Mutual Impacts and Interactions of Antibiotic Resistance Genes, Microcystin Synthetase Genes, Graphene Oxide, and Microcystis Aeruginosa in Synthetic Wastewater

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**Mutual impacts and interactions of antibiotic resistance genes, microcystin synthetase
genes, graphene oxide, and *Microcystis aeruginosa* in synthetic wastewater**

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14 **Abstract**

15 The physiological impacts and interactions of ARGs abundance, microcystin synthetase genes
16 expression, GO, and *M. aeruginosa* in synthetic wastewater were investigated. The results
17 demonstrated that the absolute abundance of *sul1*, *sul2*, *tetW*, and *tetM* in synthetic wastewater
18 dramatically increased to 365.2%, 427.1%, 375.2%, and 231.7%, respectively, when the GO
19 concentration was 0.01 mg/L. Even more interesting is that the sum gene copy numbers of *mcyA-J*
20 also increased to 243.2%. The appearance of GO made the significant correlation exist between
21 ARGs abundance and *mcyA-J* expression. Furthermore, *M. aeruginosa* displayed better
22 photosynthetic performance and more MCs production at 0.01 mg/L GO. There were 65 pairs of
23 positive correlations between the intracellular differential metabolites of *M. aeruginosa* and the
24 abundance of *sul1*, *sul2*, *tetM*, and *tetW* with various GO concentrations. The GO will impact the
25 metabolites and metabolic pathway in *M. aeruginosa*. The metabolic changes impacted the ARGs,
26 microcystin synthetase genes, and physiological characters in algal cells. Furthermore, there were
27 complex correlations among *sul1*, *sul2*, *tetM*, *tetW*, *mcyA-J*, MCs, photosynthetic performance
28 parameters, and ROS. The different concentration of GO will aggravate the hazards of *M.*
29 *aeruginosa* by promoting the expression of *mcyA-J*, producing more MCs, simultaneously, it may
30 cause the spread of ARGs.

31 **Keywords:** Interactions, Antibiotic resistance genes, Microcystin synthetase gene, *Microcystic*
32 *aeruginosa*, Graphene oxide

1. Introduction

Antibiotic resistance genes (ARGs) pollution has become knotty problem that has attracted much attention around the world ([Conley et al. 2009](#)). The overuse of antibiotics in medicine, cultivation, and aquaculture field has caused the accumulation of ARGs in aquatic environment ([Komijani et al. 2021](#)). The ARGs have been detected in many water bodies in China, especially ARGs of sulfonamides and tetracyclines are ubiquitous and with high concentration ([Sun et al. 2017](#)). The average level of ARGs in natural waters reached 1.2×10^8 gene copies/mL ([Sun et al. 2017](#)). There are not only large amounts of ARGs remaining in general water bodies, but also the problem of *Microcystis aeruginosa* (*M. aeruginosa*) should not be ignored. The overgrowth of *M. aeruginosa* not only caused harmful algae bloom but also produced very potent microcystins (MCs) which can stimulate oxidative stress of the cell, which would produce a large amount of reactive oxygen species (ROS) ([Chen et al. 2016](#), [McLellan & Manderville 2017](#)). The ROS would promote the change of cell permeability, which accelerate the release of intracellular substances ([Jiang et al. 2021](#)). The increase in membrane permeability is one of the fundamental reasons for increasing the transfer efficiency of ARGs ([Guo et al. 2021](#), [Lu et al. 2020b](#), [Sun et al. 2018](#)). And then, to make matters worse, the graphene oxide (GO) would inevitably be released into the aquatic environment with its extensive application ([Yang et al. 2019](#), [Zhu et al. 2019](#)). The presence of GO might have a certain impact on the growth of *M. aeruginosa*, microcystin synthetase genes, and MCs production ([Yang et al. 2019](#)). Aquatic environment is an important medium for the release and diffusion of MCs, ARGs, and GO. These pollutants can invade the human food chain by the water cycle, posing a serious threat to the aquatic ecological environment and human health ([Avant et al. 2019](#), [Jiang et al. 2020](#)).

It is known that the pollutants in natural water bodies are very complicated, and there are many kinds of pollutants such as ARGs, *M. aeruginosa*, and nano-pollutants ([Rzymiski et al. 2020](#)). The GO might affect the microcystin synthetase genes expression and MCs production when its concentration reaches a certain level ([Wang et al. 2020a](#), [Yin et al. 2020](#)). People formerly believed

that the abuse of antibiotics was the main reason for global accumulation and spread of ARGs (Sola 2020). However, more and more studies have shown that natural-occurring substances and some kinds of nano-pollutants in the aquatic environment can promote the spread of ARGs (Sun et al. 2021). Studies have shown that extractive of *M. aeruginosa* and pure MCs and nanometer materials can cause the spread of ARGs in aquatic environment (Fan et al. 2021, Xu et al. 2021). Some researchers have inferred that MCs and nanometer materials might change the permeability and surface functional groups of microbial cells and accelerate the rate which ARGs genetic material enters cells (Fan et al. 2021).

