

Article

# Effect of Phenyl-Acyl Compounds on the Growth, Morphology, and Toxin Production of *Microcystis aeruginosa* Kützing

Natalia Herrera <sup>1,\*</sup>, Maria Teresa Florez <sup>2</sup>, Juan Pablo Velasquez <sup>1</sup> and Fernando Echeverri <sup>1,\*</sup>

<sup>1</sup> Organic Chemistry Natural Products Group, Institute of Chemistry, Universidad de Antioquia, Calle 67 No. 53-108, 050010 Medellín, Colombia; juanp.velasquez@udea.edu.co

<sup>2</sup> Environmental Management and Modeling Research Group (GAIA), Universidad de Antioquia, Calle 67 No. 53-108, 050010 Medellín, Colombia; mariateresa.florez@gmail.com

\* Correspondence: nahelo241980@gmail.com (N.H.); fernando.echeverri@udea.edu.co (F.E.); Tel.: +57-4219-6595 (F.E.)

Received: 20 November 2018; Accepted: 16 January 2019; Published: 30 January 2019



**Abstract:** The proliferation of cyanobacteria and, consequently, the production of cyanotoxins is a serious public health concern; for their control, several alternatives have been proposed, including physical, chemical, and biological methods. In the search for new alternatives and a greater understanding of the biochemical process involved in the blooms' formation, we report here the effect of eight phenyl-acyl compounds in the growth of *Microcystis aeruginosa* Kützing (assessed as cell density/count and Chl *a* fluorescence concentration) morphology, and production of the toxin microcystin-LR (MC-LR). Caffeic acid and eugenol decreased the growth of *M. aeruginosa* Kützing and the levels of Chl *a*. However, 3,5-dimethoxybenzoic acid and syringic acid caused the opposite effect in the growth; 2' and 4' only affected the Chl *a*. A reduction in the concentration of the MC-LR toxin was detected after treatment with syringic acid, caffeic acid, and eugenol. According to HPLC/MS (High Performance Liquid Chromatography coupled to Mass Spectrometry), a redox process possibly occurs between caffeic acid and MC-LR. The optical microscopy and Scanning Electron Microscopy analyses revealed morphological changes that had been exposed to caffeic acid and vanillin, specifically in the cell division and presence of mucilage. Finally, assays in *Daphnia pulex* De Geer neonates indicated that caffeic acid had a non-toxic effect at concentrations as high as 100 mg/L at 48 h.

**Keywords:** blooms; cyanobacteria; control; toxins; phenyl-acyl compounds; caffeic acid; non-toxic; redox microcystin LR

## 1. Introduction

Currently, water bodies are threatened with eutrophication, which leads to an increased risk of blooms of potentially toxic cyanobacteria. The recognition of its impacts on ecosystems, and the implementation of sustainability programs in water management and bloom prevention, requires new alternatives to, and knowledge of, water treatment to avoid health risks [1]. In particular, the effects of cyanotoxins on human health are well-known. For example, 53 patients died due to intravenous exposure to several microcystins (MCs) during a hemodialysis treatment in Caruaru, Brazil [2].

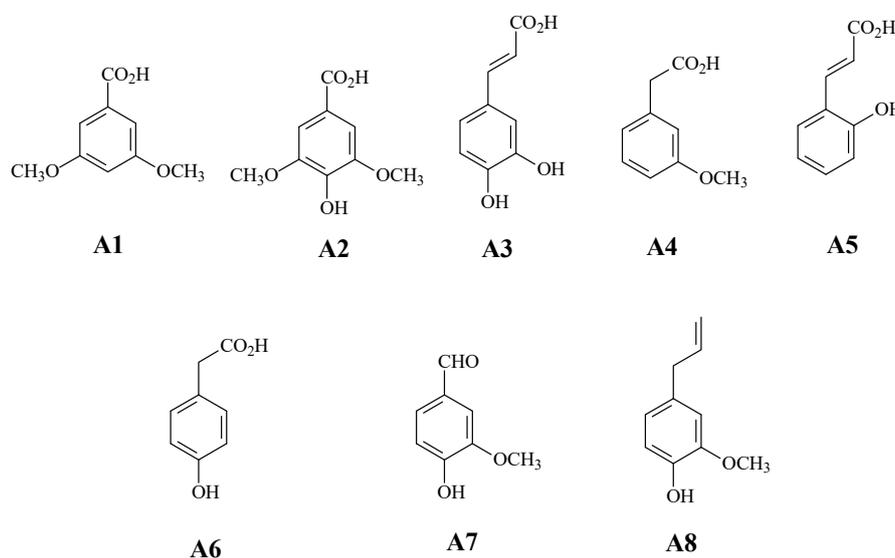
To eliminate intact cyanobacteria and prevent the release of intracellular toxins, different approaches to water treatment have been used, such as flocculation, filtration, and coagulation. However, these methodologies sometimes do not remove all these compounds.

Some algicides, such as copper sulfate, potassium permanganate, and chlorine, are widely used. However, they are highly contaminating and lack specificity, as they cause the death of other organisms

present in the same environment [3]. Copper sulfate is the most commonly used algicide because of its cost, ease of application, and effectiveness [4]. Nevertheless, its intensive application in lakes and reservoirs is a great problem due to copper accumulation and the consequent toxicity [5]. Therefore, new substances and organisms have been sought to control these blooms. For example, vitamin C has been applied to inhibit *Microcystis aeruginosa* Kützing [6]. Besides this, aquatic macrophytes affect photosynthesis and the antioxidant system of this cyanobacteria [7]. Other methods include adsorbent sediments [8], chitosan as a coagulant [9], and coagulation-flotation methods with pre-oxidation assistance [10].

It has been proposed that toxins, such as MCs, stimulate the formation of colonies of *M. aeruginosa* Kützing; thus, they may play a role in their persistence and dominance [11]. Therefore, the inhibition of the production of these toxins could be an alternative to control bloom formation as well avoid harmful effects in animals.

In this study, we assessed the effect of eight phenyl-acyl compounds, derived from natural sources (Figure 1), on the growth and morphology of *M. aeruginosa* Kützing blooms, as well as on the levels of the microcystin-LR (MC-LR) toxin. We also analyzed the toxicity of these compounds to *Daphnia pulex* De Geer.



**Figure 1.** The phenyl-acyl compounds that were assayed in this work: 3,5-dimethoxybenzoic acid (A1, the code in this text), syringic acid (A2), caffeic acid (A3), 3-methoxyphenylacetic acid (A4), 2'-hydroxycinnamic acid (A5), 4'-hydroxyphenylacetic acid (A6), vanillin (A7), eugenol (A8), and, for copper sulfate,  $\text{Cu}(\text{SO})_4$  was assigned code A9.

