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RESEARCH ARTICLE

Effects of artemisinin sustained-release granules on mixed alga growth and microcystins production and release

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Abstract To safely and effectively apply artemisinin sustained-release granules to control and prevent algal water-blooms, the effects of artemisinin and its sustained-release granules on freshwater alga (*Scenedesmus obliquus* (*S. obliquus*) and *Microcystis aeruginosa* (*M. aeruginosa*)), as well as the production and release of microcystins (MCs) were studied. The results showed that artemisinin sustained-release granules inhibited the growth of *M. aeruginosa* (above 95 % IR) and *S. obliquus* (about 90 % IR), with *M. aeruginosa* more sensitive. The artemisinin sustained-release granules had a longer inhibition effect on growth of pure algae and algal coexistence than direct artemisinin dosing. The artemisinin sustained-release granules could decrease the production and release of algal toxins due to the continued stress of artemisinin released from artemisinin sustained-release granules. There was no increase in the total amount of MC–LR in the algal cell culture medium.

Keywords Algal coexistence · Artemisinin sustained-release granules · Microcystins · Production · Release

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Introduction

Artemisinin, a new plant allelochemical, extracted from *Atremisia annua*, which had been found that it had very strong inhibition effect on *Microcystis aeruginosa* (*M. aeruginosa*) in our previous study (Ni et al. 2012). In order to improve the availability of artemisinin, we prepared the sustained-release granules with incorporation of artemisinin into alginate-chitosan and found artemisinin granules had a potent algal control effect inhibiting *M. aeruginosa* growth to the non-growth state and can release artemisinin sustainably to 150 days in theory (Ni et al. 2013). However, the effects of artemisinin sustained-release granules on the mixed alga and the production and release of microcystins in aquatic ecosystems have not been studied.

In most studies, single-species cultures have been used to study the effects of macrophyte allelochemicals on phytoplankton (Nakai et al. 2000; Zhang et al. 2009). Cyanobacteria, especially *Microcystis spp*., are the dominant species of harmful algal blooms (O'Neil et al. 2012). About 91.9 % of cyanobacteria accounted for by *Microcystis* were up to 32.7 % of the total phytoplankton biomass in Meiliang Bay of Taihu Lake in China (Ke et al. 2008). However, species of *Scenedesmus* are one of the most common phytoplankton constituents in shallow eutrophic freshwater lakes (Vanormelingen et al. 2009). Our previous study has verified the strong inhibition effect of artemisinin sustained-release granules on *M. aeruginosa* (Ni et al. 2013), however, whether artemisinin sustained-release granules also have good inhibition effects on coexisted alga in eutrophic lakes was greatly worthy of further study.

Numerous types of water-bloom-causing algae can produce toxins, cyanobacterial microcystins (MCs) exhibit detrimental effects on organisms from zooplankton to humans including gastroenteritis and liver damage, with reports linking

MCs to human hepatocellular carcinoma (Ueno et al. 1996; Chorus 2001). The impact of allelochemicals on the production and release of algal toxins is a strong determinant of the ecological safety of the application of allelochemicals for algal control. This has become a serious concern of researchers (Boylan and Morris, 2003; Fistarol et al. 2003). Several researches showed that the application of allelochemical has certain influence on the MCs (Jang et al. 2003; Gross et al. 1996). Allelochemical extracted from water lettuce can effectively inhibit the proliferation of algal cells without increasing the release of cyanotoxin (Wu et al. 2013). However, Boylan and Morris (2003) found that the application of anti-algal reagents in controlling toxin-producing algae might cause the microcystin–LR (MC–LR) concentration in the water significantly increased. Only a limited number of studies have focused on the impact of allelochemicals on the production and release of algal toxins from bloom-forming, toxin-producing algae. A systemic and comprehensive research is greatly needed to clarify whether artemisinin sustained-release granules with good algal inhibitory effects on *M. aeruginosa* (Ni et al. 2013) will cause the aforementioned problems.

