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Bioaccumulation and toxicity of perfluorooctanoic acid and perfluorooctane sulfonate in marine algae *Chlorella sp.*

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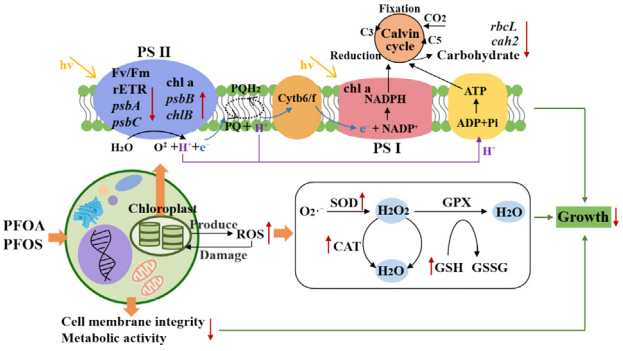
HIGHLIGHTS

- Low doses stimulate *Chlorella sp.* reproduction but high doses inhibit growth.
- PFOA and PFOS increase chlorophyll *a* content but reduce photosynthesis activity.
- PFOA and PFOS cause membrane damage, metabolic disorder and oxidative stress.
- PFOS shows higher toxicity and bioaccumulation than PFOA.

GRAPHICAL ABSTRACT



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ABSTRACT

The ocean is an important sink for perfluorinated alkyl acids (PFAAs), but the toxic mechanisms of PFAAs to marine organisms have not been clearly studied. In this study, the growth rate, photosynthetic activity, oxidative stress and bioaccumulation were investigated using marine algae *Chlorella sp.* after the exposure of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate acid (PFOS). The results showed that PFOA of <40 mg/L and PFOS of <20 mg/L stimulated algal reproduction, and high doses inhibited the algal growth. The absorbed PFOA and PFOS by algal cells damaged cell membrane and caused metabolic disorder. The photosynthesis activity was inhibited, which was revealed by the significantly reduced maximal quantum yield (Fv/Fm), relative electron transfer rate (rETR) and carbohydrate synthesis. However, the chlorophyll *a* content increased along with the up-regulation of its encoding genes (*psbB* and *chlB*), probably due to an overcompensation effect. The increase of ROS and antioxidant substances (SOD, CAT and GSH) indicated that PFOA and PFOS caused oxidative stress. The BCF of marine algae *Chlorella sp.* to PFOA and PFOS was calculated to be between 82 and 200, confirming the bioaccumulation of PFOA and PFOS in marine algae. In summary, PFOA and PFOS can accumulate in *Chlorella sp.* cells, disrupt photosynthesis, trigger oxidative stress and inhibit algal growth. PFOS shows higher toxicity and bioaccumulation than PFOA. The information is important to evaluate the environmental risks of PFAAs.

1. Introduction

Perfluoroalkyl acids (PFAAs) are a group of organic acids with hydrophobic perfluoroalkyl chains and hydrophilic carboxylate or sulfonate functional groups. They show unique physico-chemical characteristics, such as thermochemical stability, lipophobic and hydrophobicity properties (O'Hagan, 2008; Conder et al., 2008). Therefore, PFAAs have been widely used in leather, paper, fabric, packaging and furniture since the 1950s (Giesy and Kannan, 2002). After use, PFAAs are mainly discharged into aquatic environments through product degradation, wastewater discharge and surface runoff and cause concern because of their persistence, bioaccumulation and toxicity (Lee et al., 2020).

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Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are two main PFAAs in aquatic environments. The occurrence of PFOA and PFOS in drinking water, groundwater, surface water and ocean water has been discovered in many studies (Yamashita et al., 2005; Hoelzer et al., 2009; Munoz et al., 2017). As the primary producer in aquatic ecosystems, algae are vital to the ecological balance. It has been reported that PFOA and PFOS have toxic effects on freshwater algae. PFOA increased the lipid peroxidation of *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* (Hu et al., 2014). PFOS decreased cell membrane permeability, damaged the photosynthesis, and induced oxidative stress of *Chlorella vulgaris* (Xu et al., 2017). Marine algae are the basis of the marine food web and an important part of the productivity in planet (Thiagarajan et al., 2019b). At present, few studies on the toxicity of PFOA and PFOS to marine algae and have only focused on growth. However, the toxic mechanisms of cell membrane damage, metabolic disturbance, photosynthesis decrease and oxidative stress of PFOA and PFOS in marine algae have not been reported. Previous studies found that the growth (reflected by light output) of the bioluminescent dinoflagellate *Pyrocystis lunula* decreased after exposure to PFOA and PFOS, and the 24 h-EC50 were 18 mg/L for PFOA and 4.9 mg/L for PFOS, respectively (Hayman et al., 2021). The 72 h-EC50 for the green algae *Chlorella vulgaris*, the diatom *Skeletonema marinoi* and the blue algae *Geitlerinema amphibium* to PFOA were 2.36 mM, 0.89 mM and 0.6 mM, respectively (Latała et al., 2009). Green algae were far less sensitive to PFAAs than diatoms and blue algae because the indigestible cellulose forms cell wall, which is far stronger and less sensitive to PFAAs than the silica surrounding diatoms or the lipopolysaccharides in the outer layer of blue algae (Barsanti and Gualtieri, 2006). The longer the carbon chain of PFAAs, the more severe the inhibition of algal growth. Moreover, researchers have found that marine algae and freshwater algae show different sensitivities to pollutants. For example, the 96 h-EC50 of TiO_2 for freshwater Chlorella and marine Chlorella were 9.1 mg/L and 80 mg/L, respectively (Middepogu et al., 2018; Xia et al., 2018). Naphthalene (under N, P-enriched condition, < 10 mg/L) and microplastics (PE, < 200 mg/L) were reported to stimulate the growth of freshwater Chlorella (Kong et al., 2011; Song et al., 2020), but to inhibit the growth of marine Chlorella (Kong et al., 2010; Su et al., 2022). The different toxic effects of pollutants on marine algae and freshwater algae suggest different toxic mechanisms.

In addition, the high bioaccumulation of PFOA and PFOS in freshwater algae suggested that PFOA and PFOS can cause toxic effects on higher trophic level organisms (Kannan et al., 2005). PFOA and PFOS were reported to transfer through trophic levels, accumulate in mussels and fish and cause toxic effects to them (Xu et al., 2014; Menger et al., 2020; Renzi et al., 2013). PFOA and PFOS in marine fish species and invertebrate species suggested that food also contributed partly to the bioaccumulation of PFOA and PFOS in higher trophic level organisms (Du et al., 2021). However, the uptake and absorption of PFOA and PFOS in marine algae have not been reported.

Therefore, it is necessary to study the toxic mechanisms and bioaccumulation of PFOA and PFOS in marine algae. As marine algae *Chlorella sp.* is one of the dominant green algae in the ocean (Niu et al., 2019), it was selected as the research object. This study aims to analyze the toxic mechanism and investigate the bioaccumulation characteristics of PFOA and PFOS in marine algae *Chlorella sp.* The results will make up for the deficiency of PFAAs toxicity to marine algae. It is of great significance to evaluate the ecotoxicity of PFOA and PFOS in ocean systems and their impact on human health.

