Toxicity Effects of Microplastics and Nanoplastics with Cadmium on the Alga *Microcystis Aeruginosa*

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Abstract

The objective of this paper is to present a thorough analysis of the toxicity of microplastics (MPs) and nanoplastics (NPs) with the heavy metal cadmium (Cd). These components were studied separately and combined to determine how these environmental toxins affect *Microcystis aeruginosa* (*M. aeruginosa*) in fresh water. The combined toxicity of MPs or NPs and Cd to *M. aeruginosa* showed an aggressive and negative effect after 96 h of exposure. Due to the higher adsorption ability of NPs, the accumulation of Cd inside cells with NPs was lower than that found inside the cells with MPs. But the difference in toxicity between the MPs and NPs was not significant. Meanwhile, the more produce of the extracellular polymeric substance (EPS) in the presence of NPs, the more complex effect of EPS bonded to heavy metals, which reduce the toxic effect on the algal cells. Notably, the production of microcystin-LR (MC-LR) under different treatments has demonstrated that the presence of combined MPs/NPs with Cd can potentially raise some of the toxin risks harming the aquatic environment. Our findings highlight the great potential ecological risks of the combined Cd and MPs/NPs in the aquatic system.

Introduction

With the sustained consumption of plastic products, the release of plastic waste into the environment has become an environmental hazard that increases with each passing decade. Jambeck et al. (2015) estimated that in 2010, 4.8 to 12.7 million metric tons of plasticwaste from 192 countries ended up in the Atlantic, Pacific, and Indian oceans as well as the Mediterranean and Black seas. Microplastics (MPs) are defined as having a particle size smaller than 5 mm. This parameter was put forward for the first time in a meeting hosted by NOAA (National Oceanic and Atmospheric Administration) (Hartmann et al. 2019). Nanoplastics (NPs) are commonly defined as having a particle diameter of less than 100 nm (Rist et al. 2017). They have been reported to have originated from the breaking down of plastic debris and polyethylene microbeads contained in commonly used chemical-based products (Peng et al. 2020), including personal care products (Hernandez et al. 2017). The abundance of MPs in surface water ranges from 379 to 7,924 particles/m^3 in industrial port waters (Lin et al. 2018). Even higher amounts (102,550) of particles/m^3 have been found by Prata et al. (2019).

The persistence of MPs in fresh water highly affects the ecosystem with negative effects. Canniff (2018) revealed that MPs could serve as substrates for *Raphidocelis subcapitata* (a green algae) growth and provide energy for reproduction in the aquatic organism *Daphnia magna* (*D. magna*), a small planktonic crustacean. However, Wu et al. (2019) reported that MPs showed a negative effect on chlorophyll-a (*Chl-a*) contents and the photosynthetic activity of the freshwater algae *Chlorella pyrenoidosa* and *Microcystis flos-aquae*, due to the accumulation of intracellular reactive oxygen species (ROS) and the interruption of photosynthetic electron transport. With the exposure of polyvinyl chloride (PVC) MPs, the highest inhibition of growth rate (I_r) of 45.8% was recorded by Ting et al. (2019) based on the marine dinoflagellate, *Karenia mikimotoi*, after 24 hours of contact time. This was due to physical blockage and aggregation.

During MP exposure in the environment, other contaminants containing organic matter and metal ions correlated with MPs, resulting in a complex effect on the aquatic ecosystem. Increased toxicity was reported by Sandra et al. (2018) based on the effects of combined MPs and chemical contaminants (PCB, BFRs, PFCs and methylmercury) on zebrafish. These effects on the zebrafish were revealed and compared with each contaminant's effect separately. Lu et al. (2018) also found that the presence of MPs could increase cadmium (Cd) accumulation and cause oxidative damage in tissues, which eventually led to an enhanced toxicity to zebrafish. However, because of the strong adsorption capacity of glyphosate on amino-modified NPs, antagonistic effects appeared to inhibit the growth of the cyanobacterium, *Microcystis aeruginosa* (*M. aeruginosa*), a species of blue-green algae. The growth inhibition was attributed to the combined presence of NPs with pesticide (Zhang et al. 2018). Meanwhile, Ma et al. (2016) noticed that NPs showed more significant toxicity and physical damage to *D.magna* ((1.5–5.0 mm) than MPs due to the higher adsorption of phenanthrene on the smaller size NPs.

Extracellular polymeric substance (EPS) consists of a complex mixture of carbohydrates, proteins, humic acid substances and deoxyribonucleic acid (DNA). EPS originates from microorganisms. After exposure to external stress, EPS produced by algae often easily interact with other contaminants and changes the ecological effects. Protein present in EPS can interact with NPs and change the surface charge and specific surface area of NPs (Summers et al. 2018). Moreover, the protein corona presented on the altered surface of polystyrene (PS) NPs incubated with copper and EPS produced by the freshwater microalgae *R.subcapitata*, could eventually change the ecological effects in the combined system (Bellingeri et al. 2019).

For this study, the authors chose the heavy metal ion Cd as a typical metal ion found in fresh water. Cd is a non-essential metal and is presented as a highly toxic contaminant for biological functions in aquatic ecosystems (Samadani et al. 2018a). We investigated both PS MPs with an average size of 50 μm and PS NPs, which averaged 80 nm. Our research objectives were to compare the influence of MPs and NPs on the growth of freshwater algae *M. aeruginosa* with and without Cd. *M. aeruginosa* is a species of freshwater cyanobacteria best known for its ecologically devastating algal blooms. The I_r, *Chl-a* contents, and enzymatic activity were recorded to assess the toxic effects under the stress of PS and Cd in single and combined systems. EPS production by algae was analyzed under different treatments. Furthermore, the microcystin-LR (MC-LR) was also investigated to assess its ecological risk to fresh water. Results of this study will provide new insights for further investigating the combined effects of MPs and NPs with other pollutants in aquatic environments.