MCs are synthesized by the megazyme complex through non-ribosomal pathways (Yang et al. 2015). This type of complex includes peptide synthase, polyketide synthase and some other modified enzymes (Wei et al. 2020). By sequencing the gene cluster encoding synthase, it was found that the gene cluster contained a type of mixed non-ribosomal peptide synthetase genes including *mcyA*, *mcyB*, *mcyC*, *mcyD*, *mcyE*, *mcyF*, *mcyG*, *mcyH*, *mcyI* and *mcyJ* (Lu et al. 2020a). Simultaneously, nano-pollutants in aquatic environment will also affect the production of MCs. In the presence of GO, the transcription levels of the synthetase genes *mcyA*, *mcyB* and *mcyD* are significantly increased (Grasso et al. 2020). Therefore, what can be inferred is that although the production of MCs is determined by the genes in the microcystin-producing cells, the nano-pollutants such as GO in environment can also regulate their gene expression, thereby affecting the synthesis of MCs. The presence of MCs may increase the abundance of ARGs, and the GO in the aquatic environment might make the transcription of MCs synthase genes increase. While, there is still no result that can effectively verify this inference, this work is trying to prove this. What are the mutual impacts and interactions of ARGs, microcystin synthetase genes, MCs, GO, and *M. aeruginosa*, the researches on this aspect were currently rare.

The abundance changes of ARGs including *sulI*, *sul2*, *tetW*, *tetM* and the gene copy numbers of MCs microcystin synthetase genes including *mcyA*, *mcyB*, *mcyC*, *mcyD*, *mcyE*, *mcyF*, *mcyG*, *mcyH*, *mcyI*, *mcyJ* in GO-exposed *M. aeruginosa* with different concentrations were investigated in this study. Meanwhile, the correlativity between ARGs abundance and *mcyA-J* expression quantity was evaluated. Moreover, the effect of GO with different concentrations on the

ultrastructure, photosynthesis, metabonomics characters of *M. aeruginosa* were also studied. These results will reveal the mutual impacts and interactions of ARGs, microcystin synthetase genes, graphene oxide, and *Microcystis aeruginosa* in synthetic wastewater, which will provide some basics for the studies of multi-component pollutants in aquatic environment.

2. Materials and methods

2.1 Experimental design

M. aeruginosa (FACHB-315) was purchased from the Institute of Wuhan Hydrobiology, Chinese Academy of Sciences and cultured in pH 7.0 BG11 medium (Table S1). The GO was purchased from the Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences. The GO sheets diameter was 0.1-10 μm in size with average thickness of 1.5 μm . The plasmids of ARGs including *sul1*, *sul2*, *tetW*, *tetM* were prepared by Genesis Biotechnology Co., Ltd. The gene sequences of *sul1*, *sul2*, *tetW*, *tetM* were listed in Table S2.

For the incubation experiment, photobioreactors as shown in Fig. 1 was used. The GO particles were dispersed in synthetic wastewater (Table S3) with initial concentrations of 0, 0.01, 0.1, 1, and 10 mg/L, and for the control group, the GO concentration was 0 mg/L. The GO concentration range was set close to that found in natural water (Zhao et al. 2020b). In these systems, GO particles had hydrodynamic diameters of 250-300 nm and zeta potentials ranges from -30 to 50 mV, indicating that the GO particles had been stably dispersed in the synthetic wastewater at the four concentrations (Monteil et al. 2014). *M. aeruginosa* was inoculated at density of 1.0×10^5 cells/mL and the reactors were placed in an illumination incubator (MGC-300A, China) at $28.0 \pm 0.5^\circ\text{C}$ and 75% humidity. At the beginning (0 h) and after 0, 24, 48, 96 h of incubation, samples were taken from each reactor and centrifuged at $8000 \times g$ for 10 min. The supernatants were used for analyses of ARGs abundance, total nitrogen (TN), ammoniacal nitrogen ($\text{NH}_3\text{-N}$), phosphate phosphorus ($\text{PO}_4^{3-}\text{-P}$), chemical oxygen demand (COD), and extracellular MCs (MC-LR and MC-RR). The precipitates were collected for intracellular MCs and reactive oxygen species (ROS) quantification, metabolic responses, *mcyA-J* gene expression analyses, and transmission electron

microscopy (TEM) observation. Only samples taken at the end of the experiment (96 h) from the control, 0.01, and 10 mg/L GO were used for metabonomic analysis.

