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

1 <sup>1</sup> University of Palermo, Dept. of Earth and Marine Science DISTEM, Laboratory of Marine  
2 biochemistry and ecotoxicology. Via Barlotta 4, 91100, Trapani, Italy.

3 <sup>2</sup>Consorzio Universitario della Provincia di Trapani, Marine Biology Institute. Via Barlotta 4, 91100,  
4 Trapani, Italy

## 5 \* Correspondence:

6 Concetta Maria Messina

7 concetta.messina@unipa.it

8 **Keywords: polybrominated, bivalves, biotransformation, oxidative stress, bioaccumulation.**

## 9 Abstract

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

## 27 1 Introduction

28 Polybrominated diphenyl ethers (PBDEs) are used as flame retardants, being widely added to  
29 building materials, electronic products, textiles and foams. These compounds were introduced in the  
30 70s, becoming the most popular class of flame retardants in the last decades (Covaci et al., 2011).  
31 PBDEs are characterized by high stability and environmental persistence (Eljarrat and Barceló,  
32 2011), and they are known to accumulate in sediments, marine and terrestrial animals, including  
33 humans (Darnerud et al., 2001; Horri et al., 2018). Among PBDEs, commonly found compounds  
34 include the 2, 2', 4, 4'-tetrabromodiphenyl ether (BDE-47), and the 2, 2', 4, 4', 5-pentabromodiphenyl  
35 ether (BDE-99) (Bi et al., 2007; Leung et al., 2006). The effects of PBDEs have been studied for  
36 many years in several biological models (Chao et al., 2017; Espinosa et al., 2019a, 2019b;  
37 Manuguerra et al., 2019; Mercado-Feliciano and Bigsby, 2008; Tang et al., 2018; Zhang et al., 2017),

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

41 Marine mussels are frequently used as bioindicators for monitoring programs due to their capacity of  
42 accumulate and tolerate high levels of environmental pollutants, allowing to identify both temporal  
43 and geographical trends (Booij et al., 2002). The effects of PBDEs on mussels have been previously  
44 reported on hemocytes and gills damage (Ji et al., 2013; Riva et al., 2007; Vidal-Liñán et al., 2016),  
45 or immune responses (Jiang et al., 2017a, 2017b). The different effects of the pollutants in the  
46 organisms not only determine direct effects in their immunity, reproductive or endocrine systems, but  
47 could entail several alterations in bioenergetics and tissues/specimens development (Canesi et al.,  
48 2003; Luster et al., 1988).

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

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

## 64 **2 Material and Methods**

### 65 **2.1 Mussels maintenance**

66 Specimens of *Mytilus galloprovincialis* were adults (shell length:  $6.5 \pm 0.46$  cm), obtained in May  
67 2018 from a mussel farm of Messina (Italy), and acclimated to laboratory conditions for a month.  
68 Organisms were maintained in 10 L tanks containing filtered and aerated sea water under semi static  
69 conditions. The pH and salinity were adjusted to  $7.95 \pm 0.05$  and  $37.82 \pm 0.24$  ‰, respectively,  
70 temperature was kept at  $22.6 \pm 0.5$  °C with a 12:12 dark-light cycle, according to local environmental  
71 factors and the optimal condition for mussel's culture (Barón et al., 2016; Ji et al., 2013; Parrino et  
72 al., 2019). Mussels were fed with *Nannochloropsis sp.* at an approximate concentration of  $1.2 \times 10^6$   
73 cells mL<sup>-1</sup>. The seawater was renewed periodically to maintain constant conditions.

