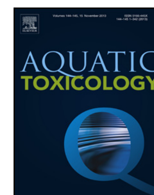
Aquatic Toxicology 185 (2017) 67–75

Contents lists available at ScienceDirect

Aquatic Toxicology

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



6:2 Chlorinated polyfluorinated ether sulfonate, a PFOS alternative, induces embryotoxicity and disrupts cardiac development in zebrafish embryos

Guohui Shi [a], Qianqian Cui [a], Yitao Pan [a], Nan Sheng [a], Sujie Sun [a], Yong Guo [b], Jiayin Dai [a,*]

[a] Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, PR China
[b] Key Laboratory of Organofluorine Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, PR China

ARTICLE INFO

Article history:
Received 1 November 2016
Received in revised form 25 January 2017
Accepted 2 February 2017
Available online 3 February 2017

Keywords:
Zebrafish
F-53B
Developmental toxicity
Cardiac development
Wnt signaling pathway

ABSTRACT

As an alternative to perfluorooctanesulfonate (PFOS), 6:2 chlorinated polyfluorinated ether sulfonate (commercial name: F-53B) has been used as a mist suppressant in Chinese electroplating industries for over 30 years. It has been found in the environment and fish, and one acute assay indicated F–53B was moderately toxic. However, the toxicological information on this compound was incomplete and insufficient for assessment of their environment impact. The object of this study was to examine the developmental toxicity of F–53B using zebrafish embryos. Zebrafish embryos were incubated in 6-well plates with various concentrations of F–53B (1.5, 3, 6, and 12 mg/L) from 6 to 132 h post fertilization (hpf). Results showed that F–53B exposure induced developmental toxicity, including delayed hatching, increased occurrence of malformations, and reduced survival. Malformations, including pericardial and yolk sac edemas, abnormal spines, bent tails, and uninflated swim bladders, appeared at 84 hpf, and increased with time course and dose. A decrease in survival percentages was noted in the 6 and 12 mg/L F–53B-treated groups at 132 hpf. Continuous exposure to 3 mg/L F–53B resulted in high accumulation levels in zebrafish embryos, suggesting an inability for embryos to eliminate this compound and a high cumulative risk to fish. We also examined the cardiac function of embryos at specific developmental stages following exposure to different concentrations, and found that F–53B induced cardiac toxicity and reduced heart rate. Even under low F–53B concentration, o-dianisidine staining results showed significant decrease of relative erythrocyte number at 72 hpf before the appearance of observed effects of F–53B on the heart. To elucidate the underlying molecular changes, genes involved in normal cardiac development were analyzed using real-time qPCR in the whole-body of zebrafish embryos. F–53B inhibited the mRNA expression of *β-catenin* (*ctnnb2*) and *wnt3a*. The mRNA levels of β-catenin targeted genes (*nkx2.5* and *sox9b*), which play critical roles in cardiogenesis, were also reduced after exposure. Thus, exposure to F–53B impaired the development of zebrafish embryos and disrupted cardiac development, which might be mediated by effects on the Wnt signaling pathway and decrease of erythrocyte numbers.

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1. Introduction

As persistent contaminants of anthropogenic origin, perfluorooctane sulfonate acid and its salts (PFOS, $C_8F_{17}SO_3^-$) are regulated under the Stockholm Convention (Fujii et al., 2007; UNEP, 2009). Given the regulatory pressure to reduce the production of PFOS and its importance in modern society, the demand for fluorinated alternatives has increased in recent years. To date, fluorinated alternatives are short-chain homologues with four or six atoms or functionalized perfluoropolyethers (PFPEs). The chemical structure of PFPEs is similar to that of per- and polyfluoroalkyl substances (PFASs), with ether linkage(s) between perfluoroalkyl chains or fluorine atoms replaced by chlorine and hydrogen (Wang et al., 2013a, 2013b, 2015). While some alternative substances, such as 6:2 fluorotelomer sulfonic acid salts (6:2 FTSA, $C_6F_{13}C_2H_4SO_3H$), are being tested at trial phase (UNEP, 2012), some perfluoroether sulfonic acids (PFESAs) have a relatively longer production history. One important example is chlorinated polyfluorinated ether sulfonate (commercial name F-53B, Cl-$C_6F_{12}OCF_2CF_2SO_3K$, CAS No. 73606-19-6), which has been widely used in decorative and hard metal plating since the late 1970s (Wang et al., 2013a, 2013b; Wang et al., 2014a, 2014b).

* Corresponding author.
E-mail address: daijy@ioz.ac.cn (J. Dai).

http://dx.doi.org/10.1016/j.aquatox.2017.02.002
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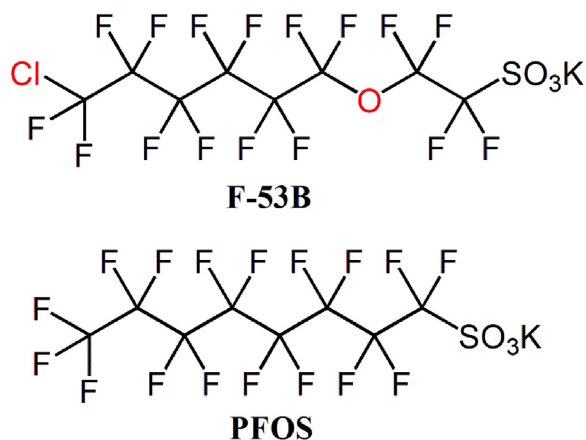

Fig. 1. The molecular structural differences between F–53B and PFOS, red color indicates atoms different from PFOS. (The reader is referred to the web version of this article to view the figure in color.)

