Environmental Science and Pollution Research (2024) 31:26646–26664
https://doi.org/10.1007/s11356-024-32705-3

RESEARCH ARTICLE

Ecotoxicity of perfluorooctanoic acid and perfluorooctane sulfonate on aquatic plant *Vallisneria natans*

Zhen Lin[1] · Wei Zhou[1] · Zhen Ke[1] · Zhonghua Wu[1]

Received: 18 December 2023 / Accepted: 25 February 2024 / Published online: 7 March 2024

Abstract

Perfluorinated compounds (PFCs) are persistent organic contaminants that are highly toxic to the environment and bioaccumulate, but their ecotoxic effects on aquatic plants remain unclear. In this study, the submerged plant *Vallisneria natans* was treated with short-term (7 days) and long-term (21 days) exposures to perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) at concentrations of 0, 0.01, 0.1, 1.0, 5.0, and 10 mg/L, respectively. The results showed that both high concentrations of PFOA and PFOS inhibited the growth of *V. natans* and triggered the increase in photosynthetic pigment content in leaves. The oxidative damage occurred mainly in leaves, but both leaves and roots gradually built up tolerance during the stress process without serious membrane damage. Both leaves and roots replied to short-term stress by activating superoxide dismutase (SOD), catalase (CAT) and polyphenol oxidase (PPO), while peroxidase (POD) was involved under high concentration stress with increasing exposure time. Leaves showed a dose–effect relationship in integrated biomarker response (IBR) values under short-term exposure, and the sensitivity of roots and leaves to PFOS was higher than that of PFOA. Our findings help to increase knowledge of the toxic effects of PFCs and have important reference value for risk assessment and environmental remediation of PFCs in the aquatic ecosystem.

Keywords PFOA · PFOS · Ecotoxic effects · Submerged plants · Oxidative stress · IBR

Introduction

Perfluorinated compounds (PFCs) are a class of synthetic organic composites in which the hydrogen atoms attached to the carbon in the molecule are replaced by fluorine atoms, they are known for their persistent and hard-to-degrade nature, causing environmental and human health concerns worldwide (Pérez et al. 2014; Chen et al. 2022). Due to the long carbon chains of some PFCs, they produce more types of pollutants through their manufacture, transportation, use, and replacement in the environment uncontrollably, causing further pollution (Eriksson et al. 2017). Many countries and regions have reported the concentrations and rates of detection of PFCs in diverse environments such as the atmosphere (Rodríguez-Varela et al. 2021), soil (Lu et al. 2020) and water (Zhang et al. 2013). The bioaccumulation and biomagnification capacity of PFCs allows their enrichment into humans along the food chain through multiple exposure pathways (Islam et al. 2018). Meanwhile, PFCs have been shown to be strongly carcinogenic (Zhang et al. 2011), immunotoxic (Grandjean and Budtz-Jørgensen 2013), and developmentally toxic (Lau et al. 2004) in numerous animal toxicology experiments. Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are two typical PFCs and have gained significant attention. Research shows that there has been more than 700 tons of PFOA released into the environment so far. Similarly, the total direct and indirect emissions of PFOS have exceeded 4930 tons in 1958–2015 (Wang et al. 2017).

There are two main sources of PFOA and PFOS in the aquatic ecosystem, one is the direct discharge of PFOA and PFOS containing effluents, and the other is generated by the decomposition or transformation of precursors originally present in the natural environment, such as the degradation of long-chain fluoroalkanes (Eriksson et al. 2017),

Responsible Editor: Gangrong Shi

Zhen Lin and Wei Zhou contributed equally to this work.

✉ Zhonghua Wu
wuzhonghua@whu.edu.cn

[1] Water Pollution Ecology Laboratory, College of Life Sciences, Wuhan University, Wuhan 430072, People's Republic of China

and the former accounts for a greater proportion (Filipovic et al. 2013). Currently, PFOA and PFOS have been detected worldwide in various water sources such as surface water, wastewater, and groundwater (Dreyer et al. 2010). In China, the detection rate and concentration of PFOA and PFOS in water maintain high, the study of Lu (2018) showed that the detected concentration of PFOA near the fluorine Industrial Park had reached 613 μg/L, causing pollution to the water environment. Similarly, the PFOS in wastewater intake from a per- and polyfluoroalkyl substances (PFASs) manufacturing facility had reached as high as 1021 mg/L (Wang et al. 2010). Previous studies have shown that PFOA and PFOS concentrations were essentially 1 μg/L to 100 mg/L (Jeong et al. 2016; Li et al. 2017). Since the environmental detection concentration characterizes their most direct toxic effects on the subjects, and both PFOA and PFOS can remain stable in the environment for a long time, it is important to conduct studies on the toxicological effects of PFOA and PFOS at higher concentrations and long-term contact to accurately assess their ecotoxicological effects.

Exposure to PFOA or PFOS causes oxidative damage in aquatic plants, inducing the production and accumulation of ROS such as superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical (-OH). The accumulation of ROS triggers the activation of antioxidant defense systems, including enzymatic and non-enzymatic antioxidant systems. The enzymatic antioxidants play a crucial role in the plant's antioxidant defense system. Multiple antioxidant enzymes in the cell, such as catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO), and superoxide dismutase (SOD), work together to protect the plant body from damage by resisting cellular peroxidation. The response of enzymatic antioxidants to PFOA and PFOS in plants reported in the literature varies widely and has not been harmonized.

Biomarkers are signal indicators of abnormalities at different biological levels (molecular, cellular, individual, etc.) caused by environmental pollutants before they cause serious damage to organisms. The value can directly reflect the damage caused by exogenous pollutants, such as heavy metals, microplastics, nano-pollutants, and organic pollutants, to biological life activities in the environment (Wang et al. 2008). Changes in the structure and function of cellular molecules, abnormalities in biochemical metabolism and physiologically active substances, and abnormal changes in individual behavior and populations or communities can be used as biomarkers (Kosaka et al. 2010). Macromolecular biomarkers such as antioxidant enzymes and lipid peroxidation products have both inhibitory and inducible effects in response to exogenous compound stress, and there are spatial and temporal differences in the response of various enzymatic activities, making them one of the most commonly used markers. Therefore, macromolecular markers are often analyzed in combination with other markers to assess pollution more effectively (Faverney et al. 2001). The integrated biomarker response (IBR) has recently become an effective tool for evaluating ecological risk in aquatic environments as an indicator of environmental stress. Nonetheless, IBR assessments are less frequently conducted on aquatic plants, which may be a potential option for assessing the potential ecological risk of PFCs and their remediation (Zhao et al. 2022).

Submerged plants, such as *Vallisneria natans*, have a higher uptake capacity for absorbing various PFCs than other aquatic plants due to their well-developed root systems and leaf-water exchange (Pi et al. 2017). They are widely recognized as an environmentally friendly and sustainable solution for ecological remediation due to their simplicity, low cost, and effectiveness in removing pollutants (Bai et al. 2020). Therefore, *V. natans* was chosen as the test species in this experiment. We investigated the physiological and biochemical responses as well as their differences of leaves and roots to single exposures of PFOA and PFOS in *V. natans*, respectively, and evaluated their ecotoxic effects comprehensively with the help of IBR. The results help to systematically explore the ecotoxicity of PFCs on submerged plants to make a complement and provide a reference basis for subsequent assessment of the phytoremediation potential of submerged plants and the risk to aquatic ecosystems.

Materials and methods

Plant material and experimental design

The aquatic plant *V. natans* was collected from the ecological station of Liangzi Island, Wuhan, Hubei Province (30°33.41′N, 114°22.59′E). PFOA (96% purity) was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China), while PFOS (98% purity) from Maya Reagent Co., Ltd. (Zhejiang, China).

Healthy plants were selected and soaked in 2% citric acid solution for 10 min to sterilize them. They were then transplanted into a light incubator at 27 ± 5 °C with a 12/12 light/dark cycle and a light intensity of 2160 lx for 15 days to acclimatize. After 7 days of incubation, *V. natans* plants with good growth and similar morphology were selected, and distilled water was used to remove the impurities attached to them. The same procedures were repeated after 15 days to minimize differences. The PFOA and PFOS were dissolved in pure water heated to about 50 °C to prepare the solution. The concentrations of PFOA and PFOS were set according to environmental concentration, river pollution concentration, fluoride site effluent concentration, spill concentration, and laboratory test concentration. In addition to the control group, the other 10 treatment groups were exposed to low

(0.01 mg/L), middle (0.1 and 1.0 mg/L), and high (5.0 and 10.0 mg/L) concentrations of PFOA and PFOS.

The plants were exposed to 2-L 10% Hoagland's solution environment for short-term (7 days) and long-term (21 days) periods. Toxicant solutions were changed every 48 h to maintain the consistency of toxicants and nutrients. Fresh weight and morphological parameters were measured before the first addition of toxicants, and again after 7 days and 21 days. The parameters of every group were selected randomly to measure the parameters below: biomass and morphological parameters (fresh weight, number of leaf blades, maximum leaf length, maximum root length, number of tillers, number of flowers), physiological parameters (photosynthetic pigment content, malondialdehyde content, soluble protein content, soluble sugar content, and H_2O_2 content), and biochemical parameters (SOD, CAT, POD, and PPO activity).

Measurement of plant growth

The fresh weights (FW) of the plants under test were measured on the 7th and 21st day after the addition of the toxicant by the method of Wang (2004). Plant biomass was calculated as fresh weight (g). The effect on plant growth was assessed by the relative growth rate (RGR) of the plants. The RGR was calculated as:

$$RGR(\%) = \left[\ln\left(FW_2\right) - \ln\left(FW_1\right)\right]/\Delta t\left(g^{-1}day^{-1}\right)$$

where FW_1 and FW_2 represent the initial and final fresh weights, and Δt indicates the corresponding time intervals.

Measurement of photosynthetic pigment content

Photosynthesis provides energy for the growth, development and reproduction of aquatic plants, where photosynthetic pigments are important basic substances for this process. The photosynthetic pigment contents were measured by using the method of Jampeetong and Brix (2009). The absorbance values were measured at 470 nm, 649 nm, and 665 nm. All spectrophotometric analyses in this experiment were conducted by MAPADA UV-1200 spectrophotometer (Shanghai Meipuda Instrument Co. Ltd., Shanghai, China).

Measurement of MDA and soluble sugar content

Soluble sugars and soluble proteins are important nutrients in the plant and help maintain cellular osmotic pressure balance under stress conditions to resist damage, and are commonly used as indicators of resistance. Weigh 0.1 g of plant leaves and add 5 mL of 10% trichloroacetic acid (TCA) to grind into a homogenate, then centrifuge at 12,000 r/min for 10 min. The supernatant was mixed with 0.6% 2-thiobarbituric acid (TBA), boiled on a water bath at 100 °C for 30 min, cooled to room temperature and then centrifuged again. Take the supernatant and determined the absorbance values at 450 nm, 532 nm, and 600 nm, the soluble sugar content and malondialdehyde content were calculated according to the method of Liu (2019).

