Effects of fluoxetine on behavior, antioxidant enzyme systems, and multixenobiotic resistance in the Asian clam *Corbicula fluminea*

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HIGHLIGHTS

- GR, TR, ABCB1, ABCC1, and ABCG2 genes were identified in the clam.
- The siphoning behavior of the clam was inhibited by FLX.
- FLX exposure induced oxidative effects to the clam.
- The expressions of MXR system genes were altered by FLX.

ARTICLE INFO

Article history:

Received 4 April 2014

Received in revised form 19 August 2014

Accepted 21 August 2014

Available online 19 September 2014

Handling Editor: Caroline Gaus

Keywords:

Fluoxetine

C. fluminea

Siphoning behavior

Antioxidant enzymes

Multixenobiotic resistance

ABSTRACT

Fluoxetine (FLX), a selective serotonin reuptake inhibitor, is widespread in aquatic environments. Despite its reported effects on behavior and reproduction in aquatic species, little is known about the effects of FLX on cellular detoxification and defense system in bivalves. Here, the adult Asian clam (*Corbicula fluminea*) was exposed to 0.5, 5, and 50 $\mu\text{g L}^{-1}$ FLX for 30 d. Siphoning behavior was inhibited by treatment with 50 $\mu\text{g L}^{-1}$ FLX. Additionally, superoxide dismutase (SOD) activity in the gills and digestive glands significantly decreased ($p < 0.05$) with 5 and 50 $\mu\text{g L}^{-1}$ FLX treatments, whereas catalase (CAT) activity and malondialdehyde (MDA) content markedly increased ($p < 0.05$). Moreover, transcription of thioredoxin reductase (TR), glutathione peroxidase (GPx) and Glutathione S-transferase pi class (GSTpi) was significantly upregulated ($p < 0.05$), whereas glutathione reductase (GR) was markedly downregulated ($p < 0.05$). These findings suggest that FLX affects behavior and induces oxidative stress in *C. fluminea*. The downregulation of ATP-binding cassette (ABC) transporter genes (ABCB1, ABCC1 and ABCG2) transporter genes indicated that FLX might suppress the multixenobiotic resistance (MXR) system in *C. fluminea*. Our results provide new insights into the adverse effects of FLX on the cellular detoxification and MXR system of *C. fluminea*.

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1. Introduction

Pharmaceuticals are a large and diverse collection of bioactive chemicals that are used in human and veterinary medicine (Kim

et al., 2007). Due to human use, large quantities of pharmaceuticals have been discharged into aquatic environments (Blair et al., 2013; Liu and Wong, 2013). Serotonin reuptake inhibitors (SSRIs), a class of antidepressant, are the most studied pharmaceuticals in the context of aquatic systems (Fent et al., 2006). These drugs have been reported to affect reproduction, foraging, stress responses, and aquatic organism locomotion (Brooks et al., 2003). Fluoxetine (FLX) is the most widely prescribed SSRI drug, and FLX contamination is widespread in rivers, surface water, and sewage treatment plant effluents in areas where it is used (Gonzalez-Rey and Bebianno, 2013). Reported FLX concentrations in surface water in Canada (Metcalf et al., 2003), Croatia (Gros et al., 2006), and the USA (Bringolf et al., 2010) were approximately 46, 66, and 111 ng L^{-1} , respectively. Recently, FLX was detected in 25% of the water samples collected from various locations in the USA at an average concentration of 65 ng L^{-1} (Ferrer and Thurman, 2012).

Abbreviations: ABC, ATP-binding cassette; ABCB1 or MDR1, multidrug resistance protein 1; ABCC1 or MRP1, multidrug resistance-associated protein 1; ABCG2, breast cancer resistance protein; AChE, acetylcholinesterase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSTpi, Glutathione S-transferase pi class; GSH, glutathione; MDA, malondialdehyde; MXR, multixenobiotic resistance; P-gp, P-glycoprotein; ROS, reactive oxygen species; SOD, superoxide dismutase; TR, thioredoxin reductase.

