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RESEARCH ARTICLE

Comparison of waterborne and in ovo nanoinjection exposures to assess effects of PFOS on zebrafish embryos

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Abstract Since perfluorooctane sulfonate (PFOS) had been detected in eggs of seabirds and fish, toxicity of waterborne PFOS to embryonic development of zebrafish (*Danio rerio*) was investigated. However, because assessment of effects by use of dietary exposure of adults is time-consuming and expensive, a study was conducted to compare effects on embryos via nanoinjection and waterborne exposure. Nanoinjection, in which small amounts of chemicals are injected into developing eggs, was used to incorporate PFOS into the yolk sac of embryos of zebrafish. Effects of PFOS during the period of development of the embryo were assessed within 96 h post-fertilization (hpf). PFOS significantly retarded development of embryos of zebrafish and resulted in abnormalities as well as lethality of developing embryos. Both methods of exposure, waterborne and nanoinjection, resulted in similar dose-dependent effects. Some sublethal effects, such as edema at 48 hpf, delayed hatching, and curvature of the spine was observed after 72 hpf. In ovo, nanoinjection was deemed to be an accurate method of exposure for controlling the actual internal dose for study of adverse effects, which closely mimicked responses to waterborne exposure of zebrafish embryo to PFOS.

Keywords Embryonic development · Fish · Toxicity · Juvenile · Teratogenesis · Perfluorinated

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Introduction

Assessing effects of organic compounds in water to aquatic organisms is complicated by differences in route of exposure (Escher and Hermens 2004). Waterborne exposure is most appropriate to test substances that are soluble in water. Due to characteristics of the chorion and vitelline membranes of fishes, soon after fertilization, water hardening will limit permeability to organic molecules, and thus traditional waterborne exposure of some chemicals does not result in accumulation from water into the embryo (Li et al. 2007; Merle and Romig Eric 1997). Some persistent and bioaccumulative compounds are sparingly soluble in water and embryos are exposed via deposition from the adult female. Thus, it is more difficult to test the effects of compounds of lesser solubility in water, which might not be accumulated directly from water into eggs or embryos but rather be accumulated from the diet and deposited by females into their eggs under field conditions. One approach to accomplish this is referred to as prefertilization exposure, where eggs are exposed to organic chemicals prior to fertilization and subsequent water

hardening. Another approach, which allows for more accurate delivery per egg and also allows for the adjustment of timing of exposures, is nanoinjection, which was developed to allow accurate dosing of compounds with sparing solubility. To overcome the permeability barrier and thus mimic maternal exposure, chemicals can be injected directly into eggs or during early development of embryos (Papoulias et al. 2003; Pastva et al. 2001; Villalobos et al. 2000, 2003a). Nanoinjection has been used as a method to mimic longer term maternal exposures (Nassef et al. 2010). The technique is rapid and does not require feeding of adult fish and, thus, in Canada, obviates the need for institutional approval for use of vertebrate animals.

Perfluorooctane sulfonate (PFOS) is a persistent and ubiquitous environmental pollutant that is a terminal degradation product of a number of chemicals used in pharmaceuticals, cosmetics, paper coatings, fire retardants, and insecticides (Giesy and Kannan 2001; Mommaerts et al. 2011). PFOS is one of the predominant perfluorinated compounds (PFCs) detected in seawater, freshwater, and even drinking water, with concentrations in the range of nanogram per liter in water (Takagi et al. 2011; Theobald et al. 2011; Yang et al. 2011b; Zhu et al. 2011) and nanogram per gram dry mass (dm) in sediments and soils (Wang et al. 2011; Yang et al. 2011a). PFOS is moderately accumulated into animals and is often the predominant PFCs in tissues of wildlife (Giesy and Kannan 2002; Giesy et al. 2010) and can even be detected in blood of humans all over the world (Chan et al. 2011; Dong et al. 2011; Pan et al. 2010; Vassiliadou et al. 2010; Wilhelm et al. 2010; Zhang et al. 2010). Bioaccumulation factors calculated based on concentrations in liver and surface water ranged from 6,300 to 125,000 in the common shiner in a Canadian creek where a spill of firefighting foam had occurred (Moody et al. 2002).

