Environmental Chemistry

Glyphosate Accelerates the Proliferation of *Microcystis aeruginosa*, a Dominant Species in Cyanobacterial Blooms

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Abstract: Glyphosate is a commonly used herbicide known for its high performance in killing certain plants and grasses; however, its use is regulated due to its harmful effects on the aquatic environment. The present study investigated and compared the toxic mechanisms of glyphosate on *Microcystis aeruginosa* (a toxin-producing cyanobacterium) under 2 conditions: 0‰ saline media (experiment I) and 2.5‰ saline media (experiment II). The results indicated that an appropriate concentration of glyphosate provided a phosphate source for *M. aeruginosa*, resulting in an increased specific growth rate in both experimental groups compared with the controls. Glyphosate-enhanced alkaline phosphatase (ALP) activity increased by up to 1.37-fold in experiment I and 1.68-fold in experiment II. Moreover, the activities of superoxide dismutase (SOD) and catalase (CAT) decreased at glyphosate concentrations below 1.2 mg L⁻¹ but increased at concentrations greater than 1.2 mg L⁻¹ in experiment I, whereas SOD and CAT activities decreased in experiment II and declined by 64 and 49% in the 30 mg L⁻¹ treatments. Furthermore, the transcript abundances of the pyruvate carboxylase (*pcB*), microcystin synthetase B (*mcyB*), and paired-like homeobox (*phoX*) genes were up-regulated by up to 6.92-, 3.63-, and 2.27-fold in experiment I and 6.74-, 6.55-, and 4.86-fold in experiment II after 96 h of incubation. The addition of glyphosate stimulated the production of dissolved organic matter including tryptophan-like substances, fulvic acid-like substances, (marine) humic acid-like substances, and microcystin-leucine-arginine in the culture. In conclusion, glyphosate stimulates the proliferation of *M. aeruginosa* and enhances the release of dissolved organic matter in saltwater ecosystems. *Environ Toxicol Chem* 2021;40:342–351. © 2020 SETAC

Keywords: *Microcystis aeruginosa*; Microcystin; Glyphosate; Dissolved organic matter

INTRODUCTION

Glyphosate is an effective herbicide typically used for broadleaf weed and grass control, and its use has rapidly increased since the beginning of its commercialization in 1974 (Van Bruggen et al. 2018). Glyphosate leaches into surface water through soil erosion and agricultural runoff, and a growing number of studies have linked the presence of small concentrations (i.e., micrograms or milligrams) of glyphosate with glyphosate pollution worldwide (Van Bruggen et al. 2018; de Freitas-Silva et al. 2020). Broad contamination by glyphosate has led to harmful effects on microbial compositions and potential indirect effects on plant, animal, and human health (Van Bruggen et al. 2018; Gillezeau et al. 2019). Finally, glyphosate has also been applied in waterways to eliminate invading aquatic plants, which has a direct impact on the surrounding environment (Clements et al. 2017).

Microalgae and cyanobacteria are common bioindicators of pollution. Glyphosate (a nonselective herbicide) can potentially inhibit microalgae and cyanobacteria growth in aquatic ecosystems. Dabney and Patiño (2018) reported that glyphosate and glyphosate-based herbicides influenced the growth of the toxic golden alga *Prymnesium parvum*. Moreover, this toxicity was reported to be concentration and species dependent, but could be modulated by other environmental factors, including air temperature and relative humidity (Sharma and Singh 2001; Carpenter et al. 2020). Glyphosate targets the plant-specific enzyme 5-enolpyruvylshikimate-3-phosphate synthase, thereby inhibiting the growth of the target plants or weeds (Fang et al. 2018). Exposure to this compound led to a 4-fold decrease in the median effect concentration in the macrophyte *Lemna minor* and the green alga *Pseudokirchneriella subcapitata* (Cedergreen and Streibig 2005). Many studies have reported that glyphosate increases or inhibits the growth rates of microalgae and cyanobacteria, such as *Scenedesmus vacuolatus* (Iumamoto et al. 2019), *Microcystis aeruginosa* (Wu et al. 2014), and *Chlorella kessleri* (Ostera et al. 2020). The toxicity of glyphosate to algae is generally

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concentration dependent. Only a few herbicides are toxic to algae at concentrations below $1 \mu\text{g L}^{-1}$ (Cedergreen and Streibig 2005). The US aquatic life benchmark for acute toxicity in nonvascular plants is below 1.2 mg L^{-1} . As primary producers, cyanobacteria or algae are more sensitive than other microorganisms, and therefore possess specialized response mechanisms to environmental pollution. Herbicide contaminants and their residues (e.g., glyphosate) are known to consistently and predictably influence the proliferation of cyanobacteria and aggravate water quality deterioration (Zhang et al. 2016; Smedbol et al. 2017). Although the concentrations of glyphosate assessed in the aforementioned studies were much higher than those in natural environments, these findings still demonstrate the potential impacts of glyphosate on aquatic organisms (Zhang 2010; Zhang et al. 2018).

Glyphosate-based herbicides typically contain carbon (C), nitrogen (N), and phosphorus (P). The P content of glyphosate is approximately 18% in pure glyphosate ($169.014 \text{ g mol}^{-1}$) and 9% in glyphosate isopropylamine salt (346.4 g mol^{-1} ; e.g., Roundup, $228.18 \text{ g mol}^{-1}$). In 2014, the agricultural use alone was 825 800 tons of glyphosates (Statista 2016) worldwide. The degradation of glyphosate results in the production of dissolved organic phosphorus (DOP). In turn, DOP may accelerate the growth of phytoplankton, which can utilize DOP compounds as a nutrition source with the aid of alkaline phosphatase (ALP; Huang et al. 2005). Glyphosate was found to enhance the ALP activity of *Anabaena doliolum* by providing additional P (Shikha et al. 2004). Moreover, the algae *Anabaena variabilis* (CCALA007), *Chroococcus minutus* (CCALA055), *Fischerella cf. maior* (CCALA067), and *Nostoc cf. muscorum* (CCALA129) were able to utilize glyphosate as a source of P in vitro (Drzyzga and Lipok 2018). The cyanobacterium *Synechococcus* 7942 can utilize glyphosate as a P source, thus reducing its toxicity in environments with low P availability (Lu et al. 2020). Therefore, glyphosate may act as an important source of organic phosphorus to support cyanobacterial growth.