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Because the FLX concentrations measured in the environment are much lower than that which produces a therapeutic effect in humans, it seems unlikely to pose a risk of acute toxic effects (Carlsson et al., 2006). However, previous studies have reported that FLX can induce toxic effects on aquatic organisms at environmental concentrations (Brooks et al., 2003; Pery et al., 2008; Sánchez-Argüello et al., 2009; Dziewieczynski and Hebert, 2012; Hazelton et al., 2013). The acute (48 h) median lethal concentration (LC50) value for fathead minnows (*Pimephales promelas*) is approximately 2 mg L⁻¹ (Ferrari et al., 2004). Additionally, FLX can affect bivalve behavior and reproduction. Lazzara et al. (2012) reported decrease in spawning in zebra mussels (*Dreissena polymorpha*) after 6-d exposure to 20–200 ng L⁻¹ FLX. In another study, the burrowing behavior of freshwater mussels (*Lampsilis fasciola*) was inhibited after exposure to 22.3 µg L⁻¹ FLX for 67 d (Hazelton et al., 2013). Gonzalez-Rey and Bebianno (2013) reported that the acetylcholinesterase (AChE) activity of mussels (*Mytilus galloprovincialis*) was repressed after 75 ng L⁻¹ FLX treatment for 15 d. Thus far, the acute toxicity and ethology of FLX on bivalves have been well investigated, but its effects on the cellular detoxification and defense systems are not well documented.

FLX has been reported to affect antioxidant system in mice and mussel (Djordjevic et al., 2011; Gonzalez-Rey and Bebianno, 2013). Oxidative stress is characterized by the imbalance when xenobiotic-mediated enhancement of reactivity species (ROS) exceed exposed aerobic organism's antioxidant defense mechanisms (Ren et al., 2013). Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and Glutathione S-transferase (GST) protect organisms against ROS. Malondialdehyde (MDA) is a product of the decomposition of unsaturated fatty acid peroxides that are generated from ROS and is usually rapidly degraded by ROS (Marnett, 1999; Stancliffe et al., 2011). Thioredoxin reductase (TR), glutathione reductase (GR), GPx, and GST are the primary antioxidant enzymes of the thioredoxin and glutathione systems, which are found in animals, plants, and microorganisms (Meister and Anderson, 1983). The fluctuation of these antioxidant enzymes and MDA have been used successfully as oxidative stress and early-warning bio-indicators of cellular hazards in mussels species (Contardo-Jara et al., 2011; Gonzalez-Rey and Bebianno, 2013; Chen et al., 2014).

The cellular multixenobiotic resistance (MXR) system represents a broad-scale defense mechanism that protects cells and organisms against endogenous and exogenous toxicants (Epel et al., 2008). MXR is mediated by membrane transport proteins from the ATP binding cassette (ABC) protein family, which recognize a wide variety of potential xenobiotics as substrates, and pump them out of the cell in an energy-dependent, ATP-driven process (Navarro et al., 2012). The primary ABC proteins with MXR-related functions include P-gp (P-glycoprotein, gene symbol ABCB1), MRP1 (multidrug resistance-associated protein 1, gene symbol ABCC1), and BCRP (breast cancer resistance protein, gene symbol ABCG2) (Leslie et al., 2005). Evidence indicates that the MXR system acts as a cellular defense mechanism in aquatic organisms (Bard, 2000; Contardo-Jara et al., 2008).

The freshwater bivalve *Corbicula fluminea* (Müller, 1774), commonly known as the Asian clam, is native to Southeast Asia (Araujo et al., 1993). As a benthonic freshwater bivalve, *C. fluminea* is sensitive to aquatic pollutants and has been used as a test organism in field and laboratory studies to monitor environmental changes and contamination (Vasconcelos et al., 2009; Frenzel et al., 2012; Chen et al., 2013). This study mainly investigated the effects of FLX on behavior, cellular detoxification, and the MXR system in the bivalve *C. fluminea*. Adult *C. fluminea* were exposed to FLX for 30 d, after which multi-endpoints were measured, including siphoning behavior, the levels of three stress biomarkers: SOD, CAT and MDA, the expression of thioredoxin and glutathione

systems genes (TR, GR, GPx, and GSTpi), and ABC transporter genes (ABCB1, ABCC1, and ABCG2) in gills and digestive gland.

