Single and combined toxic effects of clarithromycin and levofloxacin on Microcystis aeruginosa

Yixiao Wu, Huijun Ding, Liang Wan, Weihao Zhang, Yan Zhang, Lin Yang and Chong Zhao

1. Introduction

The widespread use of antibiotics for disease treatment and prevention has caused groundwater and surface water pollution around the world [1,2]. Antibiotics can be released into the aquatic environment via either by direct excretion by humans and animals or through the runoff from agriculture and livestock areas [3]. Their residues induce resistant bacteria and have deleterious effects on plants and animals [4]. Owing to the continuous discharge into the water environment of antibiotics, aquatic organisms are exposed in their entire life cycle, causing great harm to the ecosystem [5].

Among various antibiotics, clarithromycin (CLA) has been widely applied to treat the infection of the lower and upper respiratory tracts, skin or soft tissues, and in pediatrics due to its acid stability and excellent antimicrobial and pharmacokinetic properties [6]. This drug inhibits the growth of microorganisms by hindering RNA-dependent protein synthesis by binding action sites in the nascent peptide exit tunnel, which is formed by 23SrRNA [7]. Levofloxacin (LEV), as a broad-spectrum antibacterial drug, is also used extensively to cure a variety of infections [8]. LEV can act on DNA gyrase and topoisomerase IV, and the resulting ternary complexes may block DNA replication [9]. The frequent occurrence in the environment and inefficient elimination of CLA by conventional wastewater treatment plants (WWTPs) resulted in the inclusion of the drug in the watchlist of European Union Commission Decision 495/2015 [10]. WWTPs also experience difficulty in removing LEV in water; the average removal efficiency is 53% [11]. The residual concentrations of CLA and LEV in water environment range from ng/L to µg/L [11,12]. The high-risk quotients of the two antibiotics indicate a possible ecological risk to aquatic organisms [12,13]. Therefore, studies on the toxicity of CLA and LEV to aquatic organisms are urgently needed to conduct assessment of their environmental risks.

Most of the previous studies have focused on assessing the toxicity of individual antibiotics on aquatic organisms. However, chemical contaminants are usually present collectively in the environment. Thus, the joint toxic reaction may significantly differ from that of individual drugs. Hagenbuch and Pinchney [14] discovered the synergistic toxic effect of the mixtures of tylosin/lincomycin and lincomycin/ciprofloxacin on Cylindrotheca closterium and the additive effect of mixed tylosin and ciprofloxacin. Liu et al. [15] demonstrated that the mixtures of spiramycin and amoxicillin at 1:7 and 1:1 ratios exhibited antagonistic actions on Microcystis aeruginosa. These results showed the distinct effects of different antibiotic combinations on aquatic organisms. The co-occurrence of...
CLA and LEV has been detected in various waterbodies [16], but the response of aquatic organisms, especially algae, to their exposure at the biochemical level is still unknown. Additionally, previous investigations on the joint action of antibiotics mainly aimed at their inhibition effects on cell growth and the median effective inhibition concentration (EC$_{50}$) [4,13,14]. The corresponding toxic mechanisms are seldom reported. In our early studies, erythromycin, a kind of macrolide antibiotic, showed toxic effects on the photosynthesis and antioxidant system of M. aeruginosa [17]. Other researchers have also proposed excessive reactive oxygen species (ROS) induced by antibiotics as a cause of the growth inhibition of algae [18,19]. Therefore, studying the changes in biological indicators related to photosynthesis and oxidative stress of algae under exposure to single and mixed antibiotics can help to discover the mechanisms of antibiotic toxicity to algae.

Algal blooms have caused concern in recent decades due to a series of related environmental problems, such as reduction of water transparency, effects on the growth of aquatic organisms and decline in biodiversity [20]. M. aeruginosa is regarded as one of the most common forms of blooms and can release microcystins (MCs), thereby threatening human health [21,22]. Various contaminants, including antibiotics, affect the discharge of MCs [23,24]. However, the contribution of CLA and LEV alone or in combination with the release of MCs to the aquatic environment is still unknown. In addition, common anthropogenic antibiotics are designed to inhibit bacteria by destroying cellular components and metabolic functions. Given that cyanobacteria have a similar structure to bacteria, that is, they lack the membrane enclosing the nucleus, the former are more susceptible to antibiotics than green algae [23]. As recommended by the 25, the use of cyanobacteria is more appropriate than green algae for antibiotic environmental risk assessment due to the high sensitivity of the former.

The toxic effects of these drugs on cyanobacteria and their contribution to MC release need to be evaluated to raise the understanding of ecotoxicological characteristics of antibiotics when they enter the environment simultaneously. Therefore, the potential toxicities of CLA and LEV and their combination were assessed by conducting 96 h acute toxicity test on the common bloom cyanobacterium M. aeruginosa. The biomarkers that characterize growth, photosynthesis, and oxidative stress were measured to explain the related mechanisms.

2. Materials and methods

2.1. Test chemicals and algae

CLA (CAS: 81,103–11–9, purity ≥99%) and LEV (CAS: 100,986–85–4, purity ≥98%) were purchased from Shanghai Macklin Co., Ltd (China). The stock solution of LEV was prepared using deionized water, whereas CLA was prepared in dimethyl sulfoxide (DMSO) and stored at −18°C. The DMSO concentration in test solutions did not exceed 0.01% (v/v); this concentration had no effect on the algal growth in our preliminary experiment.

The tested cyanobacterium, M. aeruginosa, was obtained from the Freshwater Algae Culture Collection, Wuhan institute of Hydrobiology, Chinese Academy of Sciences. M. aeruginosa was cultured in sterile BG11 medium in an incubator at 25°C under a white fluorescent light of 37.5 μmol·m$^{-2}$·s$^{-1}$ with a 12:12 light:dark regime.

2.2. Toxicity test

Algae cells were incubated in fresh medium for 7 days before experiments. The algal cells with an initial concentration of approximately 4 × 10$^6$ cells/mL were inoculated in 100 mL medium in 250 mL Erlenmeyer flasks and cultured using the same conditions above. The algae were exposed to pollutants during the logarithmic growth phase. The test flasks were hand shaken at least thrice daily until the end of the assays.

The antibiotic solutions were prepared by serial dilutions of the stock solutions with deionized water. The suitable antibiotic exposure concentration ranges were explored before. The concentrations of CLA were finally set to 0 (control), 1, 10, 20, 40, 60 and 100 μg/L. The concentrations of LEV were set to 0, 1, 10, 50, 100, 500, and 1000 μg/L. According to the research of 26, the joint effects of chemical mixtures are connected with the ratios of individual chemicals and become most evident at the equitoxic ratio. Therefore, the toxicity of CLA and LEV mixtures was determined with an equitoxic ratio based on the ratio of their EC$_{50}$ at 96 h. The total concentration of the mixture (sum of the concentrations of the two antibiotics) was varied from 0 μg/L to 200 μg/L. All controls and samples were prepared in triplicate.

