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Integrated assessment of biomarker responses in common carp (*Cyprinus carpio*) exposed to perfluorinated organic compounds

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A B S T R A C T

In this study, the toxicological effects of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) toward the common carp, *Cyprinus carpio*, were evaluated by assessing the responses of five biomarkers, including DNA single-strand breaks (COMET), vitellogenin (VTG) concentration, and the activities of 7-ethoxyresorufin-*O*-deethylase (EROD), acetylcholinesterase (AChE) and catalase (CAT). Upon PFOA exposure, both the VTG concentration and CAT activity were significantly increased, while there was a negligible change in the responses of other biomarkers when compared to the control. Upon PFOS exposure, a significant increase in the DNA single-strand breaks was observed, while the responses of other biomarkers were not significantly altered when compared to the control. Standardized scores of biomarker responses were visualized using star plots and computed as the integrated biomarker response (IBR). As expected from the different biomarker responses, PFOA and PFOS showed totally different patterns of star plots. Additionally, the IBR values were well correlated with the logarithmic concentrations of PFOA and PFOS ($R^2 = 0.9434$ and 0.9511, respectively). These results suggest that the IBR might be a useful tool for quantification of various biomarker responses induced by toxic chemicals.

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1. Introduction

Perfluorinated organic compounds (PFOCs) have been widely used as lubricants, paints, cosmetics and fire-fighting foams [1]. These compounds have a high-energy carbon–fluorine (C–F) bond that is resistant to hydrolysis, photolysis, microbial degradation and metabolism, which makes them environmentally persistent [2]. Among the PFOCs, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) have been detected in the environment and a variety of living organisms worldwide [3–5].

PFOA and PFOS have been found to increase the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) while decreasing the activities of glutathione peroxidase and glutathione-S-transferase (GST), suggesting that these compounds play an important role in the production of reactive oxygen species (ROS) [6]. It has also been demonstrated that PFOA exposure induced substantial DNA damage in Hep G2 (human hepatocellular liver carcinoma cell line) cells [7], and that it might alter plasma concentrations of both steroidal androgens and estrogens in fathead minnows [8]. Experiments with rats exposed to PFOS have revealed that it can decrease sperm production and increase the rate of sperm deformity in male rats [9]. However, studies of the toxicological effects of PFOA and PFOS in fish species have been limited to date [10–12].

Biochemical endpoints (biomarkers) can provide valuable information regarding the working mechanism of toxic compounds and be used as early reporters relative to endpoints at higher levels of biological organization. Consequently, various biomarkers in fish species have been used as a tool for ecotoxicological assessments [13]. Given that more than one biomarker response is generally observed by exposure to toxic compounds, the use of a battery of biomarkers is likely preferred and integration of the biomarker battery is one of the key challenges [14–16].

Therefore, the goals of this study were: (1) to evaluate the responses of five biomarkers, 7-ethoxyresorufin-*O*-deethylase (EROD), DNA single-strand breaks (COMET), acetylcholinesterase (AChE), vitellogenin (VTG) and catalase (CAT), in common carp (*Cyprinus carpio*) exposed to PFOA and PFOS and (2) to develop an integrated biomarker response (IBR) index and star plot for interpretation of those biomarker responses. The common carp (*C. carpio*) was used in this study because this fish is one of the most extensively used species for monitoring of freshwater contamination [17,18].

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Table 1
Mean length (ML), mean weight (MW) and hepatosomatic index (HSI) in *Cyprinus carpio* exposed to perfluorooctanoic acid (PFOA) for 4 days.

Nominal concentration (μg/L)	Mean ± SD (% nominal concentration)	ML ± SD (cm)	MW ± SD (g)	HSI ± SD (%)
Control	ND[a]	13.4 ± 0.9	27.8 ± 5.2	0.7 ± 0.2
50	41 ± 4 (81)	13.5 ± 0.6	28.1 ± 3.8	0.8 ± 0.2
500	483 ± 69 (97)	13.5 ± 0.7	26.4 ± 3.5	0.8 ± 0.2
5,000	6,582 ± 172 (138)	13.8 ± 0.7	29.7 ± 4.3	0.8 ± 0.2
50,000	55,565 ± 8427 (118)	13.6 ± 0.6	27.4 ± 2.9	0.8 ± 0.2

[a] Not detected.

