

Influences of small-scale oscillations on growth inhibition and ultrastructural changes of
Microcystis cells

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ABSTRACT

We investigated the effects of small-scale oscillation (SSO) on toxic *Microcystis* cells. The oscillating device was made of silicon with two axes that had a diameter of ~40 mm, and a frequency of 2.5 Hz was observed at 150 rpm. The SSO was effective in inhibiting *Microcystis* growth. Microcystin release was not observed, whereas cell density barely increased in the oscillating group. Cell size and morphology of the oscillating group were no different from the control group. However, cell quotas of chl.a and microcystin in the oscillating group were half the level of the control group. Crucially, a number of large-sized holes were observed and layered long linear thylakoids were rarely observed in the oscillating group. Therefore, SSO was found to be very effective in *Microcystis* growth inhibition, and it caused ultrastructural changes without damage to the cell membrane and subsequent microcystin release.

KEYWORDS: small-scale oscillation; *Microcystis* growth inhibition; cell size and morphology; cell quotas of chl.a and microcystin; gas vesicles and thylakoids damage

INTRODUCTION

Cyanobacterial surface blooms often occur during strong and persistently stratified conditions, which is usually during summer.^[1; 2] *Microcystis* blooms have been suggested to be caused by several factors, such as intense light, high water temperature, stability of the water column and a low N:P ratio.^[3-5]

Several species of cyanobacteria produce cyanotoxins^[6; 7], and thus the removal of cyanobacteria and/or control methods have been investigated^[3; 8-10]. Chemical addition of compounds such as aluminum sulfate and copper sulfate effectively remove cyanobacterial cells but cause toxin release problems.^[11; 12] Electrooxidation has been evaluated as a highly effective method for removing not only cyanobacterial cells but also cyanotoxins; however, the removal efficiency is limited to bodies of water with large volumes.^[13]

Regulating bloom-forming factors has also been evaluated as a cyanobacterial control method. Buoyancy is crucial for cyanobacteria, which have gas vesicles, and mixing has often been used to reduce surface blooms.^[10; 14; 15] Turbulent mixing and/or artificial destratification were found to cause a dramatic shift in dominant genera from *Microcystis* to diatoms and green algae.^[16-18] In addition, turbulent mixing affected the size of *Microcystis* colonies.^[19; 20] However, the effects of small-scale turbulent mixing on cyanobacteria growth and survival have only been presented in a few reports.^[21; 22] In addition, turbulent oscillating experiments have traditionally used shakers or oscillating grids that are customizable by changing the various conditions of the grid mesh, and the frequency and length of the stroke.^[23] Therefore, in this study, we have devised a different type of oscillating device and investigated the effects of the small-scale oscillation (SSO) generated from this device on toxic *Microcystis* cells.

MATERIALS AND METHODS

Microcystis Suspension Preparation

A unicellular laboratory culture of *Microcystis aeruginosa* strain NIES3349 (National Institute for Environmental Studies, Japan) was grown in 10 L of MA medium^[24] at 23 ± 1 °C under illumination at ca. $16 \mu\text{mol m}^{-2} \text{s}^{-1}$ and a 12:12 h light:dark cycle. Cultures were harvested during the late exponential growth phase or the early stationary growth phase.

Oscillating and Stirring Devices

The oscillating device was made of silicon with two axes that have a diameter of ~40 mm (Fig. 1). Characteristically, when rotated in the counterclockwise direction, water flows downwards underneath the oscillating device and rises upwards from the side to give even circulation throughout the chamber (Fig. 1(b)). Although growth inhibition was observed above 100 rpm, the most effective inhibition was observed at 150 rpm. In addition, a frequency of 2.5 Hz was observed at 150 rpm. Therefore, all experiments were conducted at 150 rpm.

Since stirring or mixing other than oscillation may affect growth inhibition, a stirring device was also prepared. This device was a standard magnetic bar with a diameter similar to the oscillating device that was connected to a shaft. Rotation of the devices was achieved by connecting them via the shaft to small motors.

Experimental Setup

Three acryl chambers were used with a size of $320 \times 180 \times 150$ mm (length \times width \times height). Six liters of *Microcystis* suspension were added to each chamber. Oscillating and stirring devices were subsequently added to each chamber (the control sample was with no device). Devices were placed in the upper middle portion of the chamber. Experiments were conducted in a temperature-controlled room at 27 ± 3 °C and were illuminated with fluorescent lights at $10 \pm 3 \mu\text{mol m}^{-2} \text{s}^{-1}$ under a 24 h light cycle for 10 d.

Analytical Methods

Optical density at 405 nm (OD₄₀₅)

Microcystis cell density was measured by taking the optical density at 405 nm, which shows a high correlation with cell density. OD₄₀₅ was measured daily using a spectrophotometer (V-540 UV/VIS, Jasco, Tokyo, Japan).

