Nanoplastics Promote Microcystin Synthesis and Release from Cyanobacterial *Microcystis aeruginosa*

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**ABSTRACT:** Although the fate of nanoplastics (<100 nm) in freshwater systems is increasingly well studied, much less is known about its potential threats to cyanobacterial blooms, the ultimate phenomenon of eutrophication occurrence worldwide. Previous studies have evaluated the consequences of nanoplastics increasing the membrane permeability of microbes, however, there is no direct evidence for interactions between nanoplastics and microcystin; intracellular hepatotoxins are produced by some genera of cyanobacteria. Here, we show that the amino-modified polystyrene nanoplastics (PS-NH₂) promote microcystin synthesis and release from *Microcystis aeruginosa*, a dominant species causing cyanobacterial blooms, even without the change of coloration. We demonstrate that PS-NH₂ inhibits photosystem II efficiency, reduces organic substance synthesis, and induces oxidative stress, enhancing the synthesis of microcystin. Furthermore, PS-NH₂ promotes the extracellular release of microcystin from *M. aeruginosa* via transporter protein upregulation and impaired cell membrane integrity. Our findings propose that the presence of nanoplastics in freshwater ecosystems might enhance the threat of eutrophication to aquatic ecology and human health.

**INTRODUCTION**

Plastic debris is increasingly considered a global concern because of its negative social and ecological impacts.1-4 The discarded plastics can be degraded into microplastics (0.1−5 mm in size) with different charges by abiotic and biotic factors.5 In addition, microplastics can also be directly derived from personal care and cosmetic products or textile fibers via wastewater discharge.6,7 Therefore, rivers are one of the dominant pathways for plastic debris to reach the oceans.8 Fragmentation of microplastics to nanoparticles has been performed in the laboratory using nanoparticle tracking analysis and dynamic light scattering (DLS).5,9 Based on mass conservation principles, it is estimated that the nanoplatic particle concentrations are 10¹⁴ times higher than that of the presently measured microplastic particle.10 In addition, nanoplastics with nanospecific properties have become a special concern in microplastic research.11,12 Because of their ability to penetrate cells, nanoplastics could bring about growth inhibition,13 reproductive dysfunction,14 and reduced viability15 for marine invertebrate and vertebrate animals.16,17 However, the potential threats of nanoplastics on primary producers, which form the base of the food chain in freshwater systems, have not received sufficient attention.

A handful of publications elucidated that nanoplastics reduced chlorophyll *a* in *Scenedesmus*,18 photosynthesis in *Chlorella*,18 and expression of *rbcL* genes (responsible for carbon fixation) in *Chlamydomonas*.19 In addition, the toxic potential of nanoplastics strongly varies depending on the properties of nanoplastics, such as the size and surface charge.18,20 Ecotoxicity studies of the influence of nanoplastics on aquatic organisms have exponentially increased in the last 7 years, where an unsaturated amine (−NH₂) was selected as the model for positively charged polystyrene nanoparticles.20,21 Cyanobacteria, as the main primary producers, have greatly contributed to the richness and abundance of benthic algal communities in freshwater systems.22 In addition, extensive occurrence of cyanobacterial blooms is also regarded as the ultimate phenomenon of occurrence of eutrophication worldwide.22,23 The blooms can directly diminish drinking water quality and pose a potential threat to humans and...
ecosystems. Microplastic fragments, floating in aquatic environments and are 10–100 times larger than the unicellular algae, possibly provide a favorable surface for colony establishment. In addition, planktonic cyanobacteria may attach to the surface of plastic debris and form biofilms on plastic fragments by repressing expression of extracellular components required for biofilm formation. Limited data for the interactions between cyanobacteria and 200 μm microplastics implied that microplastics might have a negative influence on cyanobacteria. However, the interaction between polystyrene nanoparticles and cyanobacteria may be different from that of micro-sized ones. Polystyrene nanoparticles, 100–1000 times smaller than algal cells, may directly cling to the algal cell. However, the potential ecological effects of nanoplastics on cyanobacteria in freshwater systems are virtually unknown.

