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Original Research Paper

The Effect of Ultrasound on *Microcystis* sp. Morphological Characteristics at Different Ultrasonic Power

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ABSTRACT

Optical microscope, environmental scanning electron microscope (ESEM) and transmission electron microscope (TEM) were applied to study the effect of different intensity ultrasound on *Microcystis* cell morphology. The results showed that after the action of low intensity ultrasound (below the cavitation threshold) on *Microcystis* cell, extracellular surface became slightly rough, and gelatinous sheath became uneven. Fragmented debris appeared among cells and intracellular thylakoids and bubbles were damaged. Low intensity ultrasonication make photosynthetic capacity and buoyancy control ability of the cell suppressed or disappeared, leading to the inhibition of growth. The main mechanism of low intensity ultrasound on *Microcystis* cell was mechanical action. After ultrasonication of high intensity, extracellular surface was paler and rough. Gelatinous sheath became more uneven, and there were many fragmented debris. Cell interior was black. All of thylakoids and bubbles were broken, dissolved and disappeared. Cells present as partial block or homogenized structure. Some algal cells appeared pyknotic, but most cell were dead. The main mechanism of high intensity ultrasound was cavitation effect, accompanied by mechanical action at the same time.

INTRODUCTION

Microcystis sp. is a class of globally distributed cyanobacteria. They can live in a variety of environments including extreme environments because of its unique set of morphological, physiological and biochemical mechanisms. Compared with other organisms, *Microcystis* sp. has some survival advantages (Hudnell 2008). *Microcystis* can grow fast in eutrophic water and gather in surface, forming visible algae aggregates which are water blooms (Heisler et al. 2008). In recent years, water blooms in some European and North American countries with good water governance have regained momentum. The frequency of water bloom shows a rising trend in many developing countries. A series of environmental and health problems caused by blooms are being highly valued around the world (Bury 2007, Fleming et al. 2002, Stewart et al. 2006). At present, the ultrasonic technology is used to remove and inhibit algae in water (Fan et al. 2013, Rajasekhar et al. 2012, Wu et al. 2012). The reaction process of sonication does not introduce other chemicals or secondary pollutants, which is recognized as "green technology" (Mason 2007). Previous researchers do a lot of research in the basic effect (Joyce et al. 2010, Kotopoulis et al. 2009, Wu et al. 2011, Wu et al. 2012, Zhang et al. 2006, Zhang et al. 2006) of using ultrasound to remove cyanobacteria blooms. Ultrasound would cause mechanical action and cavitation in water, but it is uncertain that which plays a major role on algae. After sonication, algal cell morphology showed various changes and changes of its shape are also different at different intensity, so main mechanism of algae removal can be inferred indirectly from the morphological changes.

Algal cell morphology can be observed by the ordinary optical microscope, scanning electron microscope (SEM), environmental scanning electron microscope (ESEM) and transmission electron microscope (TEM). Though ordinary optical microscope can visually observe morphological changes of the cells in general, its small magnification, a metric of 1-10μm, belongs to the category of histology, so it is unable to observe ultrastructure. However, the metric of SEM, ESEM and TEM, 1-200nm, belongs to the category of ultrastructure of submicroscopic morphology. SEM and ESEM are used to observe the morphological changes in the cell surface. TEM is used to observe the internal structure of cells and morphological changes in organelles (Ozaki et al. 2009, Young et al. 2005). ESEM is a new type of scanning electron microscope developed in recent years. Compared with the SEM, the sample of ESEM does not require metal surface treatment, and observation environment does not require vacuum degree to be lower than 10-3 Pa. The sample without pretreatment makes it possible to do both static and dynamic observation and analysis at high resolution, but also fully reflects the authenticity of the sample (Reingruber et al. 2012). In this paper, through observing the changes of *Microcystis* cell structure and shape after the treatment of

different intensity ultrasound by ordinary optical microscope, ESEM and TEM, we can reveal the mechanism of ultrasound on *Microcystis* cell in the aspect of cell morphology.

