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Toxic effects and mechanisms of PFOA and its substitute GenX on the photosynthesis of *Chlorella pyrenoidosa*

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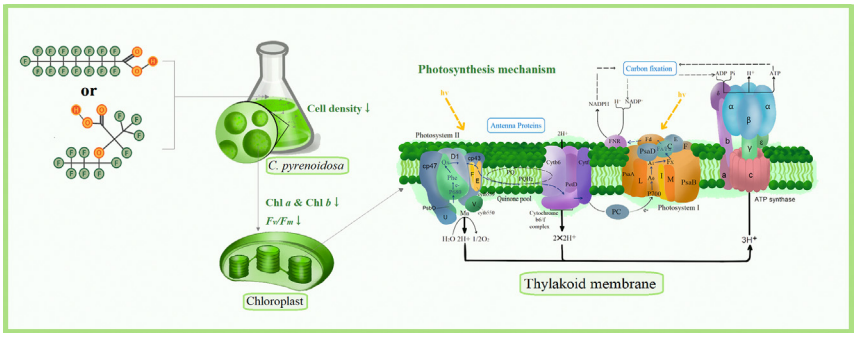
HIGHLIGHTS

- PFOA and its substitute GenX have effect on photosynthesis of *C. pyrenoidosa*.
- Transcription analysis showed that they down-regulated the photosynthetic genes.
- Both of them have similar toxicity and effect mechanisms on *C. pyrenoidosa*.

GRAPHICAL ABSTRACT



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ABSTRACT

Perfluorooctanoic acid (PFOA) and its substitute GenX are toxic chemicals that are widespread in the aquatic environment. However, there is little information about their toxicity mechanisms to aquatic organisms. In this study, *Chlorella pyrenoidosa* (*C. pyrenoidosa*) was treated with two concentrations (100 ng L^{-1} and 100 μg L^{-1}) of PFOA or GenX for 12 days. The results showed that these two concentrations of PFOA and GenX began to inhibit the growth of algae after 6 days of treatment, and the Chlorophyll content and photosynthetic activity of *C. pyrenoidosa* were also negatively affected by these two chemicals. The transcriptomic results indicated that most of the genes related to the photosynthetic metabolism of *C. pyrenoidosa* were down-regulated (in 100 ng L^{-1} treatment groups) on the 12th day. Besides, GenX and PFOA showed similar effects on algae photosynthesis including physical damage and metabolic disorders. According to this study, GenX might not be an ideal substitute for PFOA, and more attention should be paid on the management of emerging perfluoroalkyl substances.

1. Introduction

Per- and polyfluoroalkyl substances (PFASs) are a type of persistent organic pollutants (POPs). Due to their strong surface activity and stability (Li et al., 2019), they have been widely used in industry and commodities, and eventually entered into environmental media including soil and water (Brandsma et al., 2019; Domingo and Nadal, 2019; Li et al., 2020a). However, some previous studies have found that PFASs, especially those with longer carbon chains, such as perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), have the characteristics of persistence, bioaccumulation, long-distance mobility, and potential toxicity (Wang et al., 2019). This can lead to adverse human health effects (Domingo and Nadal, 2019). PFOA has been detected in

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various freshwater bodies around the world, which is directly related to the safety of drinking water and food (Bokkers et al., 2017). Moreover, PFOA has received great attention from public and scientific communities because of its potential ecological risks. Therefore, the production and use of PFOA has been subject to increasingly strict restrictions (Thompson et al., 2019), such as the PFOA management plan in the United States (2006) and *the Stockholm Convention on Persistent Organic Pollutants* held in Geneva (2019), which has led to the emergence of short-chain PFAS as PFOA alternatives (such as GenX). Initially, it was generally accepted that the PFAS with short carbon chain was less toxic and less bio-accumulative (Kudo et al., 2001), however, some recent toxicological studies about animals revealed that fluorinated alternatives (including GenX) have higher toxicity than their predecessors (Gomis et al., 2018). Although it is important to find new compounds that can replace the legacy PFAS, these alternatives also require systematic toxicology studies to confirm whether they are safe to be used in a long term.

In the past five years, studies on PFAS have mainly focused on the detection in different environment, the estimation of sources and emissions, and their toxic effects on organisms (Heydebreck et al., 2015; Wang et al., 2016; Chen et al., 2017; Gebbink et al., 2017). There is a growing concern about the potential impact of GenX on aquatic organisms (Thompson et al., 2019; Gebreab et al., 2020), driven by the detection of GenX in many surface water bodies, as well as in the drinking water (Lange et al., 2007; Flores et al., 2013; Sun et al., 2016; Gebbink et al., 2017; Domingo and Nadal, 2019). In addition, the studies on PFAS toxicity to aquatic species relevant to ecological risk assessment had been reported, including the effect on microalgae, invertebrates, fish, and amphibians, and in general, it has adverse effects in aquatic organisms when exposure to PFASs (Ankley et al., 2020). For instance, the antioxidant-related genes in green alga *Chlamydomonas reinhardtii* were affected by perfluoroalkyl phosphonic acids (PFPAs) (Sanchez et al., 2015). However, as for PFOA and its alternative GenX, their toxicity and mechanism of effect to aquatic organisms still need more research.

Microalgae play an important role in maintaining the productivity and ecological balance of the aquatic ecosystem as an important primary producer (Jin et al., 2019). PFASs upon entering the aquatic ecosystem might change the process of nutrient cycling, adversely affect algae and even higher trophic organisms through the food chain. *Chlorella pyrenoidosa* (*C. pyrenoidosa*), one of the most common single-celled microalgae, has been commonly used as a target cell for eco-toxicological experiments in the aquatic ecosystem due to its sensitivity to pollutants (Middepogu et al., 2018). Photosynthesis during algal growth involves a complex series of biochemical reactions that allow algae to convert natural energy into chemical energy to meet its own needs (Li et al., 2013). The content of photosynthetic pigment is closely related to growth of algae, which also can reflect the changes of its light utilizing ability. In addition, photosynthetic activity is also a sensitive indicator that has been widely used to detect and evaluate various stresses caused in algae photosystems, such as maximum photosynthetic yield (*Fv*/*Fm*) (Kumar et al., 2014). Most studies on the toxicity in microalgae caused by PFOA or GenX have focused on the physiological and biochemical levels, but from the perspective of photosynthetic metabolism, its underlying mechanism is still unclear.