2. Materials and methods

2.1. Chemical

Fluoxetine hydrochloride (product number F0750, purity > 98%, CAS No. 56296-78-7) was purchased from TCI (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan).

2.2. Experimental animals

Sexually mature clams (*C. fluminea*) were collected from Hongze Lake (Jiangsu province, China) and were transferred to the laboratory in water tanks with continuous aeration. There, clams were kept at constant temperature (20 ± 1 °C), pH (7.8 ± 0.2), and oxygen saturation (96% ± 2%) in 50 L glass aquaria (8–10 cm water depth, without sediment) on a 12 h: 12 h (light: dark) cycle. The clams were fed with single-celled algae *Chlorella vulgaris* and *Scenedesmus obliquus* once per day.

2.3. FLX exposure assays

After 14 d of acclimation, the clams (40 clams per aquaria, average length of 20.56 ± 2.05 mm) in the experimental groups were exposed to FLX. The nominal FLX concentrations in the water were 0, 0.5, 5, and 50 µg L⁻¹. All control or treated groups were in triplicate. The water was changed daily, at which time FLX was added. The mortalities of all groups were recorded during the exposure. At least 3 independent biological replicates for each treatment were harvested at the end of the exposure. The gills and digestive glands of the control and treated groups were collected and stored individually at -80 °C for future analyses. The following equation was used to estimate condition index (CI) based on the weights of 10 clams from each group (Gonzalez-Rey and Bebianno, 2013):

$$CI(\%) = (W_s/W_t) \times 100$$

where W_s is the wet weight of all soft tissue, and W_t is the wet weight of the entire body tissue, including the shell.

2.4. FLX concentrations in water

The FLX concentrations were quantified once per week immediately after tanks were dosed. FLX concentrations were measured using ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) as described by Batt et al. (2008). The detailed method was showed in Supplemental Material. The analyzed concentrations (mean ± standard deviation, and % of nominal concentration) of FLX in the test solutions during the exposure period were 0.48 ± 0.35 µg L⁻¹ (96%), 4.42 ± 0.38 µg L⁻¹ (88%), and 42.66 ± 2.35 µg L⁻¹ (85%) for the 0.5, 5, and 50 µg L⁻¹ treatments, respectively. Nominal FLX values are used in the present study.

2.5. Siphoning behavior

The siphoning rate was measured using the adapted method described by Cooper and Bidwell (2006), which is based on the loss of neutral red dye particles from the water column due to filtration by the clams. Immediately following the FLX exposure experiments, 5 clams from each exposure concentration were placed in 200 mL beakers containing 100 mL of neutral red solution (1 mg L⁻¹ in dechlorinated tap water with no added FLX) and

allowed to siphon for 2 h. Just prior to placing the clams in the solution, 1 mL aliquots of water were removed from each beaker, and the neutral red concentration was determined by measuring the optical density at 530 nm using a spectrophotometer. Standards of neutral red solution were measured along with the samples and used to generate a standard curve. The filtration rate was calculated using the equation of Cooper and Bidwell (2006):

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

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

2.6. Activity of antioxidant enzymes and MDA assay

Approximately 200 mg of the gills and digestive gland from 5 clams of each experimental group was ground in a 1 mL centrifuge tube with 0.5 mL RIPA Lysis buffer (Beyotime Institute of Biotechnology, China) using a pestle on ice. The homogenate was centrifuged at 12000 rpm for 10 min, and the supernatant was then collected into a new 1.5 mL tube. The protein content of the supernatant was determined using the BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China), and the samples were frozen at -80°C until further analysis. The activities of antioxidant enzymes SOD and CAT, and the MDA content were analyzed in the gills and digestive glands using Assay Kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's protocols. The detailed methods were showed in Supplemental Material.