MATERIALS AND METHODS

Algae species and culture: *Microcystis* sp. was provided by freshwater algae culture collection of the Institute of Hydrobiology of the Chinese Academy of Sciences (FACHB-1027). We placed the algae in a constant-light incubator to inactivate the culture. The temperature was set to 25°C, light intensity to 2000 lx, and the light-dark cycle to 14 h:10 h. The algae was initially cultivated in the prepared monoculture BG11, which was provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology. The composition of BG11 was 1.5 g/L $NaNO_3$, 0.04 g/L $K_2HPO_4 \cdot 3H_2O$, 0.075 g/L $MgSO_4 \cdot 7H_2O$, 0.036 g/L $CaCl_2 \cdot 2H_2O$, 0.006 g/L citric acid, 0.006 g/L ferric ammonium citrate, 0.001 g/L EDTA, 0.02 g/L Na_2CO_3, and 1 mL/L A_5+ Co solution. The composition of the A_5 + Co solution was 2.86 g/L H_3BO_3, 1.86 g/L $MnCl_2 \cdot 4H_2O$, 0.22 g/L $ZnSO_4 \cdot 7H_2O$, 0.39 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.08 g/L $CuSO_4 \cdot 5H_2O$, and 0.05 g/L $Co(NO_3)_2 \cdot 6H_2O$. The pH value of the BG11 medium was 7.1, which was regulated using NaOH or HCl.

Ultrasonic algae removal device: The device used in this study was developed by the Institute of Acoustics of the Chinese Academy of Sciences. The other parts of the reactor were home-made. The experimental setup is shown in Fig. 1. The ultrasound source was an ATANA AT3020 ultrasonic signal generator. The power amplifier with model number HFVA-62 was from Nanjing Foneng Co., Ltd. The oscilloscope with model number was UT2025C was produced by Hong Kong Unit Co.

Treatment of ultrasonic samples: *Microcystis* solution whose initial density was $(207.4 \pm 6) \times 10^4$cells/mL was divided into three groups. The first group has normal growth, known as the "control group". The second group was 50mL of algae cell solution through sonication at the frequency of 580kHz, the exposure time of 5min, and the power of 10W. Examination found that cavitation did not occur, regarded as 'no cavitation group', because the strength is lower than ultrasonic cavitation threshold. The third group was 50mL of algae cell through sonication at 580kHz, 5min, 100W. Cavitation occurred after testing, so the group was 'cavitation group'. Each group set three parallel samples.

Observation method of *Microcystis* cell morphology: Conventional squashing method was used when observed with an optical microscope. 0.1mL algae sample was placed in phytoplankton count box, then using optical microscope (Motic China Group Co., Ltd., Motic BA310) to observe cell morphology.

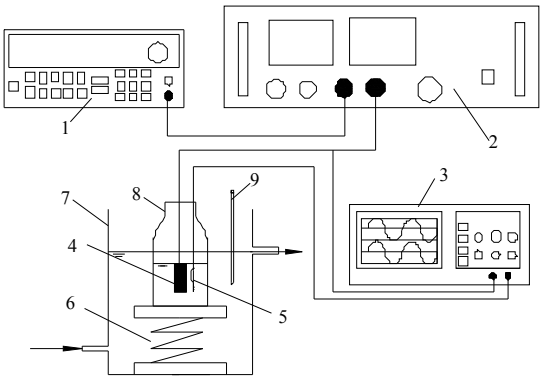

Fig. 1: Experimental ultrasonic apparatus; 1. Signal Generator; 2. Power Amplifier; 3. Oscilloscope; 4. Transducer; 5. Hydrophone; 6. Telescopic Stand; 7. Cooling Device; 8. Glass Flask 9. Thermometer

ESEM can observe samples directly without any processing, so natural forms of samples can be kept. In the experiment, sample stage temperature was 20°C, the sample chamber pressure was $(1.3 \sim 1.33) \times 10^2$Pa, and relative humidity was 75% to 80%.