The most common and most toxic cyanotoxin produced by *M. aeruginosa* was MC–LR, microcystin–RR (MC–RR), and microcystin–YR (MC–YR), which were used as representative algal toxins (Xian et al. 2006). Therefore, this research was carried out to study the inhibiting effects of artemisinin sustained-release granules on the pure *S. obliquus* and the mixed cultures of *M. aeruginosa* and *S. obliquus*, and test the production and release of algal toxins during the application of artemisinin sustained-release granules for *M. aeruginosa* control.

Materials and methods

Algal cultivation

The strains of *S. obliquus* and *M. aeruginosa* were supplied by Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB, China). *S. obliquus* and *M. aeruginosa* were precultured with sterilized Selenite Enrichment (SE) medium (Chen and Guo 2012) and BG-11 medium (Hong et al. 2008), respectively, at 25 °C under 40–60 μmol photons m^{-2} s^{-1} (12 h light/12 h dark) conditions. Cultivation was performed in batch and under exponential growth conditions.

Preparation of artemisinin sustained-release granules

The artemisinin (purity>99 %) was purchased from Nanjing Zelang Medical Technology. The optimal preparation of sustained-release granules of artemisinin was based on our previous study (Ni et al. 2013). According to the optimum preparation conditions, the encapsulation efficiency of artemisinin sustained release granules could reach 68 %, and the release content could reach about 4 mg L^{-1} in distilled water every day.

Algal inhibition test of artemisinin sustained-release granules

Algal bioassays (ISO 2004) were used to test the inhibition effectiveness of the artemisinin sustained-release granules. For the pure culture test, *S. obliquus* was inoculated into a SE culture medium in a 250-mL flask using 10^6 cells mL^{-1} as the initial algal density. The experimental groups were divided into pure artemisinin (0, 4, 12, 20, 28, and 36 mg L^{-1}) and granular artemisinin (0, 1, 5, 10, and 20 g L^{-1}). Blank granular group and the groups without artemisinin/granular artemisinin dosing were selected as control groups. For coexistence tests, mixed cultures of *M. aeruginosa* and *S. obliquus* were inoculated into a SE and BG-11 mixed culture (1:1 *v*/*v*) medium in a 250-mL flask using $2–3\times10^5$ cells mL^{-1} as the initial algal density. Then, artemisinin and granular artemisinin were added into cultures to obtain four treatment groups and a control group. There were three replicates for each group of two tests. All flasks were cultivated at 25 °C under 40–60 μmol photons m^{-2} s^{-1} (12 h light/12 h dark) conditions. Algal growth was monitored each day and cells were counted using microscopy with a hemocytometer. The percentage growth inhibition was calculated by comparing the concentration groups to control group (Hong et al. 2008).

Determination of extracellular and intracellular MCs

The concentrations of MC–LR, MC–RR, and MC–YR in cells and culture medium were detected. A solid-phase extraction method combined with high-performance liquid chromatography (HPLC) was applied to determine the amount of MCs (Song et al. 1999). The samples were first centrifuged at 9000 rpm (Mini-10 K) for 15 min at 4 °C. The supernatant after centrifugation was gathered for testing extracellular MCs. The remaining pellet was frozen and thawed three times and suspended in 5 % acetic solution for centrifugation at 10,000 rpm (Mini-10 K) for 10 min to gather the supernatant. Then the remaining pellet was resuspended in ultrapure water for centrifugation at 10,000 rpm (Mini-10 K) for 10 min, and the mixed supernatant was gathered for testing intracellular MCs. The intracellular and extracellular MCs were extracted using C18 solid phase extraction column and tested with HPLC. The detailed pretreatment method can refer to the research of Men and Hu (2007).

Statistical analysis

Data were analyzed using Excel 2010 and SPSS 17.0 software. The differences between the groups were analyzed

single factor analysis of variance (one-way ANOVA). Significant differences were established at $p<0.05$ and for certain cases at $p<0.01$.