Several cyanobacteria species can produce microcystin (MC), with Microcystis as the predominant producer in freshwater systems. Over 80 variants of MC have been identified, where MC—leucine—arginine (MC-LR) is the most common and harmful. MC has been associated with liver cancer and mortality, for example, with the death of 60 patients after renal dialysis with MC-contaminated water in Brazil. Notably, MC accumulated in animal tissues could be transferred to higher trophic levels through the aquatic food web. Previous research suggests that nanoplastics can induce surface reconstruction of cell membrane, resulting in increased membrane permeability. Hence, nanoplastics might accelerate the release of MC, which could make it more likely for aquatic organisms to be exposed to high concentrations of MC. In this study, we investigate the interaction of Microcystis aeruginosa, a dominant species causing cyanobacterial blooms, and amino-modified polystyrene nanospheres (PS-NH2), functionalized polystyrene nanoparticles to assess nanoplastic toxicity. We investigated the influence of PS-NH2 on the growth, MC generation, and release from M. aeruginosa. The iTRAQ-based comparative proteomics analysis was employed to reveal the mechanism of nanoplastics on M. aeruginosa at the level of translation. Physiochemical responses of chlorophyll a content, transcription level of key photosynthetic genes, and antioxidant systems to PS-NH2 verified the proteomics results. Furthermore, weighted gene coexpression network analysis (WGCNA) was used to explore the key parameter for intracellular generation and extracellular release of MC from M. aeruginosa. These results may contribute to a better understanding of the potential risks of nanoplastics on primary producers and control of cyanobacterial bloom.

## MATERIALS AND METHODS

### Characterization of Nanoplastics

The PS-NH2 (50 nm) particles and green fluorescently labeled 50 nm PS-NH2 particles (excitation wavelength, 475 nm; emission wavelength, 510 nm) were purchased from Bangs Laboratory (USA) and micromod Partikeltechnologie GmbH (Germany), respectively. Sulfonic acid-modified polystyrene nanoplastics (PS-SO2H) were synthesized in the laboratory through nitrogen-protected emulsion polymerization with styrene as a monomer. Before the experiment, the nanoparticles were transferred to a dialysis bag (1 kDa) for 3 days to remove redundant monomers or initiators. The diameter and morphologies of PS-NH2 were characterized by a transmission electron microscope (JEM-2100F, JEOL, Japan). The size (Z-average) and ζ-potential (mV) were determined using DLS (Zetasizer Nano ZS, Malvern, UK). The structure and composition of nanoplastics (Figure S1) were determined via a Fourier transform infrared spectrometer (Avatar, Thermo NicoLet, USA) at wavenumbers from 4000 to 400 cm⁻¹. Ultraviolet–visible (UV–vis) spectra (190–450 nm) of PS-NH2 in the aqueous phase were recorded using an ultraviolet–visible (UV–vis) spectrophotometer (UV-6100, Metash, China), and the concentration of the nanoplastics in the aqueous phase was determined by measuring the UV absorbance at 220 nm (Figure S1).

### Exposure of M. aeruginosa to Nanoplastics

M. aeruginosa FACHB 905 (single cells), FACHB 1327 (small colonies), and FACHB 1338 (large colonies), purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), were cultured in an autoclaved standard blue-green (BG-11) medium at a pH of around 7.2. PS-NH2 was added on the 10th day of single cell strain growth with 8 × 10⁶ cells/mL. The systems without PS-NH2 and those with PS-NH2 at concentrations of 3.40 and 6.80 μg/mL were set as the control, low-concentration, and high-concentration exposure treatments, respectively. All the experiments were performed in six replicates. After acute (2 days) and long-term (10 days) exposure to PS-NH2, the responses of M. aeruginosa by PS-NH2 were investigated.

### Analysis of MC, Chlorophyll a, and Cell Membrane Integrity

M. aeruginosa samples were centrifuged at 10,000g and 4 °C for 5 min. The supernatant was used to analyze the extracellular MC. The residues were resuspended in an original volume of ultrapure water and then frozen in liquid nitrogen and thawed at room temperature thrice. Then, the solution was centrifuged at 10,000g and 4 °C for 5 min. The supernatant was filtered through 0.22 μm acetate cellulose membranes for the analysis of intracellular MC. The extracellular and intracellular MC concentrations were detected using MC enzyme-linked immunosorbent assay kits (Runyu Biotechnology Co., China). Chlorophyll a analysis was performed based on the previous research. The cell membrane integrity was evaluated according to the protocol. The PS-NH2 distribution in M. aeruginosa was observed through confocal microscopy. Confocal imaging was performed using a laser-scanning confocal microscope (LSM-700, ZEISS, Japan).

### Analysis of Antioxidant Responses

M. aeruginosa samples were centrifuged at 10,000g and 4 °C for 5 min. The supernatant was discarded, and the residue was resuspended in 300 μL of ultrapure water. Then, the samples were frozen in liquid nitrogen and thawed at room temperature thrice. After centrifugation at 10,000g and 4 °C for 5 min, the supernatant was filtered through 0.22 μm acetate cellulose membranes for the analysis of the antioxidant responses of M. aeruginosa. Commercially, SOD and GSH assay kits (Nanjing Jiancheng, China) were used to determine the activities of antioxidant enzymes, using a programmable microplate reader (Infinite F50, Tecan, Switzerland). The concentrations were normalized to the cell numbers before statistical analysis.

