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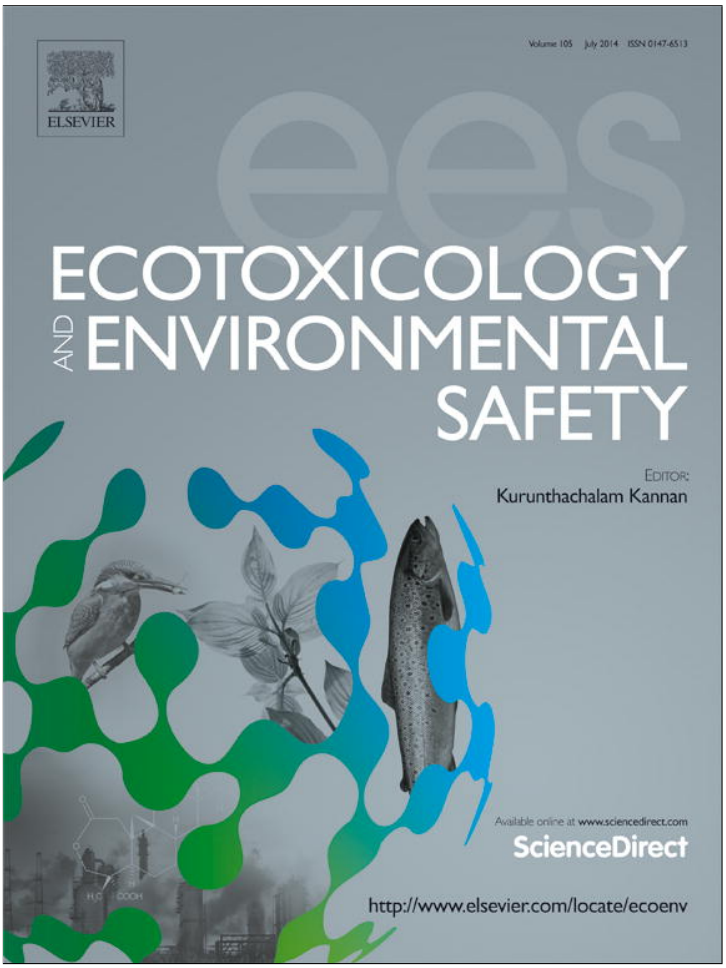

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Effects of limonene stress on the growth of and microcystin release by the freshwater cyanobacterium *Microcystis aeruginosa* FACHB-905

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ABSTRACT

The effects of limonene exposure on the growth of *Microcystis aeruginosa* and the release of toxic intracellular microcystin (MCY) were tested by evaluating the results obtained from the batch culture experiments with *M. aeruginosa* FACHB-905. The time series of cell as well as intracellular and extracellular MCY concentrations were evaluated during 5 d of the incubation. After exposure to limonene, the number of cells gradually diminished; the net log cell reduction after 5 d of the exposure was 3.0, 3.6, and 3.8 log when the initial cell densities were set at 1.6×10^7, 1.1×10^6 and 4.1×10^5 cell/mL, respectively. Limonene was found to significantly influence the production and release of MCY. As the limonene exposure could inhibit the increase in the number of cells, the increase in the total MCY concentration in the medium was also inhibited. In the presence of limonene, the intracellular MCY was gradually released into the medium through a gradual reduction in the number of cells. The extracellular MCY concentration in the medium was significantly higher in the limonene-exposed samples than in the control samples, which confirmed that limonene cannot decompose the extracellular MCY.

