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Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctanesulphonicacid (PFOS)

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Abstract

Perfluorooctanesulphonicacid (PFOS), a persistent organic contaminant, has been widely detected in the environment, wildlife and humans, but few studies have assessed its effect on aquatic organisms. The present study evaluated the effect of PFOS on zebrafish embryos. Zebrafish embryos exhibited bent spine and developmental toxicity after exposure to various PFOS concentrations (0.01-16.0 μM) from 6 to 120 hour post-fertilization (hpf). The LC_{50} at 120 hpf was 4.39 μM and the EC_{50} at 120 hpf was 2.23 μM. PFOS induced apoptosis at 24 hpf was consistently located in the brain, eye, and tail region of embryos. PFOS elevated the basal rate of swimming after 4 days of exposure, and larvae exposed to PFOS (0.5-8.0μM) for only 1 h at 6 dpf swam faster with increasing PFOS concentration. Larvae exposed to 16.0 μM PFOS for 24 h periods from 1 to 121 hpf showed the highest incidence of malformations in the 97-121 hpf window. Continuous exposure to PFOS from 1 to 121 hpf resulted in a steady accumulation with no evidence of elimination. Our results further the understanding of the health risks of PFOS to aquatic organisms and identify additional research needed on PFOS toxicology.

Keywords

zebrafish embryo; PFOS; toxicity; behavior; window; uptake

1. Introduction

Perfluorinated chemicals (PFCs), as a newly recognized class of persistent organic contaminants, have received increasing attention because of their extensive use in surfactants, lubricants, adhesives, refrigerants, paper coating, fire retardants, propellants, agrochemicals and medicines (Key et al., 1997; Kissa, 2001; Renner, 2001). Recent studies demonstrate that they are ubiquitous contaminants in the environment, wildlife, and humans (see Houde et al., 2006 for review). Perfluorooctanesulphonicacid (PFOS), an end product of the breakdown of many other PFCs, can enter soil, water and atmosphere and

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bioaccumulate in the food chain. PFOS has also been widely detected in the environment, wildlife and humans (e.g., Giesy and Kannan, 2001; Lien et al., 2008; So et al., 2006; Yeung et al., 2006) and its contamination is regarded as a global environmental problem.

The toxicology of PFOS is reported to increase liver triglyceride and cholesterol levels, decrease serum cholesterols, cause hypolipidemia, and reduce food consumption in cynomolgus monkeys (Butenhoff et al., 2002; Seacat et al., 2002). Additionally, PFOS caused development toxicity, hepatotoxicity, immunotoxicity or neurotoxicity effects in zebrafish (Du et al., 2009; Shi et al., 2008), rats or mice (Fuentes et al., 2007a; Johansson et al., 2009; Keil et al., 2008; Lau et al., 2003; Peden-Adams et al., 2008; Thibodeaux et al., 2003), and other aquatic species such as common carp (Hagenaars et al., 2008; Hoff et al., 2003), *Paramecium caudatum* (Kawamoto et al., 2008), and invertebrates and aquatic model fish medaka (Ji et al., 2008). Some of the processes affected by these toxicities are fatty acid transfer and metabolism, integrity of cell membrane, mitochondrial function, liver glutathione peroxidase and superoxide dismutase activity, and reduction of thyroid hormone levels (see Lau et al., 2004, 2007 for reviews).

Zebrafish is an appropriate vertebrate model for investigating developmental toxicity of compounds like PFOS. Zebrafish embryos exposed to 1-5 mg/l PFOS exhibit spinal curvature, anemia, impaired swim bladder inflation, reduced hatching rates, and decreased blood flow and body length (Shi et al., 2008). A few studies have evaluated behavior of PFOS exposed animals (Fuentes et al., 2007b; Kawamoto et al., 2008), while no PFOS behavioral studies in zebrafish have been reported.

In the present study, we determined the PFOS induced malformation and behavioral responses following acute and subacute exposures. The eye and vascular patterning were examined using microscopy, and tissue-specific apoptosis was examined. We report that PFOS exposure leads to vascular patterning malformations and behavioral deficits. We also quantitatively evaluated uptake of PFOS during development.

2. Materials and methods

2.1. Fish husbandry and embryo collection

For the microscopic examination of vascular patterning, we used transgenic Tg (fli1: EGFP) (“fli-1”) zebrafish, which were obtained from Dr. Brant Weinstein of the National Institute of Child Health and Human Development. In all other experiments we used wild-type (AB strain) zebrafish. All fish were raised and kept at standard laboratory conditions of 28 °C on a 14:10 dark/light photoperiod in a recirculation system according to standard zebrafish breeding protocols (Westerfield, 1995). Water supplied to the system was filtered by reverse osmosis (pH 7.0-7.5), and Instant Ocean® salt was added to the water to raise the conductivity to 450-1000 μS/cm (system water). The fish were fed three times daily with either the zebrafish diet (Zeigler, Aquatic Habitats, Apopka Florida) or live artemia (Jiahong Feed Co., Tianjin, China). Zebrafish embryos were obtained from spawning adults in tanks overnight with the sex ratio of 1:1. Spawning was induced in the morning when the light was turned on. Embryos were collected within 0.5 h of spawning and rinsed in embryo

medium (EM; Westerfield, 1995). Fertilized and normal embryos were staged under a stereomicroscope (Nikon, Japan) according to the descriptions of Kimmel et al. (1995).

2.2. PFOS stock solutions and exposure protocols

Perfluorooctanesulphonicacid (PFOS; CAS # 1763-23-1, purity >96%) was purchased from Sigma-Aldrich Chemical (St Louis, MO, USA) and PFOS stock solutions of 64 mM were prepared by dissolving it in 100% dimethyl sulfoxide (DMSO). A serial dilution was made in 100% DMSO that is 1000 times more concentrated to allow for a 1:1000 fold dilution with the EM to create a serial dilution with a final DMSO concentration of 0.1%. The control also received 0.1% DMSO (v/v in EM).

In order to determine the LC_{50}, zebrafish embryos were exposed to 0, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 μM PFOS (0, 0.25, 0.50, 1.00, 2.00, 4.00, and 8.00 mg/l, respectively) in 96-well plates from 6 to 120 h post-fertilization (hpf). The range of concentrations was selected based on our initial range-finding studies which were conducted in order to roughly estimate the LC_{50} in zebrafish embryos. Embryos were kept in sterile 96-well plates, with one embryo per well, containing 200 μl treatment or control solutions. Sealing films were put on the plates to prevent evaporation. For each exposure, five replicates of 32 embryos each were performed at 28 ± 0.5 °C in light-controlled incubator. To determine the EC_{50}, subacute toxicity 0, 0.01, 0.1, 1.0, 4.0, and 8.0 μM PFOS (0, 0.005, 0.05, 0.50, 2.00, and 4.00 mg/l, respectively) was used. The other operations were conducted the same way as the acute toxicity test. There was no renewal of PFOS during the experiment and the concentrations of PFOS in exposure water and whole-body tissues of zebrafish were measured after exposure at 120 hpf. Developmental parameters were monitored and documented daily between 24 and 120 hpf. The end points of toxicity included bent spine, malformed tail, pericardial edema, yolk sac edema, uninflated swim bladder, failed hatching, single eye, and opaque head (apparent necrosis). Results were plotted as graphs and LC_{50} and EC_{50} were determined.

2.3. Quantification of PFOS in exposure solutions and larval tissues

The PFOS concentrations were measured using combined liquid chromatography (Agilent 1200, American) mass spectrometry (Bruker Esquire HCT ion trap, Germany) (LC/MS) according to Giesy and Kannan (2001) and Saito et al. (2003) with minor modifications. Exposure water samples were taken randomly from 10 wells per treatment shortly after exposure (day 0) and at the end of the exposure tests (day 5), and were stored at 4 °C. Before analysis, the pooled water samples were diluted 1:10 with deionized water, and then vortex mixed for 30 s and passed through a 0.2 μm nylon mesh filter into an auto sampler vial.

For quantification of PFOS in larval tissues, 10 live animals per treatment were taken out from the wells after exposure, rinsed twice with 10 ml of deionized water to remove adherent solution and then transferred into 2.0 ml preweighed polypropylene tubes. Larvae wet weights were determined after blotting excess water with a paper towel. These samples (three replicates of larvae per treatment) were then frozen immediately in liquid nitrogen and stored at −80 °C until analysis. Before the analysis, the pooled zebrafish larvae were homogenized using a pestle in 200 μl deionized water, 600 μl methyl-tert-butyl ether

(MTBE) was added and the homogenate was vortexed for 30 s and centrifuged at 4 °C for 15 min at 3000×g to separate organic layers, aqueous layers, and cell debris. The aqueous mixture was rinsed with MTBE and separated three times. Every time the organic layer was transferred to 2.0 ml polypropylene tube. All rinses were combined in 2.0 ml polypropylene tube. The solvent of MTBE for extraction was selected according to Giesy and Kannan (2001) and allowed to evaporate under nitrogen at 40 °C before being reconstituted in 1 ml methanol. The extract was vortex mixed for 30 s and passed through a 0.2 μm nylon mesh filter into an auto sampler vial.

