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Effects of perfluorinated compounds on development of zebrafish embryos

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Abstract Perfluorinated compounds (PFCs) have been widely used in industrial and consumer products and frequently detected in many environmental media. Potential reproductive effects of perfluorooctanesulfonate (PFOS), perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA) have been reported in mice, rats and water birds. PFOS and PFOA were also confirmed developing toxicants towards zebrafish embryos; however, the reported effect concentrations were contradictory. Polyfluorinated alkylated phosphate ester surfactants (including FC807) are precursor of PFOS and PFOA; however, there is no published information about the effects of FC807 and PFNA on zebrafish embryos. Therefore, this study was conducted to determine the effects of these four PFCs on zebrafish embryos. Normal fertilized zebrafish embryos were selected to be exposed to several concentrations of PFOA, PFNA, PFOS or FC807 in 24-well cell culture plates. A digital camera was used to image morphological anomalies of embryos with a stereomicroscope. Embryos were observed through matching up to 96-h post-fertilization (hpf) and rates of survival and abnormalities recorded. PFCs caused lethality in a concentration-dependent manner with potential toxicity in the order of PFOS > FC807 > PFNA > PFOA based on 72-h LC_{50}. Forty-eight-hour post-fertilization pericardial edema and 72- or 96-hpf spine crooked malformation were all observed. PFOA, PFNA, PFOS and FC807 all caused structural abnormalities using early stages of development of zebrafish. The PFCs all retarded the development of zebrafish embryos. The toxicity of the PFCs was related to the length of the PFC chain and functional groups.

Keywords PFNA · PFOA · PFOS · FC807 · *In ovo* · Developmental toxicity

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Introduction

Perfluorinated compounds and their polyfluorinated precursor compounds (PFCs) have been widely produced and used as surfactants, lubricants, polishes and fire-retardants and primarily to repel both moisture and oil. These uses have resulted in global distribution of stable precursors and transformation products in humans and wildlife (Giesy and Kannan 2002), such as perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA) (Giesy and Kannan 2001; Hansen et al. 2001). At present, biomagnifications of PFCs have aroused great concern because increasing concentrations of PFCs have been detected in surface water, sediments and even in wildlife from the Arctic (Butt et al. 2010). Generally, PFOS and PFOA are the most frequently detected PFCs in environment media (Ahrens 2011). The concentrations of PFOS and PFOA are detected mostly at the level of nanograms per liter in lakes, rivers and coastal water (Saito et al. 2004; So et al. 2004; Lu et al. 2011; Zhu et al. 2011). In soils and sediments, the levels of PFOA and PFOS ranged from several nanograms per gram to a few hundred of nanograms per gram dry weight (Sepulvado et al. 2011; Wang et al. 2011). PFCs can enter bodies and adhere to proteins in the blood, liver and muscle, and mostly concentrations in the blood and liver are the highest (Giesy and Kannan 2001). PFOS and PFOA in serum of Amur tiger (*Panthera tigris altaica*), loggerhead sea turtles (*Caretta caretta*) and Kemp's ridley sea turtles (*Lepidochelys kempii*), and eggs of Herring Gulls (*Larus argentatus*) and other water birds were in the range of nanograms per milliliter (Gebbink et al. 2009; Keller et al. 2005; Li et al. 2008; Wang et al. 2008). Concentration of PFOS in fish can be up to as great as 612 ng/g dw from estuarine and coastal areas of Korea (Naile et al. 2010) and in blood plasma of bald eagles from the midwestern USA can even be up to 2.57 mg/L (Giesy and Kannan 2001), which was approximately 100,000-fold greater than the concentrations of PFOS in coastal seawaters of Hong Kong, the Pearl River Delta, including the South China Sea, and Korea (So et al. 2004). Perfluorononanoic acid (PFNA) is the largest perfluorinated carboxylic acid surfactant. It was detected in the Pacific and Atlantic Oceans at concentrations that were intermediate between PFOS and PFOA (Yamashita et al. 2005). It has also been detected in blood and liver of wildlife at the level ranging from picograms per liter to nanograms per liter (Calafat et al. 2007; Houde et al. 2005; Moon et al. 2010; Weihe et al. 2008; Yeung et al. 2006). PFOS, PFOA and PFNA can all be detected in the serum of human even in umbilical cord blood at the level of nanograms per milliliter (Monroy et al. 2008; Lien et al. 2011). The highest concentrations of PFOS and PFOA were detected in the blood sera of employees in the fluorochemical manufacturing industry at concentrations up to 12.8 and 114 mg/mL, respectively (Bossi et al. 2005).