2. Materials and methods

2.1. Algal cultivation

The *Chlorella sp.* was provided by Freshwater Algae Culture Collection at the Institute of Hydrobiology, Wuhan, China. The preparation of culture medium (Erdschreiber medium) is introduced in the Supporting Information. The algae were grown in a conical flask in an illumination incubator with a 12:12 h light (5000 lx)/dark cycle at 23 ± 1 °C. The conical flask was shaken three times per day to prevent algae from sticking to the wall. The algal solution was inoculated every two weeks to ensure good growth status. All cultivation and experiments were performed under aseptic conditions.

2.2. Growth measurement

The exposure experiments were conducted when the algae were in logarithmic growth phase. The algal cells were diluted to an initial density of 5 × 10^4 cells/mL and were exposed to 0, 5, 10, 20, 40, 80, 160, 320 mg/L PFOA (purity>98 %, Tokyo Chemical Industry Co., Ltd.) and 0, 5, 10, 20, 40, 80, 160 mg/L PFOS (purity>98 %, Beijing Mreda Technology Co., Ltd.) according to OECD guidelines. The algal cells and cell size were counted by flow cytometry (Accuri™ C6 Plus, BD, USA) every 24 h from day 1 to day 7. The growth inhibition rate was calculated according to the formula in OECD guidelines and 96 h-EC50 was fitted (Xu et al., 2013), the details were provided in the Supporting Information. In addition, the cell dry weight was measured on day 7. Algal cells were collected by centrifugation (5000 *g*, 5 min) and washed by phosphate buffered saline (PBS). The cells were dried at 100 °C and weighted. The individual cell dry weight was calculated using the number of algal cells.

2.3. Quantification of chlorophyll a fluorescence, maximal quantum yield, relative electron transfer rate and carbohydrate contents

According to the 96 h-EC50, the concentrations in the following exposure experiments were set as 0, 5, 10, 20, 40, 80 mg/L PFOA and 0, 5, 10, 20, 40 mg/L PFOS. Chlorophyll a (chl a) emits red fluorescence after receiving excitation light, hence the chlorophyll *a* fluorescence intensity in algal cells was measured on day 1, 3, 5 and 7 in the APC channel by flow cytometry (Liu et al., 2008).

Maximal quantum yield (Fv/Fm) and relative electron transfer rate (rETR) were measured on days 1, 3, 5 and 7 after exposure. 3 mL algal solution was collected and adapted in the dark for 30 min to ensure that photosystem II and all photoelectronic doors were fully open (Middepogu et al., 2018). The Junior-Pulse-Amplitude-Modulation chlorophyll fluorometer (Heinz Walz GmbH, Germany) produced measuring light and saturation pulses and calculated Fv/Fm and rETR automatically (Wang et al., 2021).

The carbohydrate contents were quantified by the anthrone-sulfuric acid method (Zheng et al., 2017). Algal cells were collected on day 7 by centrifugation (5000 *g*, 5 min), and were resuspended in PBS. The cells were disrupted in an ice box using an ultrasonic homogenizer (300 W power, 10 min). The dissolved contents were separated from the residues after centrifugation, and the residues were treated with 1.5 mL 30 % perchloric acid for 15 min to further collect the dissolved contents. The collected solution was mixed with triploid volumes of 1 g/L anthrone in 30 % sulfuric acid, and was placed in stopper tubes for a 15-min boiling water bath, followed by a 15-min ice bath. The carbohydrates were hydrolyzed into monosaccharides under the action of sulfuric acid, and dehydrated to form furfural derivatives rapidly. The furfural derivatives combined with anthrone to form colored compounds (Zheng et al., 2017). The carbohydrate contents were quantified by an ultraviolet spectrophotometer (TU-1900, Puxi, China) at 625 nm absorbance.

2.4. Reactive oxygen species and antioxidant biomarkers

Reactive oxygen species (ROS) were measured on days 1, 3, 5 and 7 after exposure. Algal cells were collected from 1 mL suspension via centrifugation (5000 *g*, 5 min), stained with 100 μM H_2DCFDA for 1 h in the dark. H_2DCFDA can enter cells and be hydrolyzed by intracellular enzymes to produce DCFH, and then the intracellular ROS oxidize DCFH to produce fluorescent DCF (Zheng et al., 2017). The stained cells were washed and resuspended in PBS, and the ROS levels were quantified in the FITC channel of flow cytometry.

For analysis of superoxide dismutase (SOD) activity, catalase (CAT) activity and glutathione (GSH) contents, algal cells were collected by centrifugation after 7-day exposure and then were re-suspended in PBS. The suspension was sonicated to disrupt algal cells in an ice box using an ultrasonic homogenizer (300 W power, 10 min). The antioxidant substances in the supernatant were collected after centrifugation (5000 *g*, 10 min). The SOD activity, CAT activity and GSH contents were quantified using the corresponding assay kits (Jiancheng Bioengineering Institute, China).

2.5. Cell membrane integrity and esterase activity

Algal cells were collected from 1 mL suspension via centrifugation (5000 *g*, 5 min) on days 1, 3, 5 and 7 after exposure. Cell membrane integrity was measured after 20-min staining with 15 μM propidium iodide (PI) in the dark (Hu et al., 2020). PI cannot pass through the intact cell membrane, but can penetrate the damaged cell membrane and enter algal cells and bind to nucleic acids to produce red fluorescence (Liu et al., 2008). The stained cells were washed and re-suspended in PBS and measured in the PE channel of flow cytometry. The esterase activity was measured after 10-min staining with 25 μM fluorescein diacetate (FDA) in the dark (Franklin et al., 2001). After being absorbed by cells, FDA can transform into fluorescein by esterase to display green fluorescence (Zhang et al., 2020). The stained cells were washed and re-suspended in PBS and measured in the FITC channel of flow cytometry.

2.6. Gene expression analysis

After the exposure to 5, 80 mg/L PFOA and 5, 40 mg/L PFOS for 7 days, polysaccharides and polyphenols were removed from the algal cells (FruitMate™, TaKaRa, China). The total RNA was extracted from 100 mg algal cells via RNAiso Plus method (TaKaRa, China). Reverse transcription of total RNA (1 μg) was performed using a Hifair® III 1st Strand cDNA Synthesis SuperMix containing gDNA digester (Yeasen, China). Real-time quantitative PCR was performed using an applied biosystems QuantStudio 5 real-time quantitative PCR system (Quantstudio 5, Thermo, USA). The primers of 6 genes (*chlB*, *psbA*, *psbB*, *psbC*, *cah2* and *rbcL*) related to photosynthesis were synthesized according to literature (Wang et al., 2019; Middepogu et al., 2018). The 18S rDNA gene was selected as a housekeeping gene (Xu et al., 2010). The relative expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Bioaccumulation of PFOA and PFOS by algae

After 7-day exposure, algal cells were collected from 40 mL suspension through centrifugation (5000 *g*, 5 min). The PFOA (or PFOS) adsorbed on cells was separated from the PFOA (or PFOS) uptake in cells through washing with 5 mL deionized water and centrifugation three times. The washing solutions were combined together (about 15 mL in total) to measure the adsorbed PFOA (or PFOS). The precipitated cells were collected and freeze-dried to measure the PFOA (or PFOS) uptake by algal cells. The amounts of PFOA and PFOS were quantified using an ultra-high-performance liquid chromatograph (Ultimate 3000, Thermo Fisher Scientific Inc., USA) equipped with a Hypersil GOLD™ C18 column (2.1 mm × 150 mm length, 3 μm particle size) coupled to a mass spectrometer (ISQ EC, Thermo Fisher Scientific Inc., USA). The sample purification and analysis were performed according to literature (Liu et al., 2017), which were introduced in detail in the Supporting Information. Information on ions, corresponding isotope-labeled standards, detection limits, quantification limits and recoveries of target compounds are summarized in Table S1 in the Supporting Information.