Experimental Methods

Algae and culture conditions

M. aeruginosa was purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB) in Hubei, China. The algae were cultured in a BG-11 medium (Table S1) and maintained in a sterile growing environment in an oscillating light incubator at a room temperature of 25

± 1 °C, with a pH value of 7.0 ± 0.1, and a rotating speed of 150 r/min. The light condition was adjusted to a light: dark cycle of 12: 12-h with the light intensity of 50 μmol photons/m^2/s. In order to reduce the influence of light intensity on the growth of algae, the flasks were randomly arranged and changed every day.

Chemicals

PS MPs were purchased from Guangdong Dongguan Xingwang Plastic Products Co., Ltd., China, with a particle size of about 50 μm. MPs need to be pretreated with 95 % ethyl alcohol and then with 5 % nitric acid to remove any residual organic or inorganic pollutants. After that, MPs were washed to attain a neutral state by ultrapure water and dried in air before usage. A certain number of MPs was weighted and dispersed into ultrapure water to prepare a concentration of 1,000 mg/L solution. Virgin PS NPs with a diameter of around of 80 nm were purchased from the BaseLine ChromTech Research Center in Tianjin, China, as a 2.5 % (w/v) suspension diffused in ethanol. The stock solution with a concentration of 1,000 mg/L was prepared by dilution with ultrapure water before the experiments.

The analytical grade cadmium chloride ($CdCl_2$) with 99 % purity was purchased from the Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). A stock solution of 1000 mg/L of Cd was prepared in ultrapure water.

Algae toxicity test

Toxicity test of single pollutants on algae

In order to evaluate single effects of MPs, NPs and Cd on *M. aeruginosa*, algae exposed with a different range of pollutants were investigated separately. A certain volume of Cd stock solution was transferred into a 250 mL conical flask containing 100 mL sterile medium until a variety of concentrations were obtained (i.e., 0.2, 0.5, 1.0, 1.5 and 2.0 mg/L). Due to the complex effect with Cd, ethylenediaminetetra acetic acid (EDTA) in the medium was removed to prevent the influence of metal ions on the toxicity of algae. MPs and NPs with concentrations of 0.5, 1.0, 2.0, and 5.0 mg/L were also studied, respectively. Before the experiments, MP and NP solutions were dispersed well by ultrasound for 30 min to avoid agglomeration and sediment. The initial density of algae in the experiment was 4×10^6 cells/mL. Toxicity experiments were carried out for 96 h, and three parallel samples were set up for each sample under different conditions.

Effects of combined MPs/NPs and Cd on algae

The effect of the combined MPs/NPs and Cd on *M. aeruginosa* was also investigated and compared with the single system. The design of the toxicity experiments in the combined system is listed in Table 1. All the experiment conditions described in front part were maintained. The adsorption experiments of Cd on the MPs and NPs were also performed to assess the adsorbing behavior of metal ions on to plastics (details given in the SI).

Table 1 Experimental design of toxicity effects of the combination of Cd and MPs/NPs

Test	Group A		Group B	
	MPs or NPs	Cd	MPs or NPs	Cd
Concentration (mg/L)	5	0.2; 0.5; 1.0; 1.5; 2.0	0.5; 1.0; 2.0; 5.0	2.0

Analytical method

Inhibition of growth rate (I_r)

The density of *M. aeruginosa* algae was counted every 24 hours by an automatic cell counter (Countstar, Shanghai RuiYu Biotech Co., Ltd. China). One mL of algae suspension fixed with 10 μL of Luger reagent was homogeneously mixed with an oscillator. 20 μL of the mixed sample was injected into a cell counting plate and automatically counted using CountStar Algae software. As described in the guideline 201 of the Organization for Economic Cooperation and Development (OECD), the I_r was calculated as:

$$\mu = \ln(N_t/N_0) \ /\Delta_t \quad (1)$$

$$I_r = (1-\mu_0/\mu_t)\times 100\% \quad (2)$$

where N_t is the number of cells/mL at t (h), and N_0 is the control group number of cells/mL, Δt is the time interval; μ_0 is the average specific growth rate of the control group, and μ_t is the average specific growth rate of the algae at t (h).

Chl-a of algae

The *Chl-a* contents under the stress of Cd and PS in single and combined system was measured to quantify the variations in the phytoplankton biomass of algae. The suspension solution was extracted by acetone solution (details given in the SI). *Chl-a* (μg/L) was measured according to Eq. 3 as follows:

$$C_a = \frac{[11.64\times(OD_{663}-OD_{750})-2.16\times(OD_{645}-OD_{750}) +0.1\times(OD_{630}-OD_{750})]\cdot V_1}{V\cdot\sigma} \quad (3)$$

where C_a is the *Chl-a* contents inside the algae cell; V and V_1 represent the sample volume and acetone-based extract (mL) volume, respectively. OD is the absorbance at a certain wavelength, and σ is the optical path of the cuvette (cm).

Accumulation of cadmium ions in algal cells

After the exposure of Cd in single and combined systems, the metal ions absorbed in algae were analyzed. Algal samples were first filtered through a 0.45 μm cellulose acetate membrane and then

washed with 5 mL of 1 mM EDTA to remove adsorbed Cd on the surface of the cell. Then, filters containing algal cells were dried at 60 °C for 24 h. Next, the filter papers containing dried biomass were digested in 1 mL of 65 % HNO_3 and 125 µL of 30 % hydrogen peroxide (H_2O_2) at 120 °C for 12 h (Samadani et al. 2018b). Quantified biomass samples were diluted by 10 % HNO_3 and measured for accurate Cd concentrations by a flame atomic absorption spectrometer (PinAAcle 900T, PerkinElmer, Germany), which provides both flame and THGA furnace atomic absorption (AA).