2.2 Measurement of ARGs abundance

The ARGs abundances of *sul1*, *sul2*, *tetW*, *tetM* were measured by HT-qPCR. The Light Cycler 480 YSBR Green I Master was used as fluorochrome. A total of 14 pairs of primers were selected, including 4 ARGs primers, 10 pairs of microcystin synthetase genes primers (Table S2). The HR-qPCR reaction system consists of 12.5 μ L PCR reaction mixture with ROX reference dye, 0.5 μ L (with concentration of 10 μ mol/L) forward and reverse primers, 10.5 μ L DNA-free water, and 10 μ L sample DNA. The operation steps of HT-qPCR were as shown as follows: ① 50°C, 2 min; ② 95°C, 5 min; ③ 95°C, 20 s; ④ annealing for 30 s; ⑤ 72°C, 30 s; ⑥ plate read, repeat the temperature of ③-⑤; ⑦ melting curve analysis, between 60-95°C, read every 0.2°C (Wu et al. 2020a).

2.3 Measurement of algal photosynthetic response and growth rate

The phytoplankton classification fluorometer (Phyto-PAM, Germany, WALZ) was used to measure the various parameters of chlorophyll fluorescence. The specific steps are as follows: ① Start the Phyto Win software, place a certain amount of sample (the volume should be uniform each time) in a cuvette for 15 min. ② Start the instrument to determine the initial measurement fluorescence yield (F_0), measure the maximum fluorescence yield (F_m) after the saturation pulse at 4000 μ mol/(m²·s). ③ Calculate the maximum light energy conversion efficiency (F_v/F_m). ④ Set the photochemistry intensity at 3 000 μ mol/(m²·s) and irradiate for 1 min until the indicator light turns green, the initial fluorescence (F_s) and maximum fluorescence (F_m') were measured when the fluorescence value was stable. The measured chlorophyll fluorescence parameters are F_0 , F_m , F_0' , F_m' , F_s . The fluorescence parameters such as F_v/F_m , F_v/F_0 , ETR_{max} were calculated as follows (Poudyal et al. 2019):

137 Maximum light conversion efficiency **Eq. (1)**: $\frac{F_v}{F_m} = \frac{F_m - F_0}{F_m}$

138 Maximum photochemical quantum yield **Eq. (2)**: $\frac{F_v}{F_0} = \frac{F_0}{F_m - F_0}$

139 Efficiency of light energy conversion **Eq. (3)**: $\phi PSII = \frac{F'_m - F_s}{F'_m}$

140 Quantum efficiency **Eq. (4)**: $Yield = \frac{F'_m - \frac{1}{\frac{1}{F_q} + \frac{1}{F_m} + \frac{1}{F'_m}}}{F'_m}$

141 Photosynthetic electron transport **Eq. (5)**: $ETR = \phi PSII \times DAR \times 0.5 \times 0.84$

142 The specific growth rate of algae is used to reflect the growth of *M. aeruginosa*. The formula is
143 shown in **Eq. (6)**:

$$\mu = \frac{\ln \left(\frac{X_n}{X_{n-1}} \right)}{t_n - t_{n-1}}$$

144

145 In **Eq. (6)**: X_n is for the cell density of *M. aeruginosa* at the end of the GO-exposure period (t_n),
146 X_{n-1} is the cell density of *M. aeruginosa* at the GO-exposure period (t_{n-1}) ([Elser et al. 2007](#)).

147 **2.4 N, P nutrients removal determination**

148 After the supernatants of samples were filtered through 0.44- μ m filters, the concentration of
149 nitrogen and phosphorus nutrients including TN, $\text{NH}_3\text{-N}$, $\text{PO}_4^{3-}\text{-P}$, and COD were determined as
150 described in their study ([Ajayan et al. 2019](#)).

151 **2.5 MCs quantification and microcystin synthetase genes expression measurement**

152 The MCs in the supernatants were extracted with Oasis HLB and determined by liquid
153 chromatography-mass spectrometry (LC-MS). Intracellular MCs (MC-LR and MC-RR) extraction
154 was performed as described in previous study ([Pinheiro et al. 2016](#)). The sample was extracted
155 with 75% methyl alcohol at 25°C for 20 min while stirring. The homogenate was centrifuged
156 (10000 $\times$ g, 10 min) to remove the pellet. The MCs in the supernatant were eluted using 80% (v/v)

methyl alcohol, concentrated at 35°C ([Pinheiro et al. 2016](#)), purified, and quantified using HPLC (Agilent 1200, USA). A reversed phase column equipped with a guard column at 45°C was used. For *mcyA-J* gene expression analysis, total RNA of *M. aeruginosa* was transcribed to cDNA for RT-qPCR analysis on a real-time PCR system (Thermo fisher, Step One Plus, USA). The qPCR amplification procedure was operated as Lee reported ([Lee et al. 2020](#)).

