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Elucidating multilevel toxicity response differences between tris (1,3-dichloro-2-propyl) phosphate and its primary metabolite in *Corbicula fluminea*

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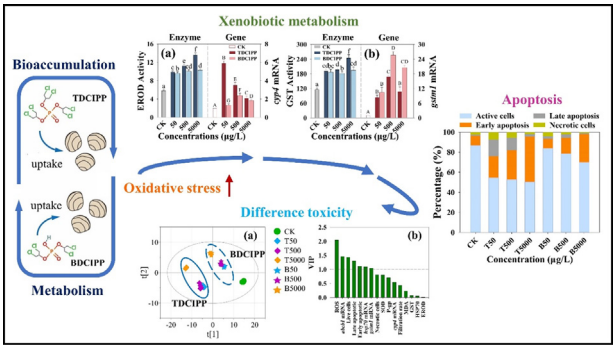
HIGHLIGHTS

- TDCIPP/BDCIPP accumulated in clams was positively related to their hydrophobicity.
- Digestive glands were the major organ for the uptake and metabolism of TDCIPP.
- *cyp4*, *gstm1*, and *abcb1* mRNA exhibited different sensitivities to TDCIPP and BDCIPP.
- TDCIPP induced more apoptotic cells in digestive glands than BDCIPP.
- The rapid metabolism of BDCIPP alleviated its biotoxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) and its primary metabolite, bis(1,3-dichloro-2-propyl) phosphate (BDCIPP) are frequently detected in aquatic environments. However, information regarding the biotoxicity of these compounds to bivalves is limited. We explored the multilevel physiological responses of *Corbicula fluminea* to TDCIPP and BDCIPP. The results indicated that TDCIPP/BDCIPP bioaccumulation in bivalves was positively correlated with their hydrophobicity. Furthermore, the higher body burden of TDCIPP in digestive glands led to significantly higher levels of ethoxyresorufin-O-deethylase (EROD), glutathione S-transferase (GST), and P-glycoprotein ($p < 0.05$). Owing to different molecular structures of inducers, upregulations of *cyp4*, *gstm1*, and *abcb1* mRNA exhibited different sensitivities to TDCIPP and BDCIPP. Although Phase-I and Phase-II biotransformation and the multixenobiotic resistance (MXR) system were activated to protect bivalves from TDCIPP or BDCIPP, digestive glands produced large amounts of reactive oxygen species (ROS). Moreover, oxidative stress, the percentage of apoptotic cells in digestive glands, and inhibition of siphoning behaviour in TDCIPP treatments were higher than those in BDCIPP treatments ($p < 0.05$), indicating that TDCIPP was more toxic to bivalves than BDCIPP. Lower bioaccumulation and rapid metabolism of BDCIPP *in vivo* may contribute to alleviating its toxicity. This research establishes a foundation for further understanding the differences between the toxic mechanisms of TDCIPP and its metabolites.

1. Introduction

Global consumption of organophosphate flame retardants (OPFRs) has been increasing sharply due to the prohibition on polybrominated diphenyl ethers (PBDEs) (Veen and Boer, 2012; Wei et al., 2015). As

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one of the main OPFRs, tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is widely applied in the manufacture of electronic equipment and upholstered furniture, in the building industry, as well as in foams and textiles (Veen and Boer, 2012). The annual production volume of TDCIPP was 4500–23,000 tons between 2011 and 2015 in the United States (Zhu et al., 2018).

As an additive flame retardant, TDCIPP could easily be released from products into surrounding environments (Wei et al., 2015), and inevitably enter aquatic environments through dry and wet deposition, surface and groundwater runoff (Cao et al., 2017; Regnery et al., 2011), and treated and untreated wastewater discharge (Kim et al., 2017). With the increase in TDCIPP usage, various aquatic environments have been contaminated with TDCIPP, including drinking water (Li et al., 2014), marine water (Chen et al., 2019a), lakes (Cao et al., 2017; Wang et al., 2018), and influents and effluents of wastewater treatment plants (Kim et al., 2017). The TDCIPP concentrations in waters ranged from nanograms per litre to milligrams per litre (Veen and Boer, 2012), and the highest level of TDCIPP (0.68–6.18 mg/L) was detected in raw water from a Japanese sea-based solid waste disposal site (Kawagoshi et al., 1999). Considering the increasing ubiquity and distribution level of TDCIPP in aquatic ecosystems, the risk posed to aquatic organisms requires urgent attention.

Bis(1,3-dichloro-2-propyl) phosphate (BDCIPP) is the primary metabolite of TDCIPP, and *in vitro* and *in vivo* studies have demonstrated rapid transformation of TDCIPP to BDCIPP (Briels et al., 2018; Wang et al., 2017a). Biotransformation of TDCIPP to BDCIPP involves cleavage of the ether bond (O-dealkylation) and oxidative dehalogenation of the terminal carbon atom to form diesters and hydroxylated metabolites (Hou et al., 2016). Furthermore, rapid depuration of BDCIPP from tissues indicated that the formed metabolites may not be persistent in organisms, and could be easily excreted (Wang et al., 2017a). Additionally, BDCIPP have been frequently detected in human urine (Hammel et al., 2016), house dust (Tan et al., 2019), wastewater, and sludge from wastewater treatment plants (Kim et al., 2017). The BDCIPP concentrations are up to 2550 ng/g in midwestern USA house dust, and exhibit significant correlations with TDCIPP (Tan et al., 2019). Moreover, the BDCIPP concentration (4550 ng/L) in influents is higher than that of TDCIPP (3150 ng/L) (Kim et al., 2017). Given the increasing production and usage of TDCIPP, BDCIPP will continue to accumulate in aquatic environments, and particular attention should be paid to its potential environmental risks.

The current investigations mainly focused on the potential toxicity of TDCIPP. Researches have confirmed that exposure to TDCIPP at environmentally relevant concentrations could cause reproductive, developmental, and neurological toxicity to zebrafish, daphnia, and Chinese rare minnow (Hong et al., 2019; Li et al., 2017; Li et al., 2020). It was observed that early-life exposure to TDCIPP could cause delayed neurotoxicity in adult zebrafish (Li et al., 2020). Li et al. (2017) demonstrated that 3000 ng/L TDCIPP significantly inhibited growth and reproduction of *Daphnia magna* and decreased survival. Another study observed that BDCIPP was a more potent teratogen by four orders of magnitude than TDCIPP, causing reduced survival and impaired development in zebrafish embryos (Noyes et al., 2015). However, BDCIPP-specific toxicity studies have received little attention despite the known adverse impacts.

Investigations on bioaccumulation and distribution are critical for evaluating the fate and potential toxicity of compounds in organisms (Wang et al., 2017a). To date, only limited research has focused on TDCIPP bioaccumulation in aquatic organisms (Wang et al., 2017a; Wang et al., 2016). It was reported that the highest TDCIPP level was detected in the liver, followed by the brain and gonads in zebrafish (Ren et al., 2018; Wang et al., 2017a). In addition, high BDCIPP levels were detected in the liver and intestine, indicating that the hepatobiliary system (liver-bile-intestine) is important for metabolism and excretion of TDCIPP in zebrafish (Wang et al., 2017a). *Corbicula fluminea* (*C. fluminea*), as a benthic organism, could accumulate various organic pollutants contaminants in its tissues, and is frequently used to reflect environmental perturbations or contaminations (Chen et al., 2012a). Nevertheless, despite a growing number of studies having been conducted to delineate the potential effects of TDCIPP to aquatic organisms, knowledge regarding the accumulation and health risks of TDCIPP and BDCIPP in *C. fluminea* remains scarce. Therefore, *C. fluminea* was selected as a biotic pollution indicator in this study.

