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The allelopathic algicides sanguinarine and berberine reduced the dominance of *Microcystis* in competition with *Chlorella*

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ABSTRACT

Gramine, sanguinarine and berberine are potential algicides that can significantly inhibit the growth of cyanobacteria. Their effects on other phytoplankton have to be considered, because other phytoplankton species and cyanobacteria usually co-exist. In this study, we have established the relationships between phytoplankton biomasses and their marker pigments. Afterwards, we have assessed the allelopathic effects of three algicides (gramine, sanguinarine and berberine) on the mono-cultured and mixed-cultured cyanobacteria *Microcystis* and green algae *Chlorella*. Our results showed that zeaxanthine and lutein can be used to determine the cell densities of *Microcystis* and *Chlorella* in the mixed cultures, respectively. Our study also demonstrated that the inhibitory effects of these three allelopathic algicides on the growths of both *Microcystis* and *Chlorella* were sanguinarine > gramine > berberine. The concentrations for 50% of the maximal effect (EC_{50}) of sanguinarine against *Microcystis* and *Chlorella* were 0.065 and 0.09 mg L^{-1}, respectively; the EC_{50} values of gramine against *Microcystis* and *Chlorella* were 1.687 and 0.870 mg L^{-1}, respectively; and the EC_{50} values of berberine against *Microcystis* and *Chlorella* were 1.969 and 2.397, respectively. The inhibitory effect of allelopathic algicides on the multiple phytoplankton species in mixed cultures was significantly greater than that in monocultures. In mixed cultures without allelopathic addition or in cultures with gramine (0.5–3 mg L^{-1}), the dominant species was *Microcystis*. However, *Chlorella* became the dominant species when the concentration of sanguinarine was 0.1 mg L^{-1} or that of berberine was greater than 0.5 mg L^{-1}. Both species of phytoplankton died completely when sanguinarine levels were greater than 0.1 mg L^{-1}. Our results suggested that the allelopathic algicides sanguinarine and berberine reduced the dominance of *Microcystis* in competition with *Chlorella*.

1. Introduction

Cyanobacteria, which is the most common harmful phytoplankton in freshwater environments cause by eutrophication [1–3], frequently forms cyanobacterial blooms in most eutrophic lakes and reservoirs, leading to severe deterioration of water quality and reduced diversity of aquatic organisms [4,5]. Some cyanobacteria species produce toxins that threaten human health [6]. There are also some alternative uses of algae [7–10]. Consequently, it is necessary to reduce cyanobacterial biomass in lakes and reservoirs for the control of bloom occurrence or beneficial use of algae biomass.

The dominance of cyanobacteria is prerequisite for producing a large amount of cyanobacterial biomass and forming blooms under natural conditions [11]. Thus, evaluation of the competition between cyanobacteria and other phytoplankton species is important to understanding the occurrence of blooms of cyanobacteria. Previous studies have investigated the effects of light intensity [12], nutrient concentrations [13,14], temperature [15] and other environmental factors [16,17] on competition between cyanobacteria and other phytoplankton species. Although great progress has been made in this field, it is still necessary to study the effects of allelopathic algicides on competition between cyanobacteria and other phytoplankton species because they were still used to reduce cyanobacteria biomass quickly in some artificial ponds but the adverse environmental effects have not been paid enough attention.

Numerous allelopathic algicides including acids, phenols and alkaloids from different sources have been reported to effectively inhibit the

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growth of cyanobacteria. Some studies have indicated that linoleic acid from *Chlorella vulgaris* [18], vanillic acid from *Vallisneria spiralis* [19], nonanoic acid and ellagic acid from *Myriophyllum spicatum* [20,21], and other fatty acids or phenolic acids significantly inhibited the growth of the cyanobacterium *Microcystis*. Phenols from *Riccia fluitans* [22] and volatiles from *Tychonema bourrellyi* [23] were also found to reduce cyanobacterial biomass. Alkaloids, which are effective allelopathic algicides, have also been shown to have inhibitory effects on the growth and cell division of *Microcystis aeruginosa* [24]. However, all of the above studies focused on the effects of allelopathic algicides on individual cyanobacterial species, while competition between cyanobacteria and other phytoplankton has not been thoroughly investigated. Accordingly, it is unclear if these allelopathic algicides work well under natural conditions in which several phytoplankton species coexist [25]. Additionally, the ecological effects of allelopathic algicides should also be assessed. Thus, it is necessary to study the effects of allelopathic algicides on competition between cyanobacteria and other phytoplankton species.

In the current study, *Microcystis* and *Chlorella* were selected as model species to study the competitive relationships between cyanobacteria and other phytoplankton species. *Microcystis* is a common cyanobacterial species that forms large blooms in summer and autumn [26–29], while the green algae *Chlorella* is widely distributed in eutrophic waters [30]. Biomass assessment of systems including these two species is a challenge because of their similarity in morphology under the microscope [31,32].

Pigments were used to assess phytoplankton in marine and freshwater ecosystems in recent years [33–35]. These studies showed that zeaxanthine and lutein could be used as marker pigments for cyanobacteria and chlorophyta, respectively [36,37]. Zhao et al. [38] also demonstrated that the concentration of zeaxanthine and lutein had good linear relationships with *Microcystis* and *Scenedesmus* biomass, respectively. Thus, zeaxanthine and lutein were used to calculate the biomass of *Microcystis* and *Chlorella* in the present study.