2.6 TEM observation and ROS determination

The algal cells of *M. aeruginosa* were added to 2.5% glutaraldehyde with the final concentration was 2.5%, then fixed for 3 h. Centrifuged at 5000×g, the supernatant was removed, and 0.1 mol/L phosphate buffer was added to wash the samples for 3 times. Then 4% osmic acid was added to fix the algal cell. The samples were centrifuged at 5000×g for 5 min after incubation overnight at 4°C, then the supernatant was removed. The acetone solutions of different concentrations of 10%, 30%, 50%, 70%, 90% and 100% were used to dehydrate. Resin was used to embed, then the sample was sectioned (EMUC7, Lycra, Austria). The 3% uranyl acetate and 2% lead citrate were used to stain. Finally, the samples were observed by transmission electron microscope (HT7700, Hitachi, Japan) ([Soares et al. 2020](#)). The ROS levels of samples were detected by ROS kit (ML Elisa0255, R&D Systems, USA) according its operating manual.

2.7 Metabonomic determination

Extraction, derivatization and GC-MS detection process of metabolites were performed as the modified method of Weckwerth ([Weckwerth et al. 2004](#)). A certain amount of sample (Grinded in liquid nitrogen) was added in 1mL pre-cooled extraction solution (volume ratio of methanol to water is 1:1) and 5 µL internal standard substance. Then the mixture was vortexed for 3 min. After centrifugation (8000×g, 5 min), 500 µL supernatant was placed in liquid nitrogen for 30 min, then the sample was freeze-dried. The 50 µL methoxyammonium hydrochloride/pyridine solution (20 mg/mL) was added, kept reacting at 40°C for 60 min. The 80 µL N-methyl-N-(trimethylsilane) trifluoroacetamide (MSTFA) was added, then reacted 80 min at 40°C. After centrifugation at 8000×g, for 10 min, the supinate was used to detection and analyzed by GC-MS.

2.8 Statistical analysis

The treatments and measurements were all performed in triplicate. Origin 8.5 was used for data processing for statistical analysis. The identification of metabolites was performed by the NIST database (2011). The metabolite data were normalized, then they were imported into SIMCA software (Version 11.5) for the PCA and PLS analysis. The HCE 3.5 software was used to perform hierarchical cluster analysis. The figures in this study were drawn by Graph pad Prism 7.0.

3. Results and discussion

3.1 Analysis of mutual impacts between ARGs and microcystin synthetase genes expressions

The absolute abundance of the total ARGs including *sul1*, *sul2*, *tetW*, *tetM* of GO-exposed *M. aeruginosa* systems at concentration of 0.01 mg/L was improved 4 times than that at concentration of 0 mg/L, especially for *sul1*, *sul2*. The highest abundance of *sul1* and *sul2* in GO-exposed *M. aeruginosa* system with concentration of 0.01 mg/L reached 4.14×10^{11} copies/L. The total genes copies of microcystin synthetase genes including *mcyA-J* reach up to 2.98×10^{10} when the concentration of GO was 0.01 mg/L. In order to explain the impacts between ARGs (*sul1*, *sul2*, *tetM*, *tetQ*) and microcystin synthetase genes (*mcyA-J*), the correlation analysis was performed and the results were shown in **Fig. 2**.

The *Pearson* correlation analysis was performed between the expression of *sul1*, *sul2*, *tetW*, *tetM* and the intracellular *mcyA-J* of *M. aeruginosa* in the synthetic wastewater when GO with concentration of 0.01, 0.1, 1, 10 mg/L. The results demonstrated that there were 30 pairs, 30 pairs, 30 pairs, and 25 pairs of correlations ($p < 0.05$) between ARGs and *mcyA-J* at 24h, 48h, 72h, 96h, respectively. It can be inferred that there was a positive correlation between the abundance of ARGs and the expression of *mcyA-J* when the GO was present. When the GO concentration is 0, there is no correlation between the abundance of *sul1*, *sul2*, *tetM*, *tetW* and *mcyA-J*, which further demonstrated that the presence of GO made the abundance of ARGs closely related to the expression of *mcyA-J*. Interestingly, when the concentration of GO was 0.01 mg/L, the expression

of *mcyA-J* was significantly increased ($P < 0.05$), and the MCs production was also significantly increased, and the expressions of *sul1*, *sul2*, *tetM*, *tetW* were also increases significantly ($P < 0.05$). The presence of GO at the concentration of 0.01 mg/L made the positive correlation between ARGs (*sul1*, *sul2*, *tetM*, *tetW*) abundance and *mcyA-J* expression further enhanced. What would be mentioned in latter section was that the photosynthesis performance of *M. aeruginosa* and MCs production were promoted when the GO concentration was 0.01 mg/L. It can be inferred that the presence of GO in aquatic environment will aggravate the overgrowth of *M. aeruginosa*, MCs production, and spread of ARGs to a certain extent ([Pan et al. 2015](#), [Wu et al. 2020b](#)).

