Chemosphere 90 (2013) 1281–1288

Contents lists available at SciVerse ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Assessment of toxic effects of triclosan on the swordtail fish (*Xiphophorus helleri*) by a multi-biomarker approach

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HIGHLIGHTS

- The toxic effects of triclosan on swordtail were assessed via a multi-biomarker approach.
- The expression levels of CYP1A, CYP3A, GST and P-gp are related to triclosan exposure.
- mRNA expression levels are more sensitive than their corresponding enzymatic activities.
- The male fish displayed higher gene expression levels and more dramatic changes in enzyme activities than the females.

ARTICLE INFO

Article history:
Received 19 June 2012
Received in revised form 24 September 2012
Accepted 25 September 2012
Available online 23 October 2012

Keywords:
Triclosan
Swordtail fish (*Xiphophorus helleri*)
Toxic effects
Gene expression
Enzymatic activities

ABSTRACT

The toxic effects of triclosan (TCS) on the swordtail fish (*Xiphophorus helleri*) were assessed based on various biomarkers including enzymatic activities of ethoxyresorufin O-deethylase (EROD), erythromycin N-demethylase (ERND) and glutathione-s-transferase (GST) and mRNA expression levels of CYP1A, CYP3A, glutathione S-transferase (GST) and P-glycoprotein (P-gp). The acute toxicity test showed the LC_{50} value of 1.47 mg L^{-1} for TCS. The mRNA expressions of CYP1A, CYP3A, GST and P-gp showed dose–effect relationships in female swordtail fish when exposed to TCS, These mRNA expression levels were found more sensitive to TCS exposure than the enzymatic activities of EROD, ERND and GST do. In addition, the male fish displayed higher gene expression levels and more dramatic changes in enzyme activities than the females did. Our data further demonstrated that TCS was a typical inducer to Phase I and Phase II metabolism enzymes and genes, suggesting it is a potential ecotoxicological risk to aquatic ecosystems.

1. Introduction

Triclosan (TCS, 5-chloro-2-(2,4-dichlorophenoxy)-phenol) as an antimicrobial agent is widely used in many personal care products, including soaps, deodorants, household cleaners, dental care products, skin care creams and textiles. The regular content in its products is between 0.1% and 0.3% by weight (Singer et al., 2002; Sabaliunas et al., 2003; Miller et al., 2008). Owing to its wide use, TCS has been frequently detected in wastewater treatment plant effluents (Halden and Paull, 2005; Ying and Kookana, 2007), surface water (Kolpin et al., 2002; Chau et al., 2008; Ramaswamy et al., 2011) and sediments (Cantwell et al., 2010; Zhao et al., 2010).

TCS in the aquatic environment can accumulate, and consequently cause adverse effects on non-target organisms (Orvos et al., 2002). For example, the average concentration of TCS in bivalves sampled was 461 ng g^{-1} in Greece, which was much higher than the concentrations in water and sediment (Gatidou et al., 2010). It was reported that TCS has potentially weak androgenic effects (Foran et al., 2000), and can alter swimming speed of fish (*Oryzias latipes*) (Nassef et al., 2010). TCS is also highly toxic to the asexual reproduction of algae (*Closterium ehrenbergii*) with an EC_{50} of 0.62 mg L^{-1}, and even causes genotoxic effects on *C. ehrenbergii* (Ciniglia et al., 2005). Yang et al. (2008) observed that TCS is the most toxic antibacterial compound (no-observed-effect concentrations (NOECs) is 200 ng L^{-1}) for the freshwater microalgae (*Pseudokirchneriella subcapitata*). Binelli et al. (2009a,b) demonstrated that TCS had potentially genotoxic and cytotoxic effects on Zebra mussel hemocytes. Moreover, TCS has been recognized as an inducer of cytochrome P450 content in rat liver microsomes (Kanetoshi et al., 1992), and an inhibitor of P450 microsomal enzymes such as ethoxyresorufin O-deethylase (EROD), pentoxy O-dealkylase (PROD) in 3-methylcholanthrene (MC)- and phenobarbital (PB)- treated rats (Hanioka et al., 1996).

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http://dx.doi.org/10.1016/j.chemosphere.2012.09.087

But paradoxically Ishibashi et al. (2004) found that TCS cannot induce EROD and PROD activity in the hepatic microsomes of female medaka, *Oryzias latipes*. This discrepancy may be due to physiological differences between mammals and fish, and/or differences in exposure conditions. In addition, TCS can act as an inhibitor and a substrate of glucuronidases and sulfatases due to its similarity in structure with polychlorobiphenols, which are potent inhibitors of Phase II enzymes (Wang et al., 2004). Canesi et al. (2007) reported that the activity of glutathione-s-transferase (GST) was increased in the digestive gland of *Mytilus galloprovincialis* exposed to TCS.