Morphologic properties

After the exposure with single pollutants (MPs (5 mg/L), NPs (5 mg/L) and Cd (2 mg/L)) and the joint system (MPs/NPs (5 mg/L) + Cd (2 mg/L)), *M. aeruginosa* cells were centrifugated at 3,000 rpm for 15 min and then mixed with 2.5 % glutaraldehyde at 4 °C for 12 h. The samples were washed by 0.1 M phosphate buffer using the pH value of 7.0 three times, followed by two hours of washing by 1 % OsO_4 in phosphate buffer before washing by a phosphate buffer (0.1 M, pH=7.0) three more times for 15 min each time. The samples were then dehydrated by gradient concentrations of ethanol (30 %, 50 %, 70 %, 80 %, 90 % and 100 %) for 15 min, successively. The prepared samples were stored in refrigerator at 4 °C in preparation for an analysis using scanning electron microscopy (SEM) (JMS-6490LV, JEOL, Japan) (Zhang et al. 2018).

Measurement of SOD activity and MDA content and ROS generation

The enzyme activities containing superoxide dismutase (SOD) and malondialdehyde (MDA) of algae under the stress of both Cd and PS in single and combined systems were assayed by using corresponding commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). The algae cells were centrifuged for 15 min at 5,000 rpm to remove the suspension. Then, the cell was smashed by an ultrasonic cell disruptor to obtain homogenates. The absorbance was detected using the Synergy H1 multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA). The SOD activity (U/mL) was monitored at 550 nm after the addition of the SOD assay kit. The MDA content (nmol/mL) was measured at 532 nm, using the MDA assay kit.

ROS were measured according to the instructions supplied with the ROS Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). The production of ROS was measured by following the conversion of the non-fluorescent probe 2', 7'-dichlorofluorescein diacetate (H_2DCF-DA) to the highly fluorescent compound 2', 7'-dichlorofluorescein (DCFH) as described by Morelli et al. (2018). Algal samples after the exposure with Cd and MPs/NPs were collected and centrifuged at 6,000 rpm for 10 min and then washed by phosphate buffer saline (PBS) solution. The washed samples were cultured in PBS buffer mixed with 10 µM DCFH-DA in the dark for 30 min. After that, samples were washed by ultrapure water twice and placed in 2 mL of PBS buffer. Then, a ZEISS Axio Vert.A1 inverted microscope (Zeiss, Oberkochen, Germany) was used to capture fluorescent images of ROS.

EPS

Dissolved EPS (S-EPS) and bound EPS (B-EPS) were extracted separately. The algae were first centrifuged at 4,000 rpm for 15 min at 4 °C. The supernatant was filtered by a 0.45 μm cellulose acetate membrane and the filtrate was determined as the S-EPS. The algae remaining on the surface of the filter membrane and at the bottom of the centrifuge tube were collected and suspended using 0.6 % NaCl solution. After that, the cells were centrifuged at 10,000 rpm for 15 min at 4 °C, and the supernatants collected after the filtration were filtered with a 0.45 μm cellulose acetate membrane for B-EPS (Chen et al. 2015).

Measurements of the protein and polysaccharide contents in S-EPS and B-EPS were analyzed, respectively. The content of proteins was determined using commercial chemical assay kits (Jiancheng Bioengineering Institute, Nanjing, China) and the polysaccharides were measured by using phenol-sulfuric acid (Wang et al. 2021).

Intracellular and extracellular microcystins (MCs)

Algae were centrifuged (10,000 rpm, 15 min, 4 °C) until the supernatant was obtained for further analysis of the extracellular MC-LR of *M. aeruginosa*. The remaining algal cells were washed with ultrapure water and then frozen and thawed three times to rupture the cells. After that, the cells were centrifuged and the obtained supernatant was tested for the intracellular MC-LR. The concentration of intracellular and extracellular MC-LR was quantitatively analyzed using a Beacon Microcystin ELISA kit (Express Technology Co., Ltd. Beijing, China) (Zhang et al. 2020).

Statistical analysis

All the experiments were conducted in triplicate and the means ± standard was obtained. Statistical analysis was performed using Origin® 9.0. We also found significant differences using the SPSS software statistical package version 22.0 ($p < 0.05$).

Results And Discussion

Effects of Cd and MPs/NPs on the growth of *M. aeruginosa*

Effects of Cd and MPs/NPs on algal growth withprolonged time

Algal density as a useful indicator to reflect the growth effect under environmental stress was analyzed in Fig. 1. The density of algal cells cultured in different treatment groups was lower than that in the control group, indicating that both MPs/NPs and Cd ions had inhibition effects on the growth of algae. The increased I_r (Fig. S1) of algal cells with the prolonged exposure time showed that the effects of each treatment group used to study the growth of algae had a cumulative effect. Both MPs and NPs had a certain inhibition effect on the growth of algae. This result was similar to that of our previous report showing that MPs had a weak effect on *M. aeruginosa* due to the adsorption of nutrients onto MPs (Wang et al. 2021). The impact of NPs alone was greater than that of MPs, which proved that the smaller

the size of the particles are, the stronger the identifiable presence of toxicity is (Anbumani and Kakkar 2018; Sjollema et al. 2016; Zhang et al. 2017). It can be determined that NPs may attach to the surface of phytoplankton and reduce the activity of microalgae by disrupting their cell morphology and organelle function (Liu et al. 2020; Wei et al. 2020). In the presence of Cd, the inhibition of the algae growth was greatly enhanced. Similar studies have shown that Cd with a concentration of 2 mg/L can significantly inhibit the growth of mixotrophic *Ochromonas gloeopara*, which may be caused by nucleic acid decomposition in alga cells by preventing the formation of phosphate (Wu et al. 2021). In addition, high concentrations of metal ions can induce the production of ROS, which can easily interact with nucleic acids and cause DNA damage in algal cells eventually (Deng et al. 2020). This DNA damage was expected since heavy metals have a reputation for destroying the permeability of cell membranes and hindering the exchange of substances and the uptake of nutrients, which affects the normal growth of algae (Wu et al. 2021).