## 2. Materials and Methods

### 2.1. Sampling and Culturing of *M. aeruginosa*

A cyanobacteria sample was collected from the Riogrande II reservoir (Antioquia, Colombia) in May 2016. *M. aeruginosa* Kützing was the most abundant cell in the blooms, accordingly microscopically analysis. For isolation, samples of 10 mL of bloom were incubated in 250 mL of Blue Green medium BG11 (SIGMA-ALDRICH, St. Louis, MO, USA) [12] in 1 L Erlenmeyer flasks with a photoperiod of 20 h/4 h light 930 lux/dark at 25 °C under continuous aeration using an air pump (Resum Air pump AC 9904, Cranbury, NJ, USA), for seven days. Then, 100 mL of culture were centrifuged at 6000 rpm for 5 min, and the supernatant was transferred to 300 mL of BG11 medium for another seven days to reduce contamination. Finally, 10 mL of the new culture were taken and incubated again in the same culture medium. For culture maintenance, the same initial conditions were followed and every 72 h the medium was replaced.

## 2.2. Substances

The following substances were obtained from SIGMA-ALDRICH (St. Louis, MO, USA): 3,5-dimethoxybenzoic acid (A1, the code in this text), syringic acid (A2), caffeic acid (A3), 3-methoxyphenylacetic acid (A4), 2'-hydroxycinnamic acid (A5), 4'-hydroxyphenylacetic acid (A6), vanillin (A7), eugenol (A8), and, for copper sulfate,  $\text{Cu}(\text{SO})_4$  was assigned code A9 (Figure 1). The MC-LR standard was obtained from MP Biomedicals, LLC (Illkirch, France).

## 2.3. The Growth Inhibition Test

The inoculants for the inhibition tests were prepared from eight-day cultures of *M. aeruginosa* Kützing in the exponential phase. For this purpose, 20 mL of the culture was taken, and then 200 mL of culture medium (BG11) containing each substance at different concentrations (100 mg/L (C3), 50 mg/L (C2), and 25 mg/L (C1)) were added; the cultures were maintained under the same initial conditions for 72 h. After that, samples were taken for growth measurements through cell counts and the Chl *a* level at 0 h and after 72 h of culture. The percentage of inhibition was calculated using the following formula: % cyanobacterial inhibition =  $(1 - \text{treatment/control}) \times 100$ , where the treatment and control are the cell densities of *M. aeruginosa* Kützing exposed and not exposed to different substances, respectively. Finally, the samples were refrigerated for 12 h before lyophilization and then extracted with methanol for the detection of MCs by HPLC/DAD (Diode Array Detector) and HPLC/MS. Each treatment was performed in triplicate, and two growth controls were prepared, one of them with copper sulfate, and the other without substances.

### 2.3.1. Cell Counts

For the cell counts, 200  $\mu\text{L}$  of *M. aeruginosa* Kützing cultures treated with substances were collected and diluted in distilled water to the desired concentration. The counts were performed using a Neubauer chamber under an optical microscope (Nikon, YS2-H, Tokyo, Japan). To calculate the number of cells per mL, the chamber dilution factor was considered. To determine the total number of cells after exposure to each substance, the number of final cells was subtracted from the initial cell number. The cell counts were evaluated by two independent observers and performed in triplicate and repeated four times.

### 2.3.2. The Chl *a* Measurement

Chl *a* fluorescence was used as a proxy for the estimation of *M. aeruginosa* Kützing biomass ( $\mu\text{g}$  Chl *a*/L) [13]. Chl *a* was measured using a Fluorometer (FluoroProbe III, bbe-Moldaenke, Schwentinal, Germany) with an integrated correction factor for colored dissolved organic matter (CDOM) to compensate for UV-LED radiation interference. This instrument measures the fluorescence of Chl *a* in the photosystem II after excitation with five light diodes (370, 470, 525, 570, 590, and 610 nm) with a resolution of 0.01  $\mu\text{g}$  Chl *a*/L.

## 2.4. Morphological Observations under the Optical Microscope and the Scanning Electron Microscope (SEM)

Each culture that had been exposed to the different substances was observed under optical microscope to identify changes in cell morphology, colony appearance, and the presence of mucilage. For this purpose, acid staining using China ink was performed [14], and *M. aeruginosa* Kützing cells were placed on 1  $\text{cm}^2$  plates and fixed with 2.5% glutaraldehyde for 12 h for a SEM (JEOL, JEOL JSM 6490 LV, Tokyo, Japan) analysis. Then, cells were washed three times consecutively with Sorensen's phosphate buffer at neutral pH, followed by distilled water, and subsequently dehydrated by washing with 50%, 75%, 95%, and 100% ethanol. After that, the samples were fixed onto a graphite tape and a thin gold coating (Au) was applied (Denton Vacuum, Denton Vacuum Desk IV equipment, Moorestown, NJ, USA). Finally, they were analyzed by scanning electron microscopy to

obtain high-resolution images. A secondary electron detector was used to evaluate the morphology and topography of the samples.

### 2.5. The Effect of Phenyl-Acyl Compounds on Toxin Concentration

The MCs were analyzed in a Gilson 305 and 306 HPLC with a diode array detector (DAD) using a Luna C-18 column (4.6 × 150 mm, 5 μm, Phenomenex, Torrance, CA, USA) with a mobile phase containing water acidified with 0.05% of TFA (trifluoroacetic acid) in pump A, and acetonitrile that had been acidified in the same way in pump B. The gradient was increased from 10% to 60% of B for 25 min, followed by 10 min at 100% of B, and, finally, returning to the initial conditions (10% of B) [15]. A 1 min flow was used, and each run lasted for 40 min. The absorbance was monitored at 238 nm, and the injection volumes were 20 μL. Each sample was injected in triplicate, and the analyses were performed using the Gilson UniPoint software v1.8 (UniPoint, Winnipeg, MB, Canada).