However, previous studies are mainly focused on the biochemical responses and antioxidant enzymes involved with toxic effects of TCS on aquatic organisms, which is not sufficient to mechanistically evaluate the effects of TCS on organisms. Indeed, responses of enzyme activities should be integrated with expression of specific genes involved in metabolism in order to clarify the detoxification pathways occurring in exposed organisms (Williams et al., 2003; George et al., 2004). In recent years, changes at the molecular or cellular levels in organisms have been used as effective early warning tools in aquatic environment monitoring (Van der Oost et al., 2003). For example, the cytochrome P450 pathway responses in aquatic organisms are well known for their responsiveness to chemical insults in aquatic organisms, and are measured as variations of transcript expression of genes and activity of proteins (Quiros et al., 2007; He et al., 2011). Since mRNA level is a snapshot of the cell activity, gene expression is not only considered to be a useful indicator for protein level, but also recognized to have potential to support or substitute classical protein biomarker (Van der Oost et al., 2003; Olsvik et al., 2005). Thus, in order to provide further insights into the action mechanisms of response to xenobiotics, there is a need to investigate the linkage between the transcriptional and catalytic effects. However, the intrinsic linkage between the gene expression and the detoxification system like Phases I and II as well as the response of corresponding enzymes activity to the exposure of TCS has not been established.

Swordtails (*Xiphophorus helleri*) has been often used in behavior ecology, genetics, and biogeography (Gutierrez-Rodriguez et al., 2007) and is considered an ideal species for the toxicological studies of endocrine-disrupting chemicals (Kwak et al., 2001) due to its great advantages, e.g., a small and easily raised species, a short generation time and distinct sexual characteristics. The aims of this study were to investigate the response of the detoxification systems including Phase I metabolism enzymes such as EROD, ERND (erythromycin N-demethylase) and Phase II metabolism enzymatic (GST) in the liver of swordfish exposed to TCS, as well as the expression levels of the counterpart genes such as CYP1A, CYP3A, GST and P-glycoprotein (P-gp). The sexual difference in gene expression and protein levels in the swordtail exposed to TCS was also assessed. Simultaneous analysis of gene expression and enzyme activity patterns in swordfish and comparison of the responses of these genes and enzymes could provide deeper insights into the action mechanisms related to the toxicity of TCS on the aquatic organisms.

2. Materials and methods

2.1. Test organism and experimental design

Three hundred swordtails at 6 months old were obtained from Pearl River Fisheries, Research Institute, Chinese Academy of Fishery Sciences in South China. The average weights and lengths were 1.50 ± 0.34 g and 4.43 ± 0.22 cm for the males, 1.79 ± 0.43 g and 4.51 ± 0.31 cm for the females. All swordtails were held at 26 ± 1 °C in de-chlorinated, aerated tap water (pH 7.5 ± 0.1) in the laboratory, under natural photoperiod for two weeks prior to TCS exposure. During this period, fish were fed daily with fish food from Pearl River Fisheries Research Institute, and half of the water was replaced daily. The swordtails used for toxicity tests were starved for 3 d before the experiments, and then no food was provided during the exposure experiments.

The target compound triclosan (TCS) was obtained from Wako (purity: >96.0%), The stock solution of TCS was prepared at a concentration of 1000 mg L^{-1} dissolved in Dimethyl Sulphoxide (DMSO), and used to prepare the test solutions by serially diluting to the required concentrations. DMSO in the test solution was kept below 0.1%.

For exposure experiments, 4-d acute toxicity tests were firstly performed to determine the LC_{50} value of TCS in swordtails Based on the preliminary experiments, the concentrations of TCS (0.86, 1.23, 1.76, and 2.52 mg L^{-1}), which could cause about 0–100% mortality, were selected for the acute toxicity tests. For concentration treatment, a group of randomly selected fishes (four males and four females) were placed in the glass aquaria (7 L of test solutions per tank). The exposure experiment was conducted in triplicate. Half of the solution in each tank was daily replaced with the fresh one. The other exposure conditions were the same as those during the acclimation period. The fish were exposed to a series of concentrations of TCS for 4 d and control groups were kept in 0.1% (v/v) DMSO solutions. The mortality of fish was recorded at 24, 48, 72, and 96 h. The LC_{50} at 96 h was calculated by the modified Kaber method (in 95% confidence interval).

The concentrations of TCS in the test solutions were measured during the exposure by high performance liquid chromatography with ultraviolet detection. The instrument used was a Hewlett–Packard 1100 LC equipped with a G1321A fluorescence detector and ultraviolet detector. The separation was achieved using an Agilent TC-C18 column (USA, 4.6 mm × 250 mm, 5 μm particle size). The mobile phase used was methanol and water (80:20, v:v) at a flow rate of 0.8 mL min^{-1}. According to the analysis of the test solutions, the degradation of TCS in the tested tanks for 24 h was lower than 2%.

