Halogen-free organophosphorus flame retardants caused oxidative stress and multixenobiotic resistance in Asian freshwater clams (Corbicula fluminea)*

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ABSTRACT

Halogen-free organophosphorus flame retardants are widespread in aquatic environments. Although it has been documented that they affect the behavior and reproduction of aquatic species, researches investigating cellular detoxification and the defense system in bivalves are scarce. In this study, adult Asian clams (C. fluminea) were exposed to tris (2-butoxyethyl) phosphate (TBEP) and tributyl phosphate (TBP) at 20, 200, and 2000 μg/L for 28 d. The results showed no noticeable difference in siphoning behavior. However, the siphoning behavior displayed a trend toward a slight decrease in the treatment groups. GR activity was markedly reduced compared with the control groups, whereas the levels of cyp4 significantly increased following the 2000 μg/L TBP treatments (p < 0.05). Moreover, the levels of gst1 and gstm1 significantly decreased following all TBEP treatments and were significantly inhibited by 20 μg/L TBP (p < 0.05). The adverse effects on antioxidant enzymes suggested that C. fluminea mainly relies on the antioxidant system to reduce damage without an increase in MDA levels following exposure to a low concentration. Moreover, mRNA expression levels of heat shock proteins (hsp 22, 40, 60, 70, and 90) were significantly down-regulated with TBEP and TBP treatments lower than 200 μg/L (p < 0.05), whereas significant up-regulations were observed for hsp 22 and hsp 70 in response to 2000 μg/L TBP treatment (p < 0.05). Up-regulation of ATP-binding cassette (ABC) transporter genes (abcb1 and abcc1) showed that TBEP and TBP could activate the multixenobiotic resistance (MXR) system to discharge xenobiotics in C. fluminea, which kept its shell closed at high concentrations to prevent xenobiotic entry. Our results provide a new insight into the different mechanisms of cellular detoxification and the MXR system of C. fluminea in response to low and high concentrations of TBEP and TBP.

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1. Introduction

Brominated flame retardants (BFRs) and polybrominated biphenyl ethers (PBDEs) have been prohibited worldwide due to their persistence, bioaccumulation, and potentially toxic effects (Covaci et al., 2011). As a result, organophosphorus flame retardants (OPFRs) are used as an alternative to BDE209 (Van der Veen and de Boer, 2012; Du et al., 2015). OPFRs have been used as flame retardants (Liu et al., 2012b) and are used in households and public buildings (Mangas et al., 2011) and as alternative additives in plastic products; consequently, the concentration of OPFRs has been increasing during the last decade (Stapleton et al., 2009). The worldwide consumption of OPFRs ranged from 500,000 t in 2011 to
680,000 t in 2015 (Van der Veen and de Boer, 2012; Hou et al., 2016). Because OPFRs are not covalently bound to flame retardants or additives (Reemtsma et al., 2008), large quantities of OPFRs enter aquatic environments (Blair et al., 2013; Liu and Wong, 2013). In particular, OPFRs such as tris (chloropropyl) phosphate (TCP) and tris (2-chloroethyl) phosphate (TCEP) persist in the environment (Watts and Linden, 2009; Regnery and Puttmann, 2010).