The joint toxicity was assessed using several joint-effect indices, including toxicity unit (TU), addition index (AI), mixed toxicity index (MTI), and combination index (CI). The TU and MTI values were calculated by using Eq. 1–4:

$$TU_i = \frac{C_i}{EC_{50i}}$$  \hspace{1cm} (1)

$$TU = \sum TU_i$$  \hspace{1cm} (2)

$$TU_0 = \frac{TU}{max (TU_i)}$$  \hspace{1cm} (3)

$$MTI = \frac{\log TU_0 - \log TU}{\log TU}$$  \hspace{1cm} (4)

where $C_i$ and $EC_{50i}$ are the concentration and EC$_{50}$ of chemical i in combination, respectively.

Based on the TU value, the joint effects are additive when $TU = 1$; antagonistic when $TU > TU_0$; synergetic
when \( TU < 1 \); independent when \( TU = TU_0 \); partly additive when \( TU_0 > TU > 1 \) [27]. For MTI judgment, the types of combined effects are considered additive, antagonistic, synergistic, or independent when \( MTI = 1, < 0, > 1, \) or \( 0, \) respectively. In addition, when \( 1 > MTI > 0 \), the joint toxicity is partly additive (Sprague). Al was computed as follows: if \( TU = 1 \), then \( AI = TU - 1 \); if \( TU < 1 \), then \( AI = 1/TU - 1 \); if \( TU > 1 \), then \( AI = -TU - 1 \). When Al is equal, lower than, or greater than 0, then the joint action is additive, antagonistic, or synergistic, respectively [28]. Cl value was calculated as \( Cl = \frac{C}{C_{cw}} \), where \( C_{cw} \) is chemical i ‘alone’ that inhibits a system \( x \%), using the CompuSyn software (www.combosyn.com). The effects on the mixture are additive, antagonistic, or synergistic when \( Cl \) is equal, lower than, or greater than 1, respectively [29].

2.3. Biochemical indicators

Biomass and Fv/Fm were tested every 24 h during the 96 h incubation. Other indicators were determined at 96 h (end of the experiments). A linear relationship was used to determine the biomass of algal cell suspension from the optical density at 680 nm. The growth rate (\( \mu \)) of algal cells was calculated as \((lnN_t - lnN_0)/(t_1 - t_0)\), and the inhibition rate (\( I \)) was calculated as \( [(\mu_0 - \mu_1)/\mu_0] \times 100\% \), where \( N_1 \) and \( N_0 \) are the cell concentrations determined at time \( 1 \) (\( t_1 \)) and 0 (\( t_0 \)), respectively. Variables \( \mu_0 \), and \( \mu_1 \) are the growth rates of the control and treatment groups, respectively.

The PEA instrument (Hansatech Instruments Ltd, UK) was used to determine Fv/Fm after a 2 mL algal solution was incubated in the dark for 15 min to complete the re-oxidation of photosystem II (PS II) electron acceptor molecules [30].

Chlorophyll a and carotenoid were extracted from algal cells in 95% ethanol at 4°C in the dark for 24 h. After centrifugation, the supernatant was measured at 470, 649, and 665 nm using a UV/VIS spectrophotometer [20]. The contents were estimated by the following formulas:

Chlorophyll a (mg/L) = 16.95A_665 - 6.88A_649

(5)

Carotenoid (mg/L) = (1000A_470 - 2.05Chla)/245

(6)

The water-soluble pigment phycobiliprotein (PBP) was extracted with phosphoric acid buffer (PBS) after freeze thawing thrice. For freeze thawing, samples were frozen at \(-80 \) °C in liquid nitrogen and melted at 30 °C. After centrifugation, the supernatant was determined at 565, 620, and 650 nm [31].

The ROS level was measured using the cell permeable indicator 2',7'-dichlorodihydrofluorescein diacetate. The algal cells were collected after centrifugation and resuspended in pure water. The cells were incubated in 10 mmol/L 2',7'-dichlorodihydrofluorescein diacetate solution in dark for 30 min. The fluorescence of each solution was determined at excitation and emission wavelengths of 524 and 535 nm, respectively [32].

The malonaldehyde (MDA) content was estimated using the thiobarbituric acid method. After centrifugation, the collected cells were resuspended in 2 mL 0.6% thiobarbituric acid and 2 mL 10% trichloroacetic acid solution and then incubated in boiling water for 20 min. The samples were finally measured at 450, 532, and 600 nm [33].

For the antioxidant enzyme determination, the cell sample withdrawn from the culture vessels was collected by centrifugation and washed with 0.05 M PBS (pH 7.8). The cells were sonicated in ice bath for disruption and centrifuged at 8000 rpm at 4°C for 15 min. The supernatant was collected for the determination of superoxide dismutase (SOD) and catalase (CAT) activities and protein assay. SOD was determined using the nitrogen blue tetrazolium method and tested at 560 nm [34]. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of nitroblue tetrazolium photooxidation reaction. CAT activity was measured by estimating the decomposition of \( H_2O_2 \) at 240 nm [35]. One unit of CAT activity was defined as the enzyme content causing a decrease in the absorbance of 0.1 per minute.

The enzyme contents were expressed on the basis of protein concentration.

Extracellular MCs were detected directly in the filtered water sample with 0.22 \( \mu \)m glass fiber filters using MC plate kits (Beacon Analytical Systems Inc., USA).

2.4. Statistical analysis

Each treatment had three replicates. One-way analysis of variance followed by Tukey’s multiple comparison tests was used for difference analysis. \( EC_{50} \) values for single chemicals and mixtures were calculated with the logarithmic concentration–response curves of the logistic model fitted in Origin 2016 (OriginLab, USA). The oxidative toxicity of the two single antibiotics and their mixture were compared by integrated biomarker response (IBR) [36].

3. Results and discussion

3.1 Effects of CLA and LEV on M. aeruginosa growth

Figures 1(a–c) present the effects of CLA and LEV, along with their combination at an equitoxic ratio, on the growth of \( M. \) aeruginosa during a 96 h culture period. All the cells in the controls and treatment groups were in logarithmic phase after 4 days of cultivation. The growth of algal cell was inhibited by single
and mixed antibiotics. The biomass was gradually restrained with the increase in time and chemical concentrations. The inhibition of CLA became significant from 72 h, whereas noteworthy growth effects were observed at 48 h of LEV and their mixture (Tables S1 to S3).

Figure 1(d) displays the concentration–response curves of two antibiotics. The $R^2$ values of the curves were all greater than 0.99, indicating that the logistic model fitted the relationship between log antibiotic concentration (mass concentration) and growth inhibition ratios well. Unlike LEV, the curve of CLA showed a platform when the CLA concentration was above 40 µg/L, that is, the growth inhibition effects did not progress under the CLA dosage with high concentration. The performance probably resulted from the regulatory function of antibiotic transporters for influx and efflux of antibiotics. A similar phenomenon was also observed by González-Pleiter et al. [13].