Sample (10 μl) was injected into a Zorbax SB-C18 column (2.1 mm×150 mm, 5 μm particle size, Agilent, American) with the mobile phase of acetonitrile (A) and 10mM ammonium acetate buffer (B) starting at 5% A. The mobile phase flow rate of 400 μl/min was utilized, and the gradient was increased to 90% A before reverting to the original conditions after 10 min. Column temperature was maintained at 35 °C. The high pressure liquid chromatography system was interfaced to electro spray tandem mass spectrometer operated in the electro spray negative ion mode. The optimized conditions were: nebulizer gas pressure 30 psi, drying nitrogen gas temperature 350 °C, drying nitrogen gas flow 10.0 1/min. The monitored ions were 499 for PFOS ([M−H] −). The calibration curves of PFOS were linear in the concentration range of 1 to 800 ng/ml. Means of two separate determinations were calculated for individual locations. Spike and recovery experiments were performed to determine the precision and accuracy of the instrumental analyses. The percentage extraction recovery of PFOS was measured using deionized water that contained known amounts of PFOS. Three independent replicates of each sample were prepared and analyzed. The concentrations of PFOS were calculated from standard curves and the average extraction efficiency of PFOS ranged from 75% to 80%.

2.4. Uptake kinetics of PFOS in embryos/larvae

Embryos at 1hpf in triplicate for each measured time point were exposed to PFOS (4.0 μM). The time points were as follows: 2, 4, 6, 10, 24, 48, 72, 96, and 120 h. After exposure, 10 embryos of each time point were selected randomly and measured according to the procedure described in Section 2.3.

2.5. Effects of PFOS on heart beat rate and spontaneous movement

To determine whether exposure to PFOS affected heart beat and spontaneous movement, larvae were exposed to PFOS at 0, 0.01, 0.1, 1.0, 4.0, and 8.0 μM beginning at 6 hpf. At 48 and 60 hpf, the larvae were mounted on a microscope slide containing a drop of EM, and heart beat for 10 s on 10 embryos from each concentration (n = 3) were recorded. From 18 to 26 hpf, spontaneous movement over 1 min of 15 embryos from each concentration (n = 3) was filmed. The time from the first to the last well was less than 10 min. All spontaneous movement and heart beat recordings started after adaptation to 27 - 28 °C for 5 min. Video was recorded via a CCD camera (Nikon, Japan) fixed on a dissection microscope.

2.6. Cell apoptosis assay using acridine orange staining

To visualize embryo cell death, control and PFOS (1.0, 4.0, and 8.0 μM) exposed embryos were incubated with acridine orange (Usenko et al., 2007). Embryos after 18 h of exposure

(from 6 to 24 hpf) were dechorionated via enzymatic digestion with 0.5 mg/ml protease E in 50 mm Petri dishes for 8 min at room temperature. Twelve larvae from each treatment were rinsed twice with EM and incubated with 5 μg/ml acridine orange dissolved in EM for 1 h at room temperature in the dark. The embryos were then washed with EM three times for 5 min each. Before examination, the live animals were anesthetized with 0.02% MS-222 for 5 min and mounted laterally on a microscope slide. All embryos were examined with a fluorescence microscope (Nikon, Japan) and representative pictures are shown.

2.7. Behavior assessment

There were two trials in this experiment. In the first trial, zebrafish embryos were exposed to 0, 0.5, 1.0, and 2.0 μM PFOS from 6 to 96 hpf. At 5 day post-fertilization (dpf), morphologically normal larvae were assessed in a ZebraLab behavior monitoring station (ViewPoint Life Sciences, Inc. Montreal, Canada). Larvae were allowed to adapt for 10 min before monitoring the swimming speed and response to dark-to-light transition stimulation. The 24-well plate containing the larvae was kept at a constant temperature of 28 °C via the water jacket circulation feature on the ZebraLab stage. Swimming speed was monitored for 30 min. The larvae were tested by light stimulation at 6dpf in the morning. The lighting parameters were 20 min light followed by a 20 min dark interval, and repeated for 120 min. Each treatment was conducted on at least two separate plates (5 larvae/treatment/plate, 20 larvae/plate), and three replicates were performed.

In the second trial, zebrafish larvae (6 dpf) were exposed to 0, 0.5, 1.0, 2.0, 4.0, and 8.0 μM PFOS for only 1 h in 24-well plates before behavior testing. Each plate also had control solutions. After a 10min adaption, locomotion monitoring was started according to the above method with infrared and visible light on. The data (frequency of movements, distances travelled and total durations of movements) were collected every 60 s and further analyzed using custom Open Office.Org 2.4 software.

2.8. PFOS exposure in fli-1 transgenic zebrafish

To determine if there were sensitivity differences between AB strain and Fli-1 embryos, Fli-1 embryos at 6 hpf were exposed to PFOS (0, 1.0, 4.0, and 8.0 μM) in 96-well plates. This experiment was replicated three times (n = 72). Mortality and malformation rate at 120hpf were analyzed. To determine if PFOS affected vascular patterning of Fli-1 fish, live larvae were removed at 96 hpf, anesthetized and fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline for 24 h at 4 °C. Larvae were then rinsed twice with 0.1 M phosphate-buffered saline and processed for microscopic analysis with a fluorescence microscope (Nikon, Japan) one or two days later. These digital images were acquired in the eye and the trunk region over the yolk sac extension.

2.9. Temporal window test

To test which phase of embryogenesis is most sensitive to PFOS, zebrafish embryos were exposed to PFOS for 24 h periods from 1 to 121 hpf (1-25, 25-49, 49-73, 73-97, and 97-121 hpf). Preliminary studies examined a range of PFOS concentrations in order to identify the concentration that would induce effects, and 16 μM was selected. At the end of each 24 h exposure period, embryos were rinsed twice, placed into EM in new plates and maintained

until 192 hpf. Malformed larvae were counted daily and the swimming speeds were determined at 6dpf. Concentrations of PFOS in embryos were also measured at the end of each 24 h exposure period using LC/MS as described in Section 2.3.

2.10. Data analysis

The sigmoidal fit for concentration–response curves which were required to determine EC50 and LC50 values was completed using origin 8.0 (OriginLab, Northampton, MA, USA). One-way ANOVA was applied to calculate statistical significance followed by Dunnett's test as a post hoc test to independently compare each exposure group to the control group. The LSD test was used as a post hoc test for multiple comparisons between groups with a P value of 0.05 for significance using SPSS 16.0 (SPSS, Chicago, IL, USA). All the data were reported as means ± standard error (SEM).

3. Results

3.1. Malformation types and concentration response curve for PFOS

Embryos were exposed to various concentrations of PFOS from 6 to 120 hpf and were monitored daily for mortality and malformation until 120 hpf. The vehicle control (0.1% DMSO) was not toxic to embryos. Malformations such as bent spine (mainly cyphosis and scoliosis), malformed tail, uninflated swim bladder, inability to stay righted, pericardial edema, yolk sac edema, and necrosis in the brain and muscle tissue were observed with increasing PFOS concentrations (Fig. 1). A lateral view of cyphosis and dorsal view of scoliosis are shown in Fig. 1c and Fig. 1d. Total observed cumulative malformations displayed concentration-dependent effects and several kinds of malformations often appeared together. The rank order of sensitivity was: bent spine > malformed tail > uninflated swim bladder and reduced ability to keep balance > pericardial and yolk sac edema, reduction of pigment, single eye. However, PFOS did not appear to affect spine morphogenesis, and uninflated swim bladder was not observed until approximately 96 hpf (data not shown).

No difference in hatch rate was observed between the control and 8μM PFOS group at 48 hpf. At 60 hpf, there was a concentration-dependent decrease in the hatching rate of the 8μM PFOS treatment group (73.8 ± 4.0%) compared to the control (88.8 ± 4.5%; $P < 0.05$). By 72 hpf, larvae in each group were nearly all hatched with no significant difference observed between PFOS and control.