Polyfluorinated alkylated phosphate ester surfactants (including FC807) are used in greaseproof food contact paper products (Begley et al. 2005; Trier et al. 2011) and have been found in wastewater, and they are also detected in blood of humans (D'eon et al. 2009). Recently, polyfluorinated dialkylated phosphate ester surfactants (diPAPS) have been found to degrade to form smaller perfluorinated carboxylic acids (PFCAs), including PFOA and PFNA (D'eon and Mabury 2007, 2011). Precursors of PFOS and PFOA have also been detected in waste water (Huset et al. 2008). Thus, FC807 with the similar structure as the other diPAPS is guessed the potential sources of PFNA, PFOA and PFOS.

Because of the persistence, potential to bioaccumulation and global distribution of PFOS and PFOA, there has been interest in their sources and potential to cause toxicity. When exposed to mice, PFOS or PFOA alters the number of circulating neutrophils and enhances the inflammatory responses of macrophages to lipopolysaccharide (LPS) (Qazi et al. 2009). PFOS and PFOA can alter behavior in mice, which is manifested as reduced and/or lack of habituation and hyperactivity (Johansson et al. 2008). The hypothalamus–pituitary–thyroid axis could be disturbed when zebrafish embryos are exposed to PFOS (Shi et al. 2009). PFOA can alter expression of peroxisomal enzymes resulting in greater formation of 8-hydroxydeoxyguanosine in rat liver (Abdellatif et al. 2003). PFOS or PFOA can also induce calcium release from storage sites, which is associated with cytokinesis in the zebrafish embryo (Liu et al. 2011). PFOA can affect mice's reproduction (Yahia et al. 2010) and causes lesser masses of both genders of *Drosophila melanogaster* W1118 stock and shorter life span of adult males (Wang et al. 2010). Besides, cell apoptosis in rates can be caused by PFNA (Fang et al. 2010). Survival rate and development were also decreased when prenatal mice were exposed to PFNA (Wolf et al. 2010). These results indicated that PFOS, PFOA and PFNA could cause a number of toxic effects in liver, the nervous system, especially on development and on reproduction. Among PFCs, PFOA and PFOS often had the greatest concentration in the eggs of fish (Gebbink et al. 2009) and in the serum of humans (Bossi et al. 2005) even in umbilical cord blood (Monroy et al. 2008; Lien et al. 2011). Superadded with the potential developmental disrupting potency of PFCs, more attention should be paid to the toxicities of PFCs to fish embryos. Huang et al. reported that PFOS induced cell death at 24 hpf in the brain, eye and tail region of zebrafish embryos and lesions in the muscle fibers with histological examination (Huang et al. 2010). Effects of PFOS and PFOA to zebrafish

embryos have been studied by several researchers, but the results were not consistent. The LC_{50} (96 h) values reported for larvae of zebrafish are lower than 500 mg/L and 71 mg/L for PFOA and PFOS (Ye et al. 2007), but Hagenaars et al. reported that the LC_{50} (96 h) values were significantly greater than 500 mg/L for PFOA and 58.47 mg/L for PFOS (Hagenaars et al. 2011). There is some information on the toxicity of PFNA to mammals (rats and mice) (Fang et al. 2010; Feng et al. 2010; Wolf et al. 2010), but there was no published available information on toxicity of PFNA and FC807 to aquatic organisms. As an excellent model to study teratogenesis, the zebrafish embryo was used in *in ovo* study to explore the developmental toxicity of PFOA, PFNA, PFOS and FC807.

