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ISSN 0792 - 156X

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PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -  
Kibbutz Ein Hamifratz, Mobile Post 25210,  
ISRAEL

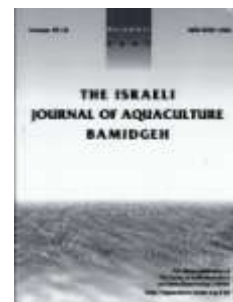
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## **Evaluation of Berberine's Algicidal Effects on Toxic *Microcystis aeruginosa* Growth using a Double Fluorescein Staining Method**

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(Received 19.12.12, Accepted 29.3.13)

**Key words:** *Microcystis aeruginosa*, fluorescein diacetate, propidium iodide, berberine

### **Abstract**

An accurate and convenient method for determining *Microcystis* cell viability is necessary to control blooms in small aquaculture water bodies. The fluorescein diacetate-propidium iodide (FDA-PI) staining method was developed to determine the viability of toxic *Microcystis aeruginosa* (FACHB-905). Live *M. aeruginosa* 905 cells, stained with FDA, emitted bright green fluorescence after excitation at 470 nm. Dead cells, stained with PI, produced bright red fluorescence after excitation at 540 nm. Berberine inhibited *M. aeruginosa* 905 growth and the inhibition rate determined by FDA-PI fluorescein staining and counting on a dark field was much higher than when determined by counting on a bright field because some berberine-killed cells were misidentified as live cells. Thus, less berberine is needed to control *Microcystis* bloom when the accurate and reliable FDA-PI fluorescein staining method is used to judge the viability of the algae cells.

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## Introduction

*Microcystis aeruginosa* is a harmful bloom-forming cyanobacterium. *Microcystis* blooms occur in small enclosed water bodies such as fishponds and in large water bodies such as reservoirs and rivers. Outbreaks of *Microcystis* blooms in fishponds always lead to water deoxygenation and fish kills. Physical, chemical, and biological measures have been proposed to control *Microcystis* blooms. Because they have specific biodegradable characteristics, plant allelopathy is an environmentally friendly method for controlling blooms in small enclosed water bodies (Nakai et al., 2006; Park et al., 2006).

Berberine can inhibit the growth of *M. aeruginosa* (Zhang et al., 2010; Dai et al., 2013). However, in previous studies, algicidal activity was not evaluated using berberine-killed algae cells because a reliable and convenient viability test for *M. aeruginosa* cells had not been developed. In algicidal assays, using inaccurate algal viability tests leads to the waste of allelochemicals or incomplete algicidal effects. For the practical control of blooms in small aquaculture water bodies, an accurate and convenient method for determining *Microcystis* cell viability is important for the economical and efficient application of berberine.

Algae viability tests using chemical dyes (Capasso et al., 2003; Tripathi et al., 2006) involve time-consuming staining, limited counting time, and subjective identification. Fluorescent dyes are more reliable indicators of cell viability than traditional colored dyes. Among the most frequently used fluorescent dyes are fluorescein diacetate (FDA) and propidium iodide (PI), often used together (Saha et al., 2003; Tsai et al., 2010), to determine viability of marine and freshwater microalgae (Franklin et al., 2001).

In this study, a double staining method using FDA and PI was developed to evaluate the viability of *M. aeruginosa* (FACHB-905) and the algicidal effects of berberine on *M. aeruginosa* 905.

## Materials and Methods

**Algae culture.** *Microcystis aeruginosa* (FACHB-905) was provided by the Institute of Hydrobiology, Chinese Academy of Sciences. Algae were cultured in conical flasks containing sterilized BG11 medium under a 12 light:12 dark cycle with a light density of 54  $\mu\text{mol}/\text{m}^2/\text{s}$  at 25°C. Cultures were repeated until the algae reached the exponential growth phase.

**Algae cell suspensions.** Suspensions of live cells were prepared as follows by centrifuging 1 ml algae at 4000 rpm for 5 min and resuspending the cells in 0.2 mol/l phosphate buffered saline (PBS; pH 7.4). To prepare dead cells, the same volume of 4% paraformaldehyde fix was added to live cell suspensions for 20 min to fix the cells; the fixed cells remained intact but lost viability. The suspensions were centrifuged at 4000 rpm for 5 min, the fixed cells were washed with 0.2 mol/l PBS three times to remove residual fix, the cells were resuspended, and the resuspension was adjusted to the necessary density. The mixture of live and dead cells was prepared at a ratio of 1:1.