The bioconcentration factor (BCF) is an index used to measure the size of bioaccumulation. BCF is calculated as follows:

$$BCF = \frac{C_a}{C_m} \tag{3}$$

where C_a and C_m are the individual concentrations of PFOA or PFOS in algae (ng/mg dry weight) and medium (mg/L) on day 7, respectively.

2.8. Statistical analysis

All experiments were performed in triplicate. Statistical analysis was operated via one-way variance (ANOVA) using the software SPSS 26. The date was expressed as the mean ± standard deviation (SD). When the $p < 0.05$, the date was considered to be significantly different.

3. Results

3.1. Effects of PFOA and PFOS on the growth of marine algae Chlorella sp.

The cell density of *Chlorella sp.* during 7-day exposure to PFOA and PFOS were shown in Fig. 1. Because the individual cell weight and cell size were not affected by the PFOA and PFOS exposure (Figs. S1 and S2), cell density according to OECD guidelines was used to characterize the cell growth (Xu et al., 2013; Hu et al., 2014; Middepogu et al., 2018). The algal cell density gradually increased from day 1 to day 7 in all treatment groups. The exposure to 5, 10, 20 and 40 mg/L PFOA promoted the algal growth (max growth with 20 mg/L PFOA), but the exposure to 80, 160 and 320 mg/L PFOA significantly inhibited algal reproduction. For PFOS, the exposure to 5, 10 and 20 mg/L PFOS also promoted the algal growth (max growth with 10 mg/L PFOS), but the algal reproduction was inhibited significantly after the exposure to 40, 60 and 80 mg/L PFOS. This result indicates that low doses of PFOA and PFOS can stimulate the cell division of *Chlorella sp.*, but high doses inhibit its growth. Moreover, the 96 h-EC50 for *Chlorella sp.* were 127.35 mg/L for PFOA and 77.62 mg/L for PFOS (Fig. S3), indicating that PFOS was more toxic than PFOA to *Chlorella sp.*

3.2. Cell membrane integrity and esterase activity

The binding of PFOA and PFOS on *Chlorella sp.* cells may damage the cell membrane (Hu et al., 2020). In this study, the algal membrane integrity was quantified by PI fluorescence (Fig. 2a & b). On day 1, a significant increase of fluorescence intensity (increased by 23.8 %) was observed only at 80 mg/L PFOA (Fig. 2a). From day 3 to day 7, high-dose PFOA caused more severe damage to cell membrane than low-dose PFOA, and the fluorescence intensity was increased by 9 %–24.5 %. PFOS caused serious membrane damage to *Chlorella sp.* during the entire exposure period, PI fluorescence increased by 10.9 %–168.1 %, indicating that PFOS was more destructive than PFOA to the cell membrane (Fig. 2b). From day 1 to day 7, the membrane damage caused by high-dose PFOS was gradually relieved, and the growth rate of fluorescence intensity in 40 mg/L treatment was reduced from 168.1 % to 80.9 %.

Studies have shown that the metabolic level of algal cells is related to cell membrane function (Xu et al., 2013; Zhang et al., 2020). The esterase activity of *Chlorella sp.* was studied using FDA straining to assess the metabolic levels (Jochem, 1999). The esterase activity of *Chlorella sp.* treated with PFOA decreased during the whole exposure period (Fig. 2c). In particular, the esterase activity became lowest on day 5, decreased to 68.5–88.2 % of control. The esterase activity in PFOS treatment groups decreased in a dose-dependent manner (Fig. 2d). The esterase activity became lowest on day 7, decreased to 62.6–85.2 % of control. Therefore, both PFOA and PFOS reduced the metabolic levels of *Chlorella sp.*, and PFOS was more destructive to metabolic activity.

3.3. Effects of PFOA and PFOS on the photosynthetic system of Chlorella sp.

Photosynthesis is an indicator of physiological characteristics and the most sensitive process under stress conditions (Zheng et al., 2017), which consists of photoreaction and dark reaction. Chlorophyll a is an antenna pigment and is considered to be the basis for estimating photoreaction rates (Liu et al., 2015). In our experiments, PFOA increased the fluorescence of chlorophyll *a* (Fig. 3a). PFOA led to stronger chlorophyll *a*

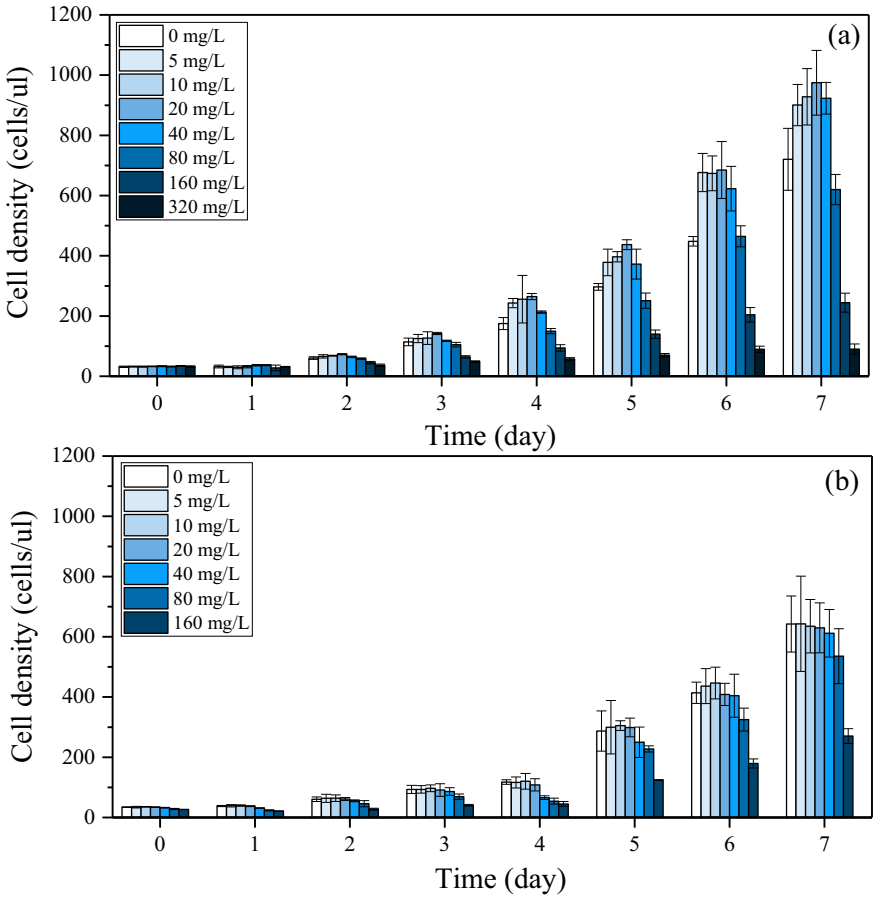

Fig. 1. The algal cell density of *Chlorella sp.* exposed to different concentrations of PFOA (a) and PFOS (b) for 7 days.