OPFRs can be divided into two types: chlorinated OPFRs and halogen-free flame retardants (HFFRs). The restriction of bromine-containing flame retardants has also prompted the development of HFFRs (Ramani and Dahoe, 2014), including tris(2-butoxyethyl) phosphate (TBEP) and tributyl phosphate (TBP). Global production of TBEP ranges from 5000 to 6000 t per year (WHO, 1998), and consumption of TBEP has been gradually increasing with the phasing out of some brominated flame retardants (Van der Veen and de Boer, 2012). In addition, TBP is commercially used as an extractant for large volumes of uranium and plutonium at nuclear fuel reprocessing facilities (~3000–5000 t/annum) (Rangu et al., 2014). TBP is very stable and persistent in natural environments and is not eliminated in conventional wastewater treatment plants (Nancharaih et al., 2015), leading to increased concerns in recent years. Studies have demonstrated that OPFRs can be detected everywhere, such as in indoor air, surface water and sediments. OPFRs, including TBEP and TBP, were detected with the highest total concentration of 14.25 μg/kg in the sediment of Taihu Lake in China (Cao et al., 2012), and they also varied from 2.6 to 7.9 ng/L in surface water and from 0.48 to 11 μg/kg in sediments in Austria (Martinez-Carballo et al., 2007). Additionally, TBP levels ranged from 5.2 to 35 μg/L in the influents of Swedish sewage treatment plants, and increased levels of up to 52 μg/L in the influent of an STP at a major airport where TBP was widely used in aircraft hydraulic fluids. The TBEP concentration ranged from 5200 to 35,000 ng/L in the influent and from 3100 to 30,000 ng/L in the effluent (Marklund et al., 2005). In water samples from Albano Lake in Italy, TBEP concentrations changed from 10 to 127 ng/L (Bacaloni et al., 2008). With the increasing use of these compounds, their effects on human health and environmental impacts cannot be overlooked (Farhat et al., 2013).

Previous studies have demonstrated that several OPFRs affect the development and endocrine system in vivo and in vitro, for example, zebrafish embryos/larvae, chicken embryos, avian hepatocytes and neuronal cells (Crump et al., 2012; Farhat et al., 2013; Fu et al., 2013; Liu et al., 2013; McGee et al., 2013; Wang et al., 2013). Some studies have also warned about the potential damage of OPFRs to the ecosystem and human health (Ren et al., 2008). For example, exposure to OPFRs has been related to adverse neurologic effects and associated with alterations of thyroid function and relative liver weight in laboratory animals (Casida and Quistad, 2005; Liu et al., 2012b). The acute toxicity of high-level OPFRs has been well documented and includes the inhibition of acetylcholine esterase, which alters functions in the nervous system. However, little is known about the effects of long-term, low-level exposure to OPFRs (Mangas et al., 2011). The effects of TBP have attracted widespread attention on account of its cholinergic toxicity and neurotoxicity (Berne et al., 2007). TBP significantly hindered algal cell growth by inducing oxidative stress and reducing photosynthesis (Song et al., 2016). Neerathilangama et al. (2010) reported that TBP disrupts Krebs cycle energy metabolism and provides a biomarker signature of TBP exposure in rats. TBP induces acute toxicity in freshwater organisms, and its chronic toxicity has been documented (Michel et al., 2004). TBEP is widely used as a flame retardant and plasticizer in different products (WHO, 1998), and it is another member of the organophosphate ester family (Han et al., 2014). Han et al. (2014) reported the developmental toxicity of TBEP exposure to zebrafish embryos and larvae. Recently, Du et al. (2015) reported that aryl organophosphate flame retardants caused cardiotoxicity during the period of zebrafish embryogenesis, and the 96-h LC50 values of TBEP and TBP in the fertilized eggs were 3.34 and 7.82 ng/L, respectively. Although the toxicity effects of TBEP and TBP have been documented, results showing their effects on the cellular detoxification and defense systems of bivalves are scarce.

C. fluminea, as a benthonic freshwater bivalve, has been popularly used to measure environmental perturbations or contaminations in field and laboratory studies (Chen et al., 2013, 2014; Ren et al., 2013). Antioxidant enzyme activities and Hsp genes in bivalves have been chosen to indicate defense system and early warning signals of pollution (Gonzalez-Rey and Bebianno, 2013; Contardo-Jara et al., 2011; Gupta et al., 2010). The expression levels of thioredoxin and glutathione system genes (GST), ABC transporter genes, and cytochrome P450 genes (CYP450) have also been investigated (Chen et al., 2014, 2015). Therefore, the environmental safety of halogen-free flame retardants with regard to freshwater bivalves (C. fluminea) was evaluated by measuring multiple toxicological endpoints. Adult C. fluminea were exposed to TBP and TBEP for 28 d at concentrations of 20, 200, and 2000 μg/L. The siphoning behavior, oxidative stress enzyme activities (SOD, CAT, GR), MDA content, and mRNA expression levels of genes (hsp, cyp, abc, gst) were measured in the digestive glands. We also focused on the shell-closing mechanism of C. fluminea exposed to various concentrations of xenobiotics.