Owing to lower production costs, F–53B has quickly dominated the Chinese mist suppressant market (Huang et al., 2010a, 2010b; Ruan et al., 2015; UNEP, 2012). Compared with PFOS, studies on the emissions, environmental occurrence, bioaccumulation, and toxic effects of F–53B are scarce. Wang et al. (2013a, 2013b) first studied the potential impacts of F–53B on the environment. The 96-h LC_{50} value (15.5 mg/L) indicates that F–53B is moderately toxic to zebrafish (*Brachydanio rerio*). Importantly, LC_{50} values within the range of 10–100 mg/L belong to Category III chemicals (harmful to aquatic life) (GSH, 2015). In addition, wastewater treatment has not yet successfully eliminated F-53B, with concentrations from wastewater effluent following treatment still found to be above 40 μg/L (Wang et al., 2013a, 2013b). Furthermore, F–53 B is widely distributed in China, and has been found ubiquitously in water and municipal sewage sludge at concentrations ranging from 2.0 to 44.2 ng/L and 0.02 to 209 ng/L, respectively (Ruan et al., 2015; Lin et al., 2016). F–53B also shows strong bioaccumulation propensity in fish (Shi et al., 2015). A recent study found that the median Log $BAF_{wholebody}$ (body bioaccumulation factors) values for F–53B (4.124–4.322) in low trophic level fish (*Carassius carassius*) exceeded regulatory bioaccumulation criteria, and were even higher than those of PFOS (3.430–3.279) (Shi et al., 2015). In 2009, F–53B production in the Chinese decorative and hard metal plating industry was estimated to be 20–30 tons, and increasing demand for F–53B as a substitute for PFOS in other sectors was foreseeable (Huang et al., 2010a, 2010b). Thus, there is urgent need for environmental hazard assessment of F-53B.

A decade of research has proven that PFOS is harmful to organisms, including to their reproduction, development, and nervous, endocrine, and immune systems (Abbott et al., 2009; Ankley et al., 2004, 2005; Arukwe and Mortensen, 2011; Austin et al., 2003; Chen et al., 2013; Shi et al., 2009; Wan et al., 2012; Wang et al., 2014a, 2014b; Wang et al., 2011; Zushi et al., 2012). Structurally similar chemicals often have similar effects on human and environmental health, and thus it is reasonable to hypothesize that F–53B might create similar biotic toxicity (Fig. 1). Except for an acute toxicity assay that determined the LC_{50} (96 h), no other data demonstrating F–53B toxicity currently exists.

Due to their high fecundity, rapid embryonic development, and optical transparency, zebrafish (*Danio rerio*) are widely used for investigating the developmental toxicity of compounds. To analyze the effects of F–53B on development, zebrafish embryos were exposed to different concentrations of F–53B (0, 1.5, 3, 6 and 12 mg/L) from 6 h post-fertilization (hpf). The percentages of hatching, survival, and malformation, heart rates, and occurrence of erythrocytes were measured to assess F–53B toxicity. To further explore the underlying mechanisms of F–53B exposure-induced toxicity, we also analyzed the expression of cardiac-related genes. Data from our study will help clarify the toxicity of F–53B and its ecological risks on aquatic organisms.

2. Materials and methods

2.1. Zebrafish maintenance and embryo collection

Adult wild-type (Tüebingen strain) zebrafish were housed in automatic flow-through feeding aquariums (ESEN, EnvironScience, China) in an environmentally controlled room (28.5 °C, 14 h/10 h light/dark cycle). The parameters of fish water were 3.5 g/L NaCl, 0.05 g/L KCl, 0.1 g/L $CaCl_2$ and 0.025 g/L $NaHCO_3$, pH: 7.2–8.0, and conductivity 500–800 μS. Adult fish were fed with live brine shrimp twice a day. Zebrafish embryos were obtained by natural breeding of adults at a sex ratio of 1:1 in breeding tanks, with spawning occurring after the light was turned on the next morning. Embryos were collected from different spawning boxes and washed three times with fish water. An optical microscope (LEICA DFC290, Germany) was used to examine embryo stages, and healthy embryos that had developed normally and reached the blastula stage were selected for subsequent experiments. All embryos were raised at similar densities. In all experiments, the solution was renewed daily with fresh test medium, and dead embryos were removed from the 6-well plates every 12 h.

2.2. Chemicals and reagents

The F–53B ($C_8ClF_{16}O_4SK$; CAS #73606-19-6, purity >96%) was provided by Dr. Guo Yong from the Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, and was dissolved in 100% dimethyl sulfoxide (DMSO). The stock solution was 64 mg/mL, and the working solution was obtained by serial dilution with fish water. The final DMSO concentration in the control and each exposure group was 0.1% (v/v).

2.3. Experiment design

According to previous PFOS studies, zebrafish embryos were exposed to 0, 1, 2, 4, 8, 16, 32 and 64 mg/L F–53B from 6 to 96 h post-fertilization (hpf) to determine the value of LC_{50}. Based on the value of LC_{50}, five concentrations of F–53B (0, 2, 4, 8, and 16 mg/L) that induced clear phenotypic effects during early zebrafish development were chosen for the further experiments.

Thirty normal 6 hpf embryos were randomly distributed in each well of a 6-well plate containing 5 mL of different concentrations of F–53B (0, 2, 4, 8, 16 mg/L). All test concentrations and the control were replicated three times, and the exposure solution was renewed daily. The developmental stage of zebrafish embryos was monitored via optical microscope (LEICA DFC290, Germany) at specific time points. Acute endpoints, such as hatching, survival, occurrence of malformations, and heart rate, were used for assessing the developmental toxic effects of F-53B. The embryos were identified as dead when coagulation of embryos, failure to develop somites, lack of heartbeat, or non-detachment of the tail from the yolk sac were observed. Abnormal morphological structures, including heart, head, eye, muscle, tail, and swim bladder, were recognized as malformations. Hatching percentages were recorded at 48, 60, and 72 hpf. We recorded the heart beat for 10 s, using 30 embryos per treatment group at 72 hpf. The mortality and malformation results were recorded every 12 h.

Table 1
Mean measured concentrations of F–53B (mg/L ± SD) in the water of replicate wells.