Materials and methods

Test species

Adult wild-type zebrafish (*Danio rerio*) were obtained from Model Animal Research Center of Nanjing University and kept in a semiautomatic rearing system, with five females and ten males in each 10-L tank at 28±1°C. Tap water was treated to remove residual ammonia, chlorine and chloramines, filtered and then treated with UV light to kill microbial pathogens. Water was exchanged at a rate of 1/3 daily. The lighting was 14/10 h light/dark and 1000 lux. Zebrafish were fed frozen blood worms and dry food twice a day. Nylon nets were used at the bottom of each tank to allow eggs to settle and kept from being eaten by the adult fish. Spawning and fertilization took place within 30 min after the lights were turned on in the morning. Eggs were transferred to a Petri dish. Clean embryos were cultured with aerated embryonic rearing water, with the following characteristics: 24.65 mg/L $MgSO_4 \cdot 7H_2O$, 58.8 mg/L $CaCl_2 \cdot 2H_2O$, 1.15 mg/L KCl, 12.5 mg/L $NaHCO_3$ with pH of 8.3±0.2, dissolved oxygen concentration of 6.07± 0.24 mg/L (Thermo Scientific Orion 5-Star Plus) at the beginning and end of experiments. The test conformed to the guidelines developed by the Organization for Economic Cooperation and Development (OECD 1996).

Test chemicals

PFOA ($C_8HF_{15}O_2$, CAS 335-67-1, 95 %) was provided by TCI (Japan). PFOS ($C_8HF_{17}O_3S$, CAS 1763-23-1, 98 %) and PFNA ($C_9HF_{17}O_2$, CAS 375-95-1, 97 %) were purchased from Alfa (USA). FC807 ($C_{27}H_{25}F_{34}N_2O_8PS_2$, molecular weight: 1,246 g/mol, chemical name: Perfluoro alkyl phosphate, trade name: FC807, 80 %) is a commercial product, one of polyfluorinated alkylated phosphate ester surfactants, and was obtained from Hubei Hengxin (China). For FC807, a stock solution was prepared by dissolving the crystals in dimethyl sulfoxide (DMSO) and stored at 4°C. No solvent was used for the other PFCs because of their high water solubility (PFOA, 9.5 g/L; PFOS, 500 mg/L; PFNA, 9.5 g/L). Exposure solutions were diluted from the stock solutions with embryonic water. Concentrations of DMSO in the greatest concentration of exposure solution were less than 0.05 % (v/v).

Experimental design

Embryos were transferred to exposure solutions immediately after fertilization and examined under a stereomicroscope. Damaged or unfertilized embryos were discarded. Zebrafish embryos were exposed in 24-well cell culture plates with 2-mL solution per well. Twenty normally shaped fertilized embryos were assigned to each treatment or control group. In each plate, the remaining four wells were filled with control solution and control eggs. For PFOS, PFOA and PFNA, the control group was embryonic culture water while for FC807, 0.05 % DMSO was included in embryonic water. Before experiment, three to four times range-finding preliminary studies were conducted to determine the effect concentrations of PFOA (0, 150, 200, 212, 225, 240, 255, 270 mg/L), PFNA (0, 6.25, 12.5, 25, 50, 100, 200 mg/L), PFOS (0, 6.25, 12.5, 25, 50, 100, 200 mg/L) and FC807 (0, 25, 44, 50, 100, 132, 200, 400 mg/L). All concentrations were repeated in triple at different days with different batches of eggs. Embryos were cultured in an incubator at 28.5°C after exposure.