PFOS can cause toxicity to aquatic organisms during development. Acute EC_{50} values for effects of PFOS on growth (cell density) of the phytoplankton *Selenastrum capricornutum* for 96 h, survival of the invertebrate *Mysidopsis bahia*, and survival of the fathead minnow (*Pimephales promelas*) were 48.2, 3.5, and 3.2 mg PFOS/L, respectively (Beach et al. 2006; Giesy et al. 2010). The most sensitive responses were alterations in yolk sac of embryos and pericardial edema in larvae (Mhadhbi et al. 2012). PFOS affected molecular expressions as well as causing deformities and lethality of marine Japanese medaka (*Oryzias melastigma*) (Wu et al. 2012). When zebrafish (*Danio rerio*) fry were exposed to 50 μg PFOS/L for 70 days followed by a further 30 days in clean water, deformations and mortality of F_1 individuals was observed (Du et al. 2009). Exposure to PFOS also caused disturbance of hormones in the hypothalamus–pituitary–thyroid axis in zebrafish larvae (Shi et al. 2010). Fry of zebrafish exposed to PFOS exhibited gross malformations during development, including epiboly deformities, hypopigmentation, edema of the yolk sac, tail and heart, as well as malformations and spinal curvature (Shi et al. 2008; Ye et al. 2007; Zheng et al. 2012).

The relationship among bioaccumulation and bioconcentration of PFOS into fish and the relative importance of dietary and waterborne sources of PFOS have been evaluated previously (Beach et al. 2006). But dietary sources of PFOS are secondary and might not significantly affect overall accumulation of PFOS by fish (Giesy et al. 2010). Therefore, most researchers have used waterborne exposures to study effects of PFOS. However, accumulation of PFOS into eggs from water is limited because of development of an impervious chorion during water hardening shortly after fertilization (Li et al. 2007). Also nominal aqueous concentrations of PFOS are depleted by sorption to the surroundings, including the walls of the aquarium, feces, food, and the test organism itself. Only the freely dissolved concentration is actually bioavailable in aquatic systems (if we exclude uptake through ingestion of food). PFOS has been found in eggs of seabirds (ppt-ppm) (Letcher et al. 2011; Yamashita et al. 2008) and eggs of fish at concentrations of hundreds of nanogram per gram weight mass (wm) (Giesy and Kannan 2001). These results indicate that accumulation of PFOS into eggs where it can affect embryos during early development is primarily due to transfer in the egg from the adult female. For these reasons, waterborne exposure of eggs of fishes to organic residues is generally inappropriate or at least not representative of accumulation from the female.

The most comprehensive way to study effects of chemicals on reproduction is exposing organisms over two generations, especially with *Daphnia magna* (Kim et al. 2012; Ortiz-Rodriguez et al. 2012). However, this protocol is more difficult with longer-lived organisms such as fishes. Moreover, partial life cycle tests that focus on sensitive life stages have been developed (Kühnert et al. 2013). Zebrafish embryos have been shown to be useful as a rapid, reproducible, and cost-effective model organism in toxicity measuring of chemicals during early stages of development (Kühnert et al. 2013; Sakurai et al. 2013). Although some toxicological information is available for PFOS, previously all studies were conducted by using waterborne exposure. However, the difficulty of exposing eggs or embryos directly to toxicants is not representative of the maternal exposure that would occur and thus effects on development during waterborne exposures might be different. If a chronically exposure egg partial or whole-life exposure is included, it is sometimes difficult to complete valid tests because chronic experiment is complicated by various parameters, good reproducibility, and even careless contaminates in females' diet. In the study, the results of which are reported here, to assess effects on development of zebrafish, PFOS was injected directly into embryos by use of nanoinjection further compared the results with traditional exposure ways (waterborne). The aim was to determine in

the vector of exposure resulted in differential effects of equivalent concentrations in the tissues of embryos.