3.2 Influence of GO on the N, P removal by *M. aeruginosa*

The nitrogen and phosphorus nutrients including total nitrogen (TN), ammoniacal nitrogen ($\text{NH}_3\text{-N}$), phosphate ($\text{PO}_4^{3-}\text{-P}$), and chemical oxygen demand (COD) removal by *M. aeruginosa* is closely related to the growth rate ([Ma et al. 2014](#)). The removal rates of TN, $\text{NH}_3\text{-N}$, $\text{PO}_4^{3-}\text{-P}$, and COD were 25%, 72%, 36.2%, and 42.9%, respectively, at 0.01 mg/L GO exposure (**Fig. 3**), indicating that 0.01 mg/L GO-exposure can effectively stimulate and promote nutrients removal by *M. aeruginosa* from the growth environment ([Aphale et al. 2015](#)). Much smaller removals of TN, $\text{NH}_3\text{-N}$, $\text{PO}_4^{3-}\text{-P}$, and COD were observed in the 10 mg/L GO treatment, which might be attributed to the negative effects of GO at high concentration on the photosynthetic rates of algal cells [40], as evident by the low F_v/F_m and ETR_{max} demonstrated in **Fig. 1**. Simultaneously, high concentration of 10 mg/L GO inhibited nutrients removal by *M. aeruginosa* ([Zhao et al. 2020a](#)).

3.3 Cellular impacts of *M. aeruginosa* and GO

Significant effects ($p < 0.05$) of GO on photosynthesis of *M. aeruginosa* was observed during the GO-exposure period at concentrations of 0.01, 0.1, 1, and 10 mg/L, respectively. The different concentration of GO-exposure also affected the growth rate significantly ($p < 0.05$). The results indicated that the intracellular production and extracellular release of MCs in GO-exposed groups were higher than that in the control (without GO exposure). As shown in **Fig. 4A**, the intracellular MCs production in *M. aeruginosa* of the 0.01mg/L GO-exposure group was the highest among all

groups during the whole exposure period. Simultaneously, the number of gene copies of *mcyA-J* in the *M. aeruginosa* were the highest among all groups, indicating that the presence of GO at concentration of 0.01 mg/L stimulated the expression of microcystin synthetase genes clusters (**Fig. 4B**). This led to a significant increase ($p < 0.05$) in the production of intracellular MCs. Since MCs are synthesized intracellularly and are released to extracellular when the algal cell ruptured ([Rincon et al. 2019](#)), the percentage of extracellular MCs release in the 0.01 mg/LGO-exposure group is lower than other groups. However, when the GO-exposure concentration was 10 mg/L, as demonstrated in **Fig. 5B**, the level of ROS in algal cell increased sharply. The level of ROS in the algal cells increased sharply with increasing GO in the media. The increased ROS and occurrence of cell rupture explain the decreased nutrients removal and significantly increased ($p < 0.05$) extracellular MCs in the 10 mg/L GO-exposure group. The TEM images in **Fig. 5A** showed obscure boundaries of the cytomembranes, indicating that severe peroxidation damage and plasmolysis of the algal cells occurred, and a large number of cells were ruptured in the high GO concentration treatments (the red circle in **Fig. 2A**). Thereupon, lots of intracellular MCs were released, and the percentage of extracellular MCs release was increased significantly ($p < 0.05$).

Furthermore, as shown in **Fig. 3A** and **Fig. 3B**, the value of F_v/F_m and ETR_{max} of *M. aeruginosa* cells were the highest among all groups during the GO-exposure was 0.01 mg/L. The F_v/F_m value reflects the potential maximum photosynthetic capacity of algal cells ([Joonas et al. 2019](#)), and the ETR_{max} value reflects the maximum transmission rate of photons in photosynthesis of *M. aeruginosa* ([Lee et al. 2019](#)). The higher F_v/F_m and ETR_{max} values would indicate the better photosynthetic performance ([Cruces et al. 2021](#)), and F_v/F_m value of normal growth of algae is about 0.7-0.8 ([Zheng et al. 2020](#)). The highest F_v/F_m value is 1.1 in group of GO-exposure at concentration of 0.01 mg/L. Therefore, the photosynthetic performance was stimulated by GO-exposure at concentration of 0.01 mg/L. In contrast, the F_v/F_m and ETR_{max} values in GO-exposure at concentration of 10 mg/L are the lowest among all groups. From another perspective, the higher the F_v/F_m and ETR_{max} values would indicate the less stressed conditions in growth environment of *M. aeruginosa*. The lower the F_v/F_m and ETR_{max} values would indicate the more stressed conditions in growth environment and the worse photosynthetic performance

([Sanz-Martín et al. 2019](#)). Photosynthetic performance, the growth rate in *M. aeruginosa* increased under 0.01 mg/L GO-exposure and decreased under 10 mg/L GO-exposure (**Fig. 6C**). These results suggested that slight GO-exposure enhanced the photosynthetic activity, growth rate, and MCs production of *M. aeruginosa* at environmentally relevant concentrations. It could reasonably be inferred that the GO pollution at environmentally relevant concentrations would aggravate the ecological hazard of *M. aeruginosa* ([Gao et al. 2019](#)).

