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6:2 fluorotelomer carboxylic acid (6:2 FTCA) exposure induces developmental toxicity and inhibits the formation of erythrocytes during zebrafish embryogenesis

Guohui Shi[a], Qianqian Cui[a], Yitao Pan[a], Nan Sheng[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 200032, PR China

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ABSTRACT

Saturated fluorotelomer carboxylic acids (FTCAs) are intermediates in the degradation of fluorotelomer alcohols (FTOHs) to perfluorinated carboxylic acids (PFCAs). Recent studies have detected FTCAs in precipitation, surface waters, and wildlife, but few studies have focused on their toxicity. In this study, zebrafish embryos were exposed to different concentrations of 6:2 FTCA (0, 4, 8, and 12 mg/L) from 6 to 120 h post-fertilization (hpf) to investigate its developmental toxicity. Results showed that 6:2 FTCA exposure decreased the hatching and survival percentages, reduced the heart rate, and increased the malformation of zebrafish embryos. The median lethal concentration of 6:2 FTCA was 7.33 mg/L at 120 hpf, which was lower than that of perfluorooctanoic acid (PFOA), thus indicating higher toxicity for zebrafish. The most common developmental malformation was pericardial edema, which appeared in the 8 and 12 mg/L 6:2 FTCA-exposed embryos from 60 hpf. Using o-dianisidine staining, we found that the hemoglobin content in embryos was reduced in a concentration-dependent manner after 6:2 FTCA exposure at 72 hpf. Based on quantitative real-time polymerase chain reaction (q-RT-PCR) and whole-mount *in situ* hybridization, the transcriptional levels of hemoglobin markers (*hbae1*, *hbbe1*, and *hbae3*) were down-regulated at 48 and 72 hpf, even though no observed malformation appeared in zebrafish at 48 hpf. Moreover, 6:2 FTCA exposure decreased the protein level of *gata1*, a principal early erythrocytic marker, in Tg (*gata1*:DsRed) transgenic zebrafish at 72 hpf. We analyzed the transcriptional level of other erythrocyte-related genes using q-RT-PCR assay. For heme formation, the transcription of *alas2*, which encodes the key enzyme for heme biosynthesis, was down-regulated after 6:2 FTCA exposure, whereas the transcription of *ho-1*, which is related to heme degradation, was up-regulated at 48 and 72 hpf. The transcriptional patterns of *gata1* and *gata2*, which are related to erythroid differentiation, differed. At 48 hpf, the mRNA level of *gata2* was significantly increased, whereas that of *gata1* exhibited no significant changes in any treatment group. At 72 hpf, the expressions of both were down-regulated in a concentration-dependent manner. Taken together, 6:2 FTCA exposure decreased the erythrocyte number and disrupted erythroid differentiation during zebrafish embryonic development. Our results suggest that 6:2 FTCA can cause developmental toxicity in zebrafish embryos, and that FTCAs exhibit greater toxicity than that of PFCAs.

1. Introduction

Perfluorinated carboxylic acids (PFCAs) are persistent global contaminants, with their main source in the environment coming from direct spills and indirect degradation of their precursors (Cousins et al., 2011; Prevedouros et al., 2006). Fluorotelomer alcohols (FTOHs) are fluorinated precursors that can result in the formation of PFCAs through serial abiotic and aerobic biodegradation in the environment (Dinglasan et al., 2004; Ellis et al., 2004; Hurley et al., 2004; Wang et al., 2005a, 2005b). As industrial raw material, FTOHs are widely produced and used as intermediates for the synthesis of FTOH-based polymeric and surfactant products (Kissa, 2001; Prevedouros et al., 2006). Degradation of FTOH-based products and fugitive emissions during manufacturing can result in the occurrence of FTOHs in the environment (Dinglasan et al., 2004; Ellis et al., 2003; Dinglasan-Panlilio and Mabury, 2006). These volatile chemicals are primarily distributed in the ambient atmosphere, with their concentration in the North American troposphere reported to range from 11 to 171 pg/m^3 (Stock et al., 2004). Precipitation, discharge from wastewater treatment plants and degradation of FTOH-based products can also result in the

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

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occurrence of FTOHs in the aquatic environment (Dinglasan et al., 2004). For example, FTOHs have been detected recently in wastewater treatment plant effluent (23.2 ng/L) and surface water (10.8 ng/L) in urban Japan (Mahmoud et al., 2009).

In the atmosphere, FTOHs are oxidized to form saturated fluorotelomer carboxylic acids (FTCAs), which are then deposited in surface water (Ellis et al., 2004; Loewen et al., 2005). For example, 8:2 FTCA and 10:2 FTCA have been detected in North American rainwater (< 10 ng/L) and Arctic freshwater lakes (Loewen et al., 2005; Scott et al., 2006; Butt et al., 2010a, 2010b). In addition to oxidation, previous studies have also found that FTOHs can undergo a series of photolysis or biodegradation processes in the aquatic environment, forming FTCA intermediates and finally transforming into PFCAs (Lange, 2002; Dinglasan et al., 2004; Gauthier and Mabury, 2005; Wang et al., 2005a, 2005b; Zhao et al., 2013). Based on the worldwide occurrence of PFCAs and their precursor FTOHs, it is reasonable to speculate on the presence of FTCAs in the environment. So far, FTCAs (including 6:2, 8:2, and 10:2) have been detected in effluent waters from wastewater treatment plants (< 8.62 μg/L, in Germany), surface waters, precipitation (< 10 ng/L, in North American), and even aquatic mammals (1.5–9.6 ng/g) (Loewen et al., 2005; Houde et al., 2005; Taniyasu et al., 2005; Scott et al., 2006; Sinclair and Kannan, 2006; Butt et al., 2007; Gremmel et al., 2017).