2.7. Identification of partial cDNA sequences from *C. fluminea*

Total RNA was isolated from the digestive gland of adult *C. fluminea* (Non-experimental groups) using the SV Total RNA Isolation System (Promega, Fitchburg, WI, USA) according to the manufacturer's protocols. The cDNA was synthesized with 2 μg RNA after RQ1 DNase treatment (Promega, USA) using random hexamers and M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions. The synthesized cDNA was stored at -20°C until further use.

To obtain cDNA fragments of the studied genes of *C. fluminea*, primer pairs were designed based on the assembled sequence of the *C. fluminea* transcriptome (Chen et al., 2013). The RT-PCR

primers are shown in Supplemental Material Table S1. For RT-PCR assays, the cDNA was amplified with GoTaq[®] GreenMaster Mix (Promega, USA) in a Veriti thermal cycle system (Life Technologies, USA). The sequences were compared to the corresponding references for mollusks in Genbank using NCBI BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

2.8. Relative quantification by real-time PCR

The total RNA was isolated, and cDNA was synthesized according to the methods described above. The expression of the tested genes (*TR*, *GR*, *GPx*, *GSTpi*, *ABCB1*, *ABCC1*, and *ABCG2*) was determined using an ABI 7500 real-time quantitative PCR system (Life Technologies, USA) according to a previously described method (Chen et al., 2013). The β -actin gene of *C. fluminea* was used as an endogenous control. Supplemental Material Table S1 lists the primer pairs used for real-time PCR. The results of real-time quantitative PCR were analyzed using the delta-delta Ct method (Schmittgen and Livak, 2008). The experiments were performed in triplicate.

2.9. Statistical analysis

Statistical analyses were performed with SPSS (version 16.0) and OriginPro (version 8.0) software. The experimental data were checked for homogeneity of variance using Levene's test. The significance of differences between exposed and control samples was determined using 1-way ANOVA followed by Dunnett's test for multiple comparisons. All quantitative data are expressed as mean $\pm$ standard error of the mean (SEM). The threshold for statistical significance was $p < 0.05$.

3. Results

3.1. Identification of partial cDNA sequences

Partial cDNA fragments of *GR*, *TR*, *ABCB1*, *ABCC1*, and *ABCG2* were isolated and cloned from adult *C. fluminea*. The sequences of the 5 genes have been submitted to the NCBI GenBank database (*GR* (KF275127, 879 bp), *TR* (KF275126, 966 bp), *ABCB1* (KJ001772, 684 bp), *ABCC1* (KJ027659, 708 bp), and *ABCG2* (KJ001773, 354 bp)). The amino acid sequences were highly conserved among all sequences analyzed (Table 1).

Table 1
Percent identities of the novel identified partial *Corbicula fluminea* genes amino acid sequences in this study with several sequences of Mollusca available in GenBank. Percent identities were obtained with NCBI Blastx.

Proteins and Species	NCBI access. No.	Description	% Identity
GR			
<i>Crassostrea gigas</i>	gb EKC35400.1	Glutathione reductase	72
TR			
<i>Crassostrea gigas</i>	gb EKC27867.1	Thioredoxinreductase 3	78
ABCB1			
<i>Crassostrea gigas</i>	gb EKC23020.1	Multidrug resistance protein 1	61
<i>Mytilus galloprovincialis</i>	gb ABO36618.1	Multidrug resistance protein	65
<i>Azumapecten farreri</i>	gb ACL80139.3	P-glycoprotein-like protein	64
<i>Perna viridis</i>	gb AFV15804.1	P-glycoprotein	58
ABCC1			
<i>Cyphoma gibbosum</i>	gb ACA53359.1	ABCC1	63
<i>Crassostrea gigas</i>	gb EKC19623.1	Multidrug resistance-associated protein 1	59
<i>Mytilus galloprovincialis</i>	emb CAX46412.2	ABCC/MRP-like protein	59
ABCG2			
<i>Mytilus galloprovincialis</i>	emb CCD42044.1	ABCG2-like	83
<i>Crassostrea gigas</i>	gb EKC19658.1	ATP-binding cassette sub-family G member 2	81