Results and discussion

Inhibition effect of artemisinin sustained-release granules on *S. obliquus*

The changes of daily algal density and inhibition rate (IR) in each concentration group are shown in Figs. 1 and 2, respectively. From Fig. 1a, artemisinin started to inhibit the growth of *S. obliquus* on the 3rd day, while the maximal inhibition rate (92 %) was achieved on about the 13th day with the concentration of 20 mg L^{-1} (Fig. 2a). Beyond this point, *S. obliquus* began to grow rapidly. The results showed that direct addition of artemisinin could inhibit *S. obliquus* growth, but its inhibition effect would decrease with the consumption of artemisinin. Algal cell growth began to recover after approximately 8 days of culturing, indicating a decrease in the inhibitory effect. This mechanism might be explained by the degradation or transformation of the effective anti-algal components in the allelochemical compounds (Kong et al. 2006). These results were consistent with our previous study (Ni et al. 2013), which showed that the direct addition of artemisinin could inhibit *M. aeruginosa* growth, but the inhibition effect would decrease dramatically with the consumption of artemisinin after a period of time. From Fig. 2a, no significant differences were found among the 20, 28, and 36 mg L^{-1} groups after 7 days of exposure. These results showed that 12–20 mg L^{-1} of pure artemisinin would be the optimal concentration range to get better inhibition of *S. obliquus* growth based on economic considerations under the laboratory conditions, while 8–12 mg L^{-1} was the optimal concentration for inhibiting *M. aeruginosa* growth well from the previous study (Ni et al. 2012; Ni et al. 2013).

From Figs. 1b and 2b, *S. obliquus* had been affected significantly by the algaecide for the entire experiment (from day zero to day 27) ($p<0.05$). The blank granules without artemisinin had weak algal inhibition effects, which indicated that the main active matter of inhibiting algae was artemisinin and not the other constituents of granules. Based on these results, it can be concluded that artemisinin sustained-release granules have better anti-algal effects than pure artemisinin and can continuously inhibit algal growth. These inhibition results for *S. obliquus* are in agreement with the inhibition results for *M. aeruginosa* in our previous study (Ni et al. 2013). In each treatment group, the inhibition rate for different concentrations of sustained-release granules of artemisinin on *S. obliquus* had no significant difference ($p>0.05$). The results showed that about 1 g L^{-1} is the optimal concentration of artemisinin anti-algal sustained-release granules to inhibit *S. obliquus* growth under the laboratory conditions in this study.

Inhibition effect of artemisinin sustained-release granules on the algal mixtures of *S. obliquus* and *M. aeruginosa*

According to the optimal concentrations of artemisinin and artemisinin sustained-release granules for inhibiting pure *S. obliquus* and *M. aeruginosa*, 8 and 16 mg L^{-1} for

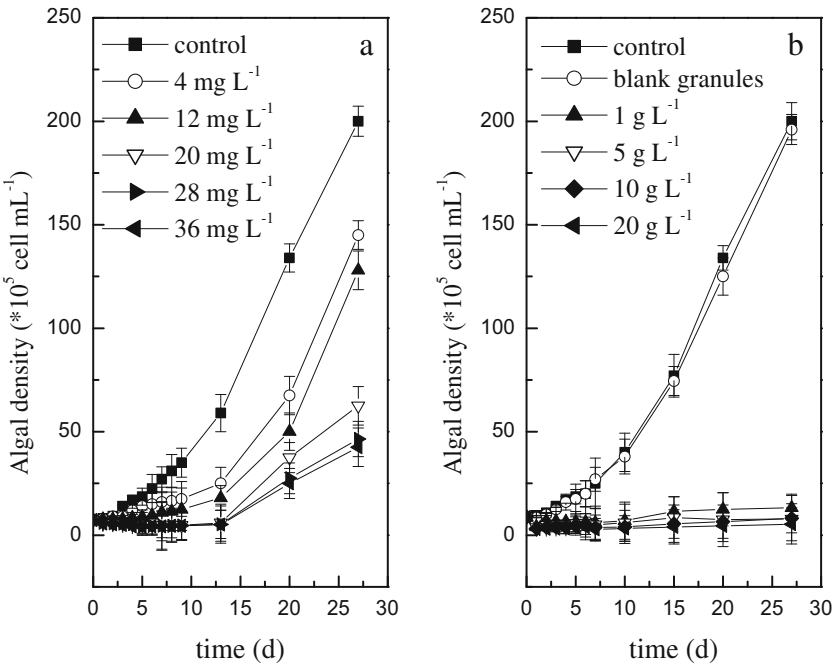

Fig. 1 Change of algal density over time for different concentration of artemisinin (**a**) and artemisinin sustained-release granules (**b**) for *S.obliquus* ($p<0.05$)