The biological approaches via transcriptomics and genomics have rapidly become practical approach for a deeper understanding of how microalgae respond and adapt when exposed to different pollutants (Jian et al., 2017). Genetic changes eventually affect the physiological and biochemical characteristics of organisms. As for this study, it is necessary to analyze the changes in protein and enzyme related-genes in *C. pyrenoidosa*, since electron transfer and photochemical reaction in photoreaction occur on the thylakoid membrane which includes photosynthesis-antenna proteins, PS II, cytochrome *b*6/f complex, PS I, and ATP synthase (Choquet and Vallon, 2000). Recently, based on RNA-seq analysis, a few studies have focused on the photo-related gene expression changes of microalgae in response to other chemicals in the photosynthesis pathways. The study of Middepogu et al. (2018) indicated that TiO_2 damaged the photosynthesis of green alga (Choquet and Vallon, 2000) by destroying the reaction center of photosystem II. Qian et al. (2018a) revealed that linoleic acid treatment significantly inhibited the transcription of genes related to photosynthesis and carbon metabolism. Therefore, using both physiology and transcriptome analysis can help elucidate the effects of these two PFASs on algae growth and photosynthesis, and can also help in comparing the underlying mechanisms that induce physiological changes.

In this study, the toxic effects of PFOA and its substitute GenX on the photosynthetic system of *C. pyrenoidosa* were compared and evaluated at the physiological level. Moreover, in order to systematically understand and accurately compare the differences in toxicity mechanism of GenX and PFOA on green algae photosynthesis, the changes of differential expression genes (DEGs) related to photosynthesis were investigated by RNA-seq analysis method. The results may assist in promoting the current comprehension of the PFASs' toxicity mechanisms in algae and its potential risks.

2. Materials and methods

2.1. Materials

The strain of *C. pyrenoidosa* (FACHB-9) was purchased from the Institute of Hydrobiology, Chinse Academy of Sciences. *C. pyrenoidosa* was pre-cultured aseptically in Erlenmeyer flasks (1000 mL) containing BG-11 medium (400 mL) at 25 ± 0.5 °C under white fluorescent light (2000 lx, 12:12 h of light: dark). The BG-11 medium was prepared as described in the supplementary material (Table S1). The cultures were shaken manually every 12 h. The culture medium and experimental vessels were autoclaved at 121 °C for 30 min. All operations (inoculation and sampling) were performed on a sterilized bench.

GenX (purity ≥ 89.5%) was purchased from AccuStandard (USA), and PFOA (purity ≥ 95%) was purchased from Sigma-Aldrich (USA). GenX and PFOA solutions were prepared using sterile water in sterile plastic tubes.

2.2. Exposure test

According to the concentration of GenX and PFOA observed in surface water bodies or groundwater of natural in worldwide (Xiao, 2017) and preliminary test, 100 ng L^{-1} and 100 μg L^{-1} were selected respectively as low exposure concentration and high exposure concentration for GenX and PFOA. These are denoted as G1 (100 ng L^{-1} GenX group), G2 (100 μg L^{-1} GenX group), P1 (100 ng L^{-1} PFOA group), and P2 (100 μg L^{-1} PFOA group). The study included a control group without PFAS, denoted as CK group. All the experiments were carried out in triplicates.

Different concentrations of two PFASs were added to the cultivated media of treatment groups, and the corresponding sterile water was added to the control group. *C. pyrenoidosa* was inoculated under a sterile condition with an initial density of 9×10^5 cells mL^{-1}. The experiment was conducted for 12 days. The variation of algal density was observed by automatic algae counter (Countstar, Shanghai) every day. The chlorophyll pigment content and photosynthetic activity in the algal cells were measured on the 3rd, 6th, 9th, and 12th days. On the 12th day, samples from the low-concentration treatment groups were selected for transcription analysis, since the pollution level of GenX in most surface aquatic environments is much closer to 100 ng L^{-1} (Wang et al., 2019).

2.3. Determination methods of photosynthetic parameters

The algae suspensions (10 mL) from different samples were centrifuged at 6000 rpm for 10 min at 4 °C. The supernatant was discarded

and algae cells were resuspended in 10 mL acetone solution (90%), and they were placed in dark in the refrigerator at 4 °C for 1 day. After incubation, the algal cells solutions were centrifuged at 6000 rpm for 10 min. The multi-mode Microplate Reader (BioTek, USA) was used to measure the absorbance (663 nm, 645 nm, and 750 nm) of the supernatant, and it was noted that the absorbance value at 750 nm did not exceed 0.005 (Marr et al., 1995).

Phytoplankton-Pulse-Amplitude-Modulated (Phyto-PAM) fluorometer (Walz, Germany) was used to measure the photosynthetic activity of *C. pyrenoidosa*. All the samples of algae cells were incubated in the dark for 25 min to ensure that the photosystem II (PS II) reaction center is open. The maximum optical quantum yield (*Fv/Fm*) of *C. pyrenoidosa* was automatically calculated by the Phyto-Win Software (V 2.13).

2.4. RNA-seq analysis

On the 12th day of the exposure period, samples from CK group, G1 group and P1 group were selected for transcriptome analysis. Each treatment was present in triplicates. The different samples were collected respectively in 50 mL centrifuge tubes and centrifuged at 8000 rpm for 10 min. The supernatant was discarded. The concentrated cells were immediately frozen using liquid nitrogen. The algal cell pellets were resuspended in lysis buffer and ground into a fine powder. The total RNA was then extracted according to the manufacturer's instructions.

Agarose gel electrophoresis was used to analyze the degree of RNA degradation and determine whether the samples were contaminated. Nanodrop spectrophotometer was used to assess the purity of the RNA. Qubit 2.0 was used to quantify the RNA concentration, and the Agilent 2100 (Agilent RNA 6000 Nano Kit) was used to detect the RNA accuracy. Nine libraries from all samples were built and tested. Then according to the effective concentration and target offline data volume requirements, different libraries were merged into the flow cell. Illumina high-throughput sequencing platform was used for paired-end (PE) sequencing after cBOT clustering. Unmapped Reads (obtained from the ribosome comparison with the reference genome) were compared with the data comparison software HISAT, which used Bowtie2 as the comparison "engine".

