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TITLE:

PFOS affects posterior swim bladder chamber inflation and swimming performance of zebrafish larvae

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ABSTRACT

Perfluorooctane sulphonate (PFOS) is one of the most commonly detected perfluorinated alkylated substances in the aquatic environment due to its persistence and the degradation of less stable compounds to PFOS. PFOS is known to cause developmental effects in fish. The main effect of PFOS in zebrafish larvae is an uninflated swim bladder. As no previous studies have focused on the effect of PFOS on zebrafish swim bladder inflation, the exact mechanisms leading to this effect are currently unknown. The objective of this study was to determine the exposure windows during early zebrafish development that are sensitive to PFOS exposure and result in impaired swim bladder inflation in order to specify the mechanisms by which this effect might be caused. Seven different time windows of exposure (1-48; 1-72; 1-120; 1-144; 48-144; 72-144; 120-144 hours post fertilization (hpf)) were tested based on the different developmental stages of the swim bladder. These seven time windows were tested for four concentrations corresponding to the EC-values of 1, 10, 80 and 95% impaired swim bladder inflation ($EC_1 = 0.70 \text{ mg.L}^{-1}$, $EC_{10} = 1.14 \text{ mg.L}^{-1}$, $EC_{80} = 3.07 \text{ mg.L}^{-1}$ and $EC_{95} = 4.28 \text{ mg.L}^{-1}$). At 6 days post fertilization, effects on survival, hatching, swim bladder inflation and size, larval length and swimming performance were assessed. For 0.70 mg.L^{-1} , no significant effects were found for the tested parameters while 1.14 mg.L^{-1} resulted in a reduction of larval length. For 3.07 and 4.28 mg.L^{-1} , the number of larvae affected and the severity of effects caused by PFOS were dependent on the time window of exposure. Exposure for 3 days or more resulted in significant reductions of swim bladder size, larval length and swimming speed with increasing severity of effects when the duration of exposure was longer, suggesting a possible effect of accumulated dose. Larvae that were only exposed early (1–48 hpf) or late (120-144 hpf) during development showed no effects on the studied endpoints. The results demonstrate that PFOS does not affect the budding phase, and does not cause deflation of already inflated swim bladders. PFOS clearly affects processes that take place during the inflation phase and might also have an effect on the formation of the tissue layers forming the swim bladder.

KEYWORDS

PFOS; zebrafish larvae; early development; swim bladder; swimming performance

HIGHLIGHTS

- PFOS caused an uninflated swim bladder and spinal curvature in zebrafish larvae.
- PFOS exposure resulted in decreased length and swimming speed.
- Effects were more severe when the exposure duration increased.
- PFOS did not affect the early formation of the swim bladder.

1. Introduction

Due to the persistent and bioaccumulative nature of perfluorinated alkylated substances (PFAS), these compounds are being detected worldwide in wildlife and humans (Sturm and Ahrens, 2010). The aquatic environment is known as an important sink for PFAS resulting in high concentrations in aquatic organisms. Despite the phase-out of perfluorooctane sulphonate (PFOS) production in 2002 by its largest producer (3M, 2000), this pollutant is still one of the most commonly detected perfluorinated alkylated substances in the aquatic environment due to its persistence and the degradation of less stable PFAS to PFOS (Galatius et al., 2013; Lutz, 2011). PFOS is currently being used as one of the most important model pollutants to study the effects and modes of action of PFAS in general.

Exposure to PFOS is known to cause developmental effects in many organisms (Lau et al., 2004; Shi et al., 2008). Several studies have described the developmental effects of PFOS in zebrafish embryos. The main morphological effects are an uninflated swim bladder and spinal curvature. Both effects have been reported in all of the studies that included these endpoints (Huang et al., 2010; Hagenaaars et al., 2011; Shi et al., 2008; Ulhaq et al., 2013a; Zheng et al., 2012). Spinal curvature or the impairment of swim bladder inflation can alter swimming performance and buoyancy in both juvenile and adult fish and therefore affect essential behaviour such as feeding, predator avoidance and reproduction (Gee, 1983). The effects of PFOS on larval swimming performance were already studied by Huang et al. (2010) and Ulhaq et al. (2013b) demonstrating a reduced swimming performance of larvae exposed to high concentrations while low concentrations resulted in hyperactivity. Although swim bladder inflation effects are sublethal, it is generally assumed that they affect apical endpoints that are traditionally considered relevant to environmental risk assessment such as feeding behaviour, predator avoidance, growth and reproduction. Such effects lower the probability of survival, especially in natural habitats where food resources are limited (Villeneuve et al., 2014).

As no previous studies have focused on the effect of PFOS on zebrafish swim bladder inflation, the exact mechanism leading to this effect is currently unknown. The swim bladder of zebrafish consists of two chambers separated by a narrow duct. The posterior chamber is

developed and inflated around 4 dpf and operates as a hydrostatic organ to regulate buoyancy while the anterior chamber is developed and inflated around 20 dpf and functions primarily as an acoustic resonator aiding hearing (Finney et al., 2006; Robertson et al., 2007). This study focusses on the effects of PFOS on swim bladder development during the embryonic and larval stages. The following hypotheses are therefore related to the inability to inflate the posterior chamber.