In the present study, the bioaccumulation and tissue distribution of TDCIPP and its primary metabolite, BDCIPP, were evaluated following exposure of *C. fluminea* to TDCIPP or BDCIPP. Concomitantly, the toxic effects of TDCIPP and BDCIPP on siphoning behaviour, apoptosis and necrosis, oxidative stress, cellular detoxification, and the multixenobiotic resistance (MXR) system of *C. fluminea* were examined. Finally, the underlying mechanism of differential toxicity of TDCIPP and BDCIPP to bivalves was proposed.

2. Materials and methods

2.1. Reagents

TDCIPP (purity >95%) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). BDCIPP (purity >99%) was acquired from Toronto Research Chemicals Inc. (Canada). All solvents were HPLC-grade and were provided by Shanghai Macklin Biochemical Co. Ltd. (China). The CNWBOND GCB/NH_2 solid-phase extraction (SPE) cartridges (6 mL, 500 mg) were supplied by CNW Technologies GmbH (Germany).

2.2. C. fluminea and exposure protocols

Healthy *C. fluminea* (approximately 2.1 ± 0.5 cm in length) were provided by the Fishery Management Office at Hongze Lake (Jiangsu, China). Prior to the exposure experiment, the specimens were acclimatised in aerated tap water at 25 ± 1 °C for one week in the dark without feeding (Li et al., 2018). Thereafter, 20 *C. fluminea* specimens were added to a 3-L beaker and exposed to a 1.5 L solution of TDCIPP and BDCIPP at concentrations of 0, 50, 500, and 5000 μg/L for 20 days, respectively. A solvent control was used, and all treatment groups received 0.01% dimethyl sulfoxide. Exposure solutions were changed daily with fresh water containing corresponding TDCIPP and BDCIPP concentrations, and each treatment included five replicate beakers. Before and after the change of exposure solutions, the concentrations of TDCIPP and BDCIPP were determined, and more detection details were provided in Supplementary information (SI). The concentrations of TDCIPP and BDCIPP in exposure solutions were highly consistent with the calculated nominal concentrations. Besides, no significant depletion was observed for TDCIPP and BDCIPP in this clam exposure system, which was consistent with the researches of Zhang et al. (2018) and Zhu et al. (2015).

The specimens were fed daily with 5 mL *Scenedesmus obliquus* algae at a concentration of 2.5×10^6 cells/mL. At the end of the exposure period, eight *C. fluminea* individuals were collected, opened by tweezers, and rinsed with Milli-Q® water. Then, the soft tissues were dissected into mantle, foot, gill, and digestive glands and frozen separately at −20 °C and −80 °C for accumulation of TDCIPP and BDCIPP, and enzyme and gene expression analyses, respectively (Li et al., 2018; Wang et al., 2019).

2.3. Quantification of TDCIPP and BDCIPP in tissues

Biota samples were freeze-dried, ground, and homogenised. Extractions and quantifications of TDCIPP and BDCIPP in the tissues of *C. fluminea* were conducted according to Wang et al. (2019) with some modifications; more details of extraction methods are described in the SI. TDCIPP and BDCIPP concentrations were determined by an ultraperformance liquid chromatography-tandem triple quadrupole mass spectrometry (Xevo TQ-S; Waters, Corporation, USA) equipped with a Waters BEH C18 column (2.1×100 mm, 1.7 μm). The quantification details can be found in the SI.

2.4. Quality assurance and quality control

All of the containers used in the experiments were composed of glass to avoid sample contamination. To compensate the matrix effects on TDCIPP and BDCIPP quantitation in bivalves, multi-level calibration curves were created based on the spiked blank tissues and high linearity ($r^2 > 0.99$) was obtained for all target compounds. Method blanks and solvent blanks were included with each batch of 12 samples to check background contamination and monitor any instrument carryover. Three quality control standard solutions (10 μg/L TDCIPP and BDCIPP standards mixture) were run to monitor sensitivity drift along with each 12 real samples. Recovery of TDCIPP and BDCIPP was evaluated by spiking the standard solution of TDCIPP and BDCIPP to different tissues (10 ng/g, dw) and water (5 μg/L) to monitor matrix effects, and recoveries of TDCIPP and BDCIPP were 98.7–126.7% and 93.2–117.3% in tissues and were 107.4% and 98.6% in water, respectively (Table S2). Method detection limits (MDLs) were defined as three times the noise, and the MDLs for TDCIPP and BDCIPP were 1.47 and 3.16 ng/g dw (dry weight) in tissue and 0.36 ng/L and 0.21 ng/L in exposure solution, respectively. None of the target compounds were detected in the blanks.

2.5. Relevant enzymes and malondialdehyde (MDA) assay

According to the method of Chen et al. (2015), approximately 200 mg of digestive glands from three *C. fluminea* specimens of the control and treated groups were ground on ice with a pestle in a centrifuge tube (1.5 mL) containing 0.5 mL RIPA Lysis buffer (Beyotime Institute of Biotechnology, China). The homogenate was centrifuged at 12,000*g* for 10 min, and the supernatant was collected. All treatment specimens were in quintuplicate. The contents of malondialdehyde (MDA), P-glycoprotein, and heat shock proteins 70 (HSP70), as well as the activities of reactive oxygen species (ROS), superoxide dismutase (SOD), ethoxyresorufin-O-deethylase (EROD), and glutathione S-transferase (GST) were determined in digestive glands using corresponding kits (Jiangsu Feiya Biological Technology Co., Ltd., China) according to the manufacturer's protocols.

2.6. Relative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated from the digestive glands of each sample using an SV Total RNA Isolation System (Promega, USA), as specified in the manufacturer's protocols. Then, 800 ng of RNA was reverse transcribed into cDNA according to the protocol described by Chen et al. (2015). The expression of each mRNA (*cyp4*, *gstm1*, *abcb1*, and *hsp70*) was analysed in quintuplicate with the LightCycler® 480 Software Setup (Roche, Switzerland). The thermal cycle of polymerase chain reaction (PCR) consisted of initial steps (5 min at 95 °C) followed by 40 amplification cycles (10 s at 95 °C, 10 s at 60 °C, and 30 s at 72 °C). The *β-actin* mRNA of *C. fluminea* was used as an endogenous control. The primer pairs are listed in Table 1. The final results were analysed using the delta-delta Ct method as described by Livak and Schmittgen (2001).

Table 1
Primer sequences used for *C. fluminea* mRNA detection.