Based on the LC_{50} at 96 h, the dosages of TCS (0.0, 0.002, 0.05 and 1.25 mg L^{-1}) were selected for chronic toxicity experiments (for 168 h). The exposure conditions were the same as mentioned above. The experiments were performed for each concentration and control group in triplicate. Three male and three female swordtails were taken randomly from each treated groups to obtain a mixture sample at the time points of 24, 72 and 168 h, respectively. Fish were sacrificed by decapitation and dissected for liver tissue, which were immediately stock-frozen in liquid nitrogen, and then stored at −80 °C refrigerator until use. No mortality was observed in all groups at different dosages during the whole exposure period.

2.2. Enzyme extraction and measurements

The methods used for enzyme extractions were adopted from a previous study (He et al., 2011). In brief, the liver tissues were homogenized in sucrose buffer (0.25 M sucrose, 0.1 M Tris–HCl, 1 mM EDTA, pH 7.4) with a ratio of 1:4 (w/v). The homogenates were centrifuged at 10 000*g* for 20 min at 4 °C, and the resulted supernatant was further centrifuged at 105 000*g* at 4 °C for 1 h. Aliquots of the final supernatant (cytosol) were further purified for GST assay in order to remove fatty layer. The microsomal pellets were re-suspended in sucrose buffer for EROD and ERND assay. The contents of liver microsomal protein were determined using the method developed by Bradford assay (1976).

EROD activities were measured using a previous method (Pohl and Fouts, 1980). The rate of resorufin production from the deethylation of substrate (7-ethoxyresorufin) mediated by EROD was determined using spectrofluorometry (HITACHI F-4500, Japan)

at the wavelengths of 550 nm (excitation) and 580 nm (emission). The reaction was initiated for 10 min by adding NADPH. A resorufin standard calibration curve was used in order to transform fluorescence values into resorufin pmol min^{-1} mg^{-1} microsomal protein.

The method for determining the activity of ERND enzyme was based on the increase in the absorbance at 420 nm caused by formaldehyde as reaction product of ERND (Nash, 1953). The product was quantified using a calibration curve of HCHO (unit, nmol min^{-1} mg^{-1} microsomal protein).

GST activity was determined by the changes of the absorbance at 340 nm due to the conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzenem (CDNB) (Lemaire et al., 1996). The assay was conducted in the 2.0 mL mixture containing 0.2 M phosphate buffer (pH 7.4), 0.2 mM CDNB, and 0.2 mM GSH. The unit was U mg^{-1} protein.

2.3. Real-time quantitative PCR analysis

RNA extraction from the liver samples was performed using Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). The quality of RNA was evaluated by electrophoresis on 1% agarose gels, and its concentration was determined by spectrophotometry at 260 and 280 nm. First-strand cDNA synthesis was performed using the Prime Script™ RT reagent Kit (TaKaRa Biotechnology, China) according to the manufacturer's instructions, and the synthesized cDNA was stored at −20 °C until real-time PCR analysis.

The real-time quantitative PCR (RT-qPCR) analysis was performed with an ABI 7500 Real-Time PCR System (Applied Biosystems, USA) using SYBR® Premix Ex Taq™ II (TaKaRa Biotechnology, China). The specific primers for CYP1A (5′-GCTCTTCC GTCATTCTTCATACC-3′; 5′-GCTTGT TGACCTCTGTGCCA-3′); CYP3A (5′-CTTGGCTTACAATCTGGC-3′; 5′-GATGACGCTGTCCA AGTAG-3′); GST (5′-CGAGGGACTGAAGGACGAGA-3′; 5′-AAGCCAAAGCGGAA-GAGG A-3′); P-gp (5′-CACGGCTTCACCTTCTCCTT-3′; 5′-ACTCCCTCC ACAT CCATCCTT-3′) and β-actin (5′-TGCTATGTTGCACTGGACTTT-GAG-3′; 5′-CCTCTCGTTTC CGATGGTGATGAC-3′) genes were designed according to the cDNA sequences of swordtail obtained in Pearl River Fisheries Research Institute (No. FJ763581 and DQ060278 for GST and β-actin, respectively; the sequences of CYP1A, CYP3A and P-gp were not published). The RT-qPCR amplifications were carried out in a 20 μL solution containing 10 μL SYBR® Premix Ex Taq™ II (2×), 0.4 μL ROX Reference Dye II (50×), 0.4 μL each of forward and reverse primer (concentration of 20 μM), 1 μL cDNA template and 7.8 μL molecular-grade water. The RT-qPCR program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 60 °C for 34 s. A melting curve analysis after reaction was performed for checking the specificity of the products. In order to verify the sequence of the PCR products, they were purified by using gel Extraction kit (OMEGA bio-tek, USA) and inserted into the PMD18-T vector (TaKaRa Biotechnology, China). The clones were sequenced by Shanghai Sangon Biotechnology Corporation (China), and the sequences were blasted in GenBank. Gene expression levels were calculated using the expression Ratio Quantitative $RQ = 2^{-\triangle\triangle CT} = 2^{-[(CT1-CT2)-(CT3-CT4)]}$ as described in reference (Ni et al., 2007), where CT1 and CT2 were the threshold cycle (CT) of the target gene and the β-actin gene (as a control gene) of the exposure group, and the CT3 and CT4 were the CT of the target gene and the β-actin gene of the control group. Differences in the CT between the target and the β-actin gene (△CT) were calculated to normalize the differences in the amount of total nucleic acid added to each reaction and the efficiency of the RT-qPCR. The gene expression levels of the exposure groups were compared with the control group for each sampling date.