Toxicological endpoints included time until hatching, whether eggs were clear or opaque at 4, 8, 12, 24, 48 and 72 hpf, edema at 48 hpf and structural malformations at 72 or 96 hpf (Table 1). Malformations of the spine crooked were defined as scoliosis and curvature of the tail.

Statistical analyses

The proportion of normal embryos in the control group was >80 %. The normality of each sample set was assessed with the Kolomogrov–Smirnov one-sample test before parametric analysis. Then Duncan's multiple comparisons test was used if appropriate. A Student *t*-test was used to test the null hypothesis that there was no significant difference between the parallelisms of each treatment. One-way ANOVA was used to test the null hypothesis that there was no significant difference between the mean of each parameter measured in the treated group and the control group. Differences were considered significant if $p<0.05$. Probit model or logistic model was used to calculate the EC_{50}/LC_{50} of the endpoints that appeared in the development stages of zebrafish embryo. The software of Sigma plot 11.0 was used to draw figures.

Table 1 Toxicological endpoints at different stages of development of zebrafish embryos (hours post-fertilization)

	4 hpf	8 hpf	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
Opaque embryo	+	+	+	+	+	+	
Gastrula not start		+					
Completion of gastrulation			+				
Extension of the tail				+	+		
Spontaneous movements within 20 s				+	+		
Development of the eye				+	+		
No heartbeat					+		
Edema					+		
Delay of hatching						+	
Malformation of hatching						+	+

"+" indicates that the morphological changes parameters are selected as toxicological endpoints at different stages of development

Results

Developmental toxicity of PFOA

PFOA was acutely toxic to zebrafish embryos, with LC_{50} values of 262 mg/L PFOA, at 8 and 72 hpf, respectively. The number of opaque embryos did not increase between 8 and 72 hpf. PFOA was more toxic during early development of zebrafish embryos and caused a non-specific lethality. At 8 hpf, the proportions of opaque and deformed embryos were approximately equal, which indicated that the lethality was the primary effect. PFOA can also bring on 48 hpf edema and series malformations after hatching. The 96-hpf EC_{50} value of spine crooked malformations was 198 mg/L.

A small amount of edema occurred at 48 hpf, and hatching delays and spine crooked malformations occurred at 72 hpf, but these effects occurred only at 200 mg/L or at greater concentrations (Fig. 1). Almost 100 % mortality occurred when exposed to 270 mg/L PFOA. The LOEC based on edema observed at 48 hpf, hatching delay and spine crooked malformations at 72 hpf were 225, 212 and 212 mg/L, respectively. At concentrations greater than these, PFOA caused significant effects relative to the controls. Spine crooked malformation was the most sensitive toxicological endpoint. At 72 hpf, the rate of spine crooked malformations increased until 96 hpf; the rate of spine crooked malformations was significantly different from that of the controls and exhibited a concentration-dependent relationship.

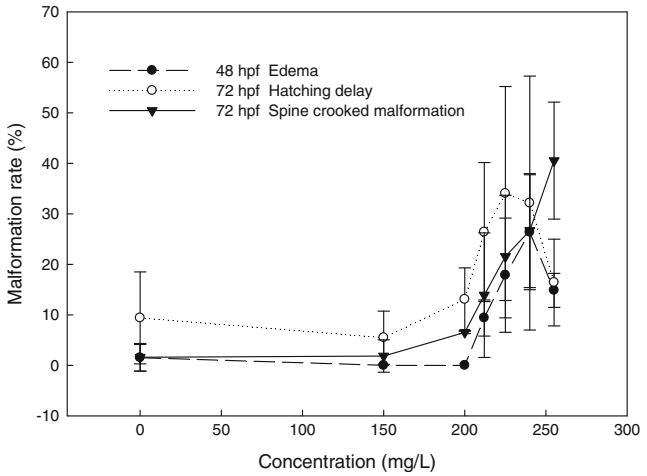

Fig. 1 A 48-hpf pericardial-sac and yolk-sac edema malformation, 72-hpf hatching delay and spine crooked malformations caused by PFOA

