Chronic Effects of Perfluorooctane Sulfonate and Ammonium Perfluorooctanoate on Biochemical Parameters, Survival and Reproduction of *Daphnia magna*

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Perfluorinated compounds are widespread in the environment, animals, and humans, and have been proposed to be included in the list of persistent organic pollutants that have the potential to cause a global pollution problem similar to that caused by existing persistent organic pollutants. *Daphnia magna* was used to evaluate the chronic effects of the ammonium salt of pentadecafluorooctanoic acid (PFOA) and the potassium salt of perfluorooctane sulfonate (PFOS) on the reproduction and biochemical responses of aquatic animals. Reproduction of *Daphnia magna* was a more sensitive response than survival or enzyme activities in daphnia exposed to PFOA or PFOS. No observed effect concentrations (NOECs) of daphnia reproduction were 1 and 10 mg/l after 21 days of exposure to PFOA and PFOS, respectively. The median lethal concentration (LC_{50}) and survival NOEC values of PFOA for *Daphnia magna* were all greater than 100 mg/l after 21 days of exposure. The LC_{50} and survival NOEC values of PFOS were 9.1 mg/l and 5 mg/l after 21 days exposure. No significant changes in cholinesterase, catalase and heme peroxidase activities were observed between controls and exposure to PFOA or PFOS. This suggests that these enzyme activities are not sensitive biomarkers of exposure to these two chemicals or their effects in daphnids after chronic treatment. Based on the results of this study and other findings published in the literature, it is suggested that current PFOS and PFOA levels in freshwater may have no harmful impact on the aquatic environment. However, more information on the long-term ecological effects of PFOS and PFOA on diverse aquatic species is still needed to provide important information for adequately assessing the ecological risks of PFOS and PFOA.

Key words —— perfluorooctanoic acid, ammonium perfluorooctanoate, *Daphnia magna*, reproduction, ecotoxicological effect

INTRODUCTION

Perfluorinated chemicals have emerged in our modern life over the past several decades as global pollutants that are widely used in numerous commercial and industrial applications as active ingredients, impurities or as degradation products of derivatives.[1,2] Among the commonly occurring perfluorinated substances, perfluorooctane sulfonate (PFOS) and pentadecafluorooctanoic acid (PFOA) are two major environmentally persistent chemicals representing final environmental degradation or metabolism compounds of other perfluorinated products.[1,3] In the past decade, these perfluorinated compounds have been discovered to be widespread in the environment, animals and humans.[4–6] Therefore, these perfluorinated chemicals have been proposed to be included in the list of persistent organic pollutants (POPs) and they have the potential to cause global pollution problems similar to those caused by existing POPs.[7]

Both PFOS- and PFOA-related substances are synthetic chemicals that do not occur naturally in the environment. In the past decade, there has been growing interest and extensive published scientific information on PFOS and PFOA research.[8,9] In particular, most of the published scientific information has involved analysis of the presence of PFOS and PFOA in various environmental media, human or biota.[4–6] On the other hand, ecotoxicological data on the effects of these compounds remain scarce.[10] Both PFOS and PFOA have been found to cause moderate acute toxicity to aquatic organisms based on the limited information available.[3,11,12] Although the acute toxic effects in aquatic animals for these two compounds often occur only at levels higher than those expected to be encountered at environment levels based on current information, it is possible that the risk posed by these compounds is not acute, but is due to long-term ef-

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fects in the aquatic ecosystem.[13] Until now, only a few published studies have investigated the chronic sublethal effects of PFOS and PFOA on aquatic species; including fish, amphibian and aquatic invertebrate.[11, 14–19] The potential long-term ecotoxicological effects of PFOS and/or PFOA are needed to provide important information to adequately assess the ecological risk of PFOS and PFOA, which can be of great concern for aquatic fauna.