2.4. Statistical analyses

Mean values and standard deviation were calculated for each group of all assays performed in triplicate. Pearson correlation (analyzed with the mean values) was conducted to determine the general relationships ($p < 0.05$) between the gene transcript and enzyme activity. One-way ANOVA (Dunnett's test) was used to evaluate the significant differences between the exposure and control groups at $p < 0.05$.

3. Results

3.1. Acute toxicity of TCS to swordtail fish

No mortality was observed in the control groups during the acute toxicity experiment. All swordtail fish exposed to 2.52 mg L^{-1} of TCS treatment group after 24 h exposure were dead. The 96 h mortalities were 83.3% and 16.7% at the concentrations of 1.76 and 1.23 mg L^{-1}, respectively. And however no dead fish was found at the lowest concentration (0.86 mg L^{-1}). The 96 h LC_{50} value of TCS was calculated as 1.48 mg L^{-1} for swordtail fish with 95% confidence interval from 1.21 to 1.80 mg L^{-1}.

3.2. Genes expression and enzyme activities

The chronic experiments showed that the mRNA gene expression levels of CYP1A the liver tissue of the females were dose-dependent (Fig. 1A). The mRNA gene expression of CYP1A was inhibited at the lowest dose (0.002 mg L^{-1}), and significantly induced at the highest dose (1.25 mg L^{-1}). However, for male, CYP1A mRNA expression levels were found to be rapidly increased ($p < 0.05$) at middle and highest dose, and exhibited a good time-dependent increase at the low dose (0.002 mg L^{-1}) during all the testing period (for 168 h). Compared to CYP1A mRNA expression, EROD enzymatic activity was less responsive to TCS exposure. EROD activities were just induced at the highest dose (1.25 mg L^{-1}) after 24-h exposure and the middle dose (0.25 mg L^{-1}) after 72-h exposure for the females (Fig. 1B). For the males, EROD activities were observed to be time-dependent, and in the lowest dose (0.002 mg L^{-1}) reached the maximum level after 168-h exposure, but in the middle and highest doses reached the maximum level after 24-h exposure.

The levels of CYP3A mRNA expression and ERND activities are also presented in Fig. 1C and D. For the females, CYP3A mRNA expression was exhibited ($p < 0.05$) dose-dependent, and inhibited at the lowest dose, but induced ($p < 0.05$) at the highest dose. For the males, CYP3A mRNA expression was induced significantly ($p < 0.05$) at all the exposure concentrations after 72 and 168 h exposure, with exception of inhibition at the lowest dose after 24 h exposure (Fig. 1C). ERND activities for the females were significantly increased ($p < 0.05$) at the middle and high dose after 72 and 168 h exposure, while for the males, a good time-dependent increase of ERND activities was observed at the lowest and highest doses (Fig. 1D). Under exposure to TCS, the CYP3A gene and ERND enzyme exhibited more dramatic variation for the males than that for the females, displaying a remarkable sexual difference.

For both male and female swordtails, the expression levels of GST mRNA showed an increasing trend associated with the exposure dose significantly (Fig. 2A). A similar trend was also observed for GST activities for the females after 24 and 168 h exposure (Fig. 2B). However, the dose dependence was intangible for the females after 72 h exposure. Regarding the males, GST activities increased gradually with the increases of exposure time and TCS concentration.