The specific goals of this study were to clarify the effects of allelopathic algicides on competition between *Chlorella* and *Microcystis*, with their biomass assessed using zeaxanthine and lutein. Three alkaloidal allelopathic algicides including gramine, sanguinarine and berberine were tested for their ability to inhibit *Microcystis*. The concentrations for 50% of the maximal effect (EC_{50}) of these three algicides against *Microcystis* have been shown to be 0.5–2.1 mg L^{-1} [39], 0.035 mg L^{-1} [40] and 0.851–1.627 mg L^{-1} [41], respectively. However, the effective concentration (EC_{50}) of sanguinarine toward *Chlorella pyrenoidosa* is 5.32 mg L^{-1} and that toward *Scenedesmus obliquus* is 9.70 mg L^{-1} [42], both of which are higher than that of *Microcystis*. Several researchers also reported that the green algae including *Chlorella* was less vulnerable to contaminants than *Microcystis* [43–46]. Thus, the competitiveness may be decreased for *Microcystis* and increased for *Chlorella* when allelopathic algicides are added. Therefore, we investigated whether allelopathic algicides could improve the competitiveness of *Chlorella* and lead to *Chlorella* becoming the dominant species in mixed cultures with *Microcystis*.

2. Materials and methods

2.1. Organisms and sources of alkaloids

Axenic strains of *Microcystis aeruginosa* (FACHB−469) and *Chlorella* sp. were used in this study. *M. aeruginosa* was obtained from the Freshwater Algae Culture Collection at the Institution of Hydrobiology, Chinese Academic of Science (Wuhan, China). *Chlorella* sp. was isolated from the Danjiangkou Reservoir. The two unicellular strains were cultured in BG11 medium for three months before the experiments.

The guaranteed reagent of gramine ($C_{11}H_{14}N_2$), sanguinarine chloride hydrate (sanguinarine; $C_{20}H_{14}C_1NO_4{\cdot}xH_2O$) and berberine chloride (berberine; $C_{20}H_{18}C_1NO_4$) were purchased from Sigma-Aldrich.

2.2. Culture conditions

M. aeruginosa and *Chlorella* sp. were inoculated into 150 mL liquid medium in a conical flask, then grown at 25 °C for 10 days in a thermostatic incubator (GXZ-800C-3, Ningbo Jiangnan Instrument Factory, Zhejiang, China). The initial biomass of *M. aeruginosa* and *Chlorella* sp. were 30×10^4 cells mL^{-1} and 5×10^4 cells mL^{-1}, respectively, because the cell volume of *M. aeruginosa* was six times smaller than that of *Chlorella* sp [32,47]. The fluorescent light intensity was 50 μmol photons m^{-2} s^{-1} with a 12: 12 Light: dark cycle. Triplicate samples were prepared and measured for each treatment, and all cultures were manually shaken three times a day.

2.3. Experimental design and cells counts

M. aeruginosa and *Chlorella* sp. were monocultured and mixed cultured in BG11 medium containing different concentrations of allelopathic algicides including gramine, sanguinarine and berberine. The cell density of *Microcystis* and *Chlorella* in monocultures was determined using ultraviolet spectrometry (UV-1780, Shimadzu, Japan) based on the relationships between biomass of *M. aeruginosa* and *Chlorella* sp. and their absorption at 680 nm (Eq. (1); Eq. (2)). Simultaneously, the concentrations of zeaxanthine unique to *Microcystis* and lutein unique to *Chlorella* were determined using High Performance Liquid Chromatography (HPLC, Agilent-1100 series, USA) to establish the relationships between biomass and their unique pigments. Thus, the biomass of *Microcystis* and *Chlorella* in mixed cultures was calculated according to the concentration of zeaxanthine and lutein, respectively. The effects of allelopathic algicides on growth and competitiveness of these two species was also assessed.

$$\text{Cell density of } \textit{Microcystis}\ \left(10^4 \text{ cells mL}^{-1}\right) = 2078 \times \text{OD}_{680} - 72\ \left(R^2 = 0.982\right) \quad (1)$$

$$\text{Cell density of } \textit{Chlorella}\ \left(10^4 \text{ cells mL}^{-1}\right) = 674.18 \times \text{OD}_{680} - 6\ \left(R^2 = 0.9946\right) \quad (2)$$

2.4. Pigment determination

A certain volume of each culture was sampled and then filtered through glass fiber filter membranes (Whatman GF/F) every day during the experimental period. The membranes were subsequently placed in a plugged test tube with 5 mL of 95% acetone. The pigments were subsequently extracted with ultrasonic cleaner (SB 25–12 DTDN, Ningbo Xinzhi Biotechnology Co., Ltd., Zhejiang, China) at 4 °C for 10 min and then stored in a freezer (Galanz BCD-180 N, Guangdong, China) at −20 °C for 24 h in darkness. Next, the extracts were filtered through 0.22 μm filters with polytetrafluoroethylene (PTEF) syringes before being transferred into a 1.5 mL brown chromatographic bottle. Finally, qualitative and quantitative analysis of the pigments was conducted by HPLC.