3.4 Analysis of interactions from metabolomic aspects

The metabolic pattern of *M. aeruginosa* under GO-exposures of 0.01 mg/L and 10 mg/L were compared with the control without GO-exposure. The GO-exposures at concentration of 0.01 mg/L and 10 mg/L were close to the low and high contamination levels of GO in aquatic environments, respectively ([Zhang et al. 2020a](#)). The metabolic profiling of *M. aeruginosa* in 0.01 and 10 mg/L GO-exposure groups are distinct (**Fig. 7A**), indicating that the metabolites in these two groups are significantly different ($p < 0.05$). A total of 64 differential metabolites were screened (**Fig. 7B**), while the relative abundance of differential metabolites (**Fig. 7C**) and significantly different metabolic pathways ($p < 0.05$) in the GO-exposed groups were analyzed (**Fig. 8**).

After 96 h of exposure to 0.01 mg/L of GO, 56 metabolites were upregulated while 8 metabolites were down-regulated (**Table 1**). The identified metabolites were involved in 4 main physiological processes according to significant enriched pathways ($p < 0.05$), including photosynthetic metabolism, glycometabolism, amino acid metabolism, and lipid metabolism. Much more metabolites were up-regulated instead of down-regulated, indicating that most physiological activities in *M. aeruginosa* were stimulated at the presence of 0.01 mg/L GO. In contrast, after 96 h of GO-exposure at concentration of 10 mg/L, 47 metabolites were up-regulated while 17 metabolites were down-regulated. More metabolites were down-regulated as compared with the 0.01 mg/L GO treatment, suggesting that the physiological activities were motivated to initiate the defensive mechanism against GO stress in the 10 mg/L GO treatment ([Zhang et al. 2019](#)). The result of enrichment analysis of KEGG pathway demonstrated that carbon fixation in photosynthetic process, valine, leucine and isoleucine biosynthesis, and galactose metabolism were significantly

enriched ($p < 0.05$) in *M. aeruginosa* exposed to GO at concentration of 0.01 mg/L.

The metabolic network map reflects the important interactions between the altered metabolic pathways (Fig. 8). Notably, in 0.01 mg/L GO-exposure group, an increase in amino acid metabolism including increase in L-threonine, L-valine, L-alanine, and L-proline, was observed. These findings are in accordance with previous studies where an increase in amino acid turnover in stimulated algal cells by low concentration of GO was reported (Ouyang et al. 2020). The other important metabolic pathway found to be altered in *M. aeruginosa* in 0.01 GO-exposure group was nucleotide metabolism, including increase of uracil and hypoxanthine. Proliferating algal cells of *M. aeruginosa* stimulated by GO often demand for nucleotides for the synthesis of cellular materials, which is fulfilled by purines and pyrimidines. Increases in nucleotides indicate that they are needed for cell proliferation (Zhang et al. 2020b). Additionally, glycometabolism and fatty acid metabolism were indicated to be alerted in *M. aeruginosa* at GO-exposure of 0.01 mg/L. Specifically, increased levels of carbohydrates and numerous unsaturated fatty acids including D-glucose, galacturonate, linoleic acid, glutaric, and tetradecanoic acid were observed. The increase of glycometabolism indicates the vigorous growth of algal cell (Zhang et al. 2018), and unsaturated fatty acid will promote the photosynthetic performance of algal cells (Anto et al. 2020).

Moreover, the reticular correlativity between differential metabolites and other results including TN, $\text{NH}_3\text{-N}$, $\text{PO}_4^{3-}\text{-P}$, COD, *mcyA-J* gene copies, MCs production, ARGs (*sul1*, *sul2*, *tetW*, *tetM*), F_v/F_m , ETR_{max} , and growth rate were calculated. Highly interconnected metabolites with high degrees play key roles in the interaction of *M. aeruginosa* and GO. According to the correlativity analysis between metabolites and other pollutants and factors (*sul1*, *sul2*, *tetM*, *tetW*, *mcyA-J*, MCs production, $\text{NH}_3\text{-N}$, TN, $\text{PO}_4^{3-}\text{-P}$, COD, F_v/F_m , ETR_{max} , growth rate, ROS, and 16S rRNA) as shown in Fig. 9. Moreover, it demonstrated that there were 23 pairs of positive correlations between the intracellular differential metabolites of *M. aeruginosa* and the abundances of *sul1*, *sul2*, *tetM*, and *tetW* with different GO concentrations. The metabolites that related ARGs abundance were mainly amino acids. The metabolites that related *mcyA-J* expression were mainly amino acids and small molecule acids. There were 40 pairs of positive correlations between these metabolites and *mcyA-J*. Furthermore, there were 28 pairs of positive correlations between the abundance of *sul1*, *sul2*, *tetM*,