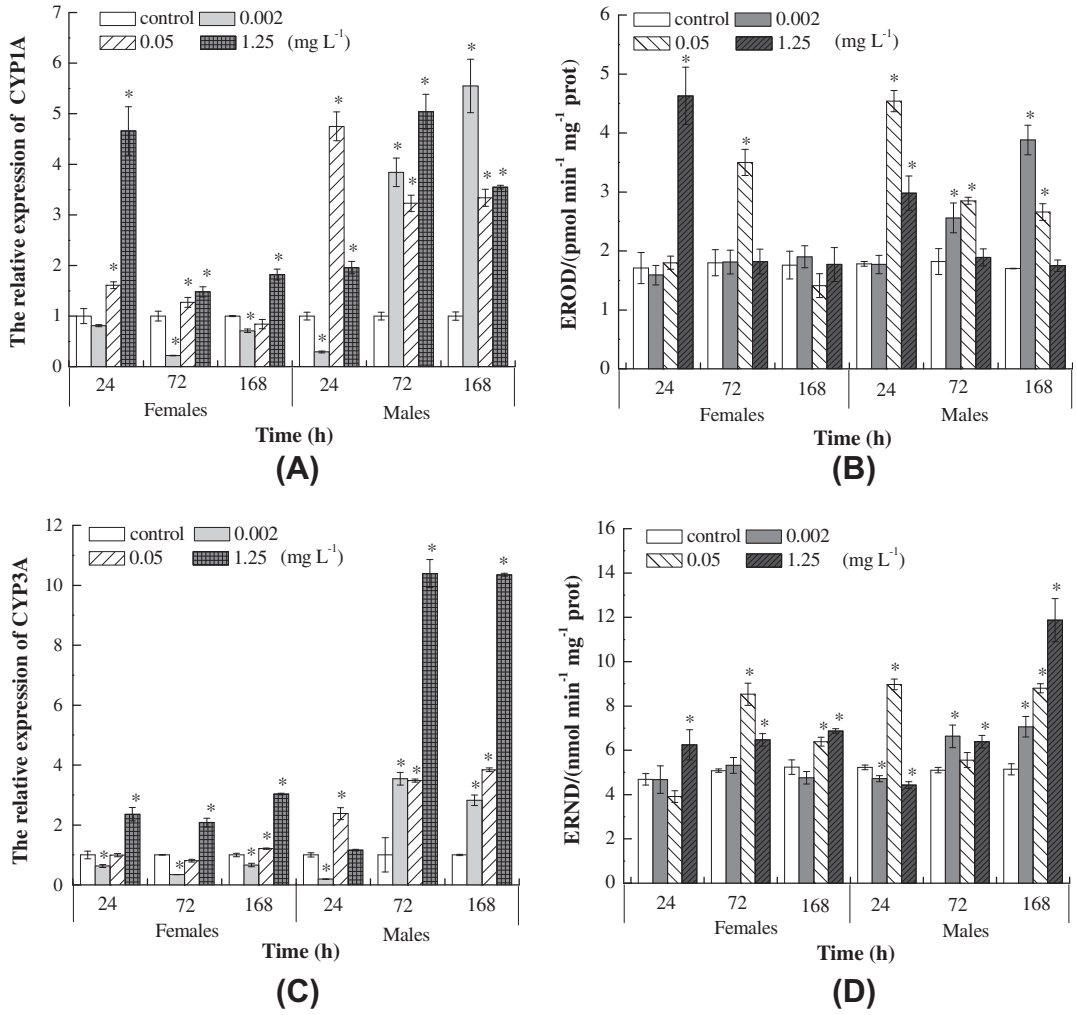

Fig. 1. CYP1A gene relative expressions (A), EROD enzyme activities (B), CYP3A gene expressions (C), and ERND enzyme activities (D) in the liver tissues of swordtail fish exposed to different concentrations of TCS. Asterisk (*) indicates significant differences from control ($p < 0.05$).

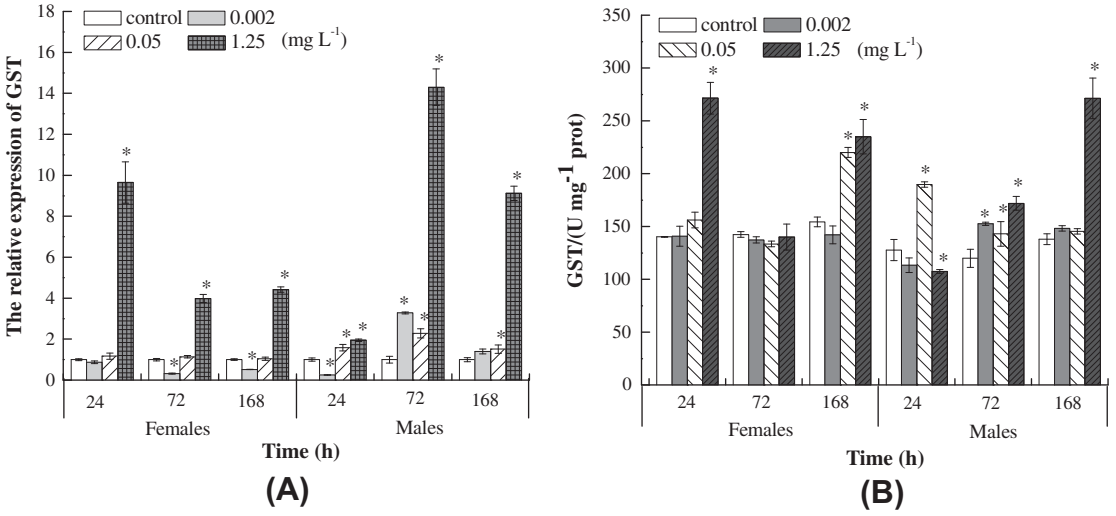

Fig. 2. GST gene relative expressions (A) and GST enzyme activities (B) in the liver tissues of swordtail fish exposed to different concentrations of TCS. Asterisk (*) indicates significant differences from control ($p < 0.05$).

