The influence of microplastics on the toxic effects and biodegradation of bisphenol A in the microalgae *Chlorella pyrenoidosa*

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Research Article

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Abstract

Bisphenol A (BPA) and Polystyrene (PS) microplastics have attracted much attention due to they were widely distributed in the environment, while their combined toxicity to aquatic organisms has rarely been studied. Therefore, this study explored that the impact of microplastics on the toxic effects and biodegradation of BPA to the microalgae *Chlorella pyrenoidosa* (*C. pyrenoidosa*). The results indicated that the presence of PS increased the growth inhibition of BPA and also accelerated the degradation efficiency of BPA in the medium of *C. pyrenoidosa*. When PS and BPA coexisted, the chlorophyll content and the Fv/Fm value decreased with the increased PS concentration due to the hermetic effect and shading effect. Moreover, this study also found that five intermediates were formed during BPA degradation process because of the presence of oxidoreductase and glycosyltransferase. The results of the study provided vital information on the effect of PS on the toxicity and biodegradation of BPA to microalgal.

1. Introduction

Microplastics are defined as a particle size less than 5 mm, which is a new type of environmental pollutant with stable chemical properties and refractory to degradation (Horton et al. 2017; Lin et al. 2020). Microplastics can be discharged into the water environment in many ways, such as direct discharge of cosmetics or fine fibers into the water during industrial production and human daily life (Herbort et al. 2018). Because of their low price and easy processing, plastics play a vital role in packaging, construction, transportation, electric power, medical devices and other fields, and have been widely used in daily life (Yang et al. 2020b). Microplastics are widely distributed in water and exist in many forms. Polypropylene (PP), polyethylene (PE), polystyrene (PS), polyvinylchloride (PVC), and polyamide (PA) were the most common types of microplastics found in the freshwater systems, marine environments and sediments (Cincinelli et al. 2017; Klein et al. 2015).

Previous studies have shown that microplastics may have a variety of negative effects on the aquatic environment. For example, microplastics can reduce energy intake and affect the fecundity and offspring performance of aquatic organisms (Sussarellu et al. 2016). Wu et al. investigated different sizes (1 μm and 100 nm) of the PS microplastics on the effect of the algae growth, the results indicated that 1 μm microplastics showing more adverse effects than 100 nm microplastics to *Microcystis aeruginosa* (Wu et al. 2021). Therefore, more research is needed to fully understand the impact of microplastics on different natural cycles, and one aspect is the microalgae degrade organic pollutants.

Microalgae-based biotechnology has been considered as a promising alternative solution to current activated sludge systems to better treat nutrients and pharmaceutical contaminants (Xiong et al. 2017a). Phytoremediation by microalgae as a form of solar-powered decontamination is considered an environmentally friendly, sustainable, cost-effective reclamation strategy and technology (Xiong et al. 2016). The biodegradation of environmental organic pollutants by algae has also been reported by some researchers (Xie et al. 2020; Xiong et al. 2020), indicating that algae have the potential to remove

pollutants in wastewater. Bisphenol A (BPA) (an endocrine disruptor compound), which is used in the production of epoxy resin and polycarbonate (PC) plastics for various food and beverage packaging, baby bottles, and dental sealants (Staples et al. 1998). In recent years, positive outcomes were obtained for the remediation of BPA-contaminated aqueous systems by green algae, including *Monoraphidium braunii* (Gattullo et al. 2012), *Chlamydomonas Mexicana* and *Chlorella vulgaris* (Ji et al. 2014) and *Desmodesmus sp.WR1* (Wang et al. 2017). However, reports on the effect of microplastics on the biodegradation of BPA during the cultivation of microalgae are still lacking.

Chlorella pyrenoidosa (*C. pyrenoidosa*) is a freshwater green algae, which is sensitive to pollutants in the aquatic environment and is usually used as a model organism in ecotoxicity tests (Li et al. 2019; Li et al. 2013; Liu et al. 2019; Zhao et al. 2017). Additionally, *C. pyrenoidosa* can accumulate and remove different pollutants in the aquatic environment (Peng et al. 2014; Wang et al. 2020; Wang et al. 2018). Therefore, in the present study, the ecotoxicological effects of different concentrations of BPA and PS microplastics on *C.pyrenoidosa* were evaluated according to the changes in microalgae growth. Moreover, further experiments were carried out to analyze the effects of PS on the biodegradation of BPA during microalgae cultivation. The kinetics of biodegradation of the BPA in combination and individually were also assessed.