Antioxidant activities and H_2O_2

Plants initiate antioxidant mechanisms to scavenge stress-induced excess reactive oxygen radicals (Bhaduri and Fulekar 2012). The antioxidant activities of this study were measured by the methods of Gao (2006) and Cang and Zhao (2013). 0.1 g of fresh leaves or roots were ground the homogenate with pre-cooled phosphate buffer (50 mM, pH 7.8, containing 1% PVP), centrifuge the grind at 4 °C, 12,000 r/min for 10 min. The samples were stored at 4 °C and used for the determination of H_2O_2, soluble protein content as well as the activities of SOD, CAT, POD, and PPO.

The SOD activity was measured by nitrogen blue tetrazolium (NBT) reduction method, which the amount of enzyme required to inhibit 50% photochemical reduction of NBT was taken as one unit (U). The CAT activity was determined by UV spectrophotometry, and one enzyme activity unit corresponded to a 0.1 decrease in absorbance at 240 nm within 1 min. The POD activity was measured by the guaiacol method, using an absorbance change of 0.01 per minute at 470 nm as a unit of enzyme activity. The PPO activity was estimated by the catechol method, and an increase of 0.01 in OD_{398} within 1 min was used as a unit. The H_2O_2 content was determined by the method of Satterfield (1955), measured OD_{410}, and then calculated from the standard curve.

Soluble protein content

The absorbance was measured at 595 nm after 0.1 mL of the sample extract and 5 mL of Kaumas Brilliant Blue G-250 protein reagent was added, shaken and mixed thoroughly for 2 min (Bradford 1976). The protein content was then calculated based on the standard curve.

Integrated biomarker response

To comprehensively assess the effects of exposure treatments on leaves and roots of *V. natans* and plant response, IBR was introduced to characterize the plant response at different concentrations and treatment times. The area covered by the star chart indicates the IBR value. The H_2O_2 content, soluble protein content, antioxidant enzymes (SOD, CAT, POD, and PPO) activity, and MDA content were selected as the biomarkers to calculate IBR. The total chlorophyll content was additionally included in the IBR calculation for leaves. The IBR method was carried

out with reference to Kim (2016). Each biomarker index should first be standardized and then scored. The magnitude of the value for each biomarker at different concentrations of different exposure time is expressed as the length of the radial line in the star chart. The IBR value for a certain exposure condition was obtained by calculating the area of the star chart.

Since the order of several biomarkers in the star chart affects the results of IBR calculation, the full ranking of several biomarkers was considered in this study and the mean of IBR values was calculated as the final value.

Statistical analysis

Data analysis was performed using SPSS 22 (IBM Inc., Chicago, IL, USA) software. After performing Levene's test to analyze homogeneity of variance then Duncan's multiple comparison method in one-way ANOVA was used to analyze the significance of multiple samples in the control and treatment groups. The experimental data were expressed as mean ± standard deviation and were specified to be considered significantly different when $p < 0.05$. Graphs were made with SigmaPlot 12.5 (Systat Software, Inc., USA) and significant differences were denoted on error bars using distinct lowercase letters, and when there were no differences in the whole group, they were not labeled. Similar statistical analysis were reported by several authors (Wilcox et al. 2015; León-Mejía et al. 2016, 2018; Gredilla et al. 2017; Nordin et al. 2018; Gasparotto et al. 2018, 2019; Ramírez et al. 2019; Rojas et al. 2019).

Results

Growth of *V. natans*

As shown in Fig. 1A, both treatment of PFOA and PFOS for 7 days reduced the RGR of *V. natans* but not significantly. Nevertheless, the RGR was reduced and significantly decreased by 33.77% and 34.72% under 10.0 mg/L of PFOA and PFOS after 21 days (Fig. 1B). These results indicated that the overall RGR of *V. natans* gradually decreased with increasing treatment concentration, but did not reduce the fresh weight of plants. Furthermore, the inhibitory effect of *V. natans* under long-term PFOA treatment increased with increasing concentration.

The results indicated that there was no significant difference in all morphological indicators of *V. natans* after 7 and 21 days, as showed in Table 1 and 2. Further analysis of each index showed that the increase of maximum leaf length was lower in both high-concentration PFOA- and PFOS-treated groups than the control after 21 days, as compared to 7 days. 50.78% increase was observed in the control after 21 days, while only 29.25% and 27.08% increase were observed separately under 10.0 mg/L PFOA and PFOS treatment.

Photosynthetic pigment content of *V. natans*

Table 3 showed that the photosynthetic pigments of *V. natans* generally increased at lower concentrations, then decreased with increasing concentration after 7-day exposure. Specifically, the contents of chlorophyll in high concentration PFOA treatment were lower than those in the

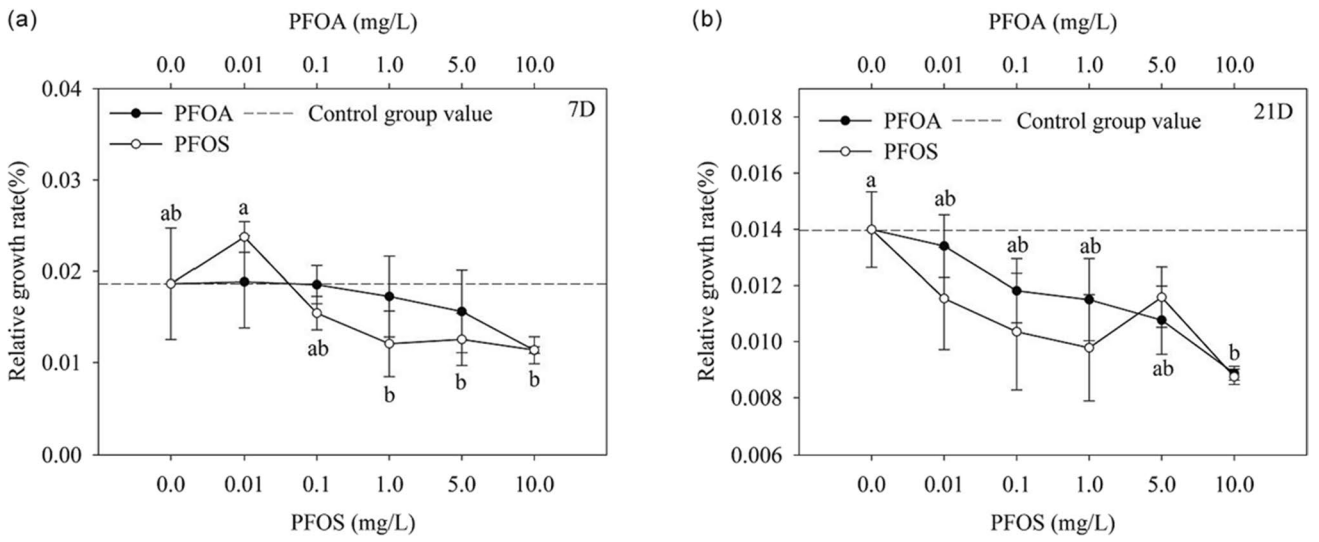

Fig. 1 RGR for treatments of PFOA and PFOS to *V. natans* after exposure for 7 (**a**) and 21 days (**b**). 7D and 21D represent 7- and 21-day treatments, respectively, the same as below

Table 1 Effects of PFOA and PFOS on morphological parameters of *V. natans* after 7 days

PFOA concentration (mg/L)	Number of leaf blades (pcs)	Maximum leaf length (cm)	Maximum root length (cm)	Number of tillers (pcs)	Number of flowers (pcs)
0	10.33 ± 1.53	8.53 ± 0.38	5.33 ± 0.58	0.67 ± 0.58	0.67 ± 0.58
0.01	10.00 ± 1.00	8.83 ± 0.91	5.20 ± 0.27	0.67 ± 0.58	0.67 ± 0.58
0.1	10.33 ± 0.58	8.43 ± 0.98	5.10 ± 0.17	1.00 ± 0.00	0.67 ± 0.58
1.0	10.67 ± 0.58	8.70 ± 1.04	5.17 ± 0.15	1.00 ± 0.00	1.00 ± 0.00
5.0	11.33 ± 0.58	9.07 ± 0.31	5.50 ± 0.50	1.00 ± 0.00	0.67 ± 0.58
10.0	11.67 ± 0.58	9.80 ± 0.44	5.67 ± 0.29	1.00 ± 0.00	1.00 ± 0.00
PFOS concentration (mg/L)	Number of leaf blades (pcs)	Maximum leaf length (cm)	Maximum root length (cm)	Number of tillers (pcs)	Number of flowers (pcs)
0	10.33 ± 1.53	8.53 ± 0.38	5.33 ± 0.58	0.67 ± 0.58	0.67 ± 0.58
0.01	11.00 ± 0.00	9.20 ± 0.76	5.33 ± 0.58	1.00 ± 0.00	0.67 ± 0.58
0.1	10.33 ± 0.58	8.90 ± 0.27	5.43 ± 0.93	0.67 ± 0.58	0.67 ± 0.58
1.0	11.00 ± 0.00	9.20 ± 0.44	5.53 ± 0.55	0.67 ± 0.58	0.67 ± 0.58
5.0	11.00 ± 0.00	8.90 ± 0.10	4.67 ± 0.58	0.67 ± 0.58	1.00 ± 0.00
10.0	10.33 ± 0.58	9.60 ± 0.95	4.80 ± 0.52	1.00 ± 0.00	1.00 ± 0.00

Pcs is pieces, a unit for the number of leaves, tillers and flowers

Table 2 Effects of PFOA and PFOS on morphological parameters of *V. natans* after 21 days