Historically, 8:2 FTOH has been used as a major raw material since the 1970s (Prevedouros et al., 2006). As 8:2 FTOH is a potential precursor of perfluorooctanoic acid (PFOA) (Wang et al., 2005a, 2005b, 2009), short chain 6:2 FTOH has been approved by regulators to replace 8:2 FTOH as a key raw material in the manufacture of FTOH-based products (Ritter, 2010; OECD, 2012). Similar to 8:2 FTOH, manufacturing emissions and residual concentrations in products might also result in the environmental release of 6:2 FTOH (Buck et al., 2011). Several metabolic studies have suggested that 6:2 FTCA is a major transient intermediate during 6:2 FTOH biotransformation in various environments, including mammalian systems (Liu et al., 2010a, 2010b; Zhang et al., 2013; Zhao et al., 2013; Russell et al., 2015). With the wide application of 6:2 FTOH, the increase in the concentration of 6:2 FTCA in the environment is highly probable. A recent study demonstrated that 6:2 FTOH in wastewater treatment plant influent can result in an increase in the concentration of FTOH transformation products in corresponding effluent, with concentrations of 6:2 FTCA as high as 8.62 μg/L detected (Gremmel et al., 2017). In addition, 6:2 FTCA has been utilized as an alternative processing aid to PFOA in China (Wang et al., 2015; Xu et al., 2011). Thus, assessment of the potential risk of 6:2 FTCA in the environment is critical.

6:2 FTCA is structurally analogous to its precursor 6:2 FTOH and legacy PFOA (Fig. 1). Although structurally similar chemicals can result in similar effects on human and environmental health, current evidence on the potential risk of FTCAs remains insufficient and only limited reports are available on their toxicity to aquatic organisms (Phillips et al., 2007, 2010; Mitchell et al., 2011; Hoke et al., 2012). Acute aquatic toxicities have been assessed on *Chironomus dilutes*, *Daphnia magna*, *Lemna gibba*, *Pseudokirchneriella subcapitata*, *Oncorhynchus mykiss*, and *Pimephales promelas* (Phillips et al., 2007; Mitchell et al., 2011; Hoke et al., 2012). Based on 50% lethal concentrations (LC_{50}) and 50% effective concentrations (EC_{50}), the toxicity of FTCAs has been found to increase with increasing fluorocarbon (FC) chain length, and precursors of PFCAs exhibit greater toxicity than PFCAs with equivalent carbon chain lengths (Phillips et al., 2007, 2010; Mitchell et al., 2011; Hoke et al., 2012). Although evidence on the toxicity of FTCAs in aquatic environments has grown, studies assessing the potential effects of FTCAs on embryo development and the underlying mechanism have not yet been conducted.

Due to their high fecundity, rapid embryonic development, and optical transparency, zebrafish (*Danio rerio*) embryos are widely used for investigating the developmental toxicity of compounds (Embry et al., 2010; Scholz et al., 2008). To assess the developmental toxicity of

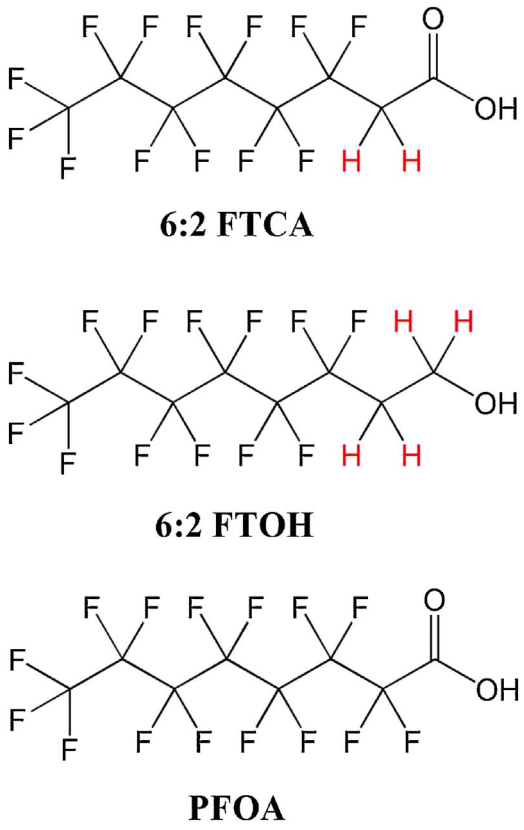


Fig. 1. Molecular structure of 6:2 FTCA, 6:2 FTOH, and PFOA. Red indicates the different atoms among them. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

6:2 FTCA, zebrafish embryos were exposed to various concentrations of 6:2 FTCA (0, 4, 8, and 12 mg/L) from 6 to 120 h post-fertilization (hpf). Different toxicity endpoints, including malformation assessment, hatching percentage, survival percentage, and heart rate, were determined. The o-dianisidine staining results showed that erythrocyte numbers were significantly reduced in 72 hpf 6:2 FTCA-exposed embryos compared with control embryos. *Gata1* is a principal early erythrocytic marker (Heicklen-Klein et al., 2005). Thus, Tg (*gata1*:DsRed) transgenic zebrafish embryos, which express DsRed in erythrocytes, were used to analyze the impact of 6:2 FTCA on erythrocytes at the protein level. To further explore the underlying molecular mechanisms of 6:2 FTCA exposure-induced toxicity, the expression of several erythrocyte-related genes was analyzed. Our study is the first to focus on the developmental toxicity and underlying molecular mechanism of FTCAs, and should be helpful for clarifying the ecological risks of FTCAs on fish.

