Differential Effects of the Allelochemical Juglone on Growth of Harmful and Non-Target Freshwater Algae

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Abstract: Allelopathy has been applied to control nuisance algae in aquatic systems, but the effects of allelochemicals on the broad spectrum of algae are not well understood. We investigate algicidal effects of the allelochemical juglone on the bloom-forming, harmful algae Microcystis aeruginosa and Stephanodiscus hantzschii, and on several non-target algal species including cyanobacteria (Anabaena flos–aquae, Oscillatoria curviceps, and Phormidium subfuscum), diatoms (Asterionella formosa, Fragilaria crotonensis, and Synedra acus), and green algae (Chlorella vulgaris, Scenedesmus ecornis, and Scenedesmus quadricauda), in laboratory and field enclosure bioassays. Under three treatment concentrations (0.01, 0.1, and 1 mg L⁻¹) of juglone, Microcystis cell density is significantly reduced by 35–93%. Concentrations of 0.1 and 1 mg L⁻¹ inhibits Stephanodiscus growth almost equally (66% and 75%, respectively). To contrast, juglone produces a stimulatory allelopathic effect on three green algae, and other tested diatoms showed hormesis. Overall, the cyanobacteria are more sensitive to juglone than the green algae and diatoms. These results indicate that the allelopathic effects of juglone on microalgae vary depending on their characteristic cellular morphology and anatomy.

Keywords: allelopathy; juglone; algicidal potential; differential effects; harmful algae; hormesis

1. Introduction

Harmful algal blooms (HABs) are a primary concern in water management, particularly regarding recreational and drinking water resources [1–5]. Although many algae are capable of blooming under suitable conditions, bloom events are generally specific to locales in which eutrophication and meteorological factors (e.g., temperature) are major driving forces [6,7]. Additionally, algal growth and development in natural fresh waters is subject to seasonal succession, depending on growth kinetics, competition, and planktonic herbivory [8]. Cyanobacteria, such as Microcystis, Anabaena, and Aphanizomenon, often proliferate during warm summers, whereas some diatoms, such as Stephanodiscus, are dominant during cold seasons in temperate regions [9,10]. Abnormal outbreaks of these algae often cause serious problems by producing harmful materials, such as toxins and off-flavors, and hindering drinking water treatment processes. Thus, effective control of these bloom-forming nuisance algae is of prime importance for water use and ecosystem management.

A variety of techniques have been developed and applied to control HABs, mostly involving physical (e.g., ultrasonic radiation, dissolved gas flotation, and nanofiltration) and chemical (e.g., copper sulphate and silver nanoparticles) methods [11–14]. These techniques are effective under certain circumstances but have shortcomings in terms of cost and ecological security [15,16]. Namely, chemical algicides (e.g., copper, chlorine, and aluminum) have been widely used in the control of
harmful algal blooms. However, most chemical algicides are nonselective, and cause general toxicity. Development of efficient, sustainable methods of controlling HABs still is challenging.

The design of environmentally safe, selective HAB inhibitors has been a focal point of study [17–20], and methods using natural compounds are of particular interest. Allelochemical control, a potential eco-friendly method of suppressing harmful algae, employs biological materials of plant origin [18–20]. Barley straw and the allelochemicals extracted from rice straw and hulls, for example, have been applied to control harmful algae over the years since the 1980s. Also, diverse higher plants (e.g., *Myriophyllum spicatum* and *Polygonatum odoratum* var. *pluriflorum*) have been studied for allelopathic inhibition on algal growth. Various allelochemicals have been demonstrated to inhibit the growth of toxic cyanobacteria, particularly including that of *Microcystis aeruginosa* [18,21,22], whereas their effects on other algal taxa, such as diatoms and green algae, are less well studied.

Pure juglone and crude walnut hull extracts have been found to inhibit the growth of a broad range of microorganisms, including bacteria, algae, and fungi [23], and prior studies have shown that juglone has allelopathic potential against some algae [24–29]. However, studies on juglone have not examined its specific effects on a broad spectrum of algal species, including diatoms and green algae. This study assesses the allelopathic effects of juglone on diverse species belonging to three algal phyla (Cyanophyceae, Chlorophyceae, and Bacillariophyceae). Particularly, we address the selective allelopathic potential of juglone on some harmful bloom-forming algae via both laboratory and field studies.

