *Mytilus galloprovincialis* fed with different diets (control-co; vehicle –acetone; D1, D2, D3 (doses from 1ng L^{-1} to 100ng L^{-1}) at T0, T15 and T30 days of treatment. **a)** results expressed as ng of BDE-47/g dry tissue); b) results expressed as ng of BDE-47/ g total lipids. Values are the mean ± SEM (n=6). Statistical differences ($P < 0.05$) between groups are indicated by different letters.

Supplemental figure 1. Tubule phases in haepatopancreas from *Mytilus galloprovincialis*: a) absorptive phase, b) digestive phase, c) disintegrating phase, d) reconstructing phase, e) holding phase. Stain: haematoxilyn + eosin.

502 **Table 1**. *Mytilus galloprovincialis* primer sequences used for real-time PCR.

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Gene	Accession number	F/R Primer sequence (5'–3')
cat		F-AACCGAGAAACTCACCTGAAGGATCC
		R-ACCTTGGTCAGTCTTGAAGTGGAAT
sod		F-AGGCGCAATCCATTTGTTAC
		R-CATGCCTTGTGTGAGCATCT
ras	AY679522	F-ATGACGGAATACAAGCT
		R- TCCTTCTCCCGTTCTCAT
pgp	EF057747	F-CATCCGACTAGGGAATCCTAATG
		R- CGTTGTTTCTGTCCTCCTGATA
cyp4Y1	AF072855	F- ACCAGTTCCCTTAATTTCAAGAC
		R- GGGTTGTGATGGAGACCA
gst	JX485635	F- ACTGCTTGAGTGCCTGTT
		R- GACAGTGGCCACATACTCTT
gst-σ1	JX485636	F- GGACGAAACTAAGAAGCCTGATA
		R- GCTTATCACCAACCAACCATTC
gst-σ2	JX485637	F- CAGCAGCTGGTGTGAAATATG
		R- CAATGGCTAGACTCTGTCCTAAG
18S	DQ640512	F- AACTTTGTGCTGATCGCACG
		R- CGTTTCTCATGCTCCCTCTC
β-actin	Ab257134	F- CTCTTGATTTCGAGCAGGAAA
		R- AGGATGGTTGGAATAGTGATT

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507 **Table 2.** Cellular activity in the hepatopancreas of *M. galloprovincialis* exposed to BDE-47. The
508 results are expressed as mean ± SEM (n=6). Significant differences (P < 0.05) are indicated by different
509 letters.

Histological character	Control	D1- 15 days	D1- 30 days	D3 - 15 days	D3 - 30 days	P-value
% Edema present in digestive tubule	8.61 ± 0.63 ^{a}	12.48 ± 1.45^{b}	7.91 ± 1.54^{a}	22.97 ± 2.54^{c}	15.68 ± 1.31 ^{b}	<0.001
Digestive tubule thickness (µm)	17.35 ± 0.79^{a}	20.31 ± 0.54^{a}	22.18 ± 0.11^{a}	27.58 ± 1.02^{b}	14.29 ± 0.35 ^{c}	<0.001
Nuclei diameter (µm)	4.31 ± 0.09 ^{a}	3.95 ± 0.14^{b}	2.87 ± 0.06^{b}	4.54 ± 0.12 ^{c}	4.01 ± 0.08 ^{b}	0.001
% Cells presence in lumen	9.75 ± 1.47 ^{a}	3.85± 0.44^{a}	2.37 ± 1.28^{a}	22.06 ± 2.4 ^{b}	20.10 ± 2.10 ^{b}	<0.001