Microcystis is the most dominant cyanobacterial genus in eutrophic water. It produces microcystins, which can harm zooplankton, fishes, and the liver of higher order animals (Mohamed et al. 2020). An understanding of the response of *M. aeruginosa* to glyphosate would help us to recognize the effects of glyphosate on toxin-producing cyanobacteria and the environmental activity of glyphosate in eutrophic environments. Considering the moderate persistence of glyphosate in marine water (Mercurio et al. 2014), the aims of the present study were: 1) to identify the effect of salinity and glyphosate exposure on *M. aeruginosa* growth, and 2) to characterize the extracellular substances produced by *M. aeruginosa* in response to glyphosate and salt.

MATERIALS AND METHODS

Experimental design

We used an *M. aeruginosa* strain that was isolated from a local pond in Yantai, China. Microcystin–leucine–arginine

(MC–LR) is the main toxin produced by *M. aeruginosa*. The strain was cultured in 500 mL of sterile modified BG-11 medium (pH 7.0; Drzyzga and Lipok 2018). The medium contained 17.6 mM NaNO_3 , 0.23 mM K_2HPO_4 , 0.3 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.24 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.031 mM citric acid- H_2O , 0.021 mM ferric ammonium citrate, 0.0027 mM $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, and 0.19 mM of trace metals (2.860 g H_3BO_3 , 1.810 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.222 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.390 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.079 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 0.0494 g $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) in 1 L of deionized water. Salinity was determined using a portable salinometer (HI98203; Hanna). The salinities we tested were selected based on the salinities typically measured in coastal rivers with cyanobacterial blooms (0 and 2.5‰) in Yantai that were suspected to be contaminated with herbicides due to surface runoff from farmlands. Two concentrations of sea salt (0 and 2.5‰; Sigma-Aldrich) were used to simulate a saline environment in experimental groups I and II, respectively.

The glyphosate (N-(phosphonomethyl)glycine; purity 99.5%; Aladdin Chemistry) was first filtered through a $0.22\text{-}\mu\text{m}$ filter (GVWP04700; Millipore). Following this, the glyphosate was added to the experimental flasks to complement K_2HPO_4 as a P nutrition source. Cultures without glyphosate were set as control-I and control-II, respectively. The glyphosate concentrations were selected based on the environmental concentrations in agricultural water streams and the biological toxicity of glyphosate. Nine concentrations of glyphosate (5 [L5], 10 [L10], 20 [L20], 0.5 [M0.5], 0.8 mg [M0.8], 1.2 [M1.2], 20 [H20], 25 [H25], and 30 mg L^{-1} [H30]) were tested for each experimental group. Glyphosate concentrations were analyzed using a Waters ACQUITY I-Class ultra-performance liquid chromatography (UPLC) device with an AB Sciex 5500 triple quadrupole mass spectrometer. The methods described by Gao et al. (2015) and the area normalization method were used to determine and calculate the glyphosate concentrations at the end of the experiments.

Prior to the experiment, *Microcystis* cells were cultured in modified media with P deficiency. Algal cells in the exponential growth phase were collected via centrifugation (5000 g, 15 min). *Microcystis* cells were washed 3 times with 0% saline media without glyphosate for experiment I. The remaining *Microcystis* cells were resuspended 3 times with 2.5‰ saline media without glyphosate for experiment II. At the beginning of the experiment, the concentration of *Microcystis* cells was set at $1 \times 10^6 \text{ cells mL}^{-1}$ in the 2 experimental groups. All culture flasks were shaken by hand 3 times/d, and the positions of the flasks within the incubator ($35 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at $28 \pm 1^\circ \text{C}$) were randomly changed to minimize light-intensity effects.

Specific growth rate and pigment analyses

The cultured samples were fixed with glutaraldehyde (1% final concentration). *Microcystis* cell counts were performed with 0.1-mL aliquots in a hemocytometer under a light microscope (Olympus CX 23). At least 400 cells were counted for each sample. For chlorophyll a (Chl-a) determination, the

10-mL samples were centrifuged at 5000 g for 15 min, and the precipitate was extracted with 90% acetone in dark conditions at 4 °C for 24 h, followed by centrifugation at 5000 g for 15 min. The supernatant, to which a droplet of 1 M HCl was added, was used for Chl-a determination at 663 and 645 nm on a UV2800 spectrophotometer (Unico). The Chl-a values were calculated according to Equation 1. A 90% acetone solution was used as a blank control for determination.

$$\text{Chl a} = 0.0127\text{OD}_{663} - 0.00269\text{OD}_{645} \quad (1)$$

Phycocyanin was extracted from *Microcystis* cells and estimated according Prabhuthas et al. (2011). The phycocyanin concentration (mg mL^{-1}) was determined as follows:

$$\text{Phycocyanin concentration} = \frac{(\text{OD}_{615} - 0.474\text{OD}_{652})}{5.34} \quad (2)$$

Measurement of ALP and microcystins

A total of 50-mL *Microcystis* cultures of each sample were filtered through a 0.45- μm membrane (Whatman), and the filtrate was used for ALP and microcystin determination. Extracellular ALP was routinely measured through colorimetric methods via hydrolysis of *p*-nitrophenylphosphate disodium hexahydrate (Sigma) to *p*-nitrophenol at 410 nm (Xu et al. 2018). To measure ALP, 1 mL of intracellular extract was mixed with 8.4 mL of Tris-HCl, after which *p*-nitrophenylphosphate disodium hexahydrate (2 mL, 2.4 mM) was added to the mixture. The mixture was incubated at 37 °C for 2 h, and the reaction was terminated by the addition of NaOH (300 μL , 4 M). The samples were then measured with a spectrophotometer (U1901; Beijing Purkinje General Instrument) using a modified procedure (Shen and Song 2007). Control-I and control-II were used as blanks, and *p*-nitrophenol was used to generate a standard curve. The ALP activity was reported as micrograms of *p*-nitrophenol/mg of Chl-a/h ($\mu\text{g Chl-a h}^{-1}$).