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1. Introduction

Cyanobacteria are known to produce a variety of metabolites that affect lake ecosystems and humans. Microcystin (MCY) is one of the most widespread toxin produced by several genera of cyanobacteria (Chianella et al., 2003). MCY has been classified as a potential carcinogen to humans (Class 2B) by the International Agency for Research on Cancer (IARC, 2007). It can also cause hepatic lymphocyte infiltration, fatty degeneration, and cancer cell migration in low concentrations (< 0.1 μM) (Ito et al., 1997; Zhang et al., 2012). Previous studies have suggested that MCY may be accumulated in the tissues of blue crabs and then transferred to consumers at higher trophic levels, including humans (Deblois et al., 2011; Garcia et al., 2010; Kozlowsky-Suzuki et al., 2012). Although humans do not directly consume cyanobacteria, they may be regularly exposed to sub-lethal dosages of extracellular MCY through drinking water or the consumption of aquatic products from cyanobacteria-contaminated lakes and reservoirs. In February 1996, the largest MCY outbreak occurred in Brazil, in which 88 people died and 131 people experienced visual disturbances, nausea, vomiting, and muscle weakness (Azevedo et al., 2002). In late May 2007, a massive bloom of the toxin producing cyanobacteria in Taihu Lake caused a drinking water crisis, leaving approximately two million people without drinking water for at least a week (Qin et al., 2010).

Several different physical and environmental parameters such as nutrition (Wang et al., 2010; Yang et al., 2012), trace metals (Lukač and Aegerter, 1993; Zeng et al., 2009), growth temperature (Zeng and Wang, 2011), light (Hesse et al., 2001), and pH (Banares-Espana et al., 2006; Van der Westhuizen and Eloff, 1985) have been found to influence the growth of toxic cyanobacteria and production of MCY. One of the conventional treatments to inhibit the growth of cyanobacteria involves the use of copper sulfate. However, excess copper is potentially hazardous to human health because it can participate in the Fenton reaction, producing radical species such as hydroxyl radicals (OH) (Arnal et al., 2012). Other treatments include the use of biofilm (Li et al., 2011) and graphene oxide (Pavagadhi et al., 2013); however, it is very expensive to apply such treatments to large waterbodies such as lakes or reservoirs. Therefore, alternative inexpensive in-lake treatments are needed. Monoterpenes extracted from plant essential oils are known to have antibacterial activity (Okoh et al., 2010; Pavithra et al., 2009; Ragasa et al., 1997; Saddiq and Khayyat, 2010; Tyagi and Malik, 2011). In this study, limonene, a monoterpene, was tested as an algicide against the excessive growth of *Microcystis aeruginosa*.

Limonene is a major aromatic compound biosynthesized by many plants, such as *Citrus maxima*, *Citrus sinensis* (Singh et al., 2010), *Typha latifolia* (Parra et al., 2004), *Polygonum minus* (Yaacob, 1990), and *Nelumbo nucifera* (Omata et al., 1990). It was registered as a

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flavoring in foodstuffs by the European Commission (Burt, 2004) and has many commercial applications. It has been introduced as an environmentally acceptable solvent to replace toxic halogenated hydrocarbons (García et al., 2009; Shin and Chase, 2005), and also widely used as a biofuel (Lohrasbi et al., 2010; Pourbafrani et al., 2010), medicine (Gurgel do Vale et al., 2002), and pesticide (Hebeish et al., 2008). Limonene showed antifungal and antibacterial activities against many species of microorganisms, such as *Trichoderma viride*, *Cladosporium herbarum* and *Aspergillus flavus* (Mourey and Canillac, 2002; Ozturk and Ercisli, 2006; Singh et al., 2010). Kumar et al. (2011) reported that limonene could serve as molluscicide for the controling of harmful snails. However, the effect of limonene on the behavior of intracellular MCY has not been reported. The aim of this study is to investigate the effect of limonene on the growth of *M. aeruginosa* and the behavior of intracellular MCY during the limonene treatment. In this study, *M. aeruginosa* suspensions were incubated for 5 d after the addition of limonene, and the cell density as well as the intracellular and extracellular MCY concentrations was monitored during the incubation period.

2. Material and methods

2.1. M. aeruginosa cultivation

An axenic strain of *M. aeruginosa*, kindly provided by Freshwater Algae Culture of Hydrobiology Collection at the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), No. FACHB-905, was used in this study and cultured in sterilized MA liquid medium (Ichimura, 1979). The Cultures were kept at 25 °C under a light:dark regime of 14:10 h. The full details of the growth conditions are presented in the Supporting Information (S1).

