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Morphological and Biochemical Changes of *Microcystis aeruginosa* and *Anabaena flos-aquae* by Low Frequency Ultrasonic

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Abstract: In this study, laboratory-scale ultrasonication (20 to 200 kHz) was conducted on *Microcystis aeruginosa* and *Anabaena flos-aquae* to investigate the effect of this procedure on algal cell structure, apoptosis and necrosis of cells, release of microcystin and ultrasonication efficiency. The morphological changes were observed using a transmission electron microscope. Cell status was examined using flow cytometry. After ultrasonication, changes in cell organelles included ribosome and thylakoid disorganization, cytoplasmic vacuolation, nucleoplasm diffusion and plasmolysis. Moreover, the increased apoptosis was determined to be related to the ultrasonication of the cells.

Key words: *Anabaena flos-aquae*, low frequency ultrasonication, *Microcystis aeruginosa*

INTRODUCTION

Annually, highly eutrophic drinking water sources suffer from massive cyanobacterial blooms dominated by *Microcystis aeruginosa* and *Anabaena flos-aquae*. Freshwater algae in drinking water sources cause serious problems in various stages of the conventional water treatment system, such as poor coagulation and flocculation, poor settling, filter clogging, increased chlorine demand and unpleasant odors and tastes (De Figueiredo *et al.*, 2004; Henderson *et al.*, 2008; Hitzfeld *et al.*, 2000).

Removal of the algae and algal toxin from drinking water supplies that are affected by algal blooms has become a mandate for many metropolitan areas in China. The conventional control strategies for inhibiting cyanobacterial bloom include coagulation, flotation, clarification, filtration (Zamyadi *et al.*, 2012), application of algicides, use of chlorine (Merel *et al.*, 2010), addition of ozone (Chen *et al.*, 2009; Miao and Tao, 2009) and photolysis (Sakai *et al.*, 2009). However, these methods are usually expensive, complex and can cause other kinds of pollution that could kill plants, crustaceans, fish and other aquatic life due to the use of chemicals (Daly *et al.*, 2007; Schmidt *et al.*, 2002). Ultrasonication may provide a suitable method for reducing cyanobacterial proliferation in eutrophic waters while having environmentally significant advantages, such as safety, cleanness and energy conservation (Kotopoulos *et al.*, 2009; Wu *et al.*, 2011; Zhang *et al.*, 2009). Ultrasound was reported to

reduce the growth rate of algae effectively by collapsing the gas vesicles during cavitation, inhibiting cell division, or inflicting immediate damage on photosynthetic activities.

The study showed that the removal efficiency of *Microcystis aeruginosa* is 29.6% higher by ultrasonic floatation process. In another study (Shi *et al.*, 2011), the removal efficiency of *Microcystis aeruginosa* was reported to reach 93.9% under the ultrasonic frequency of 20 kHz for 30 sec. However, ultrasound is also known to lyse cells and release intracellular materials (Zhang *et al.*, 2006a, b). When applied to algae, ultrasound may release algal toxins into the water, which is highly undesirable. Currently, great strides have been made through studies on cyanobacterial growth inhibition and bloom control by ultrasonic irradiation. The effect of ultrasonic irradiation on cyanobacterial secondary metabolite toxins during the inhibition of cell growth and cell structure damage is also an important problem that should be studied. Such investigations are of great importance for cyanobacterial bloom control in lakes or water treatment in drinking water plants. However, no in-depth studies of the effects on cyanobacterial toxins in relation to ultrasonication are available.

Most of the previous studies on the effects of sonication on *Microcystis aeruginosa* utilized the change in intensity of chlorophyll fluorescence and chlorophyll concentration over time at different power settings as a measure of the effect on photosynthetic activity (Daly *et al.*, 2007; Zhang *et al.*, 2009). However, few

studies reported about the morphological and biochemical changes of *Microcystis aeruginosa* and *Anabaena flos-aquae* subjected to ultrasonic waves. The aim of the present study is to investigate the effect of ultrasonication using a frequency of 20 kHz on the morphology and cell viability of *Microcystis aeruginosa* and *Anabaena flos-aquae*. The study is carried out to evaluate the effect of ultrasonication on *Microcystis aeruginosa* and *Anabaena flos-aquae* and to determine the appropriate conditions in minimizing the release of toxins while rupturing gas vesicles and promoting the settling of cells.