**Fluorescence staining and cell counting.** An FDA working solution (5 mg/ml) was prepared when needed by dissolving FDA (Sigma) in reagent grade acetone. FDA was added to live cell suspensions and the final concentration was adjusted to 100  $\mu\text{g}/\text{ml}$ , mixed, and reacted with algae cells for 10 min in the dark before observing and taking micrographs under a fluorescence microscope (Nikon Ti-s, Japan) with a hemocytometer. The micrographs were taken in a bright field without fluorescence excitation as well as in a dark field with fluorescence excitation at 470 nm, and the number of algae cells was counted. A PI stock solution (10 mg/ml) was prepared by dissolving PI (Sigma) in deionized water and storing it at -20°C until use. A PI working solution (1000  $\mu\text{g}/\text{ml}$ ) was added to dead cell suspensions, and the final concentration of PI was adjusted to 60  $\mu\text{g}/\text{ml}$ . The cells were stained and counted as above except that PI-stained dead cells were excited at 540 nm in a dark field. The staining rate was calculated as no. cells counted in dark field/no. cells counted in bright field.

For double staining, final concentrations of FDA and PI were adjusted to 100  $\mu\text{g}/\text{ml}$  and 60  $\mu\text{g}/\text{ml}$ , respectively. In the dark field, two micrographs were taken of the doubly-stained mixture cells after excitation at 470 and 540 nm; the two micrographs were

synthesized into one with image processing software (NIS-Elements BR). Only one micrograph was taken in the bright field.

**Algicidal effects of berberine on *M. aeruginosa*.** Berberine (Northeast General Pharmaceutical Factory, China) was dissolved in heated distilled water to prepare a stock solution (10%, w/v) that was stored at 4°C until use. Berberine was added to algae cultures ( $1.0 \times 10^6$  ind/ml) to concentrations of 0.0000% (control), 0.0005%, 0.0010%, 0.0015%, and 0.0020% (w/v) with three replicate flasks per concentration. Samples were removed from the cultures at 24-h intervals (0, 24, 48, 72, 96 h) and stained with FDA-PI fluorescein. The number of cells was counted after 470 nm excitation in a bright field and 540 nm excitation in a dark field. To reduce effects related to minor differences in photon irradiance, the flasks were slightly shaken three times each day and randomly rearranged.

## Results

**Fluorescence staining rates.** The staining rate of FDA on live algal cells at the observed densities reached up to 96% while the staining rate of PI on fixed (dead) cells reached 100% (Table 1). The selected excitation wavelength for the FDA-stained cells was 470 nm because it produced a bright green fluorescence that was easy to count because of the clear-cut contours of the cells (Fig. 1). When excited at 540 nm, the PI-stained fixed cells emitted a bright red fluorescence (Fig. 2). The mixture of live and fixed cells, stained with FDA-PI, emitted a bright green and a red fluorescence, respectively (Fig. 3).

Table 1. Staining rates (%) of two fluorescent dyes on *Microcystis aeruginosa* 905 (means±SD).

Dye	Algae density (ind/ml)		
	$5.5 \times 10^5$	$1.1 \times 10^6$	$2.2 \times 10^7$
Fluorescein diacetate	96.43±3.62	97.34±2.09	98.91±0.74
Propidium iodide	100.00±0.00	100.00±0.00	100.00±0.00

**Algicidal effects of berberine.** Under the bright field, the density of *M. aeruginosa* without berberine treatment increased with culture time and decreased with the increase of berberine concentration and exposure time. Under the dark field with fluorescence excitation, a same time-dependent increase in *M. aeruginosa* density was observed in the control while algae densities in berberine-treated groups were lower than those determined in a bright field (Fig. 4). Determination of the inhibitory effect of berberine using a dark field and FDA-PI staining was much more accurate than when using a bright field, as some berberine-killed cells were misidentified as viable cells.