2.2. Chemical treatment

M. aeruginosa cells were incubated in the presence or absence of limonene to evaluate the effects of its toxicity on the living cells. To study the growth inhibition of logarithmically growing *M. aeruginosa* by limonene, initial algal densities were set to be 1.1×10^6 (Park et al., 1998) and 4.1×10^5 cell/mL (Manage et al., 1999), thereby mimicking the cell density in an actual algal bloom. To study the influence of limonene on the growth of cells in the stationary phase, the initial cell densities were set to be 1.6×10^7 cell/mL. The culture medium (600 mL) was transferred to a 1 L Erlenmeyer flask, after which limonene (purity ≥ 97 percent, Sigma-Aldrich, 5.0 mL) was added to the medium. The concentration of limonene in medium was 12 mg/L, determined by the gas chromatography analysis. Full details are shown in the Supporting Information (S2) in Appendix A. The undissolved limonene was not removed from the system to maintain its concentration in water during the 5 d of incubation.

2.3. Cell density

The algal cell density was counted using a hemocytometer under a light microscope. The counted cell numbers were expressed as the cell density (cell/mL) in the following figures, neglecting the physically destroyed cells.

The morphological and structural changes in the algal cells during the logarithmic growth phase (1.1×10^6 cell/mL) were observed using a scanning electron microscope (SEM, JEOL JSM-6700 F, Tokyo, Japan).

2.4. MCY analysis

The intracellular and extracellular MCY concentrations were determined using MCY enzyme-linked immunosorbent assay (ELISA) analysis kits (MCY total-kit, J & Q Environmental Technologies, China), respectively. The MCY concentration in each of the samples was determined by taking the average of all the results from the two wells in the 96-well plates. To determine the extracellular MCY concentration, each of the samples was filtered using a 0.2 μm glass fiber filter (Whatman) and then subjected to ELISA analysis. To determine the intracellular MCY concentration, the algal cells (from the 10 mL medium) were harvested and dispersed in Milli-Q water (Millipore, 18.2 MΩ cm, 10 mL) and then disrupted in liquid nitrogen. The homogenate was then centrifuged (10,000*g*, 10 min, 4 °C) prior to the ELISA analysis. The Milli-Q water served as the control sample to prevent false positives.

2.5. Statistical analysis

The responses of *M. aeruginosa* to the limonene stress were analyzed by a one-way ANOVA. If the statistical test was found to be significant at $p < 0.05$, a Tukey test was then employed to observe where the difference occurred (Zar, 1996). All the tests were carried out using the Statistical Package for Social Science (SPSS 20.0 for Mac OS X). Each of the results shown in the figures represents the average of the three independent replicate treatments. The results were considered to be significantly different only if the probability (p) was less than 0.05.

3. Results

3.1. Cell density

Algal growth can be divided into four stages: the lag, logarithmic, stationary, and aging phases. During the lag and aging phases, the algae concentration was found to be too low for accurate measurements; therefore, a comparison was carried out between the logarithmic growth and stable stages. Fig. 1 shows the profiles