Genes	Function	Primer sequences (5′-3′)	Accession no.
β-Actin	Cytoskeletal gene	CGCCATCCAGGCTGTGCTTTCA[a] ATGGCGTGTGGAAGGGCGTA[b]	EF446608.1
cyp4	Phase-I metabolism	GCGAAACCAAGCGAGAGGT[a] ATCCCATAACAACTGCGGGT[b]	KF218340
gstm1	Phase-II metabolism	GGGCCTGGTGGAAGATATG[a] ACAAGTCGGCCAATGTGAG[b]	KJ001774
abcb1	Xenobiotics excretion	ATCCTGGTTGATGGCACTGA[a] AGGTTCCTGGCTCACAATACC[b]	KJ001772
hsp70	Warning bioindicator	CGCCGACGCTGATTACCTTA[a] AAACGGTTGATAGGACGCAAG[b]	KC979064

[a] Forward primer.
[b] Reverse primer.

2.7. Apoptosis and necrosis assays

To evaluate apoptosis and necrosis of the digestive glands of *C. fluminea* after exposure to TDCIPP and BDCIPP, an Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, MO, USA) was used according to the manufacturer's protocol. Briefly, fresh digestive glands from each treatment were dissected and ground into pieces of approximately 1 mm^3 in a sterile atmosphere and washed twice with cold 0.01 M phosphate buffer saline (PBS). After treatment with proteinase K for 30 min, the suspension was filtered through a 40μm nylon mesh and centrifuged at 250*g* for 5 min. Cells were washed twice with 0.01 M PBS and resuspended in 1 × binding buffer at a concentration of 1×10^6 cells/mL. Subsequently, 500 μL of the cell suspension was incubated with 5 μL of Annexin V FITC conjugate and 10 μL of propidium iodide solution for exactly 10 min at 25 °C in the dark. Cell apoptosis and necrosis were detected by flow cytometry (Becton Coulter, Inc.). The contour plots were chosen to present the results of the flow cytometry results, because it could not only exhibit the percentages of live cells, apoptosis rates and necrotic cells, but also provide a good indication of the frequency of cells in given region, as the contours in these plots are computed so that an equal percentage of the cells are bounded by each contour (Tung et al., 2004).

2.8. Siphoning behaviour

Immediately after 20 days chemical exposure, five *C. fluminea* from each treatment were placed in a 250-mL beaker containing 100 mL neutral red solution (1 mg/L) for 3 h to measure the siphoning rates. Prior to placing the clams in the solution, and after the 3-h exposure, 1 mL water was sampled from each beaker to determine the absorbance of neutral red solution with a spectrophotometer at 530 nm. Absorbance can be transformed into the neutral red concentration based on a standard curve of the concentration of standard solutions of neutral red (Chen et al., 2015; Yan et al., 2017). The filtration rates, m (mL/animal/h), in each treatment group were calculated according to the equation of Cooper and Bidwell (2006):

$$m = \left[\frac{M}{nt}\right] \log\left(\frac{C_0}{C_t}\right), \quad (1)$$

where 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.

2.9. Statistical analysis

One-way analysis of variance was conducted to examine significant differences between the same biomarker response in different treatment groups. The least significance difference (LSD) were applied to test for significant ($p < 0.05$). Statistical analysis was carried out using SPSS 20.0. Graphs were plotted in SigmaPlot 12.5. Partial least-squares-discriminant analysis (PLS-DA) was run using SIMCA-P 14.1.

3. Results

3.1. Quantification of TDCIPP and BDCIPP in C. fluminea

The TDCIPP and BDCIPP contents in clams in the control group were below the detection limit of the method. After 20 days of exposure, the total bioaccumulations of TDCIPP in soft tissues of *C. fluminea* were 39.73 ± 0.07, 120.91 ± 6.08, 498.79 ± 3.28 nmol/g dry weight (dw) in the 50, 500, and 5000 μg/L TDCIPP treatments, respectively (Fig. 1a). The highest TDCIPP concentration was observed in digestive glands, followed by gill, mantle, and foot, while gills accumulated more pollutants in the 50 μg/L TDCIPP group. Besides, the concentrations of BDCIPP transformed by TDCIPP in tissues were also detected

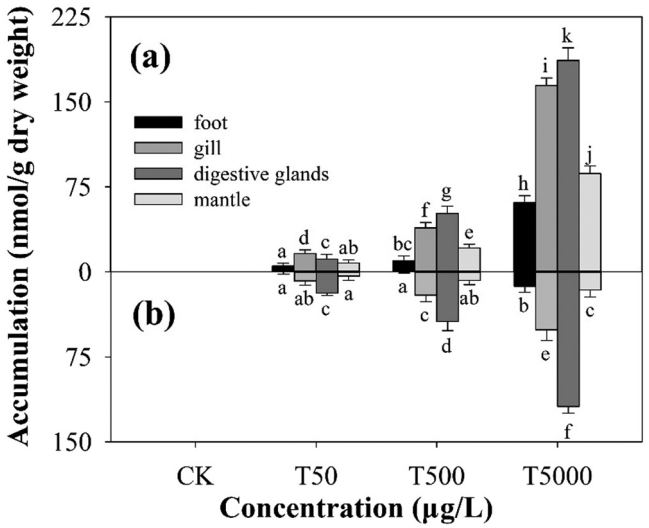

Fig. 1. Concentrations of (a) TDCIPP and its primary metabolite, (b) BDCIPP in different tissues of *C. fluminea* after 20 days exposure to the control (CK) and various TDCIPP concentrations. Values are the mean ± SE (n = 5). Bars with different letters are significantly different based on LSD ($p < 0.05$).

after exposure, which were 30.54 ± 0.35, 71.83 ± 5.50, 198.42 ± 13.73 nmol/g dw in the 50, 500, and 5000 μg/L TDCIPP treatments, respectively, and accounted for 76.9, 59.4, and 39.8%, respectively, of the TDCIPP accumulation at mole mass. The peak BDCIPP concentration in tissues was also observed in digestive glands, and the order of BDCIPP bioaccumulation in tissues was similar to that of TDCIPP.

3.2. Effects on oxidative stress

In Fig. 2, the activity of ROS, SOD, and MDA in the digestive glands of *C. fluminea* were increased by both TDCIPP and BDCIPP after 20 days exposure ($p < 0.05$). Furthermore, the levels of ROS, SOD, and MDA in the TDCIPP treatments were higher than those in the BDCIPP groups exposed to the same concentration ($p < 0.05$). Changes in the activity of ROS, SOD, and MDA exhibited consistent patterns in the presence of TDCIPP or BDCIPP, and larger increases were generally observed in organisms exposed to high concentrations.

3.3. Effects on xenobiotic metabolism

As shown in Fig. 3, the activity of EROD and GST, and the contents of P-glycoprotein and HSP70 increased with increasing TDCIPP concentrations. Following exposure to BDCIPP, upregulations of EROD, GST, P-glycoprotein, and HSP70 were lower than in the TDCIPP treatments ($p < 0.05$). Besides, the P-glycoprotein and HSP70 contents exhibited a concentration dependence with respect to BDCIPP, while the relationship between the activity of EROD and GST and the BDCIPP concentration was not obvious.