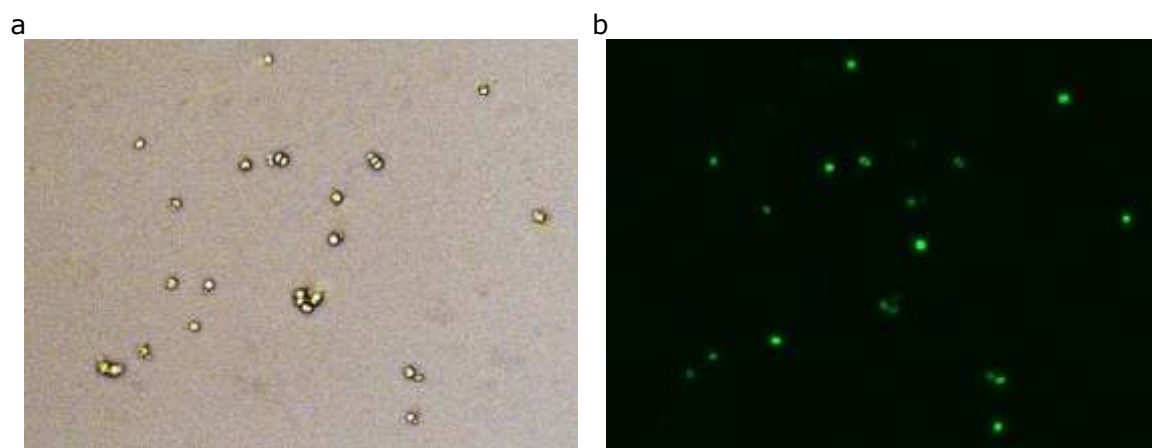


Fig. 1. Micrographs of live *Microcystis aeruginosa* 905 cells stained by fluorescein diacetate (FDA) and photographed in (a) bright and (b) dark fields.

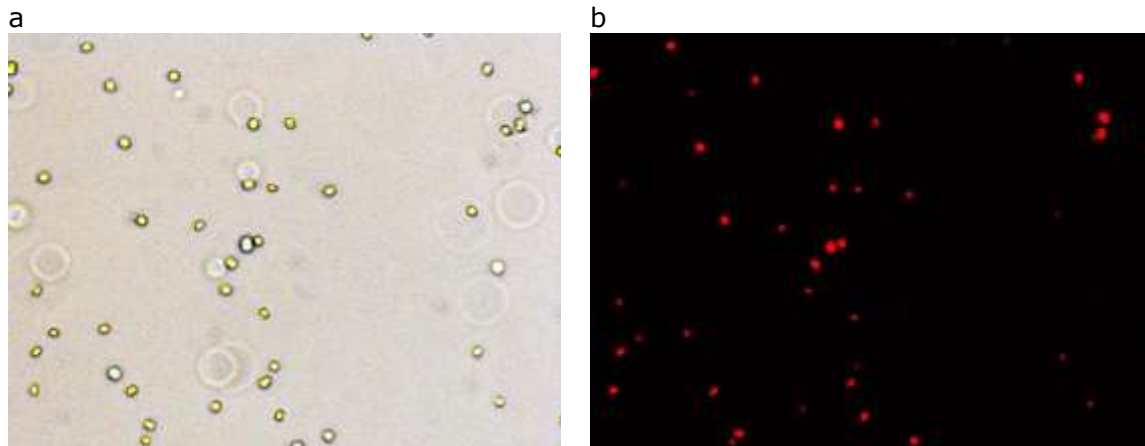


Fig. 2. Micrographs of dead *Microcystis aeruginosa* 905 cells stained by propidium iodide (PI) and photographed in (a) bright and (b) dark fields.