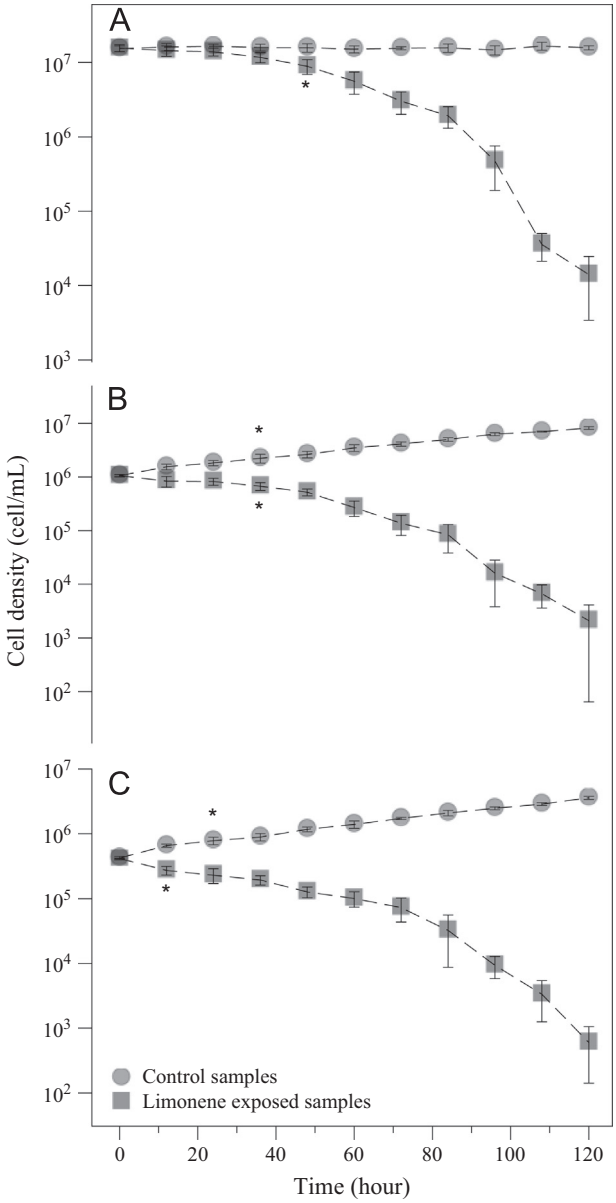

Fig. 1. Effect of limonene on the number of viable cells: (A) initial cell density was 1.6×10^7 cell/mL; (B) initial cell density was 1.1×10^6 cell/mL; and (C) initial cell density was 4.1×10^5 cell/mL. *$p < 0.05$.

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of the number of *M. aeruginosa* cells during the incubation in the presence of limonene. The inhibitory effect of limonene on the algae growth was calculated as the net log cell reduction by taking the difference in the number of cells between the control and limonene-exposed samples. A constant decease in the cell density was observed in the presence of limonene during the stationary phase (Fig. 1(A)). The number of *M. aeruginosa* cells was gradually diminished by limonene. A significant change was observed with a net 3.0 log cell reduction after 5 d of exposure ($p < 0.05$). As shown in Fig. 1(B) and (C), when the algal cells were in the logarithmic growing phase, the cell density increased rapidly in the control samples and decreased gradually in the limonene-exposed samples. The net log cell reduction after 5 d of exposure was 3.6 and 3.8 log, respectively, which is a significant change ($p < 0.05$).

3.2. Change of surface morphology of algal cells

The cells of *M. aeruginosa* in the control sample were found to be round and plump with the average size of about 2.6 μm, similar to the normal cells (Fig. 2(A)). However, after the addition of limonene, the surface morphologies of algal cells changed. After reaction for 24 h, the size of the algal cells did not change significantly but they were distorted from their normal spherical shape and appeared flattened, and the surfaces became less smooth. After further contact time, some of the cells cracked, the cell membrane of algal cells had been lysed, and the inclusion leaked out, as shown in Fig. 2(B) and (C). Thus, limonene is responsible for causing severe damage to *M. aeruginosa*, thereby inhibiting its growth.

3.3. MCY concentration analysis

Fig. 3 shows the profiles of the MCY concentration during the incubation. As shown in Fig. 3(A), when the algal cells were in the stationary phase, the total MCY concentration in the controls did not exhibit any obvious change ($p > 0.05$) after 120 h of the incubation (727 μg/L). The extracellular MCY concentration was gradually increased by 321 μg/L after the 120 h of the incubation, and the intracellular MCY concentration was 406 μg/L ($p > 0.05$). In the limonene-exposed samples, the intracellular MCY concentrations decreased, and the extracellular MCY concentrations increased, while the total MCY concentration remained almost constant during the 120 h of the exposure (Fig. 3(B)). A significant change was observed after 48 h of the exposure as the concentration of the intracellular MCY decreased to 213 μg/L and the extracellular MCY level increased to 435 μg/L ($p < 0.05$).