### 2.6. The Analysis of the Redox Effect of Caffeic Acid on MC-LR by LC/Q-TOF (Liquid Chromatography/Quadrupole-Time of Flight)

To establish the mechanism of action of caffeic acid, 50 mg of pure caffeic acid was added to 200 mL of Milli-Q water containing 50 mL of a MC-LR toxin sample that had been extracted from the culture of *M. aeruginosa* Kützing; the mixture was maintained for 72 h with constant agitation. Then, a 30 mL sample was taken at 0, 24, and 72 h. The samples were adsorbed in to solid phase extraction C18 cartridges, eluted with methanol, concentrated under reduced pressure, and injected into an HPLC/HR-MS using a UHR-QTOF Impact II-Bruker (Ultrahigh Resolution Quadrupole Time-of-Flight, Bruker Corporation, Billerica, MA, USA). The extracts were separated using a Chromolith High Resolution C18 column, Germany (50 × 4.6 mm), which was maintained at 40 °C. The mobile phase comprised Milli-Q water plus 0.1% formic acid (A) and acetonitrile plus formic acid 0.1% (B). The separation was performed using a gradient that increased from 5% B to 10% B for 1 min, followed by 50% (B) up to 4 min and up to 95% (B) at 6 min, and that finally returned to the initial conditions at 8 min. The auto-sampler was maintained at 10 °C always. Data were acquired in positive ion electrospray scanning mode from 50 to 2000 m/z with a 2 s/scan and a 0.1 s inter-scan delay. The ion source capillary and sampling cone parameters were 2.9 and 25 V, respectively. The desolvation temperature was 220 °C, and the source temperature was 80 °C. The cone gas and desolvation gas flows were 8 L h<sup>-1</sup>. A tuning mix solution was used as a calibrant. Instrument control and data acquisition (centroid) and processing were performed using the Bruker Compass DataAnalysis 4.3 software (Bruker Corporation, Billerica, MA, USA).

### 2.7. The Acute Toxicity Tests with *D. pulex*

Assays were carried out by exposing five neonates of *D. pulex*, aged <24 h old, to phenyl acyl compounds and copper sulfate. A total of 30 mL of growth medium, mixed with the substances, was added to each container containing *D. pulex* to a final concentration of 25, 50, and 100 mg/L. After 24, 48, and 72 h of exposure, the number of survivors in each test vessel was counted to identify the lethal concentration 50 (LC<sub>50</sub>). Analyses were done in triplicate for a total of 15 neonates; in addition, water was used as a negative control.

### 2.8. Data Analysis

Initially, a goodness-of-fit test (the Kolmogorov–Smirnov test) was performed to establish statistically significant differences in the cell density measurements that were obtained from the tests using different substances. A parametric variance analysis with a significance level of 0.05 was applied. In cases wherein statistically significant differences were found, a comparison analysis of multiple ranges was performed to determine which samples were different from each other. To establish correlations between different cell density measurements, a Spearman correlation analysis with

a significance level of 0.05 was applied using the statistical package Statgraphics Centurion XVI (Statgraphics.Net, Madrid, Spain).

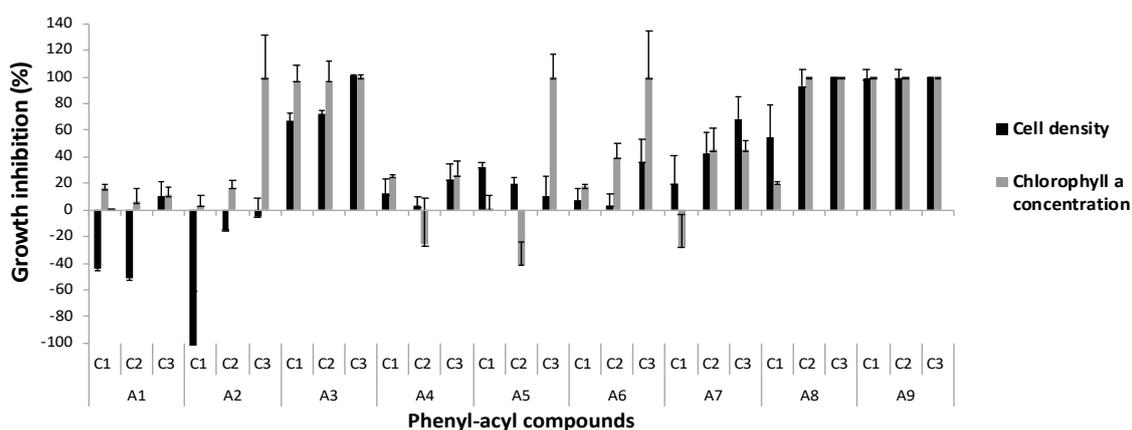
The  $LC_{50}$  was determined from the acute toxicity results, and the IBM SPSS Statistics 21 software (IBM, Armonk, NY, USA) was used as an analytical tool.

### 3. Results

#### 3.1. Growth Inhibition of *M. aeruginosa* Kützing

The effects of the substances on the cultures of *M. aeruginosa* Kützing were assessed in two ways (Figure 2): cell density measurements and Chl *a* quantities. Caffeic acid and eugenol were powerful inhibitors in both parameters at 50–100 mg/L; vanillin exhibited a medium inhibitory effect at both 50 mg/L and 100 mg/L.

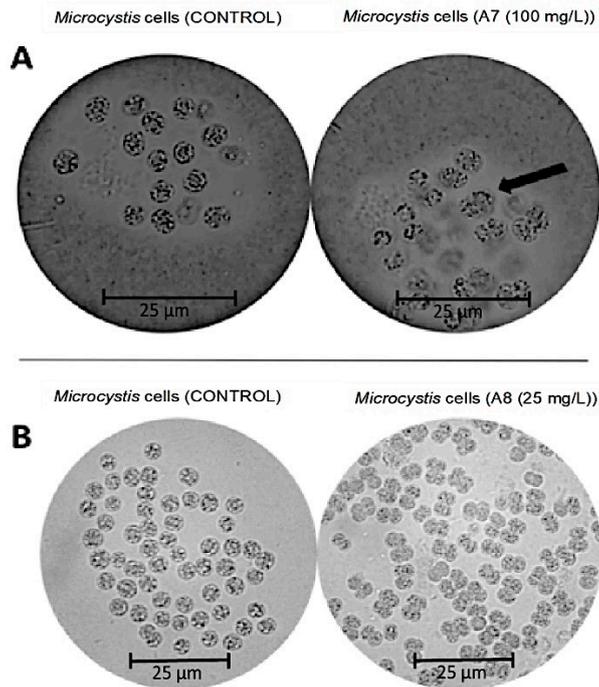
However, a differential response was noticed when Chl *a* levels were considered. Syringic acid, 2'-hydroxycinnamic acid, and 4'-hydroxyphenylacetic acid were strong inhibitors of Chl *a* at the highest concentration. This seems to indicate a specific action in the levels of Chl *a*. Strangely, at a lower concentration, 3,5-dimethoxybenzoic acid, and especially syringic acid, were powerful promoters of *M. aeruginosa* Kützing growth. As might be expected, copper sulfate caused 100% inhibition; this compound has been conventionally used to control blooms in reservoirs for several years [16].