With its short lifespan and reproductive capabilities, the water flea *Daphnia magna* has been commonly used as a standard aquatic test species for many years.[20] In particular, *Daphnia magna* can rapidly respond to different environmental changes and is sensitive to a vast majority of toxic substances. Moreover, it is an important trophic component of aquatic food webs as a major food source of fish and invertebrate predators and plays a vital role in transporting energy and nutrients in aquatic ecosystems. In this study, *Daphnia magna* was used to evaluate the chronic effects of the ammonium salt of PFOA and potassium salt of PFOS on the reproduction and biochemical responses of aquatic animals. Biochemical biomarkers have been widely used to reflect the exposure and effects of different environmental pollutants. Haem peroxidase activity determined in *Daphnia magna* is an indirect method to measure oxidase activity of detoxification.[21] Cholinesterase activity serves as a sensitive exposure indicator of neurotoxic pollutants. Increased oxidative stress is thought to be one possible mechanism after exposure of peroxisome proliferators. Catalase is mainly a peroxisomal enzyme and commonly considered as an indicator of oxidative stress. The combined measurement of these three enzyme activities in *Daphnia magna* can help us to understand effects of these chemicals associated with their potential toxic actions. The results of this study can also provide useful information about the chronic toxicity of PFOS and PFOA and help us to assess the potential chronic ecotoxicological effects of PFOS and PFOA in aquatic ecosystems.

MATERIALS AND METHODS

Chemicals —— PFOS (heptadecafluorooctanesulfonic acid potassium salt; > 98%) was obtained from Fluka (Steinheim, Switzerland). The ammonium salt of PFOA (pentadecafluorooctanoic acid ammonium salt; > 98%), also called ammonium perfluorooctanoate, or APFO, was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). American Society for Testing and Materials (ASTM) test medium was prepared with NANOpure DIamond pure water (Barnstead, Dubuque, IA, U.S.A.) containing 0.12 g/l $CaSO_4.2H_2O$, 0.12 g/l $MgSO_4$, 0.192 g/l $NaHCO_3$, and 0.008 g/l KCl. All stock solutions of testing chemicals were prepared in ASTM medium in using polymethyl pentene containers, because these two chemicals can be adsorbed onto glass surfaces.[16] Water solubility for PFOS was 550 mg/l at 24–25°C[22] and for PFOA was 3400 mg/l at 25°C.[23] In this study, stock solutions of PFOS and PFOA were 400 mg/l and 1000 mg/l, respectively. Based on the water solubility of test chemicals and previous acute toxicity results, nominal test concentrations were in the range of 0.5–20 mg/l for PFOS and 1–100 mg/l for PFOA.[12] All biochemical materials for enzyme assays and chemicals for daphnia culture medium were purchased from Sigma-Aldrich.

Test Organisms and Husbandry —— Water flea (*Daphnia magna*) were purchased from local suppliers and maintained in the laboratory for more than one year. Water fleas were fed green algae (*Chlorella* spp.) and half of the medium was renewed with dechlorinated water twice a week. *Chlorella* spp. were obtained from Tungkang Biotechnology Research Center (Fisheries Research Institute, Taiwan). The animals used in the experiments were juveniles less than 24 hours-old originating from the second to fourth brood of stock culture daphnids.

Daphnia Reproduction Test —— The daphnia reproduction test was performed as a semi-static test according to Organization for Economic Cooperation and Development (OECD) test guideline 211.[24] At the start of each test, 10 animals (<24 hr old) were exposed to PFOS at nominal concentrations ranging from 0.5 to 20 mg/l or PFOA at nominal concentrations ranging from 1 to 100 mg/l for a period of 21 days. Five concentrations of the test chemicals and the control (ASTM medium only) were prepared, with 10 replicates for each concentration. Each concentration was prepared by diluting stock solutions with fresh ASTM medium. Each concentration consisted of ten 50-ml polypropylene culture tubes containing 50 ml of test solution and a single test organism. Test solutions were renewed three times weekly. Daphnids were fed *Chlorella* spp. at 1.5×10^7 cells per animal per day. The number of neonates was counted daily by removing the neonates from the tube. Culture tubes were covered

with polyester plastic lids at 20 ± 1°C with a 16 : 8 light : dark cycle in a temperature incubator. Each PFOS or PFOA treatment was conducted three different times. The total test duration was 21 days.