### Analysis of Gene Expression

Total RNA was extracted according to the procedures of Bacteria Total RNA Isolation Kit (Sangon Biotech, China). The RNA concentration and purity were quantified by a nucleic acid analyzer. Before reverse transcription, a Primerscript RT reagent kit (TaKaRa, China) with a gDNA Eraser was used to remove genomic DNA contamination in RNA samples. The cDNA was then
synthesized through reverse transcription polymerase chain reaction and stored at \(-20^\circ\text{C}\) until real-time qPCR analysis.38

**Analysis of Proteomic Responses.** 
*M. aeruginosa* cells were sampled in three biological replicates for the control and experimental groups during two phases: acute exposure (2 days) and long-term exposure (10 days). Protein extraction was performed via the trichloroacetic acid/acetone precipitation and the SDT-Lysis procedure.39 After quantification, the protein extracts were digested according to the filter-aided sample preparation protocol procedure.40 The iTRAQ-labeled peptides were fractionated by strong cation exchange chromatography using the AKTA purifier system. The collected fractions were desalted on C18 Cartridges and injected for nanoliquid chromatography–mass spectrometry (LC–MS)/MS analysis. LC–MS/MS analysis was performed on a Q-Exactive mass spectrometer coupled to an EASY-nLC. Protein identification was performed using the MASCOT engine embedded into Proteome Discoverer 1.4. To reduce the probability of false peptide and protein identification, the cutoff global false discovery rate was set to 0.01. Diﬀerentially expressed proteins (DEPs) were defined based on fold changes of >1.2 or <0.83 and a p value of <0.05 in all three replicates.41 The protein–protein interaction (PPI) information of DEPs was retrieved from the IntAct molecular interaction database using the STRING software, and the results were visualized via Cytoscape5 software (version 3.2.1).

Furthermore, the degree of each protein was calculated to evaluate the importance of the protein in the PPI network. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identiﬁer PXD011664. All other data are available from the corresponding author on reasonable request.

**RESULTS AND DISCUSSION**

**Promotion of MC Synthesis and Release from *M. aeruginosa* by PS-NH2.** *M. aeruginosa* was cultured in BG-11 medium containing polystyrene nanoplastics with diﬀerential surface charges to simulate changes caused by weathering of plastics and adsorption of natural organic matter.20 The negative-charged sulfonic acid modiﬁed polystyrene nanoplastics (PS-SO3H) showed no obvious inhibitory eﬀect even when the exposure concentration was 100 μg/mL (Figure S2A). However, positive-charged amino-modiﬁed polystyrene nanoplastics (PS-NH2) had a greater inﬂuence on *M. aeruginosa* than negatively charged plastics (Figure 1A), consistent with previous results.20 This could be due to...
electrostatic repulsion, where positively charged nanoparticles interact easily with negatively charged membrane residuals. During the acute 48 h exposure, growth inhibition of *M. aeruginosa* by low (3.40 μg/mL) and high (6.80 μg/mL) concentrations of PS-NH₂ was 23.57 and 46.10%, respectively, with the normally green *M. aeruginosa* turning yellow (Figure 1B,C), in parallel with a significant reduction in the chlorophyll a content (Figure 1D). This inhibition was significantly reduced upon long-term exposure (10 days), with *M. aeruginosa* regaining its green coloration (Figure 1C), indicating that the interaction of nanoplastics and cyanobacteria was dynamic and without persistence within the experimental period. However, the synthesis of MC in per cell increased significantly both under acute and long-term exposure of PS-NH₂, compared to the control group (Figure 1E). In addition, MC–leucine–arginine (MC-LR), the most common and harmful MC in freshwater environments, also increased significantly (Figure S3A). Furthermore, both exposures of PS-NH₂ significantly stimulated the extracellular release of MC and MC-LR from *M. aeruginosa* (Figures 1F; S3B). Conversely, the negatively charged PS-SO₃H was less likely to attach the negative-charged cell membrane with the effect of electrostatic repulsion, and the acute exposure of 100 μg/mL PS-SO₃H negligibly affected the extracellular release of MC and MC-LR from *M. aeruginosa* (Figure S4B). Although PS-NH₂ inhibited the growth of *M. aeruginosa*, during the long-term exposure of PS-NH₂, the concentration of total MC in medium of low concentration group (900.73 μg/L) was significantly higher than the control group with 851.85 μg/L (*p* < 0.01, Figure S5).}