The LC50 of PFOS at 120 hpf was calculated to be 4.39 μM (Fig. 2A). Concentrations of PFOS at or above 2.0 μM were lethal to embryos (defined as an opaque egg or the absence of a heartbeat) while embryos exposed to 0.5 and 1.0 μM PFOS showed no increased mortality over the exposure time (data not shown). The calculated EC50 at 120 hpf was 2.23 μM (Fig. 2B).

3.2. Quantification of PFOS in exposure water and larval tissues

To determine the static bath PFOS concentration and the larval uptake, samples were collected for PFOS measurement shortly after exposure (day 0) and at the end of the

experiment (day 5). The PFOS in exposure water at day 5 was slightly higher than the original solutions (day 0) (Table 1). This was likely due to evaporation of the water in the well. Thereafter, the plate covers were sealed with parafilm to avoid the evaporation.

Levels of PFOS (ng/g wet weight or ng/fish) in whole-body tissue homogenates of larvae (day 5) exposed to PFOS increased with exposure concentrations (Table 1). Larvae exposed to PFOS at 1.0, 4.0, and 8.0 μM (0.50, 2.00, and 4.00 mg/l, respectively) accumulated PFOS at concentrations of 15.2 ± 2.4, 52.8 ± 2.1, and 66.1 ± 2.4 ng/embryo, respectively.

3.3. Uptake kinetics of PFOS in embryos/larvae

PFOS at 4.0 μM (2.00 mg/l) was accumulated in larvae during the entire course of exposure (Fig. 3). PFOS penetrated the chorion and reached the embryo within 2 h (0.9 ± 0.4 ng/embryo), followed by a slow absorption up to 24 h (4.6 ± 0.5 ng/embryo). A relatively rapid absorption occurred over the next 24 h (12.3 ± 1.4 ng/embryo). Between 48 and 120 h the uptake of PFOS continued more rapidly and larvae accumulated 53.7 ± 2.6 ng/embryo after exposure for 120 h (Fig. 3).

3.4. PFOS decreased heart rate and affected spontaneous movement

Heart rate over a 10s period in 48 and 60 hpf PFOS exposed embryos decreased significantly compared to controls (Fig. 4). At 48 hpf, exposure to 1, 4, and 8 μM PFOS resulted in 25.9 ± 0.2, 24.7 ± 0.3, and 23.7 ± 0.3 beats /10 s, respectively, compared with 28.0 ± 0.4 beats /10 s in control ($P < 0.001$). At 60 hpf, heart rates in the 1, 4, and 8 μM PFOS group were also significantly inhibited having 31.5 ± 0.3, 31.1 ± 0.6, and 30.3 ± 0.3 beats/10 s, respectively, compared with 34.5 ± 0.3 in control ($P < 0.001$).

The tail bend frequency of control embryos averaged 8.87 ± 0.53 bends/min at 18 hpf, declining to 3.96 ± 0.29 bends/min at 25 hpf (Fig. 5). By comparison, the 8.0 μM PFOS group did not reach its peak frequency of 7.88 ± 0.54 bends/ min until 19 hpf, and this frequency was significantly lower than the control frequency ($P<0.05$; Fig. 5). We also found that PFOS may have had a prolonged stimulating effect on tail bending as 5.43 ± 0.45 bends/min were observed in 8.0μM PFOS exposed embryos at 25 hpf and this was significantly higher than the average control frequency at 25 hpf ($P<0.05$; Fig. 5).

3.5. PFOS induced cellular death

Cellular death assays were performed to determine if exposure to PFOS affected cellular death in specific cells or tissues. Dechorionated embryos exposed from 6 to 24 hpf were exposed to acridine orange for 1 h. Only a few apoptotic cells were observed in the control larvae or the 1.0 μM PFOS group, but considerable numbers of apoptotic cells were consistently found in the brain, eye, and tail region of embryos treated with 4.0 or 8.0 μM PFOS (Fig. 6)

3.6. PFOS affected larval behavior

The average swimming speed of 5 dpf larvae exposed to 1.0 μM PFOS (2.24 ± 0.11 mm/s) from 6 to 96 hpf was faster than the controls (1.91 ± 0.09 mm/s; $P<0.05$; Fig. 7A). Larvae exposed to increasing PFOS concentrations for only 1 h at 6 dpf swam significantly faster at

8μM PFOS (1.75 ± 0.13 mm/s) than in the controls (1.44 ± 0.12 mm/s; P<0.05; Fig. 7B). In the light stimulation test a rapid transition from light-to-dark resulted in a similar, brief burst of swimming in both exposed and control larvae (Fig. 8). Consistent with the data in Fig. 7, longer duration of 1uM PFOS exposures elicited a higher basal swim rate than both the control or 0.5 and 2.0μM PFOS (Fig. 8).

3.7. PFOS altered vascular patterning of blood vessels in Fli-1 transgenic zebrafish

To determine if PFOS induced malformations were related to vascular defects, we observed the vascular structure in fli-1 transgenic larvae. Larvae with PFOS induced malformations typically exhibited dorsal-ventral trunk axis vascular defects (Fig. 9). Vascular defects in the eyes were more strongly manifested as fusion, absence, and faintness (Fig. 10). Eye vascular defects were significantly more prevalent than defects of blood vessels in the trunk (P<0.05; Fig. 11).

3.8. Temporal window test

To determine if zebrafish were differentially sensitive to PFOS during development, embryos were exposed to PFOS (16.0 μM) for 24 h periods from 1 to 121 hpf and subsequently rinsed and raised in EM. Larvae exposed for 24 h showed an increase in malformations with no reversal through 192hpf for all groups (Fig. 12A). Malformations were most pronounced in embryos exposed from 97 to 121 hpf (P < 0.001). For the 97-121 hpf exposure window, larvae developed a curved spine (mainly cyphosis) and uninflated swim bladder (Fig. 12D). All abnormal larvae had an opaque head (apparent necrosis) (Fig. 12D). Average swim speed at 6dpf from the window test indicated that PFOS exposure from 97-121 hpf resulted in significantly slower speed than did exposure from 25-49 hpf or 49-73 hpf (P < 0.05; Fig. 12B). Measurement of tissue levels of PFOS at the end of each 24 h exposure period using LC/MS indicates the uptake of PFOS (16.0 μM) in zebrafish embryos during each 24 h period (Fig. 12C). The uptake of PFOS was the greatest between 97 and 121 hpf (P<0.001), which is consistent with the prevalence of malformations during this window. We also analyzed the concentrations of PFOS in EM in the recover duration and there were no elimination (data not shown).

4. Discussion

PFOS is a mid-level toxin according to the WHO classification criteria for acute toxicity of compounds. In the present study, the various PFOS induced malformations including bent spine (mainly cyphosis and scoliosis), uninflated swim bladder, and decreased hatching rates are in agreement with the previous findings (Shi et al., 2008), although the parameters are slightly dissimilar. For example, embryos in all treatment groups in the present study hatched at 72 hpf (P > 0.05), while Shi et al. (2008) reported a significant dose-dependent decrease in the hatching rate of the PFOS-treated groups (1, 3, and 5 mg/l) compared with the hatching rate of the control group at 72 hpf (P < 0.05). Differences between studies could be related to different starting exposure time and container. In the current study, 96-well plates were utilized for a more rapid approach to screening. The calculated LC_{50} of PFOS in this study was 4.39 μM (2.20 mg/l), much lower than the LC_{50} value of 17.95 mg/l in water flea (Ji et al., 2008).

Likely no single mechanism can account for the PFOS malformations, but hypotheses have been proposed. Cheng et al. (2000) attributed the spinal deformities to a reduction in both myosin and myotome formation necessary for the healthy musculoskeletal system development. We hypothesize that the bent spine and tail may relate to the muscle or skeleton as we have observed disorganized, broken, and loosen array muscle fibers (data not shown), however, this needs to be confirmed by further investigation.

Although PFOS is mostly found in the environment with low concentrations over a range of 0.1~100 ng/l, concentrations up to 600 ng/l have been reported in the Tennessee River downstream of a fluorochemical manufacturing facility (Hansen et al., 2002). The total PFCs downstream of spills can range up to 17000 μg/l (Moody et al., 2002). No studies have reported a PFOS metabolite, and its elimination by organisms has not been characterized. But PFOS does bioaccumulate to high concentrations in upper trophic levels (e.g. 3680 ng/g wet liver weight in mink) (Giesy and Kannan, 2001). PFOS has been shown to accumulate in wildlife by binding to proteins in the blood and liver tissues (Giesy and Kannan, 2001). In rats, PFOS is not metabolized, but enters the liver and remains there (Johnson et al., 1984). This may be due to the stability of the carbon–fluorine bond and the high electro negativity of perfluorinated alkyl acids. Seacat et al. (2002) reported an elimination half-life of 200 days for PFOS in male and female cynomolgus monkeys following 6 months of daily oral dosing with 0.75 mg/kg PFOS. Humans also appear to have a long half-life (5.4 years) in serum elimination of PFOS (Olsen et al., 2007). Thus, high concentrations used in exposures are important for us to determine the possible toxic mechanisms (Lema et al., 2007). So the high concentrations of PFOS used herein allowed us to characterize PFOS developmental toxicity in a vertebrate model.