**Figure 2.** The effects of phenyl-acyl compounds on the growth of *Microcystis aeruginosa* Kützing at 72 h of growth according to cell density measurements and chlorophyll *a* concentration. 3,5-dimethoxybenzoic acid (A1), syringic acid (A2), caffeic acid (A3), 3-methoxyphenylacetic acid (A4), 2'-hydroxycinnamic acid (A5), 4-hydroxyphenylacetic acid (A6), vanillin (A7), eugenol (A8), and for copper sulfate, a  $Cu(SO_4)$  control (A9). The concentrations assayed were: 100 mg/L (C3), 50 mg/L (C2), and 25 mg/L (C1). The letters and numbers refer to the structures shown in Figure 1.

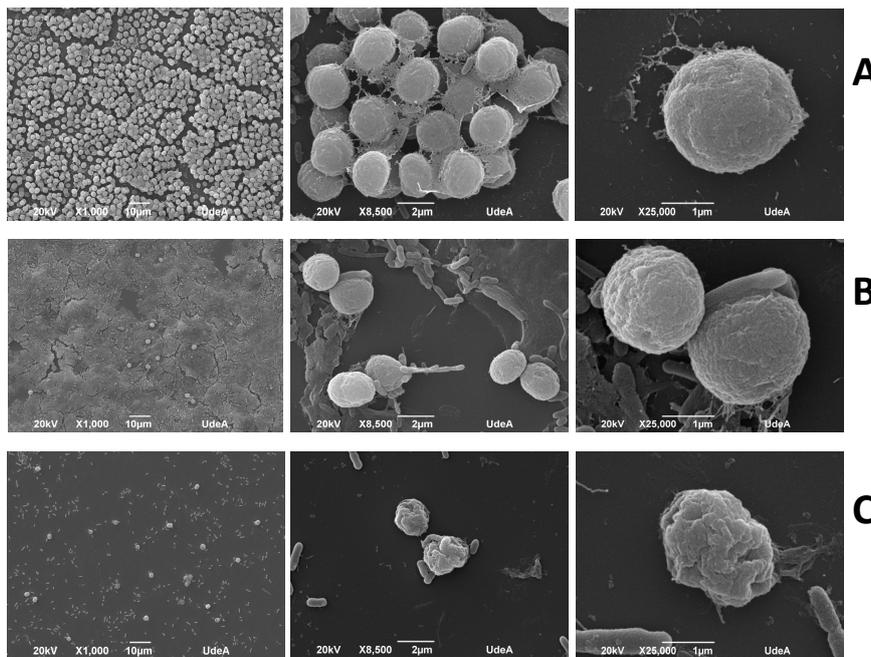
#### 3.2. The Analysis of Morphology of *M. aeruginosa* Kützing by Microscopy

The effect of the more active compounds (caffeic acid, eugenol, and vanillin) on *M. aeruginosa* Kützing was identified through a microscopy analysis. Several changes in the structure of the *M. aeruginosa* Kützing cells were observed under optical microscopy. A delayed cell division behavior was noticed 72 h after treatments with vanillin (Figure 3A) and eugenol (Figure 3B) at 100 mg/L. Moreover, optical microscopy evidenced changes in the cellular division of *M. aeruginosa* Kützing, since normal conditions produce symmetrical sister cells, but environmental perturbations increase the asymmetry between sister cells; when cells of *M. aeruginosa* Kützing were exposed to vanillin and eugenol, this effect was noticed as shown in Figure 3.



**Figure 3.** Aspects of *M. aeruginosa* Kützing cells under an optical microscope (40x) after treatment with vanillin (A) and eugenol (B) for 72 h. Scale bar: 25 µm.

In the SEM images, *M. aeruginosa* Kützing cultures treated with vanillin and caffeic acid showed significant changes in their morphology. In the first instance the control sample (Figure 4A) displayed well-formed and homogeneous cells embedded in a mucilage. In samples treated with vanillin (Figure 4B), the colony had partially lost its integrity and scattered cells can be seen in small groupings on a disintegrated mucilage. In cultures treated with caffeic acid (Figure 4C), the effects were more severe because mucilage was not appreciated, and the cells seem to have been lysed.