The first hypothesis involves the interference with the formation of the swim bladder tissue leading to the impairment of swim bladder inflation. PFOS might affect the early development of the swim bladder which originates from an evagination of the foregut epithelium from 30 to 48 hours post fertilization (hpf, see Figure 1). Different signalling pathways are then involved in the differentiation, organization and growth of three tissue layers (epithelium, mesenchymal layer differentiating into smooth muscle and an outer mesothelial layer) during the pre-inflation phase from 48 to 72 hpf. Hedgehog signalling is required for the growth of the swim bladder epithelial cells and the differentiation of the mesenchymal cells while the Wnt/ β -catenin signalling is required for the organization and growth of all three tissue layers. PFOS might disturb these signalling pathways and affect swim bladder structure (Lindsey et al., 2010; Robertson et al., 2007; Winata et al., 2009; Winata et al., 2010; Yin et al., 2011).

A second hypothesis is interference with the initial inflation of the swim bladder. Prior to swim bladder inflation, a sequence of behavioural patterns including adherence to the tank walls, detachment, tail flicking for upward movement and reattachment are seen in zebrafish larvae of 3 to 4 dpf (days post fertilization). These behaviours are necessary to reach the air-water interface after which the larvae gulp air that is transmitted by peristaltic movement through the foregut and the pneumatic duct into the gas bladder lumen to inflate the swim bladder by 4 to 5 dpf (Goolish and Okutake, 1999; Lindsey et al., 2010). Several studies describe tail malformations such as a spinal curvature, apoptosis in the tail region and muscle lesions after PFOS exposure (Huang et al., 2010; Hagenaars et al., 2011; Shi et al., 2008; Ulhaq et al., 2013a; Zhang et al., 2011; Zheng et al., 2012). Ji et al. (2008) demonstrated that the swim-up success in medaka larvae was decreased when the parent generation was exposed to PFOS. These effects might preclude the swim-up behaviour to

reach the air-water interface and therefore result in the inability of the larvae to inflate the swim bladder. Furthermore, PFOS forms a layer on the air-water interface with the hydrophobic tails of PFOS reaching out of the water. It is known that zebrafish larvae grown in closed chambers without air-water interface show very small inflation rates (Goolish and Okutake, 1999). If the tension of the PFOS layer at the air-water interface is high enough to prevent the larvae from surface air-gulping, the effects seen on swim bladder inflation might be attributed to this. Also, we suggested in a previous study that the inflation of the swim bladder might be directly perturbed by the surfactant properties of PFOS (Hagenaars et al., 2011) as the production of surfactant is crucial to maintaining the surface tension necessary for swim bladder inflation (Prem et al., 2000).

After initial inflation by the intake of air through the pneumatic duct, further inflation and maintenance of the swim bladder volume occurs by diffusion of oxygen from the blood (Finney et al., 2006). In this process, lactic acid production by the epithelial cells of the swim bladder results in a local blood acidification which in turn promotes oxygen release from haemoglobin by the Root effect (Umezawa et al., 2012). Lactate is produced by anaerobic glycolysis and in one of our previous studies we demonstrated that PFOS interferes with the glucose metabolism and could disturb the aerobic scope (Hagenaars et al., 2008).

Finally, deflation of the swim bladder might occur during post-inflation phases by the interference of PFOS with mechanisms that maintain the swim bladder volume.

The objective of this study was to determine the time window at which PFOS exposure results in impaired swim bladder inflation in zebrafish in order to evaluate these hypotheses. We want to distinguish between effects that precede swim bladder inflation (i.e. early formation of the swim bladder), effects that lead to the impairment of the processes necessary for inflation (i.e. reaching the air-water interface, potential surfactant effects or further inflation by diffusion of oxygen) and effects after the inflation (i.e. deflation of already inflated swim bladders). To that end, zebrafish embryos were exposed to PFOS during different time windows corresponding to the different stages in swim bladder development and inflation described by Winata et al. (2009) and Lindsey et al.

(2010). Knowledge of the sensitive time windows during development is essential to differentiate among the hypothesized mechanisms.

2. Materials and Methods

2.1 Test chemical

Perfluorooctane sulphonate potassium salt (CAS 2795-39-3, Sigma-Aldrich, Saint Louis, US) had a purity $\geq 98\%$. PFOS was fully soluble in water in the tested concentrations (maximum solubility: 680 mg.L^{-1} at $24\text{--}25^\circ\text{C}$; 3M, 2003) and no solvents were therefore used. The pH of all solutions was 7.5 ± 0.2 .

2.2 Egg production

Non-exposed adult wildtype zebrafish (*Danio rerio*) were used for egg production. Fish were kept in a ZebTEC zebrafish housing system (Tecniplast, Buguggiate, Italy) containing reconstituted fresh water made with Instant Ocean® Sea Salt (Blacksburg, VA, US) at a 14h/10h light/dark cycle. Temperature ($28 \pm 0.2^\circ\text{C}$), conductivity ($500 \pm 15 \mu\text{S/cm}$) and pH (7.5 ± 0.3) were monitored continuously and kept constant using Instant Ocean Salt and NaHCO_3 . Ammonium, nitrite and nitrate levels were measured daily with TetraTest kits (Tetra Werke, Melle, Germany) and were always lower than 0.25; 0.3 and 12.5 mg.L^{-1} respectively. The breeding stock was fed four times a day: twice daily with granulated food (Biogran medium, Prodac International, Cittadella, Italy) at a rate of 1.5% of their mean wet weight and twice daily with thawed and rinsed *Artemia* sp. nauplii, *Daphnia* sp., Chironomidae larvae and Chaoborus larvae alternately. Male and female fish were separated in breeding tanks with a perforated bottom using a divider. The divider was removed when the lights turned on in the morning. Within 45 minutes after the lights turned on, spawning and fertilization took place and eggs were collected after this period using a plastic disposable hose. Eggs were then transferred to clean reconstituted fresh water with the same composition as in the breeding tanks, and rinsed.

