

A flow cytometric technique to assess viable and membrane compromised cells of *Microcystis aeruginosa* following exposure to a biological control agent: *Bacillus mycooides*

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Abstract

We report a flow cytometric (FCM) technique to assess viable and membrane compromised cells of *Microcystis aeruginosa* following exposure to a biological control agent *Bacillus mycooides*. Using copper and *B. mycooides* treated samples, the majority of *Microcystis* cells were membrane-damaged cells. This was further confirmed by microscopic studies. In the control samples, the majority of cells had intact membranes and a functional esterase activity. The biological control agent *B. mycooides* appears to cause *Microcystis* cell lysis resulting in cells with compromised membranes with a FCM cytogram signature equivalent to a copper generated cytogram. *B. mycooides* could therefore be considered as biological control agent for the control of harmful algal blooms dominated by *Microcystis*.

Keywords: *Microcystis aeruginosa*, Flow cytometry, esterase activity, biological control, *Bacillus mycooides*.

INTRODUCTION

The technique of flow cytometry coupled with use of fluorogenic probes is now well developed and is applied to the counting and viability assessment of aquatic microorganisms and cyanobacteria in particular (Brookes *et al.*, 2000; Franklin *et al.*, 2004; Latour *et al.*, 2004; Regel *et al.*, 2004; Regel *et al.*, 2002; Phinney *et al.*, 1989). Flow cytometry is a rapid, sensitive and precise technique that is used to count thousands of cells per second as they are carried within a fast moving fluid that passes a focused light beam (Franklin *et al.*, 2004).

Fluorescence emission and excitation characteristics are used to distinguish cyanobacteria with different sub-populations (heterogeneous) and from other microorganisms such as bacteria based on accessory pigments (Franklin *et al.*, 2004). Thus flow cytometry targets populations of interest and rapidly measures different optical signals as morphological parameters (side scatter and forward scatter) (Latour *et al.*, 2004). This is used to distinguish cyanobacteria between sub-populations and other microorganisms. This has led to the development of a tool to quantify viability in phytoplankton, in particular *Microcystis* following exposure to different environmental stress factors such as nutrient limitation (Brookes *et al.*, 2000), nutrient enrichment (Latour *et al.*, 2004), copper toxicity (Franklin *et al.*, 2004), turbulence (Regel *et al.*, 2004), acid mine drainage exposure (Regel *et al.*, 2002), ultrasonic irradiation (Lee *et al.*, 2000), viral (Brussaard *et al.*, (2001) and bacterial infection (this study).

The viability of *Microcystis* cells was assessed by flow cytometric analysis of two cellular functions, i.e. esterase activity and membrane integrity, after staining with fluorescein diacetate (FDA) and propidium iodide (PI) respectively. FDA diffuses across cells with intact membranes due to esterase activity. However once within active cells, the FDA substrate is cleaved by non-specific esterases releasing a polar fluorescein product that is retained inside cells with an intact membrane and the cells are stained green (Lebaron and Joux, 2000).

Propidium iodide (PI) is a polar substance that easily penetrates only inactive or damaged cell membranes. Once inside the cell, PI binds to double strand nucleic acids with intercalation and fluoresces bright red under blue light excitation (Yamaguchi and Nasu, 1997).

We report the use of a flow cytometric (FCM) technique to assess viable and membrane compromised cells of *Microcystis aeruginosa* following exposure to a potential biological control agent: *B. mycooides*.

MATERIALS AND METHODS

Host cyanobacteria

Batch cultures of *Microcystis aeruginosa* PCC7806 were grown in modified BG 11 medium (Krüger and Eloff, 1977) at room temperature and under continuous light (2000 lux) and shaking, 80rpm. Cultures were used when in an exponential growth phase (10 d). The cyanobacterial suspensions were then used as prey in the subsequent experiments.

Predatory bacteria

The bacteria *B. mycooides* was isolated from the eutrophic water of Hartbeespoort dam, South Africa. An inoculum of bacterial stock was incubated in 100ml nutrient broth on a Labcon orbital shaker, 128 rpm at 25°C for 24 h. The bacterial suspension was centrifuged (10,000 rpm, 25°C, 10 min) and washed once with quarter Ringer's solution. The pellet was re-suspended in minimum Ringer's solution and was stored at 4°C until further use.

Experimental design

The bacterial pellet was suspended in Ringer's solution (1ml) and added to 2ml of *Microcystis* suspension and incubated under the same conditions as host cyanobacteria for 25 d but no shaking. For controls, 1ml of Ringer's solution was added to 2ml of *Microcystis* suspension and incubated as above.

Exposure to copper sulphate solutions

For positive controls, 1ml of copper sulphate (10mg/ml) was added to 2ml of *Microcystis* suspension and incubated as above.

