Effects of Perfluorooctane sulfonate on immobilization, heartbeat, reproductive and biochemical performance of *Daphnia magna*

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HIGHLIGHTS

- Heartbeat of *Daphnia magna* exposed to PFOS 48 h was stimulated significantly.
- 48h-EC₅₀ was 79.35 mg L⁻¹, the toxicity of PFOS was classified as intermediate.
- Intrinsic rate of natural increase was a sensitive parameter.
- Reproductive, antioxidant and neurological responses were significantly inhibited.
- At 8 mg L⁻¹ concentration, most evaluated endpoints have sensitive effects.

GRAPHICAL ABSTRACT

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ABSTRACT

In recent years, Perfluorooctane sulfonate (PFOS) was widely detected in Yellow-Bohai Sea and other areas, causing a series of adverse effects in aquatic organisms. However, present studies of its chronic and acute toxicity on aquatic organisms were far more inadequate. Therefore, in the present study, *Daphnia magna* was used to investigate PFOS toxicity on their immobilization, heartbeat, reproductive and biochemical performance in acute, subchronic and chronic exposure. The results showed that the 48h-EC₅₀ value for immobilization was 79.35 mg L⁻¹ and the toxicity was classified as intermediate. Heartbeat was significantly stimulated and reproductive parameters were significantly suppressed by PFOS, which can be used to reflect the toxicological effects on individuals. On the other hand, intrinsic rate of natural increase was more sensitive than reproductive parameters, which indicated negative responses on population dynamics of *Daphnia magna*. In addition, there were different degrees of inhibition on GST, CAT and ChE activity, which indicated three types of enzyme could become biomarkers to chronic PFOS

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exposure. Most of selected and evaluated endpoints have significant sensitivity to PFOS at the concentration of 8 mg L⁻¹ during subchronic and chronic exposure.

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1. Introduction

Perfluorooctane sulfonate (PFOS) is one of the most difficult to degrade synthetic chemicals that has become a ubiquitous environmental contaminant. PFOS is used as a chemical material in diverse industrial products and daily necessity, including textiles, papermaking, electronics and pharmaceutical industry (Lau et al., 2007; Supreeyasunthorn et al., 2016). Based on the properties of stability, persistence, biological accumulation and toxicity, PFOS was listed in Stockholm Convention on Persistent Organic Pollutants (Wang et al., 2009; Chen et al., 2014; Jantzen et al., 2016; Salgado et al., 2016). Although PFOS was hydrophobic, it has the water-soluble functional groups, which differs from other typical persistent organic pollutants.

This feature of PFOS leads that PFOS has high solubility in water and it can exist in aquatic ecosystem for a long time. Previous published scientific studies reported that PFOS exists widely in rivers, lakes and oceans (Yamashita et al., 2005; Munoz et al., 2015; Sakurai et al., 2016). Considering Yellow-Bohai Sea as a case area, the maximal concentration in sea water of PFOS was reported to be about 30.9 ng L⁻¹ in coastal areas of Bohai Sea (Wang et al., 2011). The findings of previous research also reported that PFOS was detected from 0.35 to 47 ng L⁻¹ along the West Coast of Korea (Naile et al., 2010, 2013). In recent decades, elevated levels of PFOS has been detected in many freshwater animals, which has accumulated and biomagnified in the marine food chain (Bossi et al., 2005; Yeung et al., 2009; Renzi et al., 2013). Therefore, toxic effects of PFOS on aquatic organisms were significant for providing scientific basis for risk assessment.

Daphnia magna is an aquatic animal with reproductive capabilities and feeds on algae, which has been widely applied to chemicals toxicity tests. Due to its sensibility to pollutants in the environment, *Daphnia magna* was selected as an international standard experimental organisms (Seda and Petrusek, 2011). A number of current studies on *Daphnia magna* are concentrated on indicators such as immobilization, reproductive parameters and bioaccumulation of pollutants (OECD, 2004; Giesy et al., 2010; Home and Directorate, 2013). However, few studies have explored the heartbeat and intrinsic rate of nature increase of *Daphnia magna* and used heart rate and intrinsic rate as evaluating indicators. A 10 d subchronic test added to chronic exposure may reflect the toxicological process better, which is in contrast with the 21 d test duration in traditional method.