2.3 Experimental design

2.3.1 Exploratory range finding tests

Three independent range finding tests were performed, each containing 11 concentrations over a range of 0 to 100 mg.L^{-1} PFOS (nominal concentrations 0; 0.025; 0.05; 0.1; 0.5; 1; 5;

10; 25; 50; 100 mg.L⁻¹). All test solutions were made using the same reconstituted fresh water as previously described for adult fish. Eggs were transferred to the test solutions within 60 minutes after spawning. Fertilized eggs were separated from non-fertilized eggs using a stereomicroscope and eggs with anomalies or damaged membranes were discarded.

42 normally shaped fertilized eggs per exposure concentration were divided over a 48-well plate (sterile tissue culture plates, Greiner Bio-One, Frickenhausen, Germany) and each egg was placed individually in 1 ml of the test solution. The remaining 6 wells per plate were filled with reconstituted fresh water and used as internal negative control embryos. A plate was considered valid if no more than 1 internal negative control embryo showed lethal effects (OECD TG 236). Apart from the negative control embryos, a separate plate with control embryos in clean water was used in each test and this plate was used as the control for statistical comparison with exposed embryos. A test was considered valid if >90% of the controls successfully hatched and showed neither sublethal nor lethal effects (OECD TG 236). A concentration range of 0.5; 1; 2 and 4 mg.L⁻¹ 3,4-dichloroaniline (CAS 95-76-1, purity of 98%, Sigma-Aldrich) was used as a positive control (OECD TG 236) in each test to compare the sensitivity of the embryos between the different tests. The 48-well plates were covered with parafilm (Parafilm® M, Bemis Europe, Soignies, Belgium) to avoid evaporation, placed in an incubation chamber (MIR-254-PE, Panasonic, TCPS, Rotselaar, Belgium) at 28.5 ± 0.2°C and subjected to a 14/10h light/dark cycle. Every 48 hours, exposure solutions were renewed. Dead embryos as well as the remains of the chorion after hatching were removed every 24 hours.

Morphological endpoints were recorded at distinct stages of the development directly in the wells using a S8APO stereomicroscope (Leica Microsystems GmbH, Germany). Embryos were checked every 24 hours for lethality using the four endpoints (coagulation, no heart beat, no somite formation and no detachment of the tail) described in OECD TG 236. Furthermore, hatching and swim bladder inflation were recorded every 24 hours until 6 dpf (days post fertilization).

2.3.2 Swim bladder inflation range finding tests

Based on the results of the first range finding tests, two independent narrower range finding tests were performed to determine PFOS concentrations suitable to study effects on the inflation of the posterior chamber of the swim bladder during different time windows in development. Each range finding test contained 11 concentrations over a range of 0 to 5 mg.L⁻¹ (nominal concentrations 0; 0.5; 1; 1.5; 2; 2.5; 3; 3.5; 4; 4.5; 5 mg.L⁻¹). The plate design and the use of the internal negative controls and positive control were identical to what was previously described for the range finding tests. The plates were covered with parafilm, kept at 28.5 ± 0.2 °C and subjected to a 14/10h light/dark cycle. Exposure solutions were renewed every 48 hours.

Embryos were checked for lethal endpoints, hatching, swim bladder inflation and spinal curvature every 24 hours until 6 dpf using a stereomicroscope. At the end of the experiment, larvae were anesthetized using 100 mg.L⁻¹ MS-222 of pH 7.5 (tricaine methanesulfonate, Sigma-Aldrich) and the lateral view of the larvae was photographed using a camera (Canon EOS 600D, 18 megapixels) mounted on a Leica S8APO stereomicroscope.

The posterior swim bladder chamber volume is important for buoyancy and motility. Different methods for estimating the volume of this chamber based on 2D images have been used. For adult zebrafish, the swim bladder is divided into different compartments of which the volume is estimated separately and then summed to obtain an approximation of the total volume (Robertson et al., 2008; Lindsey et al., 2010). For larval zebrafish the posterior chamber of the swim bladder is usually assumed to be an ellipsoid. The volume is then estimated using the general formula for ellipsoid volume ($V = \frac{4}{3} \pi abc$, where a , b and c are half of the length, height and width respectively). However, since a 2D image is used, a perfect ellipsoid is assumed, hence b (measured) and c (not measured) are assumed equal, and the formula is simplified to $V = \frac{4}{3} \pi ab^2$ (Lindsey et al., 2011; Palinska-Zarska et al., 2014) and is therefore directly related to the observed surface $S = \pi ab$. Since toxicant exposure can affect swim bladder morphology, the shape of the swim bladder cannot always be assumed to be a perfect ellipsoid. Digital images were analysed using the ImageJ software (available at <http://rsbweb.nih.gov/ij/>) to determine the exact surface of the swim

bladder (i.e. not by calculation based on a and b , but based on the actual circumference). We did also calculate the estimated volume according to the formula stated above and regression analyses between estimated volume and observed surface showed R^2 values of 0.80 ± 0.05 , depending on the dataset (different exposure concentrations). We subsequently used the observed surface of the swim bladder as the most empirically correct indicator of swim bladder volume, therefore not making assumptions on c . The same software was further used to calculate larval length (standard length). A calibrator with known length was photographed together with the larvae.