Developmental toxicity of PFNA

Exposure to PFNA caused toxicity at several time points (Table 2). The proportion of opaque embryos was proportional to concentration with an LC_{50} of 84 mg/L at 72 hpf. PFNA also delayed hatching. The EC_{50} based on hatching rate at 72 hpf was 214 mg/L. At 8 hpf, some embryos were disintegrating that embryos stopped cleavage and became

Table 2 EC_{50}/LC_{50} of toxicological endpoints of zebrafish embryo result from PFNA

Toxicological endpoints	EC_{50}/LC_{50} (mg/L)	95 % confidence interval (mg/L)
4 hpf opaque	193	150–309
8 hpf gastrula not start	126	93–197
8 hpf opaque	161	120–254
12 hpf opaque	120	95–162
12 hpf abnormal	106	92–123
24 hpf opaque	99	86–114
48 hpf opaque	86	61–129
72 hpf opaque	84	60–125
72 hpf hatch rate	214	–

opaque. There were no significant differences in rates of edema malformations at hatching between the control and treatments.

Developmental toxicity of PFOS

LC_{50} values at 24, 48 and 72 hpf for PFOS were 69, 68 and 68 mg/L, respectively (Table 3). At this range of concentrations, embryos were disintegrated by 4 hpf. The most sensitive endpoint was spine crooked malformations observed at 72 hpf, with an EC_{50} of 37 mg/L. The LOEC based on malformations was 12.5 mg/L. PFOS also caused edemas and delayed hatching but no significant difference (Fig. 2). The LOEC based on incidence of edemas at 48 hpf was 50 mg/L, and the LOEC based on hatching delay at 72 hpf was 6.5 mg/L.

Developmental toxicity of FC807

Embryos exposed to FC807 displayed no visible abnormalities from 0 to 48 hpf. The most sensitive endpoint was edema observed at 48 hpf (Table 4). At 48 hpf, 25 mg/L FC807 resulted in a significant difference of 19 % of embryos with pericardial-sac or yolk-sac edema (Fig. 3), relative to the controls. In the group exposed to 44 mg/L FC807, 61 % of embryos were abnormal. The NOEC based on edema at 48 hpf was less than 25 mg/L. The severity of edema was greater with concentrations increasing, and edema ultimately resulted in lethality. The LC_{50} based on opaqueness at 72 hpf was 211 mg/L.

Discussion

Although the concentrations of PFCs in surface water mostly ranged from several nanograms per liter to several hundreds of nanograms per liter, the level of PFCs in the area of manufacture can be very high. The total PFCs concentrations in surface water samples of discharge following perfluorinated material ranged from <10 to 17,000 μg/L (Moody et al. 2002). A bioaccumulation factor (BAF) range of 6300–125000 was calculated for PFOS based on concentrations in fish liver and surface water (Moody et al. 2002). The concentrations in human blood can even get up to hundreds of milligrams per milliliter (Bossi et al. 2005). So it was significative to study PFCs in milligrams per milliliter range. Zebrafish is considered as a model organism for the study of teratogens in vertebrates (Nagel 2002; Yang et al. 2009). Thus, for comparative purposes the concentrations studied in the study with zebrafish were appropriate.

All the PFCs caused significant effects of development in *in ovo* experiments, upon which we report here. The physical and chemical properties of PFCs determined that they can be biomagnified (Loi et al. 2011). Assuming that concentrations in our *in ovo* experiments can be compared with maximal estimated blood concentrations in humans (Bossi et al. 2005), *in ovo* developmental effects by PFCs would not be expected at current concentrations in the environment.