Our initial characterization of the absorption kinetics of PFOS indicated a slow initial uptake (likely due to chorion permeation kinetics), then a fairly steady rate of uptake over time, once embryos were out of the chorion. Our preliminary investigation of PFOS elimination suggested that clearance was negligible (data not shown), but a more thorough analytical approach to PFOS pharmacokinetics such as determination of body burden, metabolism and elimination kinetics with both higher and lower, environmentally relevant exposures are needed in future studies.

Rhythmic spontaneous tail bends are the first movements in zebrafish, originate from spinal neuron innervation, and are independent of higher brain inputs (Saint-Amant and Drapeau, 1998). Our data suggest that the onset of normal spontaneous tail bending motion in embryos was slightly but significantly delayed with exposure to PFOS. The peak bending motion frequency appeared one or two hours later than in control embryos and remained at a higher basal rate. This may be due to abnormal muscle development or innervation.

The balance of proliferation and apoptosis is a critical part of normal development and susceptible to perturbation by toxicant exposure (Cole and Ross, 2001; Ahmadi et al., 2003). Increasing PFOS exposure significantly increased apoptosis in the brain, eye and tail region at 24 hpf. It has been reported that PFOS may accumulate in brain in adult rats (Austin et al., 2003). Shi et al. (2008) also found apoptosis occurred in the zebrafish tail area at 84 hpf. Enhanced apoptosis in the tail may partly explain the observed tail malformations in the

present study. Liu et al. (2007) have demonstrated that PFOS is able to produce oxidative stress and induce apoptosis with involvement of caspases in primary cultured tilapia hepatocytes. Shi et al. (2008) showed that genes related to cell apoptosis such as p53 and Bax were both significantly up-regulated upon exposure to PFOS.

Ours is the first study to examine the behavioral effects of PFOS in larval zebrafish. PFOS elicited a higher rate of swimming after 4 days of exposure to 1.0 μM PFOS than was observed at 0, 0.5 or 2.0μM. PFOS at 0.5-8.0μM also elicited an overall higher basal rate of swimming than control after only a 1h exposure. Zebrafish showed a biorhythm (Hurd and Cahill, 2002), which illustrated that larvae become active after exposure to sudden darkness for several minutes and then slow down, as was shown in a previous study (Prober et al., 2006). We also observed this phenomenon in the present study; however, we did not see a differential swim response between PFOS and control larvae following a sudden light-to-dark transition.

While fli-1 transgenic zebrafish are generally a sensitive indicator of vascular abnormalities (Dong et al., 2008), we did not see a robust effect of PFOS on vascular development. The vasculature of the eye was only mildly affected in PFOS exposed animals while the body trunk vasculature was largely normal.

PFOS was developmentally toxic in all windows of development, but the 97-121 hpf window was the most sensitive. We also noted that peak malformation frequency and maximum PFOS uptake resulted from exposure during this same window.

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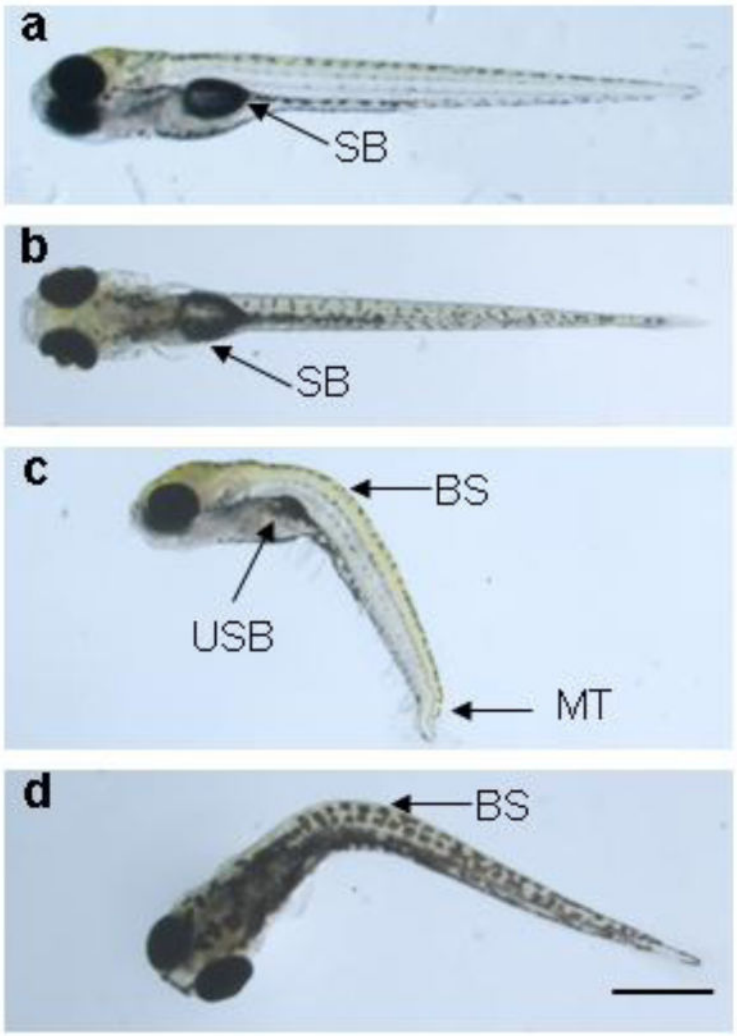

Fig. 1.

The control (a, b) and dominant malformation images of PFOS treated larvae (c, d) at 120 hpf. SB: swim bladder; BS: bent spine;USB: uninflated swim bladder; MT: malformed tail. Scale bars = 0.5 mm.

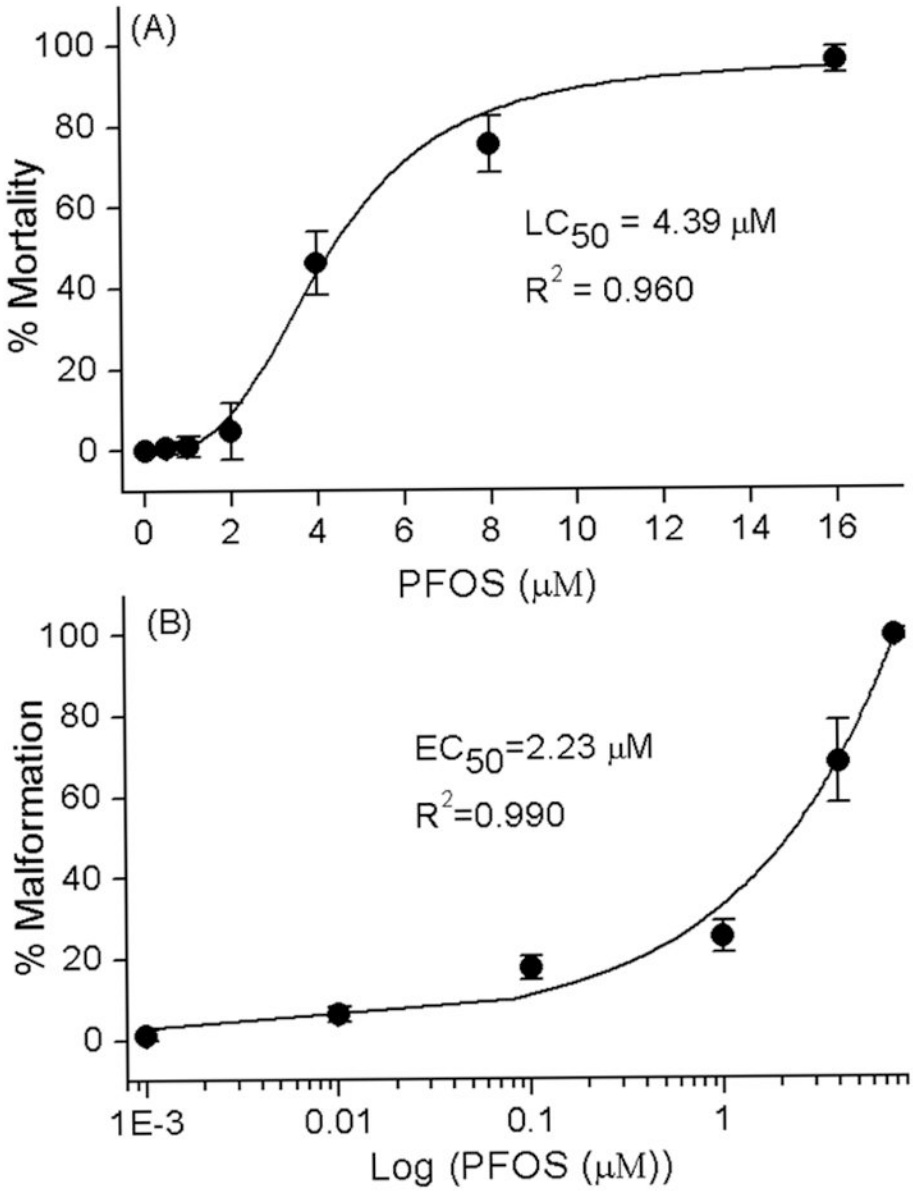

Fig. 2.