2.3.3 PFOS exposure during different developmental time windows

Four concentrations were selected based on the effects found on swim bladder inflation (EC-values for impaired swim bladder inflation) during the narrow range finding tests (see Figure 2). The selected nominal concentrations were $EC_1 = 0.70 \text{ mg.L}^{-1}$, $EC_{10} = 1.14 \text{ mg.L}^{-1}$, $EC_{80} = 3.07 \text{ mg.L}^{-1}$ and $EC_{95} = 4.28 \text{ mg.L}^{-1}$. None of these concentrations caused significant effects on survival. Effect concentrations (EC) were calculated using only uninflated swim bladder as the effect (see Data analysis for further details). These four concentrations were used to test the effects of PFOS at 6 dpf after exposure during seven different developmental time windows (Figure 1A). These time windows included different developmental stages of the swim bladder; the first phase from 1 to 48 hpf, included the swim bladder budding phase (BP in Figure 1), the subsequent phase from 48 to 72 hpf represent the pre-inflation phase (Pre-IP in Figure 1), the inflation phase from 72 to 120 hpf (IP in Figure 1) and a post-inflation phase from 120 to 144 hpf (Post-IP in Figure 1). The prolongation of all tests up to 144 hpf enabled us to determine the potential deflation of a normally inflated swim bladder on the one hand and the delayed inflation on the other hand.

The plate design and the use of the internal negative control and positive control were identical to what was previously described for the range finding tests. The plates were covered with parafilm, kept at $28.5 \pm 0.2^\circ\text{C}$ and subjected to a 14/10h light/dark cycle. Exposure solutions were renewed at 48, 72 and 120 hpf according to the scenarios described in Figure 1. For scenarios 1, 2 and 3 of Figure 1, embryos were rinsed twice with

clean reconstituted fresh water (without exposure to air) before their transition to new 48-well plates containing clean fresh water.

Embryos were checked for lethal endpoints, hatching, swim bladder inflation and spinal curvature every 24 hours until 6 dpf. At the end of the experiment, the locomotor activity (swimming speed, duration of swimming, distance travelled, angle and preference for clockwise swimming direction) of all larvae was determined in the 48-well plates using a Zebrabox video tracking device (ViewPoint, Lyon, France). Larvae were first acclimatized for 20 min to the Zebrabox environment before monitoring the swimming capacity during 30 min in 100% light. Data were analysed using the ZebraLab software version 3.20.5.104. For statistical analysis of tracking data, dead larvae were excluded. After tracking, larvae were photographed as previously described to determine swim bladder surface and larval length.

The relative swim bladder surface was calculated by dividing the measured swim bladder surface by the swim bladder surface that is expected based on the larval length:

$$\text{relative swim bladder surface} = \frac{\text{measured swim bladder surface}}{(a \cdot \text{length}) + b}$$

The parameters a and b were determined from the length – swim bladder surface relationship ($\text{swim bladder surface} = (a \cdot \text{length}) + b$) of the control larvae of all experiments and was used as a constant factor for all individual larvae.

2.4 Data analysis

The LC_{50} is a nonlinear regression estimate (variable hill slope and no constrained top and bottom) of the concentration at which 50% mortality occurs. The EC_x -values are nonlinear regression estimates of the concentrations at which $X\%$ of the embryos had no swim bladder inflation. The LC_{50} and EC_x with their 95% confidence intervals (CIs) were estimated at 6 dpf. Regression estimates were made using GraphPad Prism Version 6.00 for Windows (GraphPad Software, La Jolla California, USA).

The data for length, swim bladder surface and swimming performance were first tested for normality using the Shapiro-Wilk normality test. To determine significant effects, one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test to correct for multiple

comparisons was used for data sets following a Gaussian distribution. Data sets that had no normal distribution were analysed using the Kruskal-Wallis test followed by the Dunn's multiple comparison test. The Pearson correlation coefficient was used to estimate the correlation between length and swim bladder surface. The Spearman correlation coefficient was used to estimate the correlation between the presence of spinal curvature and presence of an uninflated swim bladder. Data were considered significantly different when p-values were <0.05 . Statistical analyses were performed using GraphPad Prism version 6.00.

The frequency data for the presence of an effect (mortality, spinal curvature and non-inflated swim bladder) were tested for statistical differences between the exposure windows using a chi-squared contingency table test with a Bonferroni post-hoc test to adjust for multiple testing. Chi-squared tests were performed in the open source statistical programming environment R (R Development Core Team, 2008). A binary logistic regression was performed using SPSS Statistics Version 22.0 for Windows (IBM Corp., NY, USA) to determine whether the significant differences were depended on the duration of exposure. Data were considered significantly different when p-values were <0.05 .