P-gp gene expression was inhibited for the females in all exposure doses and exposure time except the middle dose after the 168-h exposure (Fig. 3). In contrast, P-gp gene expression for the males exhibited a disparate pattern. Compared to the control,

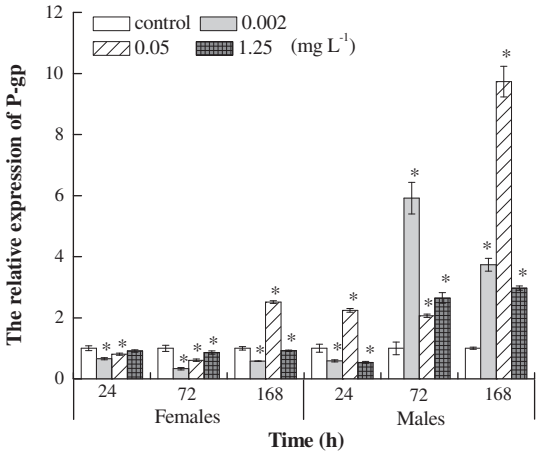

Fig. 3. P-gp gene relative expressions in the liver tissues of swordtail fish exposed to different concentrations of TCS. Asterisk (*) indicates significant differences from control ($p < 0.05$).

P-gp gene expression for all treatments were significantly induced for the males ($p < 0.05$) under 72 and 168 h exposures except for 24 h.

4. Discussion

The results from the present study showed the 96 h LC_{50} of TCS to swordtails fish to be 1.47 mg L^{-1}, suggesting that TCS was highly toxic to swordtails according to the standard evaluation procedure of US EPA for freshwater fish (Zucker, 1985). Due to the wide use of the TCS, the levels of TCS in aquatic environments ranged from ng L^{-1} to μg L^{-1}, therefore its risk to the aquatic organisms cannot be ignored (Ciniglia et al., 2005; Chau et al., 2008; Ramaswamy et al., 2011). According to the European technical guidance document (TGD) (EC, 2003), the potential environmental risk of TCS could be assessed by using risk quotient (RQ), which is calculated through the measured environmental concentration (MEC) divided by predicted no-effect concentration (PNEC). Taking into account that the highest concentrations detected so far in surface waters for TCS was 5160 ng L^{-1} (MEC) (Ramaswamy et al., 2011), and the PNEC of TCS to swordtails was 1470 ng L^{-1}, the RQ value was calculated to be 3.51 in the present study. High risks to the fish would be expected when the RQ value was higher than 1 (Hernando et al., 2006), our results indicated that the environmental concentration of TCS can pose high risk to the swordtails. Similar risk assessment results were also reported by Zhao et al. (2010) and Ramaswamy et al. (2011). In order to provide more insights into the mechanisms of TCS toxicity and improve our understanding of the intrinsic relationships between exposure stress and biological response, it is worthwhile to pay attention to the chronic toxic effects of TCS on swordtails at biochemical and molecular levels (Gao et al., 2007; An et al., 2011).

EROD enzyme and CYP1A gene are widely used as biomarkers for contamination of some environmental pollutants, such as aryl hydrocarbons including PAHs and PCBs (Bucheli and Fent, 1995; Wozny et al., 2010). PAHs and PCBs can induce the expression of CYP1A through binding with the intracellular AhR in organisms (Hahn and Stegeman, 1994; Sarkar et al., 2006). Moreover, the expression of CYP genes can be induced by binding of xenobiotics with the special receptors, which in turn activates protein synthesis and related enzyme activity (Marionnet et al., 1997; Ortiz-Delgado et al., 2008). The activities of CYP enzymes are usually regulated by the specific receptor; therefore, the affinity of pollutants to these receptors, particularly to their chemical structure, is a predominating factor for biological responses. Interestingly, TCS and its metabolites have a similar molecular structure with PCBs or PBDEs. TCS is identified as an inducer of aryl hydrocarbon receptor (AhR), implicating the relatedness in action mechanisms and biological responses between them (Ahn et al., 2008). However, it remains to be investigated that TCS can induce CYP1A mRNA expression in aquatic organisms. The present study distinctly showed the relevance between TCS exposure and CYP1A mRNA expression, although there existed a difference in the gene expression response by the gender. However, the exact mechanism involved in the induction of CYP1A mRNA expression by TCS exposure needs to be further studied.