The samples were collected through a 0.22- μm microfiber membrane (25-mm diameter; Whatman), and the filtrate was diluted 1:5 filtrate:deionized water for extracellular microcystin determination. All samples were directly concentrated on solid-phase extraction (C18) cartridges, which had been washed with methanol (100%) and distilled water. The cartridges were eluted with 10 mL of methanol (100%). This elution was evaporated until dry and dissolved in 100 μL distilled water for subsequent qualitative analysis of MC-LR via liquid chromatography–mass spectrometry with a Symmetry C18 column (50 mm $\times$ 2.1 mm $\times$ 5 μm ; Agilent). The MC-LR standard was purchased from Sigma. Area normalization was used to calculate the concentrations of intracellular and extracellular MC-LR.

RNA extraction and gene expression

During cultivation, 50-mL culture samples were collected for RNA extraction and stored at -80°C . *Microcystis* cells were collected by centrifugation at 3500 g for 15 min at 4 °C, and then washed 3 times with phosphate buffer (pH 7.0). The cells were disrupted with glass beads using a Mini Beadbeater

(Biospec). Total RNA was first extracted with the TRIzol reagent (Invitrogen), and then purified using the RNeasy Mini kit (Qiagen). The RNA concentrations were measured using a spectrophotometer (Nanodrop Technologies), and RNA integrity was evaluated via agarose gel electrophoresis. The total RNA was reverse-transcribed to complementary (c)DNA with the SuperScript™ III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. The cDNAs were then used as templates for further analysis. Quantitative polymerase chain reaction (qPCR) was performed using a SYBR® Green I qPCR kit (Takara) on an ABI 7500 real-time PCR system using the following program: 5 min at 95 °C for denaturation, followed by 25 cycles of 1 min at 95 °C, 30 s at 55 °C, 30 s at 72 °C, and 10 min at 72 °C. The gene transcription levels were calculated as the relative messenger (m)RNA levels normalized against the 16S ribosomal (r)RNA transcript levels, and the values of the *Microcystis* transcription levels in the control-I and control-II groups were set to 1. Four pairs of specific primers were used to quantify the expression of 16S rRNA (forward: 5'-GGA CGG GTG AGT AAC GCG TA-3', reverse: 5'-CCC ATT GCG GAA AAT TCC CC-3'), and the genes pyruvate carboxylase (*pcB*; forward: 5'-GGC TGC TTG TTT ACG CGA CA-3', reverse: 5'-CCA GTA CCA CCA GCA ACT AA-3'), microcystin synthetase B (*mcyB*; forward: 5'-CCT ACC GAG CGC TTG GG-3', reverse: 5'-GAA AAT CCC CAA AGA TTC CTG AGT-3'), and paired-like homeobox (*phoX*; forward: 5'-CTG TGC TGG TGG AAC NAC NCC NTG-3', reverse: 5'-GGG TCG AYT TCC ACC ATC CAN CCR T-3'). All experiments were performed in triplicate and repeated at least twice to accurately obtain their means and standard deviations (SDs).

EEM determination and parallel factor analysis modeling

After 96 h, culture samples (50 mL) were collected from the culture flasks for the analysis of excitation–emission matrix (EEM) of dissolved organic matter (DOM). Low- (L5, L10, and L20), medium- (M0.5, M0.8, and M1.2), and high-concentration (H20, H25, and H30) glyphosate solutions were mixed with the experiment I and experiment II media in triplicate to obtain L-I and L-II, M-I and M-II, and H-I and H-II, respectively. *Microcystis* cultures were filtered through 0.45- μm membrane filters to measure DOM. The filtrate was used for EEM determination. All cuvettes were washed and ultrasonicated with 5% (w/w) HNO_3 (Xu et al. 2013). Fluorescence EEM spectra were detected using a Hitachi F-7000 fluorescence spectrometer (Hitachi High Technologies) with a 700-V xenon lamp at 25 °C. The scanning emission spectra between 250 and 580 nm were recorded in 5-nm increments. The excitation wavelengths ranged from 250 to 550 nm with 3-nm increments. The spectra were recorded at a scan rate of 1200 nm min^{-1} with a 5-nm excitation and emission bandwidth. As a blank, BG-11 medium without any fluorescent substances was used.

Parallel factor analysis (PARAFAC) modeling was performed for all EEM spectra of *M. aeruginosa* extracellular DOM (Xu et al. 2013). The PARAFAC analysis was conducted using

MATLAB Ver 7.0 (MathWorks) with the DOMFluor toolbox (Murphy et al. 2013). A total of 36 samples were selected for PARAFAC modeling and analysis. The EEM spectra were corrected via scattering removal and data arrangement. After determination and validation by residual analysis, split-half analysis, and visual inspection, 3 components of the PARAFAC models were conducted to analyze the data.

Determination of SOD and catalase

For the determination of SOD and catalase (CAT) activity, samples were collected in 50-mL tubes, and then washed with 20 mL phosphate buffer (pH 7.0). The intracellular components of cyanobacterial cells were extracted using a regulated method (Iumamoto et al. 2019). Briefly, the cells were then disrupted on ice for 10 min at 300 W using an ultrasonic cell pulveriser (1200-98; BioSafer) and then centrifuged at 5000 g for 15 min at 4 °C. The supernatant was then transferred into a new tube and stored at –80 °C until further use.

The SOD and CAT activities were measured using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Total SOD and CAT activity were respectively measured via the xanthine oxidase and ammonium molybdate spectrophotometric methods using a microplate reader (SpectraMax M2/M2e; Molecular Devices).