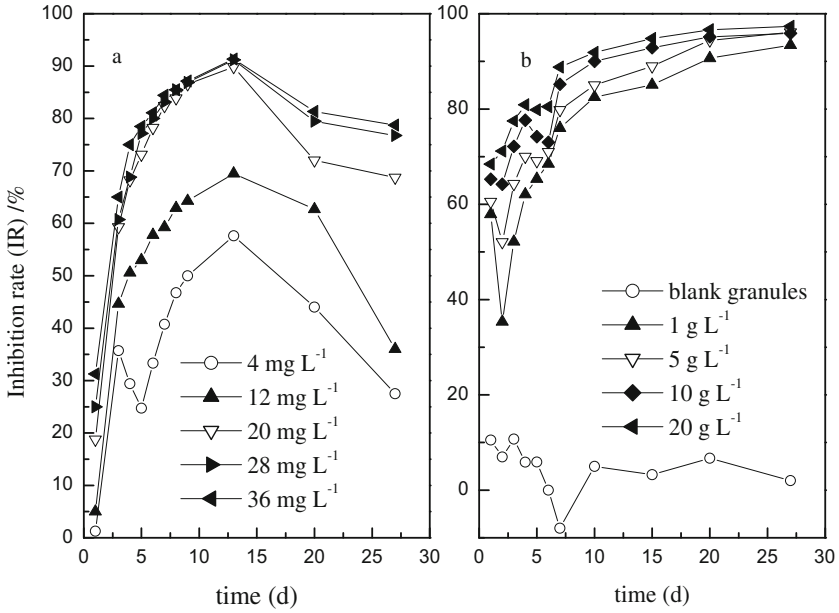

Fig. 2 Inhibition rate of different concentrations of artemisinin (**a**) and artemisinin sustained-release granules (**b**) on *S. obliquus* ($p<0.05$)

artemisinin and 1 and 5 g L^{-1} for artemisinin sustained-release granules were chosen to study the effects of artemisinin and artemisinin sustained-release granules on the algal mixtures (Fig. 3). From Fig. 3, the algal density of *M. aeruginosa* was significantly more than that of *S. obliquus* in the control group. One of the reasons might be the inter-species competition with light and nutrition (Yamamoto and Nakahara 2005). The ability of *Microcystis* sp. to photosynthesize at rates higher than green alga may facilitate the dominance of *Microcystis* in water bodies (Chen and Guo 2012). The other explanation might be that *M. aeruginosa* released allelochemicals inhibiting the growth of *S. obliquus*, because cyanobacteria could produce a wide range of secondary metabolites (although mainly intracellular) and seemed to be able to reduce the biomass of certain phytoplankton species via allelopathic mechanisms (Babica et al. 2006). In the pure artemisinin treatment groups, each concentration of artemisinin had an obvious inhibitory effect on algae during incubation which is similar with the results of our previous study (Ni et al. 2012). The density of *M. aeruginosa* was lower than that of *S. obliquus* in the first 7–9 days, which indicated the inhibitory effect of artemisinin on *M. aeruginosa* was stronger than that on *S. obliquus*. After 9 days, algal density began to increase, and *M. aeruginosa* had a greater increase rate. Similar results were obtained for other allelochemicals, while in general, cyanobacteria are considered more susceptible than green algae (Mulderij et al. 2007; Zhu et al. 2010). Although considerable differences have been