3.2. Death, CI, and behavior of *C. fluminea* in response to FLX

Over the 30 d of exposure, the mortality of each group was less than 2.5%. There was no significant difference in CI, which ranged from 33.8% to 38.2%, between the treated groups and the respective controls.

Exposure to 0.5 and 5 $\mu\text{g L}^{-1}$ FLX did not produce a statistically significant difference in siphoning behavior compared to control subjects. Filtration rates ranged from 16.52 to 19.27 mL/animal/h. The filtration rates were significantly decreased by exposure to 50 $\mu\text{g L}^{-1}$ FLX (4.32 ± 1.29 mL/animal/h) ($p < 0.05$, Fig. 1).

3.3. MDA content and activity of SOD and CAT

A significant downregulation of SOD activity in the gill and digestive gland was observed with 5 and 50 $\mu\text{g L}^{-1}$ FLX treatments, while no significant changes were observed in the 0.5 $\mu\text{g L}^{-1}$ exposure group ($p < 0.05$, Fig. 2A). Additionally, CAT activity was significantly upregulated in the gill and digestive gland in the 5 and 50 $\mu\text{g L}^{-1}$ FLX-treated groups ($p < 0.05$, Fig. 2B). Moreover, significant increases in MDA levels were observed with 5 and 50 $\mu\text{g L}^{-1}$ FLX treatments ($p < 0.05$, Fig. 2C).

3.4. Expression of thioredoxin and glutathione system-related genes

TR mRNA expression levels were elevated in the digestive gland by all FLX treatments ($p < 0.05$, Fig. 3A). However, the induction of TR mRNA in the gills was only observed with 50 $\mu\text{g L}^{-1}$ FLX treatment ($p < 0.05$, Fig. 3A). GR mRNA expression in the gills and digestive gland significantly decreased on exposure to 5 and 50 $\mu\text{g L}^{-1}$ treatments ($p < 0.05$, Fig. 3B). Additionally, significant induction of GPx mRNA was observed with 50 $\mu\text{g L}^{-1}$ treatments in the gills ($p < 0.05$), whereas significant upregulation of GPx was observed in the digestive gland for all treatments (Fig. 3C). GSTpi mRNA levels in the gills and digestive gland were upregulated across all treatments (Fig. 3D).

3.5. Transcription of ABC transporter genes

The transcript levels of 3 ABC transporter genes (*ABCB1*, *ABCC1*, and *ABCG2*) were evaluated after 30 d of exposure to FLX (Fig. 4). In

Fig. 1. Average ($n = 5$) filtration rate (mL/animal/h) of *C. fluminea* in FLX-contaminated water containing 1 mg L^{-1} neutral red solution following exposure to the indicated FLX concentrations. The experiments were performed in triplicate and repeated 3 times with similar results. Data is expressed as mean $\pm$ SEM. A statistically significant difference between treatment groups ($p < 0.05$, 1-way ANOVA) is indicated by differences in the letters above the bars.

Fig. 2. Antioxidant enzyme activities and MDA levels in the gills and digestive gland from *C. fluminea* exposed to FLX. Shown in the figure are the SOD activity (A), CAT activity (B), and MDA levels in the gills and digestive gland (C). Data is expressed as mean $\pm$ SEM ($n = 6$). A statistically significant difference between treatment groups ($p < 0.05$, 1-way ANOVA) is indicated by differences in the letters above the bars.

the gills and digestive gland, levels of *ABCB1* and *ABCC1* were significantly downregulated following exposure to 5 and 50 $\mu\text{g L}^{-1}$ FLX ($p < 0.05$, Fig. 4A and B). However, expression of the *ABCG2* transcript was significantly downregulated only in the 50 $\mu\text{g L}^{-1}$ FLX treated group ($p < 0.05$, Fig. 4C) in both tissues.