In the combined system, a similar trend was found when looking at the activity of Cd alone. But the I_r was decreased slightly in the existence of PS due to the adsorption ability of heavy metal ions. Moreover, the presence of NPs could decrease the I_r more than MPs even though there was no significant difference between them. Due to the smaller size and higher specific surface area, NPs had the higher adsorption ability of metal ions compared to MPs which could decrease the effluence of Cd ions (Bhagat et al. 2021).

Effects of different concentrations of MPs/NPs on algal growth

A various concentration of MPs/NPs was studied to explore the effect of MPs and NPs on algae growth in both stand-alone MPs and NPs as well as the combined MP/NP system. After 96 h of exposure time, the I_r gradually improved with the increasing concentration of polystyrene (PS) (Fig. 2a) in the single pollution system, due to the shielding effect and enhanced interaction between plastics and alga (Wang et al. 2020). In the joint system of Cd and PS, even more inhibitory effects to algal growth became evident compared to PS alone. The results indicated that the addition of heavy metals (particularly Cd) may greatly increase their toxic effect on algae and play a key anti-algae role in the combined system. At the same time, it was also found that the I_r of algal cells declined as the PS dose increased in the combined system. This may be related to the variances in the accumulation of Cd in cells along with the PS concentrations. Moreover, the I_r of alga treated with NPs was slightly lower in the system where the alga was also treated with MPs, even though statistically, no significant difference was found in the performance of NPs compared with MPs.

To further understand the toxicity effect of MPs/NPs in the combined system, we also investigated the accumulation of Cd in algal cells under different concentrations of PS. After 96 h of exposure time, with the increasing concentrations of MPs/NPs, the accumulation of the Cd in cells decreased obviously (Fig. 2b). Meanwhile, in the presence of NPs, Cd concentration decreased more significantly than it did when in the presence of MPs. This could be explained by the higher adsorption capacities of metal ions onto NPs than MPs (Fig. S2). Although the accumulation of Cd inside the cells after the MPs and NPs treatments

was distinct, the difference of the toxic effect on algae between them was not significant. This may be caused by the promote of higher number of EPS generated by algae under the stress of NPs, which could combine with more metal ion Cd in the solution (Shen et al. 2018). Meanwhile, the complex effect of the EPS with heavy metal ions could reduce the toxic effect of Cd in the combined system.

Effects of different concentrations of Cd on algal growth

With the increase of Cd concentration, the I_r of alga increased continuously after 96 h of exposure (Fig. 3a). The inhibition effect on alga increased gradually with the highest inhibition rate reaching up to 92.8 % under the stress of Cd (2.0 mg/L) alone. The presence of Cd may have a remarkable influence on the normal metabolic process of algae (Horcsik et al. 2007). Heavy metals have some effect on the survival of algae by disturbing their physiological processes including endocytosis and food absorption (Wu et al. 2021). Naveed et al. determined that Cd could competitively replace Ca^{2+} and Mg^{2+} by entering into cells and inhibiting the activity of enzymes that require Ca^{2+} and Mg^{2+}, thereby affecting the metabolic activity and photosynthesis of organisms (Naveed et al. 2019).

The combined system of MPs/NPs and Cd also had harmful effects to the algae. With the increasing of Cd concentration, the I_r was enhanced from 52.8% to 89% under the stress of MPs and Cd. The I_r was increased from 48.9% to 86% under the combined system of NPs with Cd after the addition of Cd. In the presence of NPs, the inhibition effect was suppressed when compared to MPs in the joint system. The main reasons could be the higher adsorption ability of NPs compared to MPs Meanwhile, the NPs could promote the algae to produce more amount of EPS, which may have complexing with metal ions (Shiu et al. 2020; Naveed et al. 2019). EPS may involve in the extracellular defense mechanism that tried to prevent the metal ions from entering into cells (Yan et al. 2021).

The accumulation of Cd in algal cells under different concentrations of Cd was also investigated (Fig. 3b). As the concentration of Cd increased, the accumulation of Cd in cells was enhanced gradually. The results indicated that Cd could enter into the cell through the cell membrane, causing a physiological disorder and toxic effects. Meanwhile, the accumulation of Cd in algal cells was lower in the presence of MPs and NPs compared to Cd alone, which indicated that the presence of MPs and NPs can inhibit Cd from entering into cells by adsorption behavior. In the combined system of NPs and Cd, the accumulation of Cd in algal cells was lower than that of MPs, which may be explained by the enhanced adsorption capacity of Cd onto NPs, which decreased the entry of Cd ions into the cell at the same time. Although the accumulation of Cd was varied greatly according to the amounts of Cd inside the cells, the difference of the toxicity between MPs and NPs in the joint system was not significant. The possible reasons were the toxic effects of cadmium which play a key role in the compound system (Liao et al. 2020), the agglomeration of nanoparticles may have some effect on the toxicity (Si et al. 2015), and the complexation of extracellular polymers with heavy metal ions (Sendra et al. 2017).

Effects of Cd and MPs/NPs on *Chl-a* with time

Chl-a is used as a standard index to reflect the growth and proliferation of algae as shown in Fig. 4. In the presence of Cd, the *Chl-a* content of algae was obviously reduced more than in the contact with MPs/NPs alone. The existence of Cd has a significant inhibitory effect on the *Chl-a* content of algae. The addition of Cd will reduce the biosynthesis and content of *Chl-a* by destroying the chloroplast structure, inhibiting the expression of photosynthesis-related genes, thereby thwarting the photosynthesis and growth of algae. The effect of NPs on the *Chl-a* content of algae was slightly greater than the effect of MPs, which is consistent with the results of Wu et al. (2019). This can be explained by the accumulation of intracellular ROS in the presence of NPs which can damage the cellular structure and hinder chlorophyll synthesis. The production of ROS can affect the function of chloroplasts, resulting in the disturbance of photosynthesis and metabolism (Zhang et al. 2020).