Materials and methods

Test chemical

PFOS ($C_8HF_{17}O_3S$, CAS: 1763-23-1, 98 %) was purchased from Alfa (USA). Solutions for in vivo waterborne exposure were formulated with medium used to rear embryos. For nanoinjection, a stock solution of 5.0×10^4 mg PFOS/L was prepared by dissolving the crystals in dimethyl sulfoxide (DMSO) and stored at 4 °C. Preliminary tests were conducted to determine exposure concentrations of 6.25, 12.50, 25.00, 50.00, 100.00, and 200.00 mg/L used in the in vivo waterborne experiment and 0, 100, 500, 1,000, 5,000, and 10,000 mg/L for use in in ovo nanoinjection. The least concentrations were similar to those reported to occur in embryos of wild fishes (Giesy and Kannan 2001).

Test organisms

Adult, wild-type zebrafish were obtained from the Institute of Hydrobiology, at the Chinese Academy of Sciences (Wuhan, China), and kept in treated tap water, in 10 L tanks with a semiautomatic rearing system at 26–29 °C. Residual ammonia, chlorine, and chloramines were removed from water by use of charcoal and UV light was to kill microbial pathogens. Fish was reared with a females/males ratio of 1:2 under 14/10-h light/dark photoperiod, with 1/3 of the water exchanged daily. Zebrafish were fed twice a day, frozen blood worms and dry fish food (Tropical Fish Food, Jianmen Porpoise Aquarium Co., Ltd. Guangdong, China). Spawning and fertilization took place within 30 min after the lights were turned on in the morning. At the end of the day, nylon nets were set at the bottom of each tank to allow embryos to settle and keep them from being eaten by adult fish. Fertilized embryos were collected and cleaned with embryo rearing water (24.65 mg/L $MgSO_4{\cdot}7H_2O$, 58.80 mg/L $CaCl_2{\cdot}2H_2O$, 1.15 mg/L KCl, 12.50 mg/L $NaHCO_3$, pH=8.3±0.2) and prepared for experiments.

In vivo waterborne exposure

Immediately after fertilization, embryos were examined under a multi-purpose zoom microscope (Nikon AZ 100, Tokyo, Japan) and damaged or unfertilized embryos were discarded. Zebrafish embryos were exposed in 24-well cell culture plates to a series of concentrations of PFOS, respectively. Twenty, normally shaped, fertilized exposed embryos were assigned to each treatment and 2 mL corresponding solution per well; the rest four wells were assigned with control embryos. Different concentration of embryos was exposed in triplicate with three plates as well as control plates. Embryos were cultured in an incubator at 28.5 °C after exposure. Toxicological endpoints included whether embryos were clear or opaque; have edema at 4, 8, 24, 48, 72, or 96 h post-fertilization (hpf); or have structural malformations at 72 or 96 hpf until hatching (Table S1). Coagulated embryos before hatching are opaque, milky white, and appear dark under the microscope. Coagulation of embryos is recorded and used for the calculation of an LC_{50} value.

In ovo nanoinjection

Embryos were nanoinjected with a series of concentrations of PFOS. Embryos were weighted and a safe amount of DMSO to be used as the carrier solvent was determined. Nanoinjection needles were obtained from glass capillaries with an internal filament (internal diameter, 0.7 mm, and external diameter, 1 mm), and micropipette puller (PN-30; Narishige, Tokyo, Japan) was used to pull the needle tip. A cover glass was used to cut the needle tip to the appropriate diameter. Embryos were injected with PFOS with the aid of a sys-PV820 micropump (World Precision Instruments, Inc. Sarasota, America). Exposure solutions were pulled into needles by use of a vacuum pump. Pressure of N_2 and duration of injection were adjusted to deliver 2 nL of PFOS-containing solution. Embryos were immobilized on agarose gel and solutions were injected into the yolk as fast as possible. A total of 60 embryos received each dose of PFOS, distributed in three 24-well cell plates, and included four control embryos in every cell plate. Controls used to determine normal anatomies during development consisted of embryos injected with 2 nL of DMSO. Each embryo was placed in a separate chamber in a 24-well cell culture plate with 0.5 % physiological saline in the constant lighting incubator at 28.5 °C. Cover fresh membrane to avoid evaporating solution. Observations were conducted until 96 h after injection, which was the same duration as that of the in vivo waterborne exposure. All tests were performed in triplicate.