fluorescence with the extension of exposure time, chlorophyll *a* fluorescence was 103.7–112.7 % of control on day 1, and was 102.7–134.9 % on day 7. PFOS also increased chlorophyll *a* fluorescence, the effect of PFOS showed dose-dependent manner on day 1, 5 and 7 (Fig. 3b). The increase in chlorophyll *a* indicated that PFOA and PFOS interfered with the photoreaction. In addition to chlorophyll *a*, the maximum photosynthetic yield (Fv/Fm) and relative electron transport rate (rETR) of *Chlorella sp.* were measured to evaluate photosynthetic capacity and to reveal the stresses. Fv/Fm began to decrease significantly on day 1 after PFOA exposure (80.9–91.4 % of control), and decreased in a dose-dependent manner on day 3 and day 5 (Fig. 3c). Meanwhile, rETR did not decrease significantly on day 1 ($p > 0.05$), but decreased severely to 68.9–76.5 % of control on day 5, and recovered to 86.6–91.4 % of control on day 7 (Fig. 3e). PFOS decreased Fv/Fm and rETR in a dose-dependent manner throughout the exposure period (Fig. 3d & f). Interestingly, the most severe inhibition of Fv/Fm (74.7–88.2 % of control) and rETR (62.6–70.7 % of control) happened on day 1. The rETR on day 7 was 90.6 % of control with 40 mg/L PFOS. The rETR on day 7 became higher than the rETR on day 3 and day 5 for PFOA and PFOS exposure, indicating that the electron transport function of *Chlorella sp.* was initially inhibited but gradually adapted to environmental stress. In addition, Fv/Fm was more sensitive to PFOS than to PFOA. The carbohydrate contents (product of the dark reaction) were measured to evaluate the function of the dark reaction. Carbohydrate contents decreased after exposing to PFOA and PFOS (Fig. 4). When PFOA and PFOS were ≥ 20 mg/L, carbohydrate contents decreased to 20.0–31.4 % of control. The reduced carbohydrate production indicated impaired carbon assimilation in the Calvin cycle.

The expression levels of genes involved in photosynthesis were measured to verify the results of the photosynthesis tests. PFOA and PFOS induced various alterations in gene expression in algal cells (Fig. 5). The expression levels of *chlB* (chlorophyll synthesis protein) and *psbB* (chlorophyll *a* apoprotein) were up-regulated after PFOA treatments. 80 mg/L PFOA increased *chlB* and *psbB* levels to 18-fold and 12-fold of the control, respectively. PFOS of 5 mg/L did not increase the expression levels of *chlB* and *psbB*, but 40 mg/L PFOS increased *chlB* and *psbB* levels to 4.9-fold and 1.7-fold of the control, respectively. In addition, the expression levels of photosystem II (PS II) reaction-related proteins (*psbA*, *psbC*) and carbon fixation-related proteins (*rbcL*, *cah2*) were generally down-regulated after PFOA and PFOS treatment. *psbA*, *psbC*, *rbcL* and *cah2* decreased to 0.45-, 0.03-, 0.20- and 0.53-fold of the control after 80 mg/L PFOA treatment, respectively. They were reduced to 0.19-, 0.24-, 0.38- and 0.15-fold of the control after 40 mg/L PFOS treatment, respectively. The results suggested that PFOA and PFOS inhibited the PS II functions and carbohydrate synthesis in algae. Furthermore, the PS II functions and carbohydrate synthesis were more sensitive to PFOS than to PFOA according to the lower *psbA*, *psbB* and *cah2* expression with PFOS treatments.

3.4. Effects of PFOA and PFOS on ROS fluorescence and antioxidant defense

Oxidative stress is one of the main causes of cell toxicity in algae (Ge et al., 2015; Sanchez et al., 2015). ROS levels and antioxidant capacity were measured to assess oxidative stress. PFOA caused a dose-dependent increase in intracellular ROS levels (Fig. 6a). The oxidative stress became more severe on day 5 and day 7, the ROS was 113.2–124.6 % of control on day 5 and 106.7–129.7 % of control on day 7. However, the PFOS induced oxidative stress was generally more severe on day 1 (162.6–240.9 % of control) compared with the longer-time exposure (Fig. 6b). The PFOS-induced oxidative stress mitigated over time, on day 7 the ROS was only 143.7–159.5 % of the control. In addition, PFOS caused more severe oxidative stress than PFOA.

Antioxidants, including SOD, CAT and GSH, are important indicators in alleviating ROS-induced oxidative damage. The activity or content of