Gene expression levels were calculated by the concept of RPKM (Reads Per Kilo bases per Million reads). After obtaining the read count expression of each sample, the TMM method was used to standardize the read count data. The Gene Ontology (GO) (http://www.geneontology.org) annotation was annotated and enriched by Cluster Profiler. The edgeR method was used for the differential expression gene analysis between every two samples. Significant enrichment analysis based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway (http://www.kegg.jp/kegg/) was used to identify the major biochemical metabolic pathways and signal transduction pathways involving differential expressed genes.

2.5. Statistical analysis

Statistical analysis was performed using IBM SPSS 22.0 (SPSS Inc., USA) and GraphPad Prism 8.0 (GraphPad Software, USA). Parametric one-way analysis of variance was used to analyze the significant differences between the control and treatment groups. Data with p-value <0.05 was considered as statistically different.

3. Results

3.1. Effects of GenX and PFOA on the growth of C. pyrenoidosa

As shown in Fig. 1, different concentrations of each PFAS have different inhibitory effects on the growth of *C. pyrenoidosa*. The influence of GenX (Fig. 1a) and PFOA (Fig. 1b) on algae growth showed similar trends. During the first half of the experimental period (from day 0 to day 6), there was no significant difference between each PFAS treatment at each sampling time. However, compared with the control group, the two PFASs gradually inhibited the growth of *C. pyrenoidosa* after 6-days of exposure. Treatment with 100 ng L^{-1} GenX or PFOA had less negative effects on algae growth as compared with their higher concentration. On the 12th day, when *C. pyrenoidosa* exposure to 100 ng L^{-1} GenX and PFOA, the cell density inhibition rates were 3.81% and 6.76%. When *C. pyrenoidosa* was exposed to 100 μg L^{-1} GenX and PFOA for 12 days, the cell density inhibition rates were 15.4% and 14.4%, respectively. The inhibition of cell density was only observed in the later period, and the inhibitory effects were still not particularly obvious. The findings from this study different from those reported in a previous study, in which the growth of *C. pyrenoidosa* exposed to higher concentrations (mg L^{-1}) of PFOA was significantly inhibited (Xu et al., 2013).

3.2. Effects of GenX and PFOA on the photosynthetic parameters of C. pyrenoidosa

After exposure to GenX and PFOA for 3 to 12 days, the changes in the photosynthetic pigments (Chl *a* and Chl *b*) of *C. pyrenoidosa* were evaluated and are presented in Figure 2 (a) and (b). On the 3rd day, the content of Chl *a* and Chl *b* of treatment groups showed no difference with that of control group ($p > 0.05$). By the 6th day, the chlorophyll pigment content in all the samples increased, but only the content of Chl *a* in G2 group and P2 group showed significant difference with CK group. After the 9th day of exposure, with the increase of cell density, the content of photosynthetic pigments increased, and the Chl *a* content of the treatment groups was significantly different from that of the CK group. At the end of the exposure time, there was a remarkable decrease in the content of Chl *a* in G2 group and P2 group, with the inhibition rates of 10.75% and 12.82%, respectively. Chl *a* content in G1 group

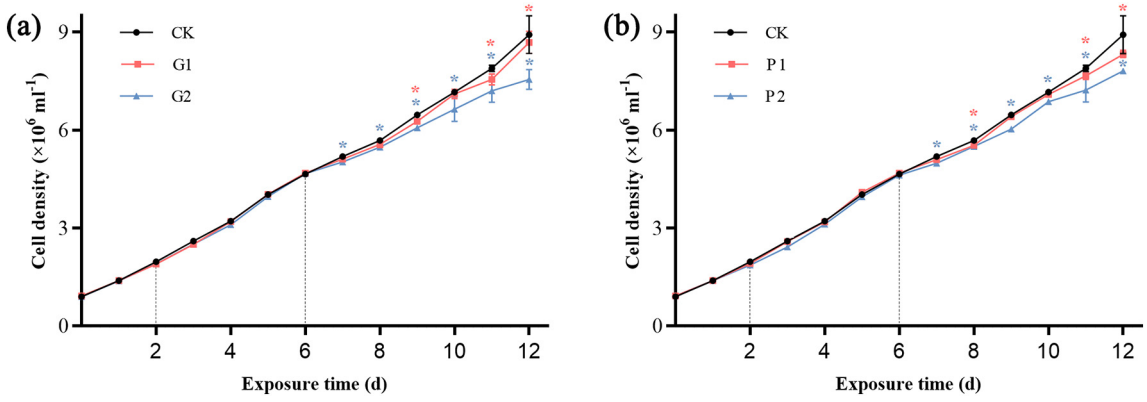


Fig. 1. The changes in algal cell density during 12 days responding to the treatments of GenX (a) and PFOA (b). Error bars indicate SEM ($n = 3$). * denotes significant differences ($p < 0.05$) from the control.

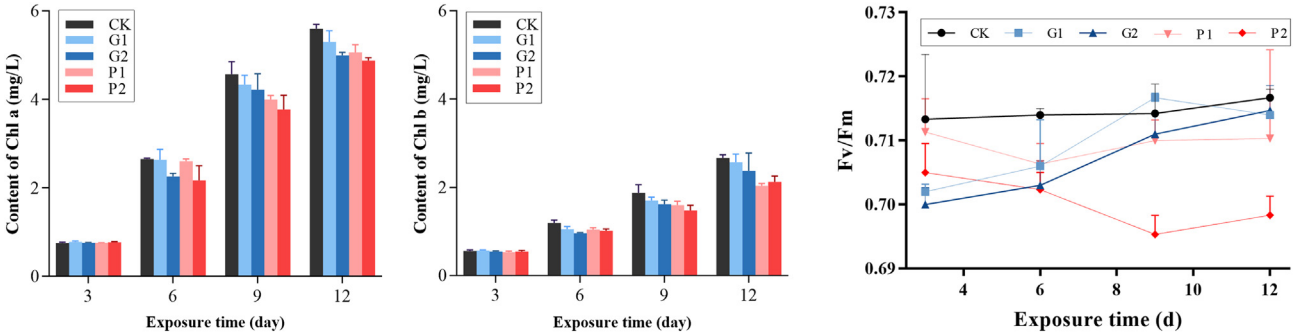


Fig. 2. (a) and (b): The content of Chl *a* and Chl *b* in the algae cells exposed to different concentrations of GenX and PFOA for the 3rd, 6th, 9th, and 12th days. (c): The *Fv/Fm* of photosystem II of *C. pyrenoidosa* under the influence of two PFASs on the 3rd, 6th, 9th, and 12th day of exposure. Error bars indicate SEM ($n = 3$). * denotes significant differences ($p < 0.05$) from the control.