Fig. 3. Fold-change in the mRNA levels of *TR* (A), *GR* (B), *GPx* (C), and *GSTpi* (D) in the gills and digestive gland of *C. fluminea* exposed to FLX. Data is expressed as mean $\pm$ SEM ($n = 6$). A statistically significant difference between treatment groups ($p < 0.05$, 1-way ANOVA) is indicated by differences in the letters above the bars.

4. Discussion

In this study, 2 thioredoxin and glutathione enzymes system genes (*TR* and *GR*) and 3 ABC transporter genes (*ABCB1*, *ABCC1*, and *ABCG2*) were cloned and identified firstly in *C. fluminea*. BLAST results revealed that the predicted amino acid sequences of *GR* and *TR* had high sequence identity with sequences from *Crassostrea gigas*. The identification of these genes provides the underlying basis for the cellular antioxidant system and cellular MXR system of *C. fluminea* in the transcript levels.

Bivalve siphons play an important role in nutritional physiology, defense, and reproductive mechanisms (Moulton et al., 1996). In the present study, the siphoning behavior of *C. fluminea* was inhibited by FLX. The inhibition of siphoning behavior observed in this study suggested that the health status of *C. fluminea* was reduced by FLX. Hazelton et al. (2013) reported that after a 67-d exposure to 22.3 µg L⁻¹ FLX, the movement and burrowing behaviors of adult mussels (*L. fasciola*) were inhibited. Previous studies have reported that 5-hydroxytryptamine (5-HT) may be a physiological regulator of siphon movements in the zebra mussel (Ram et al., 1999) and that the serotonin receptor is a primary neuroendocrine receptor in bivalves (Meechonkit et al., 2010). FLX increases extracellular 5-HT by inhibiting serotonin transport at nerve synapses (Lister et al., 2009). Therefore, inhibition of siphoning behavior in *C. fluminea* caused by FLX may due to its alteration of 5-HT signaling.

SOD and CAT are the primary enzymes of cellular antioxidant defense which can remove the ROS, such as superoxide anion and hydrogen peroxide (H₂O₂). SOD could reduce superoxide anion to H₂O₂, and then the H₂O₂ was converted to oxygen and water by CAT (Ren et al., 2013). In the present study, FLX (5 and

50 µg L⁻¹) significantly decreased SOD activity, but increased CAT activity, in the gills and digestive gland of *C. fluminea*. Inhibition of SOD activity and facilitation of CAT activity have also been observed in mice and *M. galloprovincialis* exposed to FLX (Djordjevic et al., 2011; Gonzalez-Rey and Bebianno, 2013). It is most likely that an increase in superoxide anions stimulated the consumption of SOD, and that CAT activity was induced by H₂O₂ formation in this study. We also assessed the MDA content in the clam tissues, since MDA is a biomarker of early phases of the oxidative stress response (Martin-Diaz et al., 2009). MDA content showed a significant increase in the gills and digestive gland of clams exposed to 5 and 50 µg L⁻¹ FLX. These results indicate the ROS was excess, since MDA production is stimulated when ROS levels exceed the elimination capacity of antioxidant enzymes (Ren et al., 2013).