### 74 **2.2 BDE-47 inclusion into microalgae**

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

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

### 87 **2.3 Experimental design**

88 Acclimated mussels were randomly divided into five experimental groups, each containing three  
89 aquaria (12 mussels per aquarium). Mussels from each aquarium were daily placed in separate  
90 beakers containing 1L of seawater with aeration and acclimated for 30 min, before being fed with 3  
91 ml of seawater containing 3 mg of *Nannochloropsis sp.* (approx.  $1.2 \times 10^6$  cell  $\text{mL}^{-1}$ ) treated as  
92 follow: control, acetone (vehicle control), and contaminated microalgae (0.333, 3.33 e 33.3  $\mu\text{g}$  of  
93 BDE-47 per g of microalgae). This procedure corresponded to an exposure concentration of 1, 10 and  
94 100  $\text{ng L}^{-1}$  of BDE-47 each day (D1, D2 and D3, respectively), in the same magnitude previously  
95 reported by others authors (Ji et al., 2013; Jiang et al., 2017a; Vidal-Liñán et al., 2016; Parolini and  
96 Binelli, 2012; Vidal-Liñán et al., 2016).

97 Mussels were fed with the microalgae alone (control) or the vehicle alone (acetone) and the different  
98 concentrations (D1, D2 and D3) for 1-2h until the water was clear. Clearance rate was calculated for  
99 each treatment during the experiment, to ensure the mussels fed the microalgae. After treatment,  
100 mussels were maintained in renewed sea water for 30 min, before being placed again into their  
101 respective aquaria. Mussels were daily fed with the different diets for 15 days and with control diet  
102 (microalgae alone) for the following 15 days (depuration period). Three mussels from each aquarium  
103 (9 from each treatment) were randomly chosen at the beginning, the middle and the end of  
104 experiment (on day 0, 15 and 30). Samples of digestive gland and gills were stored in PUREzol  
105 Reagent (Bio-Rad, Hercules, CA, USA) at -80°C for gene expression analysis, while digestive glands  
106 were also processed for routine light microscopy.

### 107 **2.4 Histopathological and morphometric study in hepatopancreas**

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

## 124 2.5 Gene expression

125 Total cellular RNA was isolated from the samples of digestive glands and gills in PUREzol using  
126 Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA), and the  
127 concentration was assessed spectrophotometrically at 260 nm. The absorbance ratios A260/A280 and  
128 A260/A230 were evaluated as indicators of RNA purity. Then, 1 µg of RNA were reverse-transcribed  
129 for each sample, in a volume of 20 µL, by the 5X iScript Reaction Mix Kit (Bio-Rad, Hercules, CA,  
130 USA) according to manufacturer's instructions. The amplification was performed in a total volume of  
131 20 µL, which contained: 0.4 µmol L<sup>-1</sup> of each primer, cDNA diluted 1:10 of the final reaction volume,  
132 1X IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and nuclease-free water. Conditions  
133 for real-time PCRs were optimized in a gradient cycler (C1000 Touch Thermal Cycler, Bio-Rad,  
134 USA) using the following run protocol: an initial activation step at 95°C for 3min, followed by 39  
135 cycles of 95°C for 10s and 60°C for 30s, with a single fluorescence measurement. Melting curve  
136 program was achieved at 65-95°C with heating rate of 0.5°C/cycle and a continuous fluorescence  
137 measurement. All reactions were performed in triplicate. For each PCR, we checked linear range of a  
138 standard curve of serial dilutions. The relative quantification of [*catalase (cat)*, *superoxide dismutase*  
139 (*sod*), *ras*, *multidrug resistance P-glycoprotein (pgp)*, *cytochrome p450-4Y1 (cyp4Y1)*, *glutathione-S-*  
140 *transferase (gst)*, *glutathione-S-transferase subunits σ1* and *σ2 (gst-σ1* and *gst-σ2*, respectively)] gene  
141 expression was evaluated after normalization with the reference genes. Data processing and statistical  
142 analysis were performed using CFX Manager Software (Bio-Rad, Hercules, CA, USA). The primers  
143 used are shown in Table 1. The relative expression of all genes was calculated by the 2<sup>-ΔΔCT</sup> method  
144 (Livak and Schmittgen, 2001), using *Mytilus galloprovincialis* β-actin and 18S as the endogenous  
145 reference.