PFOA concentration (mg/L)	Number of leaf blades (pcs)	Maximum leaf length (cm)	Maximum root length (cm)	Number of tillers (pcs)	Number of flowers (pcs)
0	12.33 ± 0.58	12.87 ± 0.23	6.50 ± 0.87	2.67 ± 0.58	1.00 ± 0.00
0.01	12.33 ± 0.58	12.03 ± 0.55	5.60 ± 0.36	2.67 ± 0.58	0.00 ± 0.00
0.1	12.67 ± 0.58	12.60 ± 0.53	5.87 ± 0.32	2.00 ± 1.00	1.00 ± 0.58
1.0	13.00 ± 0.00	12.75 ± 0.35	5.93 ± 0.40	2.33 ± 1.53	0.66 ± 0.58
5.0	12.67 ± 0.58	12.60 ± 0.31	6.33 ± 0.58	2.33 ± 0.58	0.00 ± 0.00
10.0	12.00 ± 0.00	12.67 ± 0.21	6.20 ± 0.52	2.67 ± 0.58	0.67 ± 0.58
PFOS concentration (mg/L)	Number of leaf blades (pcs)	Maximum leaf length (cm)	Maximum root length (cm)	Number of tillers (pcs)	Number of flowers (pcs)
0	12.33 ± 0.58	12.87 ± 0.23	6.50 ± 0.87	2.67 ± 0.58	0.00 ± 0.00
0.01	12.67 ± 0.58	12.67 ± 0.36	6.17 ± 1.04	2.67 ± 0.58	0.00 ± 0.00
0.1	12.33 ± 0.58	12.67 ± 0.45	5.83 ± 0.29	2.33 ± 0.58	0.33 ± 0.58
1.0	12.33 ± 0.58	12.00 ± 1.04	5.87 ± 0.32	1.67 ± 0.58	0.67 ± 0.58
5.0	12.00 ± 0.00	12.20 ± 0.82	5.50 ± 0.50	2.67 ± 0.58	0.67 ± 0.58
10.0	12.33 ± 0.58	12.20 ± 0.32	6.00 ± 0.50	2.33 ± 0.58	0.33 ± 0.58

control group, while PFOS eventually returned to the control level. The photosynthetic pigment content was significantly higher under single treatment with low and medium PFOS concentrations compared to the control. Chlorophyll b content was significantly increased by 52.68% under low concentration treatment of 0.01 mg/L PFOS, while chlorophyll a and carotenoid content showed significant increases of 32.63% and 40.90%, respectively.

As shown in Table 4, after 21-day exposure, the photosynthetic pigment content of *V. natans* showed a similar trend as the 7-day treatment of PFOS. It showed a maximum value at medium concentration of 0.1 mg/L PFOA and then reached a minimum value at high concentration of 5.0 mg/L. The content of chlorophyll a and total chlorophyll showed an overall increasing trend after exposing to PFOS for 21 days compared to the control group, with significant increases of 26.70% and 25.73% in 10.0-mg/L treatment group. There was no significant difference in chlorophyll b and carotenoid content.

Soluble sugar content in *V. natans*

Figure 2 indicated that leaves and roots of *V. natans* can respond to high concentrations of PFOA and PFOS stress by accumulating soluble sugars under short-term exposure but no longer respond under long-term exposure. The results showed that after 7 days, the soluble sugar content in leaves increased under both PFOA and PFOS treatments at medium

Table 3 Effect of PFOA and PFOS on photosynthetic pigment contents in leaves of *V. natans* after 7 days

PFOA concentration (mg/L)	Chlorophyll a content (mg/g FW)	Chlorophyll b content (mg/g FW)	Total chlorophyll content (mg/g FW)	Carotenoid content (mg/g FW)
0	1.09 ± 0.32^{ab}	0.41 ± 0.17^{ab}	1.50 ± 0.48^{ab}	0.39 ± 0.12
0.01	1.09 ± 0.12^{ab}	0.45 ± 0.08^{ab}	1.54 ± 0.20^{ab}	0.45 ± 0.05
0.1	1.20 ± 0.06^{a}	0.53 ± 0.00^{a}	1.73 ± 0.10^{a}	0.40 ± 0.11
1.0	1.14 ± 0.02^{a}	0.53 ± 0.01^{a}	1.67 ± 0.03^{a}	0.45 ± 0.05
5.0	0.79 ± 0.08^{b}	0.33 ± 0.06^{b}	1.13 ± 0.12^{b}	0.47 ± 0.02
10.0	0.80 ± 0.06^{b}	0.33 ± 0.06^{b}	1.13 ± 0.11^{b}	0.45 ± 0.03
PFOS concentration (mg/L)	Chlorophyll a content (mg/g FW)	Chlorophyll b content (mg/g FW)	Total chlorophyll content (mg/g FW)	Carotenoid content (mg/g FW)
0	1.09 ± 0.32^{b}	0.41 ± 0.17^{b}	1.50 ± 0.48^{b}	0.39 ± 0.06^{b}
0.01	1.33 ± 0.07^{ab}	0.63 ± 0.06^{a}	1.95 ± 0.13^{a}	0.48 ± 0.02^{ab}
0.1	1.45 ± 0.26^{a}	0.47 ± 0.17^{ab}	1.92 ± 0.43^{a}	0.51 ± 0.10^{a}
1.0	1.32 ± 0.04^{ab}	0.55 ± 0.05^{a}	1.88 ± 0.09^{a}	0.48 ± 0.00^{ab}
5.0	1.19 ± 0.06^{ab}	0.53 ± 0.04^{ab}	1.72 ± 0.09^{ab}	0.47 ± 0.02^{ab}
10.0	1.03 ± 0.10^{b}	0.44 ± 0.07^{b}	1.56 ± 0.07^{ab}	0.39 ± 0.01^{b}

Table 4 Effect of PFOA and PFOS on photosynthetic pigment contents in leaves of *V. natans* after 21 days

PFOA concentration (mg/L)	Chlorophyll a content (mg/g FW)	Chlorophyll b content (mg/g FW)	Total chlorophyll content (mg/g FW)	Carotenoid content (mg/g FW)
0	1.09 ± 0.03^{ab}	0.52 ± 0.04^{ab}	1.61 ± 0.06^{b}	0.42 ± 0.01^{ab}
0.01	1.14 ± 0.08^{ab}	0.51 ± 0.06^{ab}	1.65 ± 0.14^{b}	0.43 ± 0.02^{ab}
0.1	1.30 ± 0.04^{a}	0.58 ± 0.05^{a}	1.98 ± 0.10^{a}	0.48 ± 0.01^{a}
1.0	1.17 ± 0.09^{ab}	0.48 ± 0.08^{ab}	1.65 ± 0.17^{b}	0.43 ± 0.05^{ab}
5.0	1.06 ± 0.09^{b}	0.47 ± 0.02^{b}	1.53 ± 0.06^{b}	0.40 ± 0.02^{b}
10.0	1.12 ± 0.24^{ab}	0.50 ± 0.11^{ab}	1.61 ± 0.34^{b}	0.43 ± 0.07^{ab}
PFOS concentration (mg/L)	Chlorophyll a content (mg/g FW)	Chlorophyll b content (mg/g FW)	Total chlorophyll content (mg/g FW)	Carotenoid content (mg/g FW)
0	1.09 ± 0.03^{c}	0.52 ± 0.04	1.61 ± 0.06^{b}	0.46 ± 0.03
0.01	1.22 ± 0.02^{bc}	0.53 ± 0.05	1.75 ± 0.07^{b}	0.44 ± 0.03
0.1	1.21 ± 0.05^{bc}	0.51 ± 0.03	1.76 ± 0.07^{b}	0.44 ± 0.02
1.0	1.22 ± 0.17^{bc}	0.52 ± 0.10	1.73 ± 0.26^{b}	0.43 ± 0.04
5.0	1.27 ± 0.06^{ab}	0.57 ± 0.04	1.83 ± 0.09^{ab}	0.44 ± 0.01
10.0	1.38 ± 0.01^{a}	0.55 ± 0.04	2.02 ± 0.05^{a}	0.45 ± 0.02

and high concentrations (Fig. 2A). The soluble sugar content in leaves decreased in all groups after 21 days compared to 7 days and there was no significantly difference, as shown in Fig. 2B. After 7 days, the soluble sugar content of roots increased significantly in the high concentration treatment, with a significant increase of 42.35% in 10.0 mg/L PFOA treatment group (Fig. 2C). After 21 days, there was no significant difference in different concentration treatment groups (Fig. 2D).

Soluble protein content in *V. natans*

Under the treatments of PFOA and PFOS, the soluble protein content in leaves of *V. natans* increased at medium concentration after 7 days and then returned to the control level at high concentration. In 1.0-mg/L PFOA and PFOS treatments, there was a significant increase of 21.80% and 11.85% compared to the control (Fig. 3A). Alternatively, the contents in high concentration PFOA- and PFOS-treated groups decreased after 21 days. The treatment groups with a high concentration of 10.0 mg/L PFOA and PFOS were reduced significantly by 10.02% and 11.89%, respectively (Fig. 3B). There were no significant differences in PFOA and PFOS in roots (Fig. 3C, D).

MDA in *V. natans*

The results showed that MDA content in leaves of *V. natans* increased under high concentrations of both PFOA and PFOS treatment after 7 days (Fig. 4A, B). After 7