Preparation of cytometric stains

The FDA staining technique for *Microcystis aeruginosa* developed by Brookes *et al.* (2000) was followed in this study. An FDA (Sigma Chemicals F7378) stock solution was prepared by dissolving (50mg FDA) in 5ml reagent grade acetone and stored in the dark at -20°C until further use. Esterase activity was determined by adding 100µl of FDA working solution (80 µg/ml) to 100µl of a *Microcystis* sub sample in a 10ml centrifuge tube and incubated at room temperature for 7 min in the dark. The final FDA concentration was 40µM. The stained sample was now ready for flow cytometric analysis.

A procedure similar to that of Ross *et al.* (1989) and Franklin *et al.* (2001) was followed in the development of a PI staining technique for *M. aeruginosa*. A PI (Sigma Chemicals 81845) stock solution was prepared by dissolving (25mg PI) in 5ml distilled water and was stored at 4°C until further use. Cell viability was determined by 100µl of PI working solution (40µg/ml) to 100µl of *Microcystis* sub sample in a 10ml centrifuge tube and incubated at room temperature for 1 min. The final PI concentration was 20µM. The stained sample was now ready for flow cytometric analysis.

Flow cytometric analysis

An Epics ALTRA Beckman Coulter flow cytometer (excitation: argon laser 15 mW, 488 nm) with the standard filter set up was used. The *Microcystis* cells crossed the laser beam and short flashes of their fluorescence and scattered light were emitted through a set of filters onto photomultipliers (PMT). The green fluorescein fluorescence was measured in channel B (PMT 2 log, 553 voltage) and red PI fluorescence was measured in channel D (PMT 4 log, 740 voltage).

The *Microcystis* cells were distinguished from other particles by gating on two parameter plots of forward scatter (FSC) indicative of cell size and positive chlorophyll *a* red autofluorescence (630nm). Approximately 10,000 events or 300 voltages (which ever came first) were used in recording of flow cytometric data. The forward and side light scatter signals were used to derive 2-parameter cytograms,

which clearly defined the two sub populations of cells, subsequently defined as membrane compromised and non-viable.

Scanning Electron Microscopy

The procedure developed by Laboratory of Microanalysis and Microscopy, University of Pretoria was followed in the preparation of samples for SEM.

RESULTS AND DISCUSSION

Effect of Ringer's solution on *Microcystis* cells

The dual but separate staining of *Microcystis* cells revealed two sub-populations: living (viable), compromised and dead cells (non-viable) (Figure 3). After 25 d of incubation the percentage of gated non-treated (controls) cells was greater for FDA (negative 95% and standard controls 44%) (Figure 1a and b) than for PI (negative 12% and standard controls 1%)(Figures 1c and d). This may indicate that the majority of *Microcystis* cells were viable (intact membrane and an active esterase). Therefore Ringer's solution appears not to have an effect on *Microcystis* cells. The microscopic studies confirmed that presence of intact *Microcystis* membranes (Figure 4c).