146

## 147 2.6 Extraction and analysis of BDE-47 in tissues

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

158

## 159 2.7 Statistical analysis

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

## 165 3 Results

### 166 3.1 Effect of sub-lethal PBDEs concentrations on survival and feeding

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

### 170 3.2 Histology analyses

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

178 Our results revealed that exposure to BDE-47 significantly affected the percentage of edema in  
179 digestive tubules in absorptive phase, increasing from  $8.61 \pm 0.63\%$  in the control group, to  
180  $12.48 \pm 1.45\%$  in mussels fed the D1 and  $22.97 \pm 2.54\%$  in mussels fed the D3 after 15 days.  
181 Furthermore, the percentage of edema was recovered after 30 days in D1 exposed mussels  
182 ( $7.91 \pm 1.54\%$ ), but remained elevated in D3 exposed mussels ( $15.68 \pm 1.31\%$ ) ( $P < 0.001$ ). The presence  
183 of cells in the lumen of digestive tubules significantly increased in mussels fed the D3 both at 15 and  
184 30 days ( $22.06 \pm 2.4\%$  and  $20.1 \pm 2.1\%$ , respectively) compared to the control ( $9.75 \pm 1.47\%$ ), while  
185 mussels fed the D1 showed lower percentages both at 15 and 30 days ( $3.85 \pm 0.44\%$  and  $2.37 \pm 1.28$ ,  
186 respectively).

187 A significant increase of digestive tubule thickness was observed in mussels fed the D3 for 15 days  
188 ( $27.58 \pm 1.02 \mu\text{m}$ ) with respect to the controls ( $17.35 \pm 0.79 \mu\text{m}$ ) ( $P < 0.001$ ); however, this effect was  
189 significantly reduced after 30 days ( $14.29 \pm 0.35 \mu\text{m}$ ). No significant changes on digestive tubule  
190 thickness appeared in mussels fed the D1 both at 15 and 30 days ( $20.31 \pm 0.54$  and  $22.18 \pm 0.11 \mu\text{m}$ ,  
191 respectively). Our results showed a significant increase of nuclei diameter in cells of mussels fed the  
192 D3 at 15 days ( $4.51 \pm 0.12 \mu\text{m}$ ) compared to the control group ( $4.31 \pm 0.09 \mu\text{m}$ ) ( $P < 0.001$ ), while a  
193 reduction occurred at 30 days ( $4.01 \pm 0.08 \mu\text{m}$ ). A significant reduction of nuclei diameters was also  
194 measured in mussels fed the D1, both at 15 and 30 days ( $3.95 \pm 0.14$  and  $2.87 \pm 0.06 \mu\text{m}$ , respectively)  
195 ( $P < 0.001$ ).

### 196 3.3 Gene expression

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

209 after 15 days while, at 30 days, the mussels fed the highest dose of BDE-47 showed an increase of  
210 the same marker (fig. 5E). Also *gst* significantly decreased in mussels fed the highest dose of BDE-  
211 47 at 15 days, but no differences were found between groups at 30 days (fig. 5F). Finally, the relative  
212 expression of *gst-σ1* and *gst-σ2* showed a decrease in mussels fed the D2 and D3 at 15 days, while  
213 these genes were significantly up-regulated in mussels fed the D3 at 30 days (fig 5G-H).

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

### 220 3.2 BDE quantification in tissues

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

226

## 227 4 Discussion

228 In the present study we evaluated the molecular and cellular effects of BDE-47 on *M.*  
229 *galloprovincialis* chosen as a marine organism model. To provide environmental relevance to our  
230 results, realistic exposure concentrations were used, and administered through the diet (Gu et al.,  
231 2017; Ji et al., 2013; Jiang et al., 2017a; Vidal-Liñán et al., 2016). In fact, considering the high  
232 affinity of BDE-47 for particulate organic matter ( $K_{oc}=5.74 \times 10^4 \text{ L Kg}^{-1}$  estimated by EPI Suite  
233 software of Environmental Protection Agency United States), the major route of exposure for this  
234 contaminant to mussels is expected to be via food (Gustafsson et al., 18AD; Vidal-Liñán et al.,  
235 2016).