2. Materials and methods

2.1. 6:2 FTCA stock solutions and exposure protocols

The 6:2 fluorotelomer carboxylic acid (6:2 FTCA $C_6F_{13}CH_2COOH$, CAS No. 53826-12-3, purity > 96%) was provided by Dr. Guo Yong (Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences), and was dissolved in 100% dimethyl sulfoxide (DMSO). To our knowledge, FTCA toxicity testing using zebrafish has not been conducted previously, thus preliminary experiments were performed based on the LogKow (4.636) and predicted LC_{50} values for fish (13.36 mg/L) at 96 h to establish suitable toxicity ranges and determine the LC_{50} value. Four doses of 6:2 FTCA (0, 4, 8, and 12 mg/L), which induced obvious malformation during early zebrafish embryo development, were chosen in our study. In general, relatively higher

concentrations of toxicants than those found in surface waters are used in acute laboratory experiments to explore possible toxic mechanisms (Phillips et al., 2007; Mitchell et al., 2011; Hoke et al., 2012; Hagenaars et al., 2011; Zhou et al., 2016). The stock solution was 20 mg/mL, and the exposure solution was obtained by serial dilution with fish water (3.5 g/L NaCl, 0.05 g/L KCl, 0.1 g/L $CaCl_2$, 0.025 g/L $NaHCO_3$, pH 6.8–7.2) (Liu et al., 2015; Westerfield, 1995). The final DMSO concentration in the control and each exposure group was 0.01% (v/v).

2.2. Zebrafish maintenance and embryo collection

Adult wild-type zebrafish (Tuebingen strain), as well as the transgenic line Tg (*gata1*:DsRed) (provided by Dr. Feng Liu from the Institute of Zoology, Chinese Academy of Sciences), were housed in automatic flow-through feeding aquaria (ESEN, EnvironScience, China) and kept according to standard protocols (Westerfield, 1995). Embryos were obtained by natural breeding. An optical microscope (LEICA DFC290, Germany) was used to examine embryo stages, and fertilized embryos were selected and maintained at 28 ± 0.5 °C for subsequent experiments. The ages of embryos and larvae were defined as hours-post-fertilization (hpf).

2.3. Phenotype observation

Healthy embryos that reached the blastula stage at 6 hpf were randomly distributed into 6-well plates (30 embryos in 5 mL of exposure solution/well). Zebrafish embryos were treated with different concentrations of 6:2 FTCA (0, 4, 8, and 12 mg/L) from 6 to 120 hpf, with three replicates of the control and each treatment group. The developmental stages of the zebrafish embryos were observed using an optical microscope (LEICA DFC290, Germany). The exposure solution was renewed daily over the entire experimental period, and dead embryos were removed in a timely manner. Toxicity endpoints, including hatching, survival, malformation, and heart rate, were used to assess the developmental toxicity effects of 6:2 FTCA. Hatching percentages were recorded at 72 hpf. Heart rates of 30 embryos per group were recorded at 48 and 72 hpf for 10 s (five animals per replicate and six replicates per time point). The mortality and malformation results were recorded every 12 h. Photoshop (Adobe Systems, USA) was used to calculate the pixels of the pericardium area to determine pericardial edema.

2.4. Quantification of 6:2 FTCA in exposure solution

Water (2 mL) from each well of the treated groups was sampled at the beginning of exposure (T_0) and prior to the first water replacement (T_{24}). All water samples were stored at −20 °C. Before analysis, the water samples were diluted to 1:10 with deionized water. Sample analysis was performed as per our previous study (Shi et al., 2017), with minor modification. The native and mass-labeled standards of 6:2 FTCA were purchased from Wellington Laboratories (Guelph, Canada).

2.5. O-Dianisidine staining of erythrocytes

O-Dianisidine staining was performed as reported previously (Detrich et al., 1995). Briefly, 20 live embryos were randomly collected from each group at 72 hpf, and incubated in the dark in o-dianisidine working solution (0.6 mg/mL of o-dianisidine, 10 mM sodium acetate (pH 4.5), 0.65% H_2O_2, and 40% (vol/vol) ethanol) for 15 min at 28 °C. After washing three times with phosphate buffered saline containing 0.05% Tween 20, the embryos were observed under a stereomicroscope (Nikon, Japan). The treatment embryos with thin colored precipitates were defined as embryos with decreased erythrocytes (hemoglobin). The percentage of embryos exhibiting decreased hemoglobin was calculated, as reported previously (Zhou et al., 2016).

2.6. Erythrocyte testing using Tg (gata1:DsRed) zebrafish embryos

After exposure to different concentrations of 6:2 FTCA (0, 4, 8, and 12 mg/L), Tg (*gata1*:DsRed) zebrafish embryos were observed under an Eclipse Ti-S microscope (Nikon, Japan) at 72 hpf. Image-Pro Plus software (Media Cybernetics, USA) was used to calculate average integrated optical density (IOD), which can represent the relative number of erythrocytes.

2.7. Quantitative real-time polymerase chain reaction (q-RT-PCR)

Total mRNA was isolated from 48 and 72 hpf whole embryos using Trizol Reagent (Ambion, Life Technologies, USA), with 30 embryos per replicate and six replicates per group. The RNA concentration was measured by absorbance at 260 nm using a UV1240 spectrophotometer (Shimadzu, Japan), and quality was checked 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). Real-time PCR was monitored with the Stratagene Mx3000P q-PCR system (Stratagene, USA). A SYBR Green Real Master Mix (Tiangen, China) was used for quantification of gene expression.

The transcriptional changes of several erythrocyte-related genes were examined using q-RT-PCR. The primer sequences for hemoglobin markers (*hbae1*, *hbbe1*, and *hbae3*), and genes encoding aminolevulinate, delta-, synthase2 (*alas2*), GATA binding protein 1 and 2 (*gata1* and *gata2*), and solute carrier family 4, member 1a (*scf4*) were used as described previously (Hu et al., 2014; Zhou et al., 2016). The primers for the gene encoding heme oxygenase-1 (*ho-1*) were designed using Primer 5.0. The primers for the constitutive genes encodinf ribosomal protein L13 alpha (*rpl13a*) and β-actin were from Tang et al. (2007). All primers are listed in Supplementary Table S1. *β-actin* and *rpl13a* were used as the internal controls. The q-RT-PCR assay was performed as described previously (Zhou et al., 2016). The fold change of target genes was calculated using the $2^{-\mathrm{DACt}}$ method (Pfaffl, 2001).