Since PFOS is frequently accumulated in aquatic ecosystem, it is necessary to understand the real aquatic ecological risks and toxicological effects of PFOS. This research attempts to use acute and chronic toxicity test methods to investigate PFOS toxicity on *Daphnia magna* after different exposure periods, including a 48 h acute toxicity test, 10 d subchronic and 21 d chronic toxicity tests. Afterwards, immobilization and heart rate were measured after acute exposure. Intrinsic rate of nature increase was calculated to reflect that population of *Daphnia magna* made responses to PFOS. Moreover, reproductive parameters and enzyme activities of glutathione-S-transferase (GST), catalase (CAT) and cholinesterase (ChE) were determined in order to explore chronic toxicological effects of PFOS. The result of the present study is expected to provide additional useful information for environmental monitoring

and the effective prevention of water pollution.

2. Materials and method

2.1. Experimental chemicals and reagents

Perfluorooctane sulfonic acid potassium salt (CAS: No. 2795-39-3, ≥98% purity) was purchased from Fluka (Sigma-Aldrich, Switzerland. Tris), CDNB and Insect AChE ELISA Kit (REF: E-59928) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). GSH (CAS: No.70-18-8) was purchased from JKHD. Tissue protein extraction kit was obtained from CWBIO. BG11 and M4 culture medium were prepared by analysis of reagents of Sinopharm group. All working stock solutions were made instantly before the tests.

2.2. Experimental organisms and culture conditions

Daphnia magna was obtained from State Key Laboratory of Environmental Aquatic Chemistry (Eco-Environmental Sciences of Chinese Academy of Sciences, Beijing). *Daphnia magna* was maintained in artificial medium M4 at 20 °C with pH 7 in the laboratory (Revel et al., 2015). Green algae (*Scenedesmus obliquus*) that was used as food for *Daphnia magna* was purchased from Institute of Hydrobiology (Chinese Academy of Sciences, Wuhan) and cultivated in artificial medium BG11 (Singh et al., 2014). The *Daphnia magna* used in this experiment was grown at a density of 1 animal per 40 mL in 1 L bottles at 20 ± 1 °C under a 16:8 h light and each one was given algal cultures at a concentration of 90,000 cells mL⁻¹. The culture medium was renewed twice a week.

2.3. Experimental design

2.3.1. Acute toxicity tests

The acute toxicity tests were conducted on the grounds of OECD guidelines that were aimed to check acute toxicity of water quality to *Daphnia magna* (OECD, 2004). Preliminary experiment was carried to determine official experimental range of concentration level. Five neonates were put in 80 mL containers with M4 medium for 48 h. PFOS (concentrations of 0, 50, 100, 150, 200 and 250 mg L⁻¹) was also added into each container. Three repetitions were carried for preliminary experiment and also to the control groups. The result of preliminary experiment showed highest concentration to be 150 mg L⁻¹. Therefore, five neonates were placed in 80 mL bottles exposed to different concentrations of PFOS (0, 30, 44, 66, 100, 150 mg L⁻¹) for 48 h. Five repetitions per treatments and control groups were carried. *Daphnia magna* were not provided with algal cultures during acute exposure. The number of immobile *Daphnia magna* was counted in order to calculate EC₅₀ of immobilization after 48 h.

2.3.2. Subchronic and chronic toxicity tests

According to the results of acute toxicity tests, six concentration gradients of PFOS (0, 1, 2, 4, 8, 16 mg L⁻¹) were designed for chronic tests. The experimental design requires that the highest concentration of chronic experiment is lower than EC₅₀ in acute experiment. Ten replicates with one neonate per 80 mL beaker and control groups were exposed to mentioned concentrations of PFOS

for 21 d. During this period, *Daphnia magna* were fed with algal cultures twice daily and the culture water was also renewed twice daily. The beakers were changed with new ones because of sorption of PFOS onto the glass on the tenth day. The mortality, activity, condition and reproduction of *Daphnia magna* were observed and recorded every day, and newborn were also counted and removed daily. All of test subjects were maintained under the same room temperature. After the long exposure time of 10 and 21 d, only survival *Daphnia magna* was analyzed from biological and chemical aspects and the enzymes activity of GST, CAT and ChE were tested respectively. The chronic exposure tests were repeated at least 3 times during the test period.