2. Materials And Methods

2.1. Chemicals and reagents

Bisphenol A (BPA) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and was dissolved in methanol to obtain stock solutions. Polystyrene (PS) monodisperse microspheres (with the sizes of 9 μ m in diameter) were provided by Big Goose (Tianjin) Technology Co., Ltd (Tianjin, China) with an initial concentration of 2.5% (W/V), and the stock suspension was prepared by diluting with ultrapure water. BG11 medium was supplied by Qingdao Hope Biotechnology Co., Ltd. (Qingdao, China). All of the chemical reagents used were analytical pure grade.

2.2. Algae cultivation

Microalgae *C.pyrenoidosa* were obtained from the Institute of Hydrobiology, the Chinese Academy of Science. The culture medium (BG11) and the materials used for algal cultures were autoclaved at 121°C for 30 min before use. The algae cultivation was performed at 25 $\pm$ 1°C under the illumination of 4,000 lx with a 12/12 h light/dark cycle, the flasks were manually shaken three times in the incubator every day.

2.3. Growth inhibition test

The growth inhibition of BPA on *C.pyrenoidosa* was determined by monitoring the cell growth. The algae suspension, at its exponential phase, was inoculated into 250-mL flasks containing 100 mL of exposure medium at a density of 10⁵ cells/ml. BPA stock solutions were added in the autoclaved medium to achieve the initial concentrations (1, 10, 20 and 30 mg/L), PS solution was added in the autoclaved medium to achieve the initial concentration (5 mg/L), the group without the PS and BPA algae

suspension in the flasks served as the control group. Cultivation conditions were as described above, The optical density of algal suspension was carried out at 680 nm (OD_{680}) by the Microplate Reader (Synergy H1). The algal concentration of *C.pyrenoidosa* was measured by using a blood cell counting plate under a light microscope (Ding et al. 2017). The growth inhibition rate of *C.pyrenoidosa* was based on a correlation between OD_{680} and the microalgae density. The cell numbers of *C.pyrenoidosa* during the incubation was determined by the following equation:

$$\text{Algal cell numbers of } C.pyrenoidosa \text{ (cells/ml)} = 38.854 \times OD_{680} + 0.2124 \quad (1)$$

The growth inhibition rates were calculated as follows :

$$I\% = (C_0 - C) / C_0$$

2

where C_0 is the cell numbers of the algal suspension of the control group, C is the algal cell numbers of treated groups, and I% is the growth inhibition rate.

2.4. Analysis of photosynthetic pigment and chlorophyll fluorescence

Photosynthetic pigments, namely, chlorophyll-a (Chl-a), chlorophyll-b (Chl-b) and total chlorophyll (Chl) was measured as described by Inskeep and Bloom (1985) (Inskeep and Bloom 1985). A volume of 40 mL microalgae culture was centrifuged at 5000 rpm for 10 min. The supernatant was then decanted, and the substrate was then mixed with 80% acetone and incubated at 4°C for 24 h in the dark. Next, the absorbances of the supernatant were measured at specified wavelengths of 647 and 664.5 nm. Finally, the contents were computed using the following equations.

$$Chl-a \text{ (mg/L)} = 12.63 \cdot A_{664.5} - 2.52 \cdot A_{647} \quad (3)$$

$$Chl-b \text{ (mg/L)} = 20.47 \cdot A_{647} - 4.73 \cdot A_{664.5} \quad (4)$$

$$Chl \text{ (mg/L)} = 17.95 \cdot A_{647} + 7.9 \cdot A_{664.5}$$

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The pulse amplitude modulation fluorometer AquaPen AP 110-C (photosystem instrument, Czech Republic) equipped with FluorPen 1.0 software was used to measure the photosynthetic activity of *C. pyrenoidosa*. Fluorescence parameters were calculated according to the method described by Wang et al. (Wang et al. 2011). F_v/F_m represents the maximal photochemical quantum yield and is a symbol for the light energy conversion efficiency of Photosystem II (PSII).