Nominal concentration (mg/L)	Measured concentration (mg/L) and deviation (%)[a]		
	T_0	T_{24}	deviation (%)
Control	n.d.[b]	n.d.	n.d.
2	1.43 ± 0.07	1.26 ± 0.05	−11.6
4	2.94 ± 0.38	2.73 ± 0.28	−7.1
8	6.00 ± 0.74	5.40 ± 0.37	−10.0
16	12.11 ± 0.98	11.25 ± 0.19	−7.1

[a] Deviation = (actual content (T_0) – actual content(T_{24}))/actual content (T_0) × 100%.
[b] n.d. = not detected.

2.4. Quantification of F–53B in exposure solution and zebrafish embryos

Exposure media were renewed daily, and 2 mL of water was sampled at the beginning of exposure (T_0) and prior to the first water replacement (T_{24}). Fifteen embryos from the 4 mg/L F–53B treatment group were sampled randomly at 24, 48, 72, and 96 h post fertilization (hpf) to analyze uptake kinetics of F–53B by zebrafish embryos. Embryos were washed with deionized water, and wet weights were then determined. All samples were stored at −20 °C until further analysis. Before analysis, the water samples were diluted to 1:1000 with deionized water, and the embryos were homogenized by sonication in 1 mL of deionized water.

Sample extraction was performed as per Hansen et al. (2001), with some modifications. Briefly, 200 μL of diluted water or homogenized embryos was spiked with 0.4 ng of internal standard ($^{13}C_4$-PFOS), 2 mL of $NaHCO_3/Na_2CO_3$ buffer solution (pH = 6.5), and 1 mL of tetra-*n*-butylammonium hydrogen sulfate solution (TBAS, 0.5 M), and then extracted using 4 mL of methyl tert-butyl ether (MTBE). The mixtures were vortexed for 10 s, shaken at 250 rpm for 20 min, centrifuged at 4000 rpm for 15 min, and the supernatant was transferred to a new vial. The extracts were concentrated to dryness under nitrogen gas, dissolved in 200 μL of methanol, and then transferred to a polypropylene auto sampler vial.

The samples (2 μL) were injected into an Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm) at 40 °C, with a mobile phase of 10 mM ammonium acetate in water (A) and methanol (B). The mobile phase flow rate was 0.4 mL/min. The gradient programming began with 10% B, then increased to 90% B with time, and finally reverted to 10% B. The ESI ionization was performed at 150 °C in negative mode with a capillary voltage of 0.5 kV. Multiple reaction monitoring (MRM) transitions were 427 → 407 for F–53 B and 429 → 409 for $^{13}C_4$-PFOS. Quantification was performed by the internal standard approach using $^{13}C_4$-PFOS for F-53B. Two blanks and two matrix-spiked samples were added as quality assurance measures in each batch.

F–53B concentrations in the exposure solutions are shown in Table 1. Due to experimental error, the measured concentration was lower than the nominal concentration (Table 1). Therefore, the measured concentrations (1.5, 3, 6 and 12 mg/L) were used in the following experiment. In the exposure groups, the measured concentrations at T_{24} were slightly lower than those at T_0, and the decrease was likely due to the uptake of F–53B by zebrafish embryos. Therefore, the exposure solution was renewed every 24 h to maintain a constant concentration.

2.5. O-Dianisidine staining of erythrocytes

At 72 hpf, 20 live embryos were collected randomly from each group. The embryos were washed with phosphate buffered saline containing 0.05% Tween 20 (PBST), and then stained in working solution (0.6 mg/mL of o-dianisidine, 10 mM sodium acetate, 40% ethanol, and 0.65% H_2O_2) for 20 min in a dark place. After staining, the working solution was also washed with PBST, and the results were observed under a stereomicroscope (Nikon, Japan). Average integrated optical density (IOD) was calculated to quantitative relative erythrocyte number using Image-pro Plus software (Media Cybernetics, USA).

2.6. Whole mount in situ hybridization (WISH)

Whole mount *in situ* hybridization was performed as described previously (Thisse and Thisse, 2008), with minor modifications. Digoxigenin-labelled antisense RNA specific for *ctnnb2* was used for *in situ* hybridization. A 617 bp fragment of *ctnnb2* was amplified from embryonic zebrafish cDNA, with primer information given in the Supplementary Material. Zebrafish embryos were collected at 72 hpf, fixed overnight in 4% paraformaldehyde solution, and then dehydrated using a gradient concentration of methanol. Hybridization and washing were carried out at 70 °C. To detect hybridized RNA probes, embryos were incubated with an anti-digoxigenin Fab fragment, which conjugated to alkaline phosphatase. As an alkaline phosphatase substrate, BCIP/NBT was used in the color detection reaction. Results were observed under a stereomicroscope (Nikon, Japan).

2.7. Quantitative real-time PCR assays

Embryos were collected at 72 hpf and washed three times with PBS. Total RNA of 30 homogenized zebrafish embryos was extracted using Trizol Reagent (Ambion, Life Technologies, USA) following the manufacturer's instructions. The concentration of each sample was measured by absorbance at 260 nm using a UV1240 spectrophotometer (Shimadzu, Japan) and RNA quality was verified by *260/280* ratios. The cDNA was synthesized via reverse transcription (RT) using an oligo-$(dT)_{15}$ primer and M-MLV reverse transcriptase (Promega, Madison, USA) per the manufacturer's instructions. Real-time PCR reactions were performed with the Stratagene Mx3000P q-PCR system (Stratagene, USA). The SYBR Green Real Master Mix (Tiangen, China) was used for quantification of gene expression.

To evaluate the F–53B effect on the heart and erythrocyte development, concentration-related transcriptional changes were determined. The primer sequences for *ctnnb2, wnt3a, BMP4, myca, sox9b, nkx2.5,* and *axin2,* and amplification protocols were based on previous studies (Nery et al., 2014; Zhang et al., 2016). A dissociation curve was used to differentiate between the desired PCR products and primer-dimers or DNA contaminants. The fold change of target genes was analyzed using the $2^{-D\Delta Ct}$ method (Pfaffl, 2001), and *β-actin* was used as an internal control.