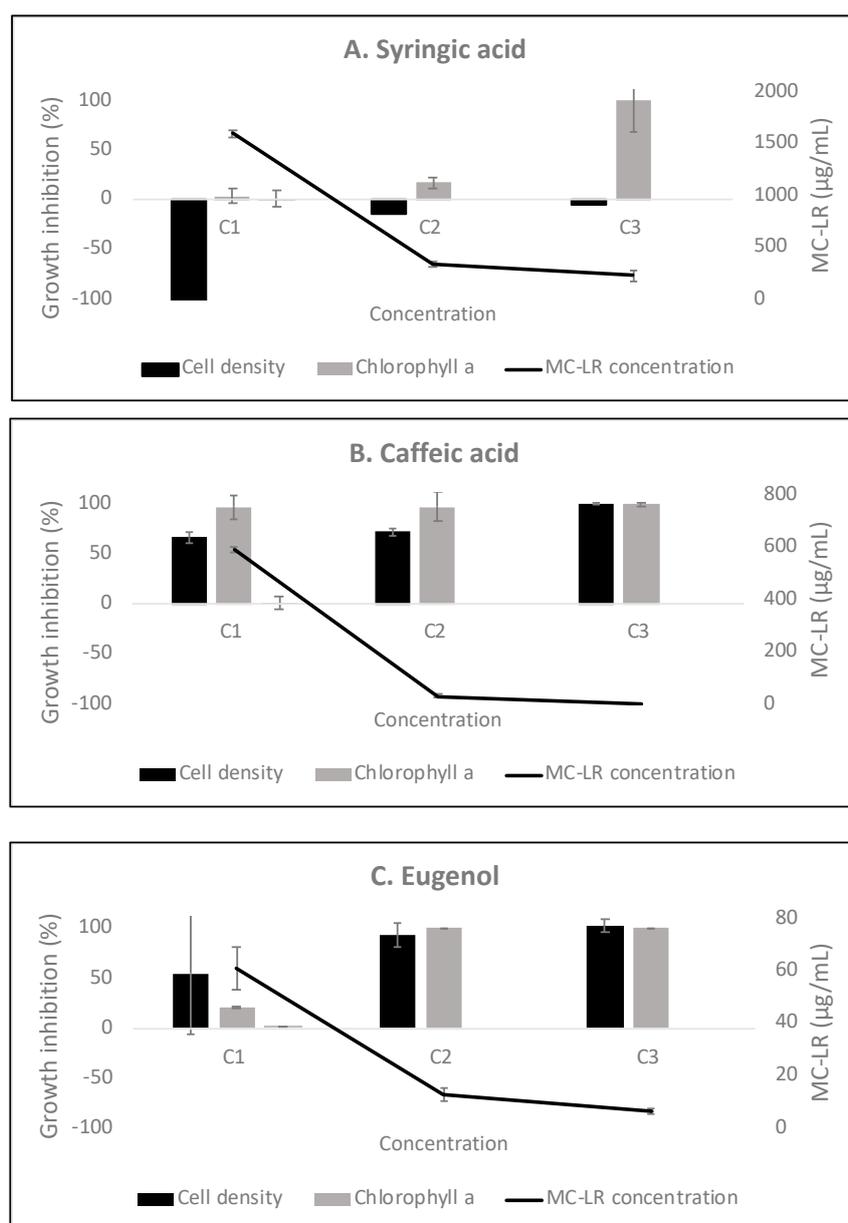


**Figure 4.** Scanning Electron Microscope (SEM) images showing *M. aeruginosa* Kützing colonies without treatment (A) and exposed to vanillin (B) and caffeic acid (C).

### 3.3. The Effect of Phenyl-Acyl Compounds on MC-LR Production

Through an HPLC-DAD analysis, the main toxin in *M. aeruginosa* Kützing was found to be MC-LR. To establish the effect of the compounds on the MC-LR level, we chose three compounds: syringic acid at non-growth inhibitory concentrations and caffeic acid and eugenol, which are powerful growth inhibitors and were found to reduce the levels of Chl *a*.

MC-LR concentrations were reduced after exposure to syringic acid by up to 77% at 50 mg/L and 84% at 100 mg/L, to caffeic acid by up to 89% at 50 mg/L and 100% at 100 mg/L, and after exposure to eugenol by up to 27% at 25 mg/L and 85% and 93% at 50 and 100 mg/L, respectively (Figure 5). A decrease in the toxin level could be explained based on the reduction of *M. aeruginosa* Kützing growth.

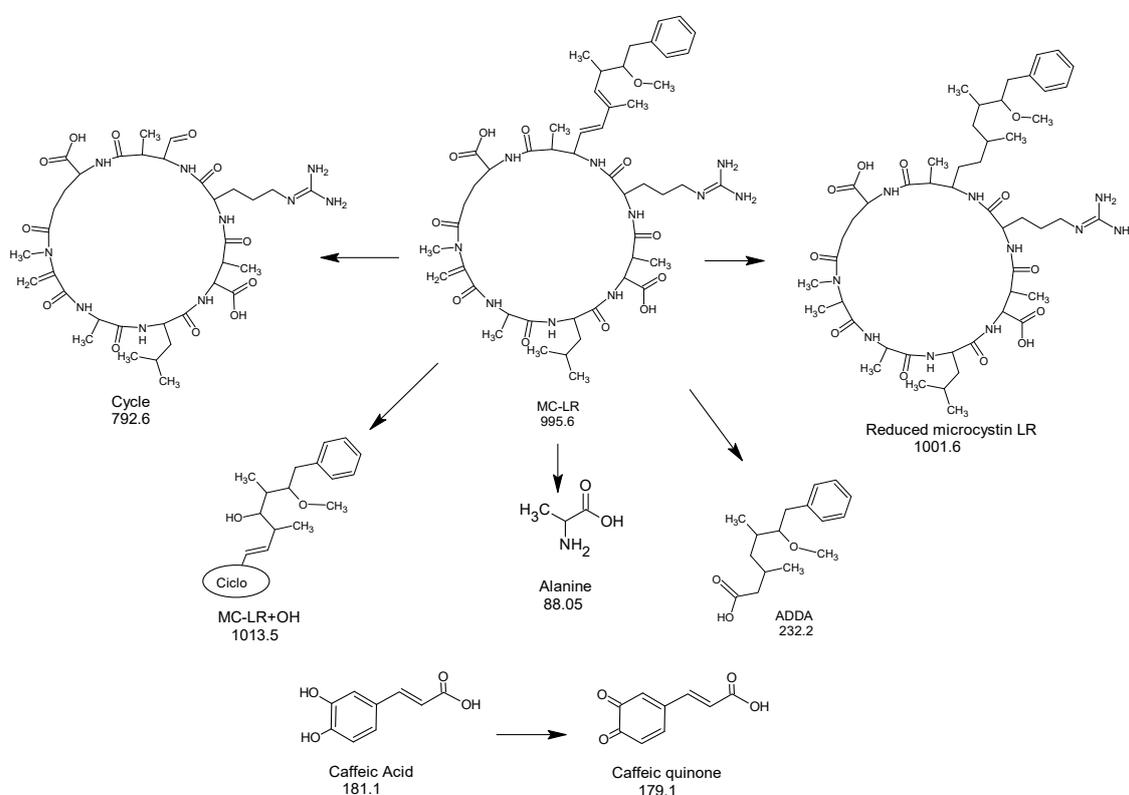


**Figure 5.** The effect of syringic acid (A), caffeic acid (B), and eugenol (C) on the concentration of microcystin-LR (MC-LR) in cultures of *M. aeruginosa* Kützing. The concentrations assayed were 100 mg/L (C3), 50 mg/L (C2), and 25 mg/L (C1).

### 3.4. The Analysis of the Redox Effect of Caffeic Acid on MC-LR by LC/Q-TOF

It is widely known that phenols possess a high redox capability, and because of that are tested like antioxidants. Several hours after mixing caffeic acid with MC-LR toxin, a grayish color had developed in the solution, which intensified over time, and changes in both compounds were observed through HPLC/MS. After 72 h, the presence of caffeic acid was detected by a peak at  $m/z$  181.1; however, another substance with a peak at  $m/z$  179.1 was also observed that corresponded to the quinone of caffeic acid by oxidation of the *ortho*-dihydroxy system. Similarly, a peak at  $m/z$  995.6 was assigned to the MC-LR toxin, and a peak at  $m/z$  1001.5 to a new compound formed by reduction of the quinone of caffeic acid (Figure 6).