Pigments were analyzed by a high performance liquid chromatography (HPLC, Agilent-1100 series, USA) equiped a Diode-Array Detector using mobile phase A and B under a constant maximum pressure limit of 400 bar. Mobile phase A was a mixture of 2:1 methanol: 0.5 mol L^{-1} ammonium acetate, while mobile phase B was 30% methanol and 70% acetonitrile. The injection volume was 100 μL, while the flow rate (mL min^{-1}) and ratios of mobile phase A and B changed over time (Table S1). Chromatographic column temperature and sample injection volume were 30 °C and 100 μL, respectively, and the detection wavelengths were 440 nm, 266 nm, and 300 nm.

2.5. Data analysis

Data in this study were expressed as the means ± SD (standard

deviation). Differences among treatments were identified by one-way variance (ANOVA) using the IBM SPSS Statistics 22 software.

The specific growth rates (μ, day^{-1}) of *M. aeruginosa* and *Chlorella* sp. in monocultures and mixed cultures were calculated (Eq. (3)). In this formula, B represents the biomass of phytoplankton, t indicates the culture time (day), and b is the initial biomass (cells mL^{-1}).

$$\ln B = \mu \bullet t + \ln b \tag{3}$$

The logistic function (Eq. (4)) was used to fit growth curves, where N, K and r represent biomass, carrying capacity and intrinsic growth rate of phytoplankton, respectively. The inflexion of time (t_p, day) means the time of initial growth inhibition (Eq. (5)).

$$N = K / (1 + e^{a - r \bullet t}) \tag{4}$$

$$t_p = (a - ln\ 2) / r \tag{5}$$

2.6. Lotka-Volterra model of competition

The population dynamics of *M. aeruginosa* and *Chlorella* sp. in mixed cultures were evaluated using the Lotka-Volterra competitive model [48], where α and β are competition coefficients indicating the effects of *M. aeruginosa* on *Chlorella* sp. and the effects of *Chlorella* sp. on *M. aeruginosa*, respectively. In this model (Eq. (6)), N_s (N_{s-1}) and N_m (N_{m-1}) are the biomass of *M. aeruginosa* and *Chlorella* sp. at t_n (t_{n-1}) hours, while r_s and r_m are the intrinsic growth rate of *M. aeruginosa* and *Chlorella* sp., respectively.

$$(N_s - N_{s-1}) / (t_n - t_{n-1}) = r_s \bullet N_{s-1} \bullet (K_s - N_{s-1} - \alpha \bullet N_{m-1}) / K_s$$

$$(N_m - N_{m-1}) / (t_n - t_{n-1}) = r_m \bullet N_{m-1} \bullet (K_m - N_{m-1} - \beta \bullet N_{s-1}) / K_m \tag{6}$$

3. Results

3.1. Method establishment for determination of phytoplankton biomass

There were good linear relationships between phytoplankton and their pigments (Fig. 1). The regression coefficients (R^2) were 0.7586 between *M. aeruginosa* and zeaxanthine and 0.8071 between *Chlorella* sp. and lutein, indicating that the cell density of *M. aeruginosa* and *Chlorella* sp. in mixed cultures can be calculated according to their respective marker pigments. The slopes in the formulas were 1873.7 with an intercept of 115.05 for *Microcystis* and 156.21 with an intercept of 16.768 for *Chlorella*.

3.2. Competition experiment

The cell density of *M. aeruginosa* in mixed cultures was less than that in monocultures, while the opposite was observed for *Chlorella* sp. (Fig. S1). After 10 days of incubation, the biomass of *Microcystis* and *Chlorella* in monocultures was 1.91×10^7 cells mL^{-1} and 0.48×10^7 cells mL^{-1}, respectively. In mixed cultures, the biomass of *Microcystis* and *Chlorella* was 0.84×10^7 cells mL^{-1} and 0.53×10^7 cells mL^{-1}, respectively. When compared with monocultures, cell density in mixed cultures decreased by 55.93% for *Microcystis* and increased by 9.36% for *Chlorella*.

Allelopathic algicides of gramine, sanguinarine and berberine had significant inhibitory effects on the growth and specific growth rate (μ) of *Microcystis* and *Chlorella* (Table 1). As the concentration of these three algicides increased, the cell density and specific growth rate (μ) of *Microcystis* and *Chlorella* in monocultures or mixed cultures decreased dramatically (Figs. S1–S3).

Gramine, sanguinarine and berberine had different inhibitory effects on the growth of *Microcystis* and *Chlorella*. Specifically, the growth inhibition of gramine toward *Microcystis* was lower than that toward *Chlorella*. When the gramine concentration was 3 mg L^{-1}, the cell densities of *Microcystis* and *Chlorella* in monoculture were 0.29×10^7 cells mL^{-1} and 0.04×10^7 cells mL^{-1}, respectively, which were 85% and 91% lower than those of the control. When cultured in mixed conditions, gramine reduced the *Microcystis* by 91.58%. There was an identical inhibitory effect of sanguinarine on the two phytoplankton species. The biomass of *Microcystis* and *Chlorella* in monocultures was 0.049×10^7 cells mL^{-1} and 0.014×10^7 cells mL^{-1}, respectively, when 0.5 mg L^{-1} of sanguinarine was added, and both strains decreased by 97% compared with the control treatment. The inhibition ratios of different berberine concentrations toward *Microcystis* were all greater than those toward *Chlorella* in monocultures or mixed cultures, which was contrary to the changes in gramine.