2. Materials and Methods

2.1. Preparation of Juglone and Algal Strains

Juglone (5-hydroxy-1,4-naphthoquinone) was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Due to the poor solubility of juglone in water, we prepared an experimental concentration of juglone using an organic solvent (methanol). The very small amount of methanol (ca. 1/1000, v/v) applied to the experiment in the final content did not harm normal algal growth, including the control.

Four diatom species, including *Asterionella formosa*, *Fragilaria crotonensis*, *Stephanodiscus hantzschii*, and *Synedra acus*, were isolated from the region of the water intake facility in Lake Paldang (Han River, South Korea; 37°31′18″ N, 127°16′52″ E) in March, 2016 (with the water temperature approximately 10 °C). Single strains were isolated from the collected samples using the capillary method [30]. Cells of the single-strain isolates were washed with distilled water and then inoculated into a diatom medium (DM) [31]. The cells were initially grown in 96-well microplates, and then gradually scaled up to 120-mL T-flasks at 10 °C under a 14:10 h light–dark cycle of 100 µmol photons m⁻² s⁻¹ provided by cool-white fluorescent lamps. The grown cells were stored in a refrigerator. The cyanobacterium *Microcystis aeruginosa* (NIER 10010) was obtained from the National Institute of Environmental Research (Ministry of Environment, Incheon, South Korea). *M. aeruginosa* exhibited a unicellular form under the culture conditions. The green algae *Scenedesmus quadricauda* (AG 10003) and *Chlorella vulgaris* (UTEX 265) were obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, South Korea) and the Culture Collection of Algae (University of Texas at Austin, Austin, TX, USA), respectively. Diatom strains were grown in a DM medium at 10 °C, while *M. aeruginosa* and the green algae were grown in in Allen medium at 25 °C. Recipes for the DM and Allen medium can be found in Beakes et al. [32] and Allen [33], respectively. Test algae were stored under a 14:10 h light–dark cycle of 100 µmol photons m⁻² s⁻¹ provided by cool-white fluorescent lamps in a shaking incubator for 2 weeks.

2.2. Laboratory Bioassays

The allelopathic effects of juglone were investigated on the growth of two harmful algal species, *S. hantzschii* and *M. aeruginosa*, and on several selected non-target algae, *A. formosa*, *F. crotonensis*,
S. acus, S. quadricauda, and C. vulgaris, at three concentrations: 0.01, 0.1, and 1 mg L\(^{-1}\). The strains were cultured using pre-filtered (Whatman GF/F filter, pore size 0.7 µm; Whatman plc, Maidstone, UK), sterile (autoclaved for 20 min at 121 °C), eutrophic lake water (Lake Ilgam, Seoul, South Korea; 37°32’23” N, 127°04’36” E) in which in situ enclosure bioassays were conducted. To conduct the test, S. hantzschii, M. aeruginosa, A. formosa, F. crotonensis, S. acus, S. quadricauda, and C. vulgaris, were inoculated at 16,800 cells mL\(^{-1}\), 810,000 cells mL\(^{-1}\), 1460 cells mL\(^{-1}\), 1830 cells mL\(^{-1}\), 1860 cells mL\(^{-1}\), 132,000 cells mL\(^{-1}\), and 875,000 cells mL\(^{-1}\) of the initial cell density, respectively. Diatom strains were grown at 10 °C, while M. aeruginosa and green algae were grown at 25 °C, respectively. These strains were grown under a 14:10 h light–dark cycle of 100 µmol photons m\(^{-2}\) s\(^{-1}\) provided by cool-white fluorescent lamps in a shaking incubator for 7 days. During all experiments, 10 mL of algal samples were added to 250 mL test flasks containing 90 mL filtered sterile lake water and then juglone was added in concentrations of 0.01, 0.1, and 1 mg L\(^{-1}\). All experiments were conducted in triplicate. Regarding cell counting, 1 mL aliquots out of 100 mL of test cultures were taken from thoroughly mixed test flasks and fixed with Lugol’s solution (2% final concentration, v/v).