236 In our study, histopathological parameters related to health and welfare of marine organisms revealed  
237 that mussels fed in presence of BDE-47 exhibit slight signs of inflammation at 15 days (edema in  
238 digestive tubule, increase of tubule thickness, change on nuclei diameters and presence of cells debris  
239 in tubule lumen), as previously described in mussels exposed to sub-lethal concentrations of  
240 dimethoate (Kumar et al., 2011; Tunali and Erkan, 2008). Both the edema and increase of tubule  
241 thickness are markers of inflammation, as cellular and nuclear hypertrophy is considered prognostic  
242 of toxicity, while the presence of cells debris in the lumen of the digestive tubule reflects the death of  
243 digestive cells (Kumar et al., 2011). These alterations have been described to be produced by  
244 increased sensibility, oxidative stress and excessive metabolic detoxification of organic xenobiotics,  
245 thus being considered as defense reactions (Kumar et al., 2011). The BDE-47 has been reported to  
246 produce oxidative stress and cytotoxicity in mussels (Jiang et al., 2017a; Parolini and Binelli, 2012),  
247 in line with our observations on both histopathological effects and accumulation of this compound.  
248 After 15 days of depuration (30 days of experiment), most of these alterations were disappeared in  
249 mussels exposed to D1, suggesting a certain capacity of recovery but also a limited bioaccumulation.  
250 Conversely, in mussels fed the higher dose of BDE-47, all the analyzed histopathological parameters  
251 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<sub>2</sub>O<sub>2</sub> 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*, *gst $\sigma$ 1* and *gst $\sigma$ 2* 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



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

## 302 **5 Conflict of interest**

303 The authors declare that they have no conflicts

## 304 **6 Author Contributions**

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

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### 316 **8.1 Life Science Identifiers**

317 Life Science Identifiers (LSIDs) for ZOOBANK registered names or nomenclatural acts should be  
318 listed in the manuscript before the keywords with the following format:

319 urn:lsid:<Authority>:<Namespace>:<ObjectID>[:<Version>]

320 For more information on LSIDs please see [Inclusion of Zoological Nomenclature](#) section of the  
321 guidelines.

322

## 323 **9 References**

324 Barón, E., Dissanayake, A., Vilà-Cano, J., Crowther, C., Readman, J. W., Jha, A. N., et al. (2016).  
325 Evaluation of the genotoxic and physiological effects of decabromodiphenyl ether (BDE-209) and  
326 dechlorane plus (DP) flame retardants in marine mussels (*Mytilus galloprovincialis*). Environ. Sci.  
327 Technol. 50, 2700–2708. doi:10.1021/acs.est.5b05814.

328 Bi, X., Thomas, G. O., C., J. K., Weiyue, Q., Guoying, S., Martin, F. L., et al. (2007). Exposure of  
329 electronics dismantling workers to polybrominated diphenyl ethers, polychlorinated biphenyls, and