2. Materials and methods

2.1. Test chemicals and fish

Perfluorooctanoic acid (PFOA, 99.8% pure) and perfluorooctane sulfonate (PFOS, 100.3% pure) were obtained from Sigma–Aldrich. Stock solutions of the PFOA and PFOS were prepared in N,N-dimethylformamide (<100 mg/L) and diluted with carbon-filtered and dechlorinated tap water to give nominal concentrations of 50, 500, 5000 and 50,000 μg/L. Dechlorinated tap water was used as a control. The exposure concentrations of PFOA and PFOS ranged from 81 to 138% and 90 to 124% of the nominal concentrations, respectively (Tables 1 and 2). Because not all concentrations were within ±20% of the nominal concentrations, the average of the measured concentrations of PFOA and PFOS was used when appropriate [19].

Common carp (*C. carpio*) were obtained from the Chungcheongnam-do Experimental Station for Inland Waters Development (Nonsan City, Republic of Korea) and held in 2000 L tanks with flowing water at 23 ± 2 °C. The fish in the culture tanks were fed once a day with commercial fish food (Fishtop feed No. 2®, Woosung Feed, Republic of Korea). The fish were starved at least for 24 h to ensure gut clearance before the exposure experiments.

2.2. Exposure experiment

A flow-through system receiving carbon-filtered and dechlorinated tap water (pH, 6.9; alkalinity, 28.0 mg/L as $CaCO_3$; total hardness, 47.8 mg/L as $CaCO_3$) was used in this study. In each aquarium (100 L), the water flow was set at a rate to achieve at least two complete turnovers per day. To avoid any effects from chemicals other than the tested compounds, all exposure systems were made with glass, Teflon® and stainless steel components. PFOA and PFOS were delivered to the aquaria from the concentrated stock solutions using syringe pumps (Kloen Co. Ltd., USA). The flow of the PFOA and PFOS into the test vessels was regulated to maintain the nominal concentrations. Ten fish were held in each exposure tank under a 16 h:8 h light: dark photoperiod and the water temperature was maintained at 23 ± 1 °C. Fish were not fed during the tests to minimize the loss of chemical concentrations in the water via adsorption to organic particulates.

After 4 days of exposure, all fish were removed from the tanks and then blotted on filter papers, weighed (total weight) and measured (total length). The liver tissues were dissected and weights were taken to determine the individual fish hepatosomatic index (HSI). The HSI was calculated as the liver weight (g)/whole fish weight (g) × 100. The sex of each fish was determined by observing the gonad. Liver, brain, and blood samples from each fish were taken and stored in eppendorf tubes at −80 °C.

2.3. PFOA and PFOS analyses

PFOA and PFOS concentrations were measured using combined liquid chromatography–mass–mass spectrometry (LC/MS/MS) according to Giesy and Kannan [4]. HPLC was conducted using a RRL system (Agilent, USA) connected to a 6410B triple quadruple mass spectrometer (Agilent, USA). Aliquots of 5 μL were loaded onto a C18 guard column (5 μm, 2.1 mm × 7.5 mm, Alltech, USA). The analysis was conducted using a Zorbax Eclipse XDB C_{18} column (5 μm, 2.1 mm × 150 mm, Alltech, USA) at a flow rate of 0.4 mL/min. The mobile phase was 10 mM NH_4OAc (A)/CH_3OH (B). A gradient elution was used starting at 5% B and going to 95% B in 3 min. After 5 min, the initial conditions were resumed. PFOA and PFOS were measured under negative electrospray ionization using multiple reactant monitoring (MRM, m/z 413 → 369/499 → 80). The dwell time was 0.1 s and the gragmentor was set at 90 V for PFOA and 200 V for PFOS, while the collision energy was 2 V for PFOA and 55 V for PFOS. The ES-capillary voltage was set at −3.5 kV, while the gas temperature was 350 °C, the gas flow was 10 L/min and the nebulizer pressure was 40 psi.