Concentration-response curves at 120 hpf for PFOS exposure. Embryos were exposed to various concentrations of PFOS from 6 to 120 hpf.

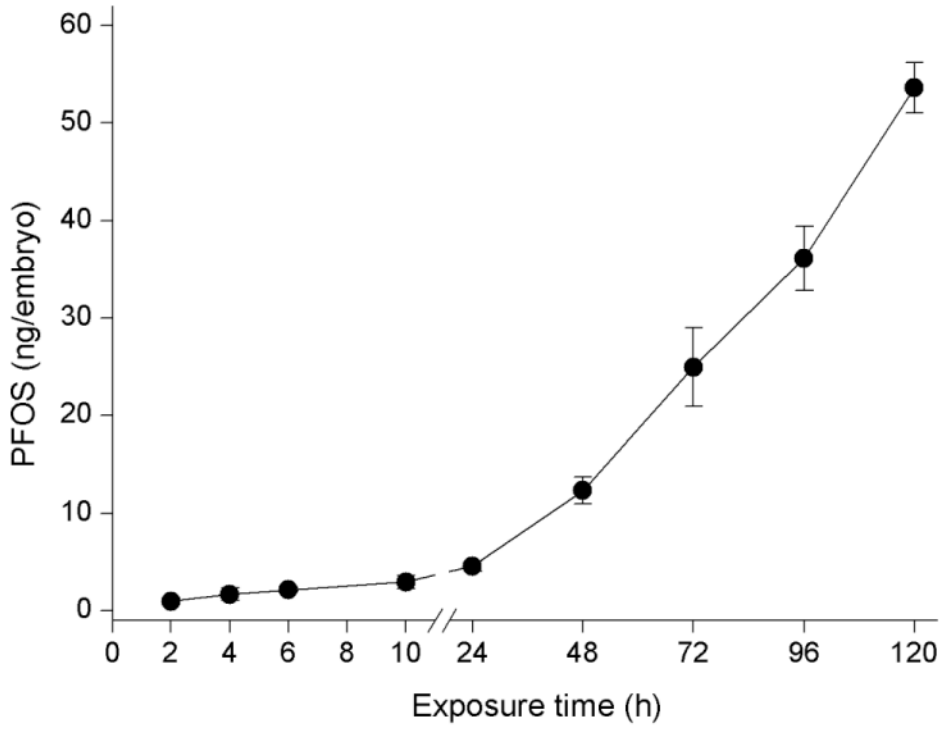

Fig. 3.

Uptake of PFOS (4.0 μM) in zebrafish embryos exposed from 1 to 121 hpf. The values are presented as mean±SEM (n=3).

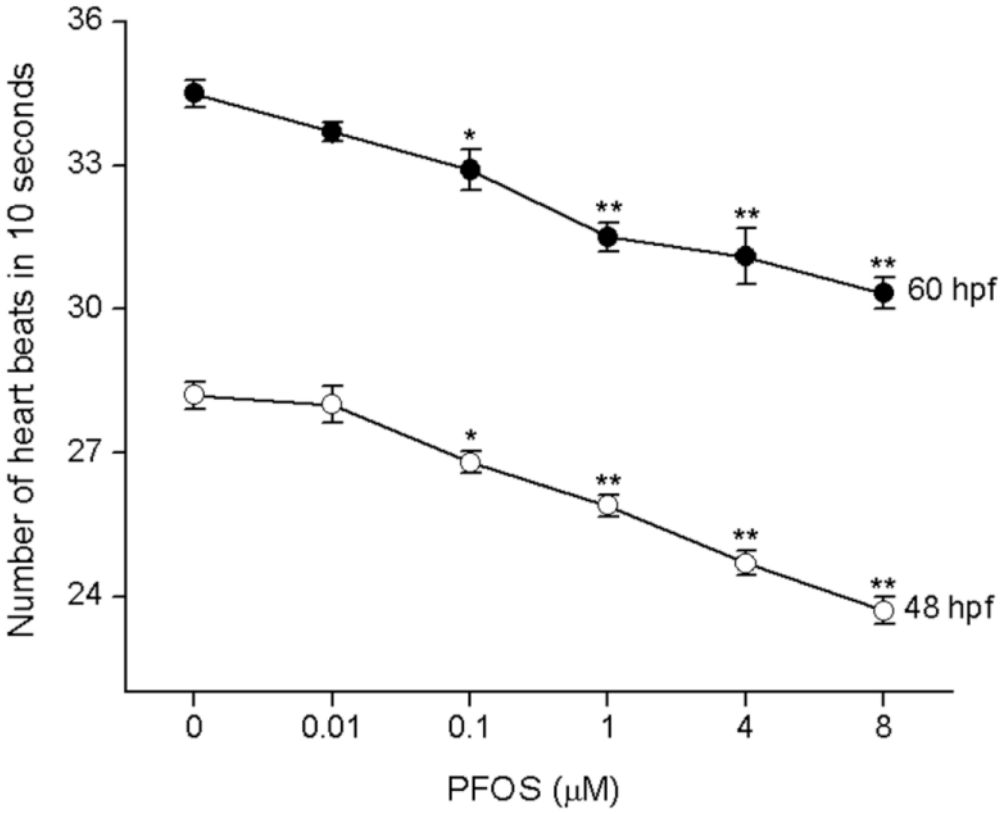

Fig. 4.

The number of heart beats over a 10 second period for zebrafish embryos at 48 and 60 hpf after exposure to PFOS at various concentrations. Asterisks indicate a statistically significant difference from control (*P < 0.05; **P<0.001). Values represent the mean±SEM of three replicates of 10 embryos each.