Statistical analyses

The rate of survival of embryos in control group was >80 %. SPSS 12.0 was used for statistical analyses. Normality was confirmed by the Kolmogorov-Smirnov test and homogeneity of variance was confirmed by use of Levine's test. If assumptions were met, one-way analysis of variance (ANOVA) followed by LSD test was used to evaluate the differences between the treatments. Values of all measured parameters were not significantly different among controls. Thus, the triplicates were pooled to provide greater power for further statistical analyses. Differences were considered significant at $p<0.05$. Concentration/dose–response relationships observed

for PFOS were summarized by the mean of the median effective concentrations (EC_{50})/lethality concentration (LC_{50}) with 95 % confidence intervals, by use of probit analysis. The lowest observable effect concentration (LOEC) was calculated based on the combined abnormalities observed at 96 hpf. LC_{50} values were calculated based on both lethality and sublethal endpoints.

Results

The average mass of embryos was 0.545 mg wet mass (wm). So the final concentrations of PFOS for nanoinjection were 0, 367, 1,834, 3,668, 18,338, and 36,676 ng/g wm.

Concentration/dose–lethality relationship

PFOS affected development of embryos and decreased survival. Lethality of embryos was observed in both the in vivo waterborne and in ovo nanoinjection exposures (Figs. 1 and 2). The number of coagulated embryos was directly proportional to concentrations/dose and duration of exposure. Lethality was the primary effect observed for each of the types of exposure.

Incidences of abnormalities were directly proportional to concentrations of PFOS. Waterborne exposure to PFOS caused incomplete epiboly of embryos that ultimately died between 8 and 12 hpf. LC_{50} (95 % confidence intervals, CI) values were 182 (143–191) and 69 (52–98)mg/L 12 and 24 hpf, respectively (concentration-dependent change with confidence intervals indicated detail in Table S2). After longer durations of exposure, the incidence of coagulated embryos did not increase. LC_{50} values were consistent among durations of 48, 72, and 96 hpf, with the same concentrations of 68 mg PFOS/L, respectively. Similarly, when exposed via nanoinjection, LC_{50} (95 % CI) values were comparable at all durations of exposure with values of 31,266 (17,421–210,887), 10,893 (6,547–239,677), and 10,893 (6,547–239,677)ng/g wm 48, 72, and 96 hpf, respectively (Table S3). No value for the LC_{50} could be calculated for durations less than 24 hpf for nanoinjection exposure because the 95 % confidence interval was too large. Concentrations of PFOS in the embryos via the two exposure routes caused the similar effect.

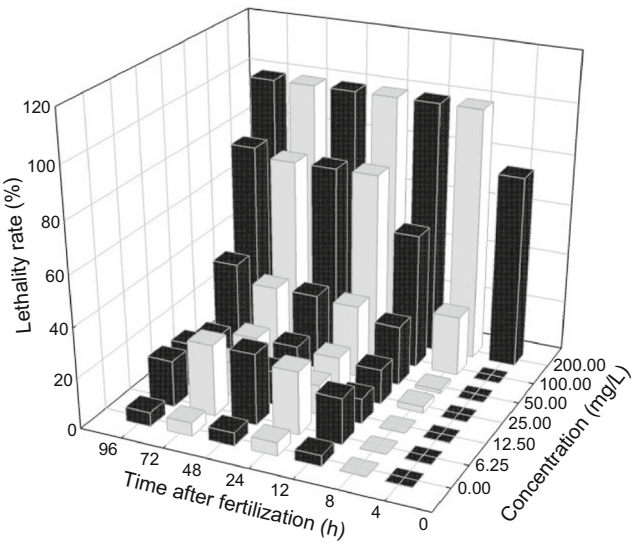

Fig. 1 The lethality rate of embryos affected by PFOS in vivo waterborne exposure