2.8. Whole-mount in situ hybridization

To verify the location and expression levels of *hbae1*, *hbbe1*, and *hbae3* in whole embryos, specific digoxigenin-labeled antisense RNA probes were amplified using the primers listed in Hu et al. (2014). Whole-mount *in situ* hybridizations were conducted as described previously (Thisse and Thisse, 2008). At 48 and 72 hpf, 20 zebrafish embryos were collected for *in situ* hybridization. Hybridization and washing were carried out at 65 °C. For the color detection reaction, we used BCIP/NBT to detect the hybridized RNA probes. The specifically labeled cells were purple in the embryos, and the relative intensity of the color indicated the transcriptional level of the target gene.

2.9. Statistical analysis

Statistical analyses were conducted using SPSS 17.0 software. All data were reported as means ± standard error (SE). The differences were analyzed by one-way analysis of variance (ANOVA), and $P < 0.05$ was considered to show statistically significant differences between groups. The LC_{50} value was calculated using Origin 8.5.0 software with a nonlinear curve fit.

3. Results

3.1. Concentration of 6:2 FTCA in exposure media

The measured 6:2 FTCA concentrations in exposure media are shown in Table 1. The concentration of 6:2 FTCA at 24 hpf (T_{24}) was slightly higher than that of the original solution (T_0). This was likely due to evaporation of water. Therefore, we renewed the exposure media daily to maintain a stable concentration. Due to the challenges in the

Table 1
Mean measured concentrations of 6:2 FTCA (mg/L ± SD) in the water of replicate wells.

Nominal concentration (mg/L)	Measured concentration (mg/L)	
	T_0	T_{24}
Control	n.d.[a]	n.d.
4	3.88 ± 0.02	3.97 ± 0.09
8	7.78 ± 0.52	7.95 ± 0.64
12	11.73 ± 0.38	11.87 ± 0.47

[a] n.d. = not detection.

current analytical methodology for quantifying 6:2 FTCA in biota, measurement of concentrations in fish tissue could not be done.

3.2. Effects of 6:2 FTCA on hatching and survival percentages

Under normal circumstances, the zebrafish embryo hatching began at 48 hpf and finished at 72 hpf. The hatching percentage was recorded at 72 hpf, and was reduced in a concentration-dependent manner in the 6:2 FTCA-exposed groups (Fig. 2A). However, the hatching percentages of the 4 and 8 mg/L 6:2 FTCA-exposed embryos were only slightly lower than those of the control. Exposure to 12 mg/L of 6:2 FTCA significantly decreased the hatching percentage (70.0 ± 2.9%) compared with that of the control (93.3 ± 3.3%).

The number of dead embryos was recorded every 12 h. As shown in Fig. 2B, there were no significant differences in the survival percentages between the exposed and control groups before 72 hpf. However, a sharp decrease in survivorship in the 12 mg/L 6:2 FTCA-treated group appeared between 84 and 108 hpf, with 100% mortality at 120 hpf. In addition, an increase in mortality was found in embryos exposed to 8 mg/L of 6:2 FTCA at 108 and 120 hpf. The survival percentage of zebrafish embryos in the 4 mg/L 6:2 FTCA-treated group was 92.2 ± 1.92% at 120 hpf, which was not significantly different from that of the control group (94.4 ± 3.8%). The 72 h-LC_{50} and 120 h-LC_{50} values of 6:2 FTCA on zebrafish embryos were 25.1 ± 1.5 mg/L and 7.33 ± 0.50 mg/L, respectively (Fig. 2C, D).

3.3. Effect of 6:2 FTCA on heart morphology and heartbeat rate of zebrafish embryos

To examine the morphological defects caused by 6:2 FTCA, the malformations of embryos exposed to 6:2 FTCA was analyzed from 48 hpf. The most sensitive malformation type in 6:2 FTCA-exposed embryos was edema (pericardial edema and yolk sac edema), which first appeared from 60 hpf (Fig. 3A, B). Following exposure to 6:2 FTCA, pericardial edema, yolk sac edema, and uninflated swim bladder increased in concentration-dependent manner (Fig. 2B). Quantitative data indicated that the embryos experienced severe pericardial edema in the higher exposure groups (8 and 12 mg/L) at 72 hpf (Fig. 4A, B). At 84 hpf, 100% heart malformation was observed in live larvae from the 12 mg/L 6:2 FTCA group, and 72.2% heart malformation was found in the 8 mg/L 6:2 FTCA group (Fig. 4C). However, exposure to a lower concentration of 6:2 FTCA (4 mg/L) did not induce heart malformation over the entire experimental period, uninflated swim bladders were found to be the prominent malformation type in this group (data not shown).

The heart rates of the 6:2 FTCA-exposed embryos at 48 and 72 hpf decreased compared with those of the control (Fig. 4D). At 48 hpf, exposure to 4, 8, and 12 mg/L of 6:2 FTCA resulted in 94 ± 4.9, 83 ± 5.9, and 78 ± 5.4 beats/min, respectively, compared with 101 ± 4.5 beats/min in the control. At 72 hpf, the heart rates in the 4, 8, and 12 mg/L 6:2 FTCA groups were still significantly inhibited at 96 ± 5.4, 80 ± 4.9, and 53 ± 7.0 beats/min, respectively, compared with 135 ± 6.3 beats/min in the control. These results suggest that 6:2 FTCA can induce cardiac malformation in zebrafish embryos