Determination of Enzyme Activities —— After 21 days of PFOS or PFOA treatment, surviving female adults for each concentration were divided into three groups for different enzyme measurements. Each daphnid was individually stored in a microcentrifuge tube and immediately frozen at −80°C until enzyme assay, which was usually within one week. Each daphnid was homogenized in 70 ul 0.1 M phosphate buffer containing 1 mM EDTA and 0.5% Tritox X-100 (pH 7.5) and centrifuged at 12000 × *g* for 20 min at 4°C. The supernatants were used to measure cholinesterase activity. Measurements of cholinesterase activity were determined by a modification of the Ellman method for a microplate.[25] In brief, the reaction mixture contained 30 µl of enzyme extract in 150 µl of 0.05 M sodium phosphate buffer (pH 8.0) containing dithiobisnitrobenzoate (DTNB) at a final concentration of 0.5 mM. After 5 minutes of incubation at room temperature (28±1°C), the reaction was triggered by the addition of 10 µl of 75 mM acetylthiocholine iodide to a 96 well microplate using a Thermo Multidrop 384 high speed automated dispenser. The rate of increase in optical density of the sample mixture was measured using a Thermo Multiskan EX plate reader at 414 nm in 10 second intervals for 120 seconds. The activities were measured in duplicate.

Each daphnid was homogenated in 70 µl of 0.05 M K-phosphate buffer (pH 7.0) with 0.005 M EDTA and centrifuged at 12000 g for 20 minutes at 4°C. The supernatant was used to measure catalase activity according to Aebi's method.[26] In brief, the reaction mixture contained 20 µl of enzyme extract in 480 µl of 0.05 M potassium phosphate buffer (pH 7.0) with 30 mM H_2O_2. The reaction was performed at room temperature (28 ± 1°C) for 60 seconds and catalase activity was determined from the rate of H_2O_2 decrease in absorbance at 240 nm in a Hitachi U2001 UV/VIS spectrophotometer using an extinction coefficient of 39.4 $M^{-1}cm^{-1}$. The activities were measured in duplicate.

Each daphnid was homogenated in 10 µl in 0.25 M sodium acetate buffer (pH 5.0) with 0.1% Triton X-100, added 60 µl of 0.25 M sodium acetate added, and then the mixture was centrifuged at 14000 g for 5 minutes at 4°C. The supernatant was used to determine haem peroxidase activity according to the method of Connon *et al.*[21] as modified from Brogdon *et al.*[27] In brief, the reaction mixture contained 10 µl of enzyme extract in 170 µl of 0.25 M sodium acetate buffer (pH 5.0) containing 3,3′,5,5′-tetramethylbenzidine at a final concentration of 2 mM. After 5 minutes of incubation at room temperature (28 ± 1°C), the reaction was triggered by addition of 20 µl of 1.5% H_2O_2 in 0.25 M sodium acetate (pH 5.0) to a 96-well microplate using a Thermo Multidrop 384 high speed automated dispenser. The rate of increase in optical density of the sample mixture was measured using a Thermo Multiskan EX plate reader at 620 nm in 10 second intervals for 60 seconds. A standard curve for heme peroxidase activities was prepared using cytochrome c. The activities were measured in duplicate and were expressed as cytochrome c equivalents.

The protein concentrations of the samples were measured using Bradford's method with bovine serum albumin as the standard.[28] All protein measurements were performed in triplicate.