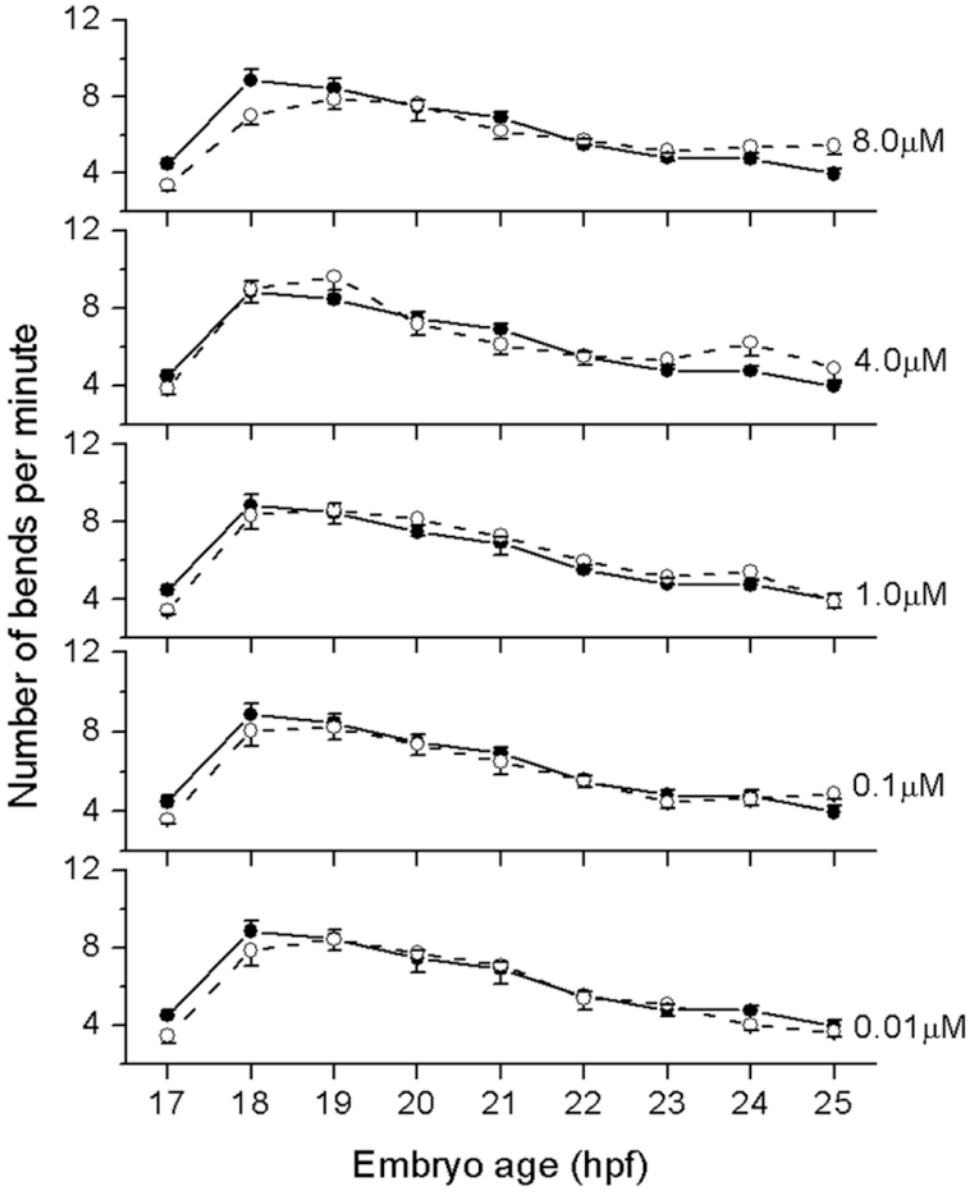

Fig. 5.

Spontaneous movements (number of bends per min) of zebrafish embryos at different times after exposure to PFOS at various concentrations (open circles) vs. controls (filled circles). Each data point represents the average bends per minute of 45 embryos±SEM (15 embryos per treatment with a total of three repeats).

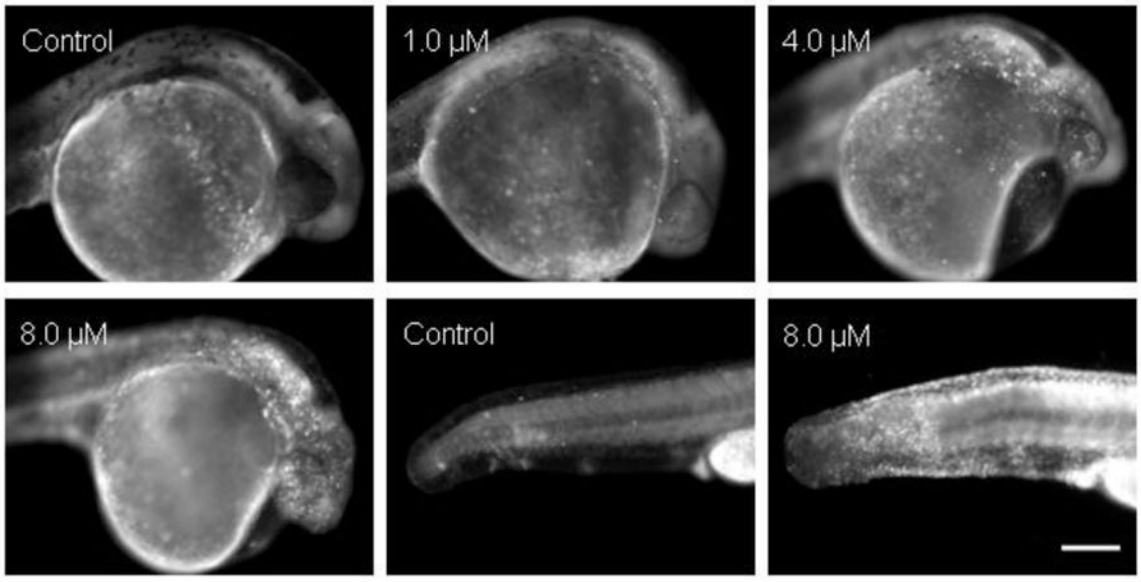

Fig. 6.

Apoptotic cells (bright white) in whole mount embryos at 24 hpf after expose to PFOS at 1.0-8.0 μM vs. controls.

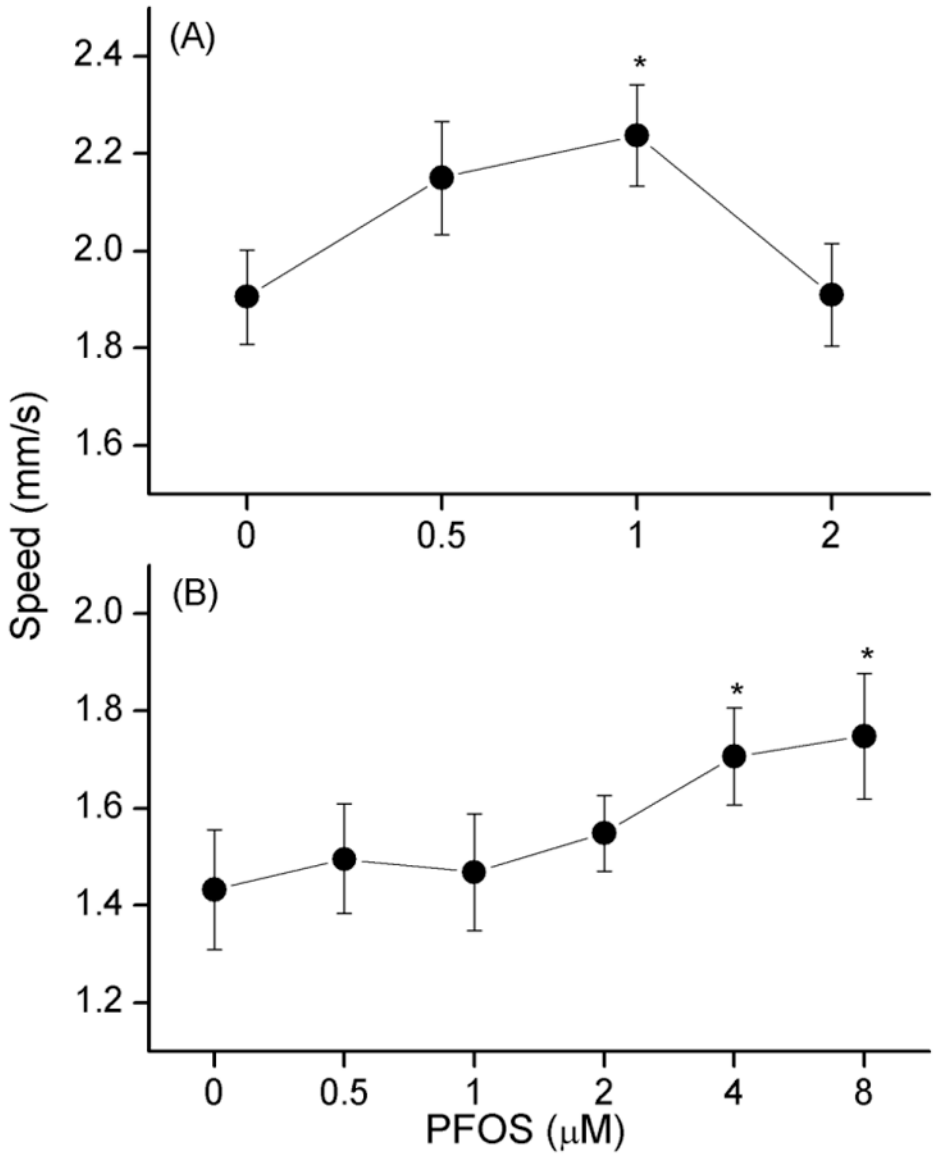

Fig. 7.

The swimming speed of 5 dpf larvae exposed to 0-2.0 μM PFOS from 6-96 hpf (A) or larvae at 6 dpf after 1h exposure to 0 - 8.0 μM PFOS (B). Asterisks indicate a significant difference from control ($P < 0.05$). Values represent the mean speed in 60 s intervals±SEM with three replicates of 8-10 larvae each.