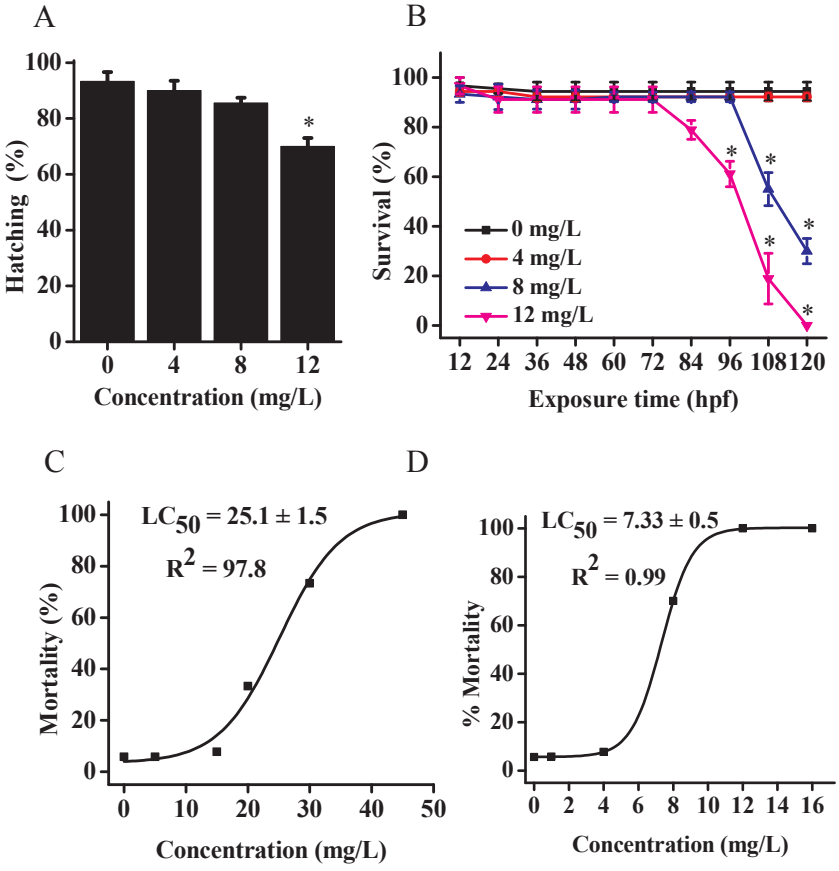


Fig. 2. Concentration-dependent effects of 6:2 FTCA on zebrafish embryo hatching percentage at 72 hpf (A), survival percentage from 12 to 120 hpf (B), LC_{50} value at 72 and 120 hpf (C). Values are means ± SE of three replicates. Asterisks indicate statistically significant difference from the control (*P < 0.05).

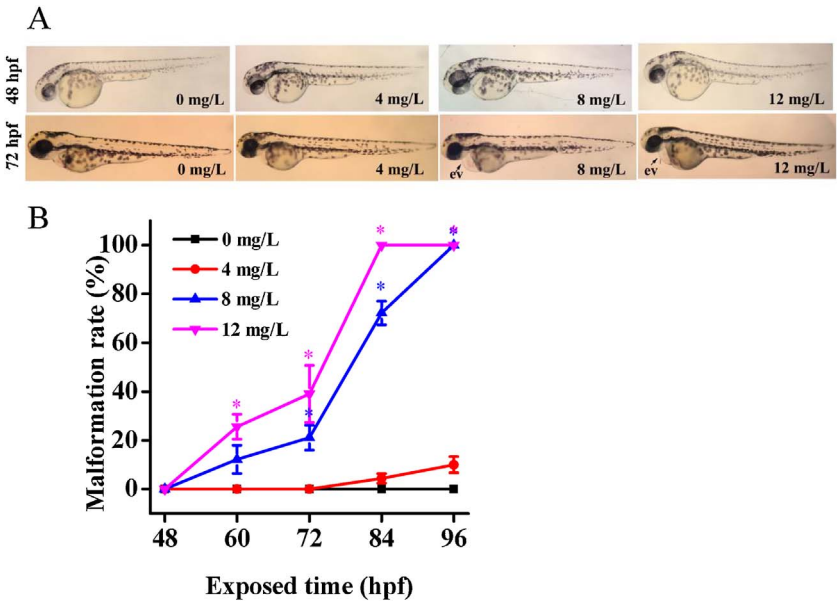


Fig. 3. Microscope photographs of zebrafish larvae in 6:2 FTCA exposure tests (A) and concentration-dependent effects of 6:2 FTCA on zebrafish embryo malformation percentage from 48 to 96 hpf (B). Values are means ± SE of three replicates. Asterisks indicate statistically significant difference from the control (*P < 0.05).