*M. aeruginosa* normally forms colonies instead of single cells under natural conditions. In order to simulate the effects of PS-NH₂ on *M. aeruginosa* in natural freshwater environments, *M. aeruginosa* FACHB 1327 and 1338 were selected as a supplementary study. *M. aeruginosa* FACHB 1327 and 1338 present small and large colonial morphology and grow on the water surface during laboratory culture (Figure S2B). After a 48 h exposure of PS-NH₂, the chlorophyll a content in both FACHB 1327 and 1338 was significantly inhibited even in low-concentration group (*p* < 0.01, Figure S2C). Although colonial *M. aeruginosa* can adapt to the bad environment, exposure of PS-NH₂ still affects their growth.

**Biological Pathways Impacted by PS-NH₂.** To identify the molecular mechanisms responsible for the effects described
above, the proteomic combining phenotypic verification experiments of *M. aeruginosa* under PS-NH₂ were performed. The proteomic results revealed that PS-NH₂ had a substantial influence on cyanobacterial protein expression (Supporting Information; Figure S6). The proteins that were significantly downregulated in both low- and high-concentration treatments suggested that PS-NH₂ may inhibit the photosynthetic activity, weaken the photosynthetic electron transport chain, and reduce carbohydrate metabolism (Figure 2; Table S1). In addition, the acute exposure of low-concentration PS-NH₂ only influenced the light reaction of photosynthesis compared with both the light and dark reactions of photosynthesis impaired by high-concentration PS-NH₂ treatment (Figure S7A). The main proteins (PsbB, PsbC, and PsbD) involved in photosystem II were all downregulated (Table S1), indicating that the photosynthetic efficiency of photosystem II was significantly inhibited by PS-NH₂. To verify the above proteomics results, we performed a qRT-PCR assay on psbD, a key gene associated with photosynthesis II. Pearson’s correlation coefficient between the qRT-PCR data (Figure 3A) and the proteomics data was 0.80, which indicates the accuracy of the proteomics data. The downregulation of PsbD protein is known to interfere with electron transport, leading to the accumulation of surplus electrons and oxidative stress.

The weakening of the photosynthetic electron transport chain under short-term exposure for low- and high-concentration PS-NH₂ leads to the accumulation of surplus electrons and induced oxidative stress (Figure S7B; Table S1). In addition, oxidative stress of *M. aeruginosa* caused by low-concentration PS-NH₂ could be gradually alleviated by detoxification enzymes. However, for high-concentration exposure, this oxidative stress of *M. aeruginosa* continued in the later period (Figure S7B; Table S1). Meanwhile, upon acute exposure of PS-NH₂, we observed a significant increase in the levels of reactive oxygen species (ROS) (Figure 3B) and superoxide dismutase (SOD) (Figure 3C), a part of the cell’s antioxidant defense system. The induction of oxidative stress was consistent with the proteomics data. Glutathione (GSH) plays a critical role in protection from multiple ROS species in cyanobacteria. The level of reduced GSH decreased significantly compared with the untreated control (Figure 3D), which was due to the oxidization of a large amount of GSH to glutathione disulfide (GSSG) in the cells. During oxidation, large amounts of ROS (H₂O₂) were eliminated to reduce oxidative stress, alkyl and lipid hydroperoxides.

The reduction of carbohydrate metabolism was consistent with decreased growth after treatment with PS-NH₂ (Figure S7C). Furthermore, the downregulation of lipopolysaccharide biosynthetic process-related proteins (Table S1), involved in
synthesizing the integral components of the membrane, indicated damage to cell membrane integrity after PS-NH₂ exposure. The exposure to both concentrations of PS-NH₂ led to the upregulation of proteins involved in the biological transport process (Figure S7D; Table S1), such as ABC transporters, which are transmembrane complexes that span both the plasma membrane and the outer membrane of *M. aeruginosa* and actively export substrates, such as macrolide antibiotics, peptides, virulence factors, and cell envelope precursors. For validation of proteomics analysis for cyanobacterial membrane transport, we used green-fluorescent PS-NH₂ to determine whether PS-NH₂ entered and accumulated in *M. aeruginosa*. The behavior of the fluorescently labeled PS-NH₂ was similar to that of PS-NH₂, both in the culture medium and in deionized water (Figure S8). The fluorescently labeled PS-NH₂ penetrated the cell membrane and accumulated inside the cells (Figures 3E; S8). The process of the nanoparticles entering the cell appeared to rupture the...
cell membrane, an effect seen under both acute and long-term exposure to PS-NH₂ which was confirmed by cell membrane permeability experiments (Figure 3F). Such a membrane rupture and transporter protein upregulation could enhance the release of intracellular materials such as MC.