2.5. Analysis of BPA by HPLC

At 0, 4, 8, 12 and 16 days after treatment, 2 ml of the algae suspension was collected and filtered by the polytetrafluoroethylene filter membrane. The concentration of BPA was monitored on a Dionex Ultimate 3000 HPLC). The HPLC system equipped with a binary pump with online solvent degasser, a diode array detector (DAD) and a TC-C18 column (Agilent, 5 µm, 250 × 4.6 mm). The temperature of the column was maintained at 35°C, The mobile phase consisted of 15% methanol and 85% water with (0.2%) acetic acid (V:V) in a flow rate of 1.0 mL/min.

The removal kinetics of BPA were determined by the first-order model as follows:

$$\ln C = -kt + \ln C_0$$

6

$$T_{1/2} = \ln 2 / k$$

7

where C_0 is the initial concentration of BPA at day 0, C is the concentration of BPA at time t , k and t are the removal rate constant (d^{-1}) and removal period in days, respectively.

3. Results And Discussion

3.1. Growth inhibition of PS and BPA to the microalgae

As showed in **Fig. 1**, The relationship between cells density and the concentration of BPA, growth inhibition and BPA in the absence and presence of 5 mg/L PS was investigated. This study found that both conditions have an adverse effect on the growth of the microalgae *C. pyrenoidosa*. Whether PS existed or not, the algae density decreased with the increase of BPA concentration. For instance, compared with the control group, in 1, 10, 20 and 30 mg/L BPA treatment, the growth inhibition rates were 12.52, 12.85, 21.75, 41.85% at 8 days of exposure, respectively. These results were consistent with research results reported by Ji et al, who found that the growth of microalgal *Chlorella vulgaris* was significantly inhibited by BPA at concentration of 50 mg/L at the same exposure time, and the bigger inhibition ratio of growth at 50 mg/L of BPA treatment was higher than that under the 25 mg/L and 10 mg/L BPA treatments in *Chlamydomonas mexicana* when exposed 7 days (Ji et al. 2014). A similar phenomenon was observed for the co-exposure of PS and BPA. For example, in 1 mg/L BPA, 1 mg/L BPA +5 mg/L PS, 10 mg/L BPA, 10 mg/L BPA +5 mg/L PS treatment, the growth inhibition rate were 7.27, 9.52, 13.48 and 16.66% at 16 days of exposure, respectively. which indicated that the co-exposure of PS and BPA was more toxic to *C. pyrenoidosa* than when treated with BPA alone. Similarly, Yi et al. reported that the presence of PS (0.55 µm) increased the toxicity of triphenyltin chloride (TPTCl) to the *C. pyrenoidosa* (Yi et al. 2019). The explanation was given in 3.2.

3.2. Effects of BPA and PS on the *C. pyrenoidosa* photosystem

The photosynthetic pigment is responsible for absorbing light energy and elevating the pigment to a higher energy state, which is known as an excited state. Photosynthetic pigments can be used as an indicator of the photosynthetic capacity of algae (Lu et al. 2018). Changes in the pigmentation system have been recognized as a defense mechanism under different types of stress conditions (Zhang et al. 2018). Common pigments in microalgae, such as chlorophyll (including chlorophyll a, chlorophyll b and total chlorophyll) and carotenoids, are usually considered to be indicators of cell adaptation. Chlorophyll plays an important role in photosynthesis. Specifically, chlorophyll plays a role in light harvesting, light energy conversion and energy transfer (Yang et al. 2020a). As observed in **Table 1**, Compared with the control group, the content of chlorophyll-a (Chl-a), chlorophyll-b (Chl-b) and total chlorophyll (Chl) in the cells increased after treatment for 16 d. As the PS concentration increased, the *C. pyrenoidosa* chlorophyll content decreased, and the low concentration of BPA (1mg/L BPA) with 5mg/L PS had the highest chlorophyll content. Although a decrease in chlorophyll content is common under stress-induced conditions, there are reports that when treated with low concentrations of pollutants, the chlorophyll content of *C. pyrenoidosa* increased and decreased at higher doses. Wan et al. Shows that when exposed to low concentrations of levofloxacin, the chlorophyll content in *Microcystis flosaquae* increased significantly, while the high concentration of levofloxacin reduced the chlorophyll content, which is due to the hermetic effect caused by antibiotic stress (Wan et al. 2014). A similar phenomenon was also observed in microalgae *Scenedesmus obliquus* when exposed to ofloxacin, the content of chlorophyll-a, chlorophyll-b, and chlorophyll, in the cells initially increased when the medium's ofloxacin was 10 mg/L (Yang et al. 2020a).