As shown in Fig. 3(C) and (E), in the absence of limonene, the cells growing logarithmically, and the intracellular MCY concentration increased as the cell density increased. After 48 h of the incubation, cell densities were 2.6×10^6 and 1.2×10^6 cell/mL, respectively, and the intracellular MCY concentration increased to 65.3 and 29.9 μg/L, respectively ($p < 0.05$). As shown in Fig. 3 (D) and (F), in the presence of limonene, the samples in logarithmic growing phase exhibited similar behavior to the sample in the stationary phase by releasing intracellular MCY. After 12 h of the incubation, the intracellular MCY concentration were 21.7 and 6.8 μg/L, respectively, and the changes were considered as significant ($p < 0.05$). In the absence of limonene, the extracellular MCY concentration increased to 5.4 and 1.8 μg/L ($p < 0.05$) after 72 h of the incubation, respectively. In the presence of limonene, the extracellular MCY concentration increased to 19.2 and 8.0 μg/L ($p < 0.05$) after 48 h of the exposure, respectively.

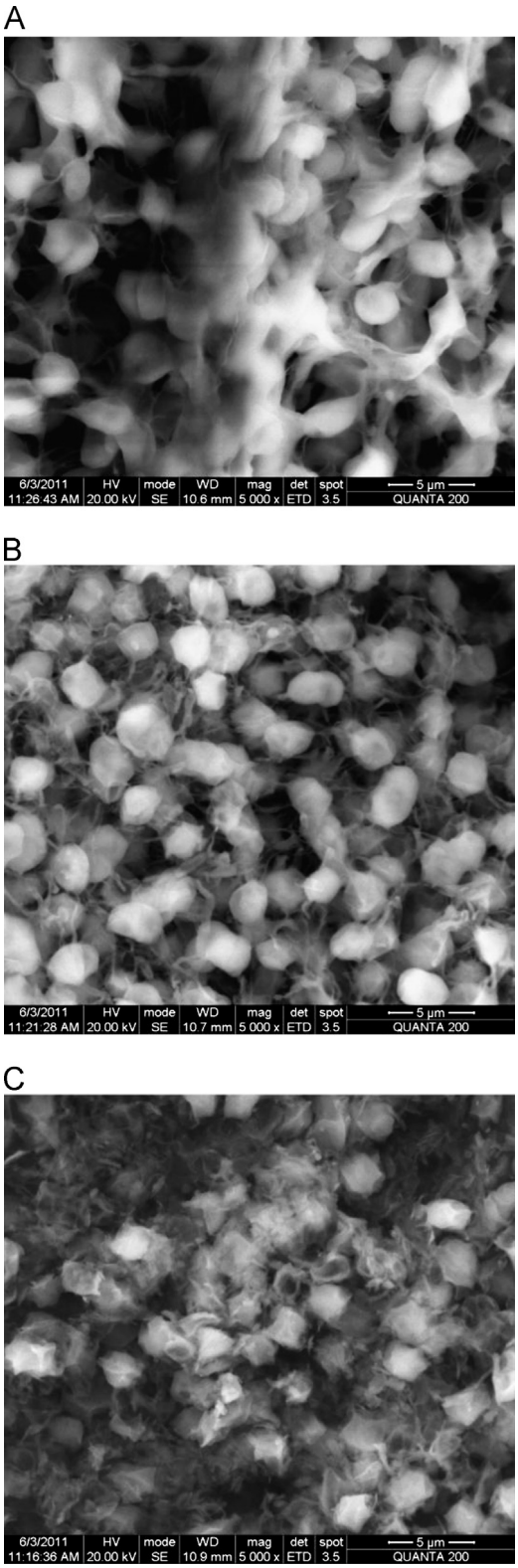

Fig. 2. SEM images: (A) control sample; (B) after exposure to limonene for 24 h; and (C) after exposure to limonene for 72 h.