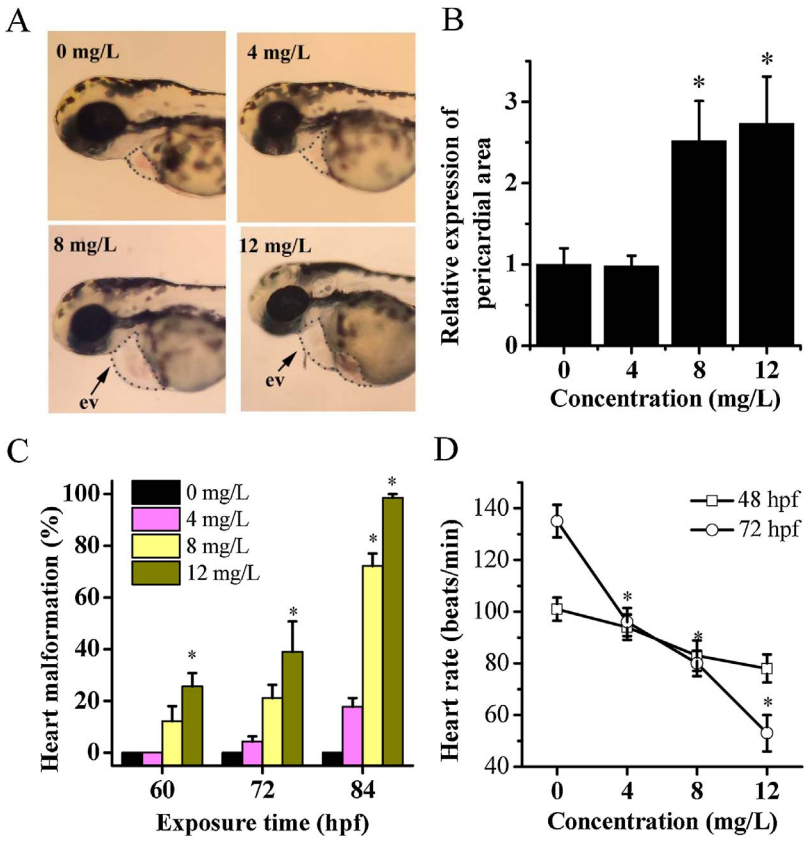


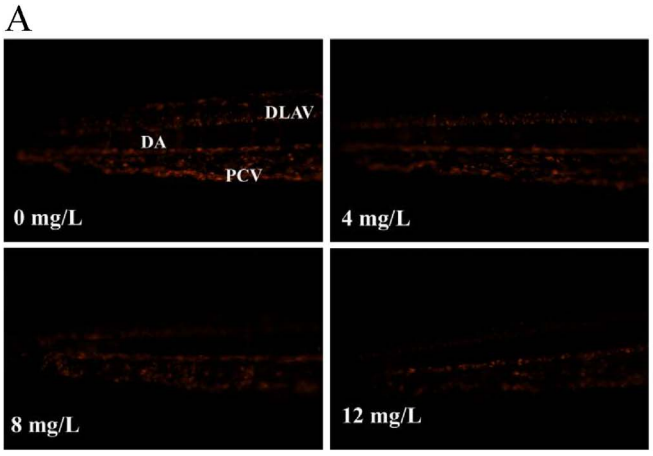
Fig. 4. Heart morphology at 72 hpf (A), quantification of pericardial edema at 72 hpf (B), heart malformations at 60, 72, and 84 hpf (C), and heart rate at 48 and 72 hpf (D) of zebrafish embryos after exposure to different concentrations of 6:2 FTCA (0, 4, 8, and 12 mg/L). Values are means ± SE of six replicates. Asterisks indicate statistically significant difference from the control (*P < 0.05).

3.4. Effects of 6:2 FTCA on erythrocytes

As hemoglobin in erythrocytes can catalyze the oxidation of o-dianisidine to rust-colored precipitates, o-dianisidine staining was used to evaluate the protein level of hemoglobin and indicate the number of erythroid cells in 72 hpf zebrafish embryos. We observed o-dianisidine stained embryonic erythrocytes at 72 hpf. The proportion of mature erythrocytes decreased in the treatment groups (Fig. 5). The percentage of embryos that exhibited reduced staining increased in a concentration-dependent manner, with 100% of embryos in the highest 6:2 FTCA-treated group presenting a decrease in the number of stained erythrocytes compared with that of the control embryos.

To further analyze the effects of 6:2 FTCA on erythrocytes *in vivo*, Tg (*gata1*:DsRed) zebrafish embryos were also exposed to different concentrations of 6:2 FTCA (0, 4, 8, and 12 mg/L) from 6 hpf. As shown in Fig. 6A, fluorescence protein intensity was obviously down-regulated after exposure to 6:2 FTCA at 72 hpf. The IOD values in the treatment groups were 0.50-, 0.28-, and 0.26-fold lower than that of the control, respectively (Fig. 6B). These results demonstrate that 6:2 FTCA exposure decreased the number of erythrocytes in a concentration-dependent manner.

Fig. 5. Reduced o-dianisidine staining of hemoglobin at 72 hpf following 6:2 FTCA exposure.



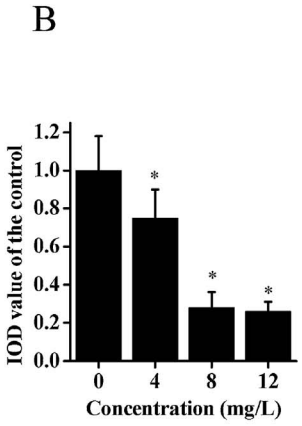


Fig. 6. At 72 hpf, the DsRed-positive cells in *gata1*:DsRed transgenic zebrafish were reduced by 6:2 FTCA exposure (A). Relative numbers of erythrocytes were determined by IOD analysis (B). Values are means ± SE of six replicates. Asterisks indicate statistically significant difference from the control (*P < 0.05).

3.5. Effects of 6:2 FTCA on erythrocyte-related genes

To investigate the possible molecular mechanisms underlying the 6:2 FTCA-induced decrease in erythrocyte number, the transcriptional levels of several key erythrocyte-related genes were examined in zebrafish embryos at 48 and 72 hpf using q-RT-PCR (Fig. 7). We first analyzed hemoglobin markers (*hbae1*, *hbbe1*, and *hbae3*), and the results indicated that the transcriptional levels of these genes were down-regulated in a concentration-dependent manner after 6:2 FTCA exposure, even though there was no observed malformation at 48 hpf. We also analyzed the transcriptional levels of two important genes of heme pathway (*alas2* and *ho-1*). The transcriptional level of *alas2* was decreased in all treatment groups at 48 and 72 hpf. The transcriptional level of *ho-1* was demonstrated to have a 2.13-fold up-regulation in the 12 mg/L 6:2 FTCA-treated group at 48 hpf, and to be up-regulated in all 6:2 FTCA-treated embryos at 72 hpf. The gene encoding the ion transport protein scf4 is specifically expressed in erythrocytes, and its transcription was inhibited in the 8 and 12 mg/L 6:2 FTCA-treated groups at 48 and 72 hpf. The genes encoding GATA transcription factor family members, i.e. *gata1* and *gata2* with products, which play crucial roles during erythroid differentiation (Moriguchi and Yamamoto, 2014), exhibited different transcriptional patterns at 48 and 72 hpf. The expression of *gata1* was not significantly changed in the 6:2 FTCA exposure groups at 48 hpf, but was down-regulated in the treatment groups at 72 hpf. The transcriptional changes in *gata1* determined by q-RT-PCR were consistent with the protein level changes observed from the Tg (*gata1*:DsRed) embryos. Unlike *gata1*, the transcriptional level of *gata2* (a marker for hematopoiesis) was up-regulated in the 6:2 FTCA exposure groups at 48 hpf, but was down-regulated in the treated zebrafish embryos at 72 hpf.