2.3. Field Enclosure Bioassays

Field enclosure bioassays were carried out in a small, eutrophic lake (Lake Ilgam, Seoul, South Korea; 37°32’23” N, 127°04’36” E) over 7 d in August (with water temperature between 28–33 °C and pH between 8.6–10.2). Six enclosures were made from uncovered, cylindrical, opaque, plastic containers (height: 0.7 m, diameter: 0.6 m), and each was filled with 150 L of ambient lake water. The containers were open and, thus, precipitation and sunlight penetration were permitted during the experimental period. Juglone was added at two concentrations of 0.1 and 1 mg L\(^{-1}\) to the test enclosures. The enclosure bioassays were performed in duplicate. Concerning algal identification and counting, 100 mL of water was taken from each enclosure after mixing its whole water using a wooden paddle and fixed with Lugol’s solution (2% final concentration, v/v). Finally, a 1 mL aliquot was taken from a 200 mL sample for cell counting.

2.4. Algal Enumeration and Identification

Algal growth responses to juglone treatments were monitored at 1 d intervals for 7 d in both the laboratory and field experiments. Algal cells were enumerated (cell density:cells mL\(^{-1}\)) using a Sedgewick–Rafter (SR) counting chamber and phase-contrast microscope (Axioplan; Carl Zeiss AG, Oberkochen, Germany) under 200x magnification and identified to the species level according to the criteria of Hirose et al. [34] and Chung [35]. One mL of Lugol-fixed sample taken from each culture flask was transferred to a gridded SR chamber (Graticules S52 Sedgewick Rafter Counting Chamber, Structure Probe, Inc., West Chester, USA). The number of cells contained in 200 grids out of a total of 1000 grids in the chamber was counted. Algal cell density was determined by multiplying a total cell number in 200 grids by a conversion factor of 5. Algae appearing as a group of cells (i.e., coenobia and colonies) were counted as single cells. Cells of colony-formed Microcystis were counted based on the estimated area in which cell numbers were approximated for the measured area [36]. Algal growth responses were expressed as percentages of that of the control. All algal cell density data were presented as mean values and uncertainty was provided with standard errors.

2.5. Data Analysis

All algal cell density data were analyzed to estimate any significant relationship between treatments and algal cell density measured over the duration of the experiment. All algal cell density data achieved normality and variance homogeneity. One-way analysis of variance (Scheffé and Games–Howell Post Hoc analysis) was used to test for differences in algal cell density among the different levels of juglone. The difference between samples was considered significant at p <0.05. All statistical analyses were run using PASW®Statistic v. 18 (SPSS Inc., Chicago, IL, USA).
3. Results

3.1. Allelopathic Effects of Juglone in the Laboratory Bioassays

When different levels of juglone were applied, the growth of two harmful algae, *S. hantzschii* and *M. aeruginosa*, was inhibited by juglone, but different levels of juglone affected the two species differently (Figure 1). Growth of *S. hantzschii* was significantly inhibited by juglone concentrations of 0.1 mg L\(^{-1}\) and higher \((F(3, 20) = 10.240, p < 0.05)\) when compared to the control, while the lowest test concentration \((0.01 \text{ mg L}\(^{-1}\)) exerted no inhibition \((p = 0.995)\) (Figure 1a). The algicidal effect on the growth of *M. aeruginosa* was only prominent at the highest concentration \((1 \text{ mg L}\(^{-1}\)) \((F(3, 20) = 15.383, p < 0.001)\) (Figure 1b). Concentrations of 0.01–0.1 mg L\(^{-1}\) did not significantly suppress cellular growth of *M. aeruginosa* compared to the control throughout the experimental period \((p > 0.05)\).

![Figure 1](image-url)

*Figure 1.* Changes in cell density of *Stephanodiscus hantzschii* (A) and *Microcystis aeruginosa* (B) at three juglone concentrations during the 7-day experimental period. Data are presented as averages with standard errors (bars). Lower case letters on the right side of the lines indicate significant differences \((p < 0.05)\).