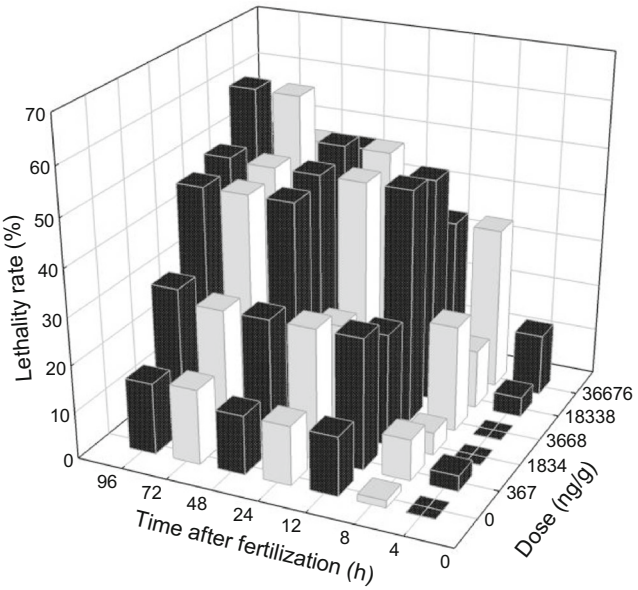

Fig. 2 The lethality rate of embryos affected by PFOS in ovo nanoinjection exposure

Greater concentrations of PFOS caused abnormal development within early stages of development. The concentration/dose–effect curve of the two exposure routines is shown in Fig.3. For waterborne exposure, approximately 76 % embryos died when exposed to 200 mg PFOS/L by 4 hpf while 100 % after 8 hpf. After 8 hpf, 24 % of embryos exposed to 100 mg PFOS/L died. Similarly, when exposed via nanoinjection, significant mortality occurred at concentrations greater than 18,338 ng PFOS/g wm after 4 hpf and at 8 hpf. Coagulation and deformities of embryos exposed to 3,668 ng PFOS/g wm were also significantly greater than that of controls. From 4 to 8 hpf, some embryos disintegrated. The density of cells decreased and later resulted in coagulation. Rates of dissolved embryos were dose-dependent with 8 hpf LC_{50} 113 mg PFOS/L and 49,513 ng PFOS/g wm for waterborne exposure and in ovo nanoinjection, respectively.

Specific teratogenicity endpoints

Statistically significant incidences of adverse effects on development were observed in embryos exposed both in vivo

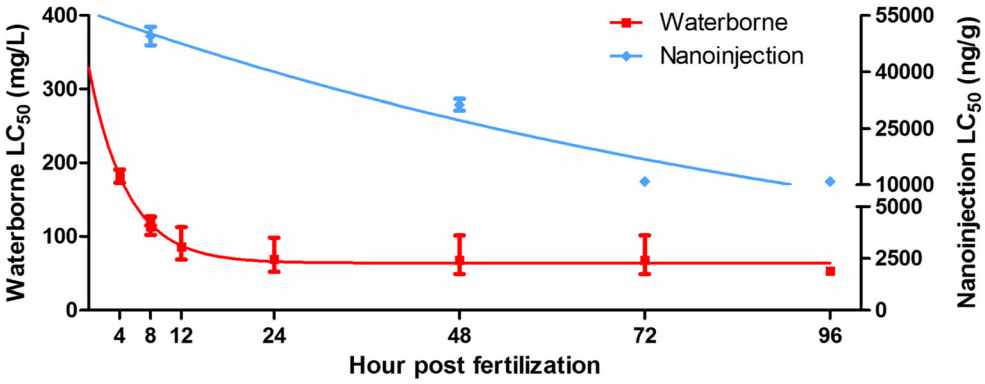

Fig. 3 The concentration/dose–effect curve of the two exposure routines, using Graphpad prism 5 software to draw scatter diagram, and then nonlinear fitted curve by use of exponential of one phase decay

waterborne and in ovo nanoinjection, relative to their respective controls. While edema was observed, the incidence at 48 hpf was small. Incidence of edema was dose-dependent at concentrations greater than 50 mg PFOS/L in the waterborne exposure, but there was no significant difference among doses by nanoinjection. After 72 hpf, hatching was delayed and curvatures of the spine were evident (Figs. 4 and 5). In the waterborne exposure, curvature of the spine was evident in embryos by 72 hpf and this was the most sensitive toxicological endpoint with an EC_{50} of 53 mg PFOS/L. The LOEC based on malformations during hatching was 12.5 mg PFOS/L. There were also malformations observed in embryos exposed via nanoinjection. While the incidence was significantly greater than the controls, but there was no dose-dependent relationship above the threshold. The threshold for effects (LOEC) for waterborne and nanoinjection exposure were 6.25 mg PFOS/L and 1,834 ng PFOS/g wm, respectively.

