



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



Xi Hu^a, Yunguo Liu^{a,b,*}, Guangming Zeng^{a,b}, Weihua Xu^{a,b}, Xinjiang Hu^a, Zhilin Zhu^b, Pingyang Zhang^a, Yaqin Wang^a

^a College of Environmental Science and Engineering, Hunan University, Changsha 410082, PR China

^b Key Laboratory of Environmental Biology and Pollution Control, Hunan University, Ministry of Education, Changsha 410082, PR China

HIGHLIGHTS

- D-Menthol could inhibit the growth of *Microcystis aeruginosa* cells effectively.
- After intracellular MCY was released, the concentration of extracellular MCY increased in the medium increased gradually.
- The D-menthol exposure could not degrade the intracellular MCY.

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ABSTRACT

The effects of D-menthol on the growth of *Microcystis aeruginosa* FACHB-905 and microcystin (MCY) concentration were evaluated by batch culture experiments. The algal biomass and the intracellular and extracellular MCY concentrations were evaluated during 5 d incubation. After the D-menthol exposure, the dry weight of the cells gradually decreased; the decrease in the dry weight after 5 d exposure was 29, 12, and 2 mg L⁻¹ when the initial cell densities were 1.4 × 10⁷, 1.2 × 10⁶, and 2.9 × 10⁵ cell mL⁻¹, respectively. The results indicate that the D-menthol exposure inhibited the cellular growth, thus also inhibiting the increase of the total MCY concentration. In the presence of D-menthol, the intracellular MCY was gradually released into the medium after the cell lysis. The extracellular MCY concentration in the medium was significantly higher in the D-menthol-exposed samples than in the control samples, confirming that D-menthol cannot decompose the extracellular MCY.

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1. Introduction

Blooms of toxigenic cyanobacteria pose a global public health and environmental concern. *Microcystis* is one of the most common bloom formers in freshwater systems on every continent except Antarctica (Fristachi et al., 2008; Amano et al., 2010). This genus can produce diverse toxic secondary metabolites (Sivonen and Jones, 1999), including microcystin (MCY), anatoxin-a, and amino β-methylamino-L-alanine (Fristachi et al., 2008; O'Neil et al., 2012). MCY has been classified by the International Agency for Research on Cancer (IARC) as a potential carcinogen to humans (Class 2B) (IARC, 2007). Previous studies have suggested that MCY may be accumulated in the tissues of blue crabs and then transferred to the consumers at higher trophic levels, including

humans (Deblois et al., 2011). 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).

The effects of physical and environmental parameters such as illumination (Tytler et al., 1984), growth temperature (Zeng and Wang, 2011), nitrogen (Yang et al., 2012), phosphorus (Wang et al., 2010), allelochemicals (Chang et al., 2012), trace metal (Hadjoudja et al., 2009; Wang et al., 2011), surfactants (Gustafsson et al., 2009), and pH (Banares-Espana et al., 2006) on the growth of toxic cyanobacteria and the secretion of MCY have been studied.

Monoterpenes extracted from plant essential oils exhibit antibacterial activity (Okoh et al., 2010). In this study, D-menthol, a cyclic monoterpene alcohol, was investigated as an algicide against the excessive growth of *Microcystis aeruginosa*, which produces MCY (Deblois and Juneau, 2010). Menthol is a naturally occurring cyclic terpene alcohol of plant origin, belonging to the Lamiaceae family (Dambolena et al., 2010), and is widely used to

* Corresponding author at: College of Environmental Science and Engineering, Hunan University, Changsha 410082, PR China. Tel.: +86 731 88649208; fax: +86 731 88822829.

E-mail address: xixixixi2046@gmail.com (Y. Liu).

decrease cold pain thresholds (Hatem et al., 2006). Further, several other studies have confirmed the antimicrobial and antifungal activity of menthol (Moleyar and Narasimham, 1986; Kishore et al., 1993; Pattnaik et al., 1997; Osawa et al., 1999; Edris and Farrag, 2003; Trombetta et al., 2005). However, few studies have reported the effect of D-menthol on cyanobacteria. In this study, we evaluated the effects of D-menthol on the growth of *M. aeruginosa* and the release of intracellular MCY during the D-menthol treatment. The *M. aeruginosa* suspensions were incubated in the presence of D-menthol, and the algal biomass and intracellular and extracellular MCY concentrations were monitored during the batch culture.