MATERIALS AND METHODS

Cell culture and conditions: *Microcystis aeruginosa* and *Anabaena flos-aquae* were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences. An axenic isolate was established by streak plate isolation on a BG-11 agar medium and then grown in batch culture in a BG-11 medium.

Ultrasonic generators: Ultrasonic generators were purchased from the Institute of Acoustics, Chinese Academy of Sciences. Electric power output was 100 W.

Flow cytometric analysis: For quantitative apoptosis analysis, the prepared cells were washed twice with cold PBS and then treated with Annexin V-FITC and PI according to the manufacturer's instruction (KGA108, Key Gen Biotech, China). The samples were then analyzed by a flow cytometer (FACS).

Analysis of membrane integrity by acridine orange and propidium iodide: *Anabaena flos-aquae* cells were treated at different ultrasound frequencies (20 to 200 kHz) for 30 sec. The pellets were resuspended in 5 μL of Acridine Orange (AO) (1 mg mL^{-1}) and 5 μL of propidium iodide (PI) (1 mg mL^{-1}).

RESULTS AND DISCUSSION

Influence of the algal cell structure by ultrasonic wave: In this study, observation through a Transmission Electron Microscope (TEM) revealed the morphological changes of the *Microcystis aeruginosa* cells subjected to ultrasonic waves (20 and 200 kHz). The cell structure was found to be damaged during ultrasonication. Three random images were used during the treatment process to verify the efficacy of the ultrasonic wave. Figure 1a and b show the TEM images of the cells that were not treated by

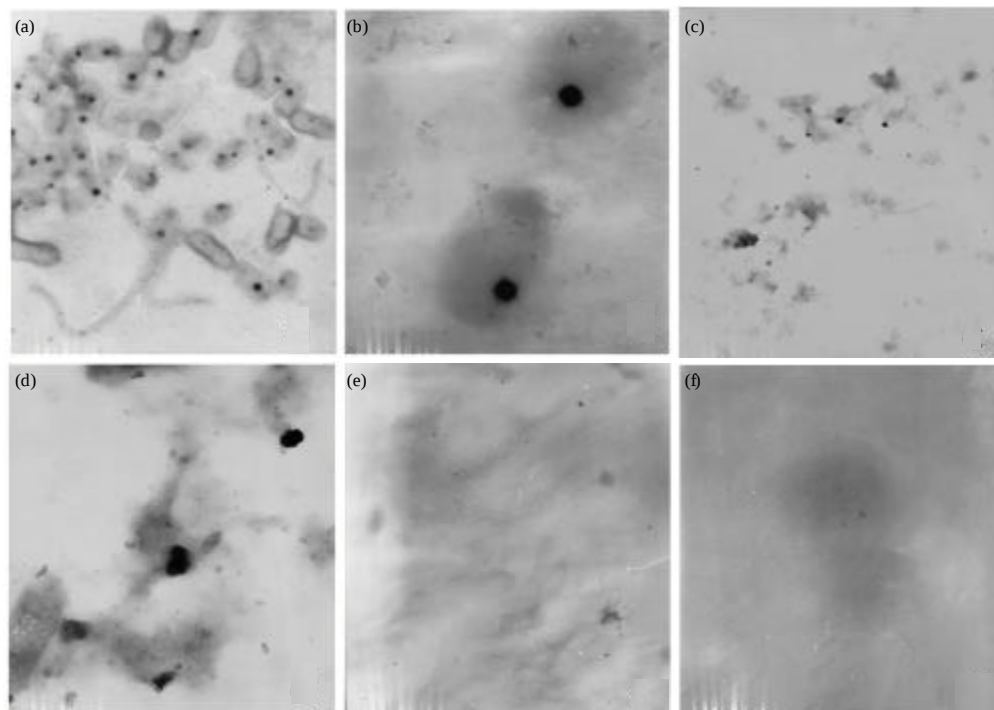


Fig. 1(a-f): TEM images of *Microcystis aeruginosa* treated with ultrasonic irradiation, (a, b) Control ($\times 10,000$), (c, d) Treated with ultrasonic irradiation at 20 kHz ($\times 10,000$) and (e, f) Treated with ultrasonic irradiation at 200 kHz ($\times 10,000$)

ultrasonic wave. *Microcystis aeruginosa* has gas vesicles, which are intracellular structures that provide the cells with buoyancy. These hollow, gas-filled structures keep the *Microcystis* cells close to the surface of the water body, which supplies optimal light and oxygen for growth. Thus, when the water column is stable, the colonies can accumulate at the water surface and then form surface water blooms. However, when cells were treated with a high frequency of ultrasonic wave (200 kHz), the cell membranes were fully disrupted into small pieces, releasing the intracellular components (Fig. 1e and f). The resulting solution could be easily collected by three-dimensional fluorescence spectrometry.