Fv/Fm indicates photosynthetic activity, which is widely used as an important indicator for monitoring algal photosystem II (PSII) activity (Yang et al. 2020b). As shown in Fig. 2, the Fv/Fm values of *C. pyrenoidosa* in the single BPA and BPA combined PS treatment at different concentrations were lower than that of the control group after 16 d, indicating that the PS had a negative effect on the PSII activity of *C. pyrenoidosa*. This negative influence was increased with concentration of the PS. The Fv/Fm values decreased by 0.2%, 5.1%, 12.1%, 6.9%, 9.5%, and 15.6% for 1mg/L BPA, 1mg/L BPA+5mg/L PS, 1mg/L BPA+100mg/L PS, 10mg/L BPA, 10mg/L+5mg/L PS and 10 mg/L BPA+100 mg/L PS, respectively. Overall, the above obtained Fv/Fm values of *C. pyrenoidosa* in 100 mg/L PS microplastics treatments were lower than those in the 5 mg/L PS microplastics treatments for both 1 mg/L and 10 mg/L of BPA exposure. This phenomenon can be attributed to the shading effect. A similar result was reported by Zhang et al. that PVC had significantly negative effects on chlorophyll content and PSII activity of microalgae *Skeletonema costatum* (Zhang et al. 2017). Mao et al. also obtained the conclusion that the bigger inhibition ratio of Fv/Fm at 100 mg/L PS microplastics (0.1 mm) treatment was higher than that under the 10 mg/L and 50 mg/L PS microplastics (0.1 mm) treatments (Mao et al. 2018).

Table 1

Photosynthetic activity of *C. pyrenoidosa* under different conditions.

Treatment	1mg/L BPA	1mg/L BPA +5mg/L PS	1mg/L BPA +100mg/L PS	10mg/L BPA	10mg/L BPA +5mg/L PS	10mg/L BPA +100mg/L PS	Control
Chl- a(mg/L)	37.96	48.98	36.58	43.43	43.48	37.49	36.01
Chl- b(mg/L)	20.62	28.01	21.54	22.37	22.97	20.87	20.26
Chl(mg/L)	58.58	76.99	58.12	65.80	66.45	58.36	56.27

3.3. Effect of PS microplastics on the *C.pyrenoidosa* removal of BPA

Due to its endogenous catabolic system, heterotrophic capacity, and role in carbon fixation and turnover, microalgae have the potential to remove organic pollutants (Xiong et al. 2017b). The removal kinetics of BPA by *C.pyrenoidosa* are shown in Fig. 3, after incubation for 16 d, the removal efficiencies of BPA were 23.76%, 39.65%, 31.80%, 34.94, 45.53% and 42.11% by incubation with this microalgae cells for 1mg/L BPA, 1mg/L BPA+5mg/L PS, 1mg/L BPA+100mg/L PS, 10mg/L BPA, 10mg/L BPA+5mg/L PS and 10 mg/L BPA+100 mg/L PS, respectively. A higher removal rate of BPA was observed at a lower exposure level with PS. Compared with the treatment without PS (exposure to 1mg/L BPA and 10 mg/L BPA), the BPA removal efficiency of lower PS exposure level (5mg/L) increased by 15.89% and 10.59%, respectively, suggesting that PS can accelerate the removal of BPA in the algae medium. These results are consistent with previous reports that the co-presence of polystyrene nanoplastic can enhance the degradation of ibuprofen in the medium of *C.pyrenoidosa*.

The kinetic analysis showed that the kinetic removal rate constant (k , d^{-1}) of 1mg/L BPA, 1mg/L BPA+5mg/L PS, 1mg/L BPA+100mg/L PS, 10mg/L BPA, 10mg/L BPA+5mg/L PS and 10 mg/L BPA+100 mg/L PS ranged from 0.0149 to 0.0374 d^{-1} , and the degradation half-life ($T_{1/2}$, d) was calculated to be 18.53-46.52 d (**Table 2**). Furthermore, the removal of BPA via abiotic factors was evaluated through the addition of BPA to the medium without any *C.pyrenoidosa* inoculation. The initial concentration of BPA in the abiotic control did not show any change throughout 16 d (**data not shown**), thus, microalgae biomass is the main factor in the removal of BPA in this study, and the contribution of abiotic factors in the removal of BPA is negligible.

Table 2

Kinetic parameters of BPA degradation during 16 d of cultivation.