The EC$_{50}$ values of CLA and LEV for *M. aeruginosa* after 96 h incubation under single antibiotic exposure calculated by the logistic model were 43.31 (95% confidence interval: 40.37–47.09 µg/L) and 437.6 µg/L (95% confidence interval: 40.37–47.09 µg/L), respectively. CLA exhibited more negative effects on algal growth than LEV. Quinolone molecules can form drug–enzyme–DNA complexes to trap gyrase and topoisomerase IV and block the passage of DNA replication apparatus [9]. As a kind of quinolone, this action of LEV may have the most pronounced effect when algal cells are dividing. However, CLA, as a protein synthesis inhibitor, can play a negative role, especially when proteins are being assembled in cells [14]. CLA may act on cells prior to LEV. In addition, the toxicity of pollutants is related to their capability for penetration through the cell membrane and thus interaction with action sites. This capability can be modeled by octanol–water partition coefficient ($K_{ow}$) [37]. Given the large log $K_{ow}$ (3.16 for CLA and 0.28 for LEV) [3], CLA may easily enter the cells and inhibit algal growth more compared with LEV.

In other published articles, the EC$_{50}$S of LEV were 58.6 mg/L for *Chlorella vulgaris* (96 h) [38], 65 mg/L for *Scenedesmus obliquus* (96 h) [39], and 12.1 mg/L for *Pseudokirchneriella subcapitata* (72 h) [40]. For CLA, the EC$_{50}$S values were 230 µg/L for *P. subcapitata* (72 h) [41] and 37.1 µg/L for *Desmodesmus subspicatus* (72 h) [42]. As reported, given the high surface/volume ratios and the lack of chloroplasts and mitochondria in

Figure 1. The cell density of *M. aeruginosa* under exposure of CLA (a), LEV (b) or combined antibiotics (c) every 24 h, and the concentration-response plots fitted by Logistic model (d) after 96 h exposure. The Y-axis is the logarithm of the cell number (cells/ml).
cyanobacteria, contaminants can enter cyanobacterial cells and act on photosynthesis and phosphorylation easier than green algae, which makes cyanobacteria more sensitive to xenobiotics [43]. Given the harm of water-blooming cyanobacteria and their sensitivity, the use of M. aeruginosa in our research was appropriate for antibiotic toxicity assessment.

The logistic model well expressed the joint action of binary mixtures on M. aeruginosa. The 96 h EC50 of combined antibiotics was 100.8 µg/L (95% confidence interval: 85.88–116.5 µg/L) and between the values of CLA and LEV. This concentration was composed of 9.164 µg/L CLA and 91.64 µg/L LEV, and the calculated inhibition rates were 3.015% and 19.38% for CLA and LEV under single antibiotic exposure, respectively. The synergistic effect means that the sum of individual effects of the two chemicals is less than the combined effect [44]; the results indicated that the mixture of CLA and LEV can exert synergistic effects on M. aeruginosa growth. Thus, the toxicity effect of LEV was remarkably enhanced by the low concentration of CLA (from 0.09091 µg/L to 18.18 µg/L). This joint effect was also proven by the results of the combined effects index (Table 1). One possible reason for the synergism was the decreased level of enzymes that metabolized LEV due to the inhibition of protein synthesis by CLA, thus allowing more LEV molecules to reach the action site [4]. Several researchers have focused on the joint effects of binary antibiotic mixtures on algae; different combinations often have dissimilar effect types. Liu et al. [15] discovered the antagonistic toxicity of spiramycin and amoxicillin mixed at equitoxic ratio on M. aeruginosa; the interaction of the two antibiotics was related to their mixture ratio. Yang et al. [4] reported the synergistic effects of binary mixtures of antibiotics, such as trimethoprim and sulfonamides or tylosin and tetracyclines, belonging to the same class and other combinations, on P. subcapitata. Hagenbuch and Pinckney [14] indicated that the combined effect types differ for various algae species; for instance, the mixtures of lincomycin and ciprofloxacin showed synergistic toxicity against Cylindrotheca Closterium but an additive one for Navicula ramosissima. The above research results demonstrate the complex joint toxicity of antibiotics. Thus, the effects of various combinations on algae need to be studied because of the co-occurrence of antibiotics in aquatic environment.

### 3.2. Effects of CLA and LEV on photosynthetic pigments and phycobiliproteins

Photosynthesis is an essential energy metabolism process for algae to satisfy their needs for growth and reproduction [45]. Four typical indicators, including Fv/Fm, chlorophyll a, carotenoid, and PBP, were selected to express the photosynthetic ability of M. aeruginosa cells. Fv/Fm represents the maximum quantum yield of PSII photochemistry and is frequently used to evaluate the efficiency of PSII in algal cells [33]. As shown in Figures 2(a–c), the Fv/Fm of M. aeruginosa was markedly affected by CLA and LEV exposure and their mixture with antibiotic concentration enrichment (p < 0.05). At a certain concentration range, the Fv/Fm values reduced continuously from the first day until the end of the test. On day 1, Fv/Fm was inhibited, but not significantly, by CLA but was notably inhibited by LEV at concentrations of 500 and 1000 µg/L (p < 0.05, Table S4 and S5). Similarly, the binary combination concentrations of 60, 100, and 200 µg/L presented constraint (Table S6). However, at this point, no effects were observed on the numbers of algae. This finding may be probably due to the stress response of algae; they can reduce the toxic effects by diluting the concentration of contaminants in cells with continuous growth [43]. The inhibition of Fv/Fm values reached the maximum at the fourth day of exposure. Statistically significant differences between treatment groups and controls were determined by post hoc tests, with values ranging from 20 µg/L to 1000 µg/L for CLA, 50 µg/L to 1000 µg/L for LEV, and 20 µg/L to 200 µg/L for their mixture. Given that Fv/Fm reflects the probability that PSII reaction centers use the available excitation energy [46], the decrease in Fv/Fm in M. aeruginosa resulting from antibiotic exposure indicates that the ability of cyanobacteria to use light energy for photochemical reactions declined [47].

Chlorophylls play important roles in various photosynthetic processes, including capturing light, delivering energy to the reaction center, and converting light energy [48]. Chlorophyll a, as a necessary substance for cell photosynthesis, is an important characteristic of phytoplankton vitality [49]. From Figures 2(d–f), CLA, LEV, and their combination all significantly affected the synthesis of chlorophyll a and carotenoid (p < 0.05). For chlorophyll a, significant inhibition appeared from 10 µg/L to 100 µg/L for CLA, from 10 µg/L to 1000 µg/L for LEV and from 20 to 200 µg/L for their mixture. For carotenoid, a remarkable suppression was observed from 40 µg/L to 100 µg/L for CLA, from 50 µg/L to 1000 µg/L for LEV and from 60 µg/L to 200 µg/L for their mixture. Compared with chlorophyll a, carotenoid was less affected under stress probably due to its antioxidant capability. This compound can protect algae against ROS by quenching singlet oxygen and the excited states of photoactivated molecules, along

### Table 1. Joint effect evaluation of CLA and LEV by different indexes.

| Evaluation methods | Values | Effect type |
|--------------------|--------|-------------|
| TU                 | 0.4099 (<1) | Synergism |
| AI                 | 1.439 (>0)  | Synergism |
| MTI                | 2.287 (>1)  | Synergism |
| CI                 | 0.3868 (<1) | Synergism |
with scavenging free radicals [50]. However, these features only make algae resistant to antibiotics with relatively low concentration. The synthesis of carotenoid can still be blocked by increased amounts of antibiotics.