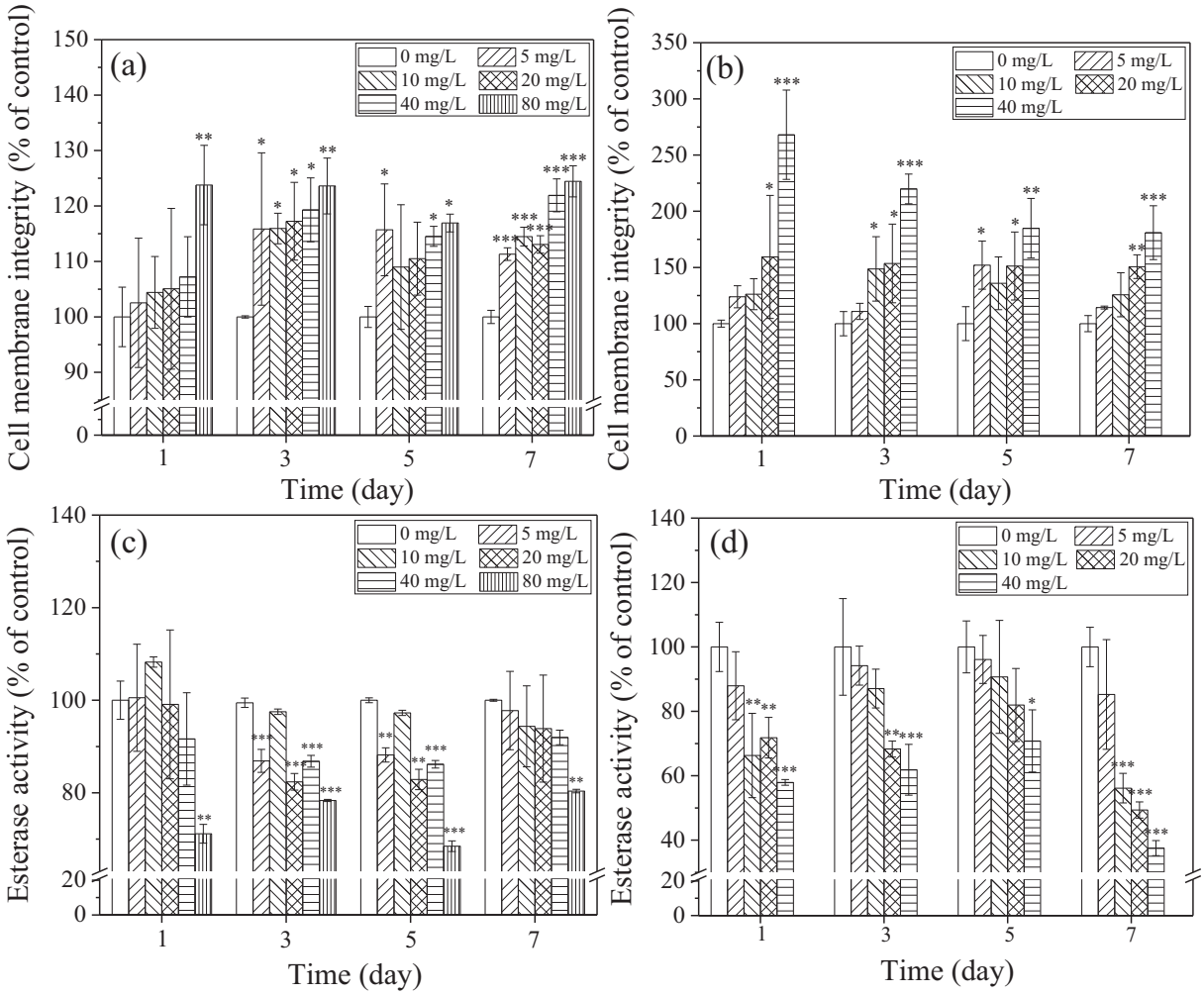

Fig. 2. Cell membrane integrity was measured by propidium iodide (PI) staining after *Chlorella sp.* cells were exposed to PFOA (a) and PFOS (b) for 1, 3, 5 and 7 days. PI penetrates the damaged cell membrane and integrates with nucleic acids to produce red fluorescence, which reflects cell membrane integrity. The esterase activity of *Chlorella sp.* was measured by fluorescein diacetate (FDA) staining after *Chlorella sp.* cells were exposed to PFOA (c) and PFOS (d) for 1, 3, 5 and 7 days. The data are expressed as percentages of the control group. Statistical significance versus the control group: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

antioxidants were measured to further verify the oxidative stress. After exposure to PFOA, SOD activity, CAT activity and GSH content increased in a dose-dependent manner, which illustrated the activation of the antioxidant system (Fig. 7a-c). PFOS caused a dose-dependent increase in CAT activity and GSH content (Fig. 7b&c), which was consistent with the results of PFOA exposure tests. PFOS stimulated SOD activity, and SOD activity achieved a maximum with 10 mg/L PFOS and then decreased (Fig. 7a), which was different from the results of PFOA exposure. The production of ROS and the activation of antioxidant system indicated that PFOA and PFOS caused the imbalance of oxidation and antioxidant effect in *Chlorella sp.*, and led to oxidative stress.

3.5. Uptake, adsorption and bioaccumulation of PFOA and PFOS by Chlorella sp.

PFOA uptake and adsorption by algal cells were positively correlated with the exposure doses (Table 1). As the PFOA doses increased from 5 to 80 mg/L, the PFOA uptake increased from 0.50 mg/g to 6.15 mg/g (dry weight), and the adsorption increased from 0.86 mg/g to 6.66 mg/g. However, the uptake and adsorption percentages of PFOA by algal cells were negatively correlated with increasing PFOA doses. The uptake percentages decreased from 0.89 % to 0.66 %, and the adsorption percentages decreased from 1.54 % to 0.71 %. For PFOS, the uptake increased from 0.78 mg/g to 3.94 mg/g (dry weight), and the adsorption increased from 1.15 mg/g to 5.19 mg/g as the PFOS doses increased from 5 to 40 mg/L. The uptake percentages decreased from 1.21 % to 0.95 %, and the adsorption percentages decreased from 1.77 % to 1.25 %. The adsorption amount of PFOA and PFOS by *Chlorella sp.* was greater than their uptake amount. The uptake percentages were decreased on the higher doses because the algal cells have limited uptake capacity. Hence, the amount of PFAAs uptake by an individual algal cell cannot keep increasing with the extracellular concentration. In addition, higher dose inhibited algal growth and resulted in less algal cells after exposure, which decreased the uptake percentages. In our experiment, the threshold concentrations to cause growth inhibition were 64.56 mg/L PFOA and 12.27 mg/L PFOS (Fig. S3), therefore the uptake percentages were decreased on the higher doses due to the lower growth.

The biological concentration factor (BCF) decreased from 117.23 to 83.11 with the increasing PFOA doses, indicating that the bioaccumulation ability of *Chlorella sp.* to PFOA decreased with increasing doses. The bioaccumulation of PFOS in *Chlorella sp.* showed the same pattern. BCF decreased from 199.75 to 146.86 as the PFOS doses increased from 5 to 40 mg/L. The data also indicate that the bioaccumulation ability of PFOS is greater than PFOA.

4. Discussion

Unicellular algae are highly sensitive to PFOA and PFOS. The injury mechanisms of PFOA and PFOS on green algae mainly include disrupting

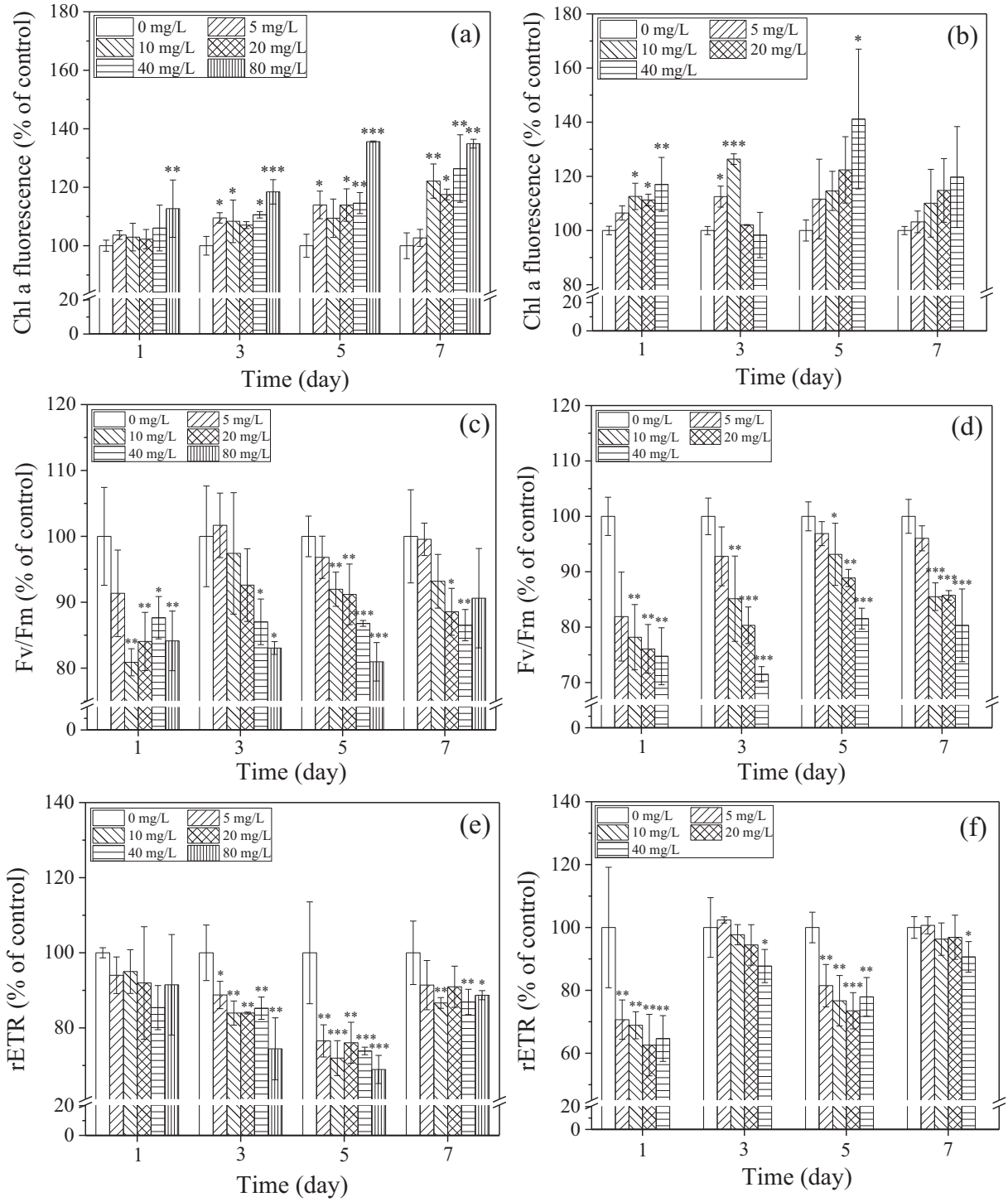

Fig. 3. Interference on the photoreaction of *Chlorella sp.* after 1, 3, 5 and 7 d exposure to PFOA and PFOS. Chl a fluorescence after the exposure to PFOA (a) and PFOS (b). Fv/Fm after the exposure to PFOA (c) and PFOS (d). rETR after the exposure to PFOA (e) and PFOS (f). The data are expressed as percentages of the control group. Statistical significance versus the control group: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