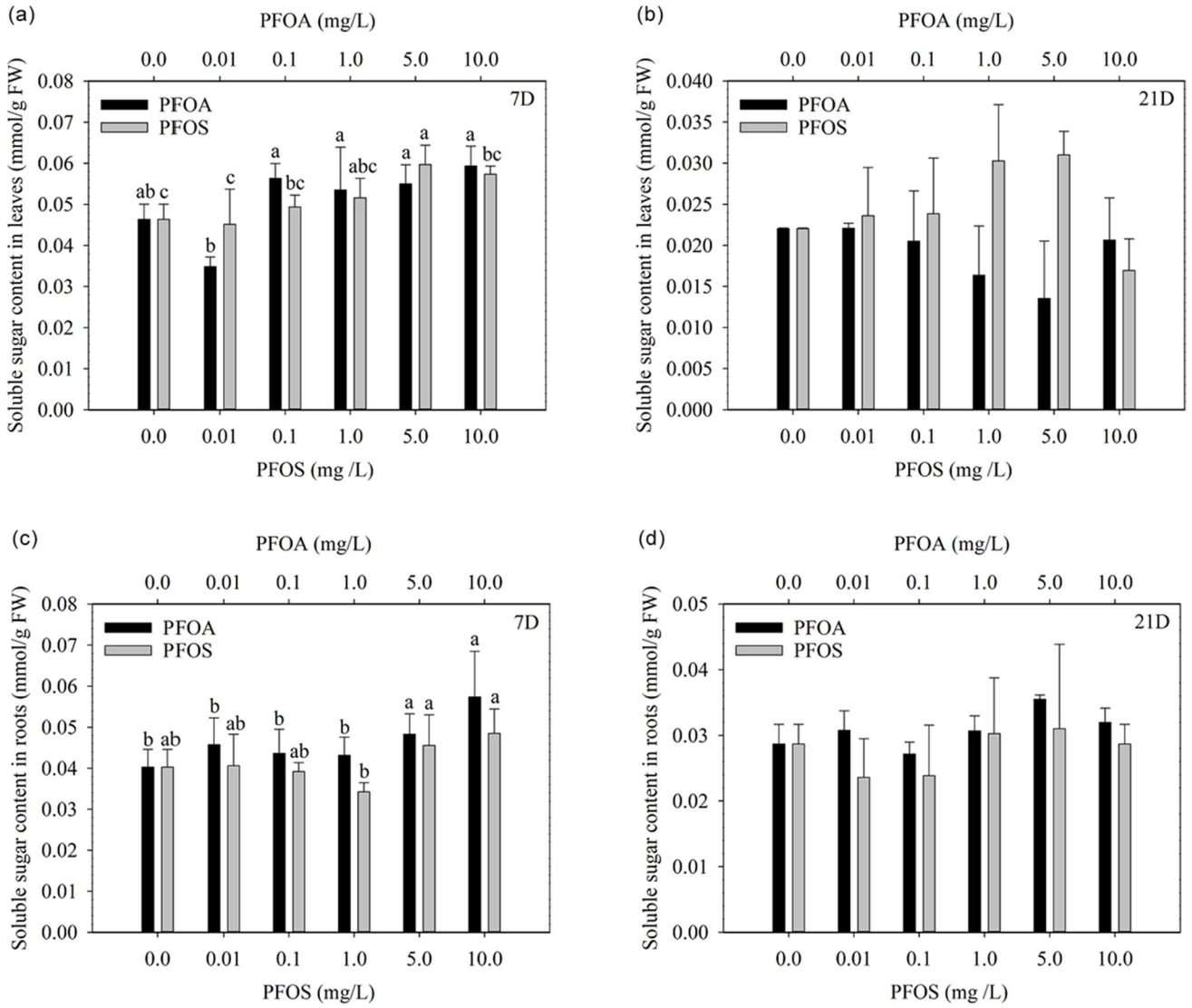

Fig. 2 Soluble sugar content for treatments of PFOA and PFOS to *V. natans* after exposure for 7 (**a**, **c**) and 21 days (**b**, **d**)

days, there was a significant increase of 69.45% and 49.13% in PFOA treatment group at concentrations of 5.0 and 10.0 mg/L, respectively. Additionally, the PFOS treated group showed and a significant increase of 37.04% and 75.70% at concentrations of 5.0 and 10.0 mg/L (Fig. 4A). There were no significant differences in both leaves and roots after 21 days, but there was an increase in MDA under prolonged high PFOS exposure (Fig. 4B, D). These results indicate that MDA accumulation in leaves of *V. natans* was only observed under short-term PFOA and PFOS mono-exposure, but increased in both leaves and roots with long-term PFOS exposure.

H_2O_2 in *V. natans*

Figure 5A showed that the H_2O_2 content in leaves of *V. natans* increased after 7 days of exposure to high concentration, in accordance with the results of MDA. At 5.0 and 10.0 mg/L, significant increase of 24.36% and 30.54% were observed in the PFOA treatment group compared to the control group, respectively. Similarly, the PFOS treatment demonstrated a significant increase of 35.47% and 27.20%. The overall H_2O_2 content in 21-day treated groups was lower than 7-day treatments, but not significantly different from the control (Fig. 5B). The H_2O_2 content in roots increased under

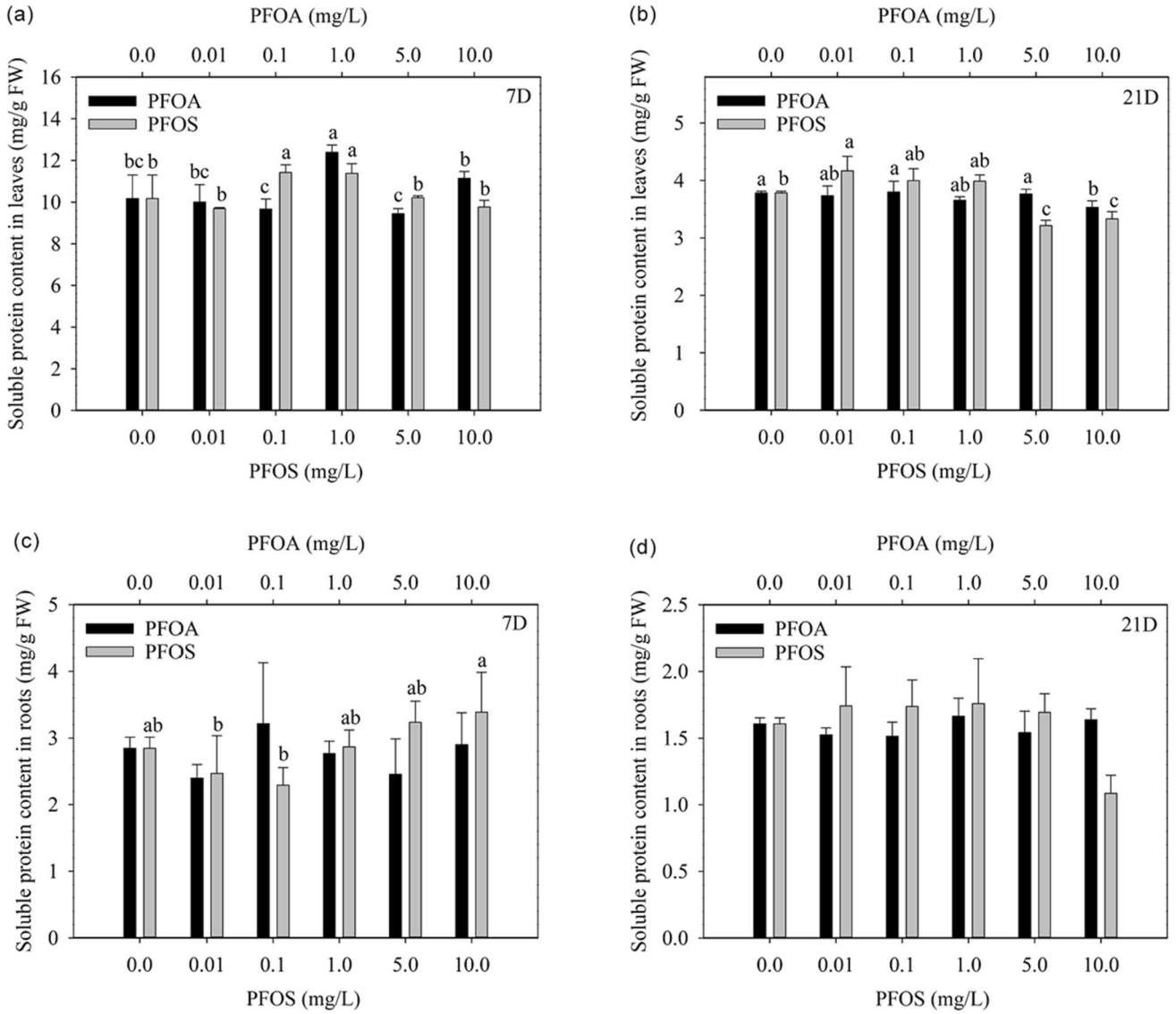

Fig. 3 Soluble protein content for treatments of PFOA and PFOS to *V. natans* after exposure for 7 (**a**, **c**) and 21 days (**b**, **d**)

high concentration of PFOA treatment, while no significant differences were observed in roots under both 7 and 21 days PFOS treatment (Fig. 5C, D).

Antioxidant defense in *V. natans*

The results indicated that both short- and long-term PFOA or PFOS exposure can increase SOD activity in leaves of *V. natans*. The results showed a noteworthy 48.22% rise in SOD activity after 7 days of 0.01 mg/L PFOS treatment, indicating that PFOS would activate SOD activity earlier, as depicted in Fig. 6A, B. The roots activate SOD activity at high PFOA concentrations in brief exposure, while SOD activity is promoted and then inhibited under long-term PFOS exposure. The SOD activity of roots increased significantly after 7 days under high PFOA concentration, while there was no significant difference in PFOS treatments (Fig. 6C). After 21 days, the SOD activity in roots showed a significant increase at medium concentration treatments, followed by a significant decrease at high concentration treatment (Fig. 6D).

Short-term high PFOA or PFOS stress increased CAT activity in leaves of *V. natans*, consistent with the H_2O_2 results, while long-term high stress inhibited it (Figs. 5A and 7A). CAT activity in leaves increased and then decreased significantly under high concentrations of PFOA and PFOS after 21 days (Fig. 7B). CAT activity in roots was inhibited by short-term high PFOA or PFOS treatment, while long-term PFOA exposure promoted an increase in activity. After 7 days, CAT activity in roots increased significantly in