2.8. Statistical analysis

Statistical analyses were performed using SPSS 17.0 software. One-way analysis of variance (ANOVA) was used to analyze differences, with $P < 0.05$ considered statistically significant. The concentration-response curves for hatching, malformation, and mortality for each time point were created by Origin 8.5.0 software. The LC_{50} values were calculated using Origin 8.5.0 software with a nonlinear curve fit.

3. Results

3.1. Developmental toxicity

Obvious embryo malformations were observed from 84 hpf (Fig. 2A). Edemas (pericardial or yolk sac edemas) appeared first, and occurred in 100% of live embryos in the highest exposure group (12 mg/L) at 96 hpf (Fig. 2B). Malformation percentage increased

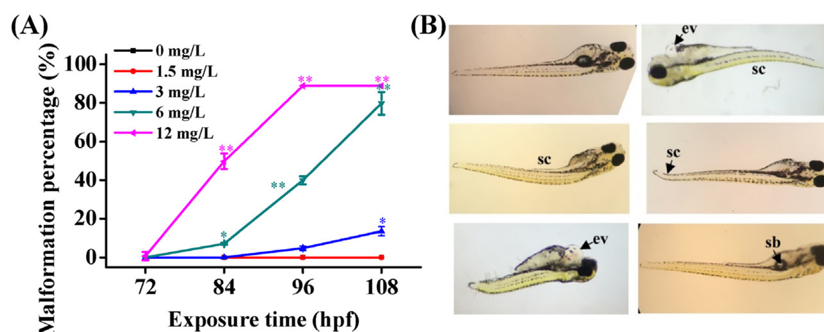

Fig. 2. (A) Cumulative malformation percentage of zebrafish embryos at different times after exposure to F–53B at various concentrations (0, 1.5, 3, 6 and 12 mg/L). Error bars indicate standard errors; *p < 0.05 and **p < 0.01 indicate significant differences between the control and exposure groups (n = 3). (B) Different malformations induced by F–53B at 120 hpf. Morphology of zebrafish in the upper left picture is normal. ev: ventricular edema; sc: malformation of the spine.

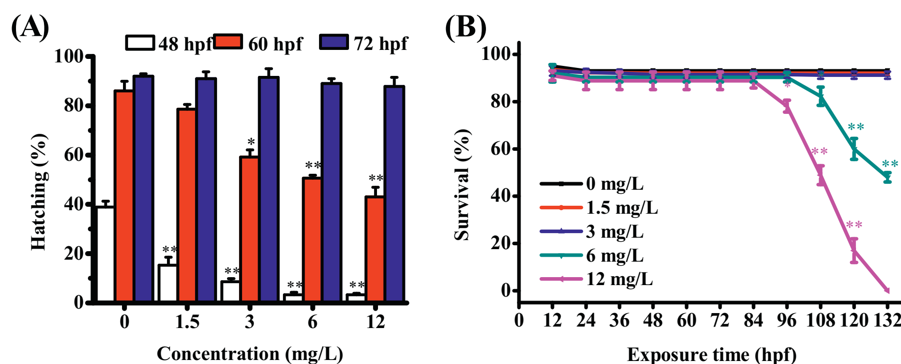

Fig. 3. (A) Hatching percentage of zebrafish embryos at 72 hpf after exposure to different concentrations of F–53B (0, 1.5, 3, 6 and 12 mg/L). (B) Cumulative survival percentage of zebrafish at different times after exposure to various concentrations of F–53B (0, 1.5, 3, 6 and 12 mg/L) (n = 3). Error bars indicate standard errors; *p < 0.05 and **p < 0.01 indicate significant differences between the control and exposure groups.

following F–53B exposure, and several other forms, such as bent spines, uninflated swim bladders, and bent tails, were also observed (Fig. 2B). Among them, edemas, curved spines, and uninflated swim bladders appeared together in the higher exposure groups (6 and 12 mg/L), whereas edemas were not observed in the 1.5 mg/L F-53B-treated group over the entire exposure period. Uninflated swim bladders was the most common morphological malformation.

Hatching is an important stage of early embryonic development. Over 90% of control zebrafish embryos hatched successfully at 72 hpf (Fig. 3A). Results showed that F–53B had no effect on the hatching percentage, and live embryos were nearly all hatched in each group, even in the highest concentration group (12 mg/L) after exposure for 72 hpf (Fig. 3A). However, a significant decrease in the hatching percentage was observed in treatment groups compared with the control group at 48 and 60 hpf (Fig. 3A). Thus, exposure to F–53B delayed the hatching time rather than influenced the hatching percentage.

The control group survival percentage was 93.0%, and exposure to 1.5 and 3 mg/L F-53B did not have significant effects on embryo survival percentage (Fig. 3B). However, the embryo survival percentage markedly decreased in the high exposure groups (6 and 12 mg/L) compared with that observed in the control group. The survival percentage of embryos in the 16 mg/L F–53B treatment group sharply decreased between 96 and 120 hpf, and 100% mortality occurred at 132 hpf, while 50% of larvae were dead at 132 hpf in the 8 mg/L F–53B group. The 96 h-LC50 value of F–53B on zebrafish embryos was 13.77 ± 3.50 mg/L (Fig. 4). The concentration-response curves of sublethal endpoints were significantly different from the concentration-response curve of mortality, which showed F–53B induced hatching delay and teratogenic effects on the zebrafish embryos (supplementary Fig. S1).