4. Discussion

Limonene can inhibit the proliferation of *M. aeruginosa* effectively during both the stationary and logarithmic growth phases. Because the concentration of limonene in this study (12 mg/L) is higher than the maximum sublethal concentration, which is

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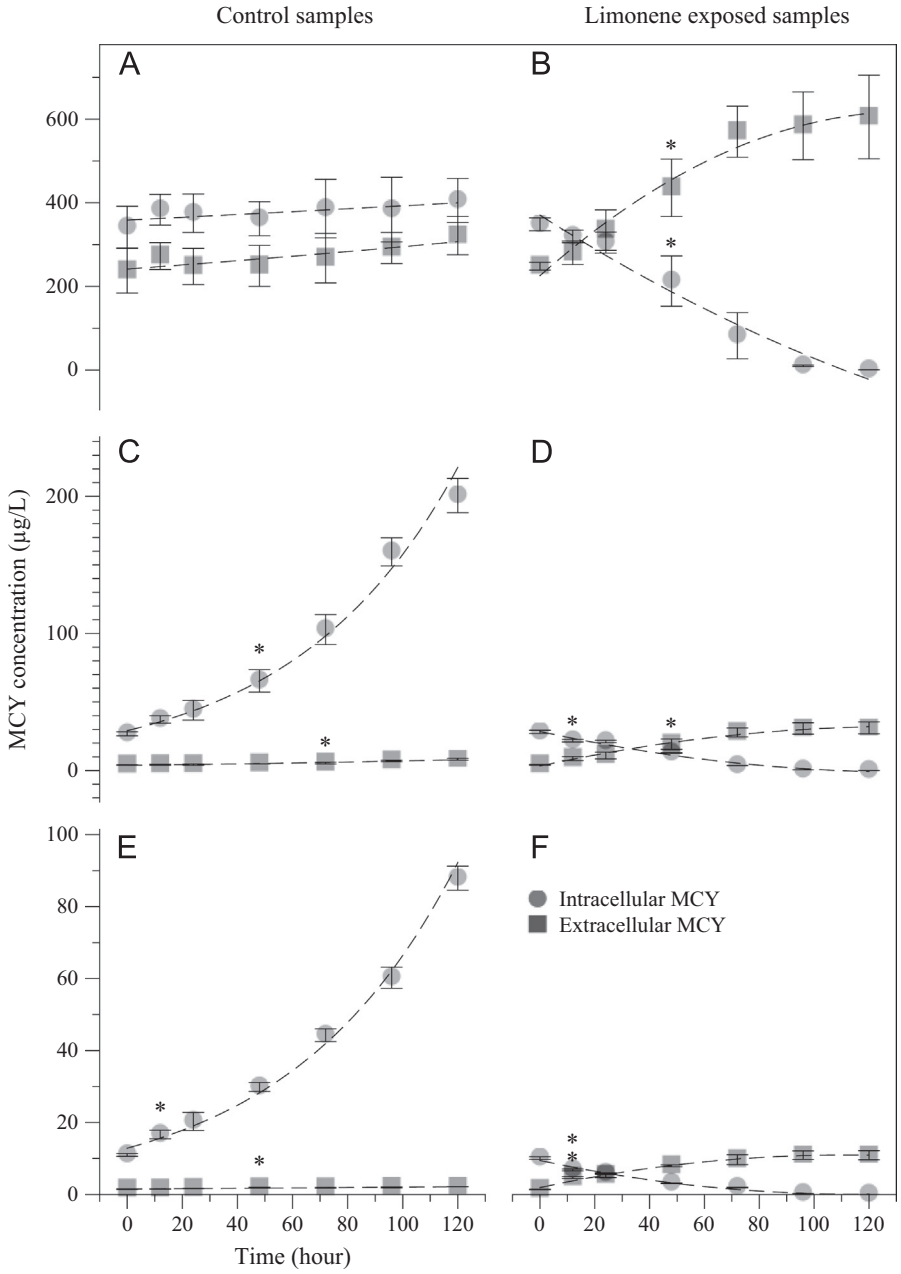

Fig. 3. Intracellular and extracellular MCY concentration: (A) initial cell density was set at 1.6×10^7 cell/mL and in the absence of limonene; (B) initial cell density was set at 1.6×10^7 cell/mL and in the presence of limonene; (C) initial cell density was set at 1.1×10^6 cell/mL and in the absence of limonene; (D) initial cell density was set at 1.1×10^6 cell/mL and in the presence of limonene; (E) initial cell density was set at 4.1×10^5 cell/mL and in the absence of limonene; and (F) initial cell density was set at 4.1×10^5 cell/mL and in the presence of limonene. *$p < 0.05$.

defined as the highest concentration of antimicrobial compound allowing the growth at the optimal temperature, of many microorganisms, such as *Escherichia coli*, *Salmonella enterica*, *Pseudomonas fluorescens*, *Brochothrix thermosphacta* and *Staphylococcus aureus* (Di Pasqua et al., 2006), a constant decease in the cell density was observed. The SEM images also confirmed that the disruption of the algal cells was due to the presence of limonene.