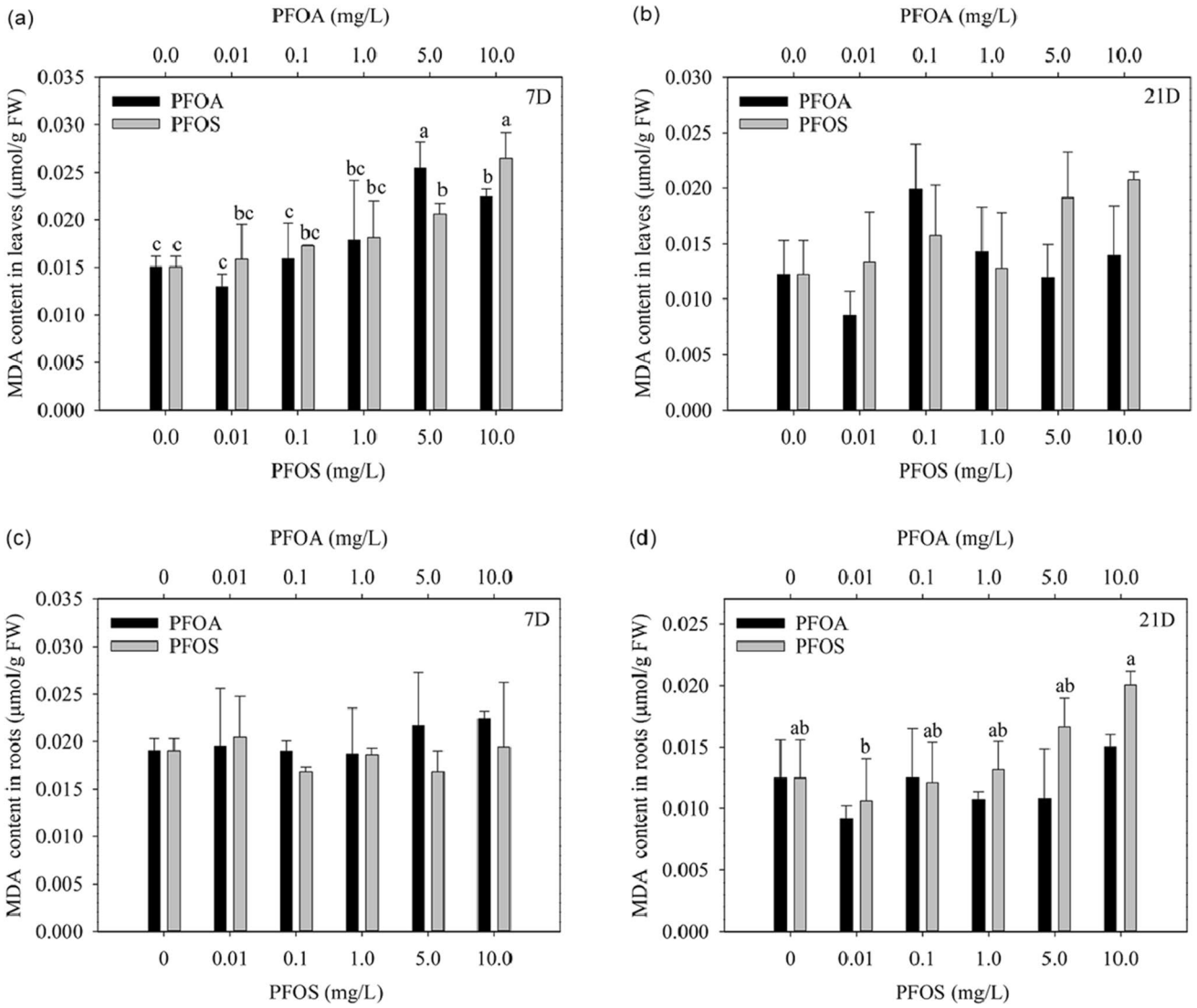

Fig. 4 MDA content for treatments of PFOA and PFOS to *V. natans* after exposure for 7 (**a**, **c**) and 21 days (**b**, **d**)

medium concentrations of PFOA treatments while decreased under high concentrations of PFOA and PFOS treatments (Fig. 7C).

POD activity in leaves of *V. natans* is inhibited by short-term high concentrations of PFOA treatment, but increased significantly under medium concentrations of PFOS treatment. It decreased significantly after 7 days of high-concentration PFOA treatment while increased significantly after 21 days, with increases of 45.46% and 47.61% at 5.0 and 10.0 mg/L, respectively (Fig. 8A, B). Under long-term treatment, medium concentrations of PFOS activate POD activity in roots while high concentrations of PFOA inhibit it. After 7 days, there was no significant difference in POD activity in roots compared to the control (Fig. 8C). After 21 days, POD activity was significantly higher both in low and medium PFOS treatments and decreased under high PFOS and PFOA treatments (Fig. 8D).

Short-term high PFOS exposure increased PPO activity in leaves and roots of *V. natans*, while long-term inhibited it. PPO activity in leaves increased significantly after 7 days at high PFOS concentrations, while there was no significant difference under PFOA treatments (Fig. 9A). After 21 days, the group treated with high PFOS concentrations exhibited significant reductions of 20.88% and 24.56% separately at 5.0 mg/L and 10.0 mg/L (Fig. 9B). Additionally, long-term high PFOA treatment contributed to increasing PPO activity in roots. PPO activity in roots increased significantly under both medium and high PFOS treatment after 7 days (Fig. 9C). After 21 days, it increased significantly under both medium and high PFOS treatments (Fig. 9D).

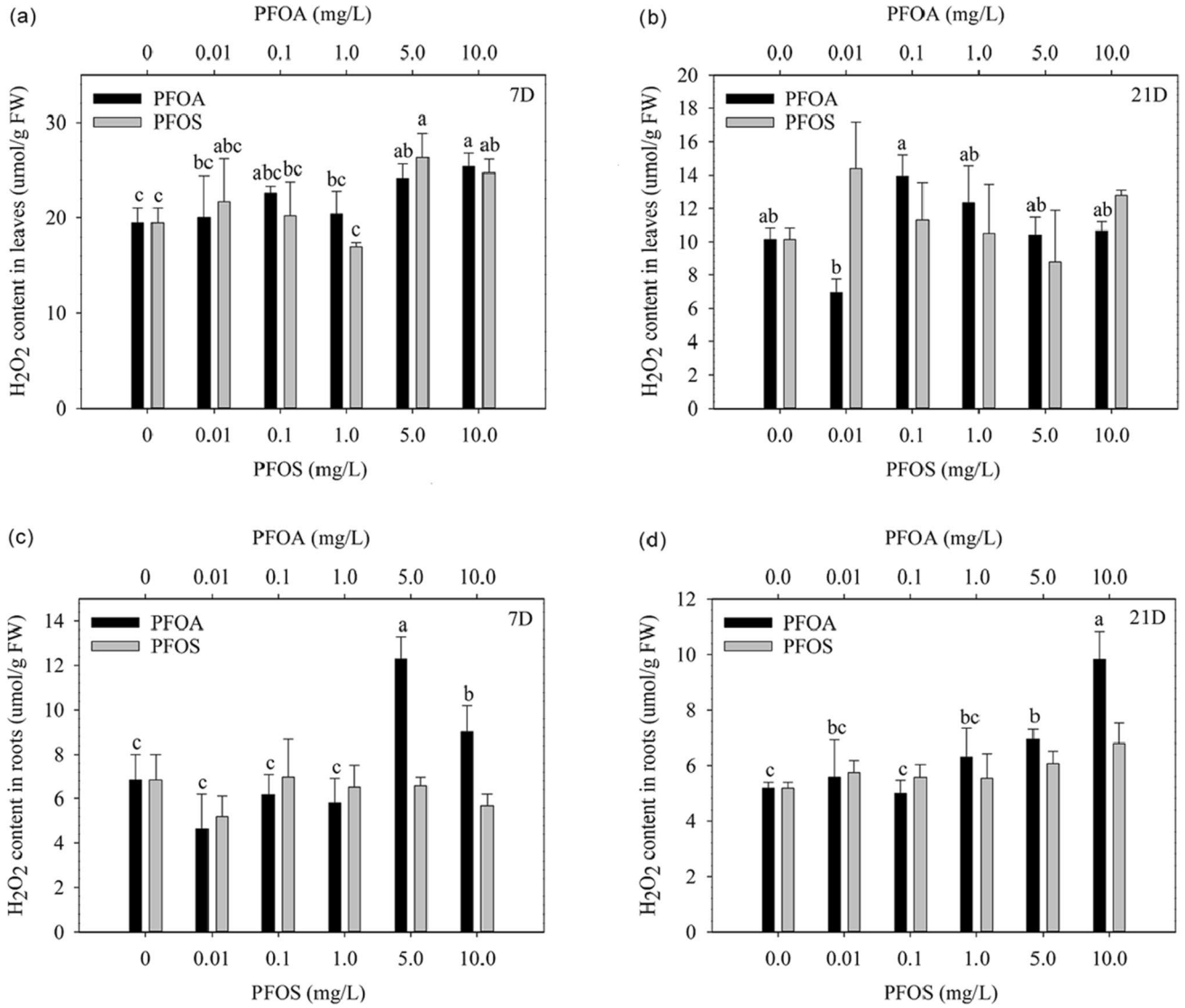

Fig. 5 H_2O_2 content for treatments of PFOA and PFOS to *V. natans* after exposure for 7 (**a**, **c**) and 21 days (**b**, **d**)

IBR of *V. natans*

After 7 days, there were higher response levels of antioxidant enzymes and MDA in leaves of *V. natans* (Fig. 10A, B), while H_2O_2 and antioxidant enzymes in roots showed high levels of response at high concentrations (Fig. 10D, E). After 21 days, roots had more pointing prominence compared to leaves, indicating that more biomarkers were elicited in response, while CAT in leaves showed higher response levels (Fig. 11A, B). H_2O_2, MDA, and antioxidant enzymes in roots showed higher response levels at high concentrations (Fig. 11D, E).

It showed that antioxidant enzymes in both leaves and roots of *V. natans* were strongly affected in the 10.0mg/L group under short and long term, with maximum IBR values (Figs. 10C, F and 11C, F). Compared to the low-concentration group and the control group, the high-concentration group had higher IBR values under PFOS treatment. While under PFOA treatment, excitation was also induced at 0.01 mg/L in both short-term treatment of roots and long-term treatment of leaves, resulting in higher response levels. Meanwhile, the area in leaves was larger compared to the roots, which indicated higher IBR values, with the short-term being more obvious (Fig. 10C, F).

Discussion

Growth and morphology are the most direct responses of aquatic plants to the external environment. The overall RGR of *V. natans* under short- and long-term PFOA and PFOS treatments showed a gradual decrease but without any