2. Materials and methods

2.1. *M. aeruginosa* cultivation

An axenic strain of *M. aeruginosa* (FACHB-905), kindly provided by the Freshwater Algae Culture of Hydrobiology Collection at the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), was used in this study. The strain of cyanobacteria was isolated from Dian Lake, China during the summer in 1998. The cultures were grown in sterilized MA medium (Ichimura, 1979) at 25 °C under a light/dark regime of 14/10 h.

2.2. Chemical treatment

M. aeruginosa cells were incubated in the presence and absence of D-menthol to evaluate its effect on the living cells at three initial algae densities: 1.4×10^7 , 1.2×10^6 , and 2.9×10^5 cell mL⁻¹. The highest concentration was 1.4×10^7 cell mL⁻¹, for the stationary phase, and 1.2×10^6 and 2.9×10^5 cell mL⁻¹, for the logarithmic growing phase. (Park et al., 1998). D-Menthol (Sigma-Aldrich, ≥99%, 2.0 g) was added to the culture medium (600 mL) in a 1 L Erlenmeyer flask. The samples without D-menthol served as the controls.

The biomass of *M. aeruginosa* was evaluated by measuring the freeze-dry weight. The cells were harvested (4.0×10^3 g, 15 min, 4 °C), triple-rinsed with Milli-Q water, and freeze-dried. The weight of the lyophilized cells was recorded.

The morphological and structural changes in the algal cells during the incubation were observed using a scanning electron microscope (SEM).

2.3. MCY analysis

The intracellular and extracellular MCY concentrations were determined using a MCY enzyme-linked immunosorbent assay (ELISA) analysis kit (MCY total-kit, J & Q Environmental Technologies, China). For the analysis of intracellular MCY concentration, the cells were harvested by centrifugation at 4.0×10^3 g at 4 °C, rinsed, freeze-dried, weighed, and stored at -70 °C until further analysis. The lyophilized cells were dispersed in Milli-Q water and then disrupted in liquid nitrogen. The homogenate was centrifuged (1.0×10^4 g, 10 min, 4 °C) prior to the ELISA analysis. To measure the extracellular MCY concentration, each of the samples was filtered through a 0.2 μm glass fiber filter (Whatman) and then subjected to the ELISA analysis. The Milli-Q water was used as the control sample to prevent false positives.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) fingerprint analyses

The lyophilized cells were suspended in Milli-Q water (1.0 g powder in 10 mL) and then disrupted in liquid nitrogen. This

procedure allows cell lysis by osmotic shock and facilitates protein extraction (Fleurence et al., 1995). The suspension was then gently stirred overnight at 4 °C. After the incubation, the suspension was centrifuged at 5.0×10^3 g for 60 min. The supernatant was filtered and freeze-dried. The freeze-dried powder was used as the raw material for the SDS–PAGE analysis, which was performed using a stacking gel of 5% and a separating gel of 12% acrylamide in 25 mM trisaminomethane (Tris)–HCl. The pH value was adjusted to 8.3, and 0.2 M glycine and 1.0 g L⁻¹ SDS were used. The separation was carried out at 120 V for 1 h (Rouxel et al., 2001).

2.5. Statistical analysis

The responses of *M. aeruginosa* to the D-Menthol stress were analyzed by a parametric one-way ANOVA test. If the statistical test was found to be significant at $p < 0.05$, a Tukey test was then used to determine the difference (Zar, 1996). All the tests were conducted using the Statistical Package for Social Science (SPSS 20.0 for Mac OS X). The results shown in the figures represent the average of three independent replicate treatments. The data obtained in this study are presented as means ± standard deviations (SD).