tetW and *mcyA-J* expression. The impacts and interactions were complicated of abundance of ARGs, *mcyA-J* expression, MCs production, photosynthesis performance of *M. aeruginosa*, intracellular ROS levels, ultrastructure, and GO. Simultaneously, there is also a close correlation among various different metabolites in *M. aeruginosa* (Kim et al. 2020).

Some chemical substances such as antibiotic contaminants and organic pollutant have been manifest to have toxic stimulant hormesis effects on algae at a certain concentration (Liu et al. 2020). Nanomaterials are also reported to have a hormesis effect on many kinds of algal cells (Agathokleous et al. 2019). The phenomenon of hormesis effect was observed in the *M. aeruginosa* in GO-exposure in the present study. Photosynthesis is the basis of a cell growth of *M. aeruginosa*, photosynthetic performance was promoted by GO-exposure at 0.01 mg/L (Wang et al. 2020b). The GO stimulated the growth rate, and then promoted the production of MCs in *M. aeruginosa* (Yu et al. 2019).

Particularly, the gene copies of microcystin synthetase (*mcyA-J*) increased. It was reasonable to conclude that the increased genetic expression of microcystin synthetase had resulted in the increased production of MCs. In contrast, the expression of *mcyA-J* was inhibited in the 10 mg/L GO- exposed group, and consequently, MCs production decreased. The results suggested that the MCs synthetic process is stimulated by low and inhibited by high concentration of GO.

The ARGs and *mcyA-J* were significantly related with photosynthetic metabolites including phytol (an essential component of chlorophyll) and 3-6-anhydro-D-glucose (photosynthetic carbon fixes important metabolites) (Zhang et al. 2018). Moreover, some studies have demonstrated that microcystin synthetase genes (*mcyA-J*) and MCs production was a kind of physiological response to environmental stressed factors (Li et al. 2019). These results confirmed that the role of MCs production and synthesis in responsive process to GO-exposure at environmental concentration (Li et al. 2019).

The *M. aeruginosa* released more MCs in the 10 mg/L GO-exposure group than in the control and the 0.01 mg/L GO treatment. The increased ROS level and membranolysis (Fig. 5) may facilitate the export of intracellular MCs (Li et al. 2019). The cells rupturing induced by GO-exposure might be an important explanation for the MCs release by *M. aeruginosa* (Li et al.

2020). With increased intracellular MCs production and *sul1*, *sul2*, *tetM*, *tetW* abundance at low concentration of GO and increased release of MCs at high concentration of GO, the hazards of *M. aeruginosa* and ARGs would be exacerbated by GO in the aquatic environment (Bandara et al. 2019). It suggested that the harm of GO by regulating the ARGs abundance, microcystin synthetase genes, and MCs production has already become an ecological problem.

During the GO-exposure period of 96 h, impacts and interactions of ARGs, microcystin synthetase genes, MCs production, photosynthesis were initiated. The relative abundance of carbohydrates related to the carbon fixation pathway in photosynthetic process in *M. aeruginosa* increased significantly ($p < 0.05$) in GO-exposure at concentration of 0.01 mg/L. Simultaneously, the expression of *mcyA-J* in *M. aeruginosa* and *sul1*, *sul2*, *tetM*, *tetW* in synthetic wastewater increased significantly ($p < 0.05$), resulting in the increase of intracellular MCs production and ARGs spread. The microcystin synthetase gene cluster of *mcyA-J* can regulate the ABC transporters (control the transportation and exchange of nutrients between extracellular and intracellular) (Han et al. 2019, Pearson et al. 2020). The result of KEGG pathway enrichment analysis suggested that the pathway of ABC transporters was significantly enriched, and the metabolites (valine, maltotriose, D-glucose, D-maltose, threonine, alanine, proline) which matched in the transporter pathway were up-regulated. It means that more extracellular nutrients (such as $\text{NH}_3\text{-N}$) and ARGs plasmid of *sul1*, *sul2*, *tetM*, *tetW* in synthetic wastewater will be transported into *M. aeruginosa* for cell growth, MCs synthesis and spread of ARGs (Yu et al. 2019). When the concentration of GO-exposure increased to 10 mg/L, cytoderm rupture occurred and large amounts of intracellular MCs was released. It indicated that hormesis mechanism would be triggered in *M. aeruginosa* and ARGs abundance when the GO presents. The presence of GO at finite concentration in aquatic environment can aggravate the harm of *M. aeruginosa* and spread of ARGs (Duan et al. 2020, Huang et al. 2020).