There is active research into MCY synthesis because it can cause serious health and environmental problems (Otten et al., 2012). When the algal cells were in the stationary phase, the cell density of the control samples did not exhibit an obvious change during incubation (Fig. 1(A)); therefore, the intracellular MCY content remained almost constant (Fig. 3(A)). When the algal cells were in the logarithmic growth phase, the number of algal cells increased logarithmically in the control samples, and the intracellular MCY concentration displayed a similar trend as the cell density. This observation indicated a strong positive correlation between the cell density and intracellular MCY concentration (Fig. 4), which is in agreement with the findings of Liu et al. (2013).

MCY is synthesized inside cells (Oberholster et al., 2004); so the increase in extracellular MCY concentrations could be due to two reasons. First, the living *M. aeruginosa* cells excreted intracellular MCY into the medium in the presence of limonene, thereby decreasing the intracellular MCY concentration in the *M. aeruginosa* cells and increasing the extracellular MCY concentration in the medium. Second, the addition of limonene caused the lysis of the *M. aeruginosa* cells, thereby releasing the intracellular MCY into the medium and thus increasing the extracellular MCY concentration. As MCY is a cyclic heptapeptide, it cannot pass naturally through the cell membrane. A transport protein is required for its

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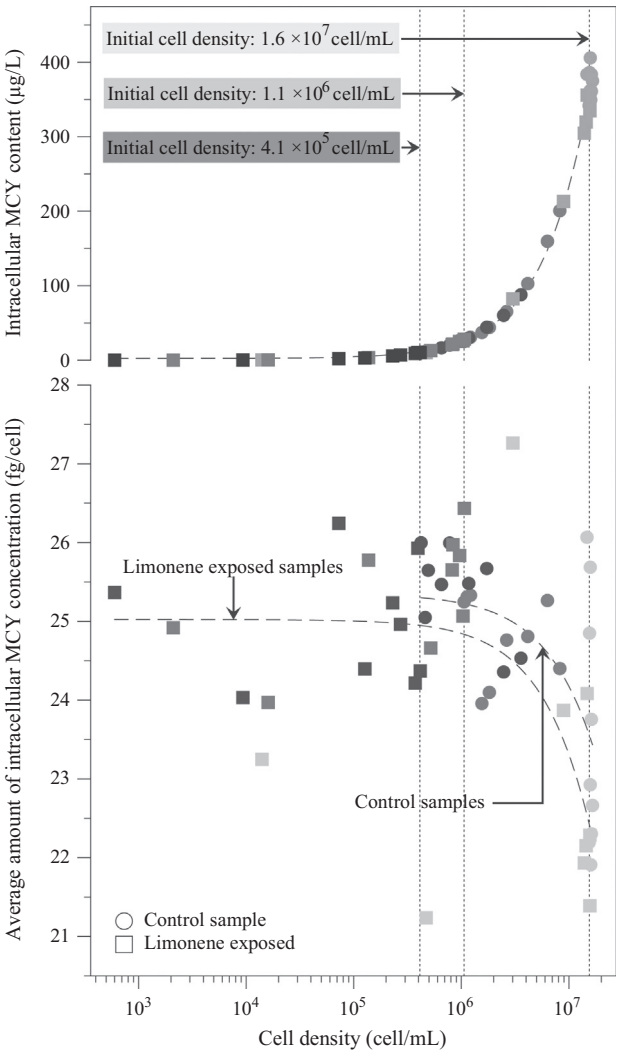

Fig. 4. Average amount of intracellular MCY concentration.

transportation across the membrane; however, no such protein has been identified (Pearson et al., 2004). This suggests that the MCY concentration in the medium increased only from the release of intracellular MCY when the cell wall was destroyed. Therefore, the extracellular MCY concentration was determined from the quantity of dead algal cells.