The combined system also had a high inhibition effect on the content of *Chl-a* which was lower than the inhibition effect of Cd alone but higher than that of PS alone. But the difference between MPs and NPs was not significant. The results demonstrated that in the presence of MPs/NPs, the combined system had a weakened effect on the photosynthesis of alga compared to Cd alone.

SOD activity and MDA content

To further investigate the toxic effects of MPs, NPs and Cd on algae, we studied the enzymatic activities of superoxide dismutase (SOD) and the content of malondialdehyde (MDA) under different treatment groups. The antioxidant enzyme SOD, which is responsible for scavenging superoxide free radicals in cells, is considered a protective characteristic to the antioxidant system (Lu et al. 2018). MDA, which is formed during the degradation of aliphatic acids, is a sign of lipid peroxidation. The higher the content of MDA is, the deeper the degree is of cell membrane lipid peroxidation (Ni et al. 2018). Both the SOD activity and MDA content expressed the highest value under the stress of a single Cd group, indicating that Cd had the strongest toxic effect on algal cells (Fig. 5). The presence of MPs and NPs alone showed a certain effect on the toxicity of algae based on the SOD and MDA values due to the physical adsorption ability of algae onto MPs/NPs which would inhibit the photosynthesis of algae cells (Wang et al. 2020). Because of the large specific surface area of NPs, it is easier to accumulate and adsorb on the surface of microalgae and cause more damage to the cell membrane compared to MPs. In addition, NPs may block the transport of external substances from entering into microalgae, thus impede the exchange of matter and energy inside and outside the cells. Moreover, NPs may also induce the production of superoxide radical (O_2^-) by microalgae that enhance its inhibitory effect on algae (Yan et al. 2021). Meanwhile the MPs had less effect on the antioxidant system of algae than the NPs.

The toxicity of the combined system was lower than a single Cd system but higher than the MPs and NPs alone, which indicated that the presence of MPs and NPs could reduce the bioavailability of Cd in the composite system. The levels of antioxidant enzymes and growth inhibition rate were significantly increased in both Cd and combined treatment groups. The disruption of photosynthesis and the increase of the level of antioxidant enzyme may lead to membrane lipid peroxidation which cause the cell damage and growth inhibition. The addition of NPs in the joint system could decrease the activity of SOD and the

content of MDA more obviously when compared to MPs, because NPs have a certain adsorption effect on Cd (Fu et al. 2019). It can also stimulate the algae to produce more EPS to complex heavy metals, thus reducing the toxic effect of Cd in the composite system (Mao et al. 2018).

ROS

Due to the detoxification mechanism of algae under the stress of pollutants, the algae may produce antioxidant enzymes to eliminate the damage caused by ROS (Wu et al. 2021). Both MPs, NPs and Cd could induce oxidative stress to cells which relate to the rise of ROS, leading to cell membrane damage and even death ultimately (Thiagarajan et al. 2019). The organism would develop an effective antioxidant defense system to maintain normal intracellular oxidative stress in response to excessive production of ROS. Therefore, the level of ROS was considered as a typical biomarker and has traditionally been used to evaluate the toxicity effect under stress (Miao et al. 2019).

As shown in Fig. 6, there was more ROS produced under the stress of Cd and MPs/NPs than the control group. The order of ROS percentages is listed as follows: Cd alone > MPs +Cd > NPs +Cd > NPs alone > MPs alone. The results confirmed that the more obvious toxicity was found in the presence of Cd compared to MPs/NPs alone, which is consistent with the SOD and MDA results. Thiagarajan et al. (2019) found that MPs could stimulate algal cells to produce ROS by restricting light irradiation and nutrient transfer. In addition, harmful metabolites are kept from entering the algal cells, which affects the normal activities of algae. NPs can hinder photosynthesis by attaching to the surface of algae, leading to the increase of ROS in algal cells (Bhattacharya et al. 2010). Moreover, NPs may interact with the enzymes in algae and affect its ecosystem function (Miao et al. 2019). In the presence of Cd, NPs can interact with algae through electrostatic adsorption and complexation, which results in the inhibition of enzymes needed for the growth and metabolism of algae, occurrence of the oxidative stress reactions and the generation of ROS—all of which can eventually produce toxic effects (Naveed et al. 2019). Therefore, in order to relieve the harmful effects of ROS, organisms will develop an effective antioxidant defense system to maintain normal levels of oxidative stress in cells and eliminate ROS by activating microbial antioxidant mechanisms. As shown in Fig. 4, the production of ROS may reflect the response to the stress of pollutants and affect algal photosynthesis and metabolism (Middepogu et al. 2018; Zhang et al. 2020). In our research, the antioxidant system of *M. aeruginosa* was seriously damaged under the stress of Cd both in single and combined system, leading to a more obvious oxidative stress response of algal cells when compared to MPs and NPs alone.

EPS

EPS make up the layer of biomacromolecular matrix covering the surface of cells produced by algae. The main components of EPS include polysaccharides and proteins, which are usually presented as a dynamic bi-layer structure composed of S-EPS and B-EPS (Naveed et al. 2019). The secretion of EPS by algae used to regulate the metabolic activities in the cells seems more like a self-protection mechanism under external stress such as heavy metals. EPS could react with metal ions by ion exchange, coordination interaction and complexation with its rich active functional groups in order to

change the migration behavior and bioavailability of metals by organisms. The adsorption capacity of heavy metals is related to the EPS content of protein and polysaccharide. Meanwhile, S-EPS and B-EPS have different antitoxic abilities under external attack. Chen et al. found that more EPSs were produced by *Chlorella vulgaris*(*C. vulgaris*) when the algae was treated with Cd, while B-EPS was more effective than S-EPS in reducing the toxicity of Cd(Chen et al. 2015).