3. Results

3.1 Exploratory range finding tests

The estimated LC_{50} value at 144 hpf was 6.25 mg.L^{-1} (95% CI: $4.48 - 8.73 \text{ mg.L}^{-1}$). Figure 2A shows the concentration-response curve for mortality. Based on the results of the three exploratory range finding tests, a narrow range of 0 to 5 mg.L^{-1} was selected to define the EC-values for the impairment of swim bladder inflation.

3.2 Swim bladder inflation range finding tests

No significant effects on survival were found up to 4.5 mg.L^{-1} compared to the control, demonstrating that this range was suitable for testing the effects on swim bladder inflation in the subsequent experiments. Figure 2A shows the concentration-response curve of the percentage of larvae that were not able to inflate their swim bladder after PFOS exposure by the time of 144 hpf in relation to the concentration-response curve for mortality on a log scale. Figure 2B shows the concentration-response curve for the percentage of embryos

with an uninflated swim bladders. The estimated EC_{50} value was 2.29 (95% CI: 1.99 - 2.64) $mg.L^{-1}$.

Four concentrations were selected for subsequent testing based on the results of the swim bladder inflation range finding tests; $EC_1 = 0.70 mg.L^{-1}$, $EC_{10} = 1.14 mg.L^{-1}$, $EC_{80} = 3.07 mg.L^{-1}$ and $EC_{95} = 4.28 mg.L^{-1}$.

PFOS caused significant effects on larval length at 144 hpf ($p < 0.001$) and multiple comparison testing demonstrated that the length of larvae was reduced in concentrations $\geq 3 mg.L^{-1}$ (Figure 2C). For concentrations $\geq 3.5 mg.L^{-1}$ the reduction in length was approximately 10 % compared to the length of control larvae. The mean surface of the swim bladder (Figure 2D) was reduced in larvae exposed to PFOS concentrations $\geq 2.5 mg.L^{-1}$ ($p < 0.001$). A chi-squared test revealed a significant increase in the proportion of larvae that was not able to inflate the swim bladder by the time of 144 hpf ($p < 0.001$). This effect was significant for concentrations $\geq 2 mg.L^{-1}$. Combining the results of length, swim bladder surface and frequency data of swim bladder inflation, the LOEC of PFOS was $2 mg.L^{-1}$ and the NOEC was $1.5 mg.L^{-1}$ at 144 hpf.

3.3 Effects of PFOS after exposure during different developmental time windows

0.70; 1.14; 3.07 and $4.28 mg.L^{-1}$ PFOS resulted in a mortality rate that was not significantly different from the control based on the chi-squared test (Figure 3). The selected concentrations were therefore considered suitable for the assessment of sublethal effects (Figure 2A). No delayed hatching or effects on hatching success were found in embryos exposed to any of these concentrations. A summary of the effects of PFOS exposure during the different time windows is presented in Figure 1B.

3.3.1 Effects on swim bladder inflation and spinal curvature

The frequency of effects on swim bladder inflation and spinal curvature caused after exposure to the four PFOS concentrations at the different time windows are presented in Figure 3. Concentrations of 0.70 and $1.14 mg.L^{-1}$ PFOS caused no significant effects on the spinal curvature or on swim bladder inflation. 3.07 and $4.28 mg.L^{-1}$ PFOS caused a similar pattern of the frequency of spinal curvature and the lack of swim bladder inflation for the

different exposure time windows. Furthermore, within each exposure condition approximately the same percentage of effect was seen for swim bladder inflation and spinal curvature and a correlation between the presence of spinal curvature and the presence of an uninflated swim bladder was found in all exposed conditions ($r = 0.79$; $p < 0.001$). No significant effects were found for embryos exposed during the swim bladder budding (1-48 hpf) and post-inflation (120-144 hpf) phases. Embryos exposed for longer periods (3 days or more) had significant effects on spinal curvature and swim bladder inflation. These effects were seen in embryos exposed from the beginning of the experiment as well as in embryos exposed from 48 and 72 hpf onwards (all p -values ≤ 0.002 in comparison to the control). Binary logistic regression showed that the effects on swim bladder inflation were more severe in groups with longer exposure duration. The effects on spinal curvature and swim bladder inflation were most severe in embryos exposed from 1 to 120 or 144 hpf (p -values < 0.001 compared to the control) with 94 and 88 % of the embryos exposed to 4.28 mg.L^{-1} suffering an uninflated swim bladder and spinal curvature respectively. A typical representation of the larvae exposed to PFOS is given in Figure 4.

3.3.2 Effects on swim bladder surface

Effects on swim bladder surface were not found for larvae exposed to 0.70 or 1.14 mg.L^{-1} . When incorporating the size of both inflated as non-inflated swim bladders in the analysis, larvae exposed to 3.07 and 4.28 mg.L^{-1} PFOS showed similar effects with significant reductions of the size of the swim bladder in all exposure windows (all p -values ≤ 0.002 compared to the control) except for larvae that were exposed only during the swim bladder budding (1-48 hpf) or post-inflation (120-144 hpf) phases.

To determine whether a decrease in swim bladder size was also found in swim bladders that did inflate, the sizes of only the inflated swim bladders of each exposure window were compared (Figure 5). In larvae exposed to 3.07 mg.L^{-1} from 1 to 144 hpf a significant reduction in swim bladder surface of the inflated swim bladders was found ($p = 0.006$). In larvae exposed to 4.28 mg.L^{-1} , a significant reduction was found in the exposure window 48 – 144 hpf ($p < 0.001$). The swim bladder surface of the 1 – 120 ($n = 1$) and 1 – 144 hpf ($n = 2$) larvae was smaller, but statistical analysis could not be performed.