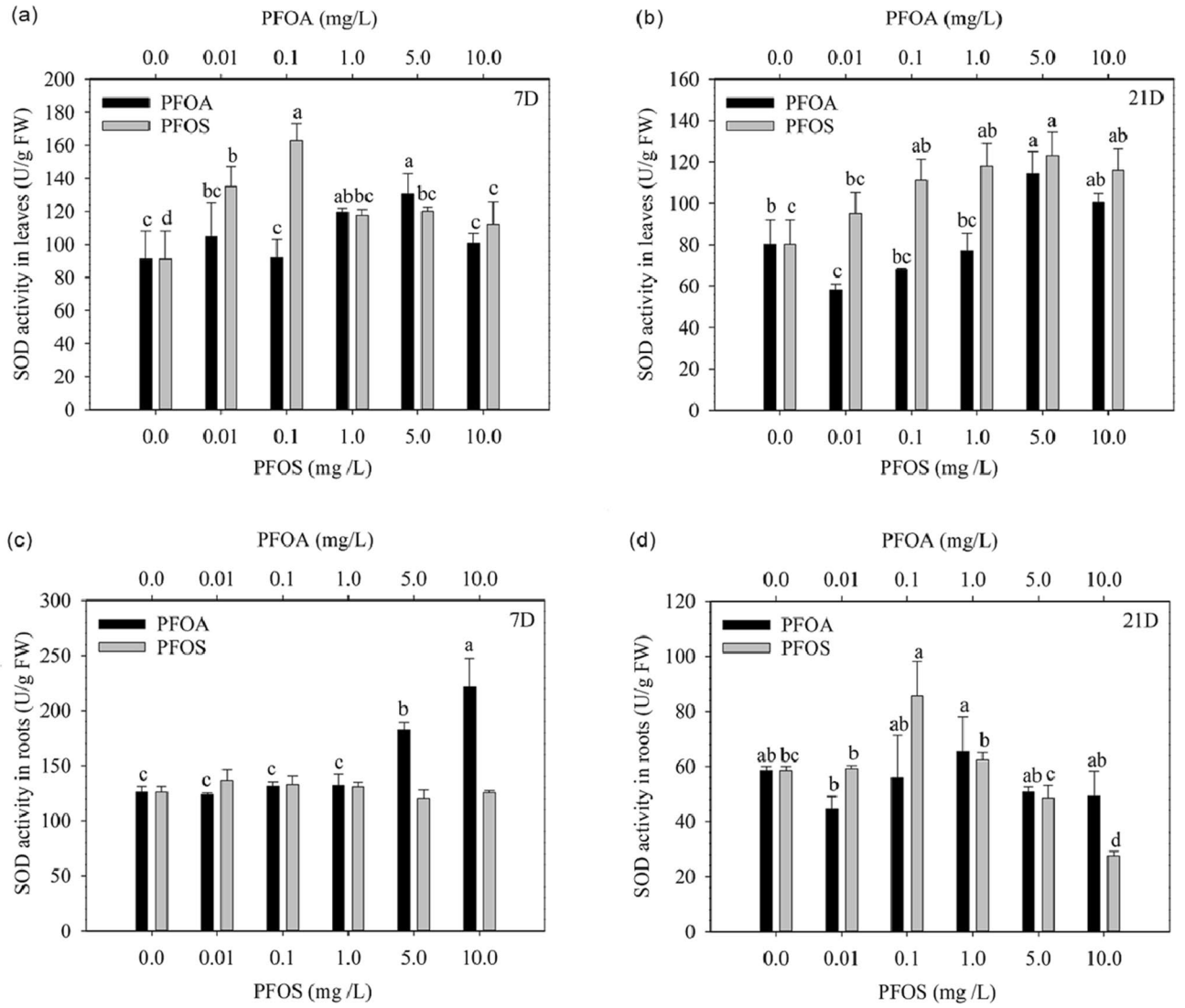

Fig. 6 SOD for single treatment of PFOA and PFOS to *V. natans* after exposure for 7 (**a**, **c**) and 21 days (**b**, **d**)

negative growth. The inhibitory effect of PFOA on *V. natans* showed a dose effect, it becoming stronger with increasing concentration. This phenomenon was also present in *Triticum aestivum*, where PFOA inhibited its seed germination in a dose-dependent manner (Zhou et al. 2016). It has also been found that PFOS induced phytotoxic responses in *Lemna gibba* proceed in a dose-dependent manner (Boudreau et al. 2003). As exposure time increased, maximum leaf length became shorter under high concentrations of PFOA and PFOS treatments, while root length did not, indicating that leaf growth may be impaired in *V. natans*. As treatment time became longer, the number of tillers was higher under high concentrations of PFOA and PFOS compared to the number of flowers, indicating that the high concentration would prompt *V. natans* to prefer asexual reproduction. *V. natans* selects for energy allocation and reproductive valence between sexual and nutritional reproduction. Under adverse circumstances, some species will actively choose a reproductively valued, less energy-intensive form of nutritional reproduction (Shen and Hu 2006).

The photosynthetic pigment content can reflect the degree of pollutant stress on aquatic plants (Deng et al. 2014). No significant appearance of yellow-green chlorosis was observed under exposure in this experiment. Further results of photosynthetic pigments of *V. natans* under short-term and long-term PFOA and PFOS treatments indicated that leaves could respond to stress by increasing photosynthetic pigment content, thus promoting photosynthesis to avoid negative biomass growth of *V. natans*. While, short-term exposure to high concentrations of PFOA may inhibit the synthesis of photosynthetic pigments, resulting in lower levels than the control group. In general agreement with the

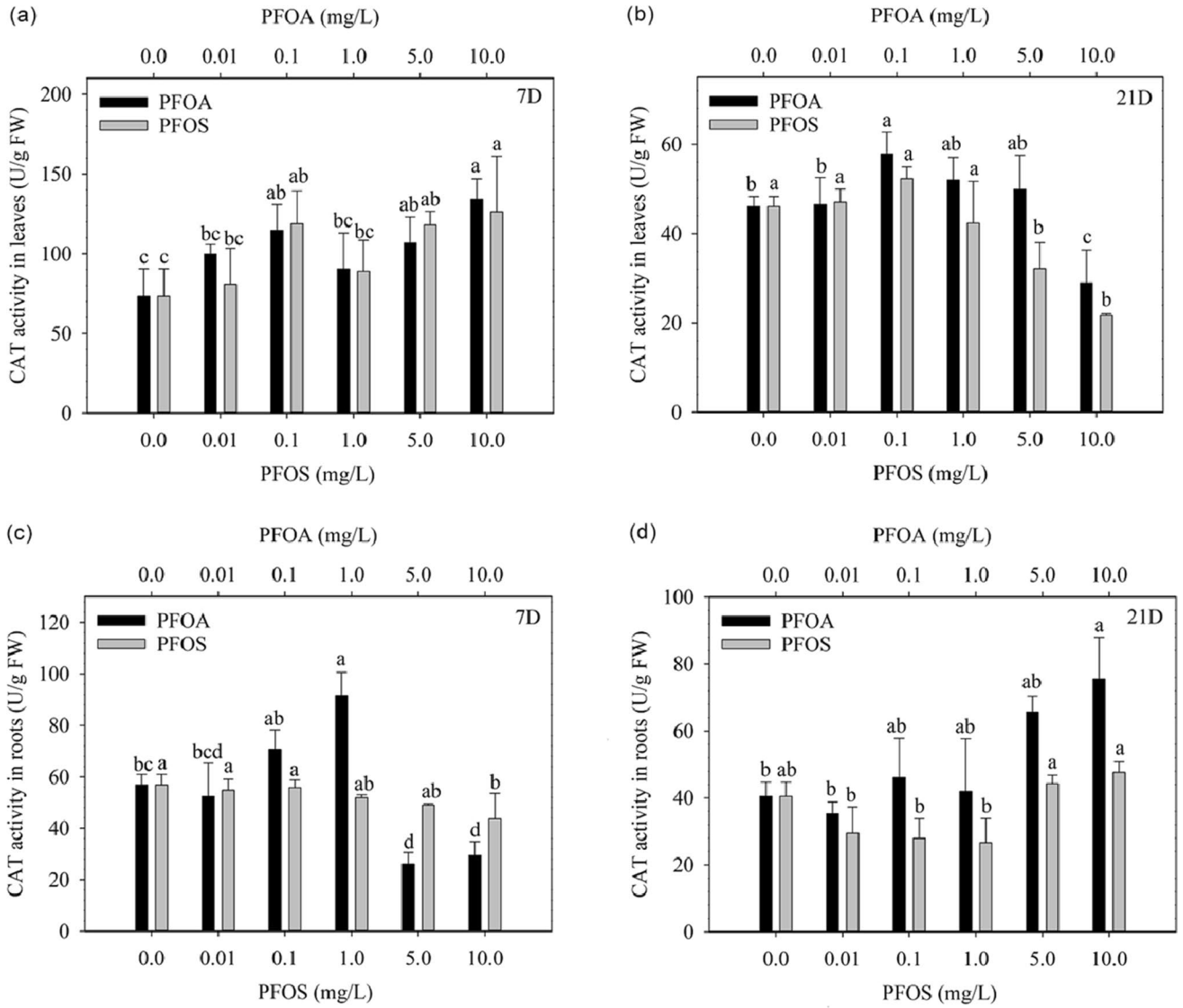

Fig. 7 CAT for single treatment of PFOA and PFOS to *V. natans* after exposure for 7 (**a**, **c**) and 21 days (**b**, **d**)

findings in *Arabidopsis thaliana*, the inhibition of photosynthesis by PFOA was greater than that by PFOS (Zhang et al. 2022). Fan et al. (2020) found that down-regulation of HEMA1, an early chlorophyll synthase gene, and FD2, a gene involved in chlorophyll biosynthesis and photosynthesis, may lead to a decrease in chlorophyll synthesis and ultimately affect photosynthesis. As for PFOS, an ecotoxicity study on the submerged plant *Ceratophyllum demersum* showed its photosynthetic pigment content was significantly higher at 10-mg/L PFOS treatment after 6 days, which is consistent with the present results. While the pigment content in *Ceratophyllum demersum* remained at a minimum when the concentration was reaching 100 mg/L, indicating that PFOS may also have an inhibitory effect on pigment synthesis in aquatic plants.

When aquatic plants are subjected to PFOA and PFOS stress, reactive oxygen species are continuously produced and accumulated in the body, triggering the antioxidant enzyme system and osmoregulatory system of the plant to regulate the stress damage caused by reactive oxygen radicals, thus alleviating the effects and damage (Fan et al. 2022). It was found that aquatic plants showed similar or even higher enrichment levels of PFOA and PFOS than animals in the same area (Shi et al. 2012; Du et al. 2021). As the time of exposure increased, the H_2O_2 content in the cell of both *Acorus calamus* and *Phragmites communis* increased significantly at high concentrations of 10- and 50-mg/L PFOS (Qian et al. 2019).

H_2O_2 is one of the main reactive oxygen species in aquatic plant bodies in response to external abiotic stresses