Juglone concentrations of 0.1 and 1 mg L\(^{-1}\) significantly reduced *S. hantzschii* cell density by 66.2 ± 8.6% and 75.1 ± 10.1%, respectively, compared to that of the control (Figure 2a). A concentration of 1 mg L\(^{-1}\) markedly inhibited the growth of *M. aeruginosa* by 92.7 ± 5.4%, whereas concentrations of 0.01 and 0.1 mg L\(^{-1}\) only reduced its cell density by 35.2 ± 6.4% and 37.7 ± 6.9%, respectively, compared to that of the control (Figure 2b). Interestingly, 0.1 mg L\(^{-1}\) of juglone showed more selective allelopathic effects among the examined diatoms than did 1 mg L\(^{-1}\). Juglone at 0.1 mg L\(^{-1}\) effectively inhibited the growth of *S. hantzschii*, while it stimulated the growth of three non-target diatoms (Figure 2a–d).
However, 1 mg L\(^{-1}\) of juglone induced 89.7 ± 5.7\%, 96.8 ± 5.1\%, and 94.9 ± 14.4\% cell density reductions in \textit{F. crotonensis}, \textit{A. formosa}, and \textit{S. acus}, respectively (Figure 2b–d). These reduction rates were higher than that of \textit{S. hantzschii}. Conversely, juglone produced marked stimulatory effects on the growth of the two green algae, \textit{S. quadricauda} and \textit{C. vulgaris}, at all three concentrations (Figure 2f,g). Moreover, there were no significant differences among the three concentrations (p > 0.05). Thus, the juglone concentration required for algicidal effects on \textit{S. hantzschii} (0.1 mg L\(^{-1}\)) was lower than that on \textit{M. aeruginosa} (1 mg L\(^{-1}\)), and this lower concentration also exerted hormetic effects on some diatoms and green algae by stimulating their growth.

![Figure 2](image)

**Figure 2.** The growth responses to juglone (% cell density relative to control) of selected freshwater algae at three different concentrations (0.01, 0.1, and 1 mg L\(^{-1}\)) after the 7-day experiment. Data are presented as averages with standard errors (bars) of triplicate experiments. Lower case letters indicate significant differences (p < 0.05). (A) \textit{Stephanodiscus hantzschii}, (B) \textit{Fragilaria crotonensis}, (C) \textit{Asterionella formosa}, (D) \textit{Syndra acus}, (E) \textit{Microcystis aeruginosa}, (F) \textit{Scenedesmus quadricauda}, (G) \textit{Chlorella vulgaris}.

### 3.2. Allelopathic Effects of Juglone in the Field Enclosure Bioassays

The results of the field enclosure bioassays were similar to those of the laboratory experiments (Figure 2e–g). Juglone concentrations of 0.1 and 1 mg L\(^{-1}\) markedly inhibited total natural algal assemblages by 60.3 ± 19.3\% and 79.9 ± 3.7\%, respectively, after 7 d (Figure 3a). Particularly, juglone decreased dominant cyanobacterial volume, but increased green algae volume in the field enclosures.
The composition ratios of cyanobacteria and green algae in the control enclosure were 97.2 and 2.5%, while these respective values changed to 84.2 and 15.5% with 0.1 mg L$^{-1}$ juglone and 81.7 and 17.1% with 1 mg L$^{-1}$ juglone on day 7 (Figure 3b–d).

Figure 3. Changes in total algal cell density and algal composition in the field enclosures after addition of juglone at concentrations of 0.1 and 1 mg L$^{-1}$. Data are presented as averages with standard errors (bars) of duplicate experiments. (A) Total algae density, (B) algal composition in the control, (C) algal composition after treatment with juglone (0.1 mg L$^{-1}$), (D) algal composition after treatment with juglone (1 mg L$^{-1}$). Lower case letters on the right side of the lines in 3-(A) indicate significant differences (p < 0.05).

A total of 19 algal species were observed in the enclosures during the 7-day experimental period (Table 1). Seventeen species were present on day 0, but by day 7, 16 species were present in the control enclosure, 15 in the 0.1 mg L$^{-1}$ juglone enclosure, and 13 in the 1 mg L$^{-1}$ juglone enclosure. Despite the general decrease in algal densities, the number of species had not markedly diminished in response by day 7. Moreover, the algal assemblages did not show large changes in succession with juglone addition.
Six major algae species were present in all enclosures over the 7-day period (Table 1; Figure 4). Among these species, four cyanobacteria, including *Anabaena flos-aquae* (15,000 cells mL$^{-1}$), *M. aeruginosa* (21,400 cells mL$^{-1}$), *Oscillatoria curviceps* (52,000 cells mL$^{-1}$), and *Phormidium subfuscum* (74,000 cells mL$^{-1}$), dominated the algal community (>10,000 cells mL$^{-1}$) on day 0 (Table 1). After treatment with juglone, the dominant algae (>1000 cells mL$^{-1}$) on day 7 were two green algae (*Scenedesmus eornis* and *S. quadricauda*) and four cyanobacteria (*A. flos-aquae, M. aeruginosa, O. curviceps*, and *P. subfuscum*) (Table 1).