3.3.3 Effects on larval length

Length of the larvae was significantly reduced after exposure to 1.14; 3.07 and 4.28 mg.L⁻¹ (Figure 6). In larvae exposed to 1.14 mg.L⁻¹, the reduction in length was approximately 2.8 % and was seen when the exposure started during the early developmental phases from 1 or 48 hpf onwards (all p-values ≤ 0.01 compared to the control). Length was not altered when the exposure started at 72 hpf or later. In larvae exposed to 3.07 and 4.28 mg.L⁻¹, the effect on length was more pronounced. For both exposure concentrations, length was reduced by approximately 5 % in larvae exposed during 3 days, either during the early developmental stages (1 – 72 hpf) or during the later phases (72 – 144 hpf) (all p-values ≤ 0.002 compared to the control). The reduction in length was approximately 10 and 11.5 % in larvae exposed during 4 days or more for 3.07 and 4.28 mg.L⁻¹ respectively (all p-values < 0.001 compared to the control).

3.3.4 Effects on the relative swim bladder surface

The relative swim bladder surface (measured swim bladder surface divided by the expected swim bladder surface) was not significantly different between the different exposure windows in any of the exposure concentrations. This demonstrates that the reduced swim bladder surface of larvae exposed to 3.07 mg.L⁻¹ from 1 to 144 hpf and larvae exposed to 4.28 mg.L⁻¹ from 48 to 144 hpf was in proportion to the decreased length of these larvae. For larvae exposed to 4.28 mg.L⁻¹ from 1 to 120 or 144 hpf no statistical analyses could be performed due to the small number of larvae with an inflated swim bladder.

3.3.5 Effects on swimming performance

Swimming performance (Figure 7) was not altered in larvae exposed to 0.70 or 1.14 mg.L⁻¹. In larvae exposed to 3.07 and 4.28 mg.L⁻¹, a significant reduction in swimming speed was found in the exposure windows 1 – 120, 1 – 144 and 48 – 144 hpf (all p-values ≤ 0.0006 compared to the control). A similar pattern and significant effects were found for the sum of the duration of all movements during 30 minutes and for the travelled distance within this period (data not presented).

To assess whether the reduced swimming speed of larvae exposed to 3.07 and 4.28 mg.L⁻¹ was due to the non-inflated swim bladder or spinal curvature, the swimming speed of larvae

with and without these impairments were compared per exposure window for each concentration separately. The swimming speed of larvae exposed to 3.07 mg.L⁻¹ from 1 to 144 hpf with a spinal curvature was lower compared to the larvae of this exposure condition without a spinal curvature ($p = 0.01$) while there was no significant difference of this last group compared to the control. Significant differences in swimming speed between larvae with or without non-inflated swim bladders ($p = 0.005$) and spinal curvature ($p = 0.01$) were found in the 4.28 mg.L⁻¹ 48 – 144 hpf exposure group. The swimming speed of the larvae in this exposure group without these impairments was not significantly different from the control. This demonstrates that the reduced swimming speed was due to the impairments. Almost all larvae with a non-inflated swim bladder had a spinal curvature and it could therefore not statistically be determined whether the reduced swimming speed was due to a spinal curvature, a non-inflated swim bladder or the interaction of both. For larvae exposed to 4.28 mg.L⁻¹ from 1 to 120 or 144 hpf no statistical analyses could be performed due to the small number of larvae without these impairments.

Data of angles and the preference for clockwise and anticlockwise swimming direction were first corrected per larva for the travelled distance (data presented in supplementary files). While the control larvae had little variance in these parameters, more differences in larvae within each PFOS exposure condition were found. Despite some significant differences between the control and the exposure conditions, no clear pattern in angles or in preference for clockwise and anticlockwise swimming direction was found that could aid in understanding the mechanism by which PFOS alters the swimming performance.

4. Discussion

We observed a clear relationship between the tested PFOS concentrations and the inability to inflate the posterior chamber of the swim bladder at concentrations that did not cause significant effects on mortality. The inability to inflate the swim bladder is therefore a sublethal effect of PFOS. This effect was accompanied by effects on the spinal curvature and reductions in length and swim bladder surface. The effects of PFOS on swim bladder inflation and tail morphology were previously reported as the most pronounced effects of PFOS in other studies as well (Huang 2010; Hagenaars 2011; Shi 2008; Ulhaq 2013a; Zheng

2012), but this is the first study a) demonstrating a concentration dependent response of these effects, and b) showing the importance of these effects relative to mortality.

4.1 PFOS affects the inflation phase

To evaluate the developmental phases at which PFOS affects swim bladder inflation, the effects of the different time windows were compared. The number of embryos affected and the severity of effects caused by PFOS at 144 hpf were dependent on the time window of exposure and the duration of exposure.