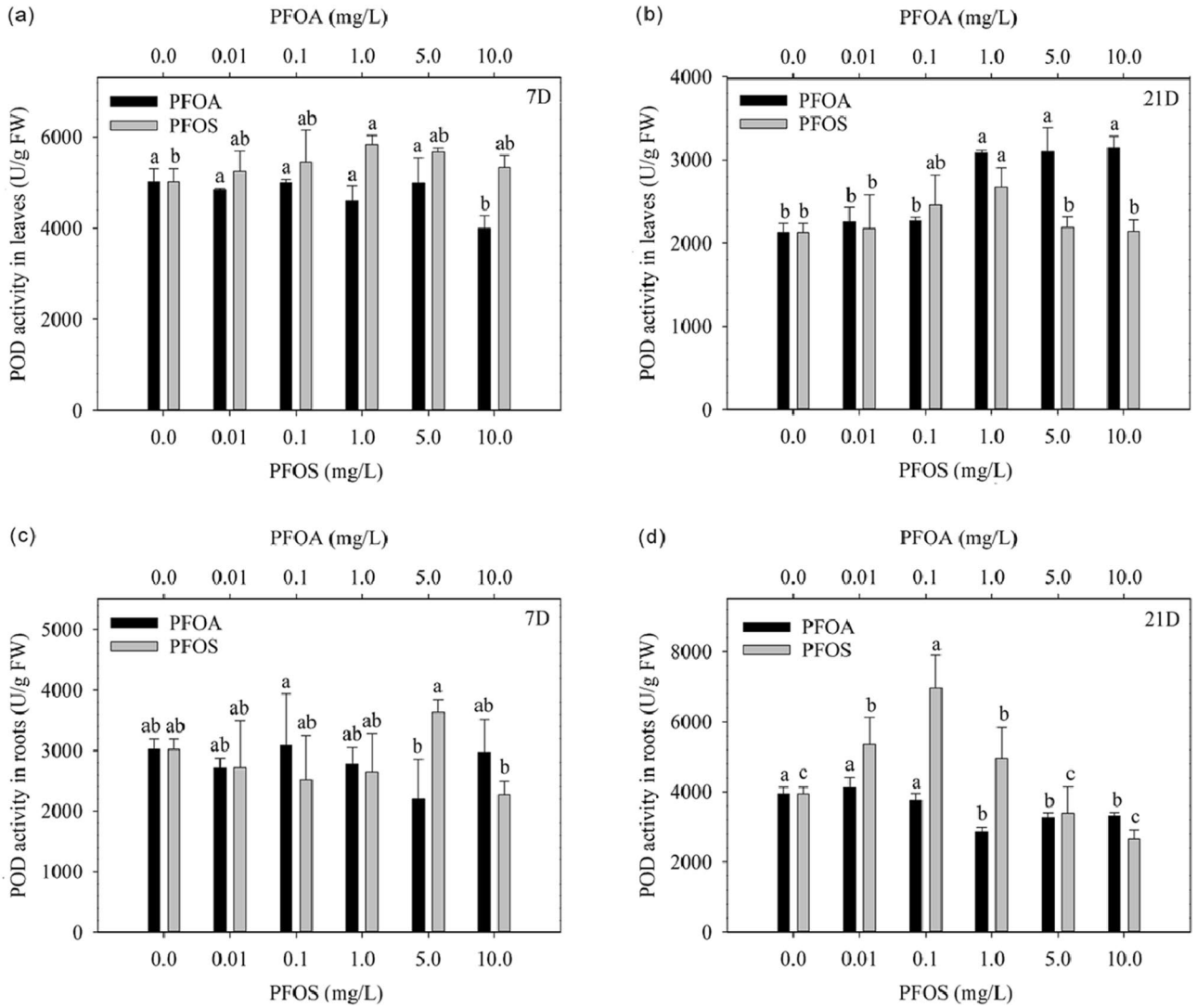

Fig. 8 POD for single treatment of PFOA and PFOS to *V. natans* after exposure for 7 (**a**, **c**) and 21 days (**b**, **d**)

and can permeate across membranes in cells thereby causing oxidative damage. The membrane lipid peroxidation product MDA is commonly used to reflect the degree of oxidation following cell damage and is an important indicator of plant stress and damage (Lee et al. 2020). In previous studies, PFOA and PFOS were found to induce H_2O_2 production (Yang et al. 2015; Li et al. 2021), and in this experiment, *V. natans* caused different H_2O_2 accumulation at different treatment times and different biomarkers responded differently to same toxicant. Short-term high concentrations of PFOA and PFOS caused an accumulation of H_2O_2 in the leaves of *V. natans*, which led to an increase in MDA content due to lipid peroxidation. After prolonged exposure, the leaves no longer accumulated H_2O_2 and the MDA content decreased, but there was still an increase of MDA content under high PFOS treatment. Only high concentrations of PFOA caused the accumulation of H_2O_2 in *V. natans* roots, but did not result in an increase in MDA content. The results above indicate that leaves are more sensitive to high short-term PFOS concentrations, while roots are more sensitive to high PFOA concentrations. Oxidative damage caused by PFOA and PFOS stress occurred mainly in leaves, and both leaves and roots gradually built up tolerance with increasing exposure time. No serious membrane damage was caused to *V. natans* under both short-term and long-term toxic exposures.

SOD, CAT, POD, and PPO are considered as key enzymes in the antioxidant enzyme system to resist peroxidation reactions, and their activities directly affect the production and elimination of reactive oxygen species. Highly active antioxidant enzymes are beneficial to improve the tolerance of aquatic plants to various PFOA and PFOS stresses (Kong et al. 2015; Hua et al. 2022). Under short-term exposure, SOD, CAT, and PPO together resisted stress induced by reactive oxygen species in roots and leaves, with

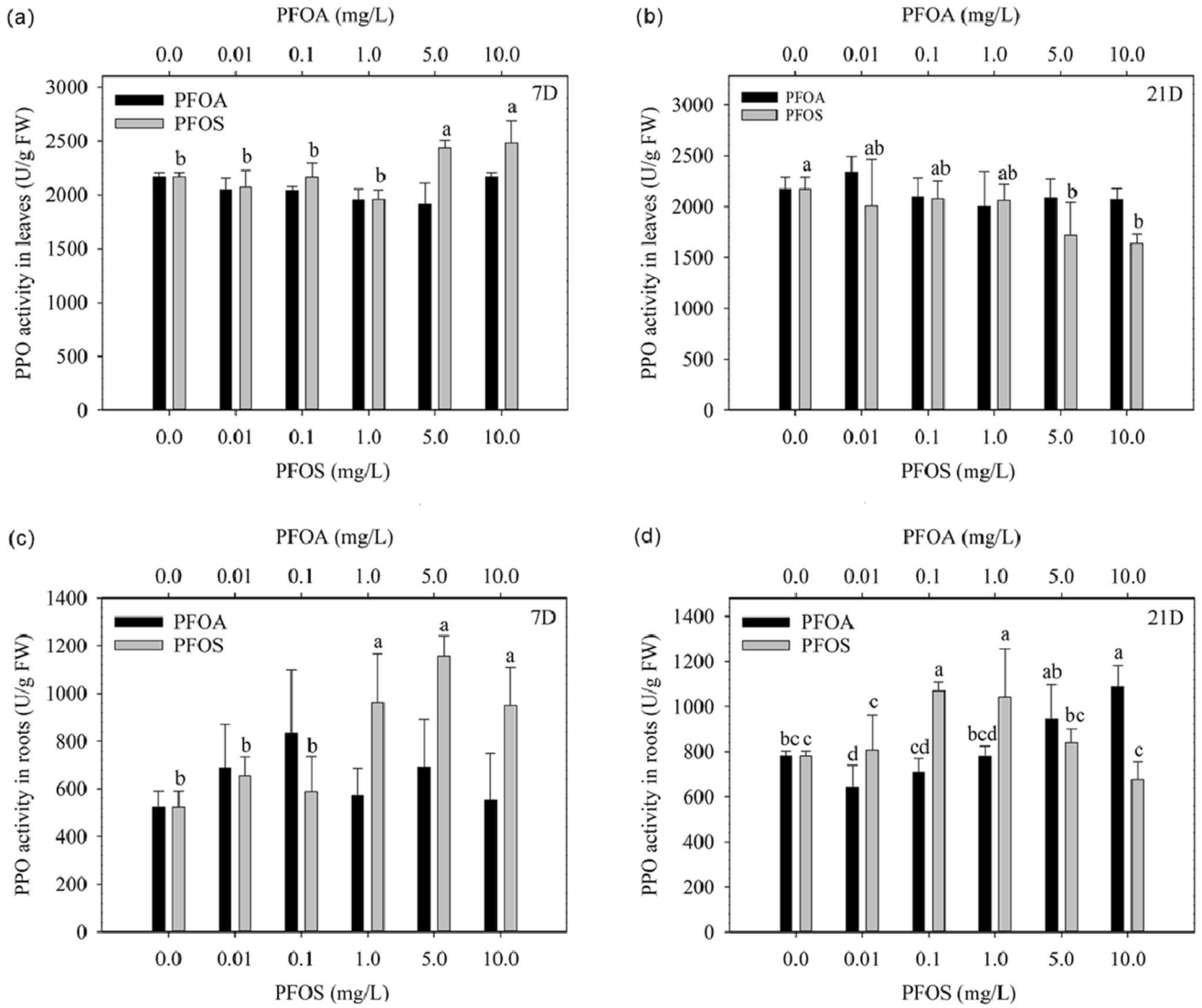

Fig. 9 PPO for single treatment of PFOA and PFOS to *V. natans* after exposure for 7 (**a**, **c**) and 21 days (**b**, **d**)

a common trend for CAT and SOD in leaves. In contrast, the induction of SOD activity and the inhibition of CAT activity usually occurred together under long-term exposure, indicating that CAT and SOD are the main protective enzymes of *V. natans*. POD act as a detoxification enzyme in the second phase of antioxidant defense system and was also involved in the elimination of H_2O_2 (Hua et al. 2022). As exposure time increased, POD joined in response to high stress concentrations. Changes in enzyme activity are influenced by a combination of toxic excitatory effects, antioxidant defense, and destruction of enzyme proteins at high concentrations. In order to adapt to the stress of PFOA and PFOS, *V. natans* integrally regulates multiple protective enzymes to form the defense function of the whole antioxidant enzyme system, maintaining the dynamic balance in the body to cope with the adverse environment and achieve the protective effect, while, the rate and duration of response of different protective enzymes to different stresses varies.

V. natans can also maintain its osmotic potential in response to adverse environment and achieve protective effects by accumulating solutes to form a long-lasting protective function of the osmoregulatory system. Similarly, the rate and duration of response of osmoregulatory substance accumulation to different concentrations of PFOA and PFOS differed. Leaves of *V. natans* mainly relying on the accumulation of soluble sugars and soluble proteins in response to short-term medium and high concentrations of PFOA and PFOS, and roots mainly relying on the accumulation of soluble sugars in response to long-term and short-term high concentrations of PFOA stress. In previous studies, the soluble protein content of *V. natans* leaves increased significantly under eutrophic water and 0.2-μg/L chloramphenicol