**Table 1.** Changes in algal species and abundance (cells mL$^{-1}$) after treatment with different concentrations of juglone in the enclosure bioassay. Data are presented as averages with standard errors of duplicate experiments.

| Classifications | Algal Species                  | Day 0 (Control) | Day 7 (0.1 mg L$^{-1}$) | Day 7 (1 mg L$^{-1}$) |
|-----------------|--------------------------------|-----------------|--------------------------|-----------------------|
| **Cyanobacteria** | *Anabaena flos-aquae*          | 15,000 ± 3000   | 29,500 ± 24,500          | 3750 ± 2250           |
|                  | *Merismopedia glauca*          | nd              | 480 ± 160                | 608 ± 332             |
|                  | *Microcystis aeruginosa*       | 21,400 ± 3000   | 27,500 ± 1300            | 24,600 ± 3600         |
|                  | *Oscillatoria curviceps*       | 52,000 ± 4000   | 17,000 ± 700             | 13,400 ± 1400         |
|                  | *Phormidium subfuscum*         | 74,000 ± 20,000 | 88,000 ± 10,000          | 13,550 ± 4450         | 3400 ± 3400 |
| **Green algae**  | *Ankistrodesmus falcatus*      | 80 ± 20         | 20 ± 20                  | 75 ± 45               | 30 ± 30       |
|                  | *Coelastrum sp.*              | nd              | 48 ± 16                  | nd                    | nd            |
|                  | *Pediastrum duplex*           | 240 ± 80        | 120 ± 40                 | 116 ± 84              | 48 ± 16       |
|                  | *Pediastrum simplex*          | 240 ± 0         | 160 ± 80                 | 320 ± 80              | 76 ± 44       |
|                  | *Scenedesmus acuminatus*      | 280 ± 200       | 180 ± 20                 | 770 ± 130             | 30 ± 30       |
|                  | *Scenedesmus quadricauda*     | 1040 ± 160      | 3320 ± 1480              | 6900 ± 1700           | 4220 ± 580    |
|                  | *Scenedesmus sp.*             | 280 ± 40        | 500 ± 100                | 1760 ± 1440           | 1300 ± 300    |
|                  | *Schroederia nitzschioides*   | 500 ± 100       | 30 ± 30                  | 410 ± 110             | 10 ± 10       |
| **Others**       | *Aulacoseira sp.*             | 340 ± 20        | nd                       | nd                    | nd            |
|                  | *Cylcotella sp.*              | 220 ± 100       | 340 ± 60                 | 100 ± 20              | 390 ± 170    |
|                  | *Ceratium hirundinella*       | 4 ± 1           | nd                       | nd                    | nd            |
|                  | *Peridinium bipes*            | 175 ± 25        | 80 ± 70                  | 25 ± 5                | 35 ± 25       |
|                  | *Cryptomonas ovata*           | 200 ± 40        | 110 ± 70                 | 10 ± 10               | nd            |
|                  | *Trachelomonas sp.*           | 15 ± 5          | nd                       | nd                    | nd            |
| Total number of species | 19 | 17 | 16 | 15 | 13 |

(Remarks) nd: not detected.

**Figure 4.** Growth responses (% cell density relative to control) of dominant algal species in the field enclosures after the addition of juglone (0.1 and 1 mg L$^{-1}$). Data are presented as averages with standard errors (bars) of duplicate experiments.
Three of these dominant cyanobacteria, *O. curviceps*, *P. subfuscum*, and *A. flos-aquae*, were inhibited by 21.2 ± 8.0%, 84.6 ± 5.0%, and 87.3 ± 6.0%, respectively, after treatment with 0.1 mg L\(^{-1}\) of juglone, and by 96.5 ± 1.5%, 96.1 ± 1.8%, and 98.3 ± 0.0%, respectively, at 1 mg L\(^{-1}\) juglone (Figure 4). The cell density of *M. aeruginosa* decreased by 10.5 ± 14.0% and 15.0 ± 9.0% after treatment with 0.1 and 1 mg L\(^{-1}\) juglone, respectively (Figure 4), and these reduction rates were much lower than those observed in the laboratory results (37.7 ± 6.9% and 92.7 ± 5.4%, respectively). The growth of two green algae, *S. quadricauda* and *S. ecornis*, was stimulated (Figure 4), as observed in the laboratory bioassays. Notably, the cell density of *S. quadricauda* remarkably increased by 350 ± 59.0% and 250 ± 65.0% compared to that of the control after treatment with 0.1 and 1 mg L\(^{-1}\) juglone, respectively. The cell density of *S. ecornis* also increased by 208 ± 26.0% and 125 ± 18.0% after treatment with 0.1 and 1 mg L\(^{-1}\) juglone, respectively.