Chlorophyll a

For chlorophyll a (chl.a) analysis, a sample of 20 mL was filtered through glass filter paper (GF/C, Whatman, Kent, UK) and the chlorophyll extracted using 90% acetone (7 mL). The absorbance of the extracts were measured at 630, 645, 663 and 750 nm using a spectrophotometer (V-540 UV/VIS, Jasco, Tokyo, Japan), and the chl.a concentration was determined using UNESCO equations.^[25]

Intra and extracellular microcystin-LR

Measurement of microcystin-LR (MC-LR) concentrations was conducted according to the method described previously by Han et al. [26] For intracellular MC-LR analysis, samples were filtered through Whatman GF/C filter paper and immediately frozen at $-30\text{ }^{\circ}\text{C}$ before being lyophilized. The dried filter was homogenized and extracted with 5% aqueous acetic acid, and the resulting supernatant was applied to an Oasis HLB cartridge (0.5 g, Waters, Milford, MA, USA) that had been conditioned previously with methanol (10 mL) and distilled water (10 mL). For extracellular MC-LR analysis, the filtered water was applied directly to an HLB cartridge, which was subsequently eluted with methanol, and the elute containing the toxin was collected. The MC-containing fraction was evaporated to dryness and the residue was reconstituted in methanol (200 μL). The resulting solution was analyzed by high-performance liquid chromatography (HPLC).

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-9A pump coupled to a SPD-10A set at 238 nm, an SPD-M10A photodiode array detector, a C-R6A integrator and an ODS column (Cosmosil 5C18-MS-II; $4.6 \times 150\text{ mm}$, Nacalai Tesque, Tokyo, Japan). The sample was separated using a mobile phase consisting of methanol:0.05 M phosphate buffer (pH 3.0) (ratio = 58:42) that was applied at a flow rate of 1 mL min^{-1} . The MC concentration was quantified against MC-LR standards (Kanto Ltd., Japan).

Flow cytometry

A small volume of the sample was withdrawn for *Microcystis* cell density and cell size measurements, and the analysis was performed using a Multisizer 4 Particle Analyzer (Beckman Coulter, Fullerton, Canada).

Scanning electron microscopy (SEM)

The samples for SEM analysis were dehydrated through a graded series of ethanol from 50 to 100%^[27; 28] and the samples were then transferred to *t*-butyl alcohol. The containers containing precipitates in *t*-butyl alcohol were placed in a refrigerator, and *t*-butyl alcohol was then frozen within a few minutes. The containers were transferred into a vacuum evaporator (JFD-310, JEOL, Tokyo, Japan), and were lyophilized for 1 h. The dried samples were coated with osmium using a sputter coater (Osmium coater Neo-AN, Meiwafoysis Co., Ltd., Tokyo, Japan). Cell morphology and element compositions were observed under a scanning electron microscope (JSM-7600F, JEOL).

Transmission electron microscopy (TEM)

The samples for TEM analysis were then fixed with 1 mL of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and stored at 4 °C until further TEM analysis. After primary fixation, the cells were centrifuged (2380 g, 15 min, 25 °C) and the pellet re-suspended in 0.1 M phosphate buffer. This process was repeated three times and the cells were post-fixed in 1% osmium tetroxide. The cells were completely dehydrated in graded alcohols (50 to 100%) and propylene oxide. The cells were placed in an epoxy mixture for 1 h and embedded in fresh epoxy resin (Epok 812) capsules and polymerized at 60 °C overnight. After resin embedding, ultrathin sections (80–100 nm) were cut and stained on uranyl acetate and lead citrate, and collected on 200 mesh copper grids. The sections were observed with a TEM (JEM-1400, JEOL) operating at 80 kV.

RESULTS

Effect of Oscillation on *Microcystis* Cell Density

Cell density barely increased in the oscillating group, whereas the cell density increased more than 2-fold in the control and stirring groups (Fig. 2). Chl.a and intracellular MC concentrations were found to mirror the cell density results.

The Effect of Oscillation on *Microcystis* Cell Damage

Microcystin is released when *Microcystis* cells are damaged. Therefore, the extracellular MC concentration can be an indirect indicator of cell damage. Extracellular MC concentrations were below the detection limit in all experimental groups (Fig. 3). This observation indicates that the oscillation does not cause *Microcystis* cell damage.

Effect of Oscillation on *Microcystis* Cell Size and Cell Morphology

Cell size variations were measured to determine cellular debris production that remained after cell membrane damage, cellular contraction, or expansion after undergoing the physical oscillating process. Cell sizes were similar in all experimental groups during the experimental period (Fig. 4). Interestingly, *Microcystis* cells did not grow substantially in the oscillating group; however, the cell size was similar to those cells in the control group.

Cell morphology of the cells in the oscillating group was also not different from cells in the control group. Intact cells with a round shape were observed for cells in the oscillating and control groups (Fig. 5). These results showed that the SSO does not cause damage to the cell membrane and/or morphology.

Effect of Oscillation on Cellular Chl.a and Microcystin Content

Since cell membrane damage was not observed, intracellular effects were evaluated such as cell quotas of chl.a and microcystin. MC cell quota variations differed between the groups, and the chl.a cell quota variations among the groups were found to correlate with the cell density results (Tables 1 and 2). MC cell quotas in the control group remained constant at ca. 40 fg cell⁻¹ during the experimental period, whereas the MC cell quotas in the oscillating group decreased to 23 fg cell⁻¹ by the 10th day (Table 2). Consequently, SSO appears to affect the cellular chl.a and microcystin contents.