2.4. Ethoxyresorufin-O-deethylase (EROD) activity

Liver samples were homogenized on ice with 5 volumes of phosphate buffer (50 mM, pH 7.8) and then centrifuged at 73,000 × *g* for 30 min at 4 °C. The supernatant was then centrifuged again at 16,000 × *g* for 60 min. Next, the pellet (microsomes) was suspended in phosphate buffer, after which the ethoxy resorufin-*O*-deethylase (EROD) activities in the microsomes were measured in the reaction product (resorufin) using a fluorescence plate reader (Fluoroskan Ascent, Thermo Labsystems, Finland) with excitation and emission filters set at 530 and 590 nm, respectively. The protein concentrations in the samples were measured by fluorescamine assay [20].

2.5. DNA single-strand breaks

The single cell gel electrophoresis assay (COMET assay) with fish blood cells was conducted following previously published methods [21]. Fish blood cells were dispersed and immobilized onto agarose gel coated on microscope slides, which were then placed in a solution to lyse and disperse the cell components, leaving the DNA immobilized in the agarose. Following electrophoresis, the slides

Table 2
Mean length (ML), mean weight (MW) and hepatosomatic index (HSI) in *Cyprinus carpio* exposed to perfluorooctane sulfonate (PFOS) for 4 days.

Nominal concentration (μg/L)	Mean ± SD (% nominal concentration)	ML ± SD (cm)	MW ± SD (g)	HSI ± SD (%)
Control	ND[a]	11.9 ± 0.5	19.0 ± 1.9	0.8 ± 0.1
50	45 ± 3 (90)	12.1 ± 0.6	21.2 ± 3.4	0.8 ± 0.1
500	620 ± 41 (124)	12.3 ± 0.5	21.0 ± 2.9	0.7 ± 0.2
5,000	5,395 ± 119 (108)	12.0 ± 0.4	19.8 ± 1.8	0.7 ± 0.1
50,000	48,242 ± 1129 (96)	12.3 ± 0.6	21.2 ± 2.6	0.7 ± 0.2

[a] Not detected.

were rinsed in a neutral buffer and the gel and its contents were fixed using ethanol. The DNA in the fixed slides was then stained with ethidium bromide. A computerized image analysis system (Komet version 4.01, Kinetic Imaging Ltd., UK) was used to determine the tail moment, which is the product of the percentage of DNA in the tail and the tail length.

2.6. Acetylcholinesterase (AChE) activity

Brain samples were thawed, homogenized in ice with 5–10 volumes of phosphate buffer (0.1 M, pH 7.6), and then centrifuged at 10,000 × g for 20 min. The supernatant (postmitochondrial supernatant, PMS) was used to assay the acetylcholinesterase (AChE) activities. AChE activities were expressed in terms of PMS protein contents determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, USA), with bovine serum albumin as a standard. Activities of the PMS toward the diagnostic substrate acetylthiocholine were assayed using the modified Ellman method [22]. A microplate reader method was used based on the 415 nm absorbance measurements.

2.7. Vitellogenin (VTG) concentration

Blood samples taken from each fish were centrifuged at 3000 × g for 60 min at 4 °C. The supernatant (plasma) was then collected and frozen at −80 °C for later ELISA analysis. The vitellogenin (VTG) concentrations were measured using a carp VTG enzyme-linked immumosorbent assay kit (Biosense Lab., Norway).

2.8. Catalase (CAT) activity

Liver samples were homogenized on ice with 5–10 volumes of phosphate buffer (50 mM, pH 7.0), after which they were centrifuged at 10,000 × g for 15 min. The supernatant was then used to assay the catalase (CAT) activity, which was measured based on the decrease in absorbance at 240 nm due to H_2O_2 consumption (ε^{mM} = 0.0436) following the method described by Aebi [23].