Statistical analysis

All data were analyzed using OriginPro Ver 9.1 and SPSS for Windows Ver 22, and were reported as the mean $\pm$ SD. Statistical analysis was conducted using one-way analysis of variance, and Student's *t* test was used to determine the differences between the control and glyphosate treatment groups. Then *p* values < 0.05 were set as statistically significant.

RESULTS

Glyphosate concentrations

During treatment, glyphosate concentrations decreased with *M. aeruginosa* incubation time. However, some glyphosate was still detectable at the end of the experiments. Only small levels of glyphosate were detected in the L5, L10, and L20 treatments in experiment I and experiment II (Figure 1). Glyphosate concentrations declined by 24, 22.7, 20.7, 11.3, 9.5, and 8.57% in the M0.5, M0.8, M1.2, H20, H25, and H30 treatments in experiment I, respectively. Moreover, the glyphosate concentrations decreased by 43.4, 27.5, 23, 18.7, 15.6, and 15.7% in the M0.5, M0.8, M1.2, H20, H25, and H30 treatments in experiment II, respectively.

Glyphosate-induced physiological variations in Microcystis

Glyphosate enhanced the specific growth rates, phycocyanin, and Chl-*a* of *M. aeruginosa* in experimental groups I

FIGURE 1: Glyphosate concentrations consumed in the different treatments after 96 h of incubation. The numbers after L (low), M (medium), and H (high) show the glyphosate concentrations in $\mu\text{g L}^{-1}$.

and II (Figure 2). However, the specific growth of *M. aeruginosa* decreased in the M1.2, H20, H25, and H30 treatments of experiment I. The lowest growth rate of *M. aeruginosa* was observed in the H30 treatment of the experiment I group, and it significantly decreased after 96 h of glyphosate incubation (Figure 2A). Phycocyanin and Chl-*a* exhibited a slight change under glyphosate treatment compared with the control (Figure 2B and C). In the experiment II groups, the specific growth rate of *M. aeruginosa* increased with glyphosate concentration (Figure 2D). The growth rate of *M. aeruginosa* in the M0.8, M1.2, H20, H25, and H30 treatments in the experiment II group increased compared with those in control-II at 96 h. Similarly, remarkable changes in Chl-*a* and phycocyanin were observed in the glyphosate-treated cells compared with control-II (Figure 2E and F). Specifically, the Chl-*a* concentration increased from 1.14 ± 0.02 to $1.62 \pm 0.03 \mu\text{g mL}^{-1}$, and phycocyanin concentrations increased from 0.55 ± 0.06 to $1.45 \pm 0.05 \mu\text{g mL}^{-1}$.

ALP activities and microcystin production of *M. aeruginosa*

The ALP activity of *M. aeruginosa* showed no significant differences in the same glyphosate treatments between experimental groups I and II (Figure 3). In experiments I and II, almost no significant change in ALP levels occurred in the L5, L10, L20, M0.5, M0.8, and M1.2 treatment groups compared with control-I after 48 h of incubation. However, a very clear increase in ALP was observed in the H20, H25, and H30 treatments after 48 h of cultivation. In the 2 experimental groups, ALP increased abruptly for all concentrations of glyphosate after 96 h of incubation. Only a slight decrease in ALP activity was recorded in the H30 treatment of the experimental I group after 96 h of incubation.

Extracellular MC-LR contents of *M. aeruginosa* were enhanced by glyphosate in groups I and II (Figure 4). Levels of MC-LR increased with glyphosate, and the cultivation time

FIGURE 2: Effects of glyphosate on the physiology of *Microcystis aeruginosa*. Specific growth rate (A), chlorophyll a [Chl-a] (B), and phycocyanin (C) of *M. aeruginosa* in the experiment I group. Specific growth rate (D), Chl-a (E), and phycocyanin (F) of *M. aeruginosa* in the experiment II group. The numbers after L (low), M (medium), and H (high) show the glyphosate concentrations in $\mu g L^{-1}$.

increased. At 96 h, extracellular MC-LR had no remarkable increase in the L5, L10, L20, M0.5, M0.8, and M1.2 treatments, but increased notably in the H20, H25, and H30 treatments compared with control-I ($F = 16.718$, $p = 0.0482$; $F = 13.718$, $p = 0.0326$; $F = 9.584$, $p = 0.0014$, respectively). The extracellular MC-LR increased at 96 h compared with at 48 h for intracellular MC-LR. In the experiment II group, extracellular MC-LR was enhanced in all groups relative to the control after 48 h of incubation. At 96 h, extracellular MC-LR reached

$0.16 \mu g L^{-1}$ and showed an increase in the H20, H25, and H30 treatments compared with the control ($F = 15.844$, $p = 0.0282$; $F = 14.813$, $p = 0.0438$; $F = 8.942$, $p = 0.0224$, respectively).

Gene transcript expression in *M. aeruginosa*

Three genes (*pcB*, *mcvB*, and *phoX*) were up-regulated after 96 h of cultivation with glyphosate addition. In the experiment I group, the *pcB*, *mcvB*, and *phoX* genes were up-regulated in response to glyphosate exposure. In the M1.2 treatment, the expressions of *pcB*, *mcvB*, and *phoX* were significantly different from the control ($F = 8.418$, $p = 0.0034$; $F = 2.814$, $p = 0.0022$; $F = 10.647$, $p = 0.0031$, respectively; Figure 5A). In the experiment II group, transcript levels of *pcB*, *mcvB*, and *phoX* were significantly enhanced by glyphosate (Figure 5B). The *pcB* and *phoX* genes were remarkably up-regulated in the H25 treatment compared with those in control-II ($F = 4.124$, $p = 0.0034$; $F = 8.584$, $p = 0.0022$, respectively). Enhanced expression of *mcvB* was also observed in the H30 treatment compared with that in control-II ($F = 12.982$, $p = 0.0046$).