Effect of Oscillation on Cellular Structures

Ultrastructural changes to *Microcystis* cells and the growth inhibition mechanism caused by oscillation were investigated using transmission electron microscopy (TEM). Healthy, normal ultrastructures, such as gas vesicles, thylakoids, glycogen granules and structured granules were observed in the control and stirring groups (Fig. 6(a)). In contrast, ultrastructural changes in gas vesicles and thylakoids were observed in the oscillating group. A number of large-sized holes were observed and were confirmed to be damaged gas vesicles (Fig. 6(b), white arrow). Furthermore, layered long linear thylakoids were rarely observed (Fig. 6(b), gray arrow).

DISCUSSION

In this study, SSO was shown to inhibit toxic *Microcystis* growth. Similar results such as a decrease or the occasional increase in cell density have been obtained in previous repetitive experiments (Fig. 7). Although there was a difference in *Microcystis* growth in the second-half of the experimental period, which was dependent on the initial state and cell density of

Microcystis, the results showed that growth inhibition of cells in the oscillating group was usually observed in all experiments (Fig. 7).

There was no significant difference between the control and experimental groups in cell density and cell quotas of chl.a and microcystin until the third day of the experiment (Fig. 2 and Table 1). Regel et al. [22] reported a delayed response after 96 h, after which, negative effects on *Microcystis aeruginosa* esterase activity, and viability at 3 and 4 Hz were observed. It is plausible that *Microcystis* cells display resistance to SSO for a period on the order of days.

Interestingly, oscillation does not cause damage to the cell membrane and/or morphology. Cell sizes were similar in all experimental groups during the experimental period and microcystin release because of cell damage was not observed (Figs. 2 and 4). Round shape *Microcystis* cells were observed in SEM analysis with no difference from the control group (Fig. 5). TEM analysis showed the presence of multilayered cell walls in both the control and oscillating groups.

Although oscillation did not cause cell membrane damage, TEM results did reveal that oscillation did cause ultrastructural changes. Damage to gas vesicles and thylakoids might be fatal to *Microcystis* cells (Fig. 6(b)). Damage to the structure of gas vesicles, which are used to modulate the buoyancy of the cell, will affect growth inhibition. In addition, relatively low chl.a and microcystin cell quotas were observed in the oscillating group (Table 1).

Understandably, reduction of chl.a affects photosynthesis and may inhibit cell growth. A positive correlation between cell division and microcystin production rates exists.^[29; 30] The cell division rate in the oscillating group (0.04 d^{-1}) was found to be lower than that of the control and stirring groups (both 0.06 d^{-1}). This may explain the lower MC cell quotas in the oscillating group (23 fg cell^{-1} on the 10th day) when compared with that of the control group. Microcystins are localized in the nucleoplasm and most abundantly associated with

thylakoids.^[31-33] In contrast to the control group, which had a constant level of ca. 40 fg cell⁻¹, the decrease of MC cell quotas by approximately 50% in the oscillating group (Table 2) could be because of damage to the thylakoids.

Based on these results, we conclude that oscillation was very effective in inhibiting *Microcystis* growth without damage to the cell membrane and subsequent microcystin release. Toxin release is a very important and troublesome factor in the development of cyanobacterial removal and/or control methods.^[34; 35] Therefore, the results of this study provide a potential viable approach to inhibit *Microcystis* growth without deleterious side effects such as toxin release.

CONCLUSION

Small-scale oscillation at 2.5 Hz, generated by an oscillating device, was highly effective in inhibiting toxic *Microcystis* cell growth; however, a tolerance period of days was observed. SSO did not affect cell size, morphology, microcystin release and cell membrane damage when compared with that of the control group. However, very low cell quotas of chl.a and microcystin were observed in the test group, and TEM results showed that SSO caused ultrastructural damage to gas vesicles and thylakoids. Damage to gas vesicles and thylakoids is fatal to cyanobacterial growth. Therefore, SSO is a very promising approach to remove and/or control toxic cyanobacterial blooms without cyanotoxin release.

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LIST OF FIGURE CAPTIONS

FIGURE 1. Characteristics of the oscillating device. (a) The oscillating device was made of silicon with two axes that have a diameter of ~40 mm. (b) Water flows downwards underneath the oscillating device and rises upwards at the sides of chamber when the axes rotate in the counterclockwise direction

FIGURE 2. Changes in cell density after SSO treatment. (a) Cell density measured by optical density at 405 nm. (b) Cell density measured by flow cytometry. White circles: control (no device); gray circles: stirring group; black circles: oscillating group.

FIGURE. 3. Changes in intra- and extracellular microcystin concentrations following SSO treatment. Bars: intracellular microcystin-LR concentration; circles: extracellular microcystin-LR concentration. White circles and bars: control (no device); gray circles and bars: stirring group; black circles and bars: oscillating group. Extracellular MC-LR concentrations were below the detection limit in all experimental groups.

FIGURE 4. Changes in cell size following SSO treatment. White circles: control (no device); gray circles: stirring group; black circles: oscillating group.