Discussion

This study was conducted partly to explore whether the effects and potencies would be different between PFOS accumulated from water and that injected directly into eggs. In the present study, both vectors of exposure to PFOS caused significant adverse effects on development, including delayed hatching, coagulation and disintegration of embryos, and edema and malformations, with curvature of the spine being the most sensitive endpoint for both exposure ways. Values for the LC_{50} determined for both exposures were similar between 24 and 96 hpf. At 72 hpf, the LC_{50} determined via waterborne exposure was 68 mg PFOS/L. An equivalent magnitude of response was observed when embryos were exposed to 10,893 ng PFOS/g wm introduced into eggs by use of nanoinjection. Some researchers have reported that magnitudes of effects were equivalent if equivalent amounts of toxicant entered the developing embryo, so that internal exposure was deemed the better predictor of effects (Escher and Hermens 2004). Similar types of effects were observed regardless of whether exposure (via the water or by nanoinjection). This result indicates that some PFOS were accumulated from the water to concentrations sufficient to cause adverse effects. Internal concentrations of compounds can be predicted from concentrations in water by use of bioaccumulation factors (BCF) or from the octanol/water partition coefficient (Kow). However, Kow is not appropriate for PFOS because PFOS is more soluble in water (Giesy et al. 2010) and tends to bind to proteins instead of partitioning into lipids as some neutral organic contaminants (Jones et al. 2003). To our knowledge, only one paper has reported concentrations of PFOS measured simultaneously in water and whole-body tissues of zebrafish larvae (Huang et al. 2010). The data from that study were used to develop a function to relate concentrations of PFOS in larvae to that in water (Fig S1). By using this relationship, it was possible to compare responses due to equivalent exposures via water and nanoinjection. At 48, 72, and 96 hpf, the LC_{50} determined for waterborne exposure was 68 mg PFOS/L. Based on the relationship between concentrations in water and larvae, this concentration in water was estimated to be equivalent to an internal dose of 75,770 ng/g, which is greater than the LC_{50} for 72 and 96 hpf of 10,893 ng PFOS/g wm determined by nanoinjection into the eggs. Thus, it can be concluded that the potency of PFOS when injected directly into the egg was greater than when it was accumulated across membranes from water. Thus, concentrations associated with thresholds for effects would be less for eggs exposed via nanoinjection.

The advantage of the nanoinjection approach is that the amount in the egg is known while the amount accumulated from water depends on the available fraction and can vary among waters due to physical-chemical properties of solutes. Effects caused by internal concentrations were reported to be independent of bioaccumulation, so the internal exposure was more predictive of effects (Escher and Hermens 2004; Escher et al. 2005). Based on these results, it is suggested that the internal dose is the more appropriate measurement of potential effects and that dose–response relationships should be reported based on internal dose.

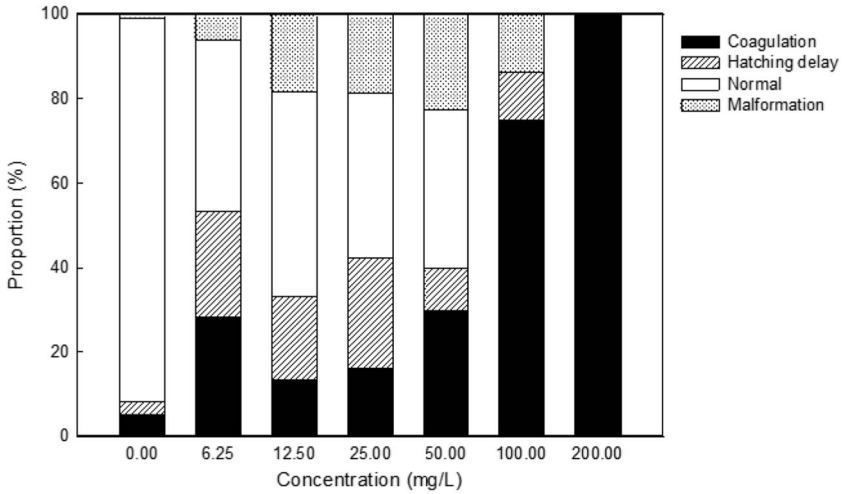

Fig. 4 Proportions of coagulation, hatching delay, normal, and malformation of embryos at 72 hpf in vivo waterborne exposure