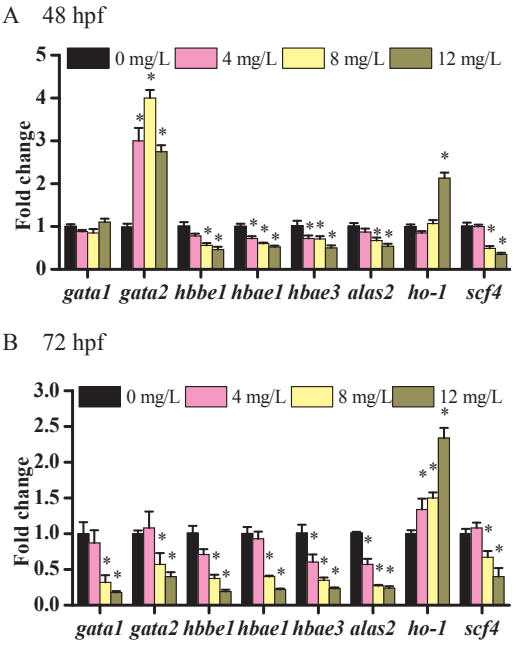


Fig. 7. Relative mRNA expressions of erythrocyte-related genes in zebrafish embryos after exposure to various concentrations of 6:2 FTCA at 48 hpf (A) and 72 hpf (B). Values are means ± SE of six replicates. Asterisks indicate statistically significant difference from the control (*P < 0.05).

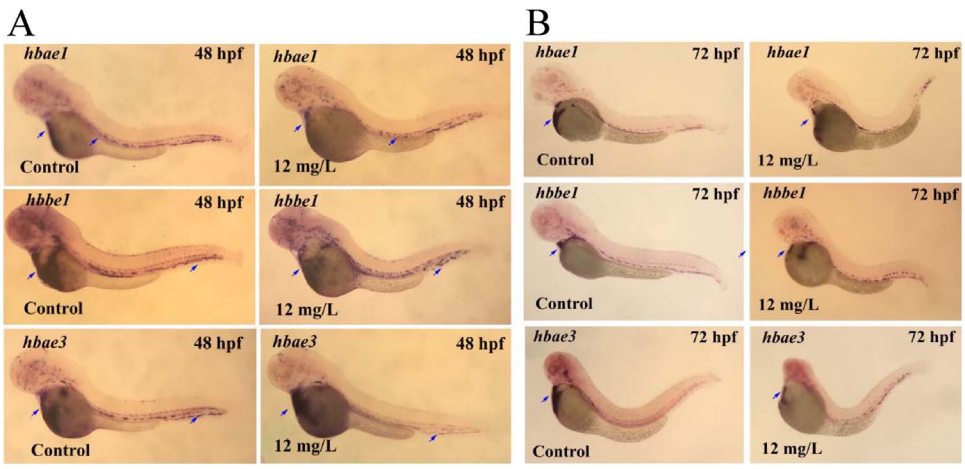


Fig. 8. Expressions of *hbae1*, *hbbe1*, and *hbae3* in the control and 6:2 FTCA (12 mg/L)-treated embryos at 48 hpf (A) and 72 hpf (B) revealed by whole-mount *in situ* hybridization.

We also applied WISH to test the location and transcriptional level of hemoglobin markers (*hbae1*, *hbbe1*, and *hbae3*) in embryos at 48 and 72 hpf (Fig. 8). Similar to the q-RT-PCR results, hemoglobin marker genes were down-regulated in the 6:2 FTCA-exposed embryos at 48 and 72 hpf compared with those in the control.

4. Discussion

Previous research has demonstrated that 6:2, 8:2, and 10:2 FTCAs can be more toxic to midges, water fleas, algae, amphipods, and fish than PFCAs with equivalent carbon chain lengths, and that the toxicity can increase with increasing carbon chain length (Phillips et al., 2007, 2010; Mitchell et al., 2011; Hoke et al., 2012). However, these earlier results were only based on LC_{50} or EC_{50} values, with the possible mode of action not investigated.

Our study is the first to examine the developmental toxicity of 6:2 FTCA using zebrafish embryos. After exposure to 6:2 FTCA, hatching success and survival were reduced, and embryo malformations increased. Moreover, exposure to 12 mg/L of 6:2 FTCA from 6 to 120 hpf significantly decreased hatching and survival percentages, although no significant effects were observed in the 4 mg/L 6:2 FTCA exposure group compared with that of the control. The median LC_{50} of 6:2 FTCA on zebrafish embryos was 25.1 ± 1.5 mg/L at 72 hpf and 7.33 ± 0.50 mg/L at 120 hpf. The 120 h-LC_{50} value was lower than that for PFOA with the same carbon chain length (> 500 mg/L) (Hagenaars et al., 2011), but higher than that for PFOS (2.20 mg/L) (Huang et al., 2010), suggesting that 6:2 FTCA is more toxic than PFOA, but less so than PFOS.