- 330 organochlorine pesticides in South China. *Environ. Sci. Technol.* 41, 5647–5653.  
331 doi:doi:10.1021/ES070346A.
- 332 Booij, K., Zegers, B. N., and Boon, J. P. (2002). Levels of some polybrominated diphenyl ether  
333 (PBDE) flame retardants along the Dutch coast as derived from their accumulation in SPMDs and  
334 blue mussels (*Mytilus edulis*). *Chemosphere* 46, 683–688.
- 335 Canesi, L., Ciacci, C., Betti, M., Scarpato, A., Citterio, B., Pruzzo, C., et al. (2003). Effects of PCB  
336 congeners on the immune function of *Mytilus* hemocytes: alterations of tyrosine kinase-mediated cell  
337 signaling. *Aquat. Toxicol.* 63, 293–306. doi:doi:10.1016/S0166-445X(02)00186-8.
- 338 Cappello, T., Maisano, M., D'Agata, A., Natalotto, A., Mauceri, A., and Fasulo, S. (2013). Effects of  
339 environmental pollution in caged mussels (*Mytilus galloprovincialis*). *Mar. Environ. Res.* 91, 52–60.  
340 doi:10.1016/j.marenvres.2012.12.010.
- 341 Chao, S. J., Huang, C. P., Chen, P. C., and Huang, C. (2017). Teratogenic responses of zebrafish  
342 embryos to decabromodiphenyl ether (BDE-209) in the presence of nano-SiO<sub>2</sub> particles.  
343 *Chemosphere* 178, 449–457. doi:10.1016/j.chemosphere.2017.03.075.
- 344 Covaci, A., Harrad, S., Abdallah, M. A.-E., Ali, N., Law, R. J., Herzke, D., et al. (2011). Novel  
345 brominated flame retardants: A review of their analysis, environmental fate and behaviour. *Environ.*  
346 *Int.* 37, 532–556. doi:10.1016/j.envint.2010.11.007.
- 347 Darnerud, P. O., Eriksen, G. S., Jóhannesson, T., Larsen, P. B., and Viluksela, M. (2001).  
348 Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. *Environ. Health*  
349 *Perspect.* 109, 49–68.
- 350 Eljarrat, E., and Barceló, D. (2011). *The Handbook of Environmental Chemistry*. doi:10.1016/0143-  
351 1471(82)90111-8.
- 352 Espinosa, C., Manuguerra, S., Cuesta, A., Esteban, M. A., Santulli, A., and Messina, C. M. (2019a).  
353 Sub-lethal doses of polybrominated diphenyl ethers affect some biomarkers involved in energy  
354 balance and cell cycle, via oxidative stress in the marine fish cell line SAF-1. *Aquat. Toxicol.*  
355 doi:10.1016/J.AQUATOX.2019.02.014.
- 356 Espinosa, C., Manuguerra, S., Cuesta, A., Santulli, A., and Messina, C. M. (2019b). Oxidative stress,  
357 induced by sub-lethal doses of BDE 209, promotes energy management and cell cycle modulation in  
358 the marine fish cell line SAF-1. *Int. J. Environ. Res. Public Health* 16, 21–26.  
359 doi:10.3390/ijerph16030474.
- 360 Fonnum, F., Mariussen, E., and Reistad, T. (2006). Molecular mechanisms involved in the toxic  
361 effects of polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs). *J. Toxicol.*  
362 *Environ. Health. A* 69, 21–35. doi:10.1080/15287390500259020.