1. Materials and methods

1.1. Chemicals

TBEP (CAS-no. 78-51-3, purity >95.0%) and TBP (CAS-no. 126-73-8, purity >99.0%) were purchased from J&K Chemical Ltd. (USA). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Acetone was used as a co-solvent in the stock solutions of TBEP and TBP. The final concentration of acetone was less than 0.01%.

1.2. Clam care and exposure

Sexually mature clams (C. fluminea) were obtained from Hongze Lake (Jiangsu Province, China), and the average length was 20.56 ± 2.05 mm. Prior to the experiment, clams were acclimatized in 5-L glass aquariums with aerated natural water for 2 weeks in the laboratory. The size of the glass aquariums was 30 cm in diameter and 15 cm in depth. The clams were exposed to TBEP and TBP at 20, 200, or 2000 μg/L for 28 d. There were three replicate aquariums in each treatment group, with each aquarium containing 30 clams. The acetone-treated group served as the solvent control, which was not significantly different from the natural water control. The conditions were as follows: temperature of 20 ± 1 °C, a 12:12-h light cycle, oxygen saturation of 96% ± 2% and pH 7.8 ± 0.2. The water was changed slowly every day without moving the clams, and the clams were sampled in triplicate at 28 d. The clams were fed daily with single-celled Chlorella vulgaris and Scenedesmus obliquus algae.

1.3. Siphoning behavior

The siphoning rate of C. fluminea was tested according to the method of Cooper and Bidwell (2006). On the 28th d of chemical exposure, five C. fluminea from one of three replicated aquariums were placed in a beaker containing 100 mL of a neutral red solution (1 mg/L) for 2 h, and the siphoning rate was assessed in triplicate.
Before the experiment, the absorbance of neutral red solution (1 mg/L) was determined with a spectrophotometer at 530 nm. At the end of the experiment, 1 mL of water was sampled from each beaker to determine the absorbance with a spectrophotometer at 530 nm. The absorbance can be transformed into the neutral red concentration based on a standard curve of the concentration of standard solutions of neutral red. The filtration rates in every treatment group were finally calculated according to Cooper and Bidwell’s (2006) equation:

\[ m = \frac{M}{nt} \log \left( \frac{C_0}{C_t} \right) \]

where \( m \) is the filtration rate (mL/animal/h), \( M \) is the volume of the test solution, \( n \) is the number of clams, \( t \) is the time in hours, \( C_0 \) is the initial concentration of the dye, and \( C_t \) is the concentration of the dye at time \( t \).

2.4. Antioxidant enzyme activity and MDA assay

Antioxidant enzyme activities and MDA contents in C. fluminea were measured according to Chen et al. (2015). Approximately 200 mg of digestive gland from each treatment group was ground on ice using a pestle in a centrifuge tube (1.5 mL) that contained RIPA Lysis buffer (Beyotime Institute of Biotechnology, China) (0.5 mL). The samples were then centrifuged at 12,000 rpm for 10 min to obtain the supernatant. Each treatment group was evaluated in triplicate. The protein content of each sample was measured according to the instructions provided with the BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China), and the supernatant was frozen at -80 °C until further analysis. According to the manufacturer’s protocols, the activities of antioxidant enzymes and MDA content were analyzed using the respective assay kits (SOD and MDA: Nanjing Jiancheng Bioengineering Institute; CAT and GR: Beyotime Institute of Biotechnology).

2.5. qRT-PCR

First, total RNA was extracted from the digestive glands of C. fluminea using an SV Total RNA Isolation System (Promega, USA) according to the manufacturer’s protocols. Second, 4 μg of RNA was reverse transcribed into cDNA according to Chen et al. (2015). The mRNA expression levels of genes (hsp, cyp, abc and gst) were detected in triplicate using the ABI 7500 real-time quantitative PCR system (Life Technologies, USA). The β-actin gene of C. fluminea was used as an endogenous control. Related information for each primer pair is listed in Table 1. The final results were analyzed according to the delta-delta Ct method as described by Livak and Schmittgen (2008).