4. Discussion

The present study showed that juglone inhibited the growth of various algae, particularly harmful, bloom-forming species such as *S. hantzschii*, a diatom that flourishes in cold seasons, and *M. aeruginosa*, a cyanobacterium abundant in warm seasons. This study also demonstrated that juglone produced positive stimulatory effects as well as hormetic effects on the growth of some green algae and diatoms, suggesting its differential allelopathic effects on different algal assemblages.

According to the broad definition, allelopathy includes both inhibitory and stimulatory effects of one plant upon another, including microorganisms [37]. During this study, the observed allelopathic effects of juglone differed among diverse algal assemblages, including diatoms, green algae, and cyanobacteria (Table 2). Notably, a low level (0.1 mg L\(^{-1}\)) of juglone selectively inhibited the growth of *S. hantzschii* by 66.2 ± 8.6%. However, the growth response of the other three diatoms (*A. formosa*, *F. crotonensis*, and *S. acus*) varied with different concentrations of juglone, showing stimulatory hormetic responses at 0.1 mg L\(^{-1}\) but inhibitory effects at 1 mg L\(^{-1}\). Hormesis, the beneficial or stimulatory effect of a toxicant at a dose lower than that causing the first detectable negative effects, also was observed in some green algae and cyanobacteria treated with low concentrations of juglone (see Table 2) [24,25]. During a study of the co-culture of two green algae, Chen et al. [38] observed similar differential allelopathic effects; they observed that the growth of *Chlorella pyrenoidosa* was inhibited by *Hydrodictyon reticulatum*, with a notable hormetic effect at low concentrations. Wendt et al. [39] also reported hormesis in the growth of macroalga *Ulva lactuca* zoospores induced by low concentrations of a biocide (triphenylborane pyridine). These studies demonstrated that hormetic responses are related to low concentrations of allelochemicals in different algal species.

Positive allelopathic effects (growth stimulation) in addition to hormesis are more likely to occur in green algae in contrast with the effects observed for cyanobacteria (Table 2). During our study, all tested green algae showed growth stimulation from all concentrations of juglone in both the laboratory and field bioassays (Figure 2; Figure 4). Similar results were reported in allelopathic studies of several green algae [38,40,41]. Additionally, previous studies demonstrated that allelochemicals (e.g., β-sitosterol-β-D-glucoside, dicyclohexanyl orizane, L-2-azetidinecarboxylic acid, lignin and hydrolyzable polyphenol) obtained from several aquatic and terrestrial plants had lower inhibitory effects on the growth of green algae than on cyanobacteria [19,22,42,43]. These results conclusively suggest that green algae have a higher resistance to allelochemicals than do cyanobacteria.

Some green algae change morphology in response to physical, chemical, and biological stressors in their environments [19]. We observed that colony formation of *S. quadricauda* and *C. vulgaris* was enhanced by juglone in the laboratory experiments. This may be a defense strategy against allelochemicals. Additionally, previous studies suggested that phenolic compounds could be metabolized by some green algal cells [44–46]. Such biodegradation abilities are another survival strategy against allelochemicals. These survival strategies stimulate the growth of green algae if allelochemical concentrations are below a certain threshold. Additionally, some organic solvents were
reported to affect algal growth in both inhibiting and stimulating manners depending on different concentrations and algae [47]. Therefore, there might be a potential effect of methanol on our results.