The extracellular MCY concentrations at time zero cannot be ignored in each of the samples, due to the culture medium not being refreshed before the limonene was added. To attain the initial cell densities, 28 d (1.6×10^7 cell/mL), 18 d (1.1×10^6 cell/mL), and 10 d (4.1×10^5 cell/mL) old samples were used, some algal cells died during this period, and thus contributed to the extracellular MCY concentration. In the control samples, the death rate of *M. aeruginosa* in stationary phase cannot be ignored; therefore, a gradual increase in the extracellular MCY concentration was observed during the 5 d of incubation. Due to the low death rate of *M. aeruginosa* in the logarithmic growth phase, a slower increase in the extracellular MCY concentration in the control sample was observed. After the limonene addition, a rapid increase in the extracellular MCY concentration was observed due to the high death rate of the algal cells. For the limonene-exposed samples, the greatest decrease in the cell density was observed during the 12–48 h corresponding to the major release of the intracellular MCY in this period. This observation is in agreement with the findings of Daly et al. (2007), who reported that although *M. aeruginosa* kept its cyanotoxins within its cells, they were dramatically released into the surrounding medium upon cell lysis.

Previous studies showed that environmental factors such as light density (Utkilen and Gjølme, 1992), temperature (Davis et al., 2009; Oberholster et al., 2004), salinity (Aguilera-Belmonte et al., 2013), and nutrition (Lee et al., 2000) can activate the production of MCY. In this study, the average intracellular MCY concentration, which is defined as the ratio of the intracellular MCY concentration to the number of cells, decreased as the cell density increased in the control sample (Fig. 4). This observation supports the generally held view that MCY production is the greatest when the conditions are favorable for its growth (Long et al., 2001). The fact that limonene cannot stimulate *M. aeruginosa* to produce MCY is corroborated by the positive linear correlation between the production of MCY and cell density, which was found by analyzing the yield of MCY from a single *M. aeruginosa* cell exposed to limonene.

The degradation of extracellular MCY was not obvious during this study, which indicated that limonene cannot promote the decomposition of MCY. (To prove this, another experiment was carried out in which limonene was added to the MCY solution, and the full details and results are shown in the Supporting Information (S3).) Thus, the application of limonene should be integrated with other methods to reduce the extracellular MCY concentration. Previous studies have shown that extracellular MCY can be degraded by many factors in the natural environment, such as UV irradiation (Sakai et al., 2007) and bacteria (Bourne et al., 2006; Nybom et al., 2012). However, the decomposition process of MCY was slow in those studies. Therefore, the rapid reduction of the MCY concentration in water is a current topic of research. Teng et al. (2013) reported that ordered mesoporous silica materials with large pore sizes of 2–12 nm could be used as the adsorbents for the rapid and efficient removal of MCY. Song et al. (2006) reported that sonolysis can effectively degrade MCY in drinking water. The photocatalytic degradation of MCY is also currently being studied (Chen et al., 2012; Feitz et al., 1998). Another main disadvantage with the use of limonene is the higher cost of limonene (approximately 0.2 US dollar per milliliter) than conventional algicides such as copper sulfate and benzalkonium chloride. However, because limonene can be extracted from plant tissue, commercial materials are not required in real applications.

5. Conclusions

The effects of limonene exposure on the release of intracellular MCY can be summarized as follows: first, as the limonene exposure could inhibit the increase in the number of cells, it inhibited the increase in the MCY concentration in the medium, regardless of whether the cells were in the logarithmic growth or stationary phases. Second, due to the gradual release of intracellular MCY caused by the gradual disruption of algal cells, the concentration of extracellular MCY increased in the medium. Third, limonene could not stimulate *M. aeruginosa* to produce MCY. Finally, exposure to limonene could not degrade the intracellular MCY. Owing to the aforementioned reasons, limonene could be used in the treatment of eutrophic water to inhibit the production of MCY, while other methods to reduce the extracellular MCY concentration are needed.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at: http://dx.doi.org/10.1016/j.ecoenv.2014.01.023.

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