In terms of induction factor, EROD activity in liver was less responsive to TCS exposure than CYP1A mRNA expression (Table 1), which is consistent with a previous study (He et al., 2011). The response of mRNA expression was faster than the enzymatic activity, which can be explained by the order of post-transcriptional processes and the protein synthesis in organisms (Williams et al., 2003). However, it could be complicated by the complex relationships between mRNA stability, protein turnover, transcriptional and translational mechanisms as well as the regulation of enzyme kinetics (Leonard et al., 2004). A limited relationship between transcriptional level and catalytic activity of cytochrome P450 was found in dab *L. limanda* from the North and Baltic Sea (Kammann et al., 2008) and European flounder from the polluted estuarine environment (George et al., 2004), while a good correlation was observed between CYP1A transcript and EROD activity in barbel (*Barbus graellsii*) from the Ebro river (Quiros et al., 2007). In the present study, the Pearson correlation value (analyzed with the mean values) between EROD activity and CYP1A mRNA expression was 0.806 for the females ($p = 0.002$) and 0.629 for the males ($p = 0.028$) (Table 2), suggesting a good correlation between EROD activity and CYP1A transcript in swordtails exposed to TCS. Furthermore, similar relationships between gene transcriptional level and catalytic activity of CYP3A and GST were observed in swordtails after exposed to TCS (Table 2), generally presenting a good correlations (most of the Pearson correlation value was higher than 0.59 ($p < 0.05$) . In this situation, it could be supposed that the gene can modulate enzyme activity via the molecular transcriptional level mechanism, and in this case, the molecular analyses exhibited more sensitivity than the biochemical responses. This hypothesis is confirmed by other research groups in which showed significant positive correlation relationships between the gene transcript and the enzyme activity (Araujo et al., 2000; James et al., 2005; Quiros et al., 2007). However, the relevance between the effects occurring at various intracellular levels is not always easy to elucidate or predict, and the transcript expression of a gene and the catalytic activity of its product might have different trends (George et al., 2004; Kammann et al., 2008), thus it is desirable to study integratedly both mRNA transcript levels and enzymes activity in future to better understand the effects of contaminants on organisms.

CYP3A is involved in the wide range of drugs metabolisms in organisms, strongly induced by a variety of structurally unrelated xenobiotics (Hegelund and Celander, 2003). As for mammals and possibly also for fish, ERND is partially linked to CYP3A, considered as an useful biomarker for evaluating the adverse effect of environmental pollutants (Vaccaro et al., 2003; Borbas et al., 2006). Previous studies have demonstrated that CYP3A expression in certain fish species could be enhanced by certain xenobiotics (Hasselberg et al., 2004; Meucci and Arukwe, 2006). In the present study, following the exposure to TCS, the CYP3A mRNA expression levels exhibited clear induction relative to that from the control at the highest dose, and a similar result was also observed in ERND activities. These results indicate that CYP3A gene and ERND enzyme can be induced by TCS for fish as well as for mammals. Some studies

Table 1
Induction factors (compared with control) of the biomarkers (CYP1A, CYP3A, GST and P-gp genes; EROD, ERND and GST enzymes) in the liver tissues of swordtails exposed to TCS.

Time (h)	TCS (mg L^{-1})	Gene				Enzyme		
		CYP1A	CYP3A	GST	P-gp	EROD	ERND	GST
Females								
24	0.002	0.81	0.63	0.87	0.66	0.93	1.00	1.00
	0.05	1.61	0.99	1.17	0.81	1.05	0.83	1.11
	1.25	4.66	2.36	9.65	0.91	2.71	1.33	1.94
72	0.002	0.22	0.34	0.32	0.33	1.01	1.05	0.96
	0.05	1.27	0.81	1.14	0.61	1.95	1.68	0.94
	1.25	1.48	2.08	3.98	0.86	1.01	1.27	0.98
168	0.002	0.71	0.66	0.52	0.58	1.08	0.91	0.92
	0.05	0.84	1.21	1.04	2.51	0.80	1.22	1.43
	1.25	1.82	3.03	4.42	0.92	1.01	1.31	1.52
Males								
24	0.002	0.29	0.19	0.25	0.59	0.99	0.90	0.89
	0.05	4.75	2.38	1.58	2.24	2.55	1.71	1.49
	1.25	1.96	1.16	1.95	0.54	1.67	0.85	0.84
72	0.002	3.84	3.54	3.28	5.92	1.41	1.30	1.27
	0.05	3.23	3.48	2.28	2.07	1.57	1.09	1.19
	1.25	5.04	10.39	14.30	2.65	1.04	1.25	1.43
168	0.002	5.55	2.82	1.40	3.74	2.28	1.37	1.07
	0.05	3.34	3.84	1.51	9.73	1.56	1.71	1.05
	1.25	3.55	10.35	9.12	2.97	1.03	2.31	1.97

Table 2
Pearson correlation value (analyzed with the mean values) between the gene expressions and their corresponding enzyme activities for swordtails exposed to TCS.

	CYP1A expression vs. EROD activity		CYP3A expression vs. ERND activity		GST expression vs. GST activity	
	Females	Males	Females	Males	Females	Males
Pearson correlation	0.806	0.629	0.409	0.667	0.779	0.598
p Value	0.002	0.028	0.187	0.018	0.003	0.040

Correlation is significant at the 0.05 level ($p < 0.05$).

have demonstrated that the induction of CYP3A expression for fish is probably regulated by the pregnane X receptor (PXR) (Meucci and Arukwe, 2006). Jacobs et al. (2005) presented in vitro evidence for TCS acting as a ligand, with a moderate affinity to the human pregnane X receptor (hPXR). Therefore, the results from the present also imply that TCS probably regulated the CYP3A gene expression via similar pathways for fish by modulating gene expression with the PXR. But further investigations are still needed to elucidate the exact pathways.