When *Microcystis aeruginosa* was exposed to ultrasonic wave (20 and 200 kHz) (Fig. 1c-e and f), tendencies of apoptosis or necrosis were observed. However, these effects were not observed in *Anabaena flos-aquae*. *Anabaena flos-aquae* is a multicellular, filamentous cyanobacterium. Under the TEM microscope, *Anabaena flos-aquae* appear as a blue-green filament that is composed of cylindrical cells arranged in branched helicoidal trichomes. These algae are motile and

glide along their axis. After ultrasonic irradiation, parts of the filaments were fractured (Fig. 2e and f), indicating damage and cell death. TEM microscopy provided the detailed structure of the cell surface. After ultrasonication, more bright and dark spots appeared on the cell surface. Bumps and holes appeared in the cell surface and the cell edge was indistinct. All these features are in contrast with those of the control (Fig. 2a). The findings indicate that the cells were damaged and their proliferation was therefore prevented. *Anabaena flos-aquae* cells also exhibited a tendency to form pseudocolonies, which are small fragments of decentralized cells (Fig. 2c and e). This effect was present throughout the entire period of the experiment and was more evident when the organisms were treated with a higher frequency of ultrasonic wave (200 kHz).

Effect of ultrasonic irradiation on *Microcystis aeruginosa* cell viability: FACS results showed that the percentages of apoptotic and necrotic cells were $0.82 \pm 0.03\%$ (Fig. 3a), $3.99 \pm 0.05\%$ (Fig. 3b) and $8.98 \pm 0.78\%$ (Fig. 3c) for the untreated