2.4. Immobilization and heart rate recording

EC₅₀ is the value of concentration of PFOS, when activity of the half *Daphnia magna* was inhibited (50%). With a glass rod gently touching the beaker, the movement distance of *Daphnia magna* is less than the length of themselves in 15 s. *Daphnia magna* was confirmed to be immobile. The number and percentage of immobile fleas were recorded to calculate EC₅₀ after 48 h acute toxic experiment. The value of EC₅₀ was determined following a modified Karber method (Hamilton et al., 1978). The formula was used to calculate EC₅₀ as follows:

$$\ln EC_{50} = X_k - d \left(\sum_i^n P_i - 0.5 \right) \quad (1)$$

where X_k is the concentration of PFOS corresponding to the immobilization rate of 100% and d is the sum of each immobilization rate of each treatment group.

After 48 h acute tests, the heart rate of *Daphnia magna* exposed to every treatment was recorded as a video (50 fps) by digital videotape that was mounted on the inverted microscope. The video played in slow motion, and the heart jump of live fleas was counted within 60 s to figure out the heart rate by watching videos.

2.5. Mortality, reproductive parameters and population growth

The long-term impacts on mortality, growth and reproduction of *Daphnia magna* exposed to PFOS were observed by checking parent animals and living neonates daily. In the course of the experiment, the days to first brood and quantity of first brood were recorded and counted. After 21 d tests, the number of dead parent fleas was counted for mortality rate.

Intrinsic rate of natural increase was regarded as an important indicator to reflect dynamic population changes. The following formula was used to calculate the intrinsic rate of natural increase of *Daphnia magna* (Jeong et al., 2016):

$$\sum l_x m_x e^{-rx} = 1 \quad (2)$$

where l_x is the proportion of individuals surviving to age x , m_x is the mean number of living later generations produced each parent at age x , and x represents the number of days in the experiment.

2.6. Enzymes activities assay

Protein concentrations and GST, CAT and ChE enzyme activities were measured on tenth day and twenty-first day in chronic toxicity tests. Tissue protein extraction kit effectively extracts protein from *Daphnia magna*. Protein concentrations were measured by the bicinchoninic acid (BCA) protein assay (Reichelt et al., 2016).

GST activity was measured using a modified version of assay

proposed by Baldwin et al. (Baldwin and Ga., 1996). GST can efficiently catalyze and reduce the combination of GSH and 1-chloride-2,4-dinitrobenzene (CDNB). The absorbance value of reaction product reached the maximum, when the wave length was 340 nm. Therefore, we put 5 mM Tris (pH 7.0), 5 μ M CDNB and 5 μ g protein samples in 96 well plates. Subsequently, an enzyme-labeled instrument was used to monitor the increasing in absorbance at 340 nm over 25 °C to get the activity of GST.

Enzymes CAT can efficiently decompose hydrogen peroxide into hydrogen and water. The absorbance value of reaction products reached the maximum, when the wave length was 240 nm. Therefore, we mixed 120 μ L Tris (pH 7.4) and 5 μ g protein samples in 96 well plates. The mixture was heated for 25 min on water bath at 37 °C and then 30 μ L hydrogen peroxide was added to plates. The samples were put in enzyme-labeled instrument under 240 nm in order to get changes of absorbance in the per unit time.

ChE activity was measured according to the ChE enzyme-linked immunosorbent assay (ELISA). Purified ChE antibody, test samples and standard products were added to the wells. Horseradish peroxidase (HRP) was also added into the wells to obtain an indicator that can gauge concentrations of ChE in samples.

2.7. Data analysis

In acute and chronic toxicity experiments for *Daphnia magna* exposed to PFOS, the data were subjected to Student's t-test and analysis of variance (ANOVA) to determine significant difference between experimental groups and control groups at 0.01 and 0.05 significance level. All statistical analyses were performed with Statistical Product and Service Solutions Ver. 13.0 (SPSS 13.0) for Windows.

3. Results and discussion

3.1. Immobilization and heartbeat

Immobilization of *Daphnia magna* affected by different PFOS concentrations were presented in Fig. 1. Increasing concentration of PFOS resulted in the increasing inhibition of *Daphnia magna*. No obvious adverse reactions of *Daphnia magna* were found, when the concentration was lower than 30 mg L⁻¹. Almost all *Daphnia magna* stayed immobile, when the concentration was higher than 150 mg L⁻¹.