2.9. Integrated biomarker response

The integrated biomarker response (IBR) was evaluated according to Beliaff and Burgeot, with some modifications [14]. Briefly, data were standardized to allow direct visual comparison of the biomarker responses at the test concentrations. The standardized data (Y) were calculated as:

$$Y = \frac{X - m}{s}$$

where X = the value of each biomarker responses; m = the mean value of the biomarker; s = the standard deviation of the biomarker.

The minimum value (min) for each biomarker was obtained from the standardized data (Y). Finally, the score (S) was computed as $S = Y + |min|$, where $S \geq 0$ and $|min|$ is the absolute value.

Star plots were then used to visualize the biomarker results [14]. A star plot radius coordinate represents the score of a given biomarker. When the S_i and the S_{i+1} are assigned as two consecutive clockwise scores of a given star plot, n is assigned as the number of radii corresponding to the biomarkers. Thus, the area A_i obtained by connecting the ith and the $(i+1)$th radius coordinates can be calculated as:

$$A_i = \frac{S_i}{2} \sin\beta(S_i \cos\beta + S_{i+1} \sin\beta)$$

where $\beta = Arc\ \tan(S_{i+1} \sin\alpha / S_i - S_{i+1} \cos\alpha)$; $\alpha = 2\prod/n$; $S_{n+1} = S_1$.

The total area corresponding to a given chemical (IBR value) was obtained as:

$$\mathrm{IBR} = \sum_{i=1}^{n} A_i$$

where n = the number of biomarkers.

2.10. Statistical analyses

Statistical analyses were conducted using the SPSS statistical package program (ver. 10.0). One-way ANOVA was used to compare variables between the control and the treatments. The significance level was set at $P < 0.05$. Duncan's multiple range test was conducted to identify significant differences among groups.

3. Results and discussion

3.1. Biomarker responses in common carp

As shown in Tables 1 and 2, there were no significant differences in the length, mass and hepatosomatic index (HSI) of common carp among treatment groups ($P < 0.05$) after 4 days of exposure to PFOA and PFOS. Du et al. demonstrated that 250 μg/L PFOS significantly reduced both the body weight and length of zebrafish after 70 days of exposure [10]. Thus it seems that PFOA and PFOS did not affect the general fitness of common carp due to the shorter duration of exposure. However, the early biochemical effects (biomarker responses) in common carp exposed to PFOA and PFOS were significant (Fig. 1). The overall results showed that PFOA markedly increased the VTG and CAT levels, while PFOS highly induced DNA single-strand breaks when compared to the control.

Under PFOA exposure, there was no substantial induction of DNA single-strand breaks when compared to the control (Fig. 1a). However, DNA single-strand breaks were significantly induced in the presence of PFOS ($P < 0.05$), as indicated by an increase to 61.4 and 93.1% at nominal concentrations of 5,000 and 50,000 μg/L, respectively. These results suggest that PFOS is likely more genotoxic than PFOA toward the common carp. These findings are consistent with those of a previous study in which PFOS was found to be able to activate the *DinD* gene, which is inducible by DNA damage in *E. coli* [24]. Additionally, Hoff et al. reported that PFOS could influence the average DNA base-pair length in *C. carpio*, suggesting that PFOS interferes with the homeostasis of DNA metabolism [12].

As shown in Fig. 1b, the hepatic EROD activity was substantially induced by PFOA, but the increase was not statistically significant ($P < 0.05$). Recently, Watanabe et al. found that 0.1, 1 and 10 μM PFOA did not affect the EROD activity in chicken embryo hepatocytes [25]. Guruge et al. also demonstrated that PFOA exposure did not induce the CYP1A gene in rats [26]. Similar to PFOA, PFOS did not have a significant effect on the EROD activity. Hu et al. also found that PFOS alone did not induce cytochrome CYP1A as measured by EROD activity [27]. In addition, Krøvel et al. reported that there was no evidence for direct aryl hydrocarbon receptor (AHR)-mediated gene expression responses of PFOS when PFOS was administered to salmon hepatocytes alone [28].