2.6. Statistical analyses

Every treatment group was assessed in triplicate. All results are shown as the mean ± SEM (standard error of the mean). All figures were drawn by Origin pro 8.0 (OriginLab, Northampton, MA, USA). All data were confirmed for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene’s tests, respectively. One-way ANOVA (\( p < 0.05 \)) followed by Dunnett’s test for multiple comparisons was applied to estimate significant differences among the treatments and control group using SPSS 17.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Effects on the siphoning behavior of C. fluminea

The effects on the siphoning behavior of C. fluminea are illustrated in Fig. 1. Although the siphoning behavior had a slightly decreasing trend with an increased exposure concentration compared with the control group, they are no significant difference following exposure to TBP and TBEP compared with the control groups.

3.2. Effects on antioxidant enzymes and MDA content

Antioxidant enzyme activities (CAT, SOD and GR) and MDA content were measured in the digestive glands, and the results are presented in Fig. 2. Significant differences were observed in CAT activity levels with the 20 μg/L TBEP and TBP treatments. SOD activity was significantly down-regulated at low concentrations of TBEP and TBP (20 and 200 μg/L). There were no significant difference in MDA content between the treatment and control groups in response to the TBEP treatments, but the MDA content greatly decreased at 20 μg/L and 2000 μg/L TBP. The levels of GR activity were not significantly different among the TBEP treatment groups but were significantly down-regulated at 2000 μg/L TBP.

3.3. Effects on gst, abc and cyp gene expression

As indicated in Fig. 3, following exposure to TBEP, the expression levels of gsts1 and gstm1 were significantly decreased, the levels of ABC transporters (abc1 and abcc1) were significantly enhanced, and the levels of cyp4 were not significantly different. Following exposure to TBP, the expression of gsts1 was greatly inhibited at 20 μg/L, the levels of abc1 and gstm1 were significantly increased and inhibited by TBP (20 and 2000 μg/L, respectively), the levels of abcc1 were significantly increased at 20 μg/L and then significantly decreased at 200 μg/L and 2000 μg/L, and the levels of cyp4 were significantly increased at high levels of TBP (2000 μg/L).

3.4. Effects on Hsp gene expression

The expression levels of five hsp genes (hsp 22, hsp 40, hsp 60, hsp 70 and hsp 90) in the digestive glands of C. fluminea exposed to TBEP and TBP are presented in Fig. 4. The expression levels of hsp 22 and hsp 40 were significantly inhibited following exposure to TBEP compared with the control groups, and the levels of hsp 60, hsp 70 and hsp 90 were significantly down-regulated at low levels of TBEP and TBP (20 μg/L and 200 μg/L). The levels of hsp 22 were significantly decreased following exposure to 20 μg/L TBP, and the expression of hsp 22 and hsp 70 was significantly increased at 2000 μg/L TBP.

4. Discussion

In the present study, C. fluminea were used to evaluate the effects of chronic exposure to TBEP and TBP by determining the siphoning behavior, activities of antioxidant enzymes, MDA content and expression levels of certain genes related to the antioxidant system and the cellular multixenobiotic resistance (MXR) system. Siphoning behavior is important for nutritional physiology, defense, and reproductive mechanisms in bivalves (Chen et al., 2015). Previous studies have shown that as residual xenobiotic metals increase in the body, C. fluminea respond by closing their valve to reduce their metabolic rate, heartbeat, and oxygen consumption (Ortmann and Grieshaber, 2003; Liao et al., 2005). Valve movement and siphoning behavior have been used as continuous bio-
monitoring indicators in aquatic environments (Chen et al., 2012). Previous studies have found that bivalve siphoning is reduced by some other compounds (Leonard et al., 2014; Chen et al., 2015). Similarly, the burrowing behavior and movement of adult *L. fasciola* were inhibited during exposure to 22.3 mg/L fluoxetine for 67 d (Hazelton et al., 2013). In the current study, a downward trend in siphoning behaviors suggested that TBEP and TBP triggered the defense system of *C. fluminea* against the pollutants. The decrease in siphoning behaviors may be associated with the accumulation of ammonia in tissues of the organism and the reduction in oxygen exchange and feeding, all of which could have implications for the survival, growth, and reproduction of bivalves (Chen et al., 2014). Therefore, we can conclude that the decreased siphoning behaviors could have negative health impacts and be an indicator of chemical stress. To respond to realistic situations, further research should seek to better understand the relationship between external and internal pollution concentrations during clam exposure to low concentrations.