3. Results

3.1. Dry weight

Compared to the control groups, the addition of D-menthol showed a negative effect on the biomass of *M. aeruginosa*. The dry weight of *M. aeruginosa* cells decreased because of the presence of D-menthol during the stationary phase. As shown in Fig. 1a, when the initial cell density was 1.4×10^7 cell mL⁻¹, a distinct decrease in the cell dry weight was observed after 72 h exposure to D-menthol (7.5 mg L⁻¹). In the controls, the cell dry weight did not exhibit a significant change ($p > 0.05$). When the initial cell density was 1.2×10^6 and 2.9×10^5 cell mL⁻¹, the dry weight of the cell increased rapidly in the controls, while it decreased gradually in the D-menthol-exposed samples (Fig. 1b and c). After 72 h incubation, the dry weight of algal cells in the controls increased to 7.3 and 1.3 mg L⁻¹ when the initial cell density was 1.2×10^6 and 2.9×10^5 cell mL⁻¹, respectively, and the dry weight of the D-menthol-exposed samples decreased to 0.8 and 0.1 mg L⁻¹ when the initial cell density was 1.2×10^6 and 2.9×10^5 cell mL⁻¹, respectively. These changes were considered as significant ($p < 0.05$).

3.2. Change in the surface morphology of algal cells

To understand the effect of D-menthol on the surface morphology of the *M. aeruginosa* cells, SEM micrographs were recorded before and after the addition of D-menthol. The cells of *M. aeruginosa* in the control sample were found to be round and plump with the average diameter of approximately 2.6 μm, intact and smooth (Fig. 2a). However, after the addition of D-menthol, the surface morphologies of the algal cells changed. After the reaction for 72 h, the algal cells were distorted from their normal spherical shape and became flattened, and the surfaces became rough. Some of the cells cracked, and the cell membrane of the algal cells lysed, thus releasing the inclusion as shown in Fig. 2b. Thus, D-menthol is responsible for causing severe damage to *M. aeruginosa*, thereby inhibiting its growth.

3.3. MCY concentration

Fig. 3 shows the MCY concentration during 120 h incubation. When the cells were in the stationary phase, the intracellular

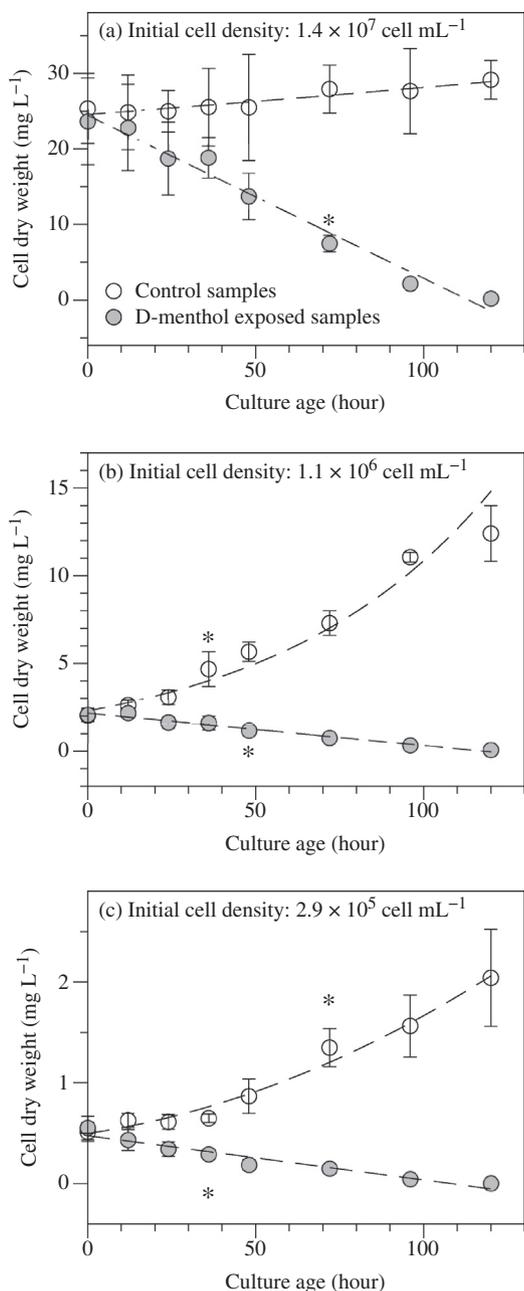


Fig. 1. Changes in the dry weight of *M. aeruginosa* in the control and D-menthol exposure over time, (a) when the initial cell density was 1.4×10^7 cell mL⁻¹; (b) when the initial cell density was 1.2×10^6 cell mL⁻¹; (c) when the initial cell density was 2.9×10^5 cell mL⁻¹. *: $p < 0.05$.