FIGURE 5. Scanning electron microscopy (SEM) micrographs of *Microcystis* cells following SSO treatment. (a) Control (normal and healthy cells); (b) SSO treated cells.

FIGURE 6. Transmission electron microscopy (TEM) micrographs of *Microcystis* cells following SSO treatment. (a) Control cell (normal and healthy cell); (b) SSO treated cell. Gray arrow: Thylakoids; black arrow: normal gas vesicles; white arrow: damaged gas

vesicles.

FIGURE 7. Changes in cell density following SSO treatment in repetitive experimental results. White circles: control (no device); gray circles: stirring group; black circles: oscillating group.

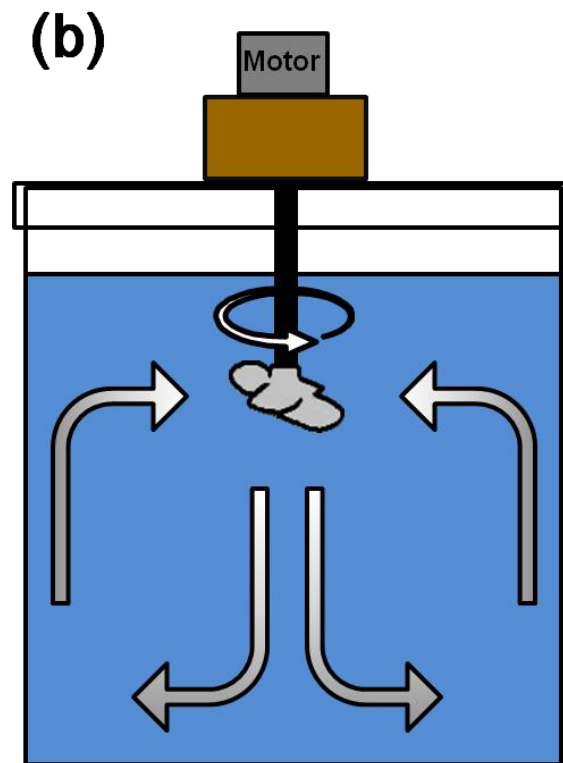
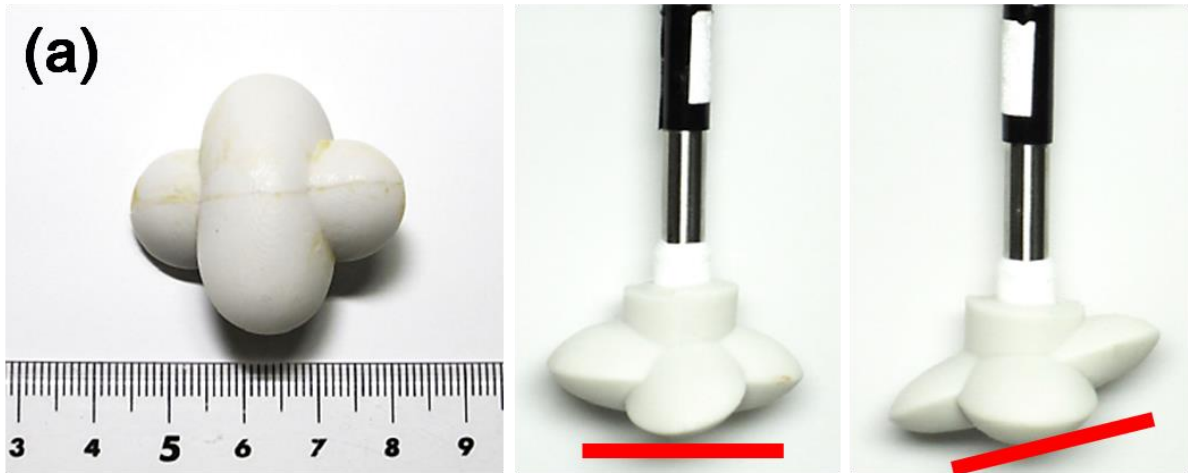