The inhibitory effects of allelochemicals toward *Microcystis* and *Chlorella* occurred in the order sanguinarine > gramine > berberine (Fig. 2). The EC_{50} of sanguinarine against *Microcystis* and *Chlorella* was 0.063–0.064, which was the lowest among the tested algicides. Sanguinarine had the lowest EC_{50}, indicating that this allelopathic algicide had the greatest inhibitory effect on the two phytoplankton species. The EC_{50} of gramine was greater than that of berberine, but less than that of sanguinarine. Berberine had the maximum EC_{50} against *Microcystis* and *Chlorella* and the lowest inhibition of the growth of the two species. The EC_{50} of berberine against *Microcystis* and *Chlorella* was 1.750 and 2.569 in monocultures, while it was 0.361 and 0.869 in mixed cultures, respectively.

The EC_{50} of gramine, sanguinarine and berberine against phytoplankton cultured in mixed conditions decreased significantly when compared with that in monoculture conditions, indicating that these three allelochemicals had greater inhibitory effects on the growth of *Microcystis* and *Chlorella* in mixed cultures (Fig. 2). The EC_{50} against *Microcystis* and *Chlorella* cultured in mixed conditions was reduced by

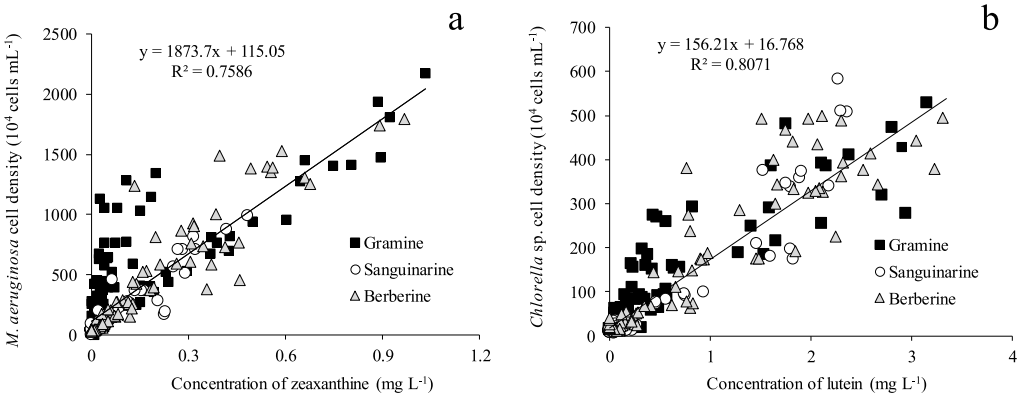

Fig. 1. Fitting curves of phytoplankton and pigments (a, *M. aeruginosa* and zeaxanthin; b *Chlorella* sp. and lutein).

Table 1
Specific growth rate (μ, day^{-1}) of *M. aeruginosa* and *Chlorella* sp. in monocultures and mixed cultures with different concentrations of gramine, sanguinarine and berberine.

Alkaloid	Concentration	Mono cultures	Mixed cultures		Mono cultures
		M. aeruginosa	M. aeruginosa	*Chlorella* sp.	*Chlorella* sp.
Gramine	0.00 mg L^{-1}	0.418 ± 0.009	0.323 ± 0.019	0.471 ± 0.011	0.459 ± 0.006
	0.50 mg L^{-1}	0.410 ± 0.008	0.177 ± 0.033	0.307 ± 0.058	0.397 ± 0.003
	1.00 mg L^{-1}	0.358 ± 0.003	0.149 ± 0.021	na	0.282 ± 0.003
	3.00 mg L^{-1}	0.226 ± 0.003	na	na	0.212 ± 0.002
Sanguinarine	0.00 mg L^{-1}	0.418 ± 0.009	0.323 ± 0.019	0.471 ± 0.011	0.459 ± 0.006
	0.05 mg L^{-1}	0.339 ± 0.010	0.297 ± 0.009	0.417 ± 0.004	0.467 ± 0.008
	0.10 mg L^{-1}	0.189 ± 0.006	na	0.378 ± 0.012	0.127 ± 0.026
	0.50 mg L^{-1}	0.049 ± 0.053	na	na	0.086 ± 0.012
Berberine	0.00 mg L^{-1}	0.413 ± 0.005	0.323 ± 0.019	0.471 ± 0.011	0.459 ± 0.006
	0.05 mg L^{-1}	0.408 ± 0.002	0.264 ± 0.016	0.464 ± 0.046	0.429 ± 0.005
	0.50 mg L^{-1}	0.389 ± 0.005	0.224 ± 0.009	0.395 ± 0.019	0.477 ± 0.010
	5.00 mg L^{-1}	0.014 ± 0.004	na	na	0.198 ± 0.017

na means not available because there were no cells in the medium.

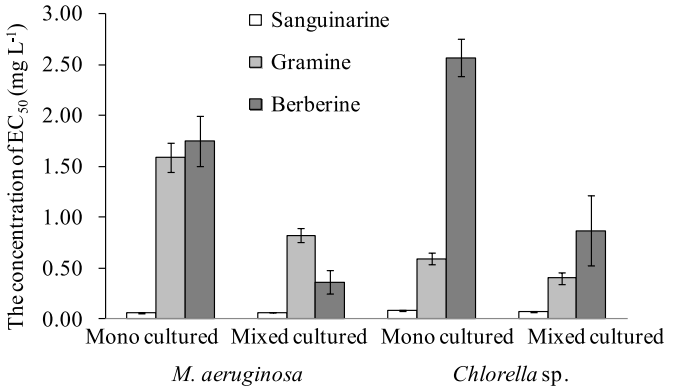

Fig. 2. Concentration for 50% maximal effect (EC_{50}) of gramine, sanguinarine and berberine on *M. aeruginosa* and *Chlorella* sp. in monocultures or mixed cultures.