The antioxidant and fatty acid metabolism systems of organisms are easily influenced by pollutants (Velisek et al., 2011; Yan et al., 2015). SOD and CAT are the primary antioxidant enzymes responsible for eliminating excess ROS. During this process, ROS is transformed to H₂O₂ by SOD, and H₂O₂ is converted to O₂ and H₂O by CAT (Ren et al., 2013). GR is responsible for glutathione disulfide (GSSG) to the sulfhydryl form GSH (Verlecar et al., 2008), which is an important cellular antioxidant. MDA is one of the decomposition products of unsaturated fatty acid peroxides and may severely damage cell membranes. Lipid peroxidation can be evaluated indirectly by measuring the MDA content. The changes detected in SOD activity and GR are in agreement with the results of Chen et al. (2014), and the CAT activity at low exposure concentrations is in agreement with the findings of Malev et al. (2012), showing a gradual reduction of CAT activity. Our results indicated that SOD and CAT could not clear excess ROS in a timely manner, leading to decreased cellular antioxidant activity that exceeded the regulatory functions of the organism, ultimately influencing the synthesis of SOD and CAT or even preventing their enzymatic activity (Lushchak, 2011). In contrast to our results, Sellami et al. (2015) reported that permethrin (PER) and anthracene (ANT) enhanced the levels of CAT activity. To some extent, siphoning behavior may lead to changes in antioxidant enzymes due to the negative effects of closed shell effects. The decreased GR activity of *C. fluminea* exposed to 2000 mg/L TBP may indicate an increase in ROS levels, which could be a result of the catalobism of TBP by cytochrome P450 enzyme (CYP450) or closed valve effects. Concurrently, the MDA content decreased following exposure to

### Table 1

| Genes     | Genes (Accession No.) | Realtime-PCR Primer sequence (5′-3′) | Product Size (bp) |
|-----------|-----------------------|--------------------------------------|-------------------|
| β-actin   | EF446608.1            | F:GCCCATCACCGCTGCTGTTTCA            | 123               |
|           |                       | R:ATGGCGTCGCTGGAGGCGCTA             |                   |
| gsts1     | KJ001775              | F:GCGGCTGCTGCTGGAGGCTATG            | 190               |
|           |                       | R:ACAAACTGGGCAATTTG                 |                   |
| gstm1     | KJ001774              | F:GCCAGGAGCAGCGCTGTTTACTG           | 103               |
|           |                       | R:CTATTGACACAAAGCACCACA             |                   |
| abch1     | KJ001772              | F:ATCCCAGCTGAGGCTGCTGACTGA          | 80                |
|           |                       | R:AGGCTTCTGCTGCTGCTGTA              |                   |
| abcc1     | KJ0027659             | F:GTGGCGGCGCTGCTGCTGTA              | 139               |
|           |                       | R:AGGGTGCTGCTGCTGCTATCC             |                   |
| cyp4      | KF218340              | F:GCCCAACAGCAGGCGAGG                | 130               |
|           |                       | R:ATCCACACAAAAGCTGGG                |                   |
| hsp22     | KF218338              | F:CTTACCCTGACGCGCTGCTGACTG          | 154               |
|           |                       | R:ACAGAGATCCGAGCTTCCACTCA           |                   |
| hsp40     | KF218339              | F:ACAGACAGCTGCCAGATTC               | 82                |
|           |                       | R:TCACGACAGCTGCCCTCC                |                   |
| hsp60     | KC979065              | F:GGACACAGCAGAAAGACACCC             | 120               |
|           |                       | R:CCGACAGCAGAAAGACACCC              |                   |
| hsp70     | KC979064              | F:GCCGAGGCGCTGGAATTCC               | 84                |
|           |                       | R:AAACAGCTGAGAGAGGCAAGCAGG          |                   |
| hsp90     | KC979063              | F:ATGGCTCTGCAATCTCTAGG              | 246               |
|           |                       | R:GCCCTCAGAAAGCAAGGACTG             |                   |