and P1 group decreased by 5.30% and 9.58%, respectively, as compared with CK group. Similar to the changes of Chl *a*, the content of Chl *b* in treatment groups was significantly decreased from CK group on the 12th day. Thus, it could be observed that the high concentration of the two PFASs had a more significant inhibitory effect, than their low concentrations on chlorophyll content in *C. pyrenoidosa*. In addition, PFOA showed a stronger inhibitory effect on chlorophyll than GenX.

The maximum quantum yield (*Fv/Fm*) of PS II in *C. pyrenoidosa* is shown in Figure 2 (c). The *Fv/Fm* in control group changed more steadily than that of values in the treatment groups after 12 days of exposure, and the value was higher than that of the treatment groups. *Fv/Fm* initially exhibited an increasing trend in G1 group, followed by a decreasing trend during the algae cultivation period. The value of *Fv/Fm* in G2 group was lower than G1 group, but there was no downward trend. After being exposed to PFOA, the values of *Fv/Fm* in *C. pyrenoidosa* showed an initial decreasing trend followed by an increase. It revealed that the significant change ($p < 0.05$) was only observed with 100 μg L^{-1} PFOA. In addition, it was shown that the higher concentration of PFOA had a greater impact on *Fv/Fm* in *C. pyrenoidosa*.

3.3. General transcriptome characteristics of C. pyrenoidosa

In order to identify the photosynthetic genes related to PFAS stress-response in *C. pyrenoidosa*, nine transcription libraries (n=3) were constructed using RNA-seq analysis of the control group (CK), 100 ng L^{-1} GenX group (G1), and 100 ng L^{-1} PFOA group (P1). According to the quality control report, Q30 of all samples was > 90%, indicating that the data was of good quality and reliable. After comparing with the ribosome database, the average remaining ratios of CK, G1, and P1 were 96.70%, 95.10%, and 96.16%, respectively. Finally, 14,721 unigenes were obtained, and the N50, GC content, length, and other statistics of the assembly results are presented in Table 1.

The BLAST software was used to annotate or classify the total unigene sequences queried from the databases of Nr (8948), Swissprot (5945), KOG (5019), and KEGG (5426) (Fig. S1). The Nr annotated unigenes were collected and compared with other species, which showed that most of the transcripts had a significant match with Chlorella (47.20% of all transcripts). Through GO-term analysis, the assembled unigenes were classified into 19 biological processes, 16 cellular components, and 13 molecular functions (Fig. S2). Metabolic process (30.59%) and cellular process (30.39%) were the most important biological processes, membrane (21.65%) was the most dominant group in the cellular components, and the most important group in molecular functions was catalytic activity (48.18%). According to the KEGG annotation results, most of the essential photo-related genes were contained in the main pathways, indicating that the sequencing depth was sufficient.

3.4. Differential expression analysis of genes in C. pyrenoidosa

According to the abundance of unigene expression, the relationship between all the samples was analyzed (Fig. S3). Correlation heat map and principal component analysis (PCA) indicated that: (Ankley et al., 2020) the parallel samples in each group were much highly correlated, (Bishop and Bishop, 1987) the samples of G1 and P1 groups showed high correlations, (Bokkers et al., 2017) the correlation between the treatment group and CK group samples was very low. According to the differential expression analysis of genes, 4394 significant DEGs were identified when G1 was compared with CK, including 1903 up-regulated genes and 2491 down-regulated genes (Fig. 3a). While, 5167 DEGs were identified when CK was compared with P1, including 2116 up-regulated genes and 3051 down-regulated genes (Fig. 3b). These DEGs were distributed in 126 KEGG pathways.

3.5. Effects of GenX and PFOA on the photosynthetic proteins of C. pyrenoidosa

Considering all the photosynthetic proteins (Fig. 4, Table S2) in *C. pyrenoidosa* treated with GenX or PFOA, most of the related genes were down-regulated. Moreover, the down-regulated genes were the same in G1/CK and P1/CK groups.

Light capture is performed by the light-harvesting complex I (LHC I) and LHC II, which can transfer the absorbed excitation energy to reaction center PS I and PS II. LHC II is also involved in the regulation of excitation energy balance between PS I and PS II, and dissipation of excess excitation energy (Li et al., 2020b). There were five down-regulated DEGs when G1 was compared with CK, which were related to the photosynthesis-antenna proteins, and there were six down-regulated DEGs between group P1 and group CK (Fig. 4a, Table S2). Except for *Lhcb7*, the down-regulated genes in G1/CK and P1/CK groups were the same, which means that low-concentrations of GenX and PFOA have an inhibitory effect on the light capturing ability of *C. pyrenoidosa*. Moreover, the progress of photochemical reactions and the inhibition principle might be same in G1/CK and P1/CK groups.

When the light energy absorbed by LCH II is transferred to the PS II reaction center, the central pigment P680 produces high-energy electrons, which are transferred to the original electron acceptor, causing

Table 1
The statistics of assembly results.