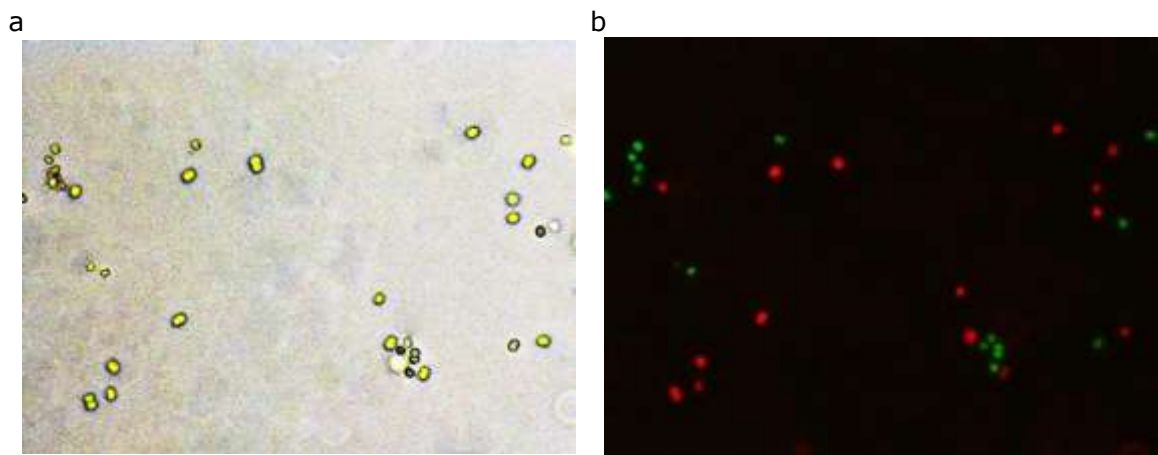


Fig. 3. Micrographs of a mixture of live and dead *Microcystis* 905 cells stained with fluorescein diacetate and propidium iodide (FDA-PI) and photographed in (a) bright and (b) dark fields.

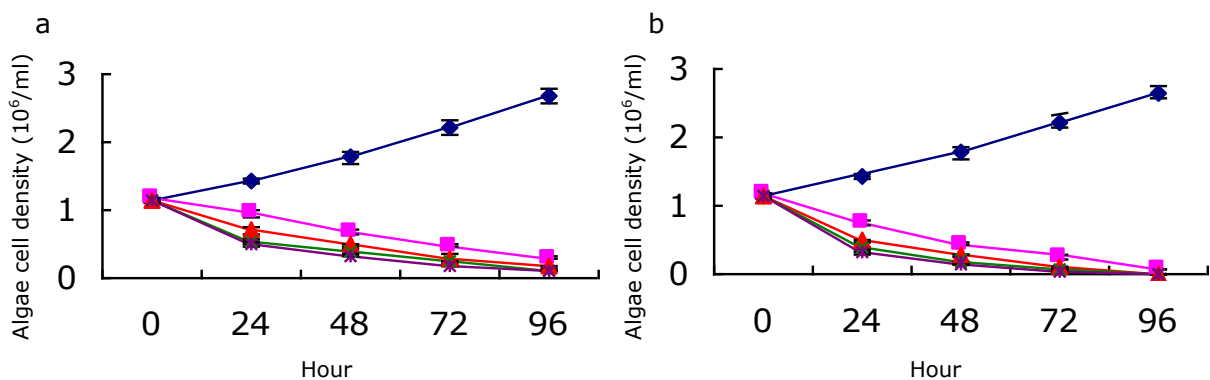


Fig. 4. Algicidal effects of berberine on *Microcystis aeruginosa* 905 observed under (a) bright field without fluorescence excitation and (b) dark field with fluorescence excitation.   
 ◆ 0.0000%    ■ 0.0005%    ▲ 0.0010%    × 0.0015%    \* 0.0020%

### Discussion

The inhibitory effect of berberine on *M. aeruginosa* 905 was better determined by counting FDA-PI fluorescein excited cells in a dark field than in a bright field because some berberine-killed cells remained intact and were miscounted as live cells in the

bright field. Thus, if the FDA-PI fluorescein staining method is used to judge algae viability, less berberine can be used to control *M. aeruginosa* blooms and the costs of the algicide can be reduced.

PI is a fully cell-membrane impermeable fluorescent dye that combines with DNA in cells after their death due to a loss of membrane integrity (Ormerod et al., 1993). FDA readily penetrates the membranes of viable cells, being hydrolyzed via non-specific esterases, and producing green fluorescence (Dorsey et al., 1989; Saha et al., 2003). Some intact cells in the bright field emitted bright red fluorescence in the dark field, suggesting that these cells had been killed and lost membrane integrity. Other intact cells observed in the bright field were not observed in the dark field because PI failed to stain them, indicating that DNA in these cells lost the ability to combine with PI. Thus, both broken cell membranes and disrupted DNA combination sites before cell lysis may be involved in the algicidal mechanism of berberine. This needs further investigation.

### Acknowledgements

The financial support provided by the National Natural Science Foundation of China (Grant Nos. 31170442 and 31300393), Natural Science Foundation of Tianjin (Grant No. 10JCZDJC18000), and Science and Technology Development Fund of Colleges and Universities in Tianjin (Grant No. 20120624) is gratefully acknowledged.

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