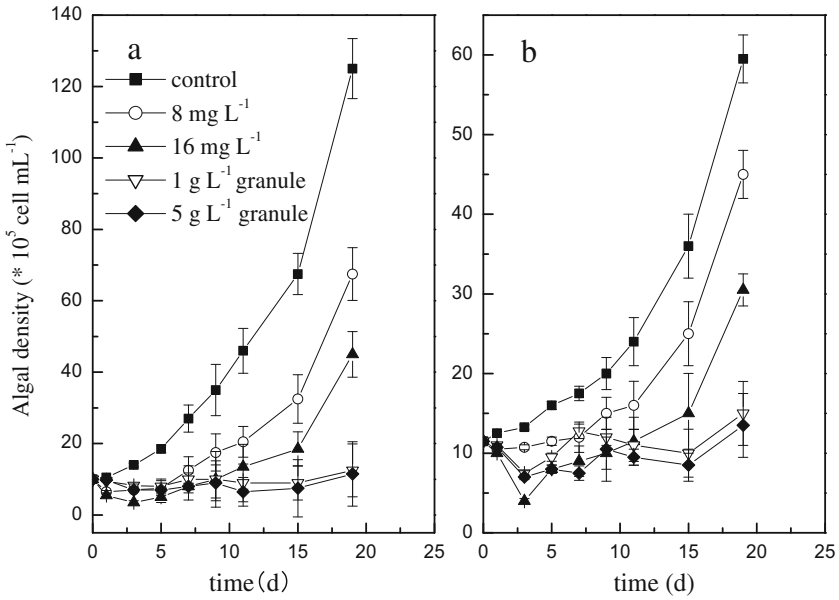

Fig. 3 Influence of different concentrations of artemisinin and artemisinin sustained-release granules on the algae density of *M. aeruginosa* (**a**) and *S. obliquus* (**b**) coexistence ($p<0.05$)

observed among different cyanobacteria (Nakai et al. 2000), as well as among green algal species (Hilt 2006), the results showed that the 16 mg L^{-1} group had a higher inhibition rate (about 60–70 %) during incubation process, and this concentration can be regarded as a good inhibition concentration.

In the artemisinin sustained-release granules treatment groups for both species (Fig. 3), the density of algae was always close to the initial inoculation density, and these results indicated that no algal growth was found, which is similar with results of Fig. 1b in this study. These results indicated that the inhibitory effect of artemisinin sustained-release granules on *M. aeruginosa* was stronger than that on *S. obliquus* because the density of *M. aeruginosa* was always lower than that of *S. obliquus*. The IR of two concentrations of artemisinin sustained-release granules on algae had little differences with each other, and the IR for both species was 90 % during the whole experimental process. The results showed that artemisinin sustained-release granules could inhibit the mixed cultures of two species to non-growth state and about 1 g L^{-1} was also the optimal concentration for inhibiting mixed cultures of algal growth under the laboratory conditions in this study.

Extracellular MCs release affected by artemisinin sustained-release granules

The MCs concentrations in culture medium of *M. aeruginosa* were detected when exposed to 8 and 16 mg L^{-1} artemisinin and 1 and 2 g L^{-1} artemisinin granules. The extracellular concentrations of MC–RR, MC–LR, and MC–YR were shown in Table 1. From Table 1, the release content of MC–LR is the highest during the algal growth process, and extracellular MC-LR of the control group was at the same level as the results reported by Robillot et al. (2000). The extracellular MC-LR concentrations of the control group were always stable during the experiment period (21 days), which suggested that few algal toxins were released into the surrounding water in a normal environment. With exposed to artemisinin (including artemisinin group and artemisinin sustained-release granule group), extracellular MC–LR contents in two groups were lower and similar with control group under the first 9 days of exposure, and then increased with the culture time. The release of extracellular MC–LR in pure artemisinin group was higher than that in artemisinin sustained-release granules group. The maximum release content of MC-LR peaked at 1.075 μg L^{-1} in 16 mg L^{-1} artemisinin at 21st day, more than 0.8455 μg L^{-1} in 1 g L^{-1} artemisinin sustained-release granules group.