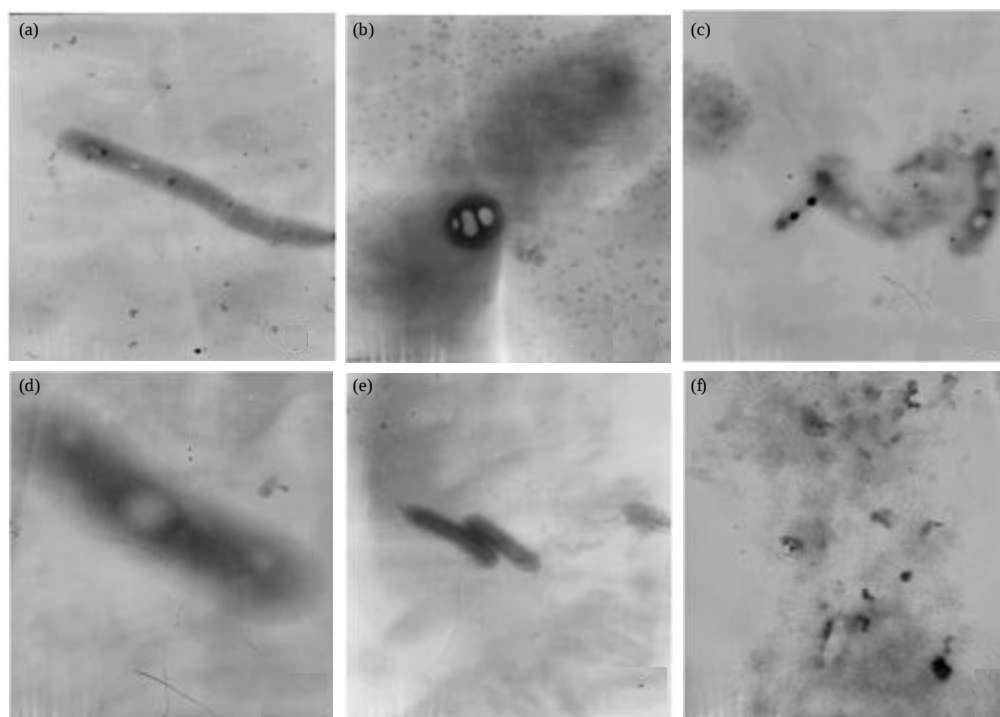


Fig. 2(a-f): TEM images of *Anabaena flos-aquae* treated with ultrasonic irradiation, (a) Control ($\times 10,000$), (b) Control ($\times 40,000$), (c) Treated with ultrasonic irradiation at 20 kHz ($\times 10,000$), (d) Treated with ultrasonic irradiation at 20 kHz ($\times 40,000$), (e) Treated with ultrasonic irradiation at 200 kHz ($\times 10,000$) and (f) Treated with ultrasonic irradiation at 200 kHz ($\times 40,000$)

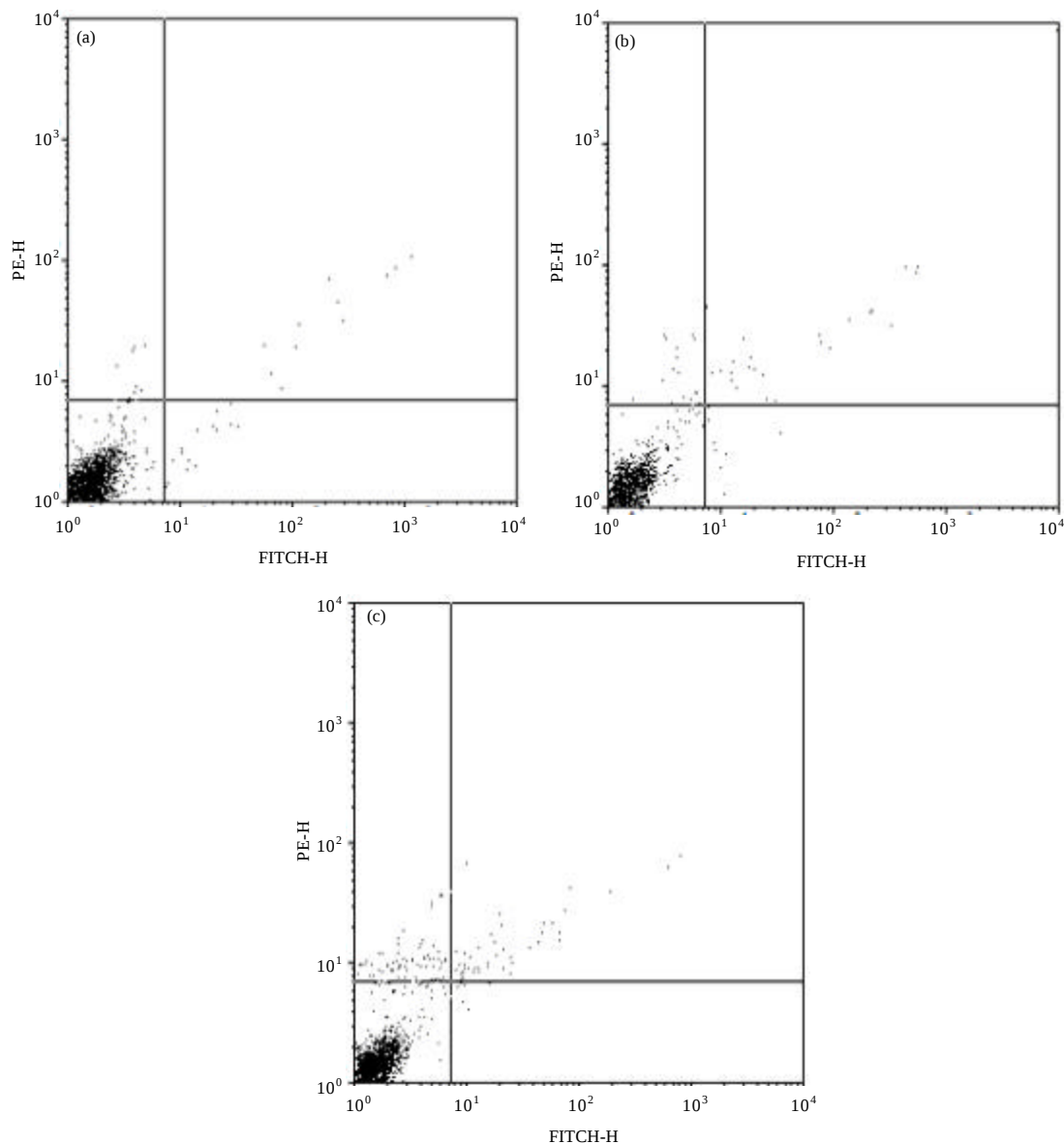


Fig. 3(a-c): Effect of ultrasonic irradiation on *Microcystis aeruginosa* cells labeled with Annexin-V using FACS analysis, (a) Control, (b) Cells treated with 20 kHz and (c) Cells treated with 200 kHz