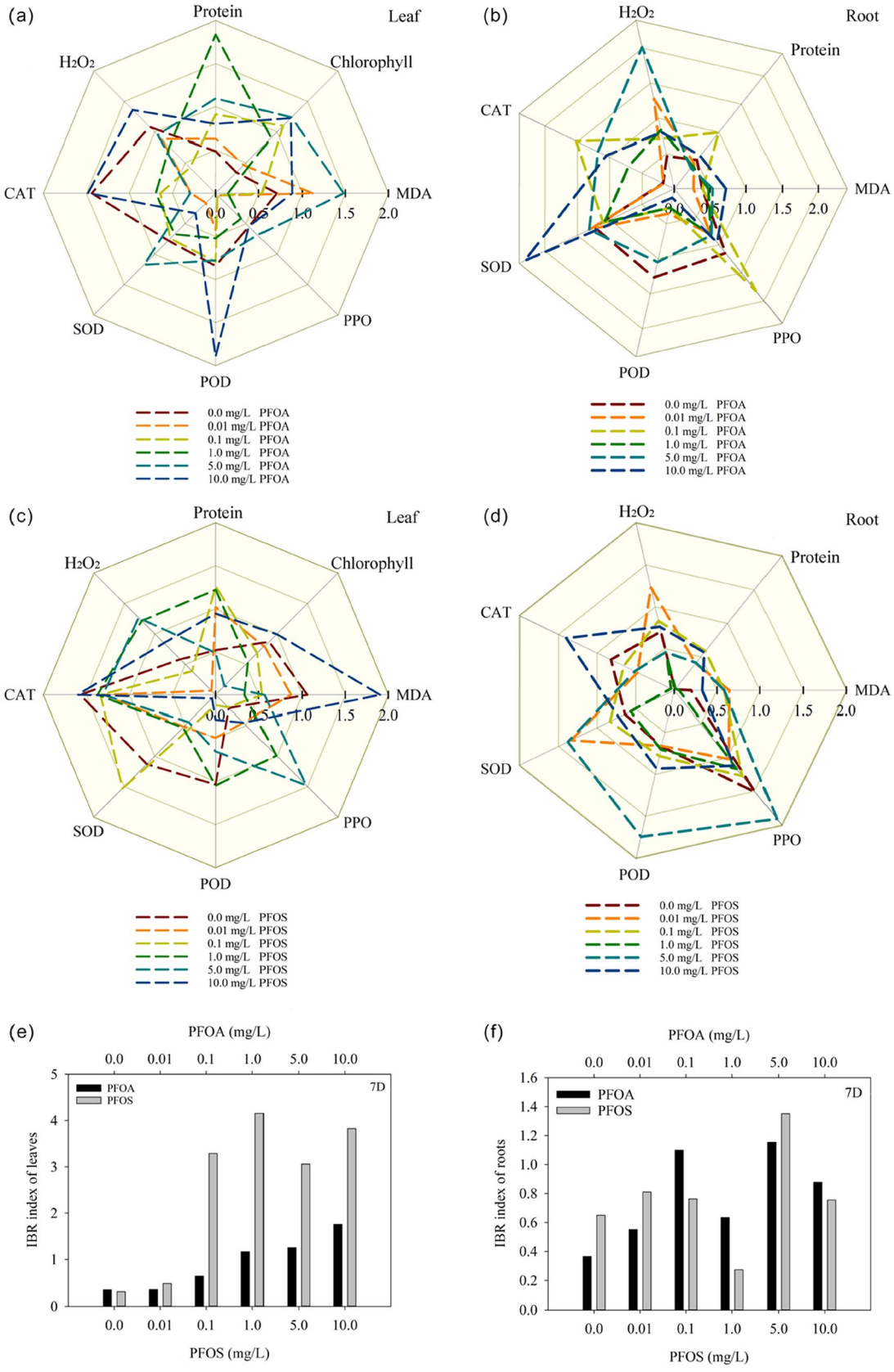

Fig. 10 IBR of single treatments of PFOA and PFOS to leaves (**a**, **c**, **e**) and roots (**b**, **d**, **f**) of *V. natans* after exposure for 7 days

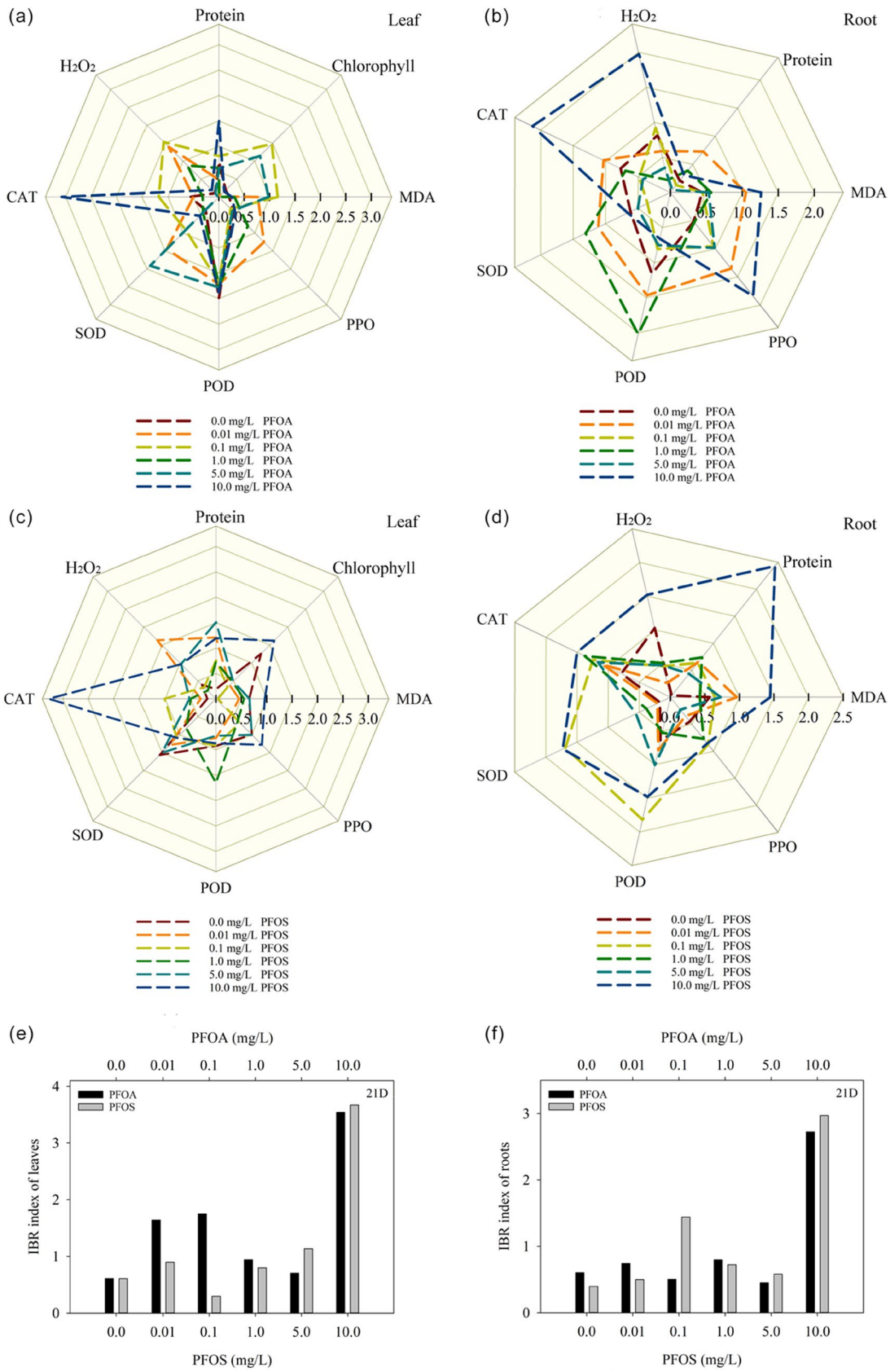

Fig. 11 IBR of single treatments of PFOA and PFOS to leaves (**a**, **c**, **e**) and roots (**b**, **d**, **f**) of *V. natans* after exposure for 21 days

stress (Hu et al. 2015), while the soluble sugar content of *V. natans* roots increased significantly under 1-mg/L BPA stress (Han et al. 2023). This is an adaptation strategy for *V. natans* to activate its osmoregulatory system to cope with external water pollution and maintain its survival.

Biomarkers such as soluble protein, SOD, CAT, POD, PPO, and MDA in leaves and roots of *V. natans* did not respond consistently to PFOA and PFOS exposure, and the ecotoxic effects could not be evaluated comprehensively using single indicator. IBR can visualize the ecotoxicological effects of *V. natans* at different exposure concentrations at different treatment times through quantitative values, thus effectively comparing their differences (Qian et al. 2019). The results indicated that the trend of IBR values for the short-term treatment of *V. natans* leaves showed a significant dose effect as well as tissue specificity: the response level of leaves increased with increasing treatment concentration and the IBR values of PFOS were greater than PFOA, and the IBR values of leaves were higher than roots at the same experimental concentrations. IBR values of roots showed no serious ecotoxic effects in short term, probably due to the large variation in the sensitivity of different biomarkers in roots to environmental stress, resulting in no correlation between IBR values and stress concentrations. It can also be further speculated that a compensatory mechanism exists between *V. natans* organism tissues, with transpiration providing some protection to root cells from greater oxidative damage, while the larger leaf-water exposure surface area allows for a higher response to PFOA and PFOS exposure. In contrast, as exposure time increased, the IBR values of both leaves and roots increased at medium and high concentrations after 21 days, reflecting the sensitivity of *V. natans* to external stimuli producing defense stress within a certain concentration range. Both leaves and roots showed maximum values of IBR at 10.0 mg/L PFOA and PFOS, indicating that long-term high concentrations of PFOA and PFOS treatment caused significant ecotoxic effects.

This result not only validates the difference in response of *V. natans* to PFOA and PFOS, but also quantifies the difference between leaves and roots, indicating that IBR can be a useful tool for quantitatively evaluating the ecotoxic effects of PFOA and PFOS.

Conclusions

This study comprehensively assessed the growth and physiological response of *V. natans* under short-term (7 days) and long-term (21 days) exposure to different concentrations of PFOA and PFOS, and the main findings are as follows: (1) both short-term and long-term high concentrations of PFOA and PFOS inhibit the relative growth rate of *V. natans*, with long-term inhibition becoming stronger but not causing negative biomass growth. (2) Most treatments can cause an increase in photosynthetic pigment content in leaves of *V. natans*. (3) In terms of oxidation, leaves were more sensitive to short-term high PFOS concentrations, while roots were more sensitive to high PFOA concentrations. Oxidative damage caused by PFOA and PFOS stress occurred mainly in leaves, both leaves and roots gradually built up tolerance with increasing exposure time during long-term oxidative stress, and no serious membrane damage was caused to *V. natans* under both short-term and long-term toxic exposures. (4) Leaves and roots mainly activate SOD, CAT and PPO in response to the accumulation of reactive oxygen species from short-term stress, while increasing exposure time activates POD in response to long-term high stress. With increasing exposure time, CAT and PPO activities were inhibited in leaves under both medium and high concentration treatments, while SOD activity was inhibited in roots. (5) The IBR values showed that under short-term exposure, leaves of *V. natans* were more sensitive than roots and more sensitive to PFOS than PFOA, while under long-term exposure at low and medium concentrations (< 5.0 mg/L), the IBR values indicated that the antioxidant system of *V. natans* was in balance and only displayed a significant ecotoxic effect at high concentrations of 10.0 mg/L. The IBR values of leaves under both short-term PFOA and PFOS exposures were dose-effective and suitable as indicators for short-term water pollution by PFCs.

Author contribution WZ: conceptualization, formal analysis, investigation, methodology. ZL: writing (original draft, review, and editing). ZK: writing (review and editing). ZW: supervision, writing (review and editing). All authors read and approved the final manuscript.

Funding This research was supported by the National Natural Science Foundation of China (No: 31270410 and 30670206).

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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