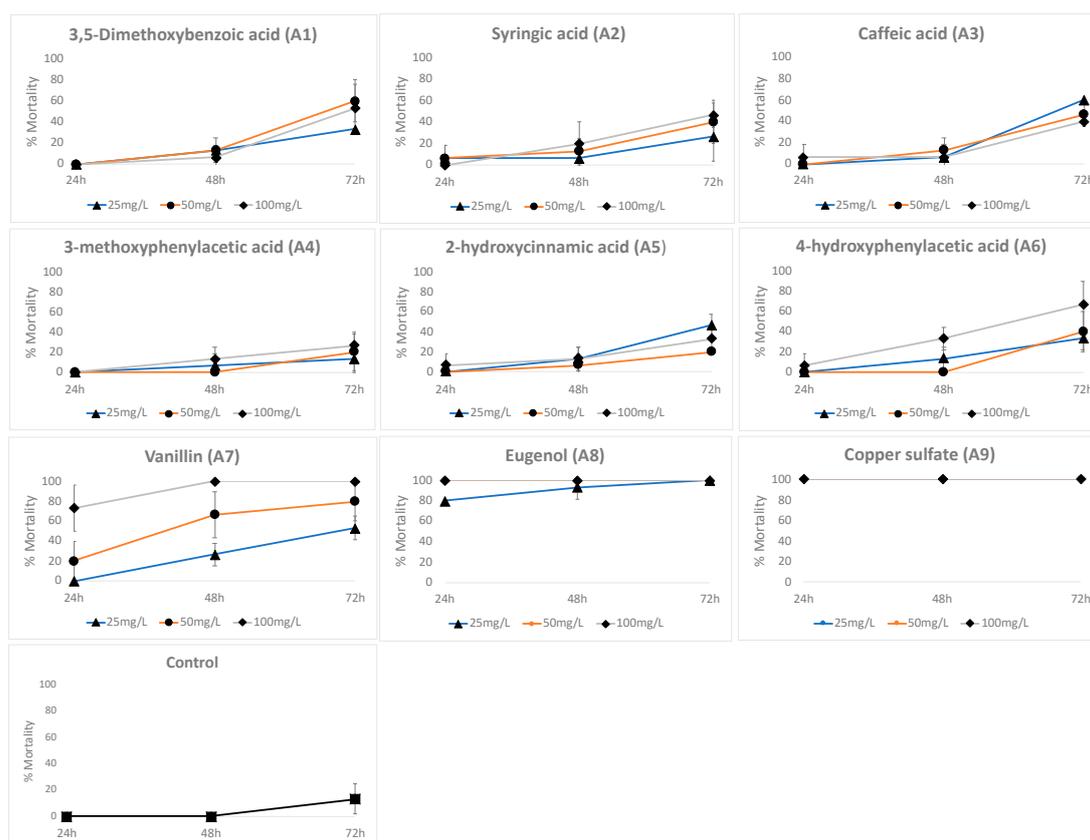
In addition, other molecules formed by degradation of the original toxin were identified, such as the peaks at  $m/z$  88.05 and 135.1, which were assignable to an alanine and a part of the side chain ADDA, respectively. Besides this, the peaks at  $m/z$  792.6 and 232.2 seem to indicate an oxidative breaking of ADDA.



**Figure 6.** A proposed oxide-reductive pathway of the decomposition of MC-LR with caffeic acid.

### 3.5. Toxicological Effects

To establish the toxicity of the phenyl-acyl compounds, *D. pulex* neonates were used. 3,5-dimethoxybenzoic acid, syringic acid, caffeic acid, 3-methoxyphenylacetic acid, 2'-hydroxycinnamic acid, and 4'-hydroxyphenylacetic acid caused less than 10% mortality in the first 24 h. After 48 h, 4'-hydroxyphenylacetic acid caused close to 40% mortality, and the other compounds caused less than 20% mortality (Figure 7). However, eugenol and copper sulfate exhibited high neonate mortality: 80% to 100% at 24 h and 100% at 48 h at the concentrations of 25, 50, and 100 mg/L. For vanillin, mortality was 100% at 48 h at 100 mg/L. Table 1 shows the  $LC_{50}$  results; some data were not estimated statistically because, under the treatment, all neonates either died (eugenol) or survived in the control (water).



**Figure 7.** The toxicity of phenyl-acyl compounds and copper sulfate on *Daphnia pulex* De Geer neonates. Control = the culture medium without a compound.

**Table 1.** LC<sub>50</sub> values for *D. pulex* De Geer neonates exposed to different phenyl-acyl compounds at 24, 48, and 72 h. The confidence intervals (95%) are given in parenthesis.

Phenyl-Acyl	LC <sub>50</sub> 24 h	LC <sub>50</sub> 48 h	LC <sub>50</sub> 72 h
A1	NE	251.854	71.8 (52.98–108.68)
A2	760.41 (NE)	146.26 (101.15–436.72)	87.49 (66.86–132.07)
A3	131.62 (NE)	228.62 (NE)	84.30 (53.52–241.42)
A4	NE	179.88 (112.21–7471.96)	125.42 (88.37–261.20)
A5	131.62 (NE)	177.83 (112.21–7471.96)	125.63 (79.01–554.19)
A6	131.62 (NE)	126.25 (93.90–242.85)	69.37 (52.58–99.22)
A7	80.42 (67.26–98.41)	40.43 (32.73–51.42)	26.62 (22.41–39.5)
A8	20.885 (NE)	13.23 (9.05–19.26)	7.1
A9	NE	NE	NE

NM, no mortality; NE, not estimated. 3,5-dimethoxybenzoic acid (A1), syringic acid (A2), caffeic acid (A3), 3-methoxyphenylacetic acid (A4), 2'-hydroxycinnamic acid (A5), 4'-hydroxyphenylacetic acid (A6), vanillin (A7), eugenol (A8), and, as a control, copper sulfate (A9).

#### 4. Discussion

Currently, several strategies are used to control *Microcystis* blooms and toxins, ranging from the application of dangerous copper salts to ultrasound and adsorption on materials such as activated carbon [17]. In this research, eight phenyl-acyl compounds were assayed as inhibitors of *M. aeruginosa* Kützing growth and toxin production. Eugenol, caffeic acid, and vanillin showed inhibition activities of 100%, 75%, and 50%, respectively, at 50 mg/L. The effects on the growth of *M. aeruginosa* Kützing were assessed through cell count and Chl *a* analysis; however, these results were not comparable. Therefore, there was a lack of correlation between cell counts and Chl *a* levels, which was possibly due to the different mechanisms of action in *M. aeruginosa* Kützing.

The production of Chl *a* was completely inhibited by syringic acid, 2'-hydroxycinnamic acid, and 4'-hydroxyphenylacetic at 100 mg/L, while, according to the cell count, there was close to 45% inhibition. A probable mechanism of action of these compounds could involve oxidative damage to Chl *a* through the inhibition of antioxidant enzymes [18]. The other substances, 3,5-dimethoxybenzoic acid and 3-methoxyphenylacetic acid, were weak growth promoters; however, there are no data on the effect of these substances as inductors of cyanobacteria growth or their role in the formation of blooms. In the same way, some phenols, such as those that originate from the decomposition of organic matter, have also shown an effect on the development of cyanobacterial blooms [11,19].