### Fig. 1

Average filtration rate (mL/animal/h) of *C. fluminea* in TBEP- or TBP-contaminated water containing 1 mg/L neutral red solution following exposure to the indicated concentrations. The results represent the mean ± SEM (n = 5). Different letters above the columns indicate significant differences at p < 0.05 between the treatment and control groups.
2000 μg/L TBP compared with the control group, potentially because *C. fluminea* close their valves to prevent the entry of higher concentrations of xenobiotic pollutants (Cooper and Bidwell, 2006). Our results showing a significant decrease in MDA content at 20 μg/L TBP also differed from those of Chen et al. (2014, 2015). This difference may be explained by the large amount of ROS elimination by depletion of SOD and CAT, which consequently could not initiate lipid peroxidation. Finally, we concluded that TBEP and TBP induced oxidative stress in clams due to changes in their antioxidant system.

GST and cytochrome P450 participate in compounding and decomposing a multitude of endogenous metabolic compounds and providing protection against oxidative stress (Ranson et al., 2002). Similar to our results, GST was significantly inhibited after exposure of yellow catfish to 500 μg/L triclosan for 168 h (Ku et al., 2014). As paralogs in the GST gene family, it is reasonable to suggest that *gsts1* and *gstm1* have similar effects and that the mRNA levels of *gsts1* and *gstm1* are similar to those of hsp genes. Cytochrome P450 enzymes can affect oxidation reactions caused by exogenous compounds (Nash et al., 2014). In the current study, there was no clear difference in *cyp4* between the TBEP treatment groups and the control groups, which is consistent with the results of Papo et al. (2014). The expression levels of *cyp4* significantly increased in response to 2000 μg/L TBP, potentially catalyzing TBP to produce ROS. The potentially increased ROS could lead to decreased levels of GST (*gsts1* and *gstm1*) and increased hsp70 in response to 2000 μg/L TBP.

Fig. 2. Antioxidant enzyme activities and MDA levels in digestive glands from different *C. fluminea* exposed to TBEP and TBP. Data represent the mean ± SEM (n = 3). Different letters above the columns indicate significant differences at p < 0.05 between the treatment and control groups.
Fig. 3. Expression levels of the gsts1, gstm1, abcb1, abcc1 and cyp4 genes in digestive glands from different C. fluminea exposed to TBEP and TBP. Data represent the mean ± SEM (n = 3). Different letters above the columns indicate significant differences at p < 0.05 between the treatment and control groups.
Fig. 4. Expression levels of \textit{hsp} genes (\textit{hsp} 22, \textit{hsp}40, \textit{hsp} 60, \textit{hsp} 70 and \textit{hsp}90) in digestive glands from different \textit{C. fluminea} exposed to TBEP and TBP. Data represent the mean ± SEM (\(n = 3\)). Different letters above the columns indicate significant differences at \(p < 0.05\) between the treatment and control groups.
TBP. The decreased levels of gst1 and gstm1 following exposure to 2000 µg/L TBP compared with the control groups could have been due to the potentially increased ROS levels, consistent with the changes in GR activity and the levels of cyp4. Similarly, Pan et al. (2011) indicated that cyp4 increased in *Rudipates philippinum* exposed to benzo(a)pyrene (B(a)P) for 10 d. It is believed that the digestive gland is the major site of action of detoxification pathways in mollusks (Zanette et al., 2013). Therefore, we presume that cyp4 participates in detoxification pathways in the *C. fluminea* digestive glands.