cells and cells treated with ultrasonic waves of 0, 20 and 200 kHz, respectively. With the increase of ultrasonic frequency, the percentage of necrotic cells gradually rose. However, the apoptotic cells treated with 20, 40, 60 and 100 kHz of ultrasonic waves were relatively stable. When the frequency increased to 200 kHz, the percentage of apoptotic cells rose to 4.4% and the highest rate of cell death was recorded.

Anabaena flos-aquae cell death induced by ultrasonic irradiation as determined by AO/PI staining: Apoptotic, necrotic and viable *Anabaena flos-aquae* cells were scored by using the AO/PI fluorescence microscopy. The control cells (untreated) were also analyzed for comparison (Fig. 4a). Early apoptosis was indicated by the intercalated AO within the fragmented DNA. In contrast, untreated cells showed a green intact nuclear structure. Blebbing, nuclear breakage and moderate apoptosis were

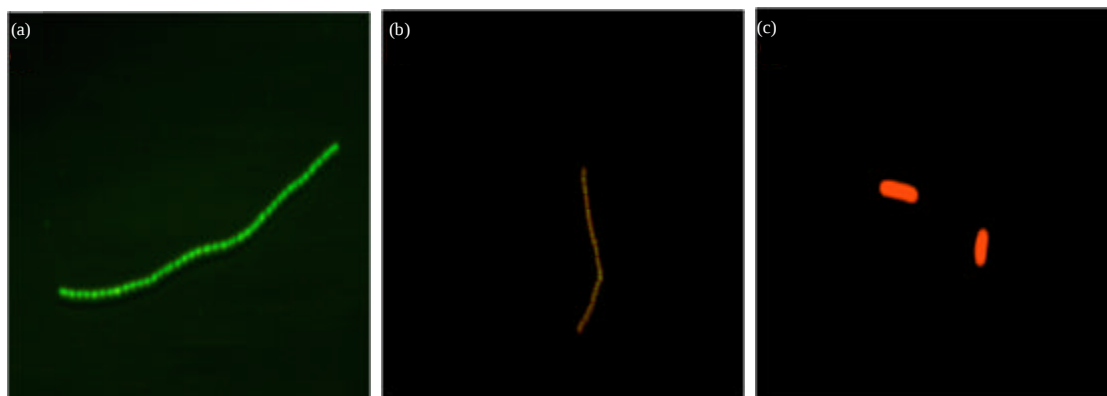


Fig. 4(a-c): Morphological changes of *Anabaena flos-aquae* cells following ultrasonic irradiation treatment as viewed under the fluorescence microscope after staining with AO/PI, (a) Control, (b) Cells treated with 20 kHz and (c) Cells treated with 200 kHz

noticed in the cells during treatment with ultrasonic waves (Fig. 4b). In the late stages of apoptosis, apoptotic body separation and the presence of reddish-orange color due to the binding of AO to denatured DNA were observed (Fig. 4c).

CONCLUSION

In conclusion, the optimal frequency of the ultrasonic wave for *Microcystis aeruginosa* and *Anabaena flos-aquae* was 20 kHz. In this frequency, the extracellular structures were destroyed, but the intracellular structure remained relatively intact. The thylakoid and simulated nuclear substance were also observed. Upon treatment with 20 kHz of ultrasound, the mortality rate of *Microcystis aeruginosa* was 3.99%, with an apoptosis rate of 2.73%. The current results indicate that *Microcystis aeruginosa* cells respond to 20 kHz of ultrasonic-induced stress through apoptosis or, ultimately, through necrosis. At the same time, this frequency keeps the cell structure intact, thereby preventing a large release of cyanobacterial toxins. The response was observed to be highly dependent on the frequency of the ultrasound. Ultrasonication at 200 kHz damaged the intracellular structure, indicating a high possibility of cyanobacterial toxin release.

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