This study presents an analysis of both S-EPS and B-EPS contents of algae in different treatment groups. As shown in Fig. 7(a), less effect on the polysaccharide content is noted at different treatment groups compared to the production of protein in S-EPS. The content of protein was significantly increased in S-EPS in the presence of Cd due to the self-protection mechanism of algae. Compared to MPs, NPs could accelerate the alga to produce more EPS due to the smaller particle size, easy agglomeration, enhanced interaction and higher toxicity (Cunha et al. 2019; Nava et al. 2021). Because of the high content of amino, carboxyl, hydroxyl and other complex functional protein groups in EPS, the EPS in algae can potentially bind with heavy metals, thereby alleviating the negative effects of Cd on algal cells (Zhou et al. 2016).

Polysaccharide and protein of B-EPS in each treatment group were also promoted when compared with the control group (as seen in Fig. 7b). The highest content of B-EPS was found in the presence of Cd alone compared to the other treatments. The binding capacity of B-EPS with Cd could prevent metal ions from entering into cells thereby reducing the toxicity of metal ions. Meanwhile, Cd can also hinder the nutrient absorption of cells and the excretion of metabolites from intracellular activities that cause certain damage to algal cells. With the coexistence of NPs, both polysaccharide and protein in B-EPS were noticed to be higher than that found in MPs. The results can be further verified by the smaller accumulation of Cd in cells in the presence of NPs than in the presence of MPs in the combined system.

Aggregation of the algae and MPs/NPs

As shown in Fig. 8(a), *M. aeruginosa* in the control group has integrity of cellular structure and easily aggregates. After contact with MPs, algae were adsorbing and surrounding the surface of MPs in order to obstruct the normal growth of algae (Fig. 8b). In the presence of NPs, lots of sticky substances had generated around the algae cells which was speculated as EPS (Wang et al. 2021; He et al. 2019) (Fig. 8c). The EPS was increased significantly by the self-protection mechanism of algae under the stress of external pollutants (Wang et al. 2021; Li et al. 2019). Under the stress of Cd alone, the most abundant EPS was noted as shown in Fig. 8f. We also deduced that the presence of Cd not only promotes the algal cells but can also produce more EPS by self-protection, which also leads to the morphological damage of cells and the reduction of the algal cell density, resulting in the greater toxic effects (Wang et al. 2021).

In the composite system (Fig. 8d, e), more obvious EPS was generated by algae after the treatment of NPs and then MPs in the presence of Cd, which was in accordance with the former results of the EPS production. Meanwhile, the morphology of algae cells was damaged in the combined system shown in the SEM images, which revealed that the combined system of MPs/NPs with Cd had more of a negative

effect on the algal cells than MPs or NPs alone. The existence of Cd with high toxicity in this system may contribution to this effect.

Extracellular and Intracellular MC-LR contents

M. aeruginosa is a representative species of *cyanobacteria* in freshwater, which is a main cause of the algal blooms. *M. aeruginosa* will release MCs that threaten the health of aquatic organisms. MC-LR, as one of the most typical types of MCs, contains both intracellular and extracellular MC-LR, which was considered in this research. The amount of extracellular MC-LR may be related to membrane lipid peroxidation and protein membrane synthesis (Zhang et al. 2020). Meanwhile, the dead algal cells also release intracellular MC-LR outside the cell, resulting in a higher extracellular MC-LR content.

As shown in Fig. 9, the maximum MC-LR both inside and outside the cells was produced under the stress of Cd, which was related to the stress reaction of cells. In addition, the osmosis of the metal ions through the membrane had the same effect on the algal metabolic activity that increased the permeability of the cell, thereby stimulating the release of the MC-LR (Xu et al. 2019). In comparison, NPs could promote more production and release of MC-LR than MPs due to the greater influence on lipid peroxidation of a cell membrane, which in turn affects the fluidity and permeability of membrane (Feng et al. 2020).

In the composite system, the generation of MC-LR was higher than in the single MPs/NPs system but lower than in the single Cd system. There was no significant difference between MPs and NPs in the combined system based on the comprehensive influencing mechanism such as the membrane lipid peroxidation caused by cell membrane damage, the size of the algal density, cell lysis and oxidative stress (Wu et al. 2020). In a word, the presence of MPs/NPs and Cd can induce MC-LR by *M. aeruginosa*, which gives rise to the *cyanobacteria* blooms and eutrophication of water especially in the existence with Cd.

Conclusions

Effect of MPs/NPs combined with Cd on *M. aeruginosa* was both investigated and compared with a single pollution system in this study. Both MPs and NPs do have a negative effect on the growth rate and antioxidant system of algae, while Cd has a significant toxic effect on algae by causing serious damage to the antioxidant system and destroying the integrity of algal cells. Compared to MPs, NPs have a greater toxic impact which has a major impact on the oxidative stress level of algae and produces more ROS in a single system. In the combined system, MPs/NPs could reduce the bioavailability of Cd to some extent, while the difference between them was not significant. This could be explained by the higher production of EPS under the stress of NPs as well as the aggregation abilities. Both MPs/NPs and Cd can stimulate the algae to produce MC-LR, while Cd as a stand-alone agent inflicted the strongest oxidative damage to algal cells, thereby generating the most abundant MC-LR. In the combined system of MPs/NPs and Cd, the generation of MC-LR was higher than in the presence of MPs/NPs alone, which may give a potential risk to the water environment.