Data Analysis —— The nominal concentrations that were lethal to 50% of *Daphnia magna* (LC_{50}) at different exposure periods were calculated using trimmed Spearman-Karber analysis.[29] Trimmed Spearman-Karber analyses were conducted using a trimmed Spearman-Karber Program (version 1.5) obtained from Environmental Monitoring Systems Laboratory (USEPA, Cincinnati, OH, U.S.A.). The no observed effect concentrations (NOECs) were determined by Dunnett's multiple comparison procedure ($p < 0.05$) using a Minitab Statistical Program (version 13.2). For comparison reasons, data were expressed as the ratio of change from the respective control value (taken as 1) for enzyme activities from three separate experiments. Although data are presented graphically as the percentage of control enzyme activities in the absence of treatments, all statistical evaluations were performed on absolute, nonreferenced data using a Minitab Statistical Program (version 13.2). Data were first tested for normality with the Kolmogorov-Smirnoff test and for equality of variance with Bartlett's test. Because most data did not meet the assumptions of normality or homogeneity of variance, differences among treatment groups were analyzed by the nonparametric Kruskal-Wallis test using the Minitab statistical program (version 13.2). If a significant result was found, the Mann-Whitney U test was used to determine which treatment groups were significantly different from the controls. Differences were considered significant if $p < 0.05$.

RESULTS

Daphnia Survival

The LC_{50} and NOEC values of *Daphnia magna* after 21-d exposure to PFOS or PFOA are summarized in Table 1. The LC_{50} and NOEC values of PFOA for *Daphnia magna* were all greater than 100 mg/l during the entire 21 days of exposure (Table 1). The LC_{50} values of PFOS for *Daphnia magna* were >20, 12.5, and 9.1 mg/l for 7, 14, and 21 days exposure respectively. On the other hand, the NOEC values of PFOS for *Daphnia magna* were 10, 5, and 5 mg/l for 7, 14, and 21 days exposure, respectively. After 21 days of exposure, the daphnid survival rate was still 66.7% at the highest PFOA concentration of 100 mg/l, but survival was significantly reduced to 3.3% at the highest PFOS concentration of 20 mg/l (Table 2).

Daphnia Reproduction

Most daphnia reproductive endpoints were significantly affected at PFOA concentrations of 32 mg/l and higher (Table 2), and all daphnia reproductive endpoints were significantly reduced at 100 mg/l (Table 2). None of the daphnids at 100 mg/l PFOA produced a fourth brood and only 3 out of 19 female daphnids at 100 mg/l PFOA produced a third brood during 21 days of exposure. There were statistically significant changes in the number of neonates produced between controls and 100 mg/l PFOA-treated groups for the first to third broods and between controls and 32 mg/l PFOA-treated groups for the first, third, fourth and fifth broods, as shown in Fig. 1 (a). Based on the total number of neonates produced per female, the NOEC of daphnia reproduction was 10 mg/l after 21 days of PFOA treatment.

Because only 1 out of 30 daphnids survived after exposure to a PFOS concentration of 20 mg/l, daphnid reproductive endpoints were determined at concentrations from 0.5–10 mg/l. Exposure of daphnids to PFOS at concentrations of 5 and

Table 1. LC_{50} and NOEC (mg/l) of *Daphnia magna* during 21 Days of PFOS or PFOA Exposure

	PFOS		PFOA	
	LC_{50}[a)] (95% CI; mg/l)	$NOEC_{survival}$[b)] (mg/l)	LC_{50}[a)] (95% CI; mg/l)	$NOEC_{survival}$[b)] (mg/l)
7 days	> 20	10	> 100	> 100
14 days	12.5 (8.1–19.5)	5	> 100	> 100
21 days	9.1 (7.3–11.5)	5	> 100	> 100

a) Determined using trimmed Spearman-Karber analysis. *b*) Determined using Dunnett's test. CI: confidence interval.