**Top Hub Proteins Related to Promote MC Synthesis and Release.** MC is synthesized by the thioredoxin function of a large, modular enzyme complex encoded within the 55 kb MC synthetase (mcy) gene cluster. As shown in Table S2, both McyB and McyG proteins were upregulated under different PS-NH₂ concentrations for short- and long-term exposure. Especially, McyG protein was significantly upregulated under high PS-NH₂ concentration for long-term exposure. In order to gain a better understanding of the proteomics and functional foundation of tolerance to environmental stressors, we built coexpression modules from our proteomic data using weighted gene correlation network analysis (WGCNA). We identified 18 coexpression modules from the data regarding the 2413 proteins from the 18 samples (Figure 4A,B) and effectively identified three groups of hub proteins (Figures 4C–E; S9), which were placed in the middle of PPI networks (Figure 4F). The top hub proteins marked with yellow in the brown module, with a negative correlation to the synthesis of intracellular MC, are tryptophan synthase C (involved in organic substance metabolic pathways). The decrease in the synthesis of organic substances caused a disadvantage for growth of *M. aeruginosa*. For protecting the cell from ROS-induced damage and enhancing the fitness of bloom populations under PS-NH₂ exposure, quotas of MC synthesis would increase. The top hub proteins in the turquoise module (PyrG) and blue module (ApcB1 and N44_03141) are positively and negatively correlated with extracellular MC, respectively (Table S3). The proteins of PyrG and ApcB1 are related to the regulation of phospholipid synthesis and thylakoid membrane by module GO enrichment, respectively. Additionally, N44_03141 is an alkaline phosphatase-like protein that is associated with the integral components of the membrane. The upregulation of PyrG protein may be a defense response against cell membrane damage. Down-regulation of apcB1 and N44_03141 was in accordance with the self-protection of thylakoid membrane and damage of cell membrane integrity, respectively. Hence, the increased synthesis of MC was a defense response to protect cells from oxidative damage and enhance the fitness of *M. aeruginosa* to the stresses caused by nanoplastics, which was observed under exposure to antibiotic, iron-limiting conditions and herbicide. The damage to membrane integrity and the upregulation of biological transport proteins were the main explanation for the stimulated release of MC.

**Environmental Significance.** Although previously reported nanoplastics could induce surface reconstruction of cell membrane, less is known about the potential threats of nanoplastics to cyanobacterial blooms underlying. The extent to which the environment is contaminated with nanoplastics remains to be quantified, given the technical challenge of detecting such small and carbon-based particles in complex natural environment. However, in the controlled laboratory, 0.3% (w/w) of a polymeric latex film formed nanoparticles with an average diameter of 196.52 nm (±89.48) after a 200 day exposure to the freshwater environment. Based on the limited reports on microplastics abundance in Three Gorges Reservoir (1597–12,611 items/m³) and midstream of the Los Angeles River (12,000 items/m³), the concentration of nanoplastics in freshwater systems might be in the level of μg/mL, not to mention the meso- or macro-plastics.

In this study, the exposure to nanoplastics in the level of μg/mL concentration promoted MC synthesis and release from *M. aeruginosa*, even without the change of coloration. In detail, PS-NH₂ may enhance the synthesis of MC by inhibiting photosystem II efficiency, reducing organic substance synthesis, and inducing oxidative stress. Furthermore, PS-NH₂ promotes the extracellular release of MC from *M. aeruginosa* via transporter protein upregulation and impaired cell membrane integrity. Cyanobacterial blooms have negative consequences for both human health and aquatic ecology. Cyanobacteria form the base of many food chains; furthermore, the accumulation of nanoplastics in cyanobacteria might have effects on other trophic levels, which could pose a potential risk to food safety.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b06085.

Structure and morphological characteristics of PS nanoparticles, effects of nanoplastics on *M. aeruginosa*, synthesis and release of MC-LR under exposure of PS-NH₂, synthesis and release of MC and MC-LR under exposure of PS-SO₃H, concentration of total MC in medium, heat maps of the DEPs, clustering results of GO enrichment biological processes, size and location of polystyrene nanoplastics, coexpression network analysis of the magenta and green modules, clustering results of GO enrichment biological processes, identified results of synthesis proteins of MCs, and changes of traits and the top hub proteins in key modules (PDF)

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Notes
The authors declare no competing financial interest.

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