In cyanobacteria photosystem, PBP and linker peptides constitute an important light-harvesting complex, phycobilisome, which can absorb and transport light energy to the reaction center and supply downstream photosynthetic processes with excitation energy [51,52]. The three main types of PBP include phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC). The energy is transferred from PE to PC, then to APC, and finally to chlorophyll a [53]. As shown in Figures 2(g–i), the single and combined antibiotics showed dose-dependent effect on the contents of the three kinds of PBP. The synthesis of PBP was stimulated by compounds with low concentration but inhibited by their elevated concentration. This induction is probably due to the oxidation resistance of PBP. PBPs are known as antioxidants and free radical scavengers [53]. In addition, given that PBP can serve as nitrogen storage compounds in cyanobacteria, algae can increase the PBP synthesis to improve the organic nitrogen reserves under stress [54].

The 96 h EC_{50} was calculated with Fv/Fm, chlorophyll a, and PBP (Table S7). CLA showed more toxicity to the photosynthesis of M. aeruginosa than LEV. Compared with the EC_{50} values calculated with growth inhibition, the values obtained for Fv/Fm and chlorophyll a were lower, indicating that the suppression of photosynthesis by both antibiotics was stronger than biomass. The inhibition of photosynthesis usually occurs prior to the decrease in algal growth [15]. The high EC_{50} of PBPs may be attributed to their excellent antioxidant capacity. In general, when cells are faced with antibiotics with low concentration, they can adjust the sizes and numbers of light-harvesting antennas to optimize the light energy utilization to protect themselves from pollutant exposure [55]. With the increase in antibiotic level, the damage to photosynthetic components and loss of photosynthetic pigments resulting from antibiotic exposure reduce the solar radiation absorption and lead to a decrease in the photochemical reaction efficiency of PSI.

Isobologram was depicted to intuitively evaluate the combined toxicity of CLA and LEV on Fv/Fm, chlorophyll a, and PBP [56]. As shown in Figure 3, the points corresponding to the three indicators were all located in the left of the line (x + y = 1), which indicates that
CLA and LEV can affect the photosystem of *M. aeruginosa* synergistically. Given the low EC$_{50}$ photosynthetic indexes were more suitable than growth to detect the combined toxicity of antibiotics. To our knowledge, the combined effects of antibiotics on PSII and PBP contents of algae were rarely researched. Similar to our results, Liu et al. [15] observed the downregulation of three photosynthesis-related genes, namely, *psbA, psbB,* and *rbcl,* in *M. aeruginosa* exposed to binary mixtures of spiramycin and amoxicillin; this finding suggests that the electron transporter was blocked, and the toxic effects to photosystem were enhanced compared with the single antibiotic exposure. Lu et al. [57] discovered that the Fv/Fm of *Chlorella pyrenoidosa* and *M. aeruginosa* is sensitive to the exposure of the mixture of copper (II) and chlortetracycline.

### 3.3. Effects of CLA and LEV on ROS and MDA

In algal cells, ROS, including superoxide (O$_2^−$), hydroxyl radicals (OH−), hydrogen peroxide (H$_2$O$_2$) and single oxygen (¹O$_2$), can be generated by respiratory machinery and photosynthetic electron transport chain [58]. In general, intracellular ROS play important roles in cellular processes, such as infection prevention and cell signaling [59]. However, the excessive ROS production under xenobiotic stress in algal cells may cause damage to biomolecules or programmed cell death [60]. In addition, ROS can attack the lipid in membranes and cause lipid peroxidation. As one of the end-products formed via the decomposition of polyunsaturated fatty acid hydroperoxides, MDA can be used as an indirect test of injury to cellular membranes and membrane integrity [61,62]. Therefore, ROS and MDA were selected to characterize oxidative damage in this study.

Figure 4 shows the significant concentration-dependent increase in ROS and MDA in *M. aeruginosa* cells caused by individual exposure to CLA and LEV. The increased levels of ROS and MDA indicate that the algal cells were all under severe oxidative stress when treated with different antibiotics. At low concentration of antibiotics, ROS and MDA contents showed non-significant increases, suggesting that the cells can still maintain homeostasis. ROS and MDA were then stimulated with increased levels of antibiotics. The relative activities of ROS and MDA contents were 222.8% and 189.3% of the control for the 100 µg/L CLA treatment groups, respectively; the values reached 138.9% (ROS) and 132.7% (MDA) for the 100 µg/L LEV treatment groups, indicating that CLA showed more oxidative damage to algae than LEV.

Antibiotics can generate O$_2^−$ in bacteria [63]. Intracellular O$_2^−$ and H$_2$O$_2$ may give rise to the release of Fenton-active ferrous iron, causing the Fenton reaction to produce OH−, which is highly reactive with DNA [58]. This mechanism of iron-mediated Fenton reactions has been suggested as the cell-killing capability of antibiotics [64]. Cyanobacteria cells contain more iron than heterotrophic bacteria. Thus, this process may be one of the generation mechanisms of ROS in cyanobacteria under antibiotic stress. Antibiotics can also interfere with the electron transport chains in the photosynthetic system, causing the formation of ROS [65]. The ROS-mediated oxidative damage to cell membranes can change the fluidity and permeability and eventually alter cellular metabolic functions [66]. Moreover, MDA, which is toxic and mutagenic to cells, can react with proteins and DNA [67]. Similar to our results, antibiotics, including ciprofloxacin, sulfamethoxazole, and streptomycin, can induce ROS and MDA production [48,68,69]. ROS accumulation is an important mechanism for antibiotics to hinder photosynthesis, damage membrane system and inhibit algal growth. The results of linear regression between ROS and other biomarkers (Table 58) prove that ROS are strongly associated with the changes in growth and photosynthesis.

For the binary combination of CLA and LEV, the levels of ROS and MDA also increased with the increase in antibiotic concentration (Figures 4c,f, respectively). The significant differences between the test groups and control for ROS were noticed when the concentration of the mixture was over 20 µg/L, whereas for MDA, the value was over 5 µg/L. The results showed that the mixture of CLA and LEV can induce ROS production and lipid oxidation at low concentrations. The ROS and MDA contents in 100 µg/L groups were 236.9% and 231.8% of the control, respectively, which were considerably higher than that of CLA or LEV alone, demonstrating that the stimulatory effects on ROS and MDA were synergistic for the two antibiotics. This synergistic induction of oxidative stress...
has also been observed in the mixture of erythromycin and enrofloxacin used on Chlorella vulgaris [70], tetrabromobisphenol A and sulfadiazine on Scenedesmus obliquus [71] and spiramycin and ampicillin on M. aeruginosa [72]. The synergistic effects in ROS production were consistent with the effects on biomass and photosynthesis, which indicated the excessive accumulation of ROS as one of the causes of synergistic growth inhibition of CLA and LEV. These results imply that the mixed antibiotics in aquatic environment may pose serious damage to the ecosystem.