- 363 Giuliani M.E., Benedetti M., Arukwe A., Regoli F. (2013). Transcriptional and catalytic responses of  
364 antioxidant and biotransformation pathways in mussels, *Mytilus galloprovincialis*, exposed to  
365 chemical mixtures. *Aquat. Toxicol.* 134–135: 120–127
- 366 Gonzalez-Rey, M., Mattos, J. J., Piazza, C. E., Bainy, A. C. D., and Bebianno, M. J. (2014). Effects  
367 of active pharmaceutical ingredients mixtures in mussel *Mytilus galloprovincialis*. *Aquat. Toxicol.*  
368 153, 12–26. doi:10.1016/j.aquatox.2014.02.006.
- 369 Gu, S. Y., Ekepeghere, K. I., Kim, H. Y., Lee, I. S., Kim, D. H., Choo, G., et al. (2017). Brominated  
370 flame retardants in marine environment focused on aquaculture area: Occurrence, source and  
371 bioaccumulation. *Sci. Total Environ.* 601–602, 1182–1191. doi:10.1016/j.scitotenv.2017.05.209.
- 372 Gustafsson, K., Björk, M., Burreau, S., and Gilek, M. (18AD). Bioaccumulation kinetics of  
373 brominated flame retardants (polybrominated diphenyl ethers) in blue mussels (*Mytilus edulis*).  
374 *Environ. Toxicol. Chem.* 1999, 1218–1224. doi:doi:10.1002/etc.5620180621.
- 375 Horri, K., Alfonso, S., Cousin, X., Munsch, C., Loizeau, V., Aroua, S., et al. (2018). Fish life-  
376 history traits are affected after chronic dietary exposure to an environmentally realistic marine  
377 mixture of PCBs and PBDEs. *Sci. Total Environ.*, 531–545.  
378 doi:doi:10.1016/J.SCITOTENV.2017.08.083.
- 379 Ji, C., Wu, H., Wei, L., Zhao, J., and Yu, J. (2013). Proteomic and metabolomic analysis reveal  
380 gender-specific responses of mussel *Mytilus galloprovincialis* to 2,2',4,4'-tetrabromodiphenyl ether  
381 (BDE 47). *Aquat. Toxicol.* 140–141, 449–457. doi:10.1016/J.AQUATOX.2013.07.009.
- 382 Jiang, Y., Tang, X., Sun, T., and Wang, Y. (2017a). BDE-47 exposure changed the immune function  
383 of haemocytes in *Mytilus edulis*: An explanation based on ROS-mediated pathway. *Aquat. Toxicol.*  
384 182, 58–66. doi:10.1016/j.aquatox.2016.11.010.
- 385 Jiang, Y., Tang, X., Zhou, B., Sun, T., Chen, H., Zhao, X., et al. (2017b). The ROS-mediated  
386 pathway coupled with the MAPK-p38 signalling pathway and antioxidant system plays roles in the  
387 responses of *Mytilus edulis* haemocytes induced by BDE-47. *Aquat. Toxicol.* 187, 55–63.  
388 doi:10.1016/j.aquatox.2017.03.011.
- 389 Kumar, S., Pandey, R. K., Das, S., and Das, V. K. (2011). Pathological changes in hepatopancreas of  
390 freshwater mussel (*Lamellidens marginalis* Lamarck) exposed to sub-lethal concentration of  
391 dimethoate. *Bull. Biosci.* 2, 18–23.
- 392 Legler, J. (2008). New insights into the endocrine disrupting effects of brominated flame retardants.  
393 *Chemosphere* 73, 216–222. doi:10.1016/J.CHEMOSPHERE.2008.04.081.
- 394 Leung, A., Cai, Z. W., and Wong, M. H. (2006). Environmental contamination from electronic waste  
395 recycling at Guiyu, southeast China. *J. Mater. Cycles Waste Manag.* 8, 21–33.  
396 doi:doi:10.1007/s10163-005-0141-6.

- 397 Li, A., Ma, J., Cao, J., and McCarron, P. (2012). Toxins in mussels (*Mytilus galloprovincialis*)  
398 associated with diarrhetic shellfish poisoning episodes in China. *Toxicon* 60, 420–425.  
399 doi:10.1016/J.TOXICON.2012.04.339.
- 400 Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time  
401 quantitative PCR and the  $2^{(-\Delta\Delta C(T))}$  Method. *Methods* 25, 402–8. doi:10.1006/meth.2001.1262.
- 402 Livingstone D.R. (1991). Towards a specific index of impact by organic pollution for marine  
403 invertebrates. *Comp. Biochem. Physiol. Part C Pharmacol.* 100, 151–155. doi:doi:10.1016/0742-  
404 8413(91)90143-H.
- 405 Luster, M. I., Munson, A. E., Thomas, P. T., Holsapple, M. P., Fenters, J. D., White, K. L., et al.  
406 (1988). Development of a testing battery to assess chemical-induced immunotoxicity. *Toxicol. Sci.*  
407 10, 2–19. doi:https://doi.org/10.1093/toxsci/10.1.2.
- 408 Malmberg, T., Athanasiadou, M., Marsh, G., Brandt, I., and Bergman, Å. (2005). Identification of  
409 hydroxylated polybrominated diphenyl ether metabolites in blood plasma from polybrominated  
410 diphenyl ether exposed rats. *Environ. Sci. Technol.* 39, 5342–5348. doi:10.1021/ES050574+.
- 411 Manuguerra, S., Espinosa, C., Santulli, A., and Messina, C. M. (2019). Sub-lethal doses of  
412 polybrominated diphenyl ethers, in vitro, promote oxidative stress and modulate molecular markers  
413 related to cell cycle, antioxidant balance and cellular energy management. *Int. J. Environ. Res. Public*  
414 *Heal.* 16, 588. doi:10.3390/ijerph16040588.
- 415 McDonald T.A. (2002). A perspective on the potential health risks of PBDEs. *Chemosphere* 46, 745–  
416 755.
- 417 Mercado-Feliciano, M., and Bigsby, R. M. (2008). The polybrominated diphenyl ether mixture DE-  
418 71 is mildly estrogenic. *Environ. Health Perspect.* 116, 605–611. doi:10.1289/ehp.10643.
- 419 Parolini, M., and Binelli, A. (2012). Cyto-genotoxic effects induced by three brominated diphenyl  
420 ether congeners on the freshwater mussel *Dreissena polymorpha*. *Ecotoxicol. Environ. Saf.* 79, 247–  
421 255. doi:10.1016/J.ECOENV.2012.01.008.
- 422 Parrino, V., Costa, G., Cannavà, C., Fazio, E., Bonsignore, M., Concetta, S., et al. (2019). Flow  
423 cytometry and micro-Raman spectroscopy: Identification of hemocyte populations in the mussel  
424 *Mytilus galloprovincialis* (Bivalvia: Mytilidae) from Faro lake and Tyrrhenian sea (Sicily, Italy). *Fish*  
425 *Shellfish Immunol.* 87, 1–8. doi:10.1016/J.FSI.2018.12.067.
- 426 Qiu, X., Mercado-Feliciano, M., Bigsby, R. M., and Hites, R. A. (2007). Measurement of  
427 polybrominated diphenyl ethers and metabolites in mouse plasma after exposure to a commercial  
428 pentabromodiphenyl ether mixture. *Environ. Health Perspect.* 115, 1052–8. doi:10.1289/ehp.10011.
- 429 Regoli F., Giuliani M.E. (2014). Oxidative pathways of chemical toxicity and oxidative stress  
430 biomarkers in marine organisms. *Mar. Environ. Res.* 93: 106-117