photosynthesis, triggering oxidative stress, inducing membrane damage and inhibiting gene expression (Li et al., 2021; Xu et al., 2017; Liu et al., 2008). In this study, we found that marine algae *Chlorella sp.* was more sensitive to PFOS than PFOA. In addition, low doses of PFOA and PFOS could stimulate the cell division of *Chlorella sp.* and high doses inhibited its growth (Fig. 1). Previous studies also found that low doses of 4-hydroxybenzoic acid and glyphosate can promote the growth of green algae *Pseudokirchneriella subcapitata* and gold algae *Prymnesium parvum*, respectively (Kamaya et al., 2006; Dabney and Patino, 2018). This may be due to the overcompensation effect caused by the reversal of homeostasis (Calabrese, 2001). Low doses of harmful substances stimulate the organisms to produce a beneficial response, hence strengthen the normal functions of the organisms and enhance the ability to resist subsequent stimuli. In this study, PFOA of <40 mg/L and PFOS of <20 mg/L stimulated algal reproduction (Fig. 1), which can be explained by the overcompensation effect. The phenomenon of low-dose PFOA and PFOS promoted algal growth was not reported in the previous studies. When the doses of PFOA and PFOS further increased to exceed the tolerance of the algae, the algae

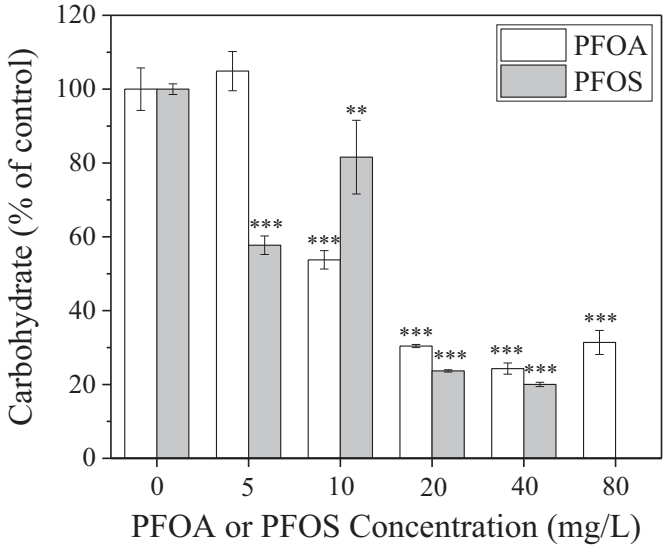

Fig. 4. Carbohydrate of *Chlorella sp.* after the exposure to PFOA and PFOS for 7 days. The data are expressed as percentages of the control group. Statistical significance versus the control group: *p < 0.05, **p < 0.01, ***p < 0.001.

showed growth inhibition (Fig. 1). Such low-dose stimulation and high-dose inhibition is called hormetic pattern (Calabrese, 2001).

PFOA and PFOS disrupted the cell membrane integrity of *Chlorella sp.* (Fig. 2a & b). PFOA and PFOS can be readily absorbed by algal cells (Table 1). Due to the high hydrophobic and oleophobic surface activity of PFOA and PFOS, they bind to cell surfaces, then the adsorbed PFOA and PFOS can interfere with the integrity and permeability of the cell membrane (Sutherland and Ralph, 2019). In addition, PFOA and PFOS may change cell membrane integrity by affecting membrane fluidity and signaling pathway of membrane proteins (Harada et al., 2005). The damage of cell membrane may disturb the normal cell functions of *Chlorella sp.*, such as metabolic levels. In this study, the metabolic levels of *Chlorella sp.* were inhibited (Fig. 2c & d). The damage of cell membrane caused an unbalanced redox state in the esterase synthesis, which decreased esterase activity and indicated a decreased level of cellular metabolism (Zhang et al., 2020). Membrane damage and reduced metabolism eventually led to reduced growth of *Chlorella sp.*

PFOA and PFOS reduced the photosynthetic activity in this study. Photosynthesis includes photoreaction and dark reaction, and the photoreaction is completed by photosystem I (PS I) and photosystem II (PS II). The decreased Fv/Fm and rETR indicated the destruction in PS II function (Fig. 3a&d). The *psbA* D1 protein forms the PS II reaction center and *psbC* protein is the component of CP43 complex in the PS II inner antenna

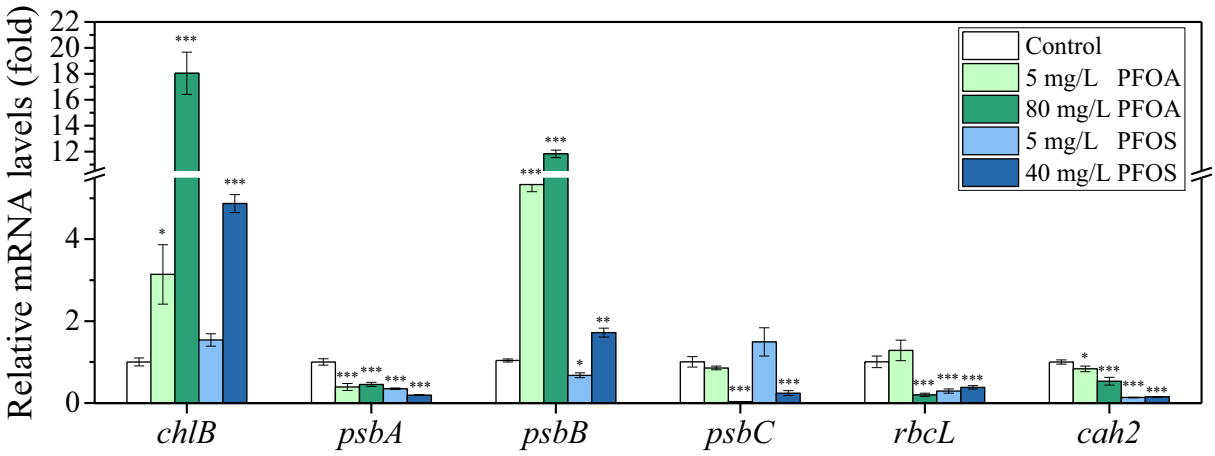

Fig. 5. Relative mRNA levels of 6 genes in the algal cells exposed to 5, 80 mg/L PFOA and 5, 40 mg/L PFOS for 7 days. Statistical significance versus the control group: *p < 0.05, **p < 0.01, ***p < 0.001.