The transcript levels of *cyp4* genes were decreased with increasing TDCIPP concentrations ($p < 0.05$), and the levels of both *gstm1* mRNA and *abcb1* mRNA were enhanced in all TDCIPP treatments ($p < 0.05$). Whereas, expression of *hsp70* transcript (5.90-fold) was significantly elevated only in the 50 μg/L TDCIPP treated group compared to the control group ($p < 0.05$). Following exposure to BDCIPP, the expressions of *cyp4*, *gstm1*, *hsp70* were upregulated ($p < 0.05$), and the levels of *gstm1* and *hsp70* were apparently higher than those in the TDCIPP treatments ($p < 0.05$). However, the significant induction of *abcb1* mRNA was only observed in the 500 μg/L BDCIPP treatment group ($p < 0.05$).

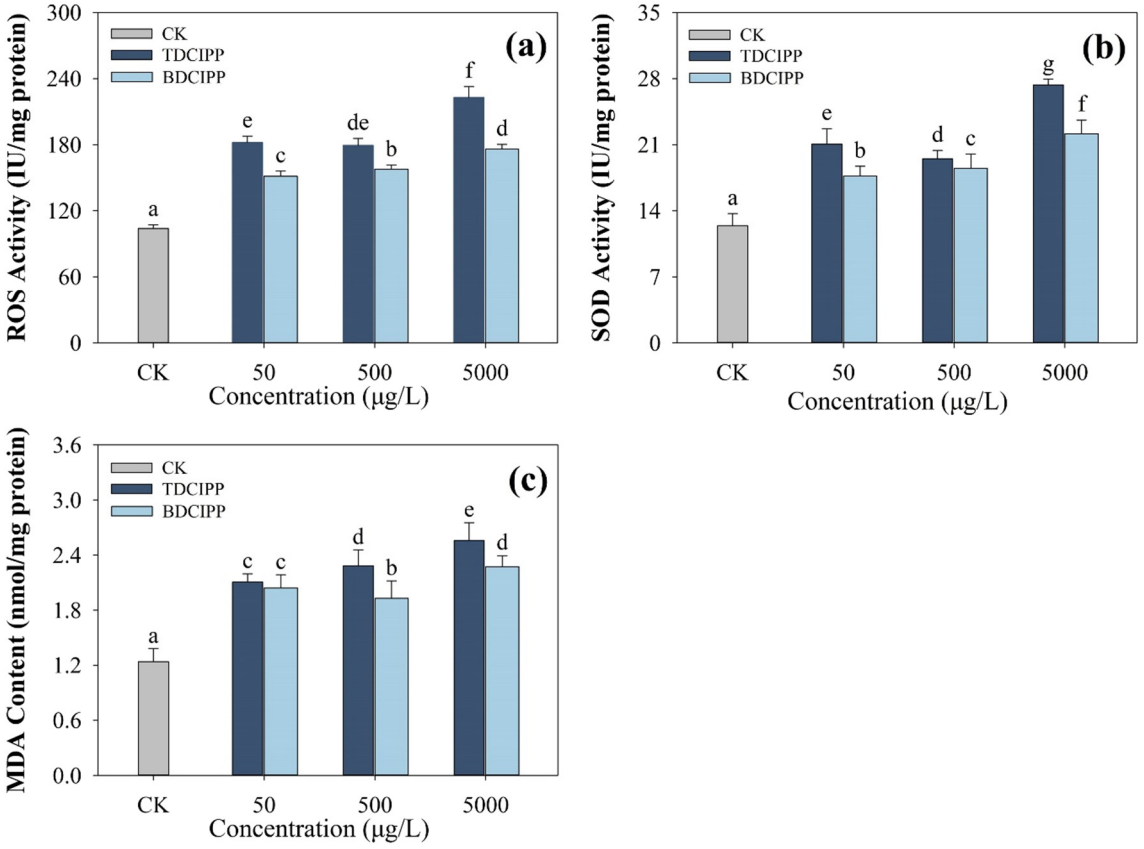

Fig. 2. Levels of (a) ROS activity, (b) SOD activity, and (c) MDA content in *C. fluminea* digestive glands after exposure to a control treatment (CK) and increasing concentrations of TDCIPP and BDCIPP for 20 days. Values are the mean ± SE (n = 5). Bars with different letters are significantly different based on LSD ($p < 0.05$).

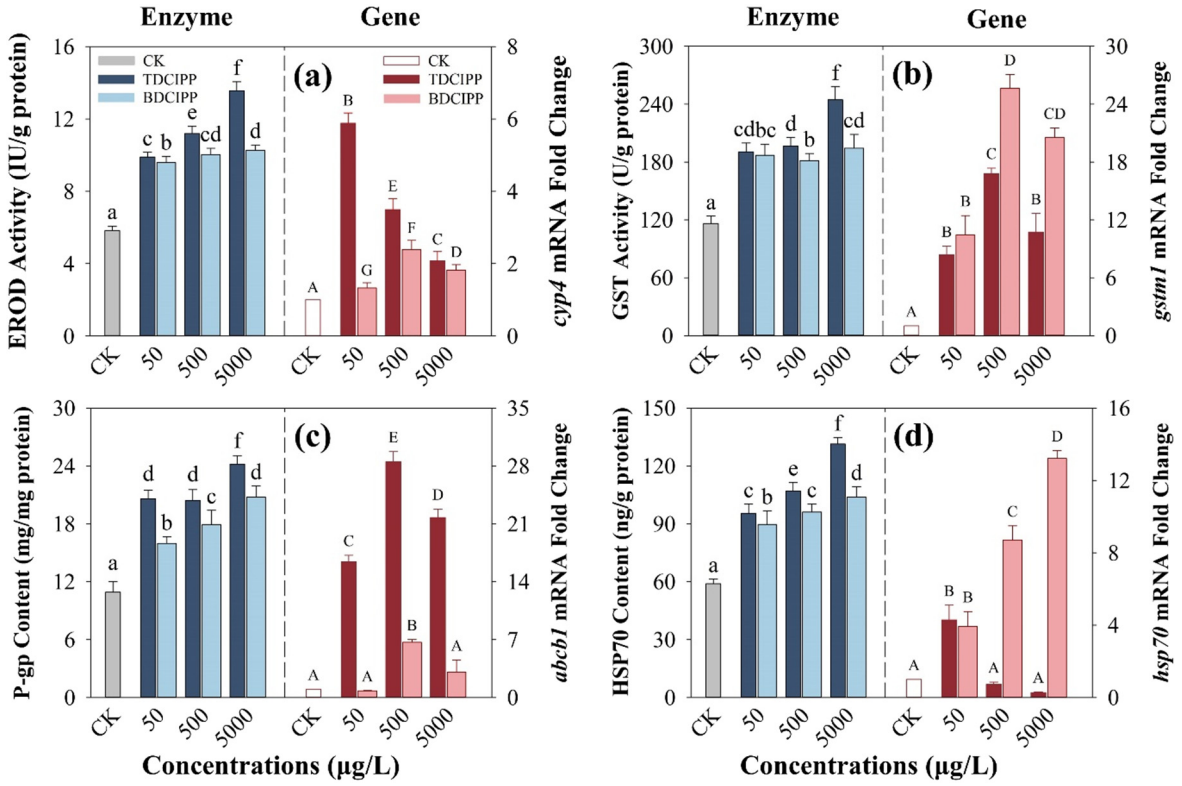

Fig. 3. Levels of (a) EROD activity, (b) GST activity, (c) P-glycoprotein (P-gp) content, and (d) HSP70 content and corresponding mRNA levels in *C. fluminea* digestive glands after exposure to a control treatment (CK) and increasing concentrations of TDCIPP and BDCIPP for 20 days. Values are the mean ± SE (n = 5). Bars with different letters are significantly different based on LSD ($p < 0.05$).