Samples that were to be seen were centrifuged for 5min at 2000r/min to collect algae in a PE tube of 1.5mL, and washed twice with a freshly prepared BG11 culture solution. Using 3% glutaraldehyde (0.1M phosphate buffer, pH 7.2 -7.4) to fix materials, washing for three times with 0.1M phosphate buffer, the solution was stored at 4°C for 3h with 1% osmium tetroxide (0.1M phosphate buffer, pH 7.4).

After dehydrating step by step with ethanol at the increasing rate of 10%, infiltrating and embedding with epoxy resin, making slices by ultra-microtome (LKB, Sweden), staining with lead citrate, the samples were observed and photographed for ultrastructure of cells with TEM (Hitachi, HITACHI-7500).

RESULTS AND DISCUSSION

Observations under ordinary light microscope and analysis: Fig. 2 is the form observed by ordinary light microscope of morphology of control group, non-cavitation and cavitation group of *Microcystis* sp. As can be seen from Fig. 2, in the control group, *Microcystis* cells grew in groups, such as spherical, oval or irregular shape. Mass showed dark green and aquamarine, and gelatinous sheath was even. Most unattached cells were spherical with radius of 2-4μm. In non-cavitation group, there are slight chances in *Microcystis* mass after sonication. Surface colour got lighter and became aquamarine, and gelatinous sheath was more uneven. Insignificant percentage of cells ruptured, and some secretion flow to the outside of the cell. In cavitation group, colour of *Microcystis* became lighter and the green disappeared. Most

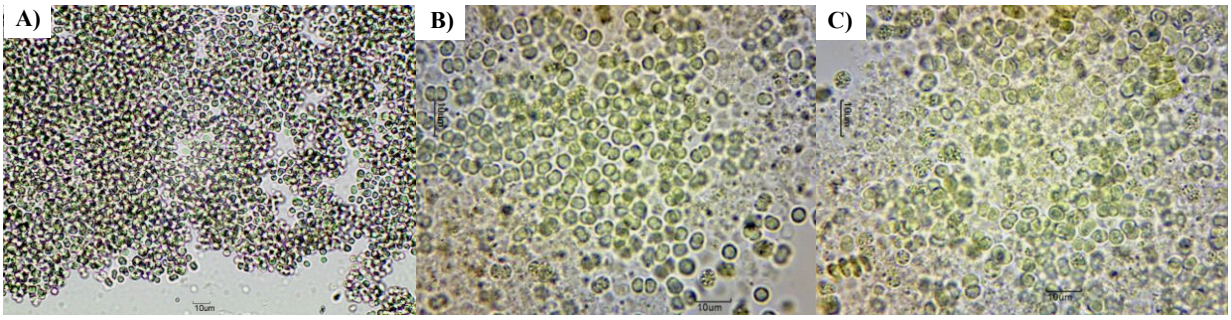

Fig. 2: The form by ordinary light microscope morphological features of *Microcystis* sp.
A. control group × 400; B. non-cavitation × 1000; C. cavitation group × 1000