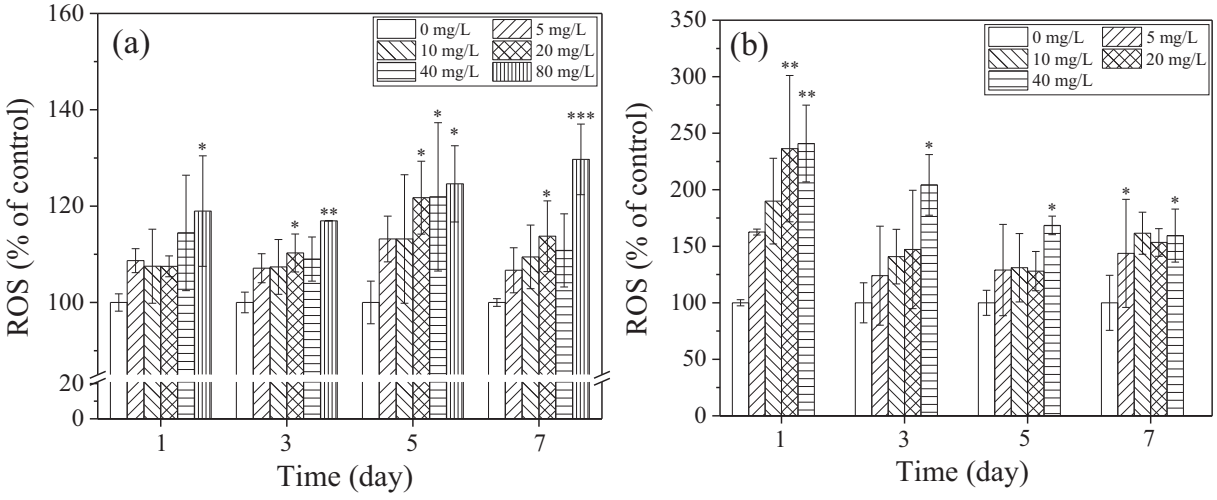

Fig. 6. Reactive Oxygen Species (ROS) was measured by H_2DCFDA staining after *Chlorella sp.* cells were exposed to PFOA (a) and PFOS (b) for 1, 3, 5 and 7 days. The data are expressed as percentages of the control group. Statistical significance versus the control group: *p < 0.05, **p < 0.01, ***p < 0.001.

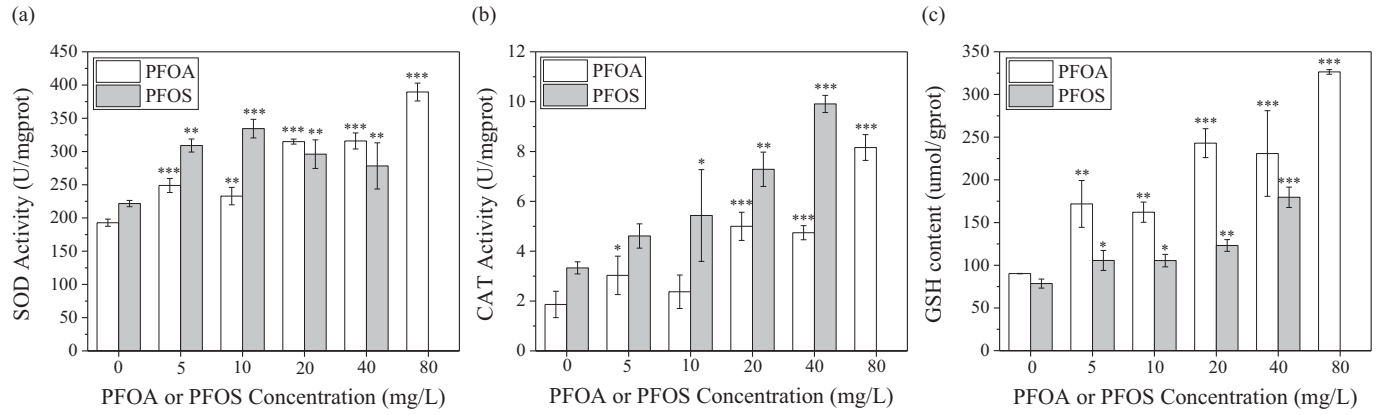

Fig. 7. Responses of the antioxidant system in *Chlorella sp.* exposed to PFOA and PFOS on day 7: (a) superoxide dismutase (SOD), (b) catalase (CAT) and (c) reduced glutathione (GSH) of *Chlorella sp.* exposed to PFOA and PFOS. Statistical significance versus the control group: *p < 0.05, **p < 0.01, ***p < 0.001.

(Adamiec et al., 2018). The two proteins play important roles in photoelectron transport and water splitting in photoreaction. In our study, the mRNA levels of *psbA* and *psbC* were down-regulated (Fig. 5), confirming that PFOA and PFOA damaged PS II reaction center, disturbed PS II energy transfer, and impaired photoreaction. Photoreaction provides energy and raw materials for ATP and NADPH synthesis. The energy for ATP synthesis is produced by the proton gradient between light trapping protein and electron transfer complex, and the electrons for NADPH synthesis is produced from water cleavage in PS II (Lu et al., 2015). In dark reactions, ATP and NADPH are required to fix carbon from CO_2 into the energy storing carbon compounds. Hence, the decrease of photoreaction should disturb carbon fixation and Calvin cycle, which was confirmed by the inhibited algal carbohydrate synthesis in our experiments (Fig. 4). In addition, *rbcL* encodes the RuBisCO small subunit and *cah2* encodes carbonic anhydrase, both are involved in carbon fixation (Razzak et al., 2017; Fukuzawa et al., 1990). Their expression levels decreased (Fig. 5), which further confirmed that PFOA and PFOS inhibited carbon fixation in algae. Therefore, the PFOA and PFOS induced growth inhibition of *Chlorella sp.* can be explained by disrupted energy transport and carbon fixation in the photosynthetic processes.

The chlorophyll *a* content was stimulated by PFOA and PFOS (Fig. 3a&b), which differs from the results using freshwater algae (chlorophyll *a* content decreased in general) (Li et al., 2021; Xu et al., 2013; Liu et al., 2018). The genes involved in chlorophyll *a* apoprotein (*psbB*) and chlorophyll synthesis protein (*chlB*) were also significantly up-regulated (Fig. 5), which was corroborated the increased chlorophyll *a* content. The increase of pigment is possibly the response of algae to resist environmental stress, which does not contribute to the higher photosynthetic activity and faster algal growth (Su et al., 2022; Middepogu et al., 2018). For example, chlorophyll is part of the signaling network pertinent to stress responses, also is involved in the production of carbon-based defensive chemicals to mitigate stress. Hence, the exposure to pollutants, such as aquaculture effluent, polybrominated diphenyl ethers and heavy metals, may stimulate the synthesis of chlorophyll (Agathokleous et al., 2020; Farzana and Tam, 2018; Nikookar et al., 2005; El-Sheekh et al., 2003). In our study, *Chlorella sp.* produced more chlorophyll *a*, which may be due to the overcompensation effect of marine algae to resist the stress of PFOA and PFOS. Freshwater algae and marine algae exhibit different responses to PFOA and PFOS, probably because they evolved into different species over time in different natural environments.