4. Conclusions

Mutual impacts and interactions of antibiotic resistance genes, microcystin synthetase genes,

graphene oxide, and *M. aeruginosa* in synthetic wastewater were investigated in the present study. There was significant correlation between the abundance of ARGs (*sul1*, *sul2*, *tetM*, *tetW*) and *mcyA-J* expression when the GO concentration was 0.01 mg/L. GO has a hormesis effect on *M. aeruginosa*, ARGs abundance, and *mcyA-J* expression. At low concentration of 0.01 mg/L, GO would stimulate the photosynthesis and growth of *M. aeruginosa*, while at high concentration of 10 mg/L, GO would induce cell rupture and MCs release of *M. aeruginosa*. The GO in aquatic environment would aggravate the ecological hazard of *M. aeruginosa* by promoting its growth, *mcyA-J* expression, MCs production, and ARGs abundance.

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Ethical Approval

This research does not involve ethical issues.

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391 **Consent to Publish**

392 All authors confirm that this paper has not been published before in any form.

393 **Authors Contributions**

394 **Xiyan Ji:** Conceptualization, Methodology, Supervision, Funding acquisition

395 **Meifang Hou:** Supervision, Conceptualization, Review & Editing

396 **Shichao Wu:** Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft

397 **Xin Li:** Resources, Data Curation, Formal analysis

398 **Jing Ye:** Review & Editing

399 **Wenwu Xu:** Project administration

400 **Rui Wang:** Visualization

401 **Competing Interests**

402 The authors declare that they have no known competing financial interests or personal relationships
403 that could have appeared to influence the work reported in this paper.

404 **Availability of data and materials**

405 The datasets used or analyzed during the current study are available from the corresponding author
406 on reasonable request.

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580

Figures

Figure 1

Schematic diagram of experimental set-up

24 h

48 h

72 h

96 h

Figure 2

Pearson correlation analysis between abundances of sul1, sul2, tetM, tetW and mcyA-J expressions

Figure 3

Changes in TN, NH₃-N, PO₄³⁻-P, and COD concentrations during the 96-h incubation of *M. aeruginosa* in BG11 medium with 0, 0.01, 0.1, 1, and 10 mg/L graphene oxide (GO)

Figure 4

A: The expression of microcystin synthase gene cluster (mcy A-J) in *M. aeruginosa* in 0.01, 0.1, 1, 10 mg/L GO-exposure groups and control group; B: The intracellular MCs production in *M. aeruginosa* in 0.01, 0.1, 1, 10 mg/L GO-exposure groups and control group; C: The percentage of extracellular MCs production in *M. aeruginosa* in 0.01, 0.1, 1, 10 mg/L GO-exposure groups and control group.

Figure 5

The TEM images and ROS levels of *M. aeruginosa* in 0.01, 0.1, 1, 10 mg/L GO-exposure groups and control group. A1-A5: ultrastructure of the *M. aeruginosa*. double-headed arrows denote plasmolysis of *M. aeruginosa*, red circles denote breakages of *M. aeruginosa*. B: ROS levels of *M. aeruginosa* in all groups.

Figure 6

The Fv/Fm, ETRmax, and growth rate of *M. aeruginosa* in 0, 0.01, 0.1, 1, 10 mg/L GO-exposure groups and control group. A: Fv/Fm of the *M. aeruginosa* in all groups, B: ETRmax of the *M. aeruginosa* in all groups, C: growth rate of the *M. aeruginosa* in all groups.

Figure 7

The metabolic analysis of *M. aeruginosa* in different concentration of GO-exposure. A: PCA analysis, B: metabolite heat map of control group, 0.01 mg/L GO-exposure, 10 mg/L GO-exposure. C: venn diagram of the differential metabolites.

Figure 8

Metabolic pathway network map of significant altered metabolites of 0.01 mg/L, 10 mg/L GO-exposure, and control group. Metabolites identified in this study are shown along with a bar plot illustrating normalized concentration differences of metabolites in control group (saffron yellow bar), 0.01 mg/L GO-exposure group (light blue bar), and 10 mg/L GO-exposure group (dark blue bar). Red metabolites are significant differential, black metabolites not identified in this study.

Figure 9

Pearson correlation network of metabolites (green), PO₄³-P, NH₃-N, COD, ROS, Fv/Fm, ETRmax, growth rate, MCs, mcyA-J (red). The blue lines represent negative correlation coefficients, while red ones represent positive correlation. Only correlation coefficients significant at $p < 0.05$ are considered ($|r| \geq 0.8$, $FDR \leq 0.05$).

Supplementary Files

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