Fig. 1

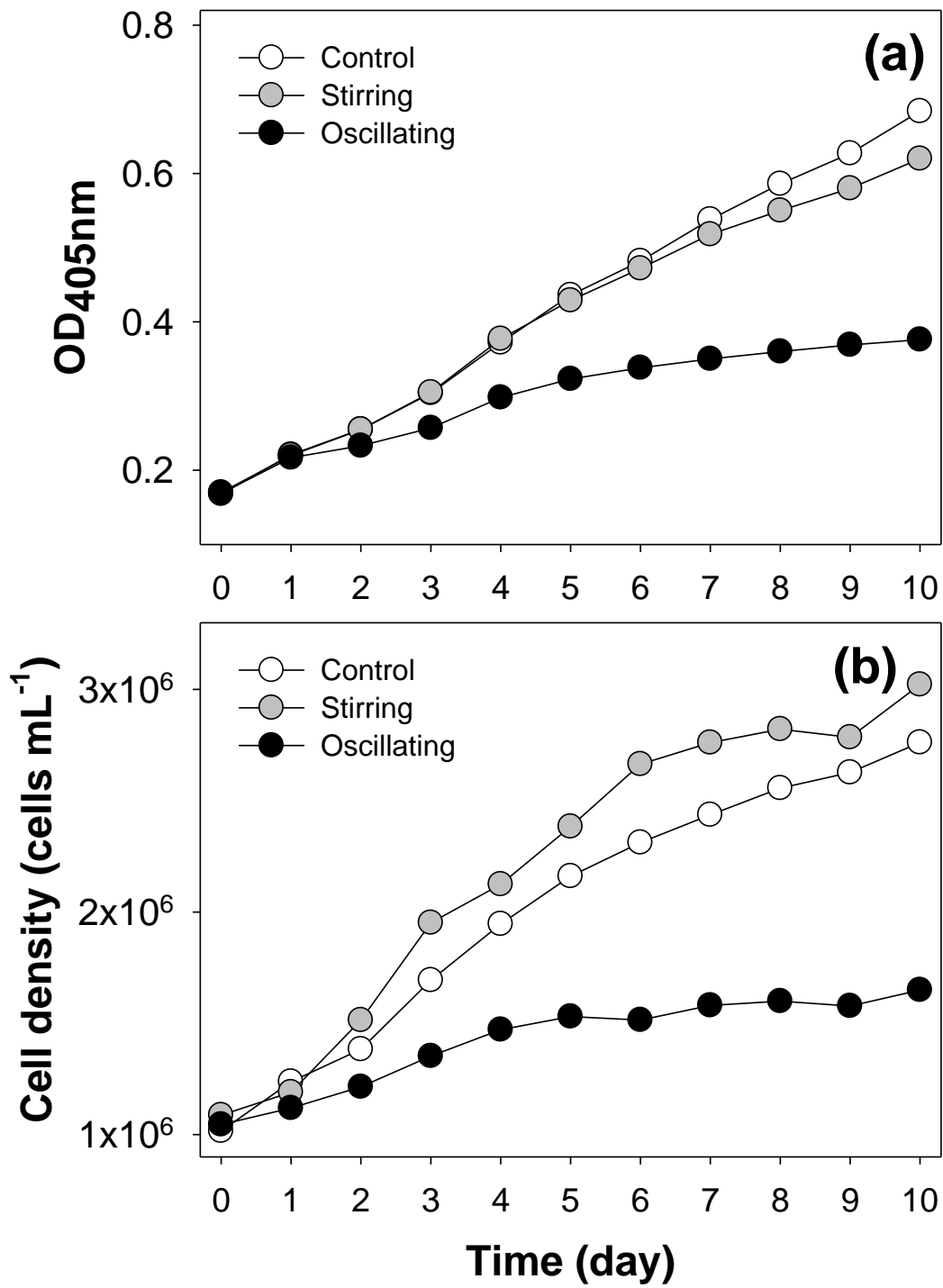


Fig. 2

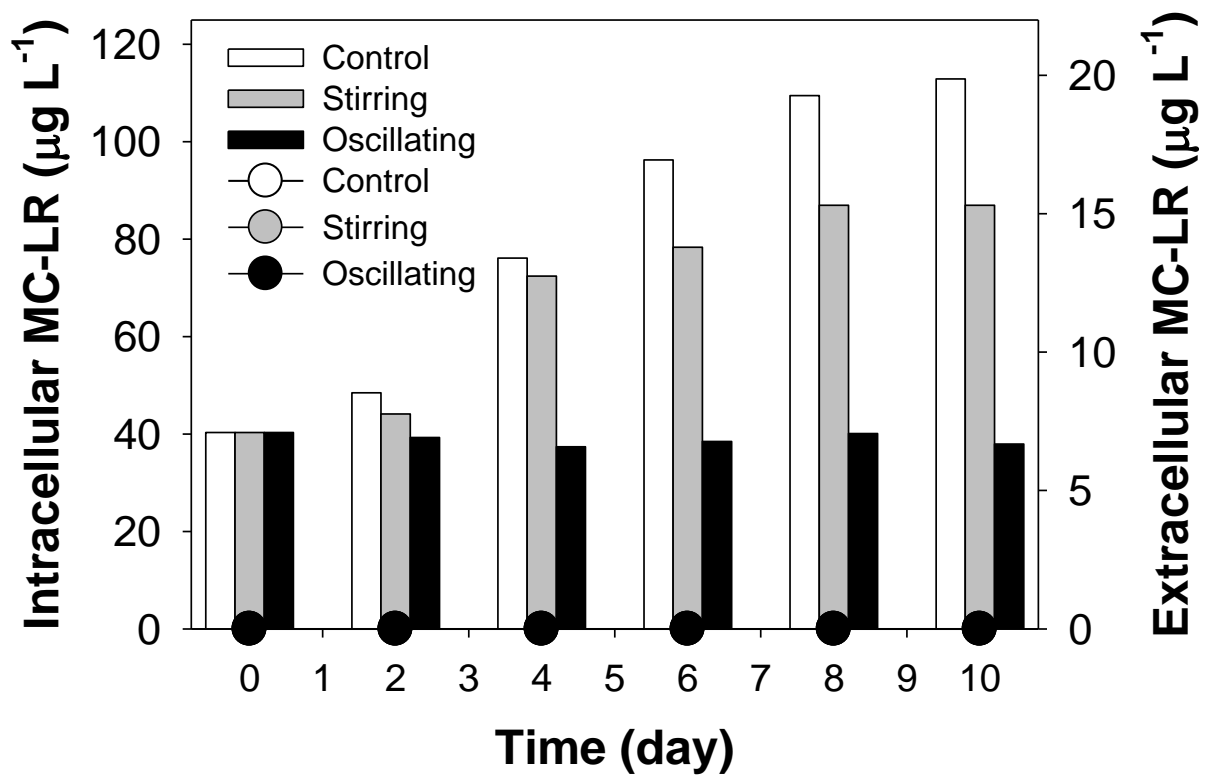


Fig. 3