PFOA and PFOS induced oxidative damage to *Chlorella sp.* by triggering the increased levels of ROS (Fig. 6). The decrease in Fv/Fm and rETR of photosystem II in algae was an important reason for the increase of ROS (Thiagarajan et al., 2019a; Costa et al., 2016), and excessive ROS can damage chloroplast structure and function (Middepogu et al., 2018). Organic pollutants may bind to D1 polypeptide in the thylakoid membrane instead of plastoquinone, which interfered with the electron transport in PS II and caused oxidative stress in algae (Peng et al., 2019). Fortunately, antioxidant enzymes, including SOD and CAT, can protect cells from excess ROS and alleviate oxidative damage. SOD catalyzes the dismutation of $\cdot O_2^-$ into O_2 and H_2O_2. CAT converts H_2O_2 into O_2 and H_2O (Xia et al., 2017). In our study, PFOA and PFOS exposure increased the SOD activity and CAT activity of *Chlorella sp.* (Fig. 7a&b). Hence algae can eliminate ROS radicals and maintain redox balance. Interestingly, PFOS stimulated SOD activity, but PFOS with the concentration of >10 mg/L mitigated the stimulation (Fig. 7a), probably because high levels of PFOS had an inhibitory effect on the antioxidant system of algae (Liu et al., 2021). Previous studies also observed that high concentrations of GenX (a substitute for PFOS) mitigated the SOD stimulation of algae (Liu et al., 2021). However, this was not the case with CAT stimulation. CAT activity exhibited an increasing trend for both PFOA and PFOS when their dosages increased (Fig. 7b). The different changing trend of SOD activity between PFOA and PFOS may be related to their different binding modes with SOD. According to molecular docking, two residues in SOD formed hydrogen bonding with PFOS, but only one

Table 1
Uptake, absorption and bioconcentration factor (BCF) of PFOA and PFOS by algal cells under different doses after 7 days of exposure.

	Dose (mg/L)	Uptake (mg/g) *	Adsorption (mg/g) *	Uptake (%)	Adsorption (%)	BCF
PFOA	5	0.50 ± 0.04^{a}	0.86 ± 0.11^{a}	0.89 ± 0.08^{a}	1.54 ± 0.20^{a}	117.23 ± 9.89^{a}
	10	0.92 ± 0.02^{a}	1.35 ± 0.11^{b}	0.83 ± 0.02^{ab}	1.21 ± 0.10^{b}	109.47 ± 2.41^{a}
	20	1.83 ± 0.03^{b}	2.69 ± 0.13^{c}	0.77 ± 0.01^{abc}	1.13 ± 0.06^{bc}	101.93 ± 1.75^{ab}
	40	3.08 ± 0.34^{c}	4.32 ± 0.11^{d}	0.67 ± 0.07^{bc}	0.95 ± 0.02^{c}	82.95 ± 9.10^{b}
	80	6.15 ± 0.83^{d}	6.66 ± 0.15^{e}	0.66 ± 0.09^{c}	0.71 ± 0.02^{d}	83.11 ± 11.18^{b}
PFOS	5	0.78 ± 0.18^{a}	1.15 ± 0.004^{a}	1.21 ± 0.27^{a}	1.77 ± 0.01^{a}	199.75 ± 45.27^{a}
	10	1.45 ± 0.15^{b}	2.18 ± 0.35^{b}	1.18 ± 0.13^{a}	1.77 ± 0.29^{a}	193.49 ± 20.58^{a}
	20	2.72 ± 0.14^{c}	3.23 ± 0.23^{c}	1.14 ± 0.06^{a}	1.35 ± 0.09^{b}	169.70 ± 8.58^{a}
	40	3.94 ± 0.19^{d}	5.19 ± 0.46^{d}	0.95 ± 0.05^{a}	1.25 ± 0.11^{b}	146.86 ± 7.02^{a}

Data are expressed as mean ± standard deviation (n = 3). Different letters display a significant difference based on LSD (p < 0.05).
* Uptake and adsorption are expressed by mg/g-dry weight.

residue formed hydrogen bonding with PFOA (Yang et al., 2019). Unlike SOD, CAT bind with PFOS and PFOA via similar binding modes (Yang et al., 2019). The increased GSH (Fig. 7c) further confirmed the PFOA and PFOS induced oxidative stress, because GSH is a key physiological antioxidant to eliminate redundant ROS (Wang and Xie, 2007). PFOA and PFOS caused the positive response of *Chlorella sp.* to the oxidative stress, but the antioxidant activities were unable to counteract radicals and resulted in greater accumulation of ROS. Oxidative stress eventually led to reduced growth of *Chlorella sp.*

The bioaccumulation capacity (BCF and uptake percentages) of algae decreased with increasing toxic substance doses (Table 1). Similar result was reported in an enrichment study of bisphenol A by *Chlorella vulgaris* (Ding et al., 2020). BCF of PFOS for *Chlorella sp.* was greater than PFOA, indicating that PFOS was more likely to accumulate in organisms and be transmitted in the food chain. Exogenous organic compounds adsorbed to cell wall components or excretive extracellular substances of algae, and then were uptake by passive diffusion or active transport, and finally bound to intracellular compounds (Sutherland and Ralph, 2019). In our study, the adsorption amount of PFOA and PFOS was greater than their uptake amount by *Chlorella sp.* (Table 1). Similar result was reported in another research that the adsorption amount of chlorinated polyfluorinated ether sulfonate was higher than its uptake amount by algae *S. obliquus* (Liu et al., 2018). As we known, the adsorbed PFAAs on algae can transfer to higher trophic levels through food chain. Such high amount of adsorption should contribute to the tropic transfer, even greater than the uptake amount. Because the small algal cells own large surface area, the amount of adsorbed contaminants on cell surface is higher compared with bigger organisms. Therefore, the adsorbed PFAAs on algae should be considered when we study the algae-related bioaccumulation, tropic transfer and risk assessment.

Although only a very small proportion of PFOA and PFOS (<3 % in total according to Table 1) were adsorbed and ingested by *Chlorella sp.*, some studies documented PFOA and PFOS in wildlife tissues and human body fluid samples (Kannan et al., 2005). Therefore, due to the bioaccumulation and biomagnification, PFOA and PFOS can cause toxic effects on high-trophic organisms, even transfer through the food chain and induce human health risks. The decrease in algal growth will lead to a decrease in the biomass of high-trophic organisms, and eventually lead to an imbalance in the ecosystem. Since photosynthesis forms the fundamental basis of the food chain, even sublethal effects on primary producers could impact energy transfer and the carbon cycle throughout the food chain.

5. Conclusion

In this study, PFOA and FPOS accumulated in marine algae *Chlorella sp.*, damaged cell membrane, triggered oxidative stress, disturbed photosynthesis and inhibited the algal growth. Interestingly, the growth of *Chlorella sp.* followed a hormetic pattern in response to PFOA and PFOS exposure, and the chlorophyll *a* content increased with the reduced photosynthesis. Both were not reported in freshwater algae. Marine algae are important primary producers, hence the photosynthesis and growth rate are involved in the carbon cycle and the ecosystem balance in ocean. The bioaccumulation and transfer of PFOA and PFOS through food webs may be initiated from microalgae, and constitute an important pathway to the organisms of higher trophic levels, even human beings. This study provides new information on the biological response of marine microalgae to PFAAs.

CRediT authorship contribution statement

Wenqian Mao: Experiments, Investigation, Data Curation, Writing - original draft.

Mingyang Li: Methodology, Data discussion, Investigation.

Xingyan Xue: Methodology, Data discussion.

Wei Cao: Experiments.

Xinfeng Wang: Methodology.

Fuliu Xu: Research design, Writing - review & editing.

Wei Jiang: Research design, Data discussion, Writing - review & editing, Supervision.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.161882.

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