3.2. Bioaccumulation of F–53B in zebrafish embryos/larvae

To determine its bioaccumulation, we measured F–53B concentrations in zebrafish embryos after exposure to 3 mg/L F–53B at 24, 48, 72, and 96 hpf (Fig. 5). F–53B accumulated slowly during the initial 24 hpf, with a concentration of 1.25 ± 0.11 ng/embryo. Relatively rapid absorption occurred from 24 to 72 hpf (36.23 ± 4.09 ng/embryo). Between 72 and 96 hpf, the uptake of F–53B continued more rapidly and reached 123.14 ± 9.1 ng/embryo. Thus, F–53B concentration in zebrafish

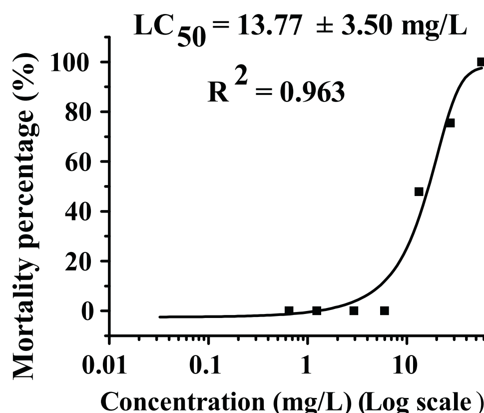

Fig. 4. Concentration-response curves at 96 hpf following F–53B exposure. Embryos were exposed to various concentrations of F–53B from 6 to 96 hpf.

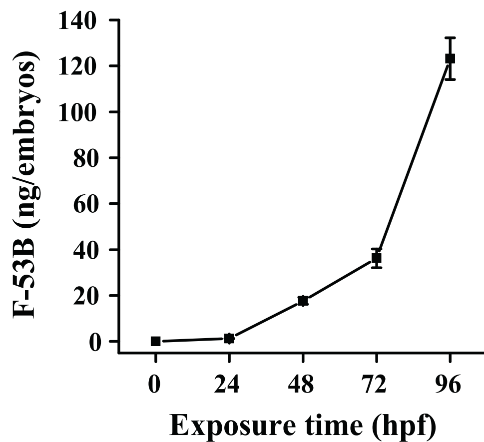

Fig. 5. Uptake of F–53B (3.0 mg/L) in zebrafish embryos following exposure for 6–96 hpf (n = 6). The values are presented as mean ± SE.

embryos significantly increased and did not achieve a steady state during the 96 hpf uptake period. Embryos were transferred into fish water after 96 hpf. F-53B concentration in zebrafish larvae was then detected at 120 hpf and was found to have decreased slightly from 123.14 to 112.30 ng/embryo, indicating that only 8.8% of F–53B was cleared from the zebrafish embryos. This suggests that zebrafish were unable to significantly eliminate F-53B.

3.3. Cardiac effects of F-53B

Embryos exposed to F–53B did not show cardiac toxicity at 72 hpf (Fig. 6A), while the percentage of cardiac teratogenesis increased remarkably between 72 and 96 hpf, with the percentage of pericardial edema in the 12 mg/L F–53B group reaching 91% (Fig. 6A and B). Embryo heart rates in the 1.5, 3, 6, and 12 mg/L groups were 156 ± 3.8, 153 ± 3.3, 149 ± 4.5, and 147 ± 3.3 beats/min, respectively, compared with 159 ± 6.3 beats/min in the control (Fig. 6C). At 96 hpf, heart rates in the 1.5, 3, 6, and 12 mg/L F–53B groups were significantly inhibited, with 153 ± 6.3, 148 ± 7.3, 88 ± 6.2, and 56 ± 7.3 beats/min, respectively, compared with 163 ± 4.5 in the control. The atrium/ventricule ratios in all groups were 1:1 (Fig. 6D), indicating F–53B did not induce atrioventricular block.

3.4. Erythrocyte detection

We used O-dianisidine staining to evaluate erythroid cell number in F-53B-exposed embryos on a protein level. Positive erythrocyte staining was tested in control and F-53B-exposed embryos at 72 hpf. As shown in Fig. 7A, F–53B exposure led to a decrease in tail erythrocyte number compared with that of the control. An indication of relative erythrocyte number in the dorsal aorta, the IOD value, was 0.56-, 0.47-, 0.30-, and 0.05-fold in the 1.5, 3, 6, and 12 mg/L F–53B treated groups compared to control group (Fig. 7B).

3.5. Effects of F–53B on gene expression

To investigate possible molecular mechanisms underlying F-53B-induced cardiac malformation and heart beat change, gene expression levels of Wnt/β-catenin pathway components, which are critical for cardiac development during early developmental stages, were analyzed at 72 and 96 hpf. We performed WISH analyses using the anti-ctnnb2 RNA probe to examine the expression of *β-catenin* (*ctnnb2*) (Fig. 8A), which is mainly distributed in the head of zebrafish embryos. The mRNA transcription level of *ctnnb2* decreased after F–53B treatment, and the results of real-time qPCR