79.37% and 66.17% for berberine, 48.41% and 31.98% for gramine and −1.59% and 19.54% for sanguinarine, respectively, when compared with monocultures.

3.3. Competition model

The logistic equation parameters of *M. aeruginosa* and *Chlorella* sp. in monocultures and mixed cultures with different concentrations of gramine (Table 2), sanguinarine (Table 3) and berberine (Table 4) were calculated. Most of the regression coefficients (R^2) of *Microcystis* and *Chlorella* were greater than 0.93, indicating that the logistic equation (Eq. (4)) fitted the growth well. Except for parameter a of *Microcystis* in mixed cultures, which increased when sangurine concentration was increasing, carry capacity (K), fitting parameter a and intrinsic growth rate (r) of *Microcystis* and *Chlorella* decreased in monocultures and mixed cultures with increasing concentrations of the three tested algicides.

The inflexion time (t_p) of *Microcystis* and *Chlorella* in monocultures was 5.60 and 4.62 days, which was greater than that in mixed cultures (5.24 and 3.60, respectively). When gramine was added, the inflexion time of *Chlorella* in monocultures or mixed cultures and *Microcystis* in monocultures first increased, then decreased, while the inflexion time of mixed cultures of *Microcystis* decreased. With the addition of sanguinarine, the inflexion time increased for *Chlorella* in monocultures or mixed cultures and for *Microcystis* in mixed cultures, but decreased for monocultured *Microcystis*. The increasing concentration of berberine caused an increase in inflexion time for monocultured *Microcystis*, while it caused a decline for monocultured *Chlorella* and for mixed cultured *Microcystis*. The inflexion time of mixed cultured *Chlorella* decreased

Table 2
Logistic equation parameters and inflexion time of *M. aeruginosa* and *Chlorella* sp. in monocultures and mixed cultures at different gramine concentrations.

Algae	Gramine (mg L^{-1})	K	a	r	R^2	Inflexion time (day)
Mono cultured *M. aeruginosa*	0.00	1913 ± 22	3.46 ± 0.03	0.50 ± 0.03	0.99 ± 0.00	5.60 ± 0.31
	0.50	1876 ± 40	3.37 ± 0.07	0.45 ± 0.02	0.99 ± 0.00	5.95 ± 0.03
	1.00	1073 ± 33	2.59 ± 0.07	0.34 ± 0.03	0.98 ± 0.01	5.54 ± 0.37
	3.00	287 ± 9	1.03 ± 0.08	0.29 ± 0.01	0.93 ± 0.02	1.18 ± 0.03
Mono cultured *Chlorella* sp.	0.00	481 ± 8	3.08 ± 0.06	0.53 ± 0.04	0.98 ± 0.02	4.62 ± 0.16
	0.50	270 ± 2	2.80 ± 0.02	0.35 ± 0.01	0.97 ± 0.01	5.98 ± 0.15
	1.00	84 ± 2	1.54 ± 0.03	0.27 ± 0.02	0.94 ± 0.01	2.98 ± 0.11
	3.00	43 ± 2	1.08 ± 0.11	0.31 ± 0.04	0.94 ± 0.02	1.43 ± 0.03
Mixed cultured *M. aeruginosa*	0.00	843 ± 40	3.37 ± 0.14	0.52 ± 0.03	0.99 ± 0.01	5.24 ± 0.11
	0.50	186 ± 10	2.57 ± 0.10	0.41 ± 0.01	0.91 ± 0.02	4.96 ± 0.28
	1.00	149 ± 4	2.18 ± 0.02	0.30 ± 0.01	0.86 ± 0.00	4.84 ± 0.04
	3.00	71 ± 2	1.25 ± 0.01	0.14 ± 0.01	0.52 ± 0.03	3.73 ± 0.00
Mixed cultured *Chlorella* sp.	0.00	526 ± 16	3.25 ± 0.03	0.65 ± 0.04	0.98 ± 0.01	3.60 ± 0.31
	0.50	78 ± 3	1.65 ± 0.25	0.19 ± 0.01	0.79 ± 0.01	6.07 ± 0.17
	1.00	45 ± 4	0.54 ± 0.24	0.35 ± 0.29	0.43 ± 0.02	4.68 ± 0.50
	3.00	37 ± 3	1.06 ± 0.20	0.15 ± 0.03	0.43 ± 0.01	5.11 ± 0.19

Table 3
Logistic equation parameters and inflexion time of *M. aeruginosa* and *Chlorella* sp. in monocultures and mixed cultures at different sanguinarine concentrations.