EEM spectra of DOM

The PARAFAC method was used to analyze the fluorescence EEM spectra of DOM in cultures. Three fluorescence peaks were observed in all treatments (Figure 6A). Peak A (275/332 nm) remained stable in the L-I, M-I, and H-I treatments of the experiment I group; the area of the green peak B (323/397 nm)

FIGURE 3: Mean ($\pm$ standard deviation) alkaline phosphatase (ALP) activity under glyphosate exposure. All treatments were performed in triplicate ($n = 3$). The numbers after L (low), M (medium), and H (high) show the glyphosate concentrations in $\mu g L^{-1}$. CK = control.

FIGURE 4: Effects of glyphosate on extracellular microcystin–leucine–arginine (MC–LR) production. The bars indicate standard deviations ($n=3$). Extracellular MC–LR in the experiment I group (A) and experiment II group (B). a, b, and c represent significant differences at $p<0.05$, $p<0.01$, $p<0.001$, respectively, between the experimental and control conditions after 48 and 96 h of exposure. The numbers after L (low), M (medium), and H (high) show the glyphosate concentrations in $\mu\text{g L}^{-1}$. CK = control.

in the glyphosate treatments was wider than that in control-I; peak C (257/457 nm) was slightly green in control-I but changed to green and light yellow in the M-I treatments and was clearly yellow in the H-I treatments. In the experiment II group, the color of peak A changed from yellow in control-I to red in the M-II and H-II treatments; peak B in control-II was light yellow and changed to dark yellow in the H-II treatment; the green color of peak C in the control-II became yellow in M-II and dark yellow in the H-II treatment.

The split-half analysis showed similar curves for each pair of halves (Figure 6B). Figure 6C illustrates the excitation/emission loadings of the 3 components identified by the DOMFluor–PARAFAC analysis. The spectral characteristics were identified by PARAFAC analysis (Table 1). Component 1 (C1) had a single excitation/emission peak at 275/332 nm, which is related to tryptophan-like substances. Component 2 (C2) was characterized by 2 peaks at excitation/emission of

FIGURE 5: Mean transcript expression levels ($\pm$ standard deviation; $n=3$) of *pcB*, *mcyB*, and *phoX* in *Microcystis aeruginosa* after 96 h of exposure. * and ** indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between the experimental and control groups after 96 h of exposure. The numbers after L (low), M (medium), and H (high) show the glyphosate concentrations in $\mu\text{g L}^{-1}$. CK = control.

(257, 362)/457 nm, related to fulvic acid-like substances and humic acid-like substances. Component 3 (C3), with a similar peak to that of C2, had 2 peaks at (251, 323)/397 nm, which were categorized as humic acid-like substances and marine humic acid substances.

SOD and CAT activities of *M. aeruginosa*

The antioxidant enzymes SOD and CAT are important protective mechanisms against reactive oxygen species (ROS) in antioxidant systems, and therefore their activities were examined to determine the toxic effects of glyphosate on the antioxidant response of *M. aeruginosa*. The SOD and CAT activities in the L5, L10, L20, M0.5, and M0.8 treatments were lower than those in control-I, whereas SOD activity was enhanced in the H20, H25, and H30 treatments compared with control-I (Figure 7A). Catalase activity was prominently up-regulated in freshwater groups with the H25 and H30 treatments. All glyphosate doses in the

FIGURE 6: Dissolved organic matter (DOM) Fluor-parallel factor analysis analysis of *Microcystis aeruginosa* culture. (A) Fluorescence excitation–emission matrix (EEM) spectra of DOM in the cultures after 96 h of incubation. (B) Split half analysis. (C) EEM excitation (dotted lines) and emission (solid lines) loading of 3 components. L, M, and H I and II represent low, medium, and high treatments in groups I and II. C1 = component 1; C2 = component 2; C3 = component 3; Em = scanning emission; Ex = excitation wavelengths.

experiment II group decreased the activities of SOD and CAT in *M. aeruginosa* compared with control-II (Figure 7B). The SOD activity declined significantly in the H20, H25, and H30 treatments of the experiment II group compared with control-II ($F = 14.135$, $p = 0.0210$; $F = 16.522$, $p = 0.0348$; $F = 8.416$, $p = 0.0085$, respectively). Catalase activity decreased remarkably, with p values of 0.0400, 0.0246, and 0.0332 in the H20, H25, and H30 treatments of the experiment II group compared with control-II, respectively.

DISCUSSION

Glyphosate water pollution can affect the balance of aquatic ecosystems, especially in eutrophic waters. In the experiment I group, the growth rate of *M. aeruginosa* increased when glyphosate concentrations were below or equal to 0.8 mg L^{-1}

but was inhibited at 1.2 mg L^{-1} or above. In contrast, glyphosate consistently enhanced the growth rate and Chl-*a* of *M. aeruginosa* in the experiment II group compared with control-II. The critical genes *pcB*, *mcyB*, and *phoX* in *M. aeruginosa* were also affected, thereby altering phycocyanin and microcystin production, as well as the activity of ALP. These results indicated that glyphosate could serve as an alternative P source and could increase the proliferation of *M. aeruginosa* in P-deficient environments regardless of experimental conditions (i.e., groups I or II).

Glyphosate utilization by *M. aeruginosa*

Dabney and Patiño (2018) reported that glyphosate can be used as a nutrient source for cyanobacterial growth or as organic carbon, providing energy for algal cell division. The ALP in membranes can directly transfer dissolved organic P to phosphate (Huang et al. 2005). Glyphosate enhances the activity of ALP of the cyanobacterium *A. doliolum* (Shikha et al. 2004). In the present study, the activities of ALP and its related gene *phoX* were up-regulated with glyphosate exposure, confirming that glyphosate was decomposed to produce phosphate as a nutrient. The bioavailable DOP (β -glycerol phosphate and d-glucose-6-phosphate) was significantly stimulated by phytoplankton to support algal cell density and the specific growth rates of algae (Mackay et al. 2020).