The activity of AChE was not influenced by PFOA or PFOS exposure (Fig. 1c). These findings are somewhat different from those of a previous study in which PFOS was related to the regulation of norepinephrine concentrations in the central nervous system of rats [29]. Furthermore, Mulkiewicz et al. reported that AChE activity was inhibited by 76.2% in response to treatment with 4,000 μM PFOA in an *in vitro* test system, while there was no substantial inhibition at lower concentrations [30].

As seen in Fig. 1d, the VTG levels in male fish exposed to PFOA significantly increased in a concentration-dependent manner when

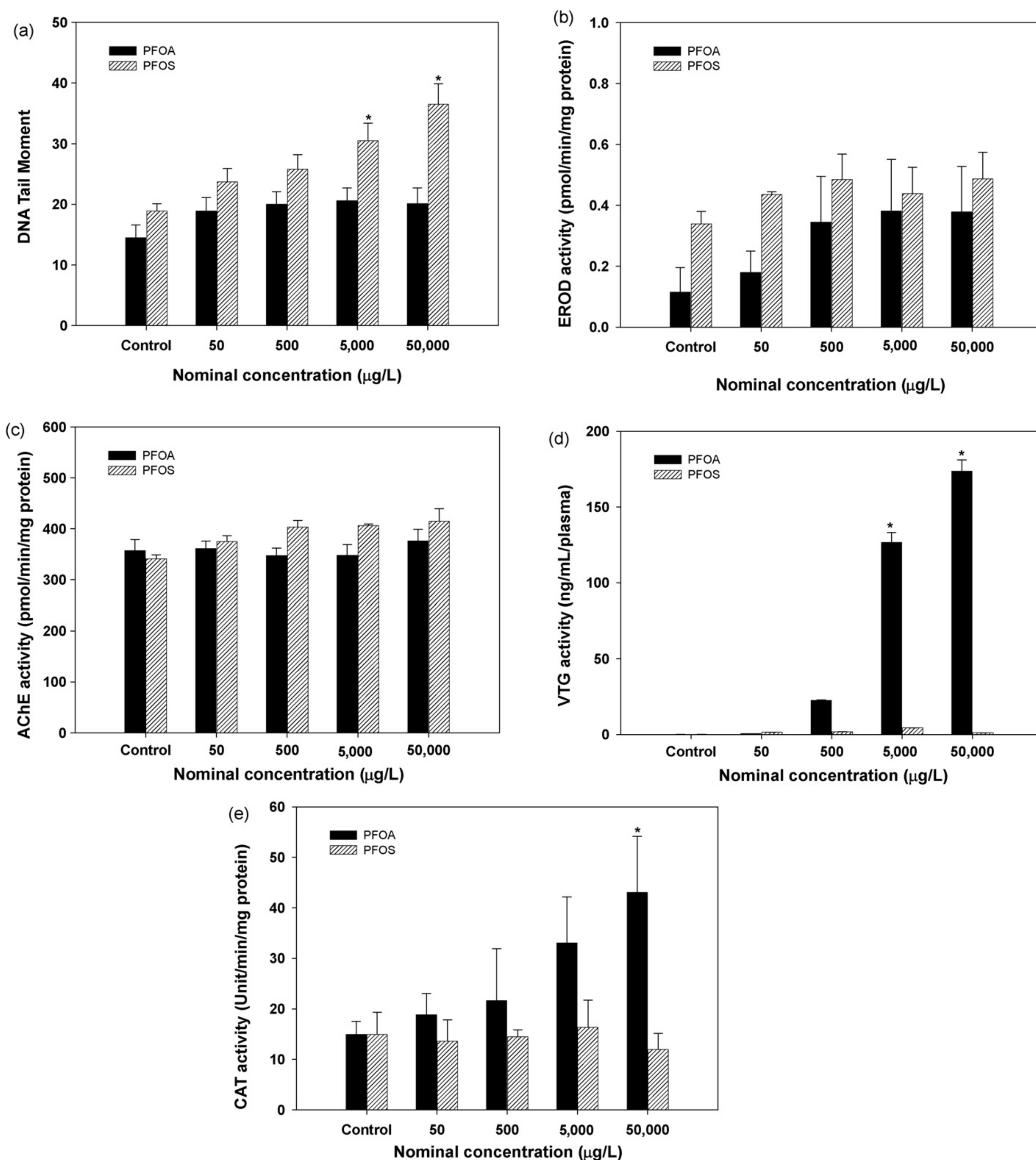

Fig. 1. Biomarker responses in *Cyprinus carpio* exposed to perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) for 4 days: (a) DNA single-strand breaks (COMET assay); (b) ethoxyresorufin-*O*-deethylase (EROD) activity; (c) acetylcholinesterase (AChE) activity; (d) vitellogenin (VTG) concentration; and (e) catalase (CAT) activity. Values represent the mean ± standard error ($n = 10$). Significant differences ($P < 0.05$) are marked with an asterisk.