According to these results, caffeic acid was the best *M. aeruginosa* Kützing growth inhibitor. It reduced the level of MC-LR and was practically a non-toxic substance, since overall survival was observed in *D. pulex* neonates, which survived when exposed to all concentrations; the effects that were noticed after 48 h may be caused by starvation. This harmless effect has been previously reported for this type of compound in *Danio rerio* [20]. However copper sulfate, which is recommended as an algicide, is toxic to many other species in aquatic environments [16,21].

Regarding the mechanism of action of these compounds, caffeic acid seems to involve a redox mechanism to degrade MC-LR, which was detected by means of direct observation of the color of the solution and HPLC/MS. Some authors have already proposed the use of phenolic acids to inhibit *M. aeruginosa* growth [20,22,23]. It has been suggested that the autoxidation of phenols induces inhibitory effects by the production of free radicals [24] with the generation of radical superoxide anions. In this way, they can induce a lipid peroxidation that affects cell membrane permeability and causes the death of *M. aeruginosa* Kützing [20]. Similarly, rice straw extracts that contain this same type of phenolic acid were shown to provoke changes in the antioxidant enzyme system in the cells of results cyanobacteria, such as *M. aeruginosa* Kützing, thereby suspending their growth and causing cell death [25].

In addition, several of the assayed molecules also seem to act at the stage of formation of the mucilage, which is mainly composed of exopolysaccharides, and whose main function is the agglutination and protection of the colonies [26,27]. These results are in accordance with the SEM images (Figure 4C) because damage to the membrane and changes in the mucilage's stability were also observed under caffeic acid treatment. In addition, vanillin and eugenol caused delays in cell division, since dividing cells were detected after 72 h of treatment (Figure 3A,B). These same substances also caused asymmetry between sister cells, an effect that has already been reported for *M. aeruginosa* Kützing under treatment of copper sulfate and the herbicide diuron, and could be an indicator of environmental disturbance [28].

About the effect of caffeic acid, the fragmentation patterns of MC-LR toxin agree with previous reports [29]. However, the exact mechanism of the MC-LR toxin's transformation needs to be elucidated, including the residues in the molecule that are reduced by caffeic acid and the kinetics of the process. These findings open new perspectives on the elimination of MC-LR toxins in potable reservoirs contaminated with cyanobacterial blooms, based on a chemical or physical redox process.

Cyanobacteria blooms develop quickly in large areas that contain millions of hectoliters of water; therefore, the application of substances for their control could be related to an early detection method. Otherwise, the amounts of required inhibitory substances would be too large and possibly affect the water's quality and increase costs. Therefore, knowledge about the effects and the mechanism of action of these types of substances can generate information about new control methods as well as provide additional data about the chemical, biological, and physical agents that trigger the blooms. Thus, exogenous substances and products from industrial activity or vegetal biomass decomposition in reservoirs could also elicit these blooms.

## 5. Conclusions

In this work, several effects were detected in *M. aeruginosa* Kützing after the application of several phenyl-acyl compounds. Some of them, such as caffeic acid, were inhibitory, while others were found

to act as growth promoters. The activity of caffeic acid included changes in cell morphology and division and the formation of mucilage, while its mechanism of action on the MC-LR toxin seems to involve a degradative redox process. Molecules with promotor effects indicate the existence of other biochemical mechanisms in the blooms' formation that are different from those that have traditionally been considered, such as edaphic factors and nutrient availability.

**Author Contributions:** Methodology: N.H., F.E.; Investigation: N.H., J.P.V., M.T.F.; Interpretations: N.H., M.T.F., F.E.; Writing: N.H., F.E.; Supervision N.H., F.E.

**Funding:** COLCIENCIAS-COLOMBIA (grant FP44842-049-2016).

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. He, X.; Wert, E.C. Colonial cell disaggregation and intracellular microcystin release following chlorination of naturally occurring *Microcystis*. *Water Res.* **2016**, *101*, 10–16. [[CrossRef](#)] [[PubMed](#)]
2. Azevedo, S.M.F.O.; Carmichael, W.W.; Jochimsen, E.M.; Rinehart, K.L.; Lau, S.; Shaw, G.R.; Eaglesham, G.K. Human intoxication by microcystins during renal dialysis treatment in Caruaru — Brazil. *Toxicology* **2002**, *181–182*, 441–446. [[CrossRef](#)]
3. Su, J.; Yang, X.; Zhou, Y.; Zheng, T. Marine bacteria antagonistic to the harmful algal bloom species *Alexandrium tamarense* (Dinophyceae). *Biol. Control* **2011**, *56*, 132–138. [[CrossRef](#)]
4. Padovesi-Fonseca, C.; Philomeno, M.G. Effects of algicide (copper sulfate) application on short-term fluctuations of phytoplankton in Lake Paranoá, central Brazil. *Braz. J. Biol.* **2004**, *64*, 819–826. [[CrossRef](#)] [[PubMed](#)]
5. Carey, C. Resilience to Blooms Resilience to Blooms. *Science* **2015**, 10–13. [[CrossRef](#)]
6. Chen, Y.; Li, J.; Wei, J.; Kawan, A.; Wang, L.; Zhang, X. Vitamin C modulates *Microcystis aeruginosa* death and toxin release by induced Fenton reaction. *J. Hazard. Mater.* **2017**, *321*, 888–895. [[CrossRef](#)] [[PubMed](#)]
7. Mohamed, Z.A. Macrophytes-cyanobacteria allelopathic interactions and their implications for water resources management — A review. *Limnologica* **2017**, *63*, 122–132. [[CrossRef](#)]
8. Herrera, N.A.; Flórez, M.T.; Echeverri, L.F. Evaluación preliminar de la reducción de microcistina-LR en muestras de florecimientos a través de sistemas sedimentarios. *Rev. Int. Contam. Ambient.* **2015**, *31*, 405–414.
9. Mucci, M.; Noyma, N.P.; de Magalhães, L.; Miranda, M.; van Oosterhout, F.; Guedes, I.A.; Huszar, V.L.M.; Marinho, M.M.; Lürling, M. Chitosan as coagulant on cyanobacteria in lake restoration management may cause rapid cell lysis. *Water Res.* **2017**, *118*, 121–130. [[CrossRef](#)]
10. Lin, J.L.; Hua, L.C.; Hung, S.K.; Huang, C. Algal removal from cyanobacteria-rich waters by preoxidation-assisted coagulation–flotation: Effect of algogenic organic matter release on algal removal and trihalomethane formation. *J. Environ. Sci. (China)* **2018**, *63*, 147–155. [[CrossRef](#)]
11. Lin, Y.; Chen, A.; Wu, G.; Peng, L.; Xu, Z.; Shao, J. Growth, microcystins synthesis, and cell viability of *Microcystis aeruginosa* FACHB905 to dissolved organic matter originated from cattle manure. *Int. Biodeterior. Biodegrad.* **2017**, *118*, 126–133. [[CrossRef](#)]
12. Stanier, R.Y.; Kunisawa, R.; Mandel, M.; Cohen-Bazire, G. Purification and properties of unicellular blue-green algae (Order Chroococcales). *Bacteriol. Rev.* **1971**, *35*, 171–205. [[CrossRef](#)] [[PubMed](#)]
13. Gregor, J.; Geriš, R.; Maršálek, B.; Heteša, J.; Marvan, P. In situ quantification of phytoplankton in reservoirs using a submersible spectrofluorometer. *Hydrobiologia* **2005**, *548*, 141–151. [[CrossRef](#)]
14. Sant'Anna, C.L.; Azevedo, M.T.D.P.; Senna, P.A.C.; Komárek, J.; Komárková, J. Planktic Cyanobacteria from São Paulo State, Brazil: Chroococcales. *Rev. Bras. Botânica* **2004**, *27*, 213–227. [[CrossRef](#)]
15. Aguate, E.C.; Gago-Martínez, A.; Leão, J.M.; Rodríguez-Vázquez, J.A.; Menàrd, C.; Lawrence, J.F. HPLC and HPCE analysis of microcystins RR, LR and YR present in cyanobacteria and water by using immunoaffinity extraction. *Talanta* **2003**, *59*, 697–705. [[CrossRef](#)]
16. Kinley, C.M.; Iwinski, K.J.; Hendrikse, M.; Geer, T.D.; Rodgers, J.H. Cell density dependence of *Microcystis aeruginosa* responses to copper algacide concentrations: Implications for microcystin-LR release. *Ecotoxicol. Environ. Saf.* **2017**, *145*, 591–596. [[CrossRef](#)]