MCY concentration in the controls did not exhibit any significant change. However, a slow increase ($p > 0.05$) in the extracellular MCY concentration was observed after this period of incubation ($31 \mu\text{g L}^{-1}$). In the D-menthol-exposed samples, the intracellular MCY concentration decreased ($2.6 \mu\text{g L}^{-1}$), and the extracellular MCY concentration increased ($428 \mu\text{g L}^{-1}$). However, the total MCY concentration remained almost constant after 120 h exposure.

In the absence of D-menthol, the logarithmic growing cells responded differently from the cells in the stationary phase because the intracellular MCY concentration increased with increasing dry weight of the cells. In the presence of D-menthol, the logarithmic growing cells exhibited a similar trend as the

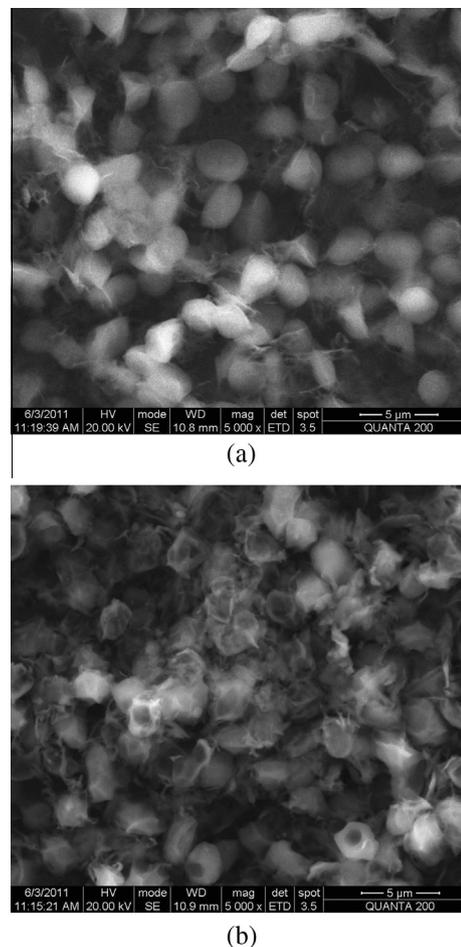


Fig. 2. SEM images: (a) control sample; (b) after the exposure to D-menthol for 72 h.

sample in the stationary phase in releasing the intracellular MCY. As shown in Fig. 3, the intracellular and extracellular MCY show opposite trends. The former decreased while the latter increased, and the total still remained relatively constant. After 72 h incubation, the intracellular MCY concentrations were 11 and $2.6 \mu\text{g L}^{-1}$, respectively, and the extracellular MCY concentrations were 24 and $7.3 \mu\text{g L}^{-1}$, respectively. The differences were significant ($p < 0.05$).

3.4. SDS-PAGE protein pattern

As shown in Fig. 4, seven protein bands are present in the control sample, i.e., 57, 51, 39, 32, 25, 19, and 15 kDa. After the D-menthol exposure, some variations in the protein pattern were observed. In particular, the appearance of two protein bands with apparent molecular weights of 55 and 49 kDa clearly indicate that the proteins with low molecular weights ($M_w < 49$ kDa) have released into the medium.

4. Discussion

In algal toxicity experiments, biomass is in general used as the indicator for growth inhibition (Nyholm, 1985). The decrease in the dry weight of the cells after 120 h D-menthol exposure was statistically significant ($p < 0.05$), indicating that D-menthol inhibited the growth of *M. aeruginosa* cells effectively. The SEM images also