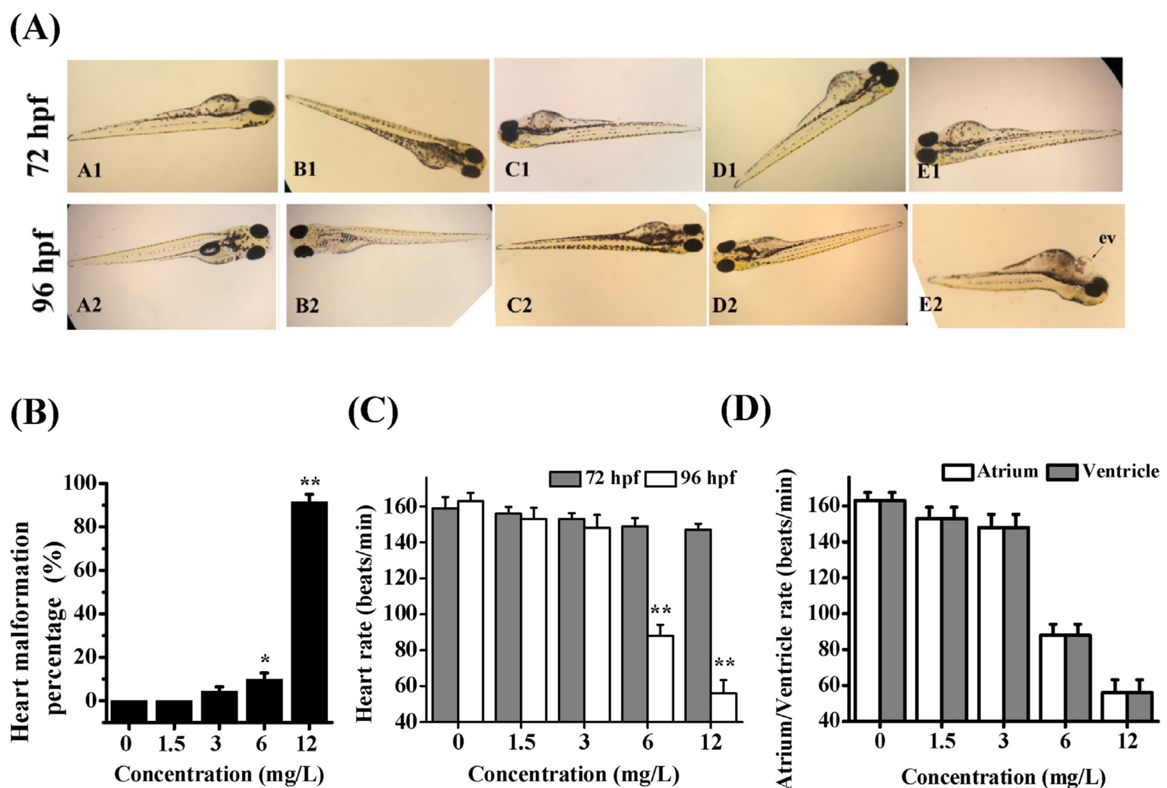

Fig. 6. Heart defects of zebrafish embryos exposed to F-53B. (A) Embryo images, (B) Heart malformation percentage at 96 hpf and (C) heart rate at 72 and 96 hpf (n = 30). (D) atrium and ventricle beat rates at 96 hpf (n = 30). Error bars indicate standard errors; *p < 0.05 and **p < 0.01 indicate significant differences between the control and exposure groups.

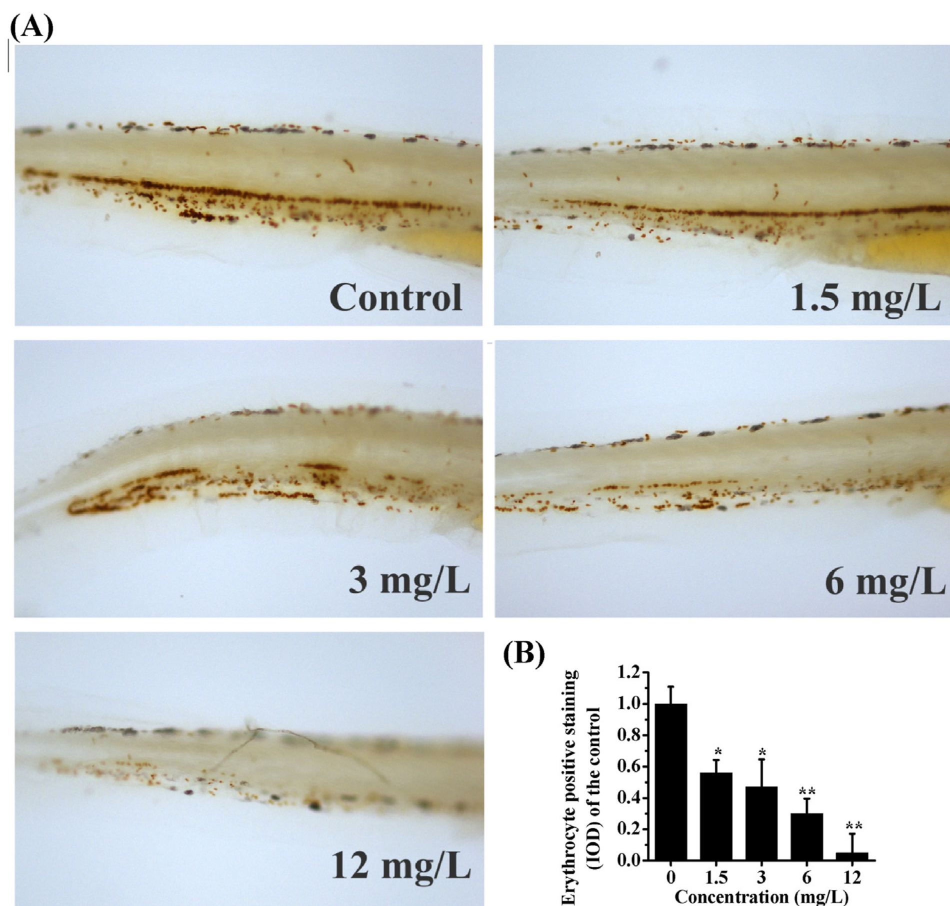

Fig. 7. Erythrocyte detection in zebrafish after exposure to F–53B at 72 hpf. (A) Erythrocytes observed by o-dianisidine staining. (B) Relative numbers of erythrocytes determined by IOD analysis (n = 20). Error bars indicate standard errors; *p < 0.05 and **p < 0.01 indicate significant differences between the control and exposure groups.

were consistent with those of WISH (Fig. 8B). The expression of *wnt3a*, a β-catenin upstream gene, was also significantly downregulated in all F-53B-treated groups at 72 and 96 hpf (Fig. 9). The expression of some β-catenin targeted genes were also analyzed. At 72 and 96 hpf, the transcriptional levels of *nkx2.5* and *sox9b* were downregulated by F-53B. The expression of *axin2* was only reduced at 96 hpf. F–53B did not significantly alter the expressions of *BMP4* and *myca* (Fig. 9).