| Classifications | Species | Growth Responses | Concentrations (mg L$^{-1}$) and Effects | References |
|-----------------|---------|------------------|----------------------------------------|------------|
| Diatoms         | Asf     | H                | 0.01(−), 0.1(+), 1(−−−−−)              | Lab        |
|                 | Frc     | H                | 0.01(−), 0.1(++++), 1(−−−−−)            | Lab        |
|                 | Sth     | A(−)             | 0.01(−), 0.1(−−−), 1(−−−−−)             | Lab        |
|                 | Sya     | H                | 0.01(−), 0.1(+), 1(−−−−−)               | Lab        |
|                 | Clv     | A(+)             | 0.01(+ + +), 0.1(+ + +), 1(+ + +)      | Lab        |
|                 | Clia    | H                | 1.74(+), 17.4(−), 174(−−−−−)            | [25]       |
|                 | Euc     | H                | 1.74(+), 17.4(−), 174(−−−−−)            | [25]       |
|                 | Mit     | H                | 1.74(+), 17.4(−), 174(−−−−−)            | [25]       |
|                 | Nes     | A(−)             | 0.52(−−−−−)                             | [27]       |
|                 | Pm      | A(−)             | 1.74(−), 17.4(−), 174(−−−−−)            | [25]       |
|                 | Sca     | H                | 0.01(+), 0.1(+), 0.5(+), 1(−−−−−), 10(−−−−−) | [24]       |
|                 | Sce     | A(+)             | 0.1(+ + + +), 1(+)                     | Field      |
|                 | Scq     | A(+)             | 0.01(+), 0.1(+), 1(+)                  | Lab        |
|                 | Scq     | A(+)             | 0.1(+ + + +), 1(+)                     | Field      |
|                 | Sec     | A(−)             | 0.174(−), 1.74(−−−−−)                  | [26]       |
|                 | Spg     | H                | 1.74(+), 17.4(−), 174(−−−−−)            | [25]       |
|                 | Anf     | H                | 0.01(+), 0.1(−), 0.5(−−−−−), 1(−−−−−), 10(−−−−−) | [24]       |
|                 | Anf     | A(−)             | 0.1(−−−−−), 1(−−−−−)                   | Field      |
|                 | Mia     | A(−)             | 0.01(−), 0.1(−−−), 1(−−−−−)             | Lab        |
|                 | Mia **  | A(−)             | 0.1(−), 1(−)                            | Field      |
|                 | Noc     | H                | 0.01(+), 0.1(+), 0.5(+), 1(−), 10(−−−−−) | [24]       |
|                 | Och     | A(−)             | 0.0174(−), 0.174(−−−−−)                | [26]       |
|                 | Ocu     | A(−)             | 0.1(−), 1(−−−−−)                       | Field      |
|                 | Phs     | A(−)             | 0.1(−−−−−), 1(−−−−−)                   | Field      |

Remarks: *: Unicellular type, **: Colonial type, Lab: This study (in laboratory), Field: This study (in field), Asf: Asterionella formosa, Frc: Fragilaria crotonensis, Sth: Stephanodiscus hantzschii, Sya: Syndera acus, Chv: Chlorella vulgaris, Clv: Closterium acerosum, Euc: Euglena california, Mit: Microcystis thomansiana, Nes: Neochloris sp., Pam: Pandorina morum, Sca: Scenedesmus acuminatus, Sce: Scenedesmus quadricauda, Sec: Selenastrum capricornutum, Spg: Spirogyra grevilleana, Anf: Anabaena floe-aquae, Mia: Microcystis aeruginosa, Noc: Nostoc commune, Och: Oscillatoria cl. chalybae, Ocu: Oscillatoria curvipes, Phs: Phormidium subfuscum, H: Hormesis, A(+) Allelopathy (stimulation of growth), A(−): Allelopathy (inhibition of growth), (+): Slight stimulation (<25%), (++): Moderate stimulation (25−50%), (+++): High stimulation (50−75%); (+++): Strong stimulation (75%), (−): Slight inhibition (<25%), (−−): Moderate inhibition (25−50%), (−−−): High inhibition (50−75%), (−−−−): Strong inhibition (>75%).

Conversely, negative allelopathic effects (growth inhibition) of juglone were common for cyanobacteria (Figure 1b; Figure 4; Table 2). The effects varied depending on juglone levels, cyanobacteria taxa, and cell type. During the present study, the growth of unicellular M. aeruginosa was inhibited more strongly by juglone than that of the colonial type (Figure 2e; Figure 4). We observed this same pattern of differential inhibitory effects on M. aeruginosa in a prior study using different allelochemicals extracted from rice hulls [18]. Notably, juglone showed a selective effect on cyanobacteria, compared to its effects on green algae and some diatoms. These results indicate that juglone has potential as an effective bacteriocide for controlling blooms of nuisance cyanobacterial species while leaving other algae less affected. Considering that M. aeruginosa generally exists in its colonial form, relatively high concentrations of juglone (>1 mg L$^{-1}$) would be necessary for effective control. A combination of juglone and other allelochemicals [18,21,22] may allow juglone to be effective even at lower concentrations. The synergistic effects of such combination treatments could not only to improve inhibition, but also reduce the hormetic or stimulatory effects induced by one allelochemical. This hypothesis warrants further investigation.