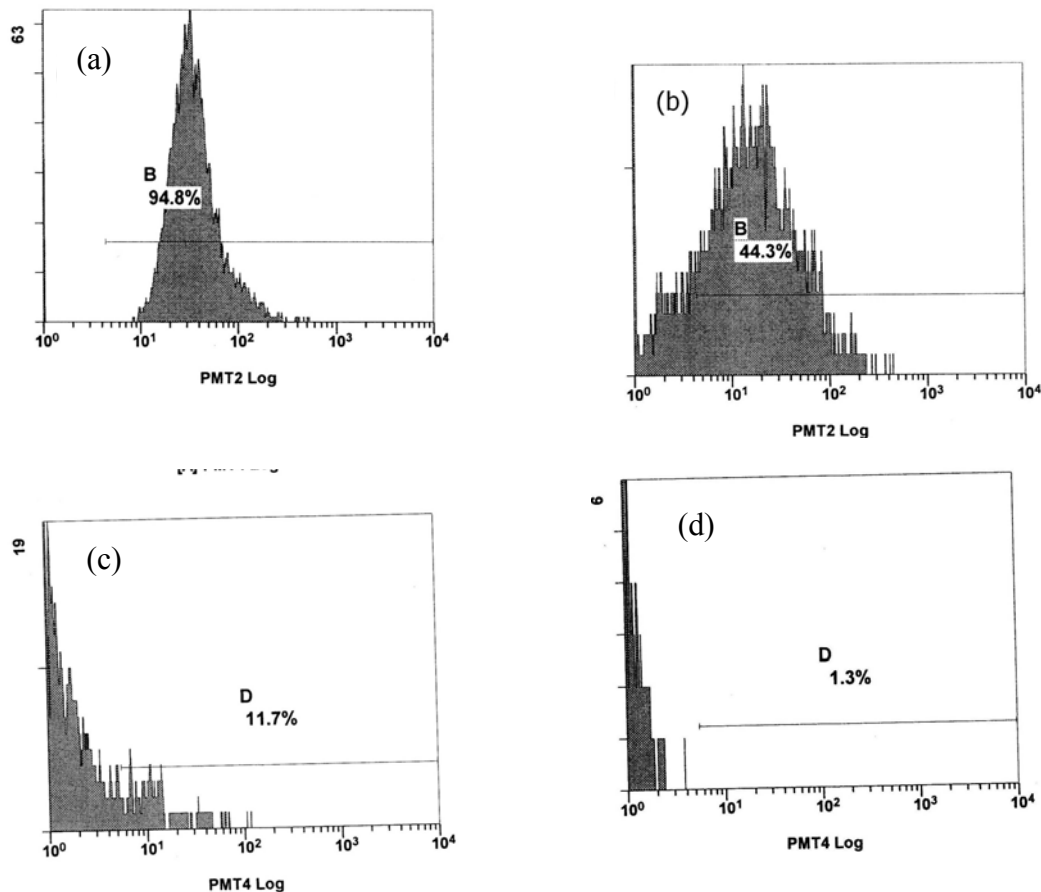


Figure 1: Gating of viable *Microcystis* cells: (a) FDA staining negative control samples with no saline, (b) FDA staining standard control samples with saline solution. Gating of membrane compromised cells: (c) PI staining negative control samples with no saline, (d) PI staining standard control samples with saline solution.

Effect of *B. mycoides* and copper on *Microcystis* cells

In bacteria treated samples, the percentage of gated cells was greater 81% (Figure 2a) than copper treated cells 58% (Figure 2b) for PI staining. In the case with FDA staining, for bacteria treated samples, percentage of gated cells was greater 27% than copper treated cells 2% (Figure 2d). Therefore in bacteria treated samples the majority of *Microcystis* cells (81% versus 27%) were non-viable (compromised or leaking membrane and less active esterase). The same is true with copper treated samples.

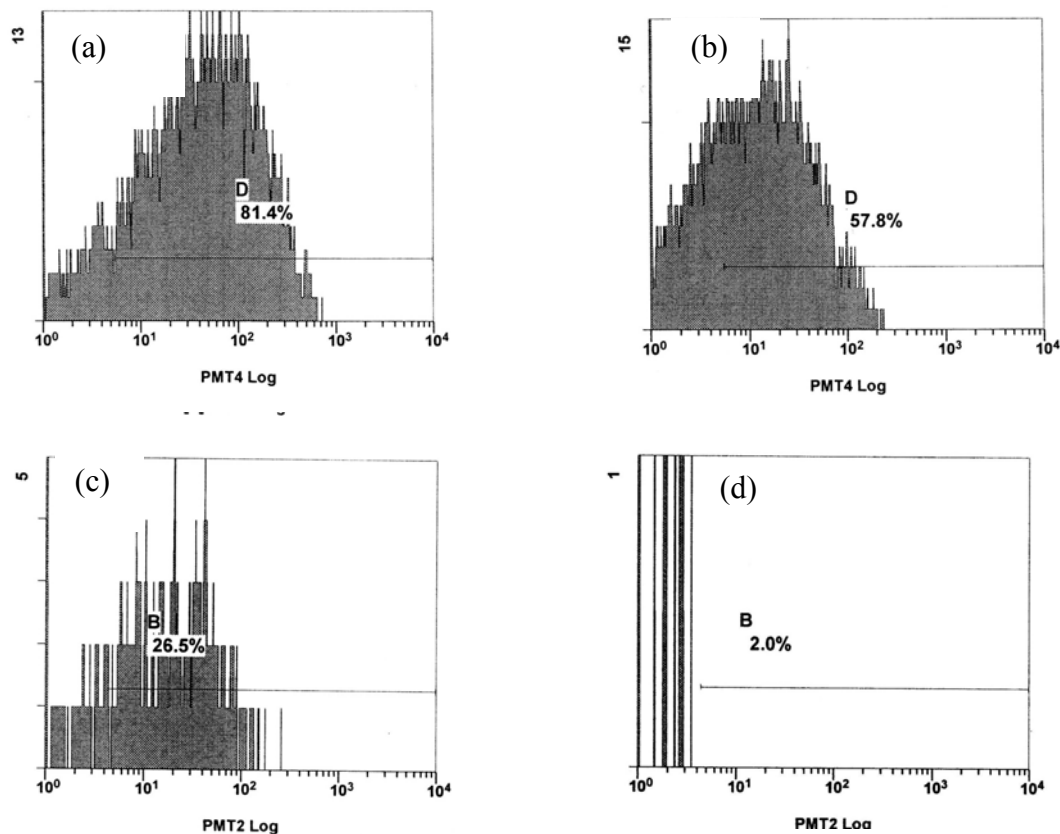


Figure 2: Gating of membrane compromised *Microcystis* cells after PI staining: (a) treated with *B. mycoides* and (b) treated with copper solution. Gating of viable *Microcystis* cells after FDA staining: (c) treated with *B. mycoides* and (d) treated with copper solution.