3.4. Effects on apoptosis and necrosis rates

As shown in the flow cytometry results (Fig. 4), the percentage of live cells of digestive glands decreased in the TDCIPP and BDCIPP treatments, and both treatments evoked apoptosis in a concentration-dependent manner. Early apoptosis rates in TDCIPP treatments were 21.53% (50 μg/L), 29.28% (500 μg/L), and 45.16% (5000 μg/L), which were higher than that of BDCIPP treatments (50 μg/L: 9.53%; 500 μg/L: 15.87%; 5000 μg/L: 28.22%). The influences of TDCIPP and BDCIPP on digestive gland necrosis were not significant compared to the control group.

3.5. Effects on siphoning behaviour

The effects of TDCIPP and BDCIPP on the siphoning behaviours of *C. fluminea* are illustrated in Fig. 5. The filtration rates of *C. fluminea* obviously decreased after exposure to TDCIPP and BDCIPP for 20 days ($p < 0.05$), and the inhibition of TDCIPP (30.3%–44.2%) on siphoning behaviour was stronger than that of BDCIPP (8.0%–15.5%). However, there was no significant correlation between the filtration rates and the exposure concentrations of TDCIPP or BDCIPP.

3.6. Partial least-squares-discriminant analysis

As PLS-DA demonstrated (Fig. 6), a distinct separation was visible among the control group, TDCIPP treatments, and BDCIPP treatments. Moreover, significant differences were also observed as a function of the exposure concentration. All samples were located within the 95% confidence interval, as indicated by the Hotelling's T2 ellipse (Fig. 6a). The values of variable importance in the projection (VIP) are presented in Fig. 6b. For *C. fluminea*, the most important biomarkers (VIP > 1) (Fig. 6b) were ROS, *abcb1* mRNA, live cells, early apoptotic cells, *hsp70* mRNA, and *gstm1* mRNA. The values of R^2Y_{cum} and Q^2_{cum} could evaluate the quality of the PLS-DA model, which were 0.655 and 0.610, respectively.

4. Discussion

4.1. Toxicity of TDCIPP to C. fluminea

As the tissue concentration could reflect the bioavailable and effective target dose more accurately than the traditional dose in the exposure media, the true potency of toxic effects should be better reflected in tissue concentration-based dose metrics (Chen et al., 2019b). After exposure to TDCIPP for 20 days, TDCIPP bioaccumulation in *C. fluminea* was dependent on the exposure concentration. The result was echoed in the finding that TDCIPP accumulated in organisms was in a dose-dependent manner (Chen et al., 2019b; Li et al., 2020). The gills are the first tissue in contact with xenobiotics (Bonnafé et al., 2015). With exposure to 50 μg/L, the bioconcentration of TDCIPP in gills was significantly higher than that in digestive glands ($p < 0.05$). There is a potential that TDCIPP could be metabolised and depurated rapidly in digestive glands at this exposure concentration. The concentration of BDCIPP transformed by TDCIPP was much lower than the bioaccumulation of TDCIPP, and this result was in accordance with the finding of Briels et al. (2018), indicating that further metabolization of BDCIPP occurred, or that TDCIPP transformed to other compounds than BDCIPP. It has generally been acknowledged that the digestive glands of bivalves are the primary place for contaminant uptake and metabolism of inorganic and organic chemicals (Zanette et al., 2013). Higher bioaccumulations of TDCIPP and BDCIPP were observed in this organ ($p < 0.05$) (Fig. 1), confirming that the digestive glands were the major organ for TDCIPP accumulation and detoxification in *C. fluminea*.

Many environmental stimuli including organic pollutants may generate high levels of ROS, which can perturb the normal redox balance, shift cells into a state of oxidative stress, and degrade proteins, DNA,