### 3.4. Effects of CLA and LEV on antioxidants enzymes

When facing oxidative stress, cells can neutralize free radicals through an elaborate antioxidant defense system to maintain normal growth [73]. SOD and CAT are important antioxidant enzymes in algal cells. SODs are a family of antioxidant that catalyze the disproportionation of two superoxide anions (O\textsubscript{2}^{-}) into hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and oxygen molecule [30]. Given that O\textsubscript{2}^{-} is the first ROS generated and remains for a long time in cells, SOD can be the first line of defense against oxidation [74]. H\textsubscript{2}O\textsubscript{2} is decomposed into oxygen and water by CAT subsequently.

The increase in SOD and CAT activities became concentration dependent after 96 h single exposure to CLA and LEV (Figure 5). The production of SOD and CAT was elevated significantly by CLA at concentrations from 40 µg/L to 100 µg/L and by LEV at concentrations from 50 µg/L to 1000 µg/L. When cells were exposed to less antibiotics, non-enzymatic antioxidants, including carotenoid and PBP, showed response prior to that of antioxidant enzymes. With the increase in
antibiotics, the photosynthetic system of *M. aeruginosa* was inhibited, and antioxidant enzymes were activated. The increase in antioxidant enzyme activity was consistent with the changes in ROS. The results of linear regression between ROS and SOD and CAT (Table S8) also proved the strong correlation between ROS and these two enzymes, indicating that increased ROS level is responsible for the activation of the cellular defense system. Moreover, the raised SOD activity verified the production of O$_{2}^•$−. The removal of superoxide by SOD can prevent the production of more radicals by hindering the development of Haber–Weiss and Fenton reactions [75]. The promotion of CAT can be attributed to the H$_2$O$_2$ directly produced under antibiotic stress and indirectly generated during the scavenging of free radicals by SOD. The increase in these antioxidant activities may also be ascribed to the up-regulation of related gene expression in cyanobacteria [76]. In addition, although algal cells can still continuously synthesize SOD and CAT when exposed to high concentrations of antibiotics, the degree of oxidative damage was not diminished. Thus, the antioxidants were no longer sufficient to protect the cells against ROS.

The synthesis of SOD and CAT of *M. aeruginosa* was also induced by the mixture of CLA and LEV (Figure 5). The activities of SOD and CAT remarkably increased starting from 20 and 5 µg/L, respectively. CLA and LEV also showed synergistic stimulation on antioxidant production, similar to the effects on oxidative stress. Unlike the continuous increase in SOD activity, the CAT levels in the 200 µg/L groups were slightly lower than

Figure 5. SOD and CAT activities in *M. aeruginosa* after 96 h exposure to CLA (a and d), LEV (b and e) and combined antibiotics (c and f). Different lowercase letters indicate differences between the treatments (P < 0.05). Line chart in blue expressed the percentage of SOD or CAT in each group to the control.
those in the 100 µg/L groups, showing that antibiotics with high concentration can inhibit CAT synthesis. Qin and Liu [77] indicated that fluoroquinolone molecules can interact with CAT through electrostatic interactions and change the conformation and function of the enzyme. This phenomenon suggests that a small amount of CLA in the mixture led to more LEV molecules entering the cells, thereby causing the inhibition of CAT. In summary, the binary combinations of CLA and LEV resulted in more severe oxidative stress than single antibiotic exposure, which hindered the photosynthetic process, damaged the membranes, stimulated the antioxidant system, and eventually inhibited cell growth.

3.5. Effects of CLA and LEV on MC release

*M. aeruginosa*, as a kind of toxigenic cyanobacteria, can produce the common toxins, MCs. The occurrence of MCs in aquatic environment is causing widespread concern because they can increase ROS and inhibit serine/threonine protein phosphatases irreversibly, thus resulting in acute to chronic health problems [78]. The release of MCs into water is regulated by various environmental factors including antibiotics. In this paper, CLA and LEV with low levels positively affected the extracellular MC contents and showed negative effects on them at the highest concentration (Figure 6). The content of extracellular MCs increased to 90.1% and 71.8% compared with the control upon exposure to 60 µg/L CLA and 500 µg/L LEV, respectively. Other contaminants, such as glufosinate [22], TiO₂ nanoparticles [24] and chlorine [79], also cause the release of MCs. MCs in *M. aeruginosa* cells can act as antioxidants against oxidative stress, stabilize the photosynthetic apparatus, and modulate proteins [80]. Therefore, the elevated extracellular MC contents may be a response of algal cells to the accumulation of ROS caused by CLA or LEV. In addition, the increased MDA level indicates cell membrane dysfunction and cell lysis. The intracellular MCs were gradually released after cell lysis [21]. This mechanism was proven by the phenomenon in which the extracellular MC contents did not decrease with the inhibition of cell growth. Meanwhile, the upregulation of MC synthesis and transport genes, including *mcyB*, *mcyA*, and *mcyH*, was discovered upon antibiotic exposure [72,81]. Thus, CLA and LEV may promote MC production and release at the genetic level. Notably, the contents of extracellular MC in each group of CLA were larger than those of LEV when the CLA concentrations were lower than those of LEV, indicating that CLA was more effective in enhancing the release of MC. The release of MCs can be enhanced by inhibiting membrane protein synthesis to increase the cell permeability due to the action of CLA [23].

The decline in MC concentrations in the medium may be related to the adsorption of nitrogen in algal cells. Nitrogen uptake modulates the MC contents [82]. The decrease in PBP due to antibiotic exposure may decrease the absorption of nitrogen, thus inhibiting MC synthesis and release. Moreover, the cell lysis caused by exposure to high concentrations of antibiotics resulted in the discharge of PBP; MCs in aquatic environments can be degraded in a photosensitive manner by PBP [83]. The similar results on promotion with low level and inhibition with high level of contaminants on extracellular MCs were also discovered by Wu et al. [84] and Yang et al. [85].

