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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 *gsts1* 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.

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

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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 (TCPP) 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 (Nancharaiah 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 grown by inducing oxidative stress and reducing photosynthesis (Song et al., 2016). Neerathilingama 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 mg/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 contamination 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.

2. Materials and methods

2.1. 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%.

2.2. 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.

2.3. 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.