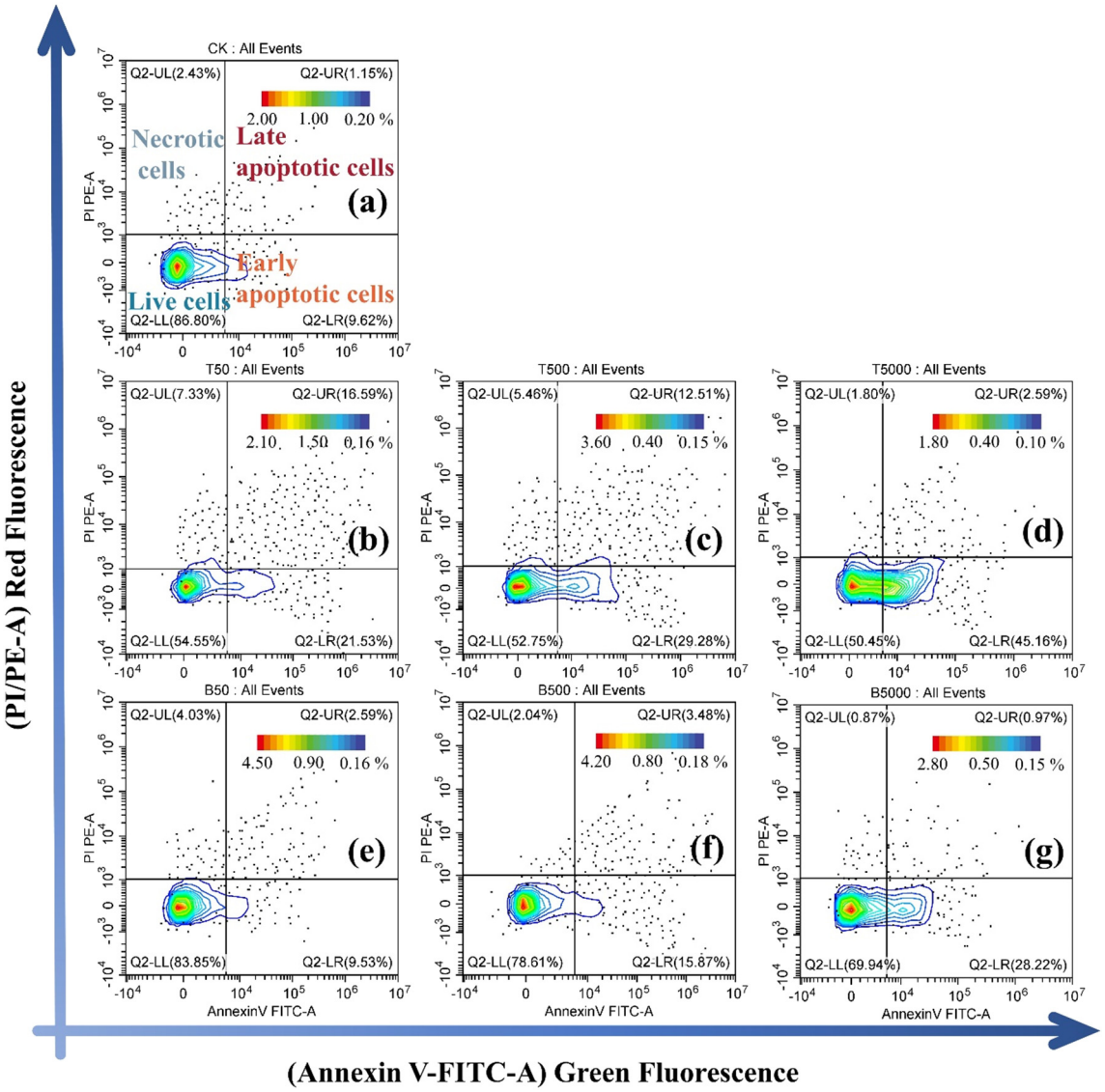

Fig. 4. Contour plots of apoptosis and necrosis in *C. fluminea* digestive glands after exposure to a control treatment (CK) and increasing concentrations of TDCIPP and BDCIPP for 20 days. Lower left quadrant, live cells; upper left quadrant, early apoptotic cells; lower right quadrant, necrotic cells; and upper right quadrant, late apoptotic cells. (a) control group (CK), (b) 50 μg/L TDCIPP, (c) 500 μg/L TDCIPP, (d) 5000 μg/L TDCIPP, (e) 50 μg/L BDCIPP, (f) 500 μg/L BDCIPP, and (g) 5000 μg/L BDCIPP.

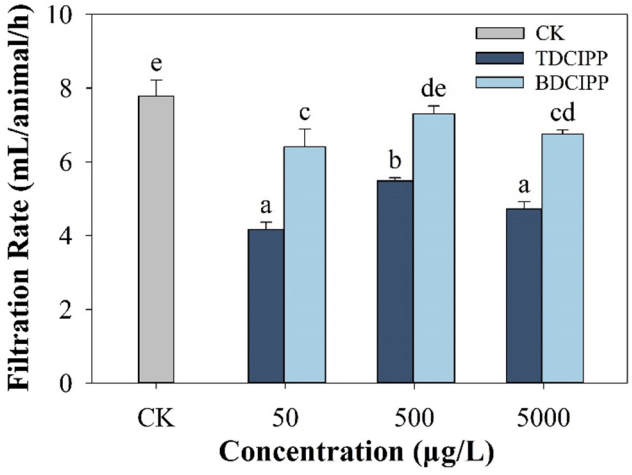

Fig. 5. Effects of TDCIPP and BDCIPP on the filtration rate (mL/animal/h) of *C. fluminea* compared to the control (CK). Values are the mean ± SE (n = 5). Bars with different letters are significantly different based on LSD ($p < 0.05$).

and lipids (Finkel and Holbrook, 2000). In the present study, the ROS activity in the digestive glands of *C. fluminea* was significantly upregulated after exposure to TDCIPP for 20 days ($p < 0.05$) (Fig. 2a). The levels of SOD activity and MDA content increased with increasing TDCIPP concentration ($p < 0.05$). As MDA is the decomposition product of unsaturated fatty acid peroxides (Yan et al., 2017), MDA production indicated that the ROS levels exceeded the elimination capacity of the antioxidant enzymes, and that cell membrane damage occurred (Li et al., 2018). The present study is consistent with the findings of Elshenawy et al. (2009), who reported that digestive cells of clams were seriously degenerated after exposure to chlorpyrifos-methyl or glyphosate. Therefore, TDCIPP may not only induce oxidative stress in clams but may also result in digestive glands dysfunction.

Upon exposure to pollutants, organisms usually attempt to metabolise and depurate directly, minimising any cellular damage they may cause (Cheung et al., 2001). Previous studies have demonstrated that OPFRs can be rapidly metabolised through Phase-I and Phase-II biotransformation to metabolites, which are more hydrophilic and more readily eliminated (Van den Eede et al., 2013). Cytochrome P450 (CYP450) is a major biotransformation enzyme (Bonnafé et al.,

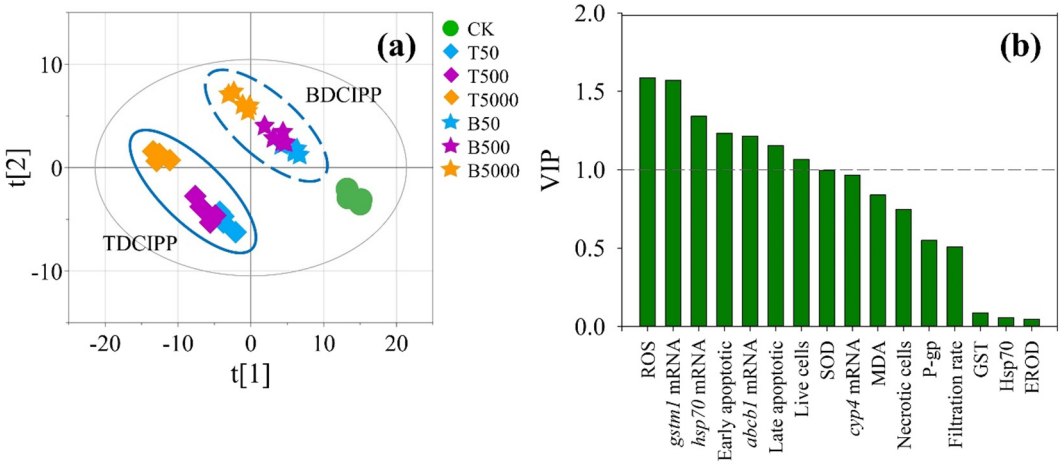

Fig. 6. (a) PLS-DA and (b) VIP of all biomarkers in *C. fluminea* after exposure to a control treatment (CK) and increasing concentrations of TDCIPP and BDCIPP for 20 days. $R^2X_{cum} = 0.974$, $R^2Y_{cum} = 0.655$, $Q^2_{cum} = 0.610$, ellipse: Hotelling's T2 (95%).