The effects of binary joint of CLA and LEV on MCs varied with different mixture concentrations (Figure 6(c)). A significant stimulation was discovered from 20 µg/L to 60 µg/L. Then, the contents of MCs decreased in the 100 and 200 µg/L groups. The combined antibiotics can promote the MC release at relatively low concentration compared with single antibiotics, which indicates the synergistic effects of CLA and LEV on MCs. This synergistic promotion effect on MCs was consistent with ROS and MDA, demonstrating that the accumulation of more ROS and serious cell rupture under joint exposure of CLA and LEV may account for the increase in MC content in the medium. Comparable outcomes were also reported by other researchers. Wang et al. [72] indicated that the combination of spiramycin

![Figure 6](image.png)

**Figure 6.** Extracellular MC contents in *M. aeruginosa* after 96 h exposure to CLA (a), LEV (b) and combined antibiotics (c). Different lowercase letters indicate differences between the treatments (P < 0.05). Line chart in blue expressed the percentage of MC in each group to the control.
and ampicillin can stimulate MC synthesis and release, especially at the equivalent ratio, and the expression of MC-synthesis-related gene is also promoted. 15, stated the synergistic inducement of the synthesis and discharge of MCs by mixed antibiotics of spiramycin and amoxicillin. Given that MCs have been proven to be liver tumor promoter, the promotion of MC discharge by the conjunction with LEV of CLA in aquatic environment may cause severe secondary risks to aquatic organisms.

3.6. IBR

As a simple multivariate graphic method, IBR is used gradually to integrate a battery of biomarkers and evaluate the adverse effects of contaminants to aquatic organisms [86–88]. The response results of antioxidant enzyme activities and ROS, MDA and MC contents after 96 h exposure were selected for the analysis by the IBR method to comprehensively assess the oxidative toxicity of CLA, LEV and their mixture to *M. aeruginosa*. The reaction of the five indicators was normalized and expressed as star plots (Figure 7). The five biomarkers were all stimulated by antibiotics and produced different patterns of star plots for each compound. The order of IBR values of the three treatments was combination (15.55) > CLA (8.742) > LEV (8.173), indicating the strong combined oxidative stress caused by CLA and LEV.

4. Conclusion

The results of the present work indicate that CLA and LEV exhibited toxic effects on the growth of *M. aeruginosa* via disruption of the photosynthetic system and production of excessive ROS to damage the cell membrane. Synergistic actions of binary joint of CLA and LEV at an equitoxic ratio were discovered on cell density and photosynthesis. The combination of CLA and LEV showed strong oxidative stress to algal cells and intense stimulation on antioxidant enzyme synthesis and MC release. Given that MCs have harmful effects on human health, the ecological effects of combined pollution of CLA and LEV in aquatic environment should be taken seriously.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**References**

[1] Na TW, Kang TW, Lee KH, et al. Distribution and ecological risk of pharmaceuticals in surface water of the Yeongsan River, Republic of Korea. Ecotox Environ Safe. 2019;181:180–186.
[2] Ortiz de García SA, Pinto Pinto G, García-Encina PA, et al. Ecotoxicity and environmental risk assessment of pharmaceuticals and personal care products in aquatic environments and wastewater treatment plants. Ecotoxicology. 2014;23(8):1517–1533.
[3] Kim JW, Ishibashi H, Yamauchi R, et al. Acute toxicity of pharmaceutical and personal care products on freshwater crustacean (*Thamnocephalus platyurus*) and fish (*Oryzias latipes*). J Toxicol Sci. 2009;34(2):227–232.
[4] Yang LH, Ying GG, Su HC, et al. Growth-inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga *Pseudokirchneriella subcapitata*. Environ Toxicol Chem. 2008;27(5):1201–1208.
[5] Fent K, Weston A, Caminada D. Ecotoxicology of human pharmaceuticals. Aquat Toxicol. 2006;76(2):122–159.
[6] Peters DH, Clissold SP. Clarithromycin – a review of its antimicrobial activity, pharmacokinetic properties and therapeutic potential. Drugs. 1992;44(1):117–164.
[7] Gaynor M, Mankin AS. Macrolide antibiotics: binding site, mechanism of action, resistance. Curr Top Med Chem. 2003;3(9):949–960.
[8] Davis R, Bryson HM. Levofloxacin – a review of its antibacterial activity, pharmacokinetics and therapeutic efficacy. Drugs. 1994;47(4):677–700.
[9] Drlica K, Malik M. Fluoroquinolones: action and resistance. Curr Top Med Chem. 2003;3(3):249–282.
[10] Barbosa-MO, Moreira NFF, Ribeiro AR, et al. Occurrence and removal of organic micropolllutants: an overview of the watch list of EU Decision 2015/495. Water Res. 2016;94:257–279.
[11] Van Doorslaer X, Dewulf J, Van Langenhove H, et al. Fluoroquinolone antibiotics: an emerging class of environmental micropolllutants. Sci Total Environ. 2014;500-501:250–269.
[12] Tran NH, Hoang L, Nghiem LD, et al. Occurrence and risk assessment of multiple classes of antibiotics in urban canals and lakes in Hanoi. Vietnam. Sci Total Environ. 2019;692:157–174.
[13] González-Pleiter M, Gonzalo S, Rodea-Palomares I, et al. Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: implications for environmental risk assessment. Water Res. 2013;47(6):2050–2064.
[14] Hagenbuch IM, Pinckney JL. Toxic effect of the combined antibiotics ciprofloxacin, lincomycin, and tylosin on two species of marine diatoms. Water Res. 2012;46(16):5028–5036.
[15] Liu Y, Zhang J, Gao BY, et al. Combined effects of two antibiotic contaminants on Microcystis aeruginosa. J Hazard Mater. 2014;279:148–155.
[16] Patel M, Kumar R, Kishor K, et al. Pharmaceuticals of emerging concern in aquatic systems: chemistry, occurrence, effects, and removal methods. Chem Rev. 2019;119(6):3510–3673.
[17] Wu XY, Wan L, Zhang WH, et al. Resistance of cyanobacteria Microcystis aeruginosa to erthyrmcyin with multiple exposure. Chemosphere. 2020;249:126147.
[18] Lin SY, Yu X, Fang JY, et al. Influences of the microcystin-lyttenzyme on cyanobacteria treatment with potassium permanganate. Water Res. 2020;177:115786.
[19] Mao YF, Yu Y, Ma ZX, et al. Azithromycin induces dual effects on microalgae: roles of photosynthetic damage and oxidative stress. Ecotoc Environ Safe. 2021;222(5):112496.
[20] Yang CY, Zhou J, Liu SL, et al. Allelochemical induces growth and photosynthesis inhibition, oxidative damage in marine diatom Phaeodactylum tricornutum. J Exp Mar Biol Ecol. 2013;444:16–23.
[21] Hu X, Liu YG, Zeng GM, et al. Effects of d-methion stress on the growth of and microcystin release by the freshwater cyanobacterium Microcystis aeruginosa FACHB-905. Chemosphere. 2014;113:30–35.
[22] Zhang Q, Song Q, Wang C, et al. Effects of gluosinate on the growth of and microcystin production by Microcystis aeruginosa at environmentally relevant concentrations. Sci Total Environ. 2017;575:513–518.
[23] Wang ZY, Chen QW, Hu LM, et al. Combined effects of binary antibiotic mixture on growth1. Environ Sci Pollut Res. 2017b;25(1):736–748.
[24] Zhang JX, Jiang LJ, Wu D, et al. Effects of environmental factors on the growth and microcystin production of Microcystis aeruginosa under TiO2 nanoparticles stress. Sci Total Environ. 2020a;734:139443.
[25] EMEA, 2018. European Chemical Agency. Guideline on the environmental risk assessment of medicinal products for human use. Doc ref: EMEA/CHMP/SWP/4447/00 Rev. 1.
[26] Lin Z, Ping Z, Kong D, et al. The ratios of individual chemicals in a mixture determine the degree of joint effect: the climax hypothesis. Arch Environ Contam Toxicol. 2005;49(1):1–8.
[27] Sprague JB, Ramsay BA. Lethal levels of mixed copper-zinc solutions for juvenile salmon. J Fish Res Board Can. 1965;22(2):425–432.
[28] Marking LL, 1977. Method for assessing additive toxicity of chemical mixtures. Aquatic toxicology and Hazard Evaluation, ASTM STP Publication 634, 99–108.
[29] Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev. 2006;58(3):621–681.
[30] Pokora W, Tukaj Z. The combined effect of anthracene and cadmium on photosynthetic activity of three desmodesmus (Chlorophyta) species. Enecot Environ Safe. 2010;73(6):1207–1213.
[31] Fan GD, Zhou JJ, Zheng XM, et al. Growth inhibition of Microcystis aeruginosa by copper-based MOF: performance and physiological effect on algal cells. Appl Organomet Chem. 2018;32(12):4600.
[32] Chen L, Mao F, Kirumba GC, et al. Changes in metabolites, antioxidant system, and gene expression in Microcystis aeruginosa under sodium chloride stress. Ecotoc Environ Safe. 2015;122:126–135.
[33] Wan L, Wu XY, Ding HJ, et al. 2020. Toxicity, biodegradation, and metabolic fate of organophosphorus pesticide trichlorfon on the freshwater algae Chlamydomonas reinhardtii. J Agric Food Chem. 2020;68(6):1645–1653.
[34] Beyer WF, Fridovich I. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem. 1987;161(2):559–566.
[35] Calmak I, Marschner H. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiol. 1992;98(4):1222–1227.
[36] Broeg K, Lehtonen KK. Indices for the assessment of environmental pollution of the Baltic Sea coasts: integrated assessment of a multi-biomarker approach. Mar Pollut Bull. 2006;53(8–9):508–522.
[37] He H, Yu J, Chen G, et al. Acute toxicity of butachlor and atrazine to freshwater green alga Scenedesmus obliquus and cladoceran Daphnia carinata. Ecotoc Environ Safe. 2012;80:91–96.
[38] Xiong QJ, Kurade MB, Jeon BH. Biodegradation of levofloxacin by an acclimated freshwater microalgae, Chlorella vulgaris. Chem Eng J. 2017a;313:1251–1257.
[39] Xiong QJ, Kurade MB, Patil DV, et al. Biodegradation and metabolic fate of levofloxacin via a freshwater green alga, Scenedesmus obliquus in synthetic saline wastewater. Algal Research-Biomass Biofuels and Bioproducts. 2017c;25:54–61.
[40] Robinson AA, Belden JB, Lydy MJ. Toxicity of fluoroquinolone antibiotics to aquatic organisms. Environ Toxicol Chem. 2005;24(2):423–430.
[41] Villain J, Mingeuez L, Halm-Lemeille MP, et al. Acute toxicities of pharmaceuticals toward green algae. Mode of action, biopharmaceutical drug disposition classification system and quantile regression models. Ecotoc Environ Safe. 2016;124:337–343.
[42] Baumann M, Weiss K, Maletzki D, et al. Aquatic toxicity of the macrolide antibiotic clarithromycin and its metabolites. Chemosphere. 2015;120:192–198.
[43] Mao F, He Y, Kushnaro A, et al. Effects of benzophenone-3 on the green alga Chlamydomonas reinhardtii and the cyanobacterium Microcystis aeruginosa. Aquat Toxicol. 2017;193:1–8.
[44] Yang GF, Jin RC. The joint inhibitory effects of phenol, copper (II), oxytetracycline (OTC) and sulfide on Anammax activity. Bioresour Technol. 2012;126:187–192.