Genes Num	GC percentage	N50	Max length	Min length	Average length	Total assembled bases
14721	67.49%	2618 bp	28,788 bp	201 bp	1663 bp	24,482,447

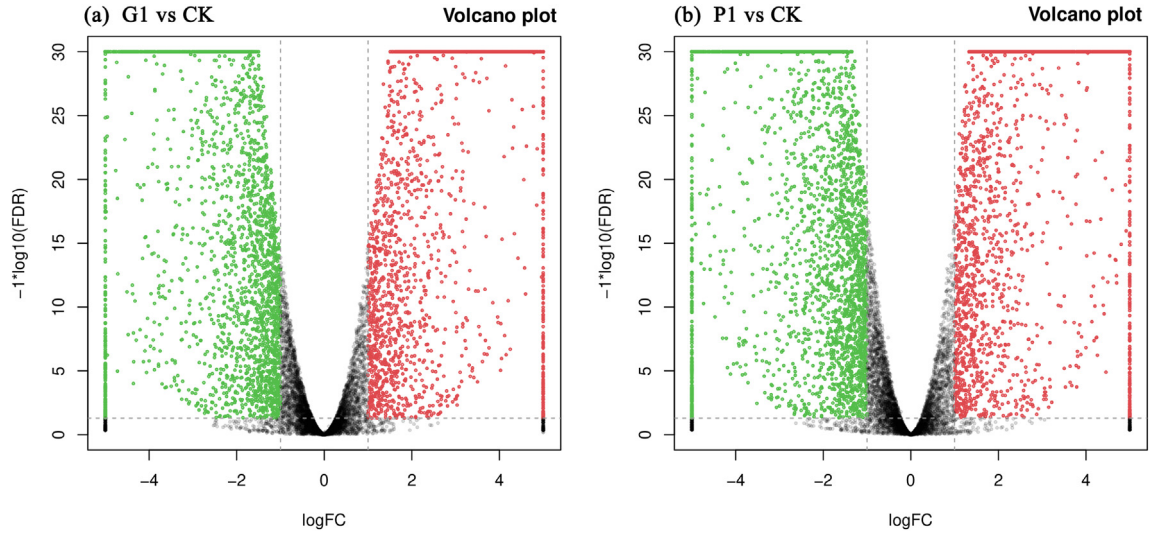


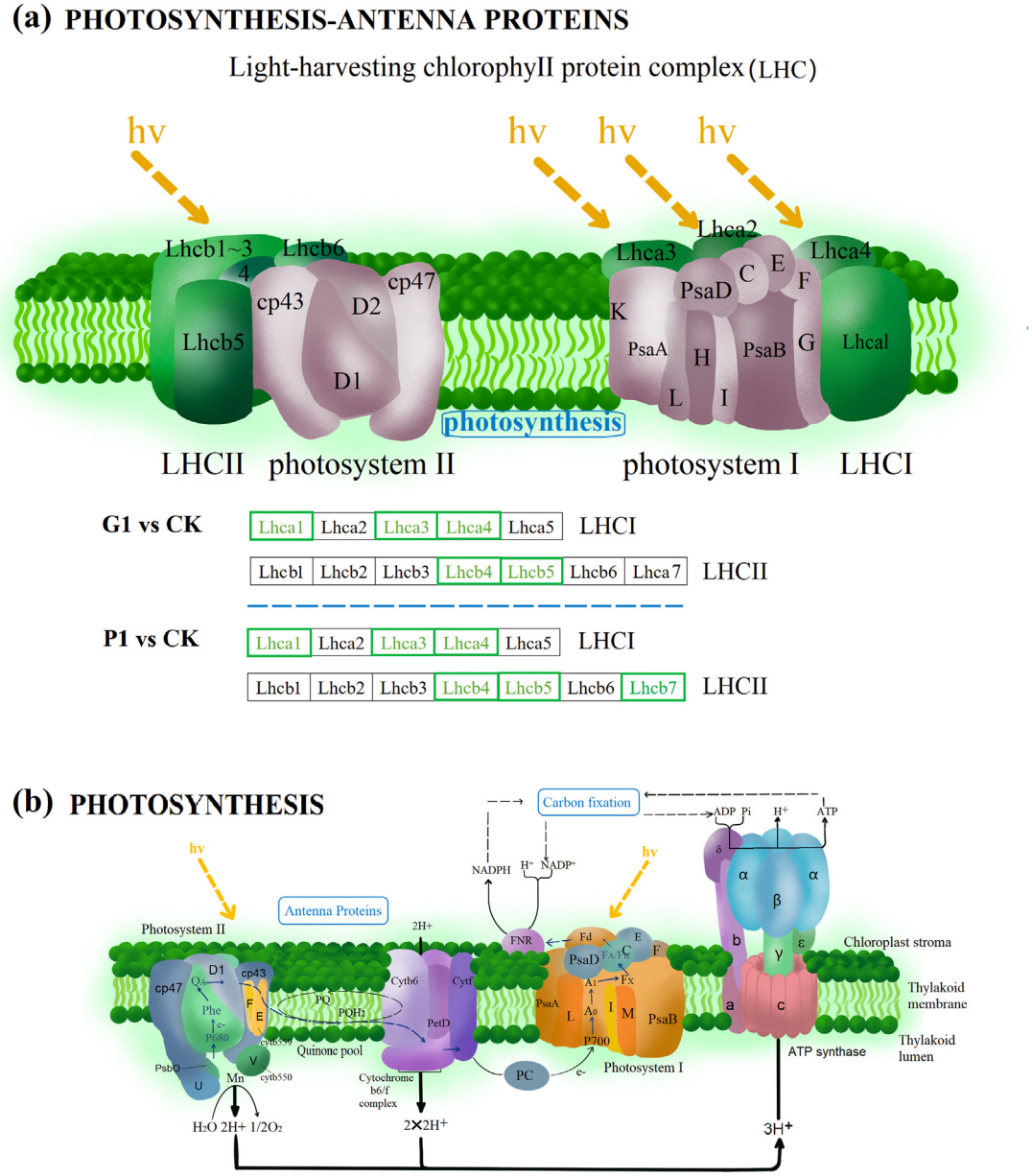
Fig. 3. Volcano plot of DEGs between (a) G1 and CK, (b) P1 and CK. Green plots represent down-regulated genes, and red plots represent up-regulated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