Effective concentration is usually used to reflect toxicity of pollutants to test organisms. EC₅₀ was chosen to observe and

Fig. 1. Inhibition rate of *Daphnia magna* exposed to PFOS after 48 h. Sample size of each treatment is 25. Values represent mean $\pm$ standard deviation; Asterisk (*) denotes a significant difference from check group ($p < 0.05$) and asterisks (**) denotes an extremely significant difference from check group ($p < 0.01$).

Fig. 2. Heart rate of *Daphnia magna* exposed to different concentrations of PFOS for 48 h. Sample size of each treatment is 25. Values represent mean $\pm$ standard deviation; Asterisk (*) denotes a significant difference from check group ($p < 0.05$) and asterisks (**) denotes an extremely significant difference from check group ($p < 0.01$).

determine the effects of pollutants on the immobilization of *Daphnia magna* following OECD guideline for the testing of Chemicals (OECD, 2004).

According to statistics of 48 h acute tests, acute toxicity effects of PFOS on the immobilization were observed and compared. The result showed that the 48h-EC₅₀ was calculated to be equal to 79.35 mg L⁻¹, which agreed with the finding of Lazhar M (EC₅₀: 80.22–95.36 mg L⁻¹) (Mhadhbi et al., 2010). Based on the guidelines for the hazard evaluation of new chemical substances, the toxicity of PFOS should be classified as intermediate. In general, the presence of PFOS in the environmental dose may not cause acute toxicity in a short period of exposure.

As shown in Fig. 2, the significance of heartbeat was also increased in contrast with control groups along the increase of the concentrations of PFOS. Furthermore, it is obvious that the heart rate reached the maximum under 66 mg L⁻¹ PFOS. When the concentration reached 100 mg L⁻¹, the frequency of heartbeat decreased. However, the frequency at 100 mg L⁻¹ was still significantly greater than control groups. Heart rate is usually used in pharmacology and toxicology studies. Increasing concentration of PFOS stimulated heartbeat, but at the high concentration state inhibits the heart rate. One of few published articles relating to heartbeat reported that Ectoine has toxic influence on *Daphnia magna* and heart rate kept being inhibited in 48 h tests (Bownik et al., 2015). Until now, researches about the impacts of PFOS on heartbeat are not enough. The previous research provided evidence that heart rate may be related to the distance between different heart chambers, which contracted cardiac muscle (Huang et al., 2011). Therefore, heart rate may be a sensitive physiological indicator that can reflect the degree of the pollutants to the blood circulation system of *Daphnia magna*. As a result, we recommend heartbeat as an indicator in toxicity evaluation of *Daphnia magna*.

3.2. Mortality, reproductive performance and population growth

After 21 d exposure of different concentrations of PFOS, the results were presented in Table 1. The mortality and reproductive performance has been influenced under different concentrations of PFOS. No death of *Daphnia magna* was observed, when the concentration of PFOS was less than 1 mg L⁻¹. With increased PFOS, almost all *Daphnia magna* died due to poisonous effects.

As shown in Table 1, the difference of days to first brood with control groups has no significance ($p > 0.05$) when PFOS concentrations were from 1 to 4 mg L⁻¹. The endpoints of most reproductive parameters were significantly suppressed by PFOS at 8 mg L⁻¹. Higher concentrations of PFOS delayed time of first litter. This tendency was consistent with earlier observations, in which toxicity usually prolonged the time of flea reproduction (Ji et al., 2008). The quantity of first brood decreased initially and then rose and fell as the concentration increased. At concentration of 2 mg L⁻¹, *Daphnia magna* continuously exposed to PFOS seems to adapt, which may cause a fractional rise.

The intrinsic rate of natural increase is a kind of ecological index, which reflects the comprehensive ability to survive and reproduce. With the increase of exposure concentration, intrinsic rate of natural increase dropped sharply at concentrations from 1 to 16 mg L⁻¹ and reached the significant level at 8 and 16 mg L⁻¹ when compared to control groups. Higher concentrations of PFOS could make intrinsic rate of natural increase lower, threatening the survival and reproduction of the *Daphnia magna* population. As time went on, self-defense mechanism failed to resist toxicity of PFOS. Intrinsic rate of *Daphnia magna* reduced significantly with increasing dose of other toxicants like Propanil, which agrees with the results of Villarroel et al. (2004). The results mentioned above suggest that the growth and reproductive performance of *Daphnia magna* are sensitive to PFOS and can be used to evaluate its toxicity in aquatic ecosystem.