TABLE 1: Spectral characteristics of the 3 component

Component	Excitation _{max} (nm)/ emission _{max} (nm)	Description
C1	275/332	Tryptophan substances
C2	(257, 362)/457	Fulvic acid-like substances, Humic acid-like substances
C3	(251, 323)/397	(Marine) humic acid-like substances

FIGURE 7: Mean (± standard deviation, $n=3$) superoxide dismutase (SOD) and catalase (CAT) activities of *Microcystis aeruginosa* after 96 h in the experiment I group (A) and experiment II group (B). At 96 h, significant differences in Student's t test values between the glyphosate experiment and control were found (*, $p < 0.05$; **, $p < 0.01$). The numbers after L (low), M (medium), and H (high) show the glyphosate concentrations in $\mu\text{g L}^{-1}$. CK = control.

Therefore, glyphosate could provide P sources for *M. aeruginosa* supersession, supporting particle stability and its transport in cyanobacteria.

Effects of glyphosate on the physiology of *M. aeruginosa*

Previous studies have demonstrated that appropriate concentrations of glyphosate could enhance the growth of some algal species, such as *Scenedesmus quadricauda* at $0.0037 \text{ mg PL}^{-1}$ (Wong 2000), *M. aeruginosa* PCC7806 at 1 mg PL^{-1} , and *Nostoc punctiforme* at 3.1 mg PL^{-1} (Forlani et al. 2008; Qiu et al. 2013). Therefore, threshold concentrations may vary depending on the glyphosate tolerance of different *Microcystis* strains. Characterizing the effects of various glyphosate concentrations on cyanobacteria is important to understand the impact of glyphosate on coastal areas, high-salinity lakes, or reservoirs. Our results indicated that the growth rate of *M. aeruginosa* was enhanced by glyphosate levels below 0.8 mg L^{-1} , whereas glyphosate concentrations

greater than 1.2 mg L^{-1} inhibited its growth rate in the experiment I group. This may occur because low glyphosate concentrations ($0.1\text{--}2 \text{ mg L}^{-1}$) provide nutrients for *M. aeruginosa* FACHB 905 (Zhang et al. 2016), whereas the toxicity of high glyphosate concentrations greatly outweighs the growth-promoting effects of low concentrations (Qiu et al. 2013). In the experiment II group, the 0 to 30 mg L^{-1} glyphosate concentrations increased the specific growth rate of *M. aeruginosa* compared with control-II, and this may result from a synergy between glyphosate (potential P source) and salt supply. Organic P may ameliorate salinity stress (Ding et al. 2020). In our study, although there were no clear salinity changes in the experiment I cultures, the organic P present in the cultures may have decreased the oxidation pressure from salt. Therefore, glyphosate addition in salty waters at appropriate concentrations stimulates the proliferation of *M. aeruginosa*, thereby increasing the ecological risks associated with this cyanobacterial species in saline water.

Glyphosate indirectly affects photosynthetic electron transport by inhibiting sink processes (Olesen and Cedergreen 2010). The addition of appropriate amounts of glyphosate stimulated the production of Chl-a of *A. doliolum* with no inorganic P addition (Shikha et al. 2004). In our study, Chl-a and phycocyanin were enhanced by appropriate glyphosate concentrations. However, in experiment I, high concentrations of glyphosate suppressed the synthesis of Chl-a and phycocyanin. Qiu et al. (2013) reported that the photosynthesis process in cyanobacteria can be recovered by cultivating them in glyphosate media, although the effects of glyphosate on photosynthesis may be temporary or may render different results depending on culture time. In summary, the variations in Chl-a and phycocyanin were consistent with the growth rate of *M. aeruginosa*, and the pigment production and growth rate were closely related to glyphosate concentration. Therefore, we concluded that the different concentrations of glyphosate we examined influenced the specific growth rate and pigment production of *M. aeruginosa*.

Changes in extracellular substances in culture

The distributions of DOM in cultures were influenced by the addition of glyphosate. The colors of the components (tryptophan-like substances, fulvic acid-like substances, and [marine] humic acid-like substances) were enhanced in both experiment I and experiment II. *Microcystis aeruginosa* can secrete tryptophan-like substances and humic acid-like substances (Ziegmann et al. 2010). Humic acid can bind to glyphosate via hydrogen bonds and have a neutralizing effect on glyphosate (Piccolo et al. 1996). The green color of the excitation/emission peak at (257/362)/457 nm and the yellow color of the excitation/emission peak of (251/323)/397 nm were identified as fulvic acid-like substances and humic acid-like substances in experiment I, indicating that the fulvic acid and humic acids produced by *M. aeruginosa* respond to glyphosate toxicity. The 3 DOM components (tryptophan-like substances, fulvic acid-like substances, and [marine] humic acid-like

substances) were enhanced in the saline media groups. Xu et al. (2013) demonstrated that tryptophan-like substances were positively correlated with *Microcystis* growth, and the production of tryptophan-like substances may enhance the survival of *M. aeruginosa* from glyphosate toxicity and promote its growth. Humic acids and fulvic acids are usually applied to reduce salt stress in saline soils (Suddarth et al. 2019). Therefore, the enhancement of humic acids, fulvic acids, and tryptophan-like substances could enable *M. aeruginosa* to tolerate environmental stress and accelerate its proliferation.

The rapid proliferation of *M. aeruginosa* and the hepatotoxic microcystin produced by several cyanobacterial genera may severely affect water quality and have adverse effects on aquatic biota and human health (Scoglio 2018; Deng et al. 2020). In the present study, the transcription of the gene encoding for microcystin (*mcyB*) was up-regulated, and the concentration of extracellular MC-LR increased from 48 to 96 h. These extracellular microcystins accumulated with glyphosate addition. A certain amount of salt could further stimulate the production of microcystins/cell (Tonk et al. 2007). Therefore, the expression levels of *mcyB* and microcystins in the experiment II group were higher than those in the experiment I group. The combined action of glyphosate and salt could enhance microcystin production, increasing the potential risk of toxicity in aquatic ecosystems.