photolysis of water. It was observed that the three important DEGs (*PsbA*, *PsbC*, and *PsbB*) of PS II were up-regulated when G1 was compared with CK (Fig. 4b, Table S2). D1, encoded by the *PsbA* gene, is one of the proteins that coordinate with P680 in PS II reaction center (Metz et al., 1989). The heterodimer D1-D2 forms the catalytic core of PS II and coordinates with most of the cofactors, which mediate electron flow from water to Q_B. *PsbB* and *PsbC* encode PS II CP47 and CP43 chlorophyll *a*poprotein. CP43 and CP47 are two important pigment-protein complexes that constitute the inner antenna of photosynthetic organisms, and mainly play a role of transmitting excitation energy in the organism. Thus the up-regulated expression of the *PsbA*, *PsbB*, and *PsbC* genes in the G1/CK group might promote electron transport and increase pigment synthesis. However, it was noted that most of the other related genes in the two groups were down-regulated, indicating that the photolysis of water was still inhibited. After LCH I absorbs photons, the photochemical reaction of PS I starts, and then transfers the excitation energy to P700. Eventually, PS I catalyzes the transfer of light-driven electron to Fd. The down-regulation of PS I related genes expression in *C. pyrenoidosa* might result in slower linear electron flow and cyclic electron flow. As a medium, Cyt b_6/f complex can transfer protons across the membrane. The expression of *PetN* gene was down-regulated in both G1/CK and P1/CK groups. For photosynthetic electron transport, genes *PetJ* and *PetF* were also down-regulated in these two groups. NADPH is produced in the last step of the electron chain of the light reactions of photosynthesis, and the NADPH-catalyzing enzymes ferredoxin-$NADP^+$ reductase (FNR) (encoded by *PetH*) was down-regulated. The gene expression encoding gene *b* of subunit CF_0 complex of F-type ATPase in G1/CK and P1/CK groups was down-regulated, which indicated that the transmembrane movement of H^+ would be affected. As a result, the energy conversion efficiency in the chloroplasts would decrease.

4. Discussion

PFOA (pKa = 2.84) and GenX (pKa = 3.8) are toxic water-soluble compounds (Mullin et al., 2019), which widely exist in the aquatic environment. There are evidences that the concentration of PFOA detected in natural freshwater falls within the range of ng L^{-1} to mg L^{-1} (Lange et al., 2007; Flores et al., 2013; Xiao, 2017). The concentration range of GenX detected in drinking water is from ng L^{-1} to μg L^{-1} (Sun et al., 2016; Xiao, 2017). Therefore, in this study, two environmentally-related concentrations (100 ng L^{-1} and 100 μg L^{-1}) were selected for the toxicity study of GenX and PFOA. In exposure experiments, one of the main parameters is cell density, which is sensitive to describe the toxicity effect of pollutants on microalgae (Boudreau et al., 2003). Xu et al. (2013) had reported that the 96 h-EC_{50} values of PFOA for *C. pyrenoidosa* and *S. capricornutum* were 207.46 mg L^{-1} and 190.99 mg L^{-1}, respectively. In another research, three levels (10 ng L^{-1}, 100 ng L^{-1}, and 1000 ng L^{-1}) of four emerging PFASs (including GenX) were selected to investigate the response of marine *Chlorella sp.* (Niu et al., 2019). The change in alga cell density observed in this research was similar to the results observed by Niu et al. (2019). These two PFASs affects the growth of algae after 6-days exposure, which indicates that they might have a chronic toxic effect on algae. The insignificant inhibitory effect under low-concentration PFAS treatments might be due to insufficient exposure time and high initial algae density. Bioaccumulation might be a reason for the increase in potential toxicity of PFAS on algae over time. Moreover, according to the preliminary experiments, the toxicity increased with the increasing PFAS concentration.

Photosynthesis plays a most basic role in green algal growth, and this research focused on the effects of two PFASs on the chlorophyll *a*nd photosynthetic activity of *C. pyrenoidosa*. Chlorophyll, which participates in the process of energy transfer through electron transfer (Qian et al., 2018b), can also be used to measure algal growth. It is obvious that when the density of algae increased during its growth phase, the content of total chlorophyll *a* and *b* also gradually increased. However, when compared with the control group, the chlorophyll content of *C. pyrenoidosa* gradually decreased under the stress after 6 days. The change in chlorophyll content was similar to the change in algae cell density. As for chlorophyll fluorescence parameters, *Fv*/*Fm* ratio was relatively stable under normal conditions, but when under stress, it seems that their changes were still not as particularly significant as that of chlorophyll content. Photosynthesis is a very complicated process. The decreased chlorophyll content does not mean decreased photosynthetic activity (Yang et al., 2020), since *Fv*/*Fm* is the theoretical maximum light quantum yield, which may be slightly different from the actual quantum yield. The fluctuation of the values in the cultivation period needs to be explored in the future. But in general, both chlorophyll pigment content and *Fv*/*Fm* exhibited a similar dose-dependent reduction (Middepogu et al., 2018), Although no significant change ($p > 0.05$) was observed in the effect of low-concentration PFASs on the *Fv*/*Fm* of *C. pyrenoidosa*, transcription analysis indicated that the process of electron transport and photosynthesis were affected because most of the relevant genes in the photosystem response center were down-regulated. This result indicated that PFASs might have a more significant effect on the physiological level of *C. pyrenoidosa* over time.



G1 vs CK photosystem II

D1	D2	cp43	cp47	cytb559		
PsbA	PsbD	PsbC	PsbB	PsbE	PsbF	

MSP OEC

PsbL	PsbJ	PsbK	PsbM	PsbH	PsbI	PsbO	PsbP
PsbQ	PsbR	PsbS	PsbT	PsbU	PsbV	PsbW	PsbX
PsbY	PsbZ	Psb27	Psb28	Psb28₂			

Photosystem I

PsaA	PsaB	PsaC	PsaD	PsaE	PsaF	PsaG	PsaH
PsaI	PsaJ	PsaK	PsaL	PsaM	PsaN	PsaO	PsaX

Cytochrome b6/f complex

PetB	PetD	PetA	PetC	PetL	PetM	PetN	PetG

Photosynthetic electron transport

PetE	PetF	PetH	PetJ

F-TYPE ATPase

beta	α	γ	δ	ε	c	a	b

P1 vs CK photosystem II

D1	D2	cp43	cp47	cytb559		
PsbA	PsbD	PsbC	PsbB	PsbE	PsbF	

MSP OEC

PsbL	PsbJ	PsbK	PsbM	PsbH	PsbI	PsbO	PsbP
PsbQ	PsbR	PsbS	PsbT	PsbU	PsbV	PsbW	PsbX
PsbY	PsbZ	Psb27	Psb28	Psb28₂			