MC–LR, as a predominantly algal toxin, is the most toxic and harmful of the known algal toxins (de Figueiredo et al. 2004). Without any external interference, the extracellular concentration of algal toxins was very low and negligible during the early culture stage of algal cells and remained at a relatively low level prior to the stable growth stage (Robillot et al. 2000),

Table 1 Extracellular release of MC–LR, MC–RR, and MC–YR of *M. aeruginosa* exposed to artemisinin and artemisinin sustained-release granules (μg L^{-1})

MC–LR test groups		3 days	9 days	15 days	21 days
Control		0.203	0.2092	0.2143	0.3403
Artemisinin	8 mg L^{-1}	0.1134	0.2230	0.2654	0.2688
	16 mg L^{-1}	0.1390	0.2939	0.4650	1.075
Artemisinin granules	1 g L^{-1}	ND	0.3013	0.3535	0.8455
	2 g L^{-1}	0.0998	0.4714	0.8047	0.5582
MC–RR test groups		3 days	9 days	15 days	21 days
Control		0.0702	0.0714	0.0727	0.0851
Artemisinin	8 mg L^{-1}	0.0598	0.0604	0.0947	0.1624
	16 mg L^{-1}	ND	0.0627	0.1549	0.1831
Artemisinin granules	1 g L^{-1}	0.0580	0.0711	0.0373	0.0551
	2 g L^{-1}	0.0382	0.0768	0.0316	0.0159
MC–YR test groups		3 days	9 days	15 days	21 days
Control		ND	ND	ND	0.1726
Artemisinin	8 mg L^{-1}	ND	ND	0.1480	0.1500
	16 mg L^{-1}	ND	0.1624	0.2045	0.1069
Artemisinin granules	1 g L^{-1}	ND	ND	ND	ND
	2 g L^{-1}	ND	ND	ND	ND

Notes: ND means beyond the detection limit

lower than the maximum limit of 1 μg L^{-1} in drinking water suggested by the World Health Organization (Codd et al. 2005; Umehara et al. 2012). However, the application of chemical algae-killing reagents causes the death and disruption of algal cells, or the cells being inactive and lysis, leading to a substantial release of intracellular algal toxins (Daly et al. 2007; Xiao et al. 2010). For example, Jones and Orr (1994) applied an algae-killing reagent (copper sulfate) to treat water exhibiting algal blooms and found that amount of MC–LR dissolved in the water (i.e., extracellular MC–LR) increased significantly and harmed water quality. In contrast, under the conditions used in this study, the utilization of pure artemisinin and artemisinin sustained-release granule to inhibit algal growth also effectively controlled the proliferation of algal cells but did not promote the extracellular release of algal toxins, and the MC–LR release content in artemisinin sustained-release granule group was lower than that in pure artemisinin group. Wu et al. (2013) also found that allelochemical extracted from water lettuce can effectively inhibit the proliferation of algal cells without increasing the release of MCs. Our study results showed that the artemisinin sustained-release granules represent a higher degree of ecological safety and can therefore be used in practical application for the water undergoing algal blooms.

Intracellular MCs production affected by artemisinin sustained-release granules

The concentrations of intracellular MC–RR and MC–YR were very low and negligible. The production contents of