- 431 Riva, C., Binelli, A., Cogni, D., and Provini, A. (2007). Evaluation of DNA damage induced by  
432 decabromodiphenyl ether (BDE-209) in hemocytes of *Dreissena polymorpha* using the comet and  
433 micronucleus assays. *Environ. Mol. Mutagen.* 48, 735–743. doi:doi:10.1002/em.20353.
- 434 Rossi, F., Palombella, S., Pirrone, C., Mancini, G., Bernardini, G., and Gornati, R. (2016). Evaluation  
435 of tissue morphology and gene expression as biomarkers of pollution in mussel *Mytilus*  
436 *galloprovincialis* caging experiment. *Aquat. Toxicol.* 181, 57–66.  
437 doi:10.1016/j.aquatox.2016.10.018.
- 438 Snyder, M. J. (1998). Cytochrome P450 enzymes belonging to the CYP4 family from marine  
439 invertebrates. *Biochem. Biophys. Res. Commun.* 249, 187–190. doi:10.1006/bbrc.1998.9104.
- 440 Stapleton, H. M., Kelly, S. M., Pei, R., Letcher, R. J., and Gunsch, C. (2009). Metabolism of  
441 polybrominated diphenyl ethers (PBDEs) by human hepatocytes *in vitro*. *Environ. Health Perspect.*  
442 117, 197–202. doi:10.1289/ehp.11807.
- 443 Szabo, D. T., Richardson, V. M., Ross, D. G., Diliberto, J. J., Kodavanti, P. R. S., and Birnbaum, L.  
444 S. (2009). Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase  
445 1 gene expression Involved in thyroid hormone metabolism in male rat pups. *Toxicol. Sci.* 107, 27–  
446 39. doi:10.1093/toxsci/kfn230.
- 447 Tang, S., Liu, H., Yin, H., Liu, X., Peng, H., Lu, G., et al. (2018). Effect of 2, 2", 4, 4"-  
448 tetrabromodiphenyl ether (BDE-47) and its metabolites on cell viability, oxidative stress, and  
449 apoptosis of HepG2. *Chemosphere* 193, 978–988. doi:10.1016/j.chemosphere.2017.11.107.
- 450 Tseng, L.-H., Li, M.-H., Tsai, S.-S., Lee, C.-W., Pan, M.-H., Yao, W.-J., et al. (2008).  
451 Developmental exposure to decabromodiphenyl ether (PBDE 209): effects on thyroid hormone and  
452 hepatic enzyme activity in male mouse offspring. *Chemosphere* 70, 640–7.  
453 doi:10.1016/j.chemosphere.2007.06.078.
- 454 Tunali, Y., and Erkan, M. (2008). A structural study of functional cells in hepatopancreas of *Mytilus*  
455 *galloprovincialis* Lamarck, 1819. *Pak. J. Zool.* 40, 109–114.
- 456 Usenko, C. Y., Abel, E. L., Kudela, M., Janise, A., and Bruce, E. D. (2015). Comparison of PBDE  
457 congeners as inducers of oxidative stress in zebrafish. *Environ. Toxicol. Chem.* 34, 1154–1160.  
458 doi:10.1002/etc.2922.
- 459 Vidal-Liñán, L., Bellas, J., Soriano, J. A., Concha-Graña, E., Muniategui, S., and Beiras, R. (2016).  
460 Bioaccumulation of PCB-153 and effects on molecular biomarkers acetylcholinesterase, glutathione-  
461 S-transferase and glutathione peroxidase in *Mytilus galloprovincialis* mussels. *Environ. Pollut.* 214,  
462 885–891. doi:10.1016/j.envpol.2016.04.083.
- 463 Vogel, C., and Marcotte, E. M. (2012). Insights into the regulation of protein abundance from  
464 proteomic and transcriptomic analyses. *Nat. Rev. Genet.* 13, 227–232. doi:10.1038/nrg3185.