Our results support the general allelopathic hypothesis that biotic sensitivity to chemicals varies according to diverse parameters, such as taxonomy, morphology, and anatomy of target organisms, as well as types and doses of chemicals [48,49]. The results of our laboratory and field assays demonstrate that algal sensitivity to juglone differed depending on algal taxa and cell types (Table 3).
First, the inhibitory effect of juglone at 0.1 mg L\(^{-1}\) was highest against the unicellular form of *Microcystis*, followed by *Anabaena, Phormidium, Oscillatoria*, and *Microcystis* in its colonial form. Second, the cyanobacteria were the taxonomic group most sensitive to juglone, followed by diatoms (aside from *S. hantzschii*) and green algae. Finally, unicellular morphology was most sensitive to juglone, followed by small chain, large chain, and colonial morphologies.

**Table 3.** Differential allelopathic sensitivity of the tested algae to juglone at 0.1 mg L\(^{-1}\). The degree of sensitivity was determined according to algal taxa, groups, and cellular morphology.

| Algal group or types | Allelopathic sensitivity to juglone (0.1 mg L\(^{-1}\)) |
|---------------------|---------------------------------------------------------|
| Cyanobacteria       | *Microcystis*(u) \(\ast\) > *Anabaena and Phormidium* > *Oscillatoria* > *Microcystis*(c) ** |
| Taxonomic groups    | cyanobacteria > diatoms > green algae                  |
| Morphology          | unicellular type > small chain type > large chain type > colonial type |

(Remarks) \(\ast\): unicellular type, **: colonial type.

Sensitivity to algicides is associated with cell morphology, cell wall thickness, and cell size among various algae [14,18,19,50,51]. During this study, algae with colonial morphology and large cell size showed a lower sensitivity to juglone than did algae with a unicellular morphology and small cell size (Figure 2; Figure 4; Table 3). Cyanobacterial cell walls are approximately 10 nm thick in algae with unicellular morphology, such as *Synechococcus*; 15–35 nm thick in algae with small chain morphology, such as *Phormidium*; and more than 700 nm thick in algae with large chain morphology, such as *Oscillatoria* [52]. Prokaryotic cells lack membrane-bound organelles, unlike eukaryotic cells, which also influences allelochemical sensitivity. The cell walls and sizes of most prokaryotes, including cyanobacteria, are considerably thinner and smaller than those of eukaryotic cells, such as green algae and diatoms. During our laboratory bioassays, the green algae formed colonial cells, unlike the diatom *S. hantzschii* and cyanobacterium *M. aeruginosa*. *S. hantzschii* and *M. aeruginosa* exhibited unicellular morphologies and were more sensitive to juglone than were other algae. However, the diatoms *F. crotonensis* and *A. formosa* exhibited chain and colonial morphologies, respectively. Filamentous chain morphologies were more resistant to low concentrations of juglone (0.1 mg L\(^{-1}\)) than was the unicellular *S. hantzschii*.

To conclude, juglone produced differential allelopathic effects and even hormesis in various kinds of algae and, in particular, it inhibited some bloom-forming harmful algae, suggesting that it can selectively control harmful nuisance algal species such as *S. hantzschii* and *M. aeruginosa*. Our study is the first to our knowledge focused on the allelopathy of juglone in diatoms, reporting interesting results on both growth inhibition and hormesis, which will improve the understanding of algal allelopathy. The hormetic responses of diatoms observed in this study suggest that low levels of juglone applied to natural ecosystems would not affect non-target diatom abundance and composition to the extent of blocking its control effects. Based on our results and those from the literature [24–27], juglone is an effective candidate for the control of harmful algal blooms. However, further studies evaluating the extent and magnitude of the synergistic effects of allelochemical combinations as well as the allelochemical effects of more specified (particularly lower) concentrations will be a necessary step in advancing the field of allelochemical control of harmful algal blooms.

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