To better understand the oxidative stress caused by FLX, the transcript levels of four antioxidant enzymes of the thioredoxin and glutathione systems, *TR*, *GR*, *GPx* and *GST* were also evaluated in this study. The sequences of *TR* and *GR* were cloned and identified in *C. fluminea* firstly. *TR* is the only enzyme known to catalyze thioredoxin reduction and is a central component of the thioredoxin system that plays an important role in antioxidant balance (Mustacich and Powis, 2000; Arner and Holmgren, 2006). After 30 d FLX exposed, *TR* transcript levels were upregulated in the gills and digestive gland of *C. fluminea*, no significant differences were detected in the gills at the low FLX concentrations (0.5 and 5 µg L⁻¹). The difference between the gills and digestive gland showed the digestive gland should play a more important role in the catalyze thioredoxin reduction. *GR* is an enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant (Meister, 1988). The observed

Fig. 4. Fold-change in the mRNA levels of *ABCB1* (A), *ABCC1* (B) and *ABCG2* (C) in the gills and digestive gland of *C. fluminea* exposed to FLX. Data is expressed as mean $\pm$ SEM ($n = 6$). A statistically significant difference between treatment groups ($p < 0.05$, 1-way ANOVA) is indicated by differences in the letters above the bars.

decrease in GR activity after FLX exposed may also indicate increased ROS levels.

Phase II GST belongs to a family of multi-functional enzymes that promote the conjugation of reduced GSH with parental electrophilic compounds, which enable its transformation to more extractable hydrophilic metabolites (Halling-Sørensen et al., 1998).

The biological role of GPx is to protect the organism from oxidative damage. It can reduce lipid hydroperoxides to their corresponding alcohols and reduce free hydrogen peroxide to water (Ren et al., 2013). Glutathione system enzyme activity can be induced by pharmaceuticals in bivalves, such as *M. galloprovincialis* and *C. fluminea* (Contardo-Jara et al., 2011; Gonzalez-Rey and Bebianno, 2012, 2014; Martin-Diaz et al., 2009). In accordance with these studies, our data indicated that GPx and GSTpi levels were increased in the gills and digestive gland of *C. fluminea* after FLX treatment. The increase of GPx gene expression in the current study also indicated the production of ROS. Take all the biomarkers studied in this paper, our findings suggests that FLX enhanced the production of ROS and induces oxidative response in *C. fluminea*.

The MXR system acts as an outwardly directed energy-dependent transport system that removes endogenous and xenobiotic chemicals from the cell (Navarro et al., 2012), and plays a role in drug resistance, in addition to protecting tissues from xenobiotic accumulation (Leslie et al., 2005). Martin-Diaz et al. (2009) reported that exposure to 0.1 and 10 $\mu\text{g L}^{-1}$ carbamazepine down-regulated *Pgp* (*ABCB1*) and *Mrp2* (*ABCC2*) expression in the gills and digestive gland of *M. galloprovincialis*. In the current study, transcripts coding for primary MXR-related genes *ABCB1*, *ABCC1*, and *ABCG2* were downregulated by FLX in the gills and digestive gland. Our data showed that *ABCB1* and *ABCC1* are more sensitive than *ABCG2* to FLX, since *ABCG2* only significant decrease at the highest FLX concentrations. Previous studies showed that some pharmaceuticals and FLX are known to be inhibitors of mammalian ABCB and ABCC transporters (Peer et al., 2004; Caminada et al., 2008). Since the MXR system providing an active defense against environmental toxins, FLX may enhance its clinical application by reducing the potentialities of the MXR system. Interestingly, no significant inhibitory effects on P-gp1 and MRP-like transporters in fish were observed after FLX exposure (Caminada et al., 2008). Further study is needed to better understand the mechanism of MXR system in aquatic organisms.

5. Conclusions

In conclusion, the identification of thioredoxin and glutathione system enzymes and ABC transporter genes of *C. fluminea* provided the foundation for the study of its cellular antioxidant and MXR systems. Short-term (30 d) exposure to FLX induced oxidative damage in *C. fluminea*. Furthermore, the significant downregulation of ABC transporter genes suggested that FLX is a potent inhibitor of multixenobiotic defense in *C. fluminea*. Our results suggest that low dose and long time expose of FLX can exert adverse effects on the cellular detoxification and defense systems of *C. fluminea*.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (21107131) and the National High-Tech R&D Program (2012AA06A302).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chemosphere.2014.08.062>.

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