Antioxidant response of *M. aeruginosa* to glyphosate

The herbicide glyphosate may have toxic effects on aquatic systems. In the experiment I group, the activities of SOD and CAT decreased in the L5, L10, L20, M0.5, and M0.8 treatments but were promoted in the M1.2, H20, H25, and H30 treatment groups compared with those in control-I, indicating that high concentration of glyphosate could induce oxidative stress and cause an antioxidant response in *M. aeruginosa*. The SOD and CAT levels were significantly decreased in green algae *S. vacuolatus* cells at glyphosate concentrations below 8 mg L⁻¹ (Iummato et al. 2019), which was consistent with the decreased SOD and CAT of *M. aeruginosa* in the L5, L10, L20, M0.5, and M0.8 treatments. The decreased activities of SOD and CAT in the M1.2, H20, H25, and H30 treatments may have been caused by the high glyphosate concentrations and its associated toxicity. Interestingly, the ROS levels of algae were enhanced by high salinity and glyphosate exposure. A similar phenomenon was reported by Iummato et al. (2019), indicating that the decreased activities of SOD and CAT in glyphosate-treated *M. aeruginosa* could be explained by the enhancement of ROS.

CONCLUSIONS

In the P-limited environments, glyphosate can provide a P source for *M. aeruginosa* to enhance its growth. However, due to the toxicity of glyphosate to algae, a high glyphosate concentration inhibited the growth of *M. aeruginosa*. The low concentration (μg L⁻¹) of glyphosate in our study was the

typical concentration in the aquatic environment, and the results indicated that glyphosate at the μg L⁻¹ levels enhanced the proliferation of *M. aeruginosa* and increased microcystin synthesis and DOM as a protective response against glyphosate toxicity. Thus, more consideration should be given to glyphosate pollution as a potential cause of environmental deterioration. Moreover, the tolerances of different phytoplankton to glyphosate were varied, and further investigations of phytoplankton responses to glyphosate are required to comprehensively understand glyphosate pollution in the ecological environment.

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REFERENCES

- Carpenter DJ, Mathiassen SK, Boutin C, Strandberg B, Casey CS, Damgaard C. 2020. Effects of herbicides on flowering. *Environ Toxicol Chem* 39:1244–1256.
- Cedergreen N, Streibig JC. 2005. The toxicity of herbicides to non-target aquatic plants and algae: Assessment of predictive factors and hazard. *Pest Manag Sci* 61:1152–1160.
- Clements D, Dugdale TM, Butler KL, Florentine SK, Sillitoe J. 2017. Herbicide efficacy for aquatic *Alternanthera philoxeroides* management in an early stage of invasion: Integrating above-ground biomass, below-ground biomass and viable stem fragmentation. *Weed Res* 57:257–266.
- Dabney BL, Patiño R. 2018. Low-dose stimulation of growth of the harmful alga, *Prymnesium parvum*, by glyphosate and glyphosate-based herbicides. *Harmful Algae* 80:130–139.
- de Freitas-Silva L, de Araújo TO, Nunes-Nesi A, Ribeiro C, Costa AC, da Silva LC. 2020. Evaluation of morphological and metabolic responses to glyphosate exposure in two neotropical plant species. *Ecol Indic* 113:106246.
- Deng XW, Chen J, Hansson LA, Zhao X, Xie P. 2020. Eco-chemical mechanisms govern phytoplankton emissions of dimethylsulfide in global surface waters. *Natl Sci Rev*. <https://doi.org/10.1093/nsr/nwaa140>
- Ding Z, Khair AMS, Ali MGM, Ali OAM, Abdelaal AIN, Lin X, Zhou ZX, Wang BZ, Liu BB, He ZL. 2020. The integrated effect of salinity, organic amendments, phosphorus fertilizers, and deficit irrigation on soil properties, phosphorus fractionation and wheat productivity. *Sci Rep* 10:2736.
- Drzyzga D, Lipok J. 2018. Glyphosate dose modulates the uptake of inorganic phosphate by freshwater cyanobacteria. *J Appl Phycol* 30:299–309.
- Fang J, Nan P, Gu Z, Ge X, Feng YQ, Lu BR. 2018. Overexpressing exogenous 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) genes increases fecundity and auxin content of transgenic arabidopsis plants. *Front Plant Sci* 9:233.
- Forlani G, Pavan M, Gramek M, Kafarski P, Lipok J. 2008. Biochemical bases for a widespread tolerance of cyanobacteria to the phosphonate herbicide glyphosate. *Plant Cell Physiol* 49:443–456.