Species	1mg/L BPA	1mg/L BPA +5mg/L PS	1mg/L BPA +100mg/L PS	10mg/L BPA	10mg/L BPA +5mg/L PS	10mg/L BPA +100 mg/L PS
k(d ⁻¹)	0.0149	0.0297	0.0199	0.0248	0.0374	0.031
T _{1/2} (d)	46.52	23.34	34.83	27.95	18.53	22.36
R ²	0.9078	0.9058	0.8165	0.8871	0.8384	0.8544
Total removal (%)	23.76	39.65	31.80	34.94	45.53	42.11
k-kinetic removal rate constant (day ⁻¹). T _{1/2} -removal half-life (day). R ² -correlation coefficient.						

3.4. Proposed metabolic pathways of BPA in *C.pyrenoidosa*

As can be seen from Fig. 4, the peak area of BPA decreased with the extension of the incubation time, and some new peaks with retention times different from that of peak-BPA were appeared after 4 d, indicating that some intermediates were formed during the *C.pyrenoidosa* degradation of BPA. Potential transformation products of BPA were identified based on the mass-to-charge ratios (m/z), and their chemical structures were confirmed by the characteristic fragment ions. The mass spectra information after microalgal degradation is shown in **Fig. S1**. Based on these results and previous literature (Wang et al. 2017), the transformation pathways for BPA are proposed in Fig. 5. P1 was formed owing to oxidoreductase activity, and P1 further lost a molecule of H₂O leading to the formation of P2. García-Rodríguez et al. revealed that phenolic compounds could be oxidized by oxidoreductases such as polyphenol oxidase and peroxidase (García-Rodríguez et al. 2015). In addition, oxidoreductase can catalyze the oxidative degradation of phenolic compounds (Taticchi et al. 2013). Therefore, our present study indicated that *C.pyrenoidosa* over-expressed oxidoreductase encoding genes to promote BPA oxidation to form hydroxyl groups on the benzene ring of BPA. P3 could be formed through another pathway via the action of oxidoreductase, indicating that the addition of a hydroxyl group to the P3. With the subsequent glycosyltransferase, P4 was further formed, a similar result was obtained for the biodegradation of BPA by the green alga *Desmodesmus* sp.WR1 (Wang et al. 2017). Hyung Ko et al (2006) demonstrated that glycosyltransferase was able to glycosylate phenolics (Hyung Ko et al. 2006). In 2003, Morohoshi et al. showed that BPA glycosylation resulted in the inability to detect the level of estrogenic activity in the yeast two-hybrid assay (Morohoshi et al. 2003). Therefore, our results also suggested that *C.pyrenoidosa* was able to transform BPA into non-toxic metabolites via glycosylation. P5 resulted from the cleavage of -CH₂OH, and the fragment m/z 406 (P6) corresponded to the subsequent loss of a hydroxyl group.

4. Conclusions

The effects of PS on the toxic effects and biodegradation and removal of BPA in *C.pyrenoidosa* were reported in this study. In conclusion, The co-exposure of PS and BPA increased the growth inhibitory effect, decreased the chlorophyll content, and enhanced the removal efficiency of BPA in the medium. In addition, five intermediates were found based on the mass-to-charge ratios (m/z) in the biodegradation process. According to this, The degradation pathway of BPA by *C.pyrenoidosa* is proposed in the presence of PS. More studies should be needed to explore the toxicity of microplastics and contaminants in the aquatic system to gain a better understanding of the detoxification mechanism behind the interactions of aquatic organisms.

Declarations

Acknowledgments

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Dandan He, Youmei Zeng, Guangming Zhou. The first draft of the manuscript was written by Dandan He and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. This study was supported by the financial support from the Central Universities (No. XDJK2019C045). The authors have no competing interests to declare that are relevant to the content of this article.

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Figures

Figure 1

The cells density of microalgae when exposed to BPA in the absence (a) and presence (c) of PS. The growth inhibition rate of microalgae when exposed to BPA in the absence (b) and presence (d) of PS.

Figure 2

The photosynthetic indices F_v/F_m of *C. pyrenoidosa* when exposed to BPA and PS microplastics for 16 d.

Figure 3

Effect of PS on the removal of BPA by incubation with *C.pyrenoidosa*.

Figure 4

Chromatograms of BPA during the *C.pyrenoidosa* degradation processes.

Figure 5

Proposed transformation pathways of BPA by *C.pyrenoidosa*.

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