Photosystem I

PsaA	PsaB	PsaC	PsaD	PsaE	PsaF	PsaG	PsaH
PsaI	PsaJ	PsaK	PsaL	PsaM	PsaN	PsaO	PsaX

Cytochrome b6/f complex

PetB	PetD	PetA	PetC	PetL	PetM	PetN	PetG

Photosynthetic electron transport

PetE	PetF	PetH	PetJ

F-TYPE ATPase

beta	α	γ	δ	ε	c	a	b

Fig. 4. (a) DEGs of photosynthesis-antenna proteins in *C. pyrenoidosa* annotated with KEGG. (b) DEGs of photosynthesis in *C. pyrenoidosa* annotated with KEGG. Genes marked with green and red represent up-regulation and down-regulation of DEG, respectively, in the G1 and P1 groups compared with the CK group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

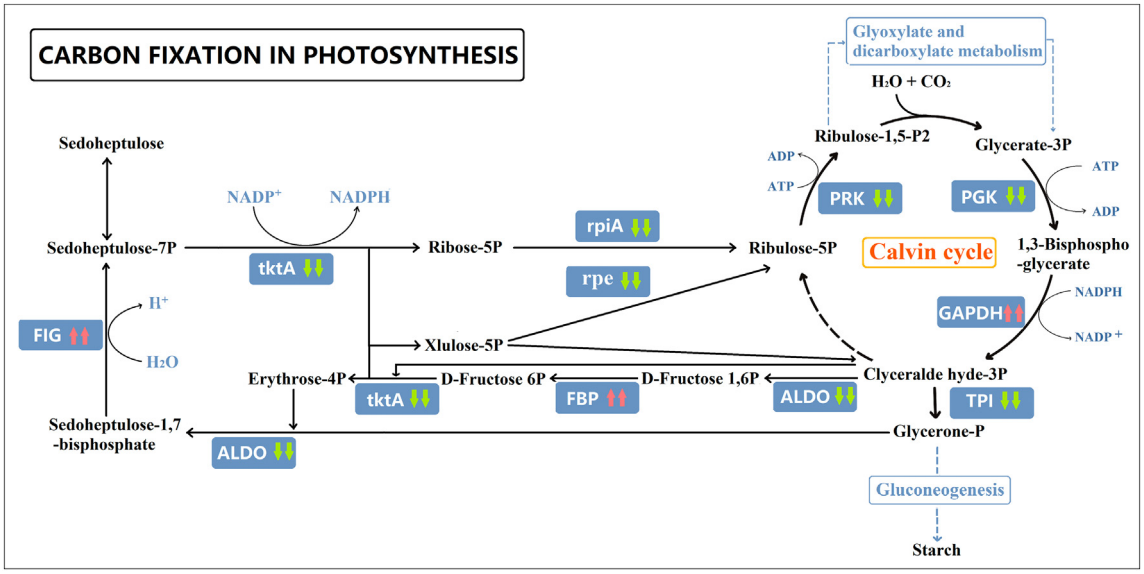


Fig. 5. KEGG carbon fixation in photosynthesis of *C. pyrenoidosa* exposed to 100 ng L^{-1} GenX or PFOA compared with CK group. The rectangular box represents the related enzyme in each process. The first and second arrow (after the enzyme) represent the DEGs of G1/CK and P1/CK, respectively. The red and green arrows represent the up-regulation and down-regulation of DEGs, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Transcription analysis of photosynthesis-related genes of PS II in *C. pyrenoidosa* showed that only *PsbA*, *PsbC*, and *PsbB* in G1/CK group were up-regulated. Correspondingly, the inhibition rate of chlorophyll content in G1 group was lower than that of the P1 group. The remodeling of LHC I and LHC II is a common adaption by photosynthetic organisms in response to environmental changes. The function of *Lhcb7* needs to be further studied in order to better explain why the *Lhcb7* gene in the P1/CK group was down-regulated. When *C. pyrenoidosa* is subjected to