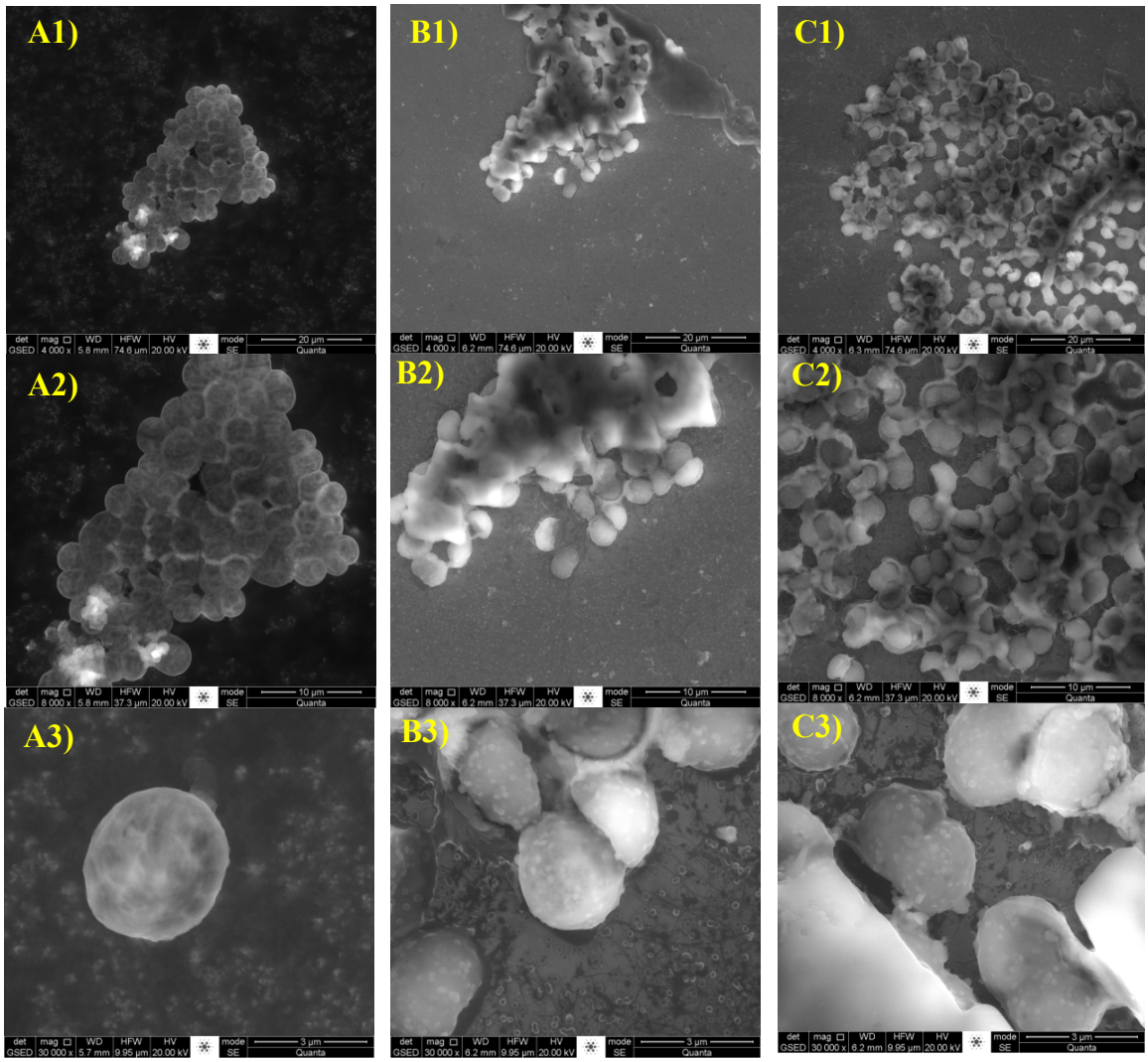

Fig. 3: The form by ESEM of *Microcystis* sp.
A1. control group × 4000; B1. non-cavitation × 4000; C1. cavitation group × 4000
A2. control group × 8000; B2. non-cavitation × 8000; C2. cavitation group × 8000
A3. control group × 30000; B3. non-cavitation × 30000; C3. cavitation group × 30000