Declarations

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Author contribution Wang Qiongjie-Conceptualization, Methodology, Writing-original draft, Data curation, Writing - review and editing. Wang Jinxiaoxue-Investigation, Formal analysis, Visualization, Software. Chen Huijuan-Formal analysis, Software, Investigation, Data. Zhang Yangyang-Investigation, Formal analysis, Visualization, Software.

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Figures

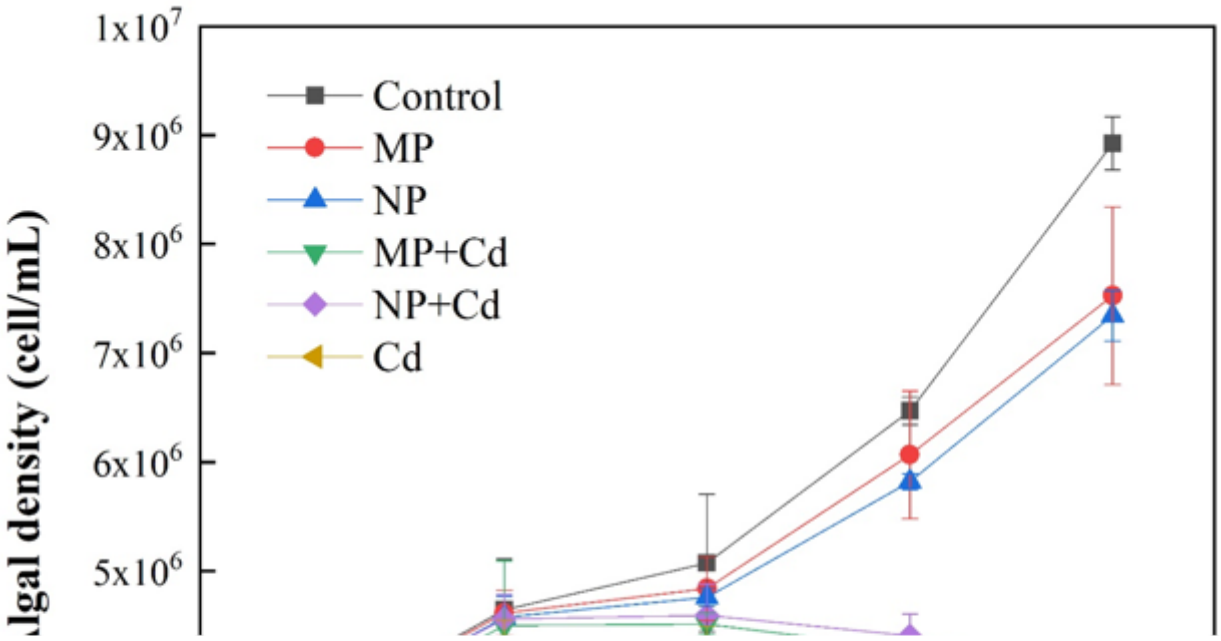

Figure 1

The algal density of *M. aeruginosa* exposed to Cd (2 mg/L) alone, MPs (5 mg/L) alone, NPs (5 mg/L) alone, MPs (5 mg/L) + Cd (2 mg/L), and NPs (5 mg/L) + Cd (2 mg/L) with exposure time prolonged from 24 h to 96 h.

Figure 2

(a) The inhibition of growth ratio of *M. aeruginosa* after 96 h exposure to MPs/NPs (5 mg/L) alone and MPs/ NPs (5 mg/L) + Cd (2 mg/L), and (b) the accumulation of Cd in *M. aeruginosa* after treated with MPs/ NPs (5 mg/L) + Cd (2 mg/L) with exposure time of 96 h.

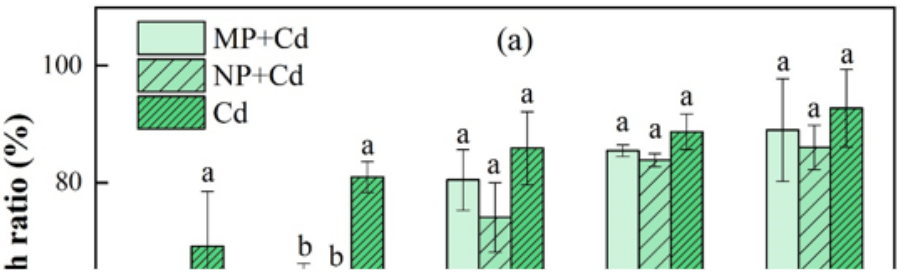

Figure 3

(a) The inhibition of growth ratio, and (b) the accumulation of Cd in *M. aeruginosa* after 96 h exposure to Cd (2 mg/L) alone and MPs/ NPs (5 mg/L) + Cd (2 mg/L).