Embryos exposed during the time window before the formation of the three layers (1 – 48 hpf) showed no effect on the studied endpoints while at least 50% of the embryos exposed from 48 hpf onwards were unable to inflate their swim bladders. This demonstrates that affecting events during the budding stage is not the mechanism by which PFOS causes impairment of swim bladder inflation. Furthermore, from 72 hpf onwards the swim bladder as an organ was visible (either inflated or non-inflated) in both exposed and non-exposed embryos of all tested time windows, which provides more evidence that PFOS exposure does not affect early swim bladder formation although we did not assess histologically whether the three layers of the swim bladder had been properly formed in all scenarios. Exposure during the post-inflation phase alone (120 – 144 hpf) had also no effect on swim bladder inflation, demonstrating that PFOS did not cause deflation of inflated swim bladders.

Significant effects were only seen in embryos exposed during the pre-inflation phase, the inflation phase or both. To investigate whether the effects on swim bladder inflation were caused by disturbing the formation and growth of the three distinct tissue layers of this organ during the pre-inflation phase, the effects in embryos exposed from 1 to 72 hpf (Pre-IP) were compared to the effects in those exposed from 72 hpf (IP) onwards. The results of the exposure window 1 – 72 hpf suggest that PFOS affects the growth of the swim bladder, as stopping the exposure after the growth phase leads to effects on inflation later on in development. On the other hand, similar percentages of embryos were affected when the exposure started only after the formation and growth of the swim bladder (72 – 144 hpf). In this scenario, the effect of PFOS can not have been caused by disturbing the formation of

the three tissue layers but by interfering with the inflation process itself. Furthermore, embryos exposed during both the pre-inflation and inflation phase (48 – 144 hpf) and embryos exposed during the inflation phase (72 – 144 hpf) showed similar effects, demonstrating that exposure during the pre-inflation phase in addition to exposure during the inflation phase does not result in additional or more severe effects compared to exposure during the inflation phase alone.

In summary, it is clear that PFOS does not affect the budding phase, and does not cause deflation of already inflated swim bladders. Further, it is equally clear that PFOS affects processes that take place during the inflation phase. This could include the initial inflation by swallowing air, surfactant effects and/or the further inflation by diffusion of oxygen from the blood, a process in which lactate production is crucial to lower blood pH causing the root effect. Whether or not PFOS also interferes with processes that take place during the pre-inflation phase remains unclear.

Overall, the severity of effects was dependent on the duration of exposure at 120 hpf (the time point by which swim bladders are normally inflated), suggesting a possible effect of accumulated dose. Huang et al. (2010) studied the uptake kinetics of PFOS in embryos and larvae and demonstrated that PFOS penetrates the chorion and accumulates in the embryo within 2 hours. The absorption of PFOS was slow up to 24 hpf, relatively rapid between 24 and 48 hpf, and steadily more rapid after hatching. The clearance of PFOS was negligible. In our experiment, the inflation of the swim bladder occurred around 120 hpf. The optimal time window for swim bladder inflation in zebrafish embryos is around 96 to 120 hpf and the chance of inflation after this period is strongly reduced (Lindsey et al., 2010). Longer exposure (1 – 144 hpf) did result in similar effects on swim bladder inflation as seen in embryos exposed from 1 to 120 hpf since the effect already occurred around 120 hpf. The effects found in embryos exposed from 1 to 72 hpf could therefore have been caused by accumulation of PFOS that only causes effects during the inflation phase. On the other hand, effects taking place at different stages of embryonic development are not necessarily additive in nature, as this depends on the similarity or difference in the mechanism of action leading to such different effects, and the relative susceptibility of an individual embryo for disturbance of the processes involved in these toxicological pathways. To further investigate

possible effects during the pre-inflation phase, a combination of measuring the internal dose, histological analysis and using molecular techniques to find potentially involved pathways will be necessary.

4.2 PFOS reduces swim bladder size and larval length

At 144 hpf, little variance of swim bladder surface was observed in the control larvae within and between each experiment. At this stage in development, there is no active control of inflation and deflation and the swim bladder remains inflated (Robertson et al., 2007). Measurement of the swim bladder at this time point seems therefore a stable parameter to estimate the ability of the larvae to achieve buoyancy. The decreased swim bladder surface in embryos exposed during 3 days or more was in the first place due to the high percentage of larvae that were unable to inflate the swim bladder which results in the inability to maintain neutral buoyancy. Additionally, comparing only the swim bladder size of those larvae with inflated swim bladders revealed that the swim bladder surface of larvae exposed for 4 days or more was smaller compared to the control. However, this reduction in swim bladder size was proportional to the smaller length of these larvae. The smaller length of zebrafish larvae exposed to PFOS was previously demonstrated (Hagenaars et al., 2011; Shi et al., 2008) and is in accordance with the results of in utero rodent studies (Lau et al., 2003; Lau et al., 2006). This reduction in length might be caused by a delayed growth which can account for the reduced size of the swim bladder. However, this effect does not explain the impaired swim bladder inflation. First inflation of the swim bladder normally occurs when reaching a body length of approximately 3.5 mm (Lindsey et al., 2010; Robertson et al., 2007) and the mean length of larvae exposed to PFOS was higher in all exposure windows.

4.3 Relevance to higher physiological effects

Larvae that fail to inflate the swim bladder use additional energy to maintain position in the water column (Lindsey et al., 2010; Marty et al., 1995). This energy expenditure might explain the decreased length and swimming speed seen in larvae exposed to PFOS. The swimming speed was significantly lower in larvae that had an uninflated swim bladder and a spinal curvature indicating that these malformations were probably the cause of this effect rather than other mechanisms by which PFOS might alter swimming speed.