- Gao L, Pan X, Zhang D, Mu S, Lee D-J, Halik U. 2015. Extracellular polymeric substances buffer against the biocidal effect of H₂O₂ on the bloom-forming cyanobacterium *Microcystis aeruginosa*. *Water Res* 69:51–58.
- Gillezeau C, van Gerwen M, Shaffer RM, Rana I, Zhang L, Sheppard L, Taioli E. 2019. The evidence of human exposure to glyphosate: A review. *Environ Health* 18:2.
- Huang B, Ou L, Hong H, Luo H, Wang D. 2005. Bioavailability of dissolved organic phosphorus compounds to typical harmful dinoflagellate *Prorocentrum donghaiense* Lu. *Mar Pollut Bull* 51:838–844.
- Iummato MM, Fassiano A, Graziano M, dos Santos Afonso M, de Molina MdCR, Juárez ÁB. 2019. Effect of glyphosate on the growth, morphology, ultrastructure and metabolism of *Scenedesmus vacuolatus*. *Ecotoxicol Environ Saf* 172:471–479.
- Lu T, Xu NH, Zhang Q, Zhang ZY, Debognies A, Zhou ZG, Sun LW, Qian HF. 2020. Understanding the influence of glyphosate on the structure and function of freshwater microbial community in a microcosm. *Environ Pollut* 260:114012.
- Mackay EB, Feuchtmayr H, De Ville MM, Thackeray SJ, Callaghan N, Marshall M, Rhodes G, Yates CA, Johnes PJ, Maberly SC. 2020. Dissolved organic nutrient uptake by riverine phytoplankton varies along a gradient of nutrient enrichment. *Sci Total Environ* 722:137837.
- Mercurio P, Flores F, Mueller JF, Carter S, Negri AP. 2014. Glyphosate persistence in seawater. *Mar Pollut Bull* 85:385–390.
- Mohamed ZA, Hashem M, Alamri S, Mostafa Y. 2020. Cyanotoxins and their environmental health risk in marine and freshwaters of Saudi Arabia. *Arab J Geosci* 13:285.
- Murphy KR, Stedmon CA, Graeber D, Bro R. 2013. Fluorescence spectroscopy and multi-way techniques. PARAFAC. *Anal Methods* 5.
- Olesen CF, Cedergreen N. 2010. Glyphosate uncouples gas exchange and chlorophyll fluorescence. *Pest Manag Sci* 66:536–542.
- Ostera JM, Malanga G, Puntarulo S. 2020. Assessment of oxidative balance in hydrophilic cellular environment in *Chlorella vulgaris* exposed to glyphosate. *Chemosphere* 248:125955.
- Piccolo A, Celano G, Conte P. 1996. Adsorption of glyphosate by humic substances. *J Agric Food Chem* 44:2442–2446.
- Prabuthas P, Majumdar S, Srivastav P, Mishra H. 2011. Standardization of rapid and economical method for nutraceuticals extraction from algae. *J Stored Prod Postharvest Res* 2:93–96.
- Qiu H, Geng J, Ren H, Xia X, Wang X, Yu Y. 2013. Physiological and biochemical responses of *Microcystis aeruginosa* to glyphosate and its Roundup® formulation. *J Hazard Mater* 248–249:172–176.
- Scoglio S. 2018. Microcystins in water and in microalgae: Do microcystins as microalgae contaminants warrant the current public alarm? *Toxicol Rep* 5:785–792.
- Sharma S, Singh M. 2001. Environmental factors affecting absorption and bio-efficacy of glyphosate in Florida beggarweed (*Desmodium tortuosum*). *Crop Prot* 20:511–516.
- Shen H, Song L. 2007. Comparative studies on physiological responses to phosphorus in two phenotypes of bloom-forming. *Microcystis*. *Hydrobiologia* 592:475–486.
- Shikha A, Singh DP, Darmwal NS. 2004. Effect of glyphosate toxicity on growth, pigment and alkaline phosphatase activity in cyanobacterium *Anabaena doliolum*: A role of inorganic phosphate in glyphosate tolerance. *Indian J Exp Biol* 42: 208–213.
- Smedbol É, Lucotte M, Labrecque M, Lepage L, Juneau P. 2017. Phytoplankton growth and PSII efficiency sensitivity to a glyphosate-based herbicide (Factor 540®). *Aquat Toxicol* 192:265–273.
- Statista. 2016. Glyphosphate use worldwide. [cited 2016 February 2]. Available from: <https://www.statista.com/statistics/567250/glyphosate-use-worldwide/>
- Suddarth S, Ferreira J, Cavalcante L, Fraga V, Anderson R, Halvorson J, Bezerra F, Medeiros S, Costa C, Dias N. 2019. Can humic substances improve soil fertility under salt stress and drought conditions? *J Environ Qual* 48:1605–1613.
- Tonk L, Bosch K, Visser PM, Huisman J. 2007. Salt tolerance of the harmful cyanobacterium *Microcystis aeruginosa*. *Aquat Microb Ecol* 46:117–123.
- Van Bruggen A, He M, Shin K, Mai V, Jeong K, Finckh M, Morris J Jr. 2018. Environmental and health effects of the herbicide glyphosate. *Sci Total Environ* 616:255–268.
- Wong PK. 2000. Effects of 2,4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of *Scenedesmus quadricauda* Berb 614. *Chemosphere* 41:177–182.
- Wu L, Wu H, Chen L, Xie S, Zang H, Borriss R, Gao X. 2014. Bacilysin from *Bacillus amyloliquefaciens* FZB42 has specific bactericidal activity against harmful algal bloom species. *Appl Environ Microbiol* 80:7512–7520.
- Xu H, Cai H, Yu G, Jiang H. 2013. Insights into extracellular polymeric substances of cyanobacterium *Microcystis aeruginosa* using fractionation procedure and parallel factor analysis. *Water Res* 47:2005–2014.
- Xu Z, Wang S, Wang Y, Zhang J. 2018. Growth, extracellular alkaline phosphatase activity, and kinetic characteristic responses of the bloom-forming toxic cyanobacterium, *Microcystis aeruginosa*, to atmospheric particulate matter (PM_{2.5}, PM_{2.5}–10, and PM > 10). *Environ Sci Pollut Res* 25:7358–7368.
- Zhang Q, Qu Q, Lu T, Ke MJ, Zhu YC, Zhang M, Zhang ZY, Du BB, Pan XL, Sun LW, Qian HF. 2018. The combined toxicity effect of nanoplastics and glyphosate on *Microcystis aeruginosa* growth. *Environ Pollut* 243:1106–1112.
- Zhang Q, Zhou H, Li Z, Zhu J, Zhou C, Zhao M. 2016. Effects of glyphosate at environmentally relevant concentrations on the growth of and microcystin production by *Microcystis aeruginosa*. *Environ Monit Assess* 188:632.
- Zhang Z. 2010. The hormesis effect of glyphosate on six kinds of marine algae. Master's thesis. Ocean University of China. Qingdao, Shandong, China. (In Chinese).
- Ziegmann M, Abert M, Muller M, Frimmel FH. 2010. Use of fluorescence fingerprints for the estimation of bloom formation and toxin production of *Microcystis aeruginosa*. *Water Res* 44:195e204.