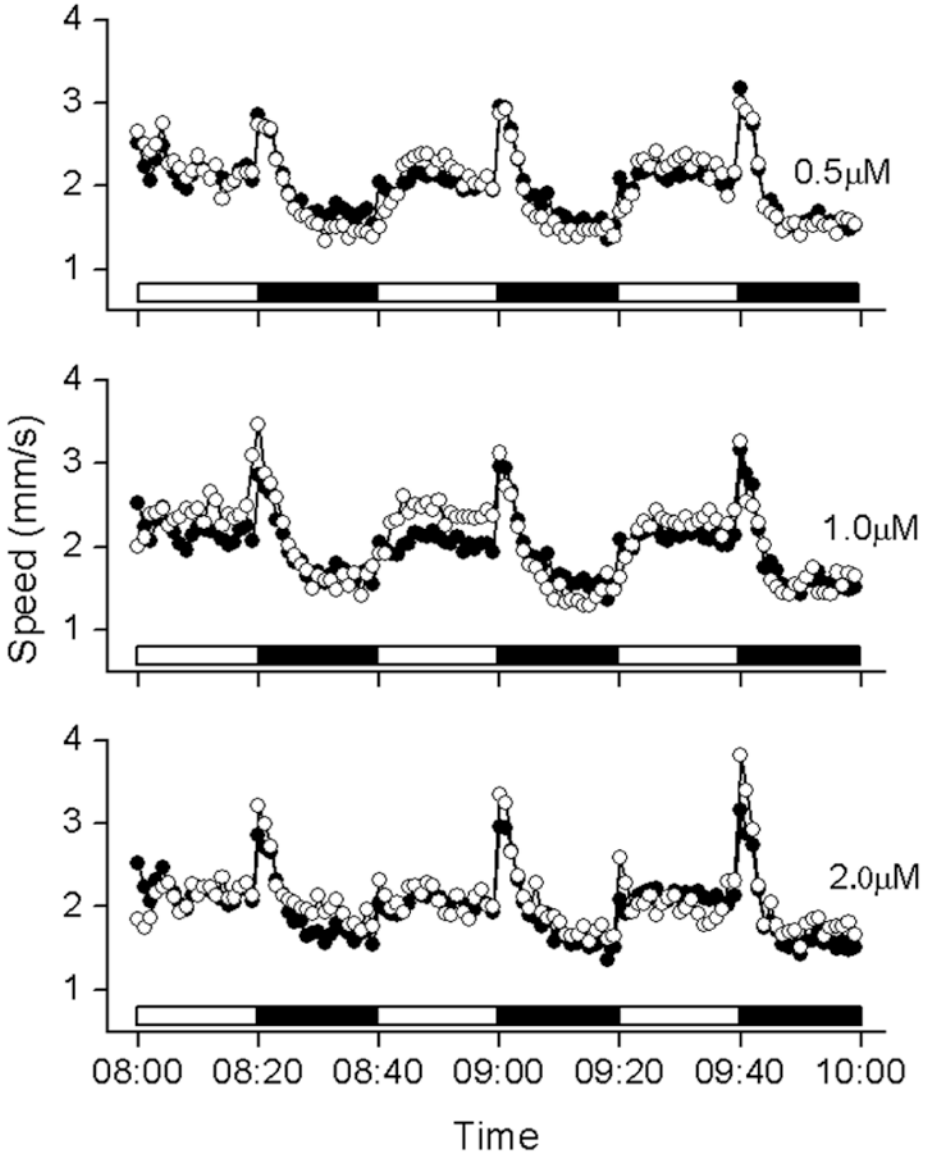

Fig. 8.

The swimming speed of larvae at 6 dpf when subject to light stimulation after exposure to PFOS at concentrations of 0.5-2.0 μM (open circles) from 6 to 96h vs. controls (filled circles). Speed was analyzed for 120 min using 20 min light (visible light) followed by a 20 min interval of dark (infrared light) in 24-well plates.

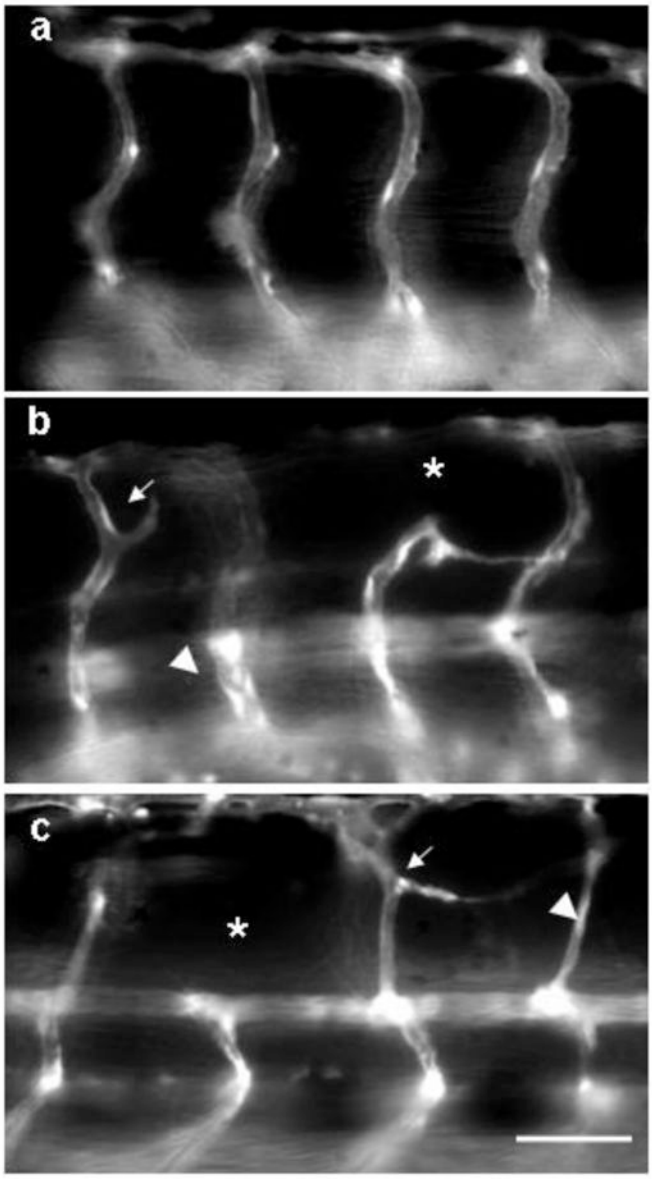

Fig. 9.

Vascular patterning of 96 hpf fli-1 zebrafish larvae in control (a) and treatment groups (b, c) exposed to 1.0-8.0 μM PFOS from 6 to 96 hpf. Aberrant patterning includes crossing (arrows), split or absent (asterisks), wider or thinner (arrowhead) blood vessels. Scale bar = 50 μm.

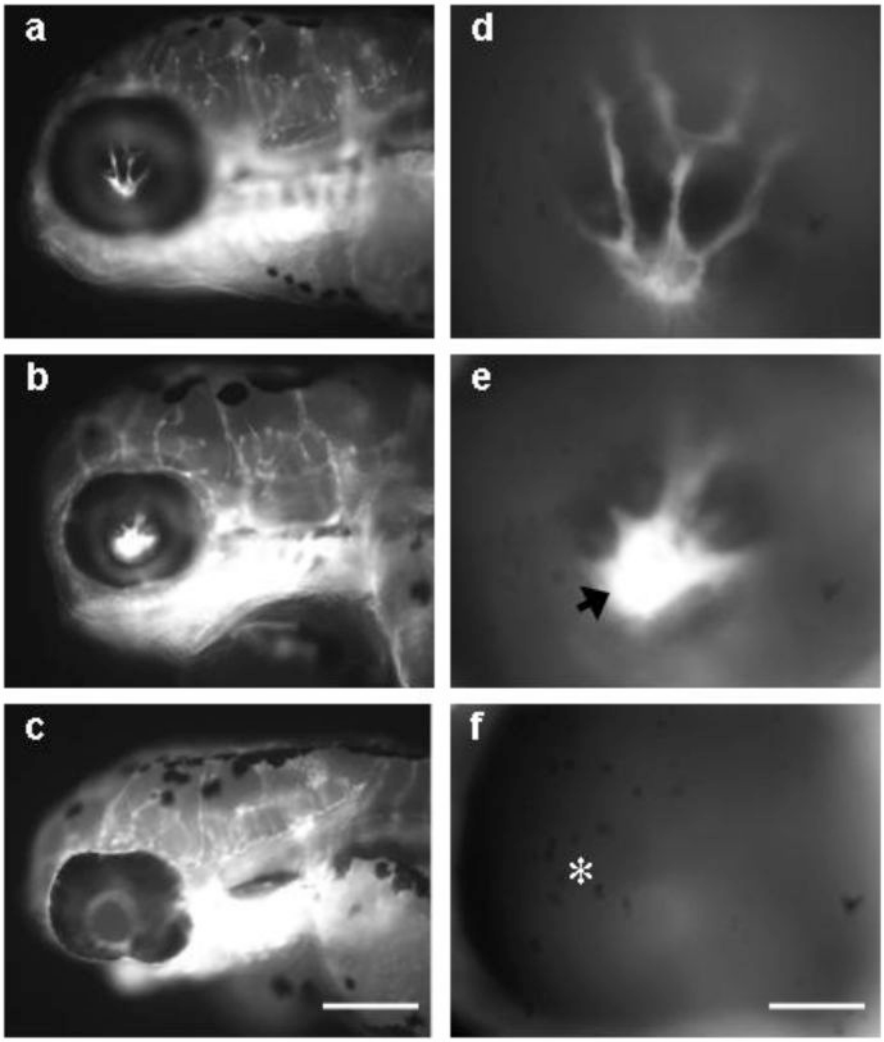

Fig. 10.