Algae	Sanguinarine (mg L^{-1})	K	a	r	R^2	Inflexion time (day)
Mono cultured	0.00	1913 ± 22	3.46 ± 0.03	0.50 ± 0.03	0.99 ± 0.00	5.60 ± 0.31
M. aeruginosa	0.05	1160 ± 39	3.31 ± 0.12	0.45 ± 0.01	0.95 ± 0.05	5.75 ± 0.31
	0.10	238 ± 23	1.62 ± 0.16	0.22 ± 0.02	0.91 ± 0.02	3.56 ± 0.43
	0.50	49 ± 2	−1.29 ± 0.20	−0.05 ± 0.03	0.86 ± 0.02	na
Mono cultured	0.00	481 ± 8	3.08 ± 0.06	0.53 ± 0.04	0.98 ± 0.02	4.62 ± 0.16
Chlorella sp.	0.05	543 ± 97	3.61 ± 0.28	0.48 ± 0.04	0.95 ± 0.02	5.50 ± 0.28
	0.10	35 ± 1	0.44 ± 0.01	0.13 ± 0.03	0.66 ± 0.04	na
	0.50	14 ± 3	−1.05 ± 1.43	0.10 ± 0.02	0.45 ± 0.10	na
Mixed cultured	0.00	843 ± 40	3.37 ± 0.14	0.52 ± 0.03	0.99 ± 0.01	5.24 ± 0.11
M. aeruginosa	0.05	502 ± 25	3.52 ± 0.41	0.44 ± 0.04	0.96 ± 0.01	6.10 ± 0.53
	0.10	122 ± 6	2.12 ± 0.40	0.27 ± 0.03	0.85 ± 0.02	na
	0.50	22 ± 5	na	na	na	na
Mixed cultured	0.00	526 ± 16	3.25 ± 0.03	0.65 ± 0.04	0.98 ± 0.01	3.60 ± 0.31
Chlorella sp.	0.05	380 ± 12	3.50 ± 0.15	0.60 ± 0.05	0.97 ± 0.01	4.69 ± 0.15
	0.10	232 ± 20	4.25 ± 0.19	0.52 ± 0.05	0.82 ± 0.02	7.15 ± 0.14
	0.50	10 ± 1	na	na	na	na

na means not available because there were no cells in the medium.

Table 4
Logistic equation parameters and inflexion time of *M. aeruginosa* and *Chlorella* sp. in monocultures and mixed cultures at different berberine concentrations.

Algae	Berberine (mg L^{-1})	K	a	r	R^2	Inflexion time (day)
Mono cultured	0.00	1913 ± 22	3.46 ± 0.03	0.50 ± 0.03	0.99 ± 0.00	5.60 ± 0.31
M. aeruginosa	0.05	1781 ± 36	3.70 ± 0.04	0.51 ± 0.03	0.97 ± 0.02	5.89 ± 0.10
	0.50	1501 ± 26	3.42 ± 0.04	0.44 ± 0.01	0.98 ± 0.01	6.27 ± 0.22
	5.00	55 ± 1	−1.46 ± 0.15	−0.16 ± 0.04	0.65 ± 0.04	na
Mono cultured	0.00	481 ± 8	3.08 ± 0.06	0.53 ± 0.04	0.98 ± 0.02	4.62 ± 0.16
Chlorella sp.	0.05	487 ± 18	3.32 ± 0.47	0.61 ± 0.04	0.94 ± 0.05	4.33 ± 0.20
	0.50	471 ± 22	3.27 ± 0.50	0.58 ± 0.06	0.95 ± 0.04	5.52 ± 0.20
	5.00	32 ± 4	−0.25 ± 2.13	0.16 ± 0.05	0.93 ± 0.03	2.70 ± 0.22
Mixed cultured	0.00	843 ± 40	3.37 ± 0.14	0.52 ± 0.03	0.99 ± 0.01	5.24 ± 0.11
M. aeruginosa	0.05	456 ± 16	3.33 ± 0.19	0.51 ± 0.03	0.96 ± 0.03	5.19 ± 0.12
	0.50	336 ± 23	3.09 ± 0.34	0.48 ± 0.01	0.94 ± 0.04	4.62 ± 0.04
	5.00	26 ± 5	−1.04 ± 1.87	−0.17 ± 0.02	0.64 ± 0.03	na
Mixed cultured *Chlorella* sp.	0.00	526 ± 16	3.25 ± 0.03	0.65 ± 0.04	0.98 ± 0.01	3.60 ± 0.31
	0.05	522 ± 23	3.36 ± 0.11	0.56 ± 0.08	0.95 ± 0.02	4.90 ± 0.27
	0.50	286 ± 12	2.67 ± 0.02	0.44 ± 0.03	0.90 ± 0.07	3.88 ± 0.26
	5.00	12 ± 3	−1.84 ± 0.08	−0.16 ± 0.13	0.22 ± 0.05	na

na means not available because there were no cells in the medium.

first, then increased in response to increasing concentrations of berberine.

The competition coefficients α (*Chlorella* against *Microcystis*) and β (*Microcystis* against *Chlorella*) are shown in Fig. 3. The growth of *Microcystis* was inhibited by *Chlorella* ($\alpha > 0$), but *Chlorella* biomass was promoted by *Microcystis* ($\beta > 0$) in mixed cultures without allelopathic algicides. When gramine was added, the inhibitory effect of *Chlorella* toward *Microcystis* was promoted, and this promotion increased with increasing concentrations of gramine. The promotion effect of *Microcystis* on *Chlorella* increased in response to low concentrations of gramine (<0.50 mg L^{-1}), then became inhibitory at high concentrations (1.00 mg L^{-1}). Sanguinarine caused an increase in mutual promotion, but intensified inhibition between *Microcystis* and *Chlorella* occurred when berberine was added.