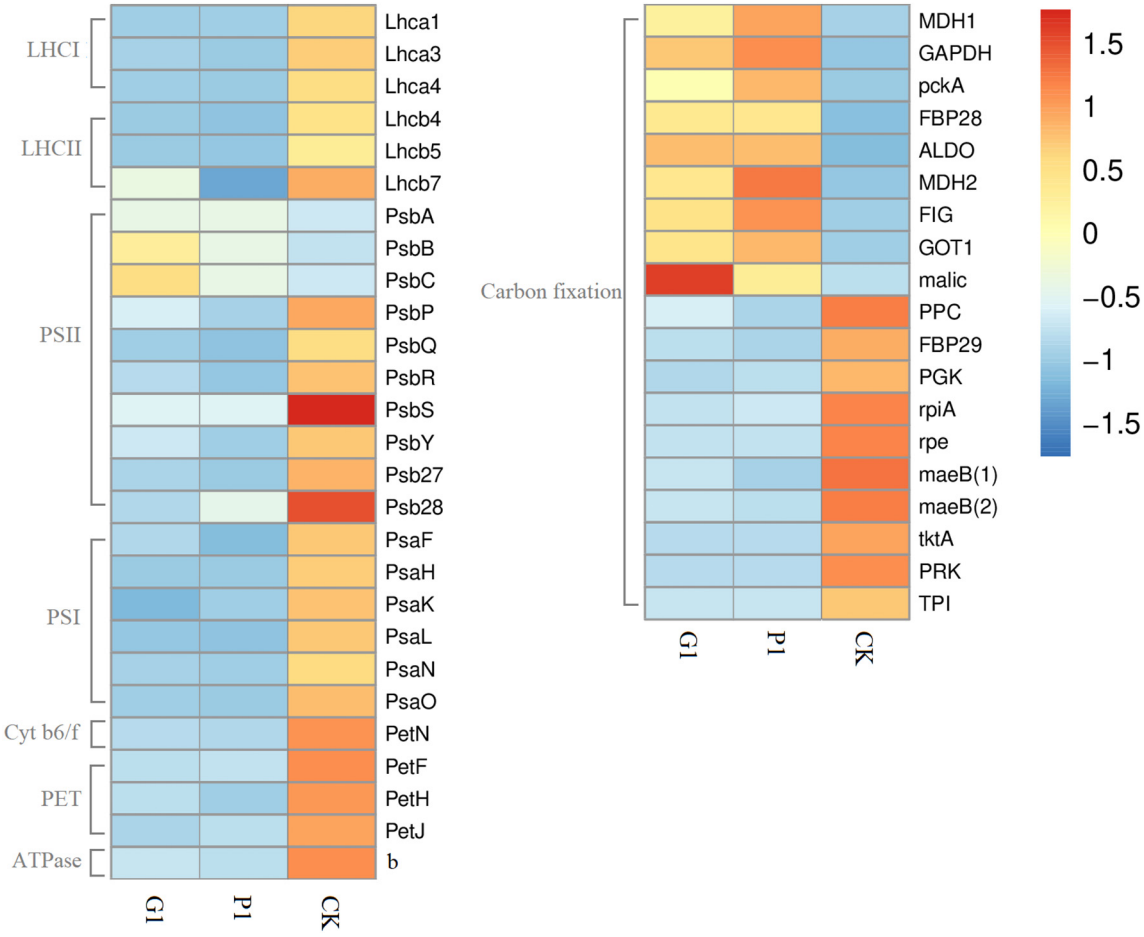


Fig. 6. Heat map of transcript proportions of KEGG level 4 in the control and treatment groups. The relative expression values are scaled by *Z*-score of each transcript.

a certain degree of external stress, it needs more complex regulation to maintain its own metabolism and growth. According to the results, most of the photosynthetic protein genes in G1/CK and P1/CK groups were down-regulated. Therefore, the oxygen, NADPH, and ATP generated through photosynthesis decreases, which finally affects the carbon fixation. Carbon fixation in photosynthesis is a conversion process of inorganic carbon to organic compounds (Bishop and Bishop, 1987), which is of great significance for the life activities in photosynthetic organisms. In G1/CK and P1/CK groups, the expression of related enzyme genes in *C. pyrenoidosa* showed the same trend of regulation, and the mRNA abundances of most enzymes in both the groups decreased, which lead to the decrease of carbon fixation (Fig. 5). In the Calvin cycle, the effect of PFASs on the fixation phase was not significant. In the reduction phase, the PGK enzyme genes involved in the conversion of ATP to ADP were down-regulated, but the gene of the GADPH enzyme was up-regulated to promote the consumption of NADPH. These gene expression changes might affect the energy storage process of photosynthesis in *C. pyrenoidosa*. As for the regeneration phase, most of the enzyme encoding genes involved in a series of carbon conversions were down-regulated, which affects the conversion efficiency of Ribulose-5P. Finally, the down-regulation of PRK enzyme gene affected the regeneration of Ribulose-1, 5-P2 (RuBP). The RuBP content in the chloroplast needs to be maintained at a certain level to ensure the normal progress of the Calvin cycle. The results showed that both PFOA and GenX could inhibit the energy conversion in chloroplasts in a similar way and affect the carbon fixation in photosynthesis.

In conclusion, the results of RNA-seq analysis indicated that at the same concentration (100 ng L^{-1}) and at the same time (the 12 days), GenX and PFOA had similar mechanism of effect on photosynthesis of *C. pyrenoidosa*, because most of the genes related to photosynthetic proteins in both the groups were down-regulated. Correspondingly, the mechanism of the effect of the two PFASs on carbon fixation in photosynthesis of *C. pyrenoidosa* was also very similar. Both GenX and PFOA can cause a decrease in the assimilation of CO_2 by influencing the photosynthetic carbon reduction cycle enzymes. However, according to the degree of down-regulation (log_2 ratio) and heat map from relative expression values of DEGs in each treatment group (Fig. 6, Table S2), most of the values of down-regulated genes in P1 group were lower than those of GenX, which means *C. pyrenoidosa* exposed to PFOA have a higher degree of gene down-regulation than those exposed to GenX.

5. Conclusions

This study provides a systematic explanation of the effects of PFOA and its substitute GenX on the photosynthesis of *C. pyrenoidosa* (including the effect on physiological level and transcription level). The results indicated that these two PFASs could inhibit the algae density and photosynthetic pigment content of *C. pyrenoidosa* after 6-days exposure. The transcription analysis of the photosynthetic mechanisms of G1/CK and P1/CK groups on day 12 showed that GenX and PFOA both significantly down-regulated most of the genes related to the photosynthetic proteins and carbon fixation pathway. In addition, the two PFASs showed similar effects on the photosynthetic metabolism of *C. pyrenoidosa*. In the long run, GenX and PFOA might have a strong inhibitory effect on the growth of algae and might affect other aquatic organisms, as well owing to their property of being hard to degrade. Therefore, it is suggested that the emerging PFASs in water bodies should be controlled more strictly, and the management or restriction of PFASs should be taken into consideration as well.

To our knowledge, this is the first report to study transcriptional changes in response to GenX and PFOA treatments in green algae. The effects of these PFASs on other pathways need to be further analyzed to form a systemic understanding of their effects on *C. pyrenoidosa*. In addition, further research should pay more attention on understanding the toxicity of emerging PFASs on other aquatic organisms and the risks they pose to the environment.

CRediT authorship contribution statement

Yanyao Li: Data curation, Formal analysis, Visualization, Writing – original draft, Project administration, Writing – review & editing. **Xianglin Liu:** Methodology, Supervision. **Xiaowei Zheng:** Investigation. **Meng Yang:** Conceptualization. **Xutao Gao:** Software. **Jingling Huang:** Software. **Liangliang Zhang:** Resources. **Zhengqiu Fan:** Funding acquisition, Writing – review & editing.

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.2020.144431.

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