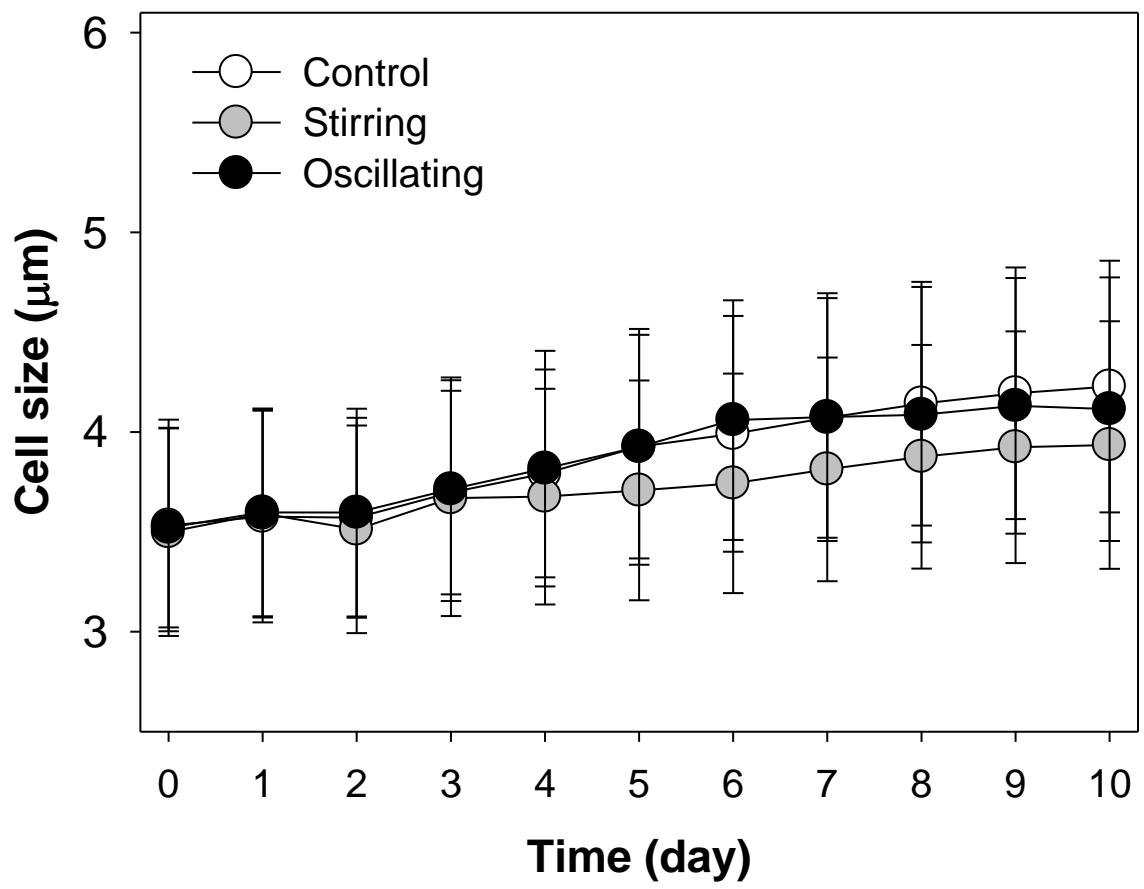


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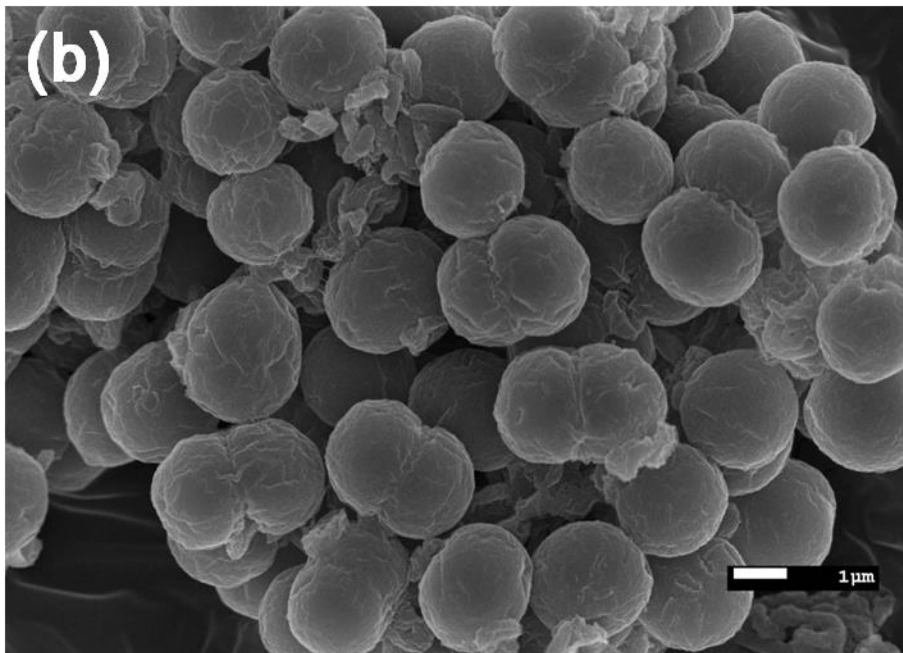