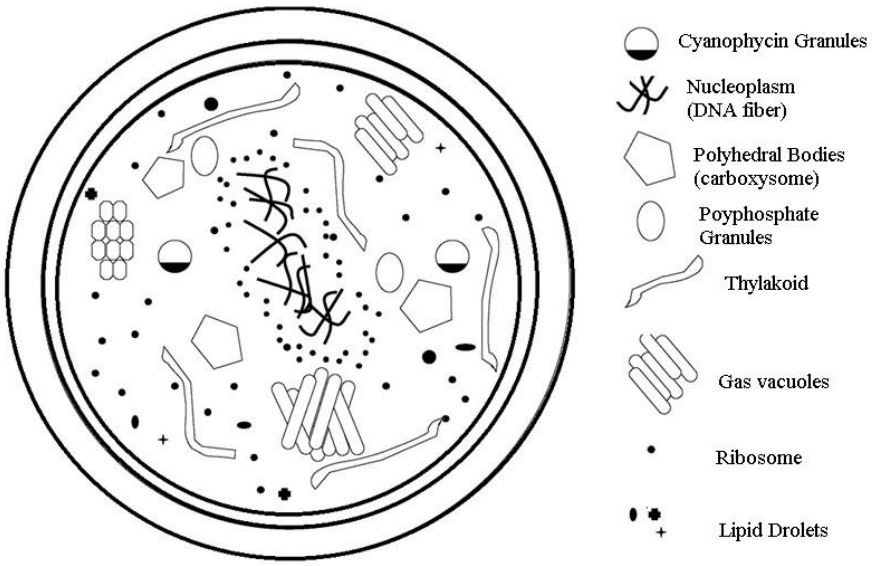

Fig. 4: Structure model of *Microcystis* sp. cell.

cells were broken, and content released into the water. Gelatinous sheath was very uneven. As the magnification of the ordinary light microscope is limited, we could not observe the change of extrasystolic surface and internal structure, so ESEM and TEM were respectively used to observe extrasystolic surface and internal structure.

Observations of ESEM and analysis: The cellular morphology by ESEM of control group, non-cavitation and cavitation group are shown in Fig. 3. The figure shows three groups of ESEM photographs that were amplified 4000 times, 8000 times and 30000 times.

In Fig. 3, the outer surface of *Microcystis* cells in control group was smooth with a clear outline of the cell wall, and coherency of mass was good. In non-cavitation group, the superficial surface became slightly rough, and there were fragments among cells, indicating that ultrasound caused slight damage to their surface. In cativation group, cell surface was rough after sonication. It is found that cluster state cells began to disperse, and fragments were more. This shows that cativation produce more mechanical damage to *Microcystis* cells than the ultrasound that does not cause cavitation.

Observations of TEM and analysis: Fig. 4 is the structure model of *Microcystis* sp. cell under normal circumstances (Li et al. 2008). In Fig. 4, a normal *Microcystis* sp. cell includes mitochondria, nucleic acids, carboxylase, polyhedral bodies, thylakoid, gas vacuoles, ribosome, lipid droplets and so on. Thylakoid, a closed and disc-shaped structure, is composed of the bilayer membrane. Phycobilisomes are attached to its outer surface. Phycobilisome is a high polymer comprised of phycoerythrin, phycocyanin and allophycocyanin. Its physiological function is to capture light excitation energy and then pass it to chlorophyll *a* on photosynthesis layers for photosynthesis. Gas vacuoles are intracellular organelles which can regulate buoyancy. When their content is moderate, the algal cells can float on water for photosynthesis. The cellular morphology by TEM of control group, non-cavitation and cavitation group is shown in Fig. 5.

In Fig. 5, the normal cells of the control group were round and oval under TEM. A large number of clear, uniform and horizontal stripe shaped photosynthesis layers arranged in cells, which were thylakoids. Meanwhile, gas vacuoles were neat and distinct. Membrane and cell wall were clearly visible.

In non-cativation group, it is noticeable to find that the thylakoids and gas vacuoles formed a cloud structure, and there was no damage or other significant changes of cell membrane. Thus, low intensity ultrasound caused damage to thylakoids and gas vacuoles, so that photosynthetic capacity and buoyancy control ability were suppressed or disappeared, leading to the inhibition of the growth of *Microcystis* cells. Main mechanism of low intensity ultrasound is mechanical action.