Generally, the order of potency based on the LC_{50} value at 72 hpf was PFOS > FC807 > PFNA > PFOA. The length of the perfluorinated tail of PFC molecules has been identified as an important factor in determining toxicity. Based on

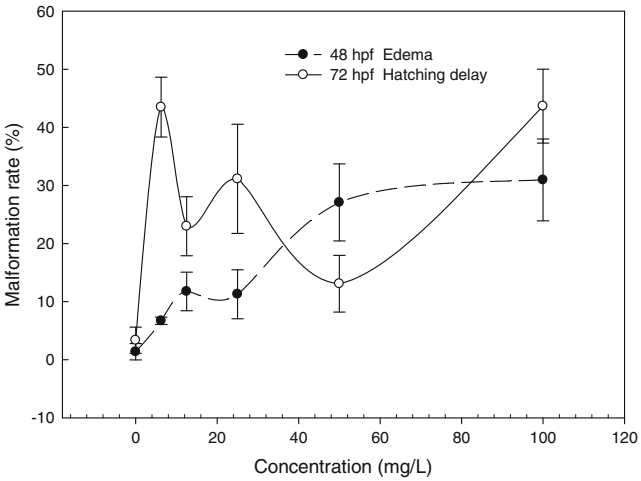

Fig. 2 A 48-hpf edema malformation and 72-hpf hatching delay caused by PFOS

Table 3 EC_{50}/LC_{50} of toxicological endpoints of zebrafish embryo result from PFOS

Toxicological endpoints	EC_{50}/LC_{50} (mg/L)	95 % confidence interval (mg/L)
4 hpf opaque	182	143–191
4 hpf abnormal	113	102–126
8 hpf opaque	121	98–160
8 hpf gastrula not start	76	56–113
12 hpf opaque	86	68–112
24 hpf opaque	69	52–98
48 hpf opaque	68	49–101
72 hpf opaque	68	49–101
72 hpf malformation	37	31–44

Table 4 EC_{50}/LC_{50} of toxicological endpoints of zebrafish embryo result from FC807

Toxicological endpoints	EC_{50}/LC_{50} (mg/L)	95 % confidence interval (mg/L)
48 hpf edema	34	32–70
72 hpf opaque	211	261–263

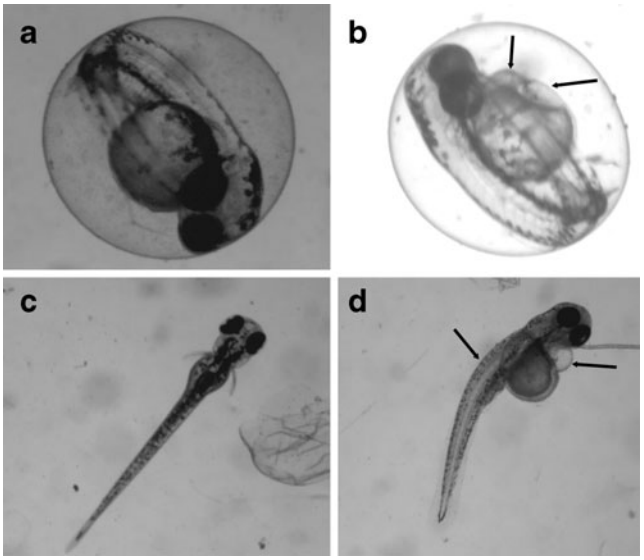

Fig. 3 Photomicrographs of embryos. **a** Normal embryo at 48 hpf. **b** Embryo with pericardial-sac edema and yolk-sac edema at 48 hpf (*arrows*). **c** Normal larva at 72 hpf. **d** Embryo with spine crooked malformation and pericardial-sac edema (*arrows*)

LC_{50}, EC_{50}, NOEC and LOEC values, PFCs with eight carbons in the PFC tail tend to be more toxic than those with four carbons (Hagenaars et al. 2011; Qin et al. 2010). Hydrophobic properties of PFCs may illustrate this phenomenon well because molecules with longer carbon chains tend to be more hydrophobic than the shorter one. In this study, PFNA, which has one more carbon than PFOA, was more toxic than PFOA.