4. Discussion

4.1. Evaluation of method

Significant linear relationships between *Microcystis* and zeaxanthine and between *Chlorella* and lutein were established in this study. Similar stable linear relationships between various phytoplankton and their pigments have been reported in previous studies. El-Sheekh et al. [49] investigated the effects of allelochemicals derived from *Microcystis* on the growth and pigments of four phytoplankton species and found that there were linear relationships between cell density of phytoplankton and their pigments, including chlorophyll-a, carotenoid and chlorophyll-b, with average regression coefficients (R^2) of 0.9400 for *Oscillatoria angutissima*, 0.9770 for *Anabaena*, 0.7492 for *Scenedesmus* and 0.7218 for *Chlorella*. Hong et al. [50] studied the effects of a novel allelochemical ethyl 2-methyl acetoacetate (EMA) on *Microcystis* and found significant correlations between *Microcystis* and carotenoids with a high regression coefficient (R^2) of 0.9504. Thus, pigments in phytoplankton can be used to study the effects of allelochemicals on the competition between *Microcystis* and *Chlorella*.

Pigments in previous studies were not only widely used to estimate biomass, but also to analyze the species composition of complex phytoplankton communities according to pigment ratios [51,52]. Additionally, dynamics of phytoplankton populations can be determined using pigments, which is a more thorough and time-saving examination method than microscopy [34]. Therefore, pigment will become one of the main indicators for quantitative and qualitative analysis of phytoplankton in natural waters.

4.2. Growth inhibition

The inhibitory effects of the three allelochemicals on the growth of *Microcystis* and *Chlorella* occurred in the order sanguinarine > gramine > berberine (Fig. 2). These findings are in accordance with those of many previous studies [53,54]. For example, Shao et al. [40] reported that the EC_{50} of sanguinarine against *Microcystis* was 34.5 ± 1.2 μg L^{-1}, while Hong et al. [39] found that the EC_{50} of gramine and berberine against *Microcystis* were 15 and 36 times higher than that of sanguinarine, respectively. However, the effects of allelopathic algicides on the

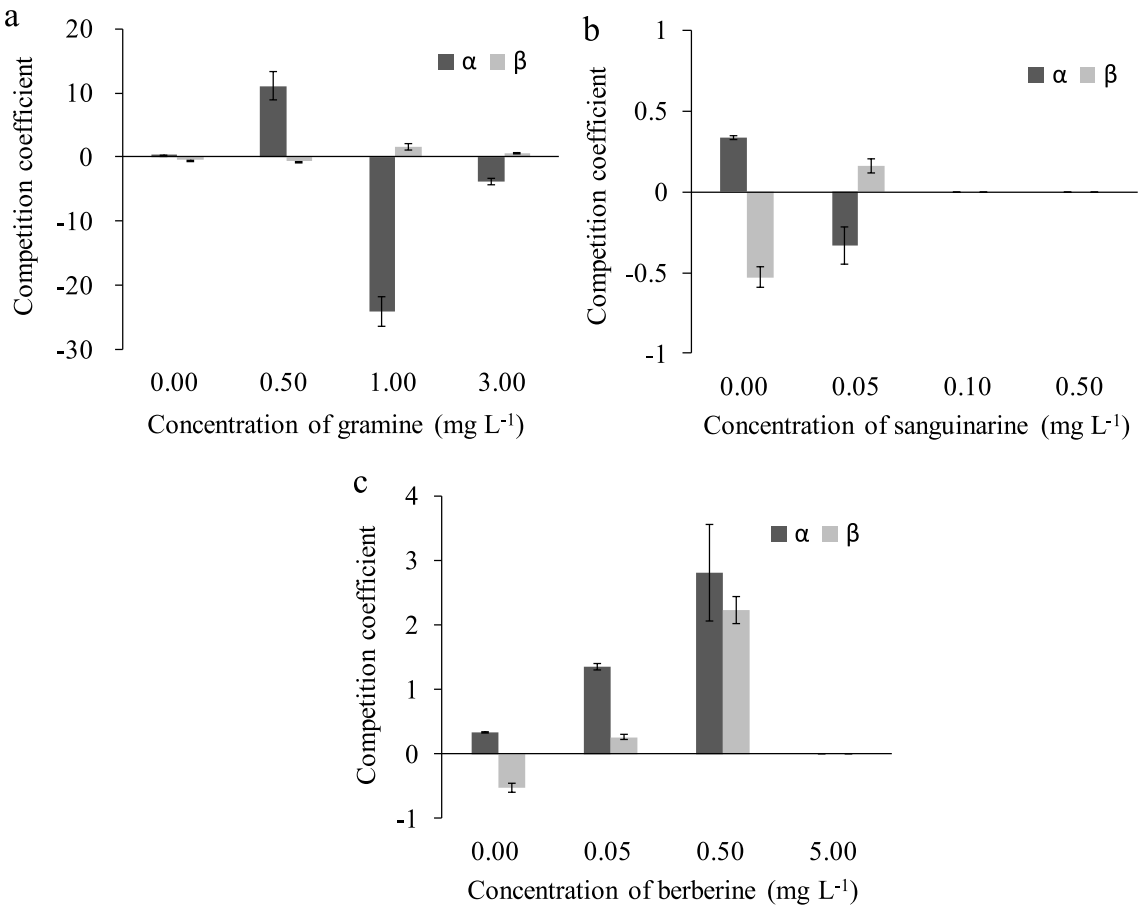

Fig. 3. Competition coefficient of *M. aeruginosa* and *Chlorella* sp. in mixed cultures treated with different concentrations of gramine (a), sanguinarine (b) and berberine (c). na means not available because there were no cells in the medium.