Table 2. Survival and Reproduction (Mean ± S.D.) of *Daphnia magna* after 21 Days of PFOS or PFOA Exposure

nominal concentration (mg/l)	survival (n = 30)	mean brood size	days to the first brood	number of brood per female	total neonates produced per female
Control	96.7%	32.5 (5.6)	8.2 (1.1)	4.7 (0.6)	154.7 (38.0)
PFOS					
0.5	93.3%	29.5 (5.9)	8.6 (1.2)	4.7 (0.5)	139.4 (31.4)
1	90.0%	32.0 (6.6)	8.5 (2.2)	4.9 (0.3)	154.9 (30.0)
5	86.7%	28.1 (9.5)	8.9 (1.7)	4.1 (0.8)*	117.0 (52.4)*
10	53.3%	27.7 (7.2)	9.5 (1.9)	4.4 (0.8)	125.1 (50.2)*
20	3.3%	—[a)]	—[a)]	—[a)]	—[a)]
PFOA					
1	96.7%	31.6 (7.0)	8.2 (0.9)	4.7 (0.4)	145.6 (46.3)
3.2	96.7%	31.6 (7.3)	8.8 (1.4)	4.8 (0.6)	150.6 (42.7)
10	83.3%	32.1 (8.6)	8.1 (1.0)	4.7 (0.6)	155.2 (47.1)
32	86.7%	24.8 (7.1)*	8.3 (1.5)	4.3 (0.5)*	106.5 (31.5)*
100	66.7%	8.3 (6.5)*	11.5 (3.0)*	1.4 (0.8)*	10.6 (8.0)*

a) No data. *$p < 0.05$; determined using Mann-Whitney U test.

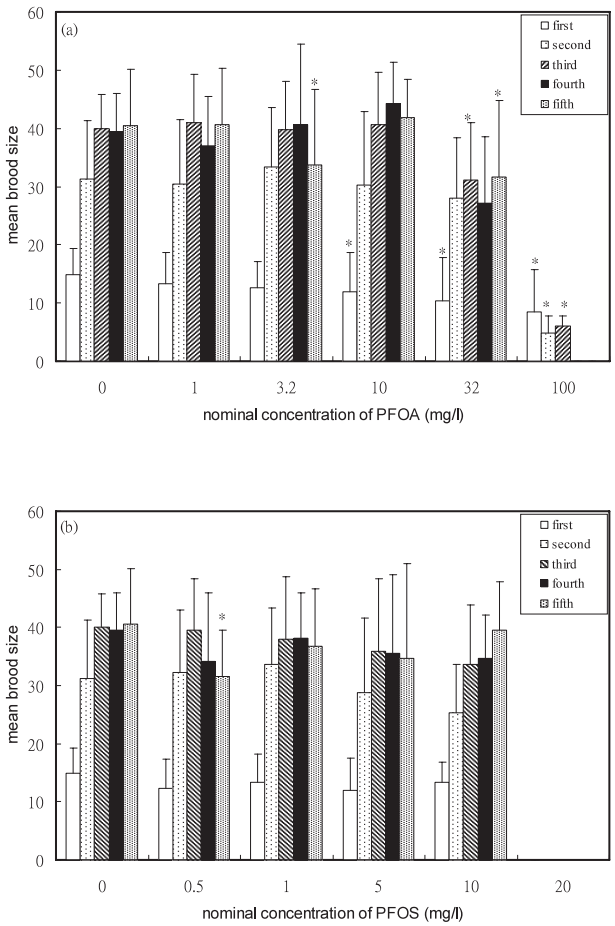

Fig. 1. Effect on Brood Size (Mean ± S.D.) of *Daphnia magna* Exposed to (a) PFOA and (b) PFOS during 21-d Treatment

Asterisks indicate a value significantly different from the control at $p < 0.05$; determined by the Mann-Whitney U test.