- 465 Yu, L., Han, Z., and Liu, C. (2015). A review on the effects of PBDEs on thyroid and reproduction  
466 systems in fish. *Gen. Comp. Endocrinol.* 219, 64–73. doi:10.1016/J.YGCEN.2014.12.010.
- 467 Zhang, L., Jin, Y., Han, Z., H. Liu, Shi, L., Hua, X., et al. (2017). Integrated *in silico* and *in vivo*  
468 approaches to investigate effects of BDE-99 mediated by the nuclear receptors on developing  
469 zebrafish. *Environ. Toxicol. Chem.* 37, 780–780. doi:doi:10.1002/etc.4000.

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

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

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

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

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

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

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

498

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

502 **Table 1.** *Mytilus galloprovincialis* primer sequences used for real-time PCR.503  
504

505

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



506

507 **Table 2.** Cellular activity in the hepatopancreas of *M. galloprovincialis* exposed to BDE-47. The  
 508 results are expressed as mean  $\pm$  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 $\pm$ 0.63 <sup>a</sup>	12.48 $\pm$ 1.45 <sup>b</sup>	7.91 $\pm$ 1.54 <sup>a</sup>	22.97 $\pm$ 2.54 <sup>c</sup>	15.68 $\pm$ 1.31 <sup>b</sup>	<0.001
Digestive tubule thickness ( $\mu$ m)	17.35 $\pm$ 0.79 <sup>a</sup>	20.31 $\pm$ 0.54 <sup>a</sup>	22.18 $\pm$ 0.11 <sup>a</sup>	27.58 $\pm$ 1.02 <sup>b</sup>	14.29 $\pm$ 0.35 <sup>c</sup>	<0.001
Nuclei diameter ( $\mu$ m)	4.31 $\pm$ 0.09 <sup>a</sup>	3.95 $\pm$ 0.14 <sup>b</sup>	2.87 $\pm$ 0.06 <sup>b</sup>	4.54 $\pm$ 0.12 <sup>c</sup>	4.01 $\pm$ 0.08 <sup>b</sup>	0.001
% Cells presence in lumen	9.75 $\pm$ 1.47 <sup>a</sup>	3.85 $\pm$ 0.44 <sup>a</sup>	2.37 $\pm$ 1.28 <sup>a</sup>	22.06 $\pm$ 2.4 <sup>b</sup>	20.10 $\pm$ 2.10 <sup>b</sup>	<0.001