17. Şengül, A.B.; Ersan, G.; Tüfekçi, N. Removal of intra- and extracellular microcystin by submerged ultrafiltration (UF) membrane combined with coagulation/flocculation and powdered activated carbon (PAC) adsorption. *J. Hazard. Mater.* **2018**, *343*, 29–35. [[CrossRef](#)]
18. Ni, L.; Rong, S.; Gu, G.; Hu, L.; Wang, P.; Li, D.; Yue, F.; Wang, N.; Wu, H.; Li, S. Inhibitory effect and mechanism of linoleic acid sustained-release microspheres on *Microcystis aeruginosa* at different growth phases. *Chemosphere* **2018**, *212*, 654–661. [[CrossRef](#)]
19. Wang, W.; Zhang, Y.; Shen, H.; Xie, P.; Yu, J. Changes in the bacterial community and extracellular compounds associated with the disaggregation of *Microcystis* colonies. *Biochem. Syst. Ecol.* **2015**, *61*, 62–66. [[CrossRef](#)]
20. Jin, P.; Wang, H.; Huang, W.; Liu, W.; Fan, Y.; Miao, W. The allelopathic effect and safety evaluation of 3,4-Dihydroxybenzalacetone on *Microcystis aeruginosa*. *Pestic. Biochem. Physiol.* **2017**. [[CrossRef](#)]
21. Zhou, C.; Huang, J.C.; Liu, F.; He, S.; Zhou, W. Effects of selenite on *Microcystis aeruginosa*: Growth, microcystin production and its relationship to toxicity under hypersalinity and copper sulfate stresses. *Environ. Pollut.* **2017**, *223*, 535–544. [[CrossRef](#)] [[PubMed](#)]
22. Mecina, G.F.; Dokkedal, A.L.; Saldanha, L.L.; Chia, M.A.; Cordeiro-Araújo, M.K.; do Carmo Bittencourt-Oliveira, M.; da Silva, R.M.G. Response of *Microcystis aeruginosa* BCCUSP 232 to barley (*Hordeum vulgare* L.) straw degradation extract and fractions. *Sci. Total Environ.* **2017**, *599–600*, 1837–1847. [[CrossRef](#)] [[PubMed](#)]
23. Zhang, C.; Ling, F.; Yi, Y.L.; Zhang, H.Y.; Wang, G.X. Algicidal activity and potential mechanisms of ginkgolic acids isolated from *Ginkgo biloba* exocarp on *Microcystis aeruginosa*. *J. Appl. Phycol.* **2014**, *26*, 323–332. [[CrossRef](#)]
24. Nakai, S.; Inoue, Y.; Hosomi, M. Algal growth inhibition effects and inducement modes by plant-producing phenols. *Water Res.* **2001**, *35*, 1855–1859. [[CrossRef](#)]
25. Hua, Q.; Liu, Y.-G.; Yan, Z.-L.; Zeng, G.-M.; Liu, S.-B.; Wang, W.-J.; Tan, X.-F.; Deng, J.-Q.; Tang, X.; Wang, Q.-P. Allelopathic effect of the rice straw aqueous extract on the growth of *Microcystis aeruginosa*. *Ecotoxicol. Environ. Saf.* **2018**, *148*, 953–959. [[CrossRef](#)]
26. Kehr, J.-C.; Dittmann, E. Biosynthesis and Function of Extracellular Glycans in Cyanobacteria. *Life* **2015**, *5*, 164–180. [[CrossRef](#)] [[PubMed](#)]
27. Pereira, S.; Zille, A.; Micheletti, E.; Moradas-Ferreira, P.; De Philippis, R.; Tamagnini, P. Complexity of cyanobacterial exopolysaccharides: Composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. *FEMS Microbiol. Rev.* **2009**, *33*, 917–941. [[CrossRef](#)]
28. Costas, E.; Lopez-Rodas, V. Copper sulphate and DCMU-herbicide treatments increase asymmetry between sister cells in the toxic cyanobacteria *Microcystis aeruginosa*: Implications for detecting environmental stress. *Water Res.* **2006**, *40*, 2447–2451. [[CrossRef](#)]
29. Duan, X.; Sanan, T.; De La Cruz, A.; He, X.; Kong, M.; Dionysiou, D.D. Susceptibility of the Algal Toxin Microcystin-LR to UV/Chlorine Process: Comparison with Chlorination. *Environ. Sci. Technol.* **2018**, *52*, 8252–8262. [[CrossRef](#)]