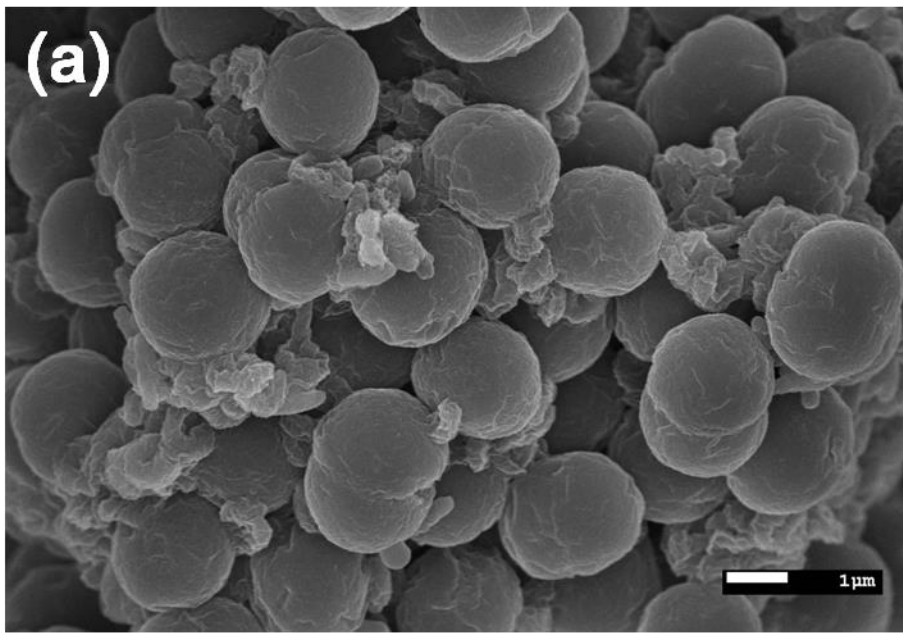


Fig. 5

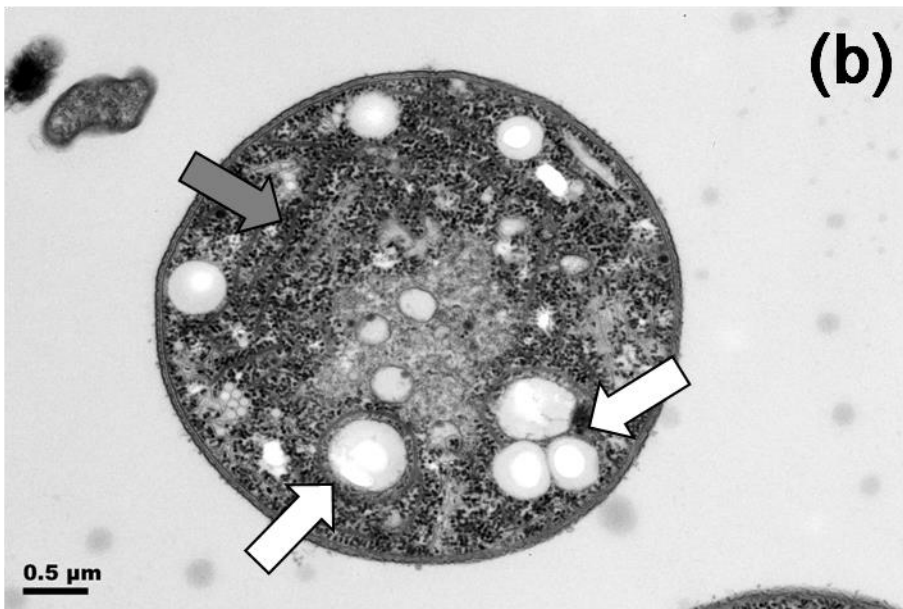
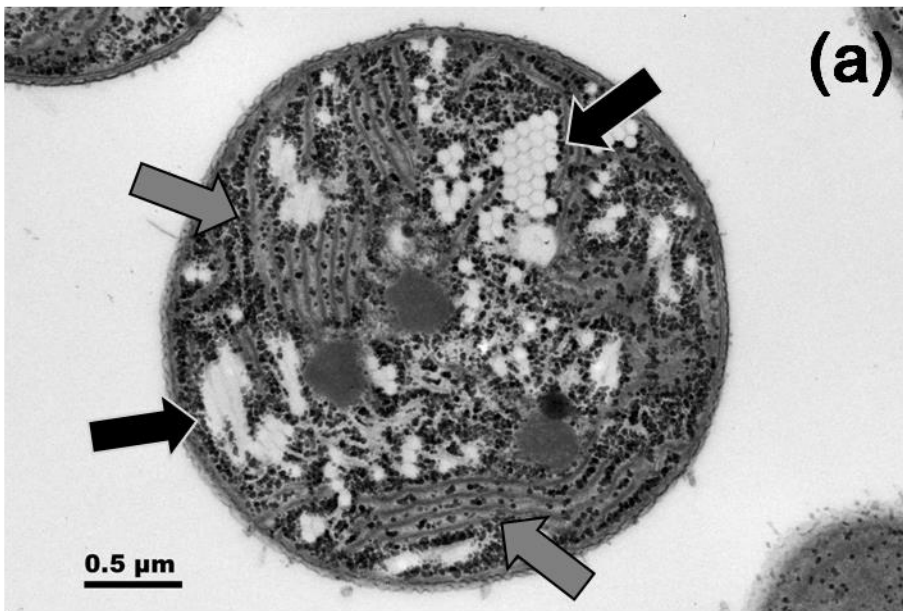