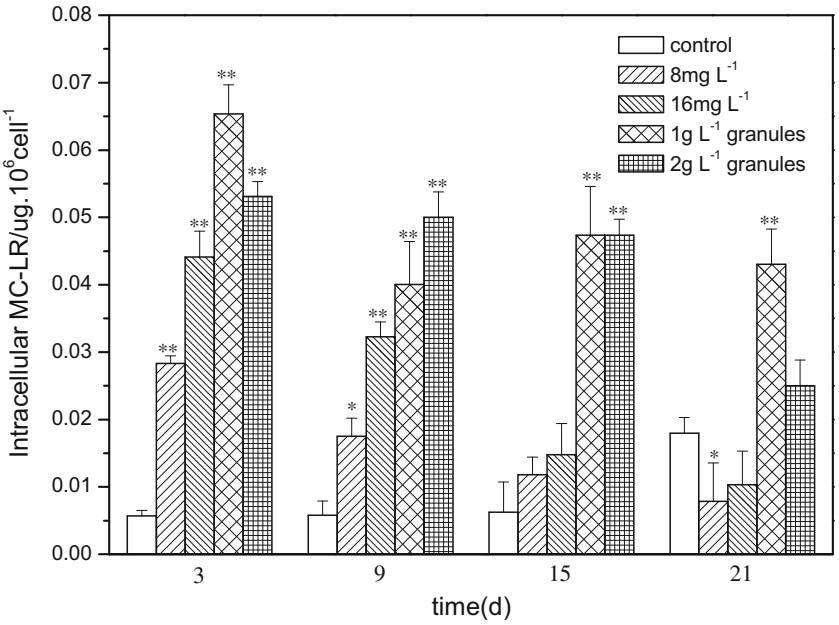

Fig. 4 Amount of intracellular MC-LR after the addition of artemisinin and artemisinin sustained-release granules, $*p<0.05$,$**p<0.01$; $n=3$

intracellular MC–LR (the mass of MC-LR per 10^6 algal cells) in *M. aeruginosa* cells with the different experimental groups (control group, pure artemisinin, and artemisinin sustained-release granules) were shown in Fig. 4. From Fig. 4, in the control group, the amount of MC–LR was at the same level as the results reported by Wiedner et al. (2003) and Downing et al. (2005). The amount of MC–LR per unit of algal cells increased in the artemisinin and artemisinin sustained-release granules groups in contrast with the control group. At first 3 days of culturing, the intracellular MC–LR level increased significantly under artemisinin stress, especially in artemisinin microspheres group. Sixteen milligrams per liter artemisinin and 1 g L^{-1} artemisinin sustained-release granules strongly inhibited *M. aeruginosa* growth within 7–9 days, and Chl–a content, SOD, and CAT activities peaked on the 3rd day and then also began to decrease ($p<0.05$) (Ni et al. 2013). These results indicated that the surviving cells would produce a greater amount of toxins due to the external stress caused by the presence of allelochemicals (artemisinin), which is in agreement with the previous researches (Robillot et al. 2000; Jang et al. 2003; Wu et al. 2008). It is generally believed that algal intracellular energy is directed primarily towards two purposes: the synthesis of the nutrients required for growth and resistance against environmental stress (Wang et al. 2011). As a type of environmental stress, allelochemicals can cause algal cells to consume more energy in the synthesis

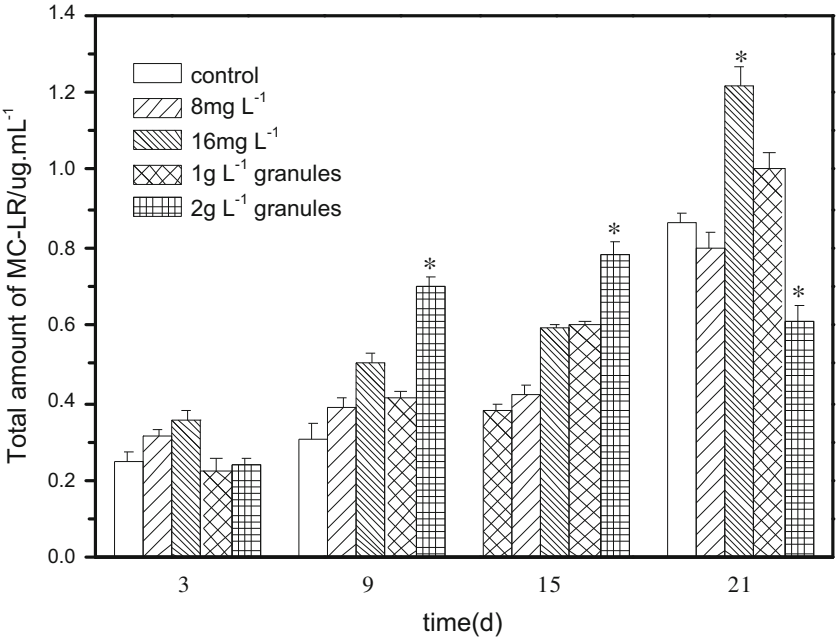

Fig. 5 Total amount of MC-LR after the addition of artemisinin and artemisinin sustained-release granules, $*p<0.05$,$**p<0.01$; $n=3$

of toxins, which is a possible reason for the slow growth of algal cells (Kearns and Hunter 2000).