The cells of cavitation group, experiencing high intensity sonication, became black. All of their thylakoids and gas vacuoles have fractured, broken, dissolved and disappeared. Cells were massive or homogenizing structure partially, and some algal cells appear pyknotic. It can be inferred that cativation make cell carbonize internally and death after high intensity ultrasound effect on *Microcystis* cells. Its main

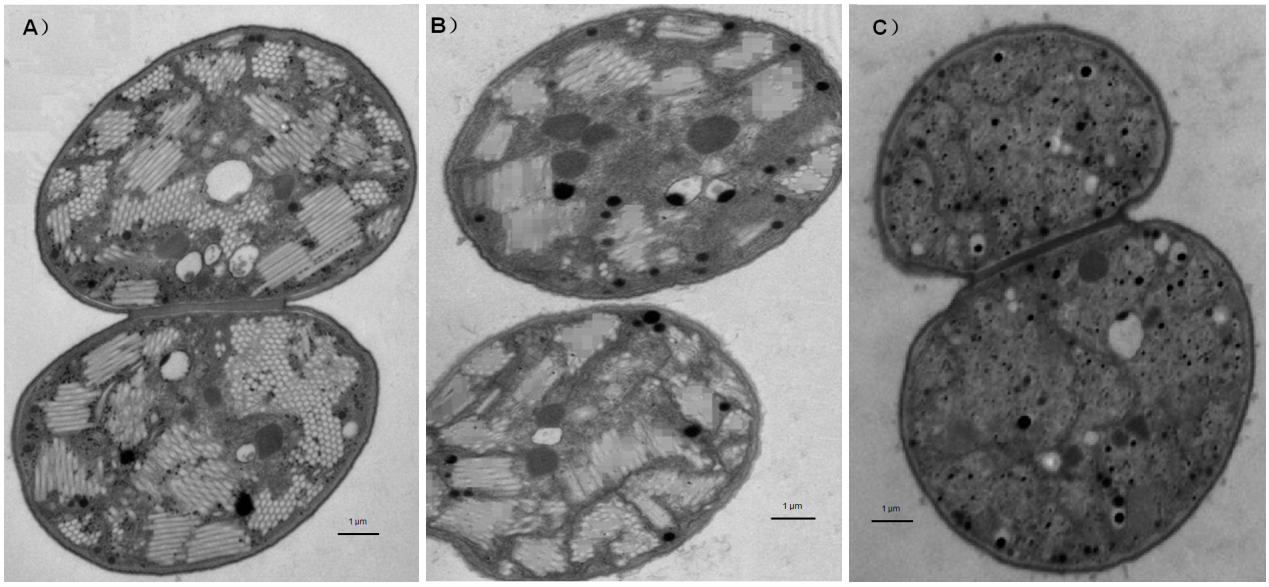

Fig. 5: The form by TEM of *Microcystis* sp.
A. control group × 10000; B. non-cavitation × 10000; C. cavitation group × 10000

mechanism is cativation, accompanied by mechanical action.

In summary, ultrasound removes algae mainly by acting on algal cells thylakoid and gas vacuoles. Ultrasound below the ultrasonic cavitation threshold makes both injured, while ultrasound higher than the threshold ruptures both. Thylakoid is a place for light reaction of photosynthesis. Damage and rupture will directly damage phycobilisome, a high polymer comprised of phycoerythrin, phycocyanin and allophycocyanin, and chlorophyll *a*, which can ultimately affect the photosynthesis of algal cells. Due to damage and fracture of gas vacuoles algal cells cannot do gas exchange smoothly. Cells lose the ability to adjust the buoyancy automatically and sink to the bottom. Cells do not get enough light, thereby affecting cell photosynthesis.

CONCLUSIONS

Ultrasound has a huge impact on *Microcystis* cell morphology. Different ultrasound power has different effects, and its mechanism is also different.

After low intensity ultrasound effect on cells, the outer surface became slightly rough with fragments among cells. Low intensity ultrasound cause damages to thylakoids and gas vacuoles, so that photosynthetic capacity and buoyancy control ability were suppressed or disappeared, leading to the inhibition of the growth of *Microcystis* cells. Main mechanism of low intensity ultrasound is mechanical action.

After ultrasonication of high intensity, extracellular surface was paler and rough. Gelatinous sheath became more uneven, and there were various fragmented debris. Cell interior was black. All of thylakoids and bubbles were broken, dissolved and disappeared. Cells were present as partial block or homogenized structure. Some algal cells appeared pyknotic, but most cell were dead. The main mechanism of high intensity ultrasound was cavitation effect, accompanied by mechanical action at the same time.

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