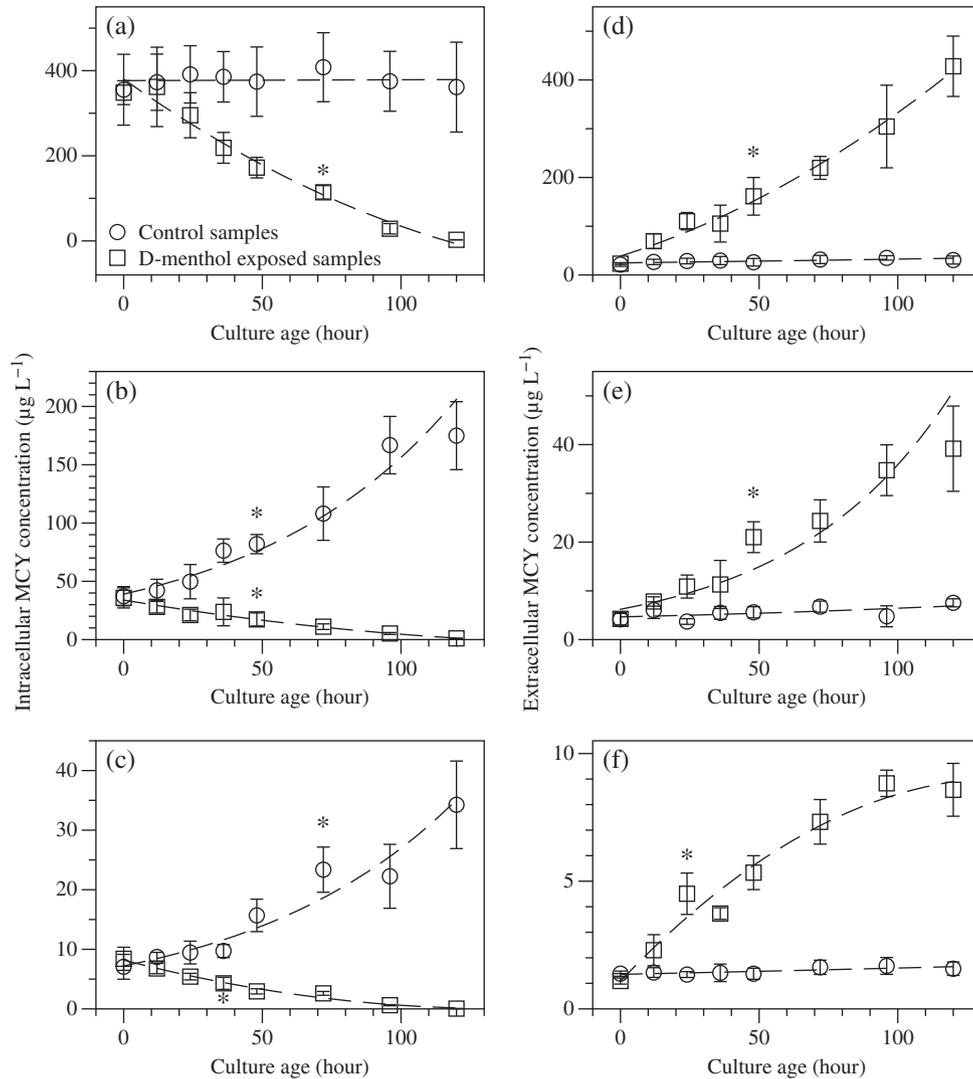


Fig. 3. Intracellular and extracellular MCY concentration; (a) intracellular MCY content when the initial cell density was 1.4×10^7 cell mL⁻¹; (b) intracellular MCY content when the initial cell density was 1.2×10^6 cell mL⁻¹; (c) intracellular MCY content when the initial cell density was 2.9×10^5 cell mL⁻¹; (d) extracellular MCY concentration when the initial cell density was 1.4×10^7 cell mL⁻¹; (e) extracellular MCY concentration when the initial cell density was 1.2×10^6 cell mL⁻¹; (f) extracellular MCY concentration when the initial cell density was 2.9×10^5 cell mL⁻¹. *: $p < 0.05$.

confirmed that the disruption of the algal cells can be attributed to the presence of D-menthol (Fig. 2).

As shown in Fig. 5a–c, logarithmic growing cells have higher intracellular MCY concentration than the cells were in stationary phase. This observation supports the generally held view that intracellular MCY concentration is the highest when the conditions are favorable for its growth (Long et al., 2001). As shown in Fig. 5d, the average amount of intracellular MCY is $14 \mu\text{g mg}^{-1}$ on dry weight basis. Our experiment results show that D-menthol cannot affect the average amount of intracellular MCY on dry weight basis, which is verified by the positive linear correlation between the intracellular concentration of MCY and the dry weight of the cells, as determined by analyzing the yield of MCY of the *M. aeruginosa* cells exposed to D-menthol (Fig. 5d).