The types and incidences of deformities, such as edema and curvatures of the spine, observed in this study with a 96-hpf LC_{50} of 68 mg PFOS/L were consistent with those reported previously (Shi et al. 2010). Expression of genes associated with motor neurons was altered when 6 hpf zebrafish embryos were exposed to 1.0 mg PFOS/L for 120 hpf (Zhang et al. 2011a). Exposure to concentrations of 4 or 16 mg PFOS/L altered rates of heart beat of zebrafish larva (Huang et al. 2011). The 96-hpf LC_{50} based on incidence of malformations and effects on heart rate of zebrafish embryos was 58.47 (54.48–62.75)mg PFOS/L (Hagenaars et al. 2011). The 96-hpf LC_{50} for zebrafish embryo was 71 mg PFOS/L (Ye et al. 2009).

To assess the potential risk posed by PFOS LOEC values based on the two routines of exposure of 6.25 mg PFOS/L and 1,834 ng PFOS/g (wm) in zebrafish embryos to concentrations of PFOS measured in surface waters. Concentrations of PFOS in water of the Liao River and Tai Lake (Ch: *Taihu*) were 0.33 (n.d. −6.6) and 26.5 (3.6–394)ng PFOS/L, respectively (Zhu et al. 2011). Mean concentrations of PFOS in the Baltic Sea were less than 10 ng PFOS/L (Theobald et al. 2011). Total concentrations of PFCs concentrations (∑PFC) in water samples from rivers in Shenyang Province averaged 5.32 ng ∑PFC/L and PFOS was one of the predominant compounds (Yang et al. 2011b). In all these cases, concentrations were in the nanogram per liter range, which is more than 1,000-fold less than the concentrations (mg/L range) that caused significant effects on zebrafish embryos exposed either in vivo through water or via nanoinjection. All hazard quotients (HQs) were less than 1.0. This indicates that concentrations of PFOS observed in surface waters were not likely to cause any adverse effects to embryos of fishes.

PFOS has been detected in embryos or eggs. While waterborne exposure on the premise of accurate estimation of bioavailability and accumulation (Escher and Hermens 2004), nanoinjection can incorporate chemicals into the yolk sac of fish embryos and mimic effects due to maternal deposition. Different transport processes are involved in accumulation of residues into eggs of fishes from water or deposition from the adult. Accumulation of residues from water must be

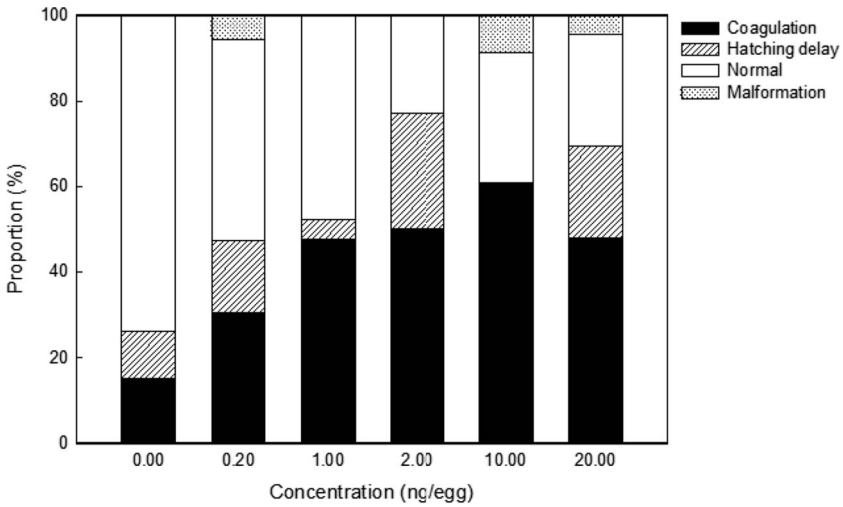

Fig. 5 Proportions of coagulation, hatching delay, normal, and malformation of embryos at 72 hpf in ovo nanoinjection exposure

able to pass the chorion of eggs and membranes of embryos. Due to these characteristics of eggs and embryos of fish, waterborne exposure often does not result in effective accumulation. Some pollutants can be transferred from adult, female fish to embryos (Letcher et al. 2011; Lien et al. 2011; Monroy et al. 2008; Noorlander et al. 2011; Zhang et al. 2010, 2011b). Nanoinjection has advantages relative to waterborne exposure and had been applied in other species (Ekman et al. 2004; Hano et al. 2009; Villalobos et al. 2003b).