[45] Sheng H, Niu XJ, Song Q, et al. Physiological and biochemical responses of Microcystis aeruginosa to phosphate. Environ Pollut. 2019;247:165–171.

[46] Parkhill JP, Mailet G, Cullen JJ. Fluorescence-based maximal quantum yield for PSI as a diagnostic of nutrient stress. J Phycol. 2001;37(4):517–529.

[47] Smedbol É, Lucotte M, Labrecque M, et al. Phytoplankton growth and PSI efficiency sensitivity to a glyphosate-based herbicide (Factor 540%). Aquat Toxicol. 2017;192:265–273.

[48] Xiong JQ, Kurade MB, Kim JR, et al. Ciprofloxacin toxicity and its co-metabolic removal by a freshwater microalga Chlamydomonas mexicana. J Hazard Mater. 2017b;323:212–219.

[49] Du DY, Wang J, Zhu ZF, et al. Comprehensive assessment of three typical antibiotics on cyanobacteria (Microcystis aeruginosa): The impact and recovery capability. Ecotoxicol Environ Saf. 2018;160:84–93. doi:10.1016/j.ecoenv.2018.05.035.

[50] Domonkos I, Kis M, Gombos Z, et al. Carotenoids, versatile components of oxygenic photosynthesis. Prog Lipid Res. 2013;52(4):539–561.

[51] Wahadoszamen M, Krüger TPJ, Ara AM, et al. Charge transfer states in phycobilisomes. Biochim Biophys Acta-Bioenerg. 2020;1861(7):148187.

[52] Xiang R, Shi JQ, Zhang HB, et al. Chlorophyll a fluorescence and transcriptome reveal the toxicological effects of bisphenol A on an invasive cyanobacterium, Cylindrospermopsis raciborskii. Aquat Toxicol. 2018;200:188–196.

[53] Manirafasha E, Ndikubwimana T, Zeng X, et al. Phycobiliprotein: potential microalgae derived pharmaceutical and biological reagent. Biochem Eng J. 2016;109:282–296.

[54] Zaccaro MC, Salazar C, de Caire GZ, et al. Lead toxicity in cyanobacterial popphyry metabolism. Environ Toxicol. 2001;16(1):61–67.

[55] Du J, Qiu B, Pedrosa Gomes M, et al. Influence of light intensity on cadmium uptake and toxicity in the cyanobacterium Synechocystis sp. PCC6803. Aquat Toxicol. 2019;211:163–172.

[56] Zhang Y, Yang RX, Wang SY, et al. Influence of humic substances on the toxic effects of cadmium and DBS to the green alga Scenedesmus obliquus. Environ Toxicol Pharmacol. 2019;68:94–100.