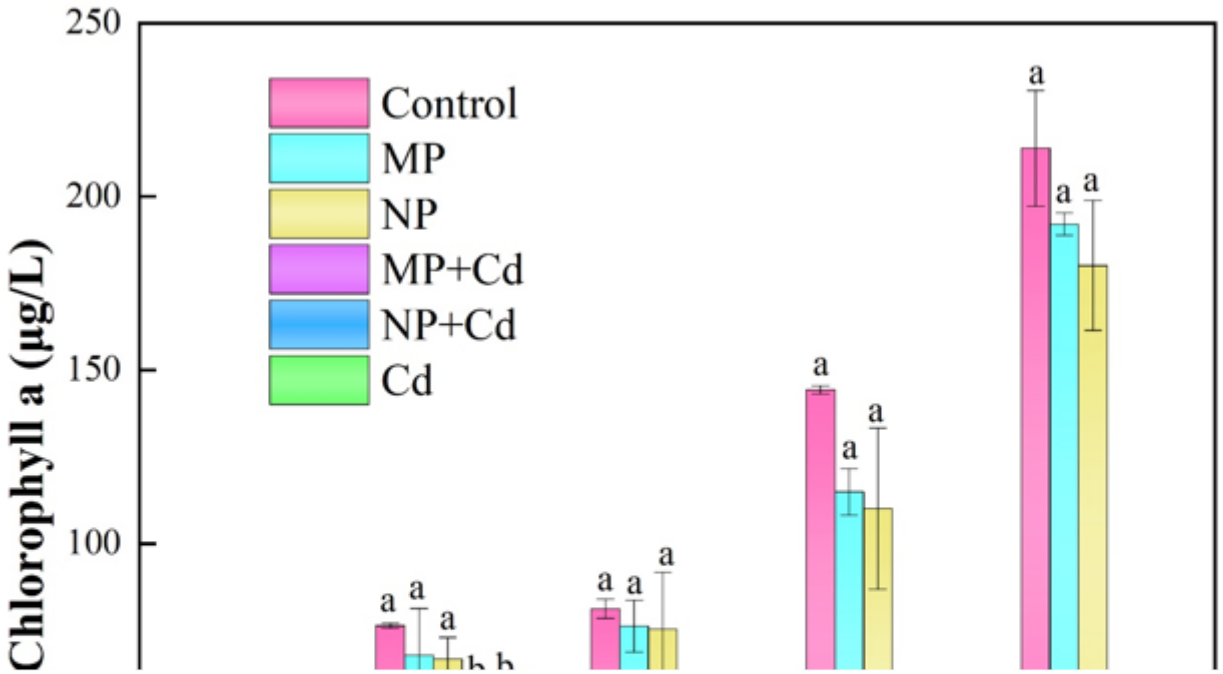

Figure 4

The content of *Chl-a* of *M. aeruginosa* exposed to Cd (2 mg/L) alone, MPs (5 mg/L) alone, NPs (5 mg/L) alone, MPs (5 mg/L) + Cd (2 mg/L), and NPs (5 mg/L) + Cd (2 mg/L) with exposure time prolonged from 24 h to 96 h.

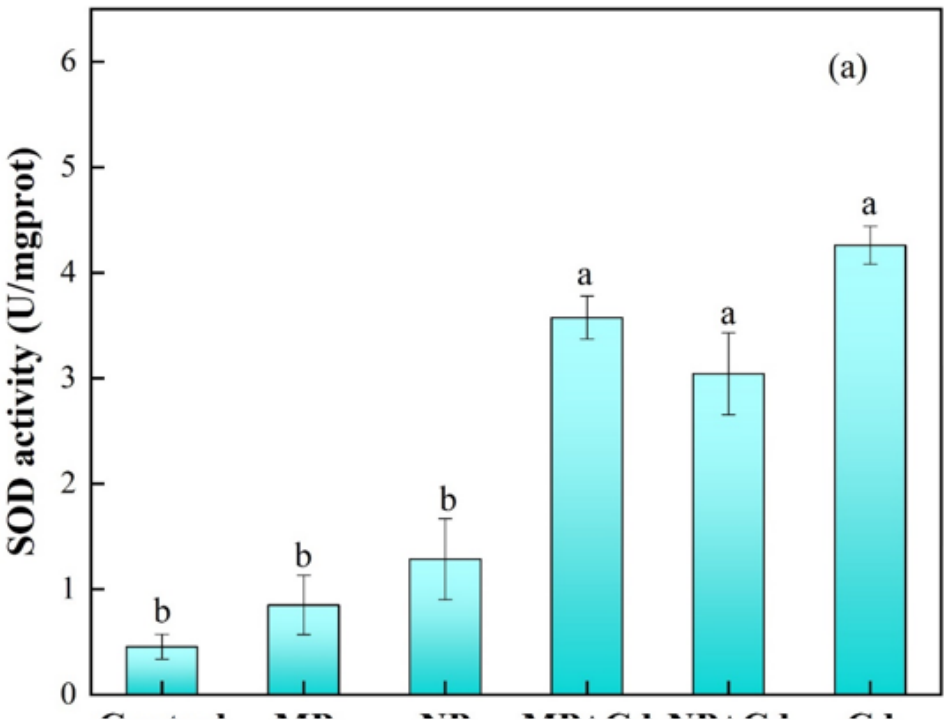

Figure 5

(a) The SOD activity, and (b) the MDA content of *M. aeruginosa* after 96 h exposure to Cd (2 mg/L) alone, MPs/NPs (5 mg/L) alone, and MPs/NPs (5 mg/L) + Cd (2 mg/L).

Figure 6

Fluorescence microscopy images of ROS of *M. aeruginosa* after 96 h exposure to: (1) the control group; (2) MPs (5 mg/L) alone; (3) NPs (5 mg/L) alone; (4) MPs (5mg/L) + Cd (2 mg/L); (5) NPs (5 mg/L) + Cd (2 mg/L) and (6) Cd (2 mg/L) alone. The fluorescent green dots represent ROS.

Figure 7

The contents of polysaccharide and protein in (a) dissolved EPS (S-EPS) and (b) combined EPS (B-EPS) of *M. aeruginosa* cells after 96 h exposure to Cd (2 mg/L) alone, MPs/NPs (5 mg/L) alone, and MPs/NPs (5mg/L) + Cd (2 mg/L).

Figure 8

SEM images of *M. aeruginosa* cells after 96 h exposure to (a) the control group; (b) MPs (5 mg/L) alone; (c) NPs (5 mg/L) alone; (d) MPs (5mg/L) + Cd (2 mg/L); (e) NPs (5 mg/L) + Cd (2 mg/L) and (f) Cd (2 mg/L) alone.

Figure 9

Extracelluar and intracelluar MC-LR of *M. aeruginosa* after 96 h exposure to Cd (2 mg/L) alone, MPs/NPs (5 mg/L) alone, and MPs/NPs (5mg/L) + Cd (2 mg/L).

Supplementary Files

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