2015), and plays a critical role in several steps of Phase-I metabolism (Hou et al., 2016). Furthermore, EROD could reflect the activity of several CYP450 involved in this metabolism (Bonnafé et al., 2015). GST are phase II detoxification enzymes and catalyse the attachment of glutathione to a variety of electrophilic species for the elimination of potentially toxic xenobiotics (Bonnafé et al., 2015). The activity of EROD and GST, as well as their corresponding gene expressions, were significantly induced in the TDCIPP treatment compared to that of the control group ($p < 0.05$) (Fig. 3a, b). Yan et al. (2017) reported that the expression levels of *cyp4* mRNA in the digestive glands of *C. fluminea* obviously increased in response to exposure to 2000 μg/L tributyl phosphate (TBP), while the expression level of *gstm1* mRNA were significantly inhibited by 2000 μg/L TBP after 28 days exposure. Furthermore, it was supposed that some products produced by xenobiotics after CYP450 metabolism may inhibit GST activity (Bonnafé et al., 2015), although this did not occur in the present study. Another crucial step in detoxification is the excretion of xenobiotics and/or their metabolites by the ATP-binding cassette transporters, named the MXR system (Bonnafé et al., 2015). P-glycoprotein (encoded by *abcb1*) is known to transport exogenous and endogenous compounds and their metabolites (Leslie et al., 2005). Higher levels of P-glycoprotein and *abcb1* mRNA were detected in the presence of TDCIPP compared to those in the control group ($p < 0.05$). This agreed with previous findings in *C. fluminea* exposed to tris(2-butoxyethyl) phosphate (TBEP) and TBP (Yan et al., 2017). The results revealed that Phase-I and Phase-II biotransformation, as well as the MXR system were activated by TDCIPP to transform and mediate the efflux of it and its metabolite in *C. fluminea*. However, the proportion of BDCIPP transformed by TDCIPP declined with increasing exposure concentrations. It was reported that the decline in transformation proportion indicated a reduction in the rates of pollutants consumed by biotransformation (Wang et al., 2016; Wang et al., 2017b), which would result in more accumulation of TDCIPP in tissues. Furthermore, the accumulation of pollutants would generate ROS, and cell death would be activated by severe oxidative stress (Finkel and Holbrook, 2000).

Cell death modes are commonly divided into apoptosis and necrosis (Dwyer et al., 2012). Apoptosis, or programmed cell death, is an important internal defence mechanism in bivalves which is involved in tissue and cellular homeostasis, and is an adaptive mechanism against environmental stresses (Gervais et al., 2015). Apoptosis can be initiated *via* intrinsic and extrinsic pathways (Kiss, 2010). The intrinsic apoptotic pathway may be activated by various of cytotoxic stimuli or environmental stressors through elevated ROS production (Kiss, 2010). In this study, the ROS content was significantly increased in a dose-dependent manner after TDCIPP exposure ($p < 0.05$), and the percentage of apoptosis cells increased with increasing TDCIPP concentrations (Fig. 4). It was reported that ROS were supposed to directly account for the loss of mitochondrial membrane potential and for phosphatidylserine exposure at the cell surface membrane (Russo and Madec, 2007). Furthermore, the annexins in the Annexin V-FITC Apoptosis Detection kit is through binding externalisation of phosphatidylserine to identify early apoptotic cells (Trotter et al., 1995). The extrinsic pathway of apoptosis is activated through death receptors and a variety of G-protein coupled receptors (Nguyen et al., 2018). The activity of Caspase-3 was induced by TDCIPP in rare minnows (Chen et al., 2019b), which could connect the extrinsic and intrinsic apoptotic pathways (Kiss, 2010). Although apoptosis enables the adequate clearance of damaged, senescent, and infected cells without inflammation (Romero et al., 2011), a large number of apoptotic cells induced by TDCIPP in digestive glands may affect organ integrity and function (Kiss, 2010).

4.2. Toxicity differences between TDCIPP and BDCIPP to C. fluminea

In the current study, high body burdens of TDCIPP in *C. fluminea* were identified, while BDCIPP bioaccumulation was not detected in any tissues. Likewise, a sudden decrease in BDCIPP was observed in *Coturnix japonica* when TDCIPP in the body was depleted (Briels et al., 2018). Furthermore, Wang et al. (2017a) reported that the metabolites may be not persistent in fish and can be easily released into aquatic environments. It was documented that the bioaccumulation of OPFRs in aquatic organisms was increased with the increasing hydrophobicity when the log K_{ow} of OPFRs was below 4.59 (Bekele et al., 2019). Lee et al. (2020) expanded the relationship to including OPFR metabolites, and pointed out that the positive associations may be explained by the potential difference in the membrane permeability of chemicals which relied on the lipophilicity. According to the PubChem Compound database (https://pubchem.ncbi.nlm.nih.gov/), the octanol-water partition coefficients of TDCIPP and BDCIPP were 3.65 and 1.50, respectively. The lower hydrophobicity of BDCIPP led to less accumulation in bivalves. The results confirmed that bioaccumulation of TDCIPP and its metabolites was mainly governed by hydrophobicity.

After exposure to BDCIPP for 20 days, the activity of EROD and GST in the digestive glands were also activated to conduct Phase-I and Phase-II biotransformation of BDCIPP ($p < 0.05$) (Fig. 3a, b), converting it into more hydrophilic and more readily eliminated substances. The levels of P-glycoprotein in the digestive glands were also elevated to excrete BDCIPP and its metabolites ($p < 0.05$) (Fig. 3c). Hu et al. (2015) reported that the activity of EROD and GST, as well as the content of P-glycoprotein in scallops showed dose-dependent manners response to

tetrabromobisphenol A. Bioaccumulation of pollutants in bivalves treated with BDCIPP was much lower than that in bivalves treated with TDCIPP. Consequently, the activity of EROD and GST, and the content of P-glycoprotein in the treatments of BDCIPP were lower than that in the TDCIPP groups ($p < 0.05$).

The levels of *cyp4* gene expression in BDCIPP treatment groups were significantly lower than those in TDCIPP treatment groups for 20 days ($p < 0.05$) (Fig. 3a), while a higher level of *gstm1* mRNA was observed in BDCIPP treatments ($p < 0.05$) (Fig. 3b). As BDCIPP was validated as one of the major Phase-I metabolites for TDCIPP (Hou et al., 2016), it appears likely that the *gstm1* gene was more easily induced by BDCIPP. Since the change of *abcb1* mRNA in the 50 μg/L TDCIPP treatment was 20-fold times greater than that in 50 μg/L BDCIPP treatment ($p < 0.05$) (Fig. 3c), the results indicated that the upregulation of *abcb1* mRNA is more sensitive to TDCIPP. Chen et al. (2012b) reported that the difference in mRNA expression may be attributed to different inducers. A stronger potency of BDCIPP toward zebrafish mortality compared to TDCIPP has also been observed (Noyes et al., 2015). However, TDCIPP had a more potent effect on circulatory failure of the hatched embryos than that of BDCIPP (Lee et al., 2020). As presented in Fig. 7, the different substituents between TDCIPP and BDCIPP not only lead to a large variation in their hydrophobicity, but also result in different affinities with different toxic endpoints (Lee et al., 2020). The differential responses of the metabolic processes to TDCIPP and BDCIPP may be complicated, justifying further intensive studies to elucidate the underlying mechanisms.

Although Phase-I and Phase-II biotransformation participated in the metabolism of BDCIPP (Fig. 2a), a large amount of ROS was still produced in digestive glands of *C. fluminea*. In addition, oxidative stress in *C. fluminea* was elevated with increasing BDCIPP concentrations. As important sentinels of chemical stress (Liu et al., 2014), the HSP70 are responsible for intracellular chaperone and extracellular immunoregulatory functions, and are sensitive to even minor assaults (Liu et al., 2014; Tukaj and Tukaj, 2010). The content of HSP70, as well as its corresponding gene expressions, were markedly increased in the presence of BDCIPP. Likewise, the levels of HSP70 exhibited a linear dose-response relationship with increasing TDCIPP concentrations, while the upregulation of *hsp70* mRNA was not significant after exposure to 500 μg/L or 5000 μg/L TDCIPP (Fig. 3d). Yan et al. (2017) observed that the expression of *hsp70* mRNA in *C. fluminea* decreased with the increasing oxidative stress. These may be a consequence of the involvement of HSP70 protein in protecting cells against oxidative stress by binding and refolding damaged proteins (Boscolo Papo et al., 2014; Otaka et al., 2006). The higher the oxidative stress induced by TDCIPP, the more HSP70 protein was translated by consuming abundant the corresponding mRNA (Li et al., 2018). Moreover, the gap in gene expression time and protein activity time would lead to the difference between variation of *hsp70* gene expression and enzyme content (Bonnafé et al., 2015). Besides, the MDA contents in BDCIPP treatments were lower than that in the TDCIPP groups ($p < 0.05$) (Fig. 2c), demonstrating that the enhancement of oxidative stress in *C. fluminea* due to BDCIPP exposure was not as serious as that of TDCIPP.