Fig. 6

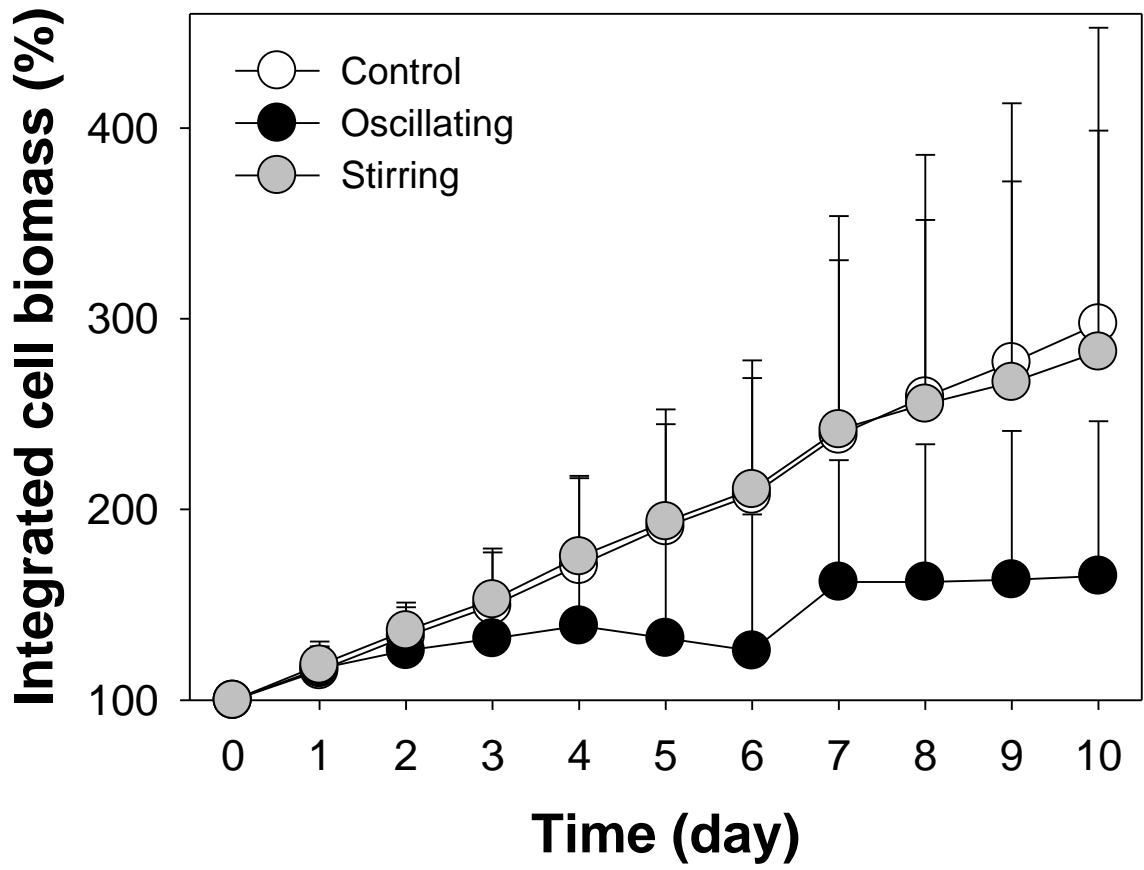


Fig. 7

TABLE 1. Variations of cellular chl.a content following SSO treatment.

(Unit: pg cell⁻¹)

Time	0 (before)	3rd	5th	9th
Control	0.23	0.27	0.32	0.42
Oscillating group	0.22	0.24	0.22	0.19

TABLE 2. Variations of cellular microcystin content following SSO treatment.

(Unit: fg cell⁻¹)

Time (day)	0 (before)	4th	6th	10th
Control	40	39	42	41
Oscillating group	37	25	25	23