From the 9th day of culturing onwards, the inhibitory effect of pure artemisinin on the growth of *M. aeruginosa* decreased, and the algal cells in the culture medium exhibited restored growth. However, the intracellular MC–LR production in the pure artemisinin group decreased with culturing time and even was lower than the control level at 21st day, indicating that, with the consumption of artemisinin and the environment stress reducing, the algae exhibited restored growth and less intracellular MC–LR produced. The intracellular MC–LR contents in 1 g L^{-1} artemisinin sustained-release granules group also began to decrease and tended to be stable, but the intracellular MC–LR production in 1 and 2 g L^{-1} artemisinin sustained-release granules groups were greatly higher than the pure artemisinin and control groups. Artemisinin sustained-release granules could continually release artemisinin to replenish the effective anti-algal component and inhibited *M. aeruginosa* to the non-growth state (Fig. 2), which led to the higher level of intracellular MC–LR production per unit algal cell for resistance against artemisinin stress.

The amount of extracellular MC–LR released was very small during culturing period (Table 1) and can be considered negligible. As a result, the total amounts of MC-LR production can be calculated based on the intracellular MC–LR amount per 10^6 cells multiplied by the detected algal density (Men and Hu 2007; Sager 2009). From Fig. 5, the total MC–LR productions in all groups increased with the culturing time. In the artemisinin group, the total amount of MC–LR treated with pure LA was more than the control group and the maximum content peaked in 16 mg L^{-1} pure artemisinin group at 21 days. Compared with algal density results (Figs. 1 and 5), the algal cell growth decreased greatly first and then began to recover after 9 days with artemisinin depletion, leading to the algal density being in rising. The total amounts of MC–LR production in artemisinin group creased with culturing time. The total amount of MC–LR production in 1 g L^{-1} artemisinin sustained-release granules group was at the similar level with the control group during the experimental process and peaked at the highest content at 21 days, however, the total amount of MC–LR production in 2 g L^{-1} artemisinin sustained-release granules group changed greatly was lowest among all groups at 21 days. This might be explained by the fact that a stable artemisinin stress from artemisinin sustained-release granules maintained continuously effective anti-algal component to ensure superior algal cell growth inhibition during culturing periods. Consequently, compared to the other groups, the algal density was the lowest in artemisinin sustained-release granules group, leading to low total MC–LR production even though the highest amount intracellular MC–LR production per 10^6 cells (Fig. 4). Therefore, the major calculation factor influencing the total amount of MC–LR production should be the detected algal density. Daly et al. (2007) found that largely numbers of intracellular MC–LR released into water as the death of algal cells. In this study, *M. aeruginosa* has been inhibited to the non-growth state using artemisinin sustained-release granules, but the total MC–LR production and extracellular MC–LR release in the cultures did not increase much in contrast with control group during culturing time. The mechanisms were being studied.

Conclusions

It was demonstrated that artemisinin and its sustained-release granules had excellent inhibition on *M. aeruginosa* (common water bloom cyanobacteria) and *S. obliquus* (common green algae) in both pure and mixed culture, with more significant effect on *M. aeruginosa*. Compared to direct dosing of artemisinin, algae could be inhibited longer and more effectively by artemisinin sustained-release granules and 1 g L^{-1} is the optimal concentration of artemisinin sustained-release granules for algal inhibition under laboratory conditions. Under the stress of artemisinin and its sustained-release granules, the artemisinin sustained-release granules demonstrated no significant impact on the extracellular release of MCs during the culturing period. The amount of intracellular MC–LR per 10^6 algal cells in artemisinin sustained-release granules group was highest among all groups during the whole experimental process. The total content of MC-LR in *M. aeruginosa* of artemisinin group increased and was higher than that of the control group. While the total content of MC–LR in 1 g L^{-1} artemisinin sustained-release granules group had no significant difference with the control group. The results of this study suggested that the artemisinin sustained-release granules may be a potential candidate for algal inhibition.

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