Bivalve siphons play important functional roles in nutritional physiology, defence, and reproduction, and siphoning behaviour has been used as a continuous biomonitoring indicator in aquatic environments (Chen et al., 2012a). Although the effects of pollutants on organisms normally occur at a later stage at toxicity (*i.e.*, are less significant) than effects at lower levels of biological organisation (Groh et al., 2015), the filtration rates of *C. fluminea* were significantly inhibited after exposure to TDCIPP and BDCIPP ($p < 0.05$) (Fig. 5). Similarly, a downward trend in siphoning behaviours of *C. fluminea* was observed during exposure to TBEP and TBP for 28 days (Yan et al., 2017). It was supposed that the decrease in siphoning behaviours may be associated with ammonia accumulation in tissues and a reduction in oxygen exchange (Chen et al., 2015). The inhibition of siphoning behaviour observed in the present study revealed that both TDCIPP and BDCIPP had negative effects on the health status of *C. fluminea*, and that the adverse effect of TDCIPP was more serious.

As presented in Fig. 6a, there was an apparent separation among the control group, the TDCIPP treatments, and BDCIPP treatments, which indicated that both TDCIPP and BDCIPP induced significant toxicity, and that *C. fluminea* did not respond in the same way. The different responses of ROS, *abcb1* mRNA, live cells, early apoptotic cells, *hsp70* mRNA, and *gstm1* mRNA (VIP > 1, Fig. 6b) in the treatment groups led to the different toxic effects on *C. fluminea*. R^2Y_{cum} is cumulative modeled variation in Y, and demonstrates the fitness of model to the experimental data; and Q^2_{cum} estimates the prediction ability of the constructed model (Mi et al., 2019). In general, a robust model has $R^2Y_{cum} > 0.5$ and $Q^2_{cum} > 0.4$ (Lee et al., 2020). The values of R^2Y_{cum} and Q^2_{cum} in this study indicated strong explanation and prediction capability of the PLS-DA model. Different toxicity between OPFRs and their metabolites was observed in previous researches. For example, it was reported that diphenyl phosphate (DPHP) was less cytotoxic than triphenyl phosphate (TPHP) *in vitro* (Mitchell et al., 2019; Su et al., 2016), and exposure to 30 mM TDCIPP induced circulatory failure in zebrafish, whereas BDCIPP did not (Lee et al., 2020). Biotransformation can be an important determinant of the toxicological effects and bioaccumulation of xenobiotics (Wang et al., 2016). BDCIPP can be metabolised rapidly *in vivo* (Wang et al., 2017a), which not only greatly reduces its accumulation in the body, but also alleviates its toxicity to organisms. In the current research, the percentage of living cells in all BDCIPP treated groups was much higher than that in TDCIPP treated groups, and the influence of BDCIPP on filtration rates was weaker than that of TDCIPP. Therefore, it is reasonable to conclude that TDCIPP was more toxic to *C. fluminea* than BDCIPP.

4.3. Implication for environmental risk

With the frequent detection of BDCIPP, there is a lack of knowledge regarding its environmental distribution, transport pathway, and

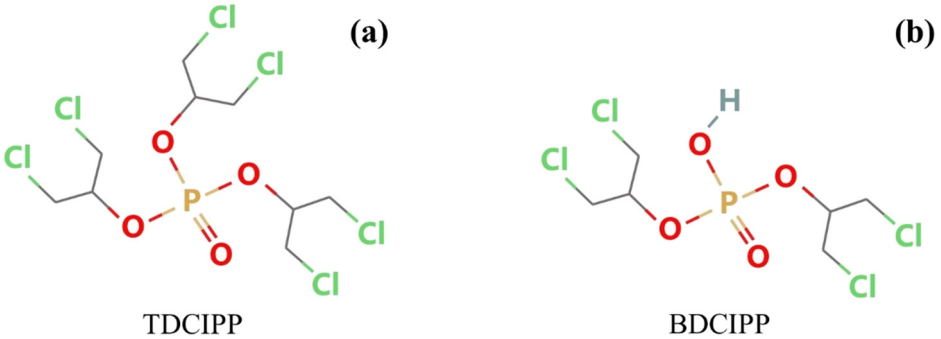

Fig. 7. Chemical structures of (a) TDCIPP and (b) BDCIPP.

biological toxicity. In the present study, both TDCIPP and BDCIPP had adverse impacts on siphoning behaviour, apoptosis rates and oxidative stress of *C. fluminea*, and TDCIPP exhibited more toxic effects than BDCIPP. As a substitute for PBDEs and emerging contaminants, the extensive production and application of TDCIPP worldwide will inevitably increase the presence of this material and BDCIPP in aquatic environments and facilitate their body burdens in organisms. Therefore, albeit the potency of BDCIPP is less than that of TDCIPP for *C. fluminea*, the potential ecological risks and biotoxicity to organisms of BDCIPP should be taken into consideration. Further investigations should use multi-omics tools to obtain more information regarding the responses of *C. fluminea* to TDCIPP and BDCIPP, as the structures of OPFRs are complex in the substitution of esters, and different structures will cause different toxic effects. Therefore, this warrants directing attention toward metabolites of other OPFRs in the environment which also pose exposure risks.

5. Conclusion

In the current study, an integrated investigation was conducted to explore the difference in the toxicity of TDCIPP and BDCIPP to *C. fluminea*; both TDCIPP and BDCIPP had adverse impacts on *C. fluminea*. Although Phase-I and Phase-II biotransformation and the MXR system were activated to alleviate the damage by TDCIPP and BDCIPP, a large amount of ROS was produced during the metabolic process, which increased the oxidative stress and led to considerable apoptosis. As the bioaccumulation of BDCIPP was less than that of TDCIPP, and BDCIPP could be metabolised rapidly *in vivo*, BDCIPP exhibited lower potency than TDCIPP in *C. fluminea*. These results provide important information for understanding the bioaccumulation and toxicology of TDCIPP and BDCIPP in aquatic organisms.

CRediT authorship contribution statement

Dandan Li: Conceptualization, Methodology, Validation, Formal analysis, Writing - original draft, Visualization. **Peifang Wang:** Conceptualization, Writing - review & editing, Funding acquisition. **Xun Wang:** Writing - review & editing. **Bin Hu:** Writing - review & editing. **Dingxin Li:** Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.142049.

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