compared to the control group ($P < 0.05$). Wei et al. reported that hepatic VTG levels were significantly elevated in male rare minnows exposed to 10 and 30 mg/L of PFOA for 14 days [31]. However, PFOS did not significantly alter the VTG levels. These results suggest that the estrogenic effect of PFOA is much greater than that of PFOS.

Similar to the results of VTG activity, the CAT activity was induced only by PFOA (Fig. 1e). These results were likely due to the production of reactive oxygen species (ROS) and subsequent antioxidative responses in hepatocytes of carp. Sohlenius et al. demonstrated that catalase activity was significantly upregulated in the liver of mice exposed to PFOA [32]. Additionally, several *in vitro* tests have shown that both PFOA and PFOS induced catalase activity [6,7]. However, opposite results or no effects were also observed in other studies [33,34].

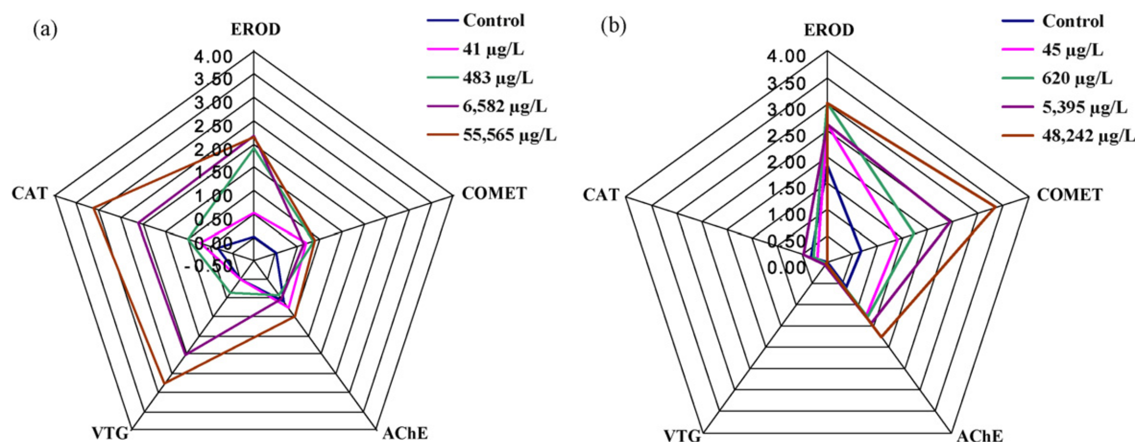

Fig. 2. Star plots for biomarker responses in *Cyprinus carpio* exposed to (a) perfluorooctanoic acid (PFOA) and (b) perfluorooctane sulfonate (PFOS) for 4 days (EROD = ethoxyresorufin-*O*-deethylase; COMET = DNA single-strand breaks; AChE = acetylcholinesterase; VTG = vitellogenin; CAT = catalase).