GST belongs to Phase II enzyme which is involved in the cellular detoxification of xenobiotics compounds and plays a fundamental role in protection against endogenous and exogenous toxic chemicals (Sheehan et al., 2001). The present study showed that GST mRNA expression presented a good dose-dependent pattern for all the exposure periods, and attained the maximum value at the highest dose for swordtails. Furthermore, a similar tendency in GST activities was observed after 24 and 168-h exposure for the females. This suggested that GST played an important role in detoxifying TCS, which is in accordance with the response of P450 mentioned above. There exists a subtle balance between Phase I and Phase II system in the metabolism process of xenobiotics for organisms. Once the balance is broken down or even disturbed the toxic intermediates will be accumulated and consequently cell damage will be happen. Therefore, when there is an increase in CYP 450 enzymes activity, it is requisite for organisms to increase Phase II enzymes activity for coping with the production of CYP 450 system and preventing cell damage (Bebianno et al., 2007). The present observations is similar to the result from Canesi et al. (2007), which showed that GST was increased in the digestive gland when *Mytilus galloprovincialis* was exposed to TCS.

P-gp is a multidrug resistance-related protein (MRP) that acts as an energy-dependent efflux flippase to prevent the cellular accumulation of wide variety of structurally and functionally diverse compounds (Gerlach et al., 1986; Achard et al., 2004). P-gp could potentially be used as one of the perspective biomarker candidate for assessing the exposure of xenobiotics (Albertus and Laine, 2001; Besse and Garric, 2008). It has been reported that P-gp is induced in vitro by CYP1A inducers in the presence of benzo[a]pyrene, 3-methylcholanthrene, and 2-acetylaminofluorene as a substrate in mammals (Gant et al., 1991; Fardel et al., 1996; Tateishi et al., 1999). In present study, a similar performance was observed. The increases of P-gp mRNA expressions were also observed after 72 and 168-h exposure for the male swordtails that displayed significant induction of CYP mRNA expression. These results indicated that variation of P-gp expression might be related to the changes of CYP mRNA expression when the male swordtail was exposed to TCS. However, P-gp mRNA expression exhibited a different response pattern from CYP mRNA at the highest dose during all the exposure period for the female, which were significantly inhibited compared with the control group. A similar result has been reported by Bard et al. (2002) that P-gp and CYP1A may play complementary roles in the cellular detoxification to protect organisms from the accumulation of moderately hydrophobic xenobiotics or toxic endogenous metabolites.

The present findings revealed that there exists a significant sexual difference in gene expressions and enzyme activities for the swordtails when exposed to TCS. Compared with the controls, the male individuals displayed higher gene expression levels of CYP1A, CYP3A, GST, and P-gp and more dramatically changes in enzyme activities than the females did. A similar observation has also been reported by other groups (Pajor et al., 1990; Haasch et al., 1993; Hegelund and Celander, 2003), they described that the male fish demonstrate higher CYP activities and gene expressions than the female. A possible explanation is that the sexual differences

in gene expressions and enzyme activities are mainly regulated by hormones. Previous studies has proved that TCS acts as a endocrine disruptor (Ishibashi et al., 2004; Ahn et al., 2008; Gee et al., 2008), affecting sexually dimorphic response by regulating hormones, due to its structure similarity with the anthropogenic estrogens (Jacobs et al., 2005). However, in the present study, whether TCS act as an endocrine disruptor to affect the sexual difference remained unclear, and the mechanism for the sexual dimorphic gene expression is still not fully understood, though it is possible that the sex steroids could be involved in it. Thus a further investigation is required to understand the endocrine disrupting effects of TCS in swordtails. In summary, our results corroborated the previous toxicity investigation (Orvos et al., 2002; Ishibashi et al., 2004; Ciniglia et al., 2005; Yang et al., 2008) that potential ecotoxicological risks of TCS to aquatic ecosystems deserve to pay much special attention.

5. Conclusion

TCS showed toxic effects on swordtails following its exposure. TCS could act as a typical inducer to increase the levels of both Phase I and Phase II genes and enzymatic activities in swordtails, whereas the molecular levels were found more sensitive to TCS exposure than the enzyme activities. The male fish displayed higher gene expression levels and more dramatic changes in enzyme activities than the females, suggesting a significant sexual difference in gene expressions and enzyme activities of swordtails when exposed to TCS. The combined biochemical and molecular parameters were considered as suitable biomarkers to improve our understanding of toxicological mechanisms of TCS to aquatic organisms.

Acknowledgments

This research was funded by the National Natural Science Foundation Committee of China (NSFC U1133005). We would like to thank our colleagues of Jinan University Analytical and Testing Centre for their assistance in HPLC analysis. We also thank to Prof. G.G. Ying from Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, China, Dr. W.H. Liu from the State University of New York at Buffalo and Dr. A.D. Han from Xiamen University, China for their helps in the language editing to the manuscript.

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