4. Discussion

Based on previous PFOS studies (Huang et al., 2010a, 2010b; Shi et al., 2008; Chen et al., 2014) and the value of LC_{50}, different concentrations of F–53B (1.5, 3, 6, and 12 mg/L) were selected for exposure. Our results showed that F–53B induced adverse effects on the early developmental stages of zebrafish. The F–53B exposure concentrations tested were higher than those found in the environment, which can range from 2 to 44.2 ng/L in riverine water and up to 78 μg/L in wastewater effluent (Wang et al., 2013a, 2013b; Lin et al., 2016). However, relatively higher concentrations of toxicants are used in laboratory experiments to explore possible toxic mechanisms (Chen et al., 2013; Hagenaars et al., 2011; Ulhaq et al., 2013; Duan et al., 2016).

Exposure to F–53B had significant effects on the survival of the hatched larvae. The death of zebrafish embryos appeared after 96 hpf, with a sharp increase over the next 24 hpf in the highest exposure group. The same effect was also found with exposure to 1.5, 3, and 6 mg/L of F–53B with time (data not shown), and has also been observed with exposure to PFOS (Shi et al., 2008). Exposure to F–53B caused hatching delay and increased occurrence of malformations. Different types of malformations were observed in the F–53B treated groups, including pericardial and yolk sac edema, curved spine, uninflated swim bladder, and bent tail. Malformations of tail and swim bladder were observed in zebrafish embryos exposed to all tested PFAAs (Hagenaars et al., 2011; Ankley et al., 2004; Shi et al., 2008; Huang et al., 2010a, 2010b; Liu et al., 2015), and pericardial and yolk sac edema were typical malformations of embryos exposed to PFOA and PFOS (Hagenaars et al., 2011).

The kinetic analysis of F–53B in organism is helpful to investigate aquatic toxicology. In our experiments, toxicokinetic data showed that the absorption of F–53B by zebrafish embryos began slowly and the rate then rapidly increased with time, with a steady state not achieved during the 96 hpf uptake period. The clearance of F–53B was negligible after 24 h depuration. The rapid absorption of F–53B by hatched larvae was consistent with a rapid increase of mortality and occurrence of malformations. Uptake kinetics of F–53B were similar to those of PFOS (Huang et al., 2010a, 2010b), and thus, F-53B, as an alternative to PFOS, might also be harmful to the aquatic environment. However, the 3 mg/L concentration of F–53B used in this study is far greater than the concentration found in the environment. Therefore, further toxicodynamic studies of F–53B using environmentally relevant concentrations are needed, as well as exposure at multiple life stages. The high bioaccumulation and negligible elimination of F–53B in zebrafish embryos supported our analysis of toxicity mechanisms using higher concentrations than those found in the environment.

The heart is one of the first functional organs during the early zebrafish embryo development. Our study demonstrated

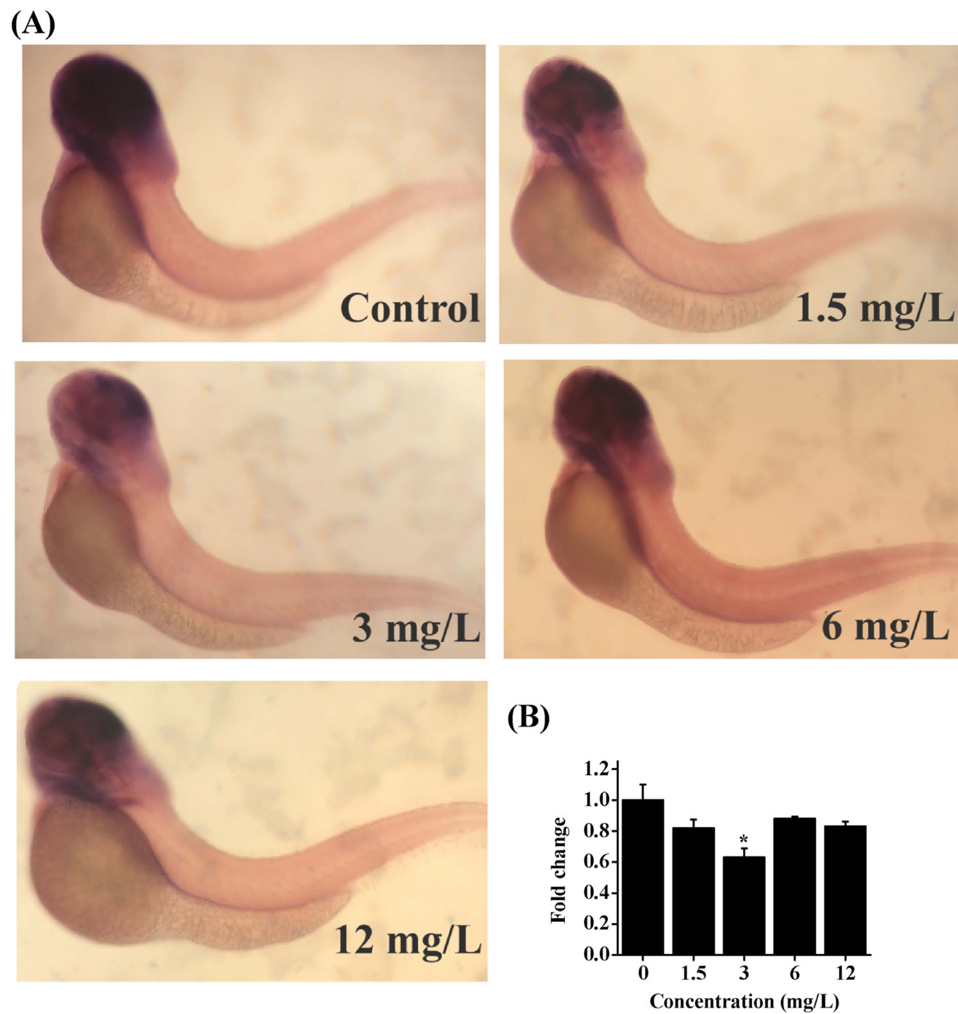

Fig. 8. Relative mRNA expression of *β-catenin* (*ctnnb2*) in zebrafish embryos after exposure to F–53B at 72 hpf. (A) WISH and (B) RT-PCR (n = 6). Error bars indicate standard errors; *p < 0.05 and **p < 0.01 indicate significant differences between the control and exposure groups.