[57] Lu L, Wu YX, Ding HJ, et al. The combined and second exposure effect of copper (II) and chlorotetracycline on fresh water algae, Chlorella pyrenoidosa and Microcystis aeruginosa. Environ Toxicol Pharmacol. 2015;40 (1):140–148.

[58] Latifi A, Ruiz M, Zhang –C-C. Oxidative stress in cyanobacteria. FEMS Microbiol Rev. 2009;33(2):258–278.

[59] Pospišil P. Production of reactive oxygen species by photosystem II. Biochim Biophys Acta-Bioenerg. 2009;1787(10):1151–1160.

[60] Ozrol A, Piotrowicz-Cieślak Al. Levofloxacin is phototoxic and modifies the protein profile of lupin seedlings. Environ Sci Pollut Res. 2017;24 (28):22226–22240.

[61] Hong Y, Hu HY, Xie X, et al. Grameine-induced growth inhibition, oxidative damage and antioxidant responses in freshwater cyanobacterium Microcystis aeruginosa. Aquat Toxicol. 2009;91(3):262–269.

[62] Riaz L, Mahmood T, Coyne MS, et al. Physiological and antioxidant response of wheat (Triticum aestivum) seedlings to fluoroquinolone antibiotics. Chemosphere. 2017;177:250–257.

[63] Albesa I, Becerra MC, Battán PC, et al. Oxidative stress involved in the antibacterial action of different antibiotics. Biochem Biophys Res Commun. 2004;317 (2):605–609.

[64] Cameron JC, Pakrasi HB. Glutathione facilitates antibiotic resistance and photosystem I stability during exposure to gentamicin in cyanobacteria. Appl Environ Microbiol. 2011;77(10):3547–3550.

[65] Nunes B, Veiga V, Frankenbach S, et al. Evaluation of physiological changes induced by the fluoroquinolone antibiotic ciprofloxacin in the freshwater macrophyte species Lemna minor and Lemna gibba. Environ Toxicol Pharmacol. 2019;72:103242.

[66] Bandyopadhyay U, Das D, Banerjee RK. Reactive oxygen species: oxidative damage and pathogenesis. Curr Sci. 1999;77(5):658–666.

[67] Marnett LJ. Lipid peroxidation – DNA damage by malondialdehyde. Mutat. Res.-Fundam. Mol Mech Mutagen. 1999;424(1–2):83–95.

[68] Lv Y, Xu JM, Xu K, et al. Accumulation characteristics and biological response of ginger to sulfamethoxazole and ofloxacin. Environ Pollut. 2020;262:114203.

[69] Qian HF, Li JJ, Pan XJ, et al. Effects of streptomycin on growth of algae Chlorella vulgaris and Microcystis aeruginosa. Environ Toxicol. 2012;27(4):229–237.

[70] Wang GX, Zhang Q, Li JL, et al. Combined effects of erythromycin and enrofloxacin on antioxidant enzymes and photosynthesis-related gene transcription in Chlorella vulgaris. Aquat Toxicol. 2019a;212:138–145.

[71] Wang S, Wang Z, Chen MD, et al. Co-exposure of freshwater microalgae to tetrabromobisphenol a and sulfadiazine: oxidative stress biomarker responses and joint toxicity prediction. Bull Environ Contam Toxicol. 2017a;99(4):438–444.

[72] Wang ZY, Chen QW, Zhang JY, et al. Long-term exposure to antibiotic mixtures favors microcystin synthesis and release in Microcystis aeruginosa with different morphologies. Chemosphere. 2019b;235:344–353.

[73] Urso ML, Clarkson PM. Oxidative stress, exercise, and antioxidant supplementation, exercise, and antioxidant supplementation. Toxicology. 2003;189 (1–2):41–54.

[74] Becerra MC, Albesa I. Oxidative stress induced by ciprofloxacin in Staphylococcus aureus. Biochem Biophys Res Commun. 2002;297(4):1003–1007.

[75] Knaer S, Knauer K. The role of reactive oxygen species in copper toxicity to two freshwater green algae. J Phycol. 2008;44(2):311–319.

[76] Peng JL, Guo JH, Lei Y, et al. Integrative analyses of transcriptomics and metabolomics in Raphidocelis subcapitata treated with chlorothymcin. Chemosphere. 2021;266:128933.

[77] Qin PF, Liu RT. Oxidative stress response of two fluoroquinolones with catalase and erythrocytes: a combined molecular and cellular study. J Hazard Mater. 2013;252-253:321–329.

[78] Khairy H, El-Sheekh M. Toxicological studies on microcystin produced by Microcystis aeruginosa: assessment and management. Egypt J Bot. 2019;59(3):551–566.

[79] Li X, Chen S, Zeng J, et al. Impact of chlorination on cell inactivation, toxin release and degradation of cyanobacteria of development and maintenance stage. Chem Eng J. 2020b;397:125378.
[80] Zhang M, Wang XC, Tao JY, et al. PAHs would alter cyanobacterial blooms by affecting the microcystin production and physiological characteristics of *Microcystis aeruginosa*. Ecotox Environ Safe. 2018;157:134–142.

[81] Liu Y, Cui MW, Zhang J, et al. Impacts of antibiotic contaminants on *Microcystis aeruginosa* during potassium permanganate treatment. Harmful Algae. 2020;92:101741.

[82] Downing TG, Meyer C, Gehringer MM, et al. Microcystin content of *Microcystis aeruginosa* is modulated by nitrogen uptake rate relative to specific growth rate or carbon fixation rate. Environ Toxicol. 2005;20(3):257–262.

[83] Liang J, Liu ZJ, Wu ZH, et al. Study on photoactivatable toxicity of phycobiliprotein from *Microcystis aeruginosa* as potential photoinsecticide. J Appl Phycol. 2015;28(4):2387–2396.

[84] Wu D, Yang SX, Du WC, et al. Effects of titanium dioxide nanoparticles on *Microcystis aeruginosa* and microcystins production and release. J Hazard Mater. 2019;377:1–7.

[85] Yang M, Fan ZQ, Xie YJ, et al. Transcriptome analysis of the effect of bisphenol A exposure on the growth, photosynthetic activity and risk of microcystin-LR release by *Microcystis aeruginosa*. J Hazard Mater. 2020;397:122746.

[86] Beliaeff B, Burgeot T. Integrated biomarker response: a useful tool for ecological risk assessment. Environ Toxicol Chem. 2002;21(6):1316–1322.

[87] Li WX, Zhu L, Du ZK, et al. Acute toxicity, oxidative stress and DNA damage of three task-specific ionic liquids ([C2NH2MIm]BF4, [MOEMIm]BF4, and [HOEMIm]BF4) to zebrafish (Danio rerio). Chemosphere. 2020a;249:126119.

[88] Zhang YQ, Zhang XY, Guo R, et al. Effects of florfenicol on growth, photosynthesis and antioxidant system of the non-target organism *Isochrysis galbana*. Comp Biochem Physiol C-Pharmacol Toxicol Endocrinol. 2020b;233:108764.