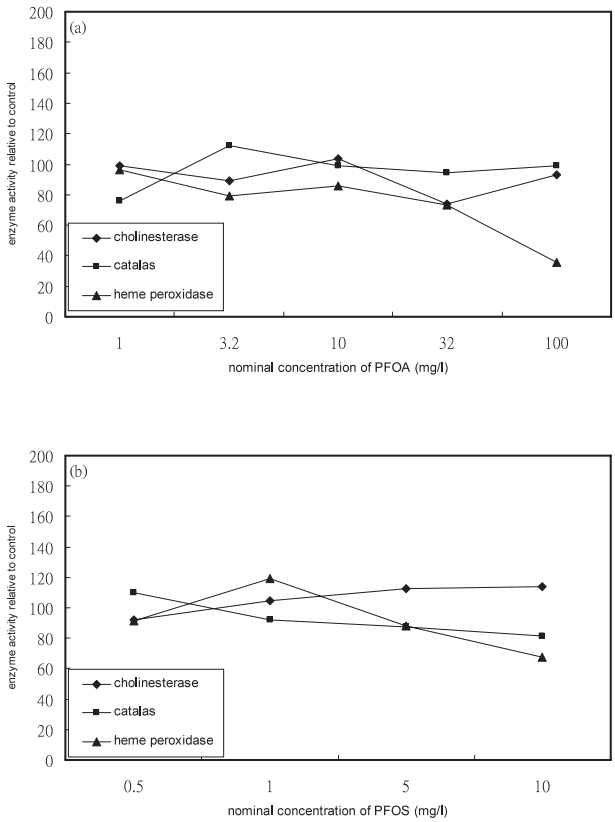

Fig. 2. Enzyme Activities in Female *Daphnia magna* Exposed to (a) PFOA and (b) PFOS after 21-d Treatment

Values are expressed as mean percentage change from the respective control values (taken equal to 100).

10 mg/l showed significant decreases in the total number of neonates produced per female after 21 days of treatment (Table 2). However, there were no significant changes in the number of neonates produced between controls and PFOS-exposed groups for the first to fifth broods, as shown in Fig. 1 (b). In addition, the mean number of broods per female was significantly reduced in *Daphnia magna* exposed to 5 mg/l of PFOS, but not for 10 mg/l of PFOS. Based on the total number of neonates produced per female, the NOEC value of daphnia reproduction was 1 mg/l after 21 days of PFOS treatment.

Biochemical Responses

No significant changes in the activity of any daphnid enzyme analyzed or in protein contents among the different PFOA and PFOS treatments were observed (Fig. 2).

DISCUSSION

The results of this study show that reproduction parameters of *Daphnia magna* were more sensitive endpoints than survival or biochemical responses in daphnia exposed to PFOA or PFOS. After 21 days of PFOA exposure, the survival NOEC was greater than 100 mg/l, while the reproduction NOEC was 10 mg/l in *Daphnia magna*. Compared to recently published studies, the reproduction NOEC value of PFOA found for *Daphnia magna* in this study was similar to the reproduction NOEC value of 20 mg/l reported by Colombo *et al.*[11] and 12.5 mg/l by Ji *et al.*[30] After 21 days of PFOS exposure, the survival NOEC was 5 mg/l, while the reproduction NOEC was 1 mg/l in *Daphnia magna*. The NOEC value of PFOS in this result is also similar to the value of 1.25 mg/l reported by Ji *et al.* for *Daphnia magna*.[30] On the other hand, there were no significant changes in daphnid cholinesterase, catalase and heme peroxidase enzyme activities after 21 days of PFOA or PFOS treatment. Based on the results of

this study, PFOA is less toxic than PFOS to aquatic invertebrates, and this finding is in a good agreement with previous studies.[19, 30)]

The 21-d LC_{50} and survival NOEC values of *Daphnia magna* exposed to PFOA in this study are greater than 100 mg/l and are similar to those reported by Colombo *et al.*[11)] In their recently published study, the EC_{50} and survival NOEC of *Daphnia magna* were reported to be greater than 88.6 mg/l after 21 days exposure. Compared with published data on the chronic toxicity of PFOS, the 21-d survival NOEC of 5 mg/l found for *Daphnia magna* in this study is similar to the value of 5.3 mg/l for *Daphnia magna* in the study of Boudreau *et al.*,[16)] but the 21-d LC_{50} of 9.1 mg/l found for *Daphnia magna* in this study is lower than the 21-d LC_{50} of 42.9 mg/l for *Daphnia magna* reported in the same study.[16)] In addition, MacDonald *et al.* reported a 10-d survival LC_{50} of 0.045 mg/l for the aquatic midge (*Chironomous tentans*).[17)] Although *Daphnia magna* is an important species for ecotoxicological study, it may not be a sensitive test species for PFOS or PFOA toxicity and some less common aquatic test species should be considered for chronic toxicity tests.