Because of the sensitivity to even minor assaults, heat shock proteins are being used as early warning bioindicators of cellular hazards (Gupta et al., 2010; Monari et al., 2011). The expression levels of hsps can be influenced by various stressors, such as physical stress and pollutants, among others (Monari et al., 2011; Yue et al., 2011; Kim et al., 2014; Liu et al., 2015). Belonging to the small heat shock protein superfamily, hsp22 can react with mimics of phosphorylated hsp27 (Benndorf et al., 2001). Hsp40 can prevent proteins from forming irreversible aggregates during the period of synthesis or at times of cellular stress, and it can participate in conditioning the ATPase activity of hsp70. As important sentinels of chemical stress, the mRNA levels of hsp60, hsp70, and hsp90 (which vary widely from bacteria to humans) (Chen et al., 2014) are frequently assessed (Hull et al., 2013). In the present study, hsp genes (hsp22, hsp40, hsp60, hsp70, and hsp90) were down-regulated at low concentrations of TBEP (20 and 200 µg/L) and TBP (20 µg/L), which is in accordance with the change in antioxidant enzyme activities. These results may be a consequence of the involvement of hsp genes (hsp22, hsp40, hsp60, hsp70, and hsp90) in repairing protein damage by oxidative stress (Otaka et al., 2006) and antioxidant enzyme reducing stress-induced increases in ROS levels by depleting SOD and CAT activities. According to Chen et al. (2014), the mRNA levels of hsp70 in digestive glands were significantly up-regulated, which is similar to our results at high concentrations of TBEP (2000 µg/L). This may be attributed to the decreased stress caused by the closed shell effects at 2000 µg/L TBP. Similar to our results at 20 µg/L TBP, Liu et al. (2012a) reported that hsp90 was decreased in *Tanichthys albonubes* following exposure to cadmium. The changes in hsp genes reflect the stress incurred by TBEP and TBP at a molecular level on the clam tissue. A multixenobiotic resistance (MXR) system protects cells and organisms from endogenous and exogenous toxicanics, and it plays an important role in the excretion of xenobiotics in cells through the ATP-binding cassette (ABC) transport protein during detoxification processes (Epel et al., 2008; Caminada et al., 2008; Babić et al., 2016). The ABCs mostly transport organic anions conjugated to glutathione, glucuronide, sulfate or other polar groups (Leslie et al., 2005) and are thought to be the first line of defense against toxicanics (Cunha et al., 2016). Previous studies have demonstrated that the most toxicologically relevant proteins in mammals include the P-glycoprotein (ABCB1) and the multidrug resistance-associated proteins 1–5 (ABCC1–5), among others. (Leslie et al., 2005; Szakacs et al., 2008). ABCC1 is known to transport moderately hydrophobic or amphiphilic substances and neutral or positively charged substances, including exogenous and endogenous compounds (Yuan et al., 2014). Herein, the abcb1 and abcc1 genes were used to evaluate the toxicity of TBEP and TBP to *C. fluminea* tissue samples. Similar to the results obtained during TBEP exposure, 2, 3, 7, and 8- tetrachlorodibenzo-p-dioxin (TCDD) have been reported to enhance human hepatic abcb1 (2-3-fold induction) and to increase protein expression levels in liver membranes (Wang et al., 2011), leading to reduced cytosolic concentrations of toxicanics to prevent damage to the organism. In contrast to our results, Chen et al. (2015) reported that the abcc1 gene significantly decreased after exposure to fluoxetine. Significantly high expression levels of abcb1 and abcc1 were observed in all TBEP treatments compared with the control groups, indicating that abcb1 and abcc1 were activated to mediate the efflux of xenobiotics in clams, which is similar to a mechanism reported in fish by Loncar et al. (2010).

5. Conclusions

In this study, we examined the effects of TBEP and TBP on siphoning behavior, stress biomarkers, and the gene expression levels of oxidative stress, heat shock proteins and the MXR system in the digestive glands of *C. fluminea*. The adverse effect of behaviors and antioxidant enzymes suggested that the multixenobiotic resistance mechanisms of *C. fluminea* differed between low and high concentrations of TBEP and TBP. *C. fluminea* mainly relies on antioxidant enzyme depletion to reduce damage without an increase in MDA levels at low exposure concentrations and on shell closure at high concentrations to prevent xenobiotic entry. Moreover, *C. fluminea* relies on increasing levels of abcb1 and abcc1 to decrease the cytosolic concentrations of xenobiotics in clams to reduce damage.

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