phytoplankton when forming colonies were not taken into account in previous studies [55]. Therefore, the inhibitory mechanisms of these three substances against *Microcystis* and *Chlorella* were investigated. Researchers found that the allelopathic algicides interfered with the transcription and expression of photosynthetic genes [41,56], reducing the synthesis of photosynthetic pigments [50,57,58]. Additionally, these algicides could induce oxidative damage [56] and destroy the ultrastructure of phytoplankton [59]. Thus, gramine, sanguinarine and berberine all had inhibitory effects on cyanobacteria or green algae growth. In addition to the above common inhibitory mechanisms, Lin et al. [60] reported that growth of algae or cyanobacteria was decreased by sanguinarine via inhibition of cell division. This may explain why sanguinarine had the greatest inhibitory effect on the growth of *Microcystis* and *Chlorella*.

The inhibition of allelopathic algicides on the multiple phytoplankton species in mixed cultures was significantly greater than that in monocultures. The synergistic effects of these two factors may be used to explain the differences in toxicity of monocultured or mixed cultured phytoplankton. One was the inhibitory effect of allelopathic algicides on these two strains [60,61]. Another was the allelopathic competition between *Microcystis* and *Chlorella* in mixed cultures [62–64].

4.3. Competition and results

An interesting phenomenon was observed when *Microcystis* and *Chlorella* were cultured together in media containing different allelochemicals; specifically, gramine increased the dominant advantage of *Microcystis*, but sanguinarine (0.1 mg L^{-1}) or berberine (>0.5 mg L^{-1}) significantly reduced the competitiveness of *Microcystis*, leading to the dominance of *Chlorella*. The different competitive results may have occurred because these three allelochemicals are different types of alkaloids. Specifically, berberine and sanguinarine are isoquinoline alkaloids [65] and natural antibacterials, respectively [66,67]. *Microcystis* is a prokaryote with a cellular structure similar to that of bacteria [68]. Therefore, the inhibitory effects of berberine and sanguinarine on *Microcystis* were greater than those on *Chlorella*, which is a eukaryote. Gramine is a toxic indole alkaloid mainly derived from giant reeds [69], barley [70], and reed canary grass [71]. Some studies have suggested that the inhibitory effects of gramine on phytoplankton are mainly because of its ability to cause accumulation of reactive oxygen species (ROS) in phytoplankton cells. This massive accumulation of ROS then inhibits the antioxidant activity of the enzyme and reduces the content of non-enzymatic antioxidants [39]. Li et al. [72] reported that cyanobacteria had stronger antioxidant capacity than green algae, especially *Chlorella*, which may explain why *Microcystis* became the dominant species in response to various concentrations of gamine.

In conclusion, gramine and berberine might be more suitable for the in-situ application to prevent and control cyanobacterial blooms. Nevertheless, there are still many problems associated with the use of these allelopathic algicides in natural aquatic systems. Zhang et al. [73] and Dai et al. [61] indicated that the amount of microcystins released into natural waters by berberine-killed cells would reduce drinking water quality and aquatic biodiversity [74,75]. Thus, a subsequent toxin removal process is needed. Gramine is a toxic allelopathic algicide; therefore, its extensive application will also inevitably cause environmental risks. The toxicity of sanguinarine toward phytoplankton was even greater than that of $CuSO_4$ [40]; therefore, it may cause devastating and irreversible damage to other phytoplankton, aquatic plants

and so on. Accordingly, the ecological safety and public health risks need to be evaluated before the application of allelopathic algicides. In addition, how to transfer the results obtained from laboratory tests to the field is also an important issue. In the natural environment, metal ions, dissolved organic matter, and some microorganisms may affect the bioavailability of these allelopathic substances. Alternative use of the removed algae biomass should also be considered in the future work to facilitate the transformation to the circular economy [76,77].

5. Conclusion

Our results demonstrated that zeaxanthine and lutein can be used to determine the cell density of *Microcystis* and *Chlorella* in mixed cultures, and therefore to investigate the competition between cyanobacteria and green algae. The inhibitory effects of three allelopathic algicides on the growth of both *Microcystis* and *Chlorella* were: sanguinarine > gramine > berberine. The EC_{50} values of allelopathic algicides against *Microcystis* and *Chlorella* in monocultures were 0.063 and 0.087 for sanguinarine, 1.590 and 0.591 for gramine, and 1.750 and 2.569 for berberine, respectively. The inhibitory effects of these allelopathic algicides toward phytoplankton in mixed cultures were significantly greater than in monocultures. In the mixed culture without allelopathic addition or the culture with gramine (0.5–3 mg L^{-1}) the dominant species was *Microcystis*. However, *Chlorella* became the dominant species when the concentration of sanguinarine was 0.1 mg L^{-1} or the berberine concentration was >0.5 mg L^{-1}. Both phytoplankton died completely in the presence of sanguinarine at >0.1 mg L^{-1}.

Author statement

Pengfei Duan: Methodology, Validation, Investigation, Writing – original draft. **Mengjiao Wei**: Investigation, Formal analysis, Validation. **Ming Li**: Conceptualization, Validation, Methodology, Writing – review & editing. **Li Gao**: Methodology, Supervision, Project administration, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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