Table 3
Standardized biomarker responses and integrated biomarker response (IBR) values in *Cyprinus carpio* exposed to perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS).

Exposure concentration (μg/L)	Score of biomarkers					IBR value
	EROD	COMET	AChE	VTG	CAT	
PFOA						
Control	0.00	0.00	0.60	0.00	0.32	0
41	0.52	0.67	0.74	0.01	0.71	0.69
483	1.92	0.84	0.40	0.36	0.99	2.33
6,582	2.15	0.62	0.50	2.01	2.12	6.34
55,565	2.13	0.85	1.00	2.76	3.12	11.42
PFOS						
Control	1.81	0.67	0.60	0.00	0.32	1.15
45	2.60	1.40	1.27	0.02	0.19	3.25
620	2.99	1.72	1.31	0.03	0.27	4.52
5,395	2.61	2.43	1.42	0.07	0.46	6.14
48,242	3.01	3.35	1.76	0.02	0.00	8.93

EROD = ethoxyresorufin-*O*-deethylase; COMET = DNA single-strand breaks; AChE = acetylcholinesterase; VTG = vitellogenin; CAT = catalase.

3.2. Integration of biomarker responses

As shown in Fig. 1, the biomarker responses in common carp were completely different in response to PFOA and PFOS. Thus, for comparison, five biomarker responses were standardized (Table 3) and presented as star plots (Fig. 2). PFOA and PFOS gave distinct patterns of star plots, which can be a useful tool for identification of these chemicals in the environment. Beliaeff and Brugeot reported that there was a reasonable agreement between the concentrations of PAH and PCB and patterns of the star plot for the Seine Estuary [14].

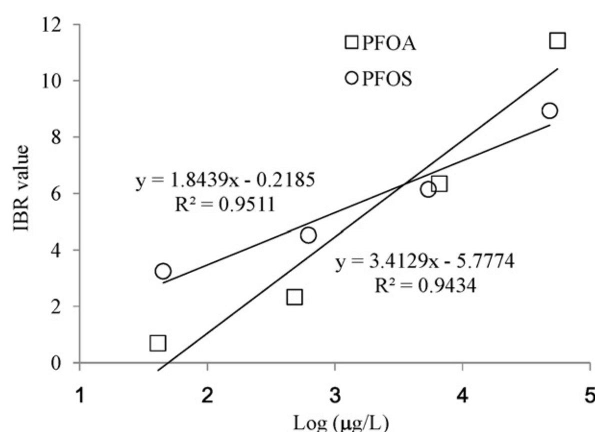

Fig. 3. Relationship between IBR values and exposure concentrations of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS).

The integrated biomarker response (IBR) values were computed as the star plot area and given in Table 3. As the exposure concentrations of PFOA and PFOS increased, the IBR values tended to increase. Given that the IBR is an indicator of environmental stress, PFOS appeared to be more stressful than PFOA toward the common carp at low exposure levels. Additionally, there was a quantitative relationship between the IBR values and PFOA and PFOS concentrations (Fig. 3). These findings suggest that the integrated biomarker responses may serve as a useful tool for quantitative monitoring of the toxicological effects of perfluorinated organic compounds toward fish.

4. Conclusions

The results of this study showed that PFOA exposure induced vitellogenesis and antioxidative stress in the common carp, *C. carpio*, while PFOS induced DNA damage, suggesting that those biological effects should be addressed in ecological risk assessments of PFOCs in fish. In addition, star plots of standardized biomarker responses and the corresponding IBR index were found to be useful for quantitative assessment of the toxicological effects of PFOA and PFOS in the common carp. Thus, further studies are needed to obtain typical biomarker profiles of different types of PFOCs and for the application of this technique in the field.

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