that F–53B exposure has an effect on the cardiac development of zebrafish embryos. Pericardial edema, as a phenotype of cardiac toxicity, was the most sensitive endpoint following exposure to F-53 B, and first appeared in higher concentrations at 84 hpf. This indicated that the developing heart was possibly an initial target for F–53B toxicity. Heart rate is an important toxicological endpoint in embryonic tests, so the measurement of heartbeat is also an important variable of interest in assessing cardiac function (De et al., 2014). In our study, 6 and 12 mg/L F–53 B exposure decreased embryo heart rate but did not induce atrioventricular block at 96 hpf. Bradycardia was the main factor that led to cardiac toxicity in zebrafish embryos exposed to F-53B. Using o-dianisidine staining, we further analyzed the effect of F–53B on erythrocytes. Our results showed that the relative erythrocyte numbers in the dorsal aorta

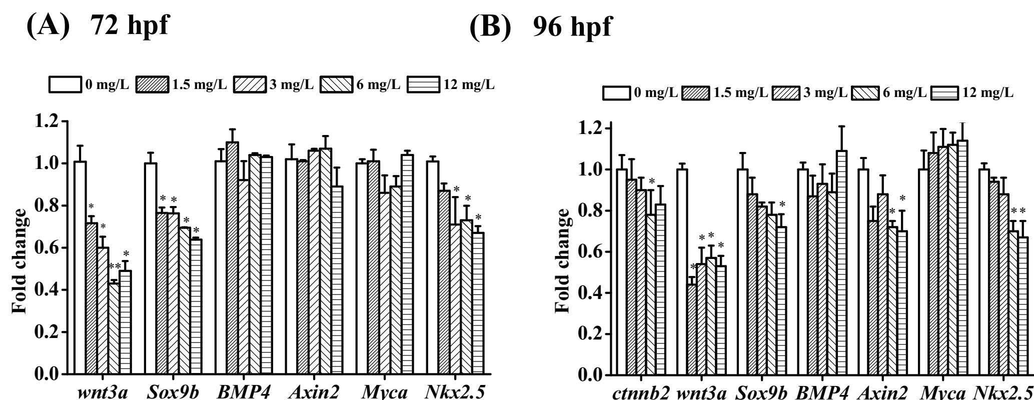

Fig. 9. Relative mRNA expressions of *ctnnb2*, *wnt3a*, *sox9b*, *BMP4*, *Axin2*, *Myca*, and *Nkx2.5* of zebrafish embryos after exposure to various concentrations of F–53B at 72 hpf (A) and 96 hpf (B) (n = 6). Error bars indicate standard errors; *p < 0.05 and **p < 0.01 indicate significant differences between the control and exposure groups.

of treated groups were significantly decreased compared with that in the control group, even though there was no observed edema at low level exposure during the whole experiment. Normal blood circulation plays an important role in early embryonic development (Korzh et al., 2008; Winata et al., 2010). Thus, the defects in cardiac function occurred prior to the morphological abnormalities in zebrafish embryos after F–53B exposure.

The Wnt/β-catenin pathway is critical for cardiac specification during early developmental stages (Ozhan and Weidinger, 2015). To explore the underlying molecular mechanism of the developmental effects of F-53B, the gene expression levels of Wnt/β-catenin pathway components were detected after exposure to F–53B for 72 hpf. The gene expressions of *wnt3a* and *ctnnb2* both decreased. The Wnts are a highly conserved family in zebrafish, and *wnt3a* can activate the Wnt canonical pathway (Clements et al., 2009). Reduction in *wnt3a* transcription was associated with abnormal migration of neural crest cells (NCCs) and defective cardiac function (Sun et al., 2008). β-catenin (the product of *ctnnb2*) is a transcriptional activator of the T-cell factor/lymphoid enhancer-binding factor (Tcf/LEFI) complex. This complex controls the expression of multiple genes, many of which play critical roles in embryo axis determination and formation of various organs. F–53B also reduced the transcription of two β-catenin target genes, *sox9b* and *nkx2.5*, which are involved in the heart development of zebrafish embryos (Hofsteen et al., 2013). Loss of *sox9b* can result in pericardial edema, elongated heart, and reduced blood circulation (Hofsteen et al., 2013). The *nkx2.5* is a gene associated with cardiac precursor cells throughout development (Chen and Fishman, 1996), and decreases in *nkx2.5* expression due to PFOS exposure have been shown to impair heart development in *Oryzias melastigma* (Huang et al., 2011). Mutations in *nkx2.5* can also cause heart malformations in zebrafish (Targoff et al., 2013). Hence, the F-53B-induced cardiac toxicity observed in this study might be via suppression of the Wnt/β-catenin pathway.

Our results demonstrated that F–53B exhibited similar toxicity to that of PFOS, and could produce similar developmental toxicity in zebrafish embryos. The kinetics experiment showed that F–53B was accumulated rapidly in zebrafish embryos, with negligible elimination. Furthermore, F–53B exposure induced developmental toxicity, including delayed hatching, increased occurrence of malformations, and reduced survival. F–53B exposure induced pronounced toxicity on the cardiovascular system, resulting in increased pericardial edemas, reduced heartbeat and erythrocyte numbers, as well as caused downregulation in the expression of key genes involved in heart development. Therefore, considering the effects of F–53B on zebrafish embryos, its appropriateness as a PFOS alternative is questionable and needs further assessment.

Competing financial interests

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB14040202) and the National Natural Science Foundation of China (31320103915 and 21377128).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.02.002.

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