In ovo nanoinjection resulted in an internal dose–response relationship and required only small volumes of toxicant and can be used for small fishes. Nanoinjection requires relatively short durations of exposure, relative to studies that expose adult female, gives reproducible results, and simulates maternal transfer without the need to chronically expose adult fish. Nanoinjection mimics the parent–larva translation process well by injecting the chemical directly into embryos. The results presented here indicate that nanoinjection was a useful way to mimic maternal transfer of contaminants.

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Comparison of Waterborne and *In Ovo* Nanoinjection Exposures to Assess Effects of PFOS on Zebrafish Embryos

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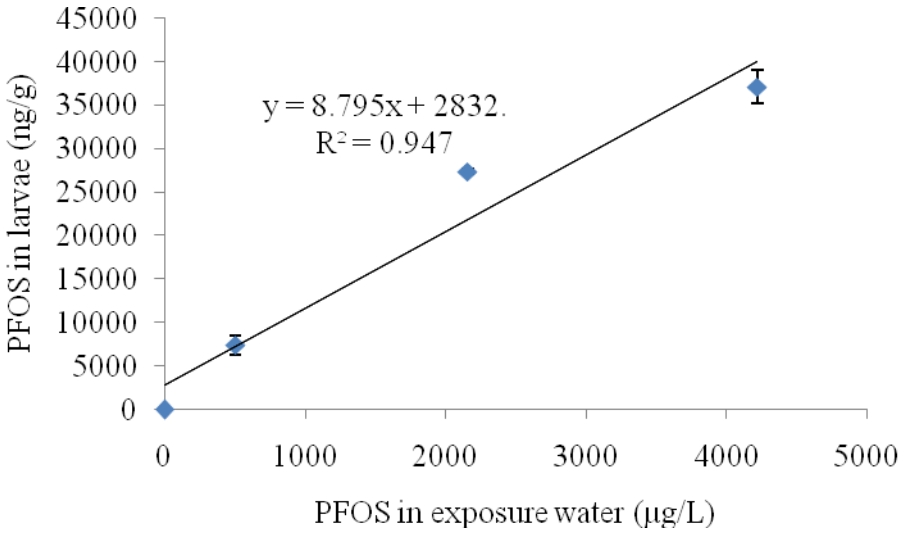

Fig S1 Relationship between concentrations of PFOS in exposure water and larvae, the data comes from the literature (Huang et al. 2010)

Table S1 Toxicological endpoints of development on zebrafish

Toxicological endpoints	Class of toxicity	4hpf	8hpf	12hpf	24hpf	48hpf	72hpf	96hpf
Coagulation of embryo	lethal	+	+	+	+	+	+	+
Gastrula not start	lethal		+					
Completion of gastrulation	lethal			+				
Extension of the tail	lethal				+	+		
No heartbeat	lethal					+	+	+
Spontaneous movements within 20 seconds	lethal				+			
Development of the eye	sublethal				+	+		
No spontaneous movements	sublethal				+			
Edema	sublethal					+		
Delay of hatching	sublethal						+	
Spinal curvature	sublethal						+	+

Table S2 Lethal concentrations (LC_{50}; mg/L) of PFOS to developing zebrafish embryos (mg/L) *in vivo* waterborne exposure.

Duration of Development	LC_{50} (mg/L)	Confidence interval (95%)
4 hpf	182	143-191
8 hpf	121	98-160
12 hpf	86	68-112
24 hpf	69	52-98
48 hpf	68	49-101
72 hpf	68	49-101
96 hpf	68	49-101

Table S3 Lethal concentrations (LC_{50}; mg/L) of PFOS to developing zebrafish embryos (mg/L) *in ovo* nanoinjection.

Duration of Development	LC_{50} (mg/L)	Confidence interval (95%)
48 hpf	31266	17421-210887
72 hpf	10893	6547-239677
96 hpf	10893	6547-239677

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