Malformations appeared from 60 hpf and increased with exposure time. Pericardial edema, as a phenotype of cardiac teratogenesis, was the most sensitive endpoint following exposure to 6:2 FTCA, and appeared first in embryos exposed to higher concentrations. Pericardial edema has also been reported in zebrafish embryos exposed to organic pollutants, including perfluoroalkyl and polyfluoroalkyl substances (PFASs) (e.g., PFOA, PFOS, and perfluorononanoic acid (PFNA)) and heavy metals, and is regarded as a cardiac toxic phenotype in zebrafish embryos (Hagenaars et al., 2011; Huang et al., 2010; Liu et al., 2015; Nijoukubo et al., 2016; Zhou et al., 2016). As an important toxicological endpoint, heart rate is often used to assess cardiac function in embryonic tests. Our results showed that 6:2 FTCA exposure suppressed the heart rates of zebrafish embryos at 48 and 72 hpf compared with those of the control group. A reduced heart rate in zebrafish embryos was also found after exposure to PFOS and PFOA at 48 hpf (Huang et al., 2010; Hagenaars et al., 2011). These observations suggest that the heart might be a priority target for 6:2 FTCA toxicity during zebrafish embryo development. However, the underlying mechanisms need further exploration.

Normal blood circulation is crucial for early embryonic development and organogenesis (Korzh et al., 2008; Winata et al., 2010). Erythrocytes are a major component in blood. Based on the o-dianisidine staining results and Tg (*gata1*:DsRed) transgenic zebrafish embryos at the protein level, erythroid cell numbers were lower in the 6:2 FTCA-exposed embryos than in the control group at 72 hpf. From the IOD values, the relative erythrocyte numbers exhibited a significant and concentration-dependent decrease in the treatment groups compared with the control group, even though there was no observed edema at low level exposure (4 mg/L) during the whole experiment. The reduced transcriptional level of *gata1* in the 6:2 FTCA-treated transgenic zebrafish embryos was further confirmed by q-RT-PCR assay. GATA1 is an essential transcription factor for the maturation of erythroid and megakaryocytic cells and can modulate many genes involved in hemoglobin subunits and heme biosynthetic enzymes (Katsumura et al., 2013). Disruption of *gata1* in mice, for example, can suppress erythroblast maturation and result in severe anemia by embryonic day 9.5 (Fujiwara et al., 1996). Zebrafish with *gata1* mutations also confirm its involvement in the regulation of erythropoiesis (Belele et al., 2009; Scott et al., 1994). In the present study, *gata2* was also analyzed in the 6:2 FTCA-exposed embryos, with its transcriptional level found to be increased at 48 hpf, but reduced in a concentration-dependent manner at 72 hpf. Different to *gata1*, *gata2* is a marker for hematopoiesis and can regulate proliferation and maintenance of hematopoietic stem and progenitor cells (Moriguchi and Yamamoto, 2014). Ectopic expression of GATA2 has been shown to promote erythroblast proliferation but block terminal erythroid differentiation (Huang et al., 2009).

The reduction in erythroid cell numbers in the 6:2 FTCA-exposed embryos was verified at the transcriptional level using WISH and q-RT-PCR. Results showed a reduction in the transcription of hemoglobin marker genes (*hbae1*, *hbbe1*, and *hbae3*) in 6:2 FTCA-exposed embryos at 48 and 72 hpf. Furthermore, 6:2 FTCA exposure decreased erythroid cell number in a concentration-dependent manner, even though no observed malformation appeared in zebrafish at 48 hpf. Decreased hemoglobin has also been found in previous studies on PFOS toxicity; for example, two genes regulating hemoglobin activity in chickens were decreased approximately two-fold after exposure to PFOS (Yeung et al., 2007). In addition, 6:2 FTCA exposure also resulted in the down-regulation in the transcription of erythropoietic marker *alas2* at 48 and 72 hpf (Davidson and Zon, 2004). The mRNA level of *ho-1*, a gene encoding a key enzyme for heme degradation, was increased in 6:2 FTCA-exposed embryos at 48 and 72 hpf (Sikorski et al., 2004). Our results indicated that 6:2 FTCA exposure might affect heme biosynthesis and degradation during early development of zebrafish embryos. As heme is an essential prosthetic group that forms the reactive core of hemoproteins and is utilized for hemoglobin formation (Ponka, 1997), the decreased heme might also be responsible for the reduced erythroid cell number in zebrafish embryos after 6:2 FTCA exposure. Compared with the control, the transcriptional level of *scf4*, a gene that encodes an ion

transport protein specifically expressed in erythrocytes (Davidson and Zon, 2004), was down-regulated after exposure to 6:2 FTCA in the higher concentration treatment groups (8 and 12 mg/L). Even though the above evidence suggests that the number of circulating red blood cells was reduced, it was not clear whether the reduced gene expression in the 6:2 FTCA exposed zebrafish embryos was caused by a reduction in cell number (erythrocytes) or by reduced gene expression in every cell, or both. In addition, some genes were only measured at the transcriptional level, which cannot directly indicate changes at the functional protein level. Thus, the possible mechanisms behind the effect of 6:2 FTCA on erythrocytes need further investigation.

Our study revealed that 6:2 FTCA exposure influenced the development of zebrafish embryos, including a reduction in the hatching and survival percentage, a decrease in heart rate, and an increase in the occurrence of cardiac malformations. Based on the LC_{50} values, 6:2 FTCA appears to be more toxic than PFOA. The reduced erythroid cell numbers in the 6:2 FTCA-exposed embryos were observed at both the transcriptional and protein levels. This study contributes to our understanding of the potential risks of FTCAs to aquatic biota, and supports the need to consider intermediates in the environmental degradation of PFCA precursors in future risk assessments.

Competing financial interests

The authors declare no conflicts of interest.

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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.2017.06.023.

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