The increase in the extracellular MCY concentrations can be attributed to two reasons. First, D-menthol can promote the living *M. aeruginosa* cells to release intracellular MCY into the medium, thus increasing the extracellular concentration. Second, the addition of D-menthol can lyse the *M. aeruginosa* cells, thus releasing the intracellular MCY into the medium. As shown in the SDS-PAGE

profile, the intracellular proteins, whose molecular weights are <49 kDa, could not be retained by the lipid membranes after the cells disrupted. The molecular weight of MCY is <49 kDa (approximately 1×10^3 g mol⁻¹); therefore, the cells can potentially release MCY from the inside to the outside of the membrane after the lysis. Further, because MCY is a cyclic heptapeptide, it cannot pass naturally through the cell membrane. A protein is needed for the transportation of MCY across the membrane; however, no such protein has been identified (Pearson et al., 2004). This indicates that the MCY concentration in the medium increased only due to the release of intracellular MCY when the cell wall was destroyed.

The extracellular MCY concentrations in the starting cannot be ignored in each of the samples. This is because the culture medium was not refreshed prior to D-menthol addition. The initial cell densities were achieved after several days, and some algal cells died during this period, thus contributing to the extracellular MCY concentration. Robillot et al. (2000) reported that without any external interference, the extracellular concentration of algal toxins was low and negligible during the early culture stage of algal cells. In

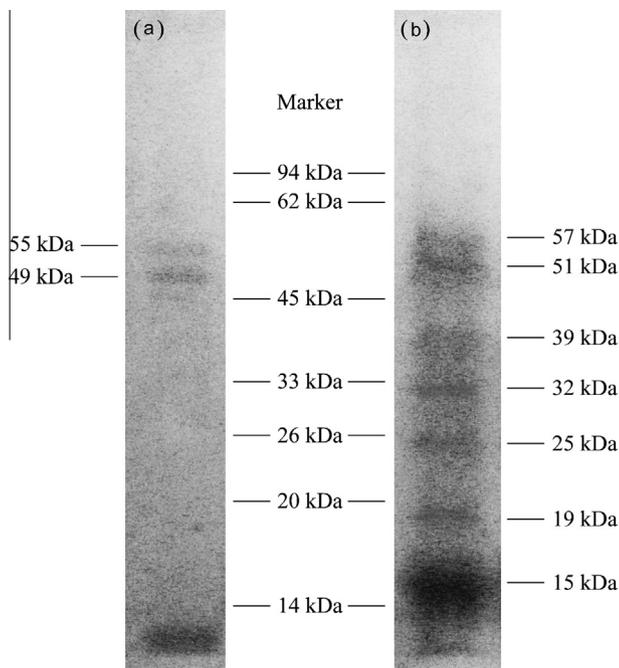


Fig. 4. Protein pattern in SDS-PAGE; (a) after 72 h exposure to D-menthol; (b) control sample.

this study, a slow increase ($p > 0.05$) in the extracellular MCY concentration in the control samples was observed when the cells were in the stationary and logarithmic growing phases.

After the addition of D-menthol, the extracellular MCY concentration continuously increased. This result indicates that D-menthol damaged the algal cells and subsequently released a large amount of MCY. In particular, the extracellular MCY concentration after 5-d exposure to D-menthol was equivalent to the initial intracellular MCY concentration (Fig. 3). Moreover, the major decrease in the dry weight of the cells was observed during 36–72 h, corresponding to the major release of the intracellular MCY in this period. This result is consistent with that of Orr and Jones (1998), who reported that although *M. aeruginosa* retained the cyanotoxins in the cells, they dramatically released into the surrounding medium upon the cell lysis.