Blood vessels in the eyes of 96 hpf fli-1 zebrafish larvae from control (a, d) and treatment groups (b-c, e-f) exposed to 1.0-8.0 μM PFOS from 6 to 96 hpf. Abnormalities include fusion (bold arrows) or absence (asterisks). Scale bars = 200 μm (a–c) and 50 μm (d-f).

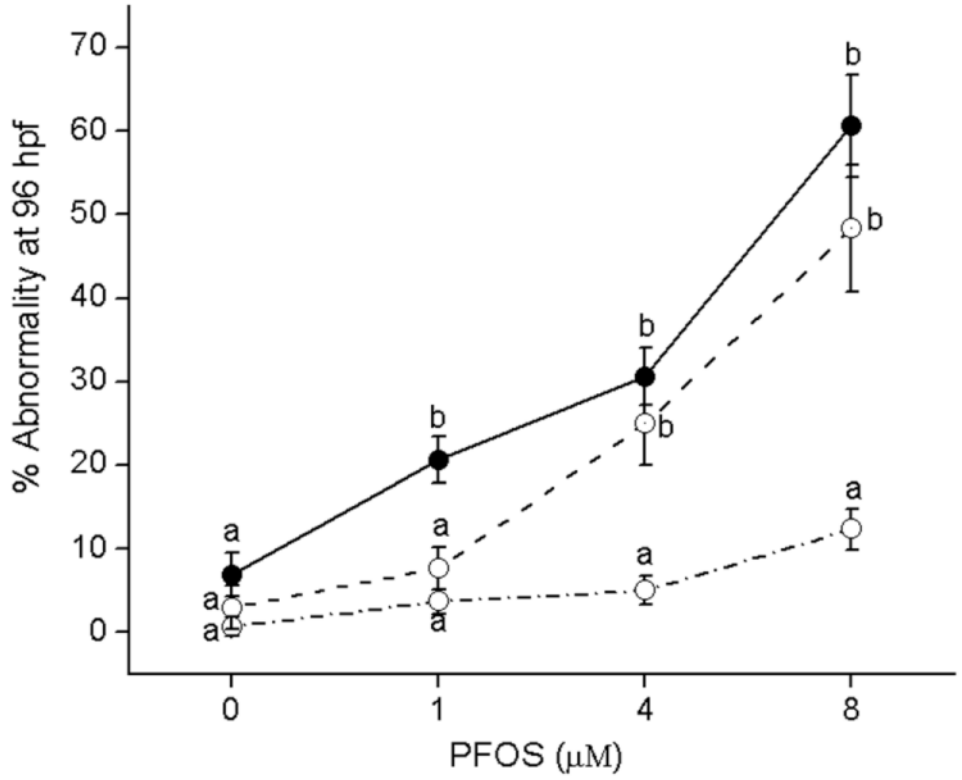

Fig. 11.

Percent of total malformation (solid line), malformation of blood vessels in eyes (dash line) or trunks (dash dot line) of fli-1 zebrafish larvae at 96 hpf after expose to 0-8.0 μM PFOS from 6 to 96 hpf. Dots sharing the same letter at the same concentration indicate no significant difference at P = 0.05. The values are presented as mean±SEM (n=3).

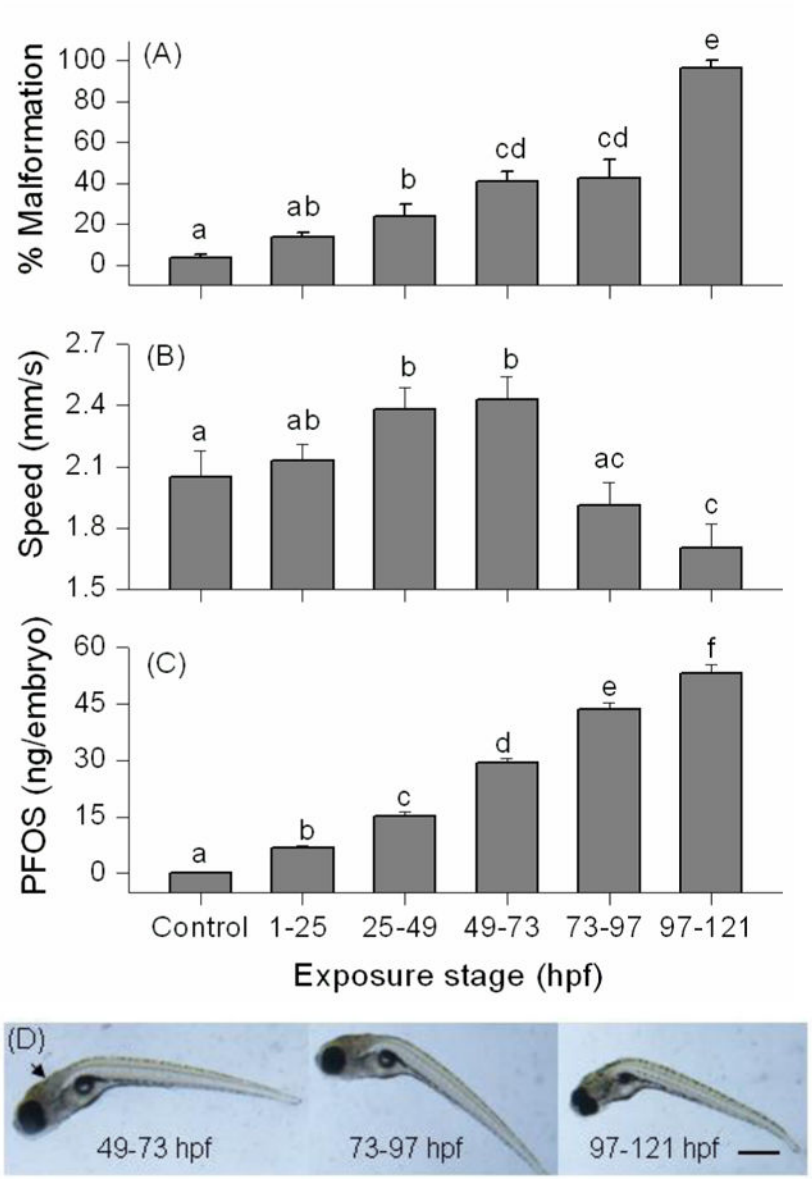

Fig. 12.

Sensitivities of zebrafish embryos exposed to 16.0 μM PFOS continuous for 24 h at different stages. (A) Percent malformation at 192 hpf; (B) Swimming speed of larvae at 6 dpf; (C) Uptake of PFOS at the end of each 24 h exposure period; (D) Typical malformation found at 144 hpf: opaque head of apparent necrosis (arrows), bent spine (cyphosis), and uninflated swim bladder. Bars sharing the same letter indicate no significant difference at P = 0.05. The values are presented as mean±SEM (n=3). Scale bar = 0.5 mm.

Table 1

Concentrations of PFOS in exposure water and whole-body tissues of zebrafish.

	Nominal	PFOS in exposure water (μg/l)[a]		PFOS in tissues (Day 5)	
μM	(μg/l)	Day 0	Day 5	ng/g wet eight	ng/fish
0	0	0.0	0.0	0.0 ± 0.0	0.0 ± 0.0
1.0	500.1	505.1	506.4	7420.0 ± 1123.2	15.2 ± 2.4
4.0	2000.5	2040.3	2160.3	27390.9 ± 234.4	52.8 ± 2.1
8.0	4001.0	4012.3	4222.8	37114.1 ± 1878.1	66.1 ± 2.4

[a]Exposure water samples were measured shortly after exposure (day 0) and at the end of the exposure tests (day 5). There was no renewal of PFOS or medium exchange during the experiment.