Peroxisome proliferators are a diverse group of environmental pollutants that can cause peroxisome proliferation in different fish or bivalve mollusces species via peroxisome-proliferator activated receptors.[31)] Both PFOS and PFOA are peroxisome proliferators in rodents,[32)] but less common in fish[14)] or aquatic invertebrates. For example, there were no significant effects on peroxisomal catalase and palmitoyl CoA oxidase activities in PFOS-treated carp after 5 days of exposure.[33)] On the other hand, significant catalase induction was found in primary cultured hepatocytes of freshwater tilapia (*Oreochromis niloticus*) after 24 hr exposure to 30 mg/l PFOS and PFOA treatments.[34)] In addition, increases in catalase activity in planaria exposed to PFOS at nominal concentrations of 5 or 10 mg/l and decreases in cholinesterase activity in planarians exposed to PFOS at a nominal concentration of 10 mg/l and to PFOA at nominal concentrations of 50 or 100 mg/l after 2-d exposure were reported.[35)] In this study, no significant changes in cholinesterase, catalase or heme peroxidase activities of *Daphnia magna* were observed, while reproductive parameters and survival rates were adversely affected by high concentrations of PFOS or PFOA. Biochemical and physiological biomarkers of organisms can provide useful information on the possible toxic actions of different compounds as well as the potential impact of contaminants on the health of organisms.[36)] This suggests that these enzyme activities are not sensitive biomarkers of exposure to these two chemicals or their effect in daphnids after chronic treatment. Alternatively, the induction or inhibition of daphnid enzyme activities may have occurred during an early period of exposure and broken down before measurements. Short-term bioassays with biochemical biomarkers may be more able to identify early responses of organisms than long-term toxicity testing under environmental pollution conditions.

PFOS is typically present in freshwater from low ng/l to hundreds of ng/l concentrations, whereas PFOA is usually detected in freshwater from high pg/l to low ng/l levels.[37, 38)] Interestingly, environmental PFOS levels are usually at least one order of magnitude higher than PFOA levels in surface water, while the toxicity of PFOS is also one order of magnitude higher than the toxicity of PFOA based on the effects on survival and reproduction in *Daphnia magna*. In relation to ecological effects of measured PFOS and PFOA levels, the risk assessment defines the predicted no-effect concentration (PNEC), which is based on standard toxicity data on LC_{50} or NOEC with the application of a safety factor, and this can be used to evaluate possible ecological effects. The 21-d reproduction NOEC values of PFOS and PFOA found in this study were 1 mg/l and 10 mg/l, respectively. Using these NOECs divided by a safety factor of 10 to calculate PNECs, the PNECs were determined to be 0.1 mg/l for PFOS and 1 mg/l for PFOA. More long-term studies on different aquatic organisms are needed before concluding that current PFOS and PFOA levels in the freshwater environment have no harmful impact on the aquatic environment. The potential of PFOS and/or PFOA for endocrine disruption and other chronically ecotoxicological effects at lower concentrations may be still of great concern for aquatic fauna and merit further investigation.

In conclusion, the reproduction of *Daphnia magna* was a more sensitive response than survival or enzyme activity in daphnia exposed to PFOA or PFOS. *Daphnia magna* is an important species for ecotoxicological study, but it may not be a sensitive test species for PFOS or PFOA toxicity. Information on the long-term ecological effects of PFOS and PFOA on diverse aquatic species is still needed to provide important data for adequately assessing the ecological risks of PFOS and PFOA.

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