4.4 Relevance to environmental risk assessment

The results of this study demonstrate that PFOS affects swim bladder inflation, spinal curvature and swimming performance although at higher concentrations than those measured in the aquatic environment which are usually in the ng.L^{-1} to $\mu\text{g.L}^{-1}$ range (Skutlarek et al., 2006; Zareitalabad et al., 2013). The tested concentrations were based on the results of range finding tests and were in the first place chosen to enable us to study the mechanisms involved in swim bladder defects. However, PFOS concentrations in fish can be up to 8850 times higher than those found in surface water (Sinclair et al., 2006) and the maternal transfer of PFOS to eggs (Sharpe et al., 2010) results in high accumulation in eggs as was demonstrated by Kannan et al. in fish collected from different rivers (2005). To determine whether PFOS causes developmental effects in environmentally relevant scenarios, comparison of PFOS concentrations found in the eggs of wild fish populations should be compared to the accumulated amounts of PFOS after exposure to concentrations that caused effects in the present study.

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Captions

Figure 1. (A) The seven different exposure windows were based on the developmental stages of the swim bladder. Dark grey boxes indicate the periods of exposure to PFOS. The seven exposure windows were tested for each of the four selected concentrations. N = 42 for each exposure window and each concentration. All experiments were ended at 144 hpf. BP: budding phase; Pre-IP: pre-inflation phase; IP: inflation phase; Post-IP: post-inflation phase. (B) A summary of the significant effects of PFOS exposure during the different time windows on the presence of swim bladder inflation (SB) and spinal curvature (SC), swim bladder surface (SBS), larval length (LL), relative swim bladder surface (RSBS) and swimming performance (SP). Asterisks represent significant effects compared to the control, 'ns' represents no significant effect and 'NA' represents the inability to perform statistical analysis due to the low number of larvae with an inflated swim bladder exposed during this time window. The illustrations on top of the timeline illustrate the different stages of the formation and inflation process (see Introduction for more details).

Figure 2. Results of the swim bladder inflation range finding tests. (A) Concentration-response curves of mortality relative to the concentration-response curve of no swim bladder inflation. The curves are based on the mean of three and two independent experiments respectively (n = 42 in each experiment). (B) Concentration-response curve of no swim bladder inflation at 144 hpf. The curve is based on the mean of two independent experiments (n = 42 in each experiment). (C) Length of larvae at 144 hpf, values are reported as means $\pm$ standard deviation of the surviving larvae (n is represented between brackets). (D) Surface of the swim bladder at 144 hpf of inflated swim bladders, values are reported as means $\pm$ standard deviation of the surviving larvae (n is represented between brackets). Bars marked by a different letter are significantly different.

Figure 3. The frequency of effects on mortality, spinal curvature and swim bladder inflation at 144 hpf. (A) $EC_1 = 0.70 \text{ mg.L}^{-1}$, (B) $EC_{10} = 1.14 \text{ mg.L}^{-1}$, (C) $EC_{80} = 3.07 \text{ mg.L}^{-1}$ and (D) $EC_{95} = 4.28 \text{ mg.L}^{-1}$. Percentage of affected larvae is indicated with the grey scale bars. For each effect (mortality, spinal curvature and no swim bladder inflation), the bars marked by a different letter are significantly different (n = 42 in each condition).

Figure 4. Photos representative for the morphology of (A) control larvae and larvae exposed to (B) 3.07 mg.L^{-1} from 1 to 144 hpf and (C) 4.28 mg.L^{-1} from 1 to 144 hpf. Arrows and arrowheads point respectively towards an inflated swim bladder and normal body shape in (A) and uninflated swim bladder and spinal curvature in (B) and (C).

Figure 5. The swim bladder surface of inflated swim bladders at 144 hpf. (A) 0.70 mg.L^{-1} , (B) 1.14 mg.L^{-1} , (C) 3.07 mg.L^{-1} and (D) 4.28 mg.L^{-1} PFOS. Values are reported as means $\pm$ standard deviation. (n) represents the number of survived larvae with an inflated swim bladder. Bars marked by a different letter are significantly different. No statistical analysis could be performed for 4.28 mg.L^{-1} PFOS 1 – 120 and 1 – 144 hpf as 'n' was too small.

Figure 6. The larval length at 144 hpf. (A) 0.70 mg.L⁻¹, (B) 1.14 mg.L⁻¹, (C) 3.07 mg.L⁻¹ and (D) 4.28 mg.L⁻¹. Values are reported as means ± standard deviation. Bars marked by a different letter are significantly different (n ≥ 36 depending on the survival within each condition).

Figure 7. The average swimming speed at 144 hpf. (A) 0.70 mg.L⁻¹, (B) 1.14 mg.L⁻¹, (C) 3.07 mg.L⁻¹ and (D) 4.28 mg.L⁻¹. Values are reported as means ± standard deviation. Bars marked by a different letter are significantly different (n ≥ 36 depending on the survival within each condition).

Supplementary Figure. The average number of clockwise and counter clockwise movements per meter at 144 hpf. Values are reported as means ± standard deviation. (n ≥ 36 depending on the survival within each condition).

Figure 1

Figure 2

Figure 3

Figure 5

(A) 0.70 mg/L PFOS

(B) 1.14 mg/L PFOS

(C) 3.07 mg/L PFOS

(D) 4.28 mg/L PFOS