The stability of the total MCY concentration in the D-menthol-exposed samples was probably due to the inhibitory effect of D-menthol on the algal growth. Although the *M. aeruginosa* cells disrupted, the extracellular MCY could not be effectively removed by D-menthol treatment. Therefore, a large amount of released MCY may have remained in the water column following D-menthol treatment. However, the released MCY may have been degraded by MCY-degrading bacteria. A large group of MCY-degrading bacteria may be prevalent in natural waters and sediments (Holst et al., 2003). The MCY-degrading bacteria, including *Sphingosinicella microcystinivorans* (Maruyama et al., 2006; Ho et al., 2007),

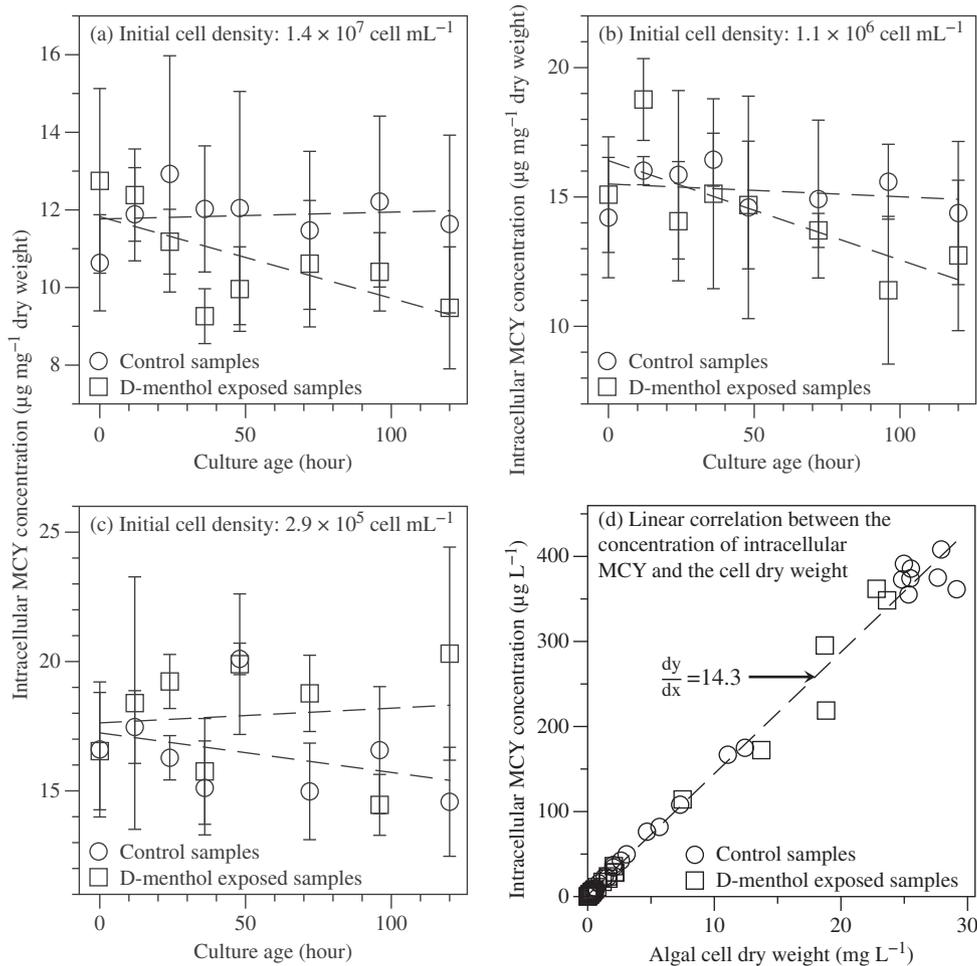


Fig. 5. Change in the intracellular MCY concentration (a); (d) linear correlation between the concentration of intracellular MCY and the dry weight of the cells.

Sphingopyxis (Ho et al., 2006), and *Sphingomonas* (Park et al., 2001) can completely degrade MCY in a few days.

5. Conclusions

The effects of *D*-menthol exposure on the release of intracellular MCY can be summarized as follows: First, the *D*-menthol exposure inhibited the increase in the number of cells, thus inhibiting the increase of MCY concentration in the medium, regardless of whether the cells were in the logarithmic growth or stationary phases. Second, because of the release of intracellular MCY caused by the disruption of algal cells, the concentration of extracellular MCY increased in the medium. Finally, the exposure to *D*-menthol could not degrade the intracellular MCY.

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