3.3. Antioxidant and neurological responses

GST and CAT were assessed to reflect the response of antioxidant enzymes to PFOS (Gómez-Oliván et al., 2014; Yu et al., 2014). While exposed to toxicants, the body produces oxidative stress and the balance of active oxygen is broken. ChE plays a crucial role in the stability of nervous system and was chosen to be measured and analyzed during 10 d and 21 d exposure periods (Liu et al., 2012).

GST is one of the most important metabolic enzymes, which is also involved in the detoxification (Liu et al., 2006). The enzymatic activity determination was presented in Fig. 3. The GST activity of *Daphnia magna* was increased after a 10 d exposure, by 3.6% compared to the control groups at the concentration of 4 mg L⁻¹. When the PFOS concentration was beyond 4 mg L⁻¹, GST activity was inhibited. While the PFOS concentration was 8 and 16 mg L⁻¹, the GST activity reached the significant level. After a 21 d exposure of *Daphnia magna* to PFOS, the GST activities of different groups

Table 1
Mortality, reproductive and population parameters of *Daphnia magna* after 21 d exposure to PFOS.

PFOS (mg L ⁻¹)	Mortality of parent flea (%)	Days to first brood (d)	Quantity of first brood	Intrinsic rate (r)
0	0	10.30 $\pm$ 0.48	20.60 $\pm$ 5.93	0.2397
1	0	10.80 $\pm$ 0.42	18.40 $\pm$ 6.11	0.2399
2	10	10.89 $\pm$ 0.33	20.78 $\pm$ 5.54	0.2363
4	10	10.89 $\pm$ 0.33	17.67 $\pm$ 5.43	0.2252
8	30	11.00 $\pm$ 0.00**	17.00 $\pm$ 3.74	0.2270**
16	90	11.00 $\pm$ 0.00	11.00 $\pm$ 0.00**	0.1965**

Note: Sample size of each treatment is 10. Values represent mean $\pm$ standard deviation; Asterisk (*) denotes a significant difference from check group ($p < 0.05$), and asterisks (**) denotes an extremely significant difference from check group ($p < 0.01$).

Fig. 3. GST activity of *Daphnia magna* exposed to various concentrations of PFOS after subchronic and chronic tests. Asterisk (*) denotes a significant difference from check group ($p < 0.05$), and asterisks (**) denotes an extremely significant difference from check group ($p < 0.01$).

were lower than corresponding 10 d results. As the exposed concentration increased, the GST activity fell continuously. At the concentration of 8 mg L^{-1} , the GST activity gets to the significant level. The reason is the dead of *Daphnia magna*, which resulted that enzyme activity could not be determined at the concentration of 16 mg L^{-1} . In 21 d chronic tests, the GST activity had the inhibition process, which is similar to 10 d chronic tests. This inhibition process of the GST activity may be explained that toxicants inhibit the production or transportation of oxidative metabolites. The decrease of GST activity was consistent with similar observations in previous findings. For example, a recent research reported that the GST activity of mice exposed to PFOS decreased significantly and agreed well with our result (Zhang et al., 2013). A reduction in GST may indicate *Daphnia magna* failed to detoxicate and resist oxidative stress in the cell (Santos et al., 2004). However, the variation of GST activity can be influenced by many factors, including pH, temperature, and toxicity (Wang et al., 2011). The result of GST assay indicated that GST may be used as a biomarker for the evaluation of organic pollutants in aquatic ecosystem.

CAT is a key enzyme in the antioxidant system and it can be increased under various environmental stress (Alberto et al., 2011). CAT decomposes hydrogen peroxide in metabolic waste and protects organs and cells from injury caused by hydrogen peroxide. After 10 d exposure, the significant changes were not found in enzyme activity of CAT with the concentrations from 1 to 8 mg L^{-1} (Fig. 4). When the concentration of PFOS reached 16 mg L^{-1} , the CAT activity was significantly lower than control groups, and the reduction was 31.5%. *Daphnia magna* has not adapted to the

Fig. 4. CAT activity of *Daphnia magna* exposed to various concentrations of PFOS after subchronic and chronic tests. Asterisk (*) denotes a significant difference from check group ($p < 0.05$), and asterisks (**) denotes an extremely significant difference from check group ($p < 0.01$).