PFOS is generally more toxic than PFOA (Ye et al. 2007; Hagenaars et al. 2011). In those studies, PFOS was more potent than PFOA and even PFCs with nine carbons in the perfluorinated tail. FC807 with two sulfonate groups was more toxic than PFOA or PFNA. Hagenaars et al. reported that PFCs with a sulfonate group have a larger toxic potential than those with a carboxyl group (Hagenaars et al. 2011). Therefore, the results of this study were consistent with those of the previously conducted studies.

FC807, which contains two PFOS precursor molecules and a phosphoric acid molecule, has been confirmed as a precursor of PFOS. There were no significant differences in the amount of edema between treated and control groups within 48 hpf, but the rates increased significantly from 48 h and embryos became opaque at 72 hpf. Although both PFOS and FC807 have sulfur element, they seemed to have different mechanisms of action. Lethality was the main toxicity of PFOS while pericardial edema and yolk-sac edema were the most severe malformation caused by FC807 (Fig. 3). It was reported that many biochemical and molecular mechanisms occur among cell, tissues and organs during embryogenesis, and a great number of pollutants could specifically influence these mechanisms (Fraysse et al. 2006). Pericardial edema was often considered as the result of heart failure or circulatory failure (Fraysse et al. 2006; Merrill 1946), so FC807 seemed to hurt heart functions significantly.

PFCs especially FC807 can also result in yolk-sac edema at 48 hpf (Fig. 3). Edema rates of PFOS, PFOA and PFNA did not follow a concentration–response relationship, but there was a standard concentration–response relationship in the effect of FC807 (data not shown). In freshwater fishes, there is a water barrier around the membrane to maintain the intracellular hyperosmotic fluids compared to the surrounding water, so a barrier must be maintained in order to minimize water entry and excrete excess water (Hill et al. 2004). FC807 might have the similar effects as TCDD to produce defects in kidney development and/or function and disturb this water permeability barrier and let water in to cells to cause yolk-sac and pericardial edemas (Hill et al. 2004). In this study, FC807 tended to cause more severe edemas than the smaller PFCs. PFOS, PFOA and FC807 can result in spine crooked malformation in hatched larva in a concentration-dependent manner (Fig. 4). Embryos exposed to PFOS showed the most severe acute toxicity that was in accordance with other reports (Hagenaars et al. 2011; Shi et al. 2008).

Conclusions

PFOA, PFNA, PFOS and FC807 were all toxic to zebrafish embryo. All of lethality, 48-hpf edemas and 72-hpf spine crooked malformations occurred throughout the duration of the study. Based on the LC_{50} value, PFOS was the most potent of the four PFCs, and PFNA was more potent than PFOA. Although all the PFCs tested caused malformations, FC807, with the larger

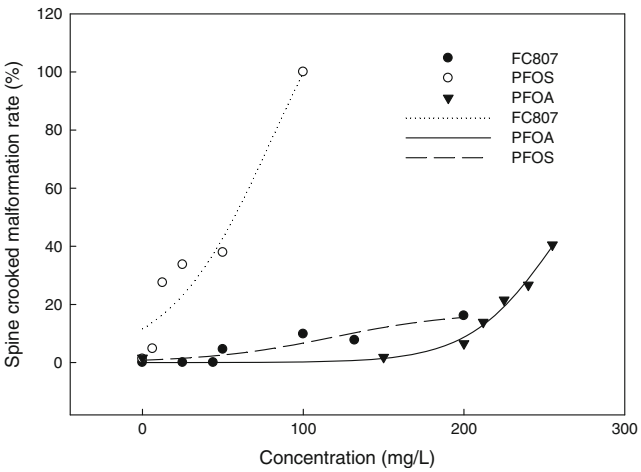

Fig. 4 Rate of spine crooked malformation at 72 hpf for PFOA, PFOS and FC807, and curves based on logistic curve

ester molecule, caused more yolk-sac and pericardial-sac edemas than other PFCs. FC807 might more easily disturb the water barrier around the embryos and disturb heart functions to cause edemas.

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