Fig. 5. ChE enzyme activity of *Daphnia magna* exposed to various concentrations of PFOS after chronic tests. Asterisk (*) denotes a significant difference from check group ($p < 0.05$), and asterisks (**) denotes an extremely significant difference from check group ($p < 0.01$).

external environmental pressure, thereby reducing CAT activity. After 21 d exposure, the CAT activity showed a trend of fluctuation. It is noteworthy that CAT activity at 4 and 8 mg L^{-1} of 21 d tests was higher than other treatments, which possibly reflected that PFOS provoked CAT to enhance the ability of scavenging free radicals. More H_2O_2 and O_2 were formed which enhanced this ability and cause a decrease in CAT activity. CAT plays an important part in antioxidant system, which guards the first defense line against oxygen toxicity (Li et al., 2011). When PFOS concentration exceeded the tolerance range, the defense process would be destroyed. However, the mechanism of antioxidant defense system in *Daphnia magna* exposed to PFOS needs to be further explored. Compared to 21 d tests, the 10 d enzyme analysis was propitious to explore dose effect of CAT activity.

The ChE activities assay was presented in Fig. 5. In 10 d exposure to various concentrations of PFOS, the ChE activities of *Daphnia magna* was decreased initially and then increased. There was no significant difference between every treatment and control groups. PFOS, accumulated in a subchronic period, has a slight effect on the conduction of nerve signals. In 21 d exposure to different concentrations of PFOS, the tendency of ChE of *Daphnia magna* activities were similar to 10 d results. In contrast with 10 d results, the change of ChE activity was easy to be observed in the 21 d tests. When the concentration was 2 and 8 mg L^{-1} , the significant decrease was achieved. To ensure neural signal transmission, ChE was hydrolyzed by increasing enzyme activity at the increase of PFOS. When PFOS concentration was higher than 16 mg L^{-1} , almost all *Daphnia magna* were died. This shows that the PFOS concentration exceeded the tolerance range of *Daphnia magna*. The results showed that the activity of ChE in *Daphnia magna* under the action of self-adjustment remains stable in the short term. At the low pollution concentration, neurotransmitter can be normal transmission by self-adjustment. However, nerve transfer process will be affected at the high concentration. Although the functions of neurobehaviours exposed to PFOS are lacking, it is proposed that interactions with neurotransmitter may be used to explain toxicological effects of PFOS on ChE (Gee and Moser, 2008). Therefore, with the support of present findings, the 21 d ChE activity may be applied to the environmental impact assessment of PFOS pollution.

4. Conclusions

Toxicological effects of PFOS on *Daphnia magna* under acute, subchronic and chronic exposure were explored and discussed in this study. In 48 h acute experiment of *Daphnia magna* to various concentrations of PFOS, EC_{50} for immobilization was calculated as

79.35 mg L⁻¹ and the toxicity was classified as intermediate. PFOS can strongly stimulate the *Daphnia Magna*'s heartbeat, making heart rate sensitive to be used as the endpoint of evaluation. In chronic exposure of *Daphnia magna* to PFOS, reproductive parameters, including days to first brood, quantity of first brood and intrinsic rate were well reflecting the toxicity of PFOS. The intrinsic rate were confirmed to be more sensitive than days and quantity of first brood. The activity of GST was significantly inhibited by high concentration of PFOS in 10 d and 21 d exposure. CAT was significantly inhibited in short exposure and antioxidant system of *Daphnia magna* was destroyed in long time exposure to PFOS. Significant inhibition of ChE occurred under the long term exposure of PFOS. At the level of 8 mg L⁻¹ PFOS concentration during sub-chronic and chronic exposure, most of selected evaluated endpoints of *Daphnia magna* have sensitive effects, which should be a call for concern. The results of the present findings may be applied to ecological evaluation of environmental risk of PFOS. It is expected that there may be potential ecological toxicity of PFOS under sufficient exposure time, and the further research on ecological risk caused by PFOS should be undertaken.

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