For copper and *B. mycoides* treated samples, the cytograms indicated the percentage of *Microcystis* cells that were membrane compromised (Figures 2a and b). The FCM cytograms for (copper and bacteria) treated *Microcystis* cells showed an increased cell granularity/complexity, which was different from the cytogram of control samples (Figures 3a and b). The microscopic studies confirmed the presence of damaged *Microcystis* cells (Figures 4a and b).

Based on *Microcystis* cell size and red autofluorescence, the FCM was able to distinguish green fluorescein fluorescence of bacterial cells from that of cyanobacteria. The technique was able to distinguish the cyanobacterial red autofluorescence and red PI fluorescence.

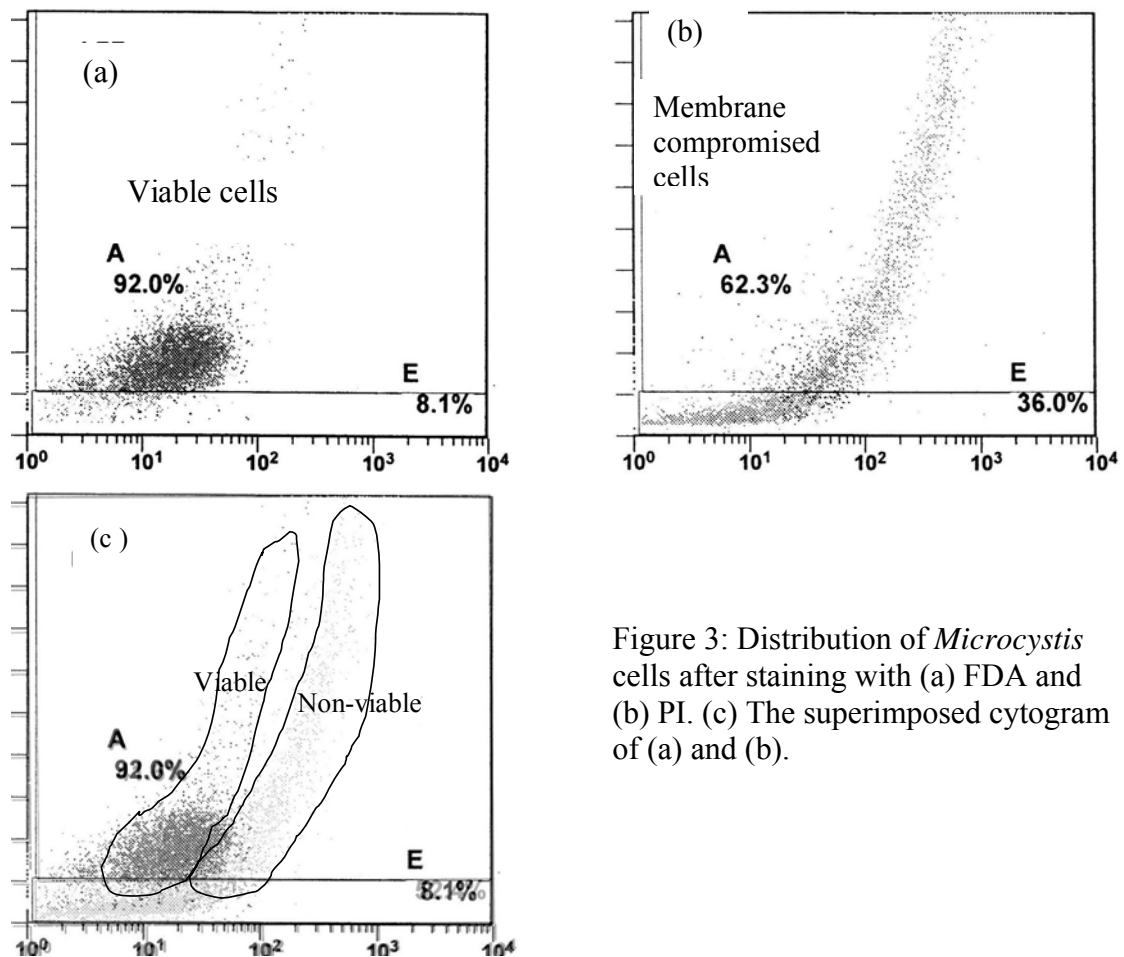


Figure 3: Distribution of *Microcystis* cells after staining with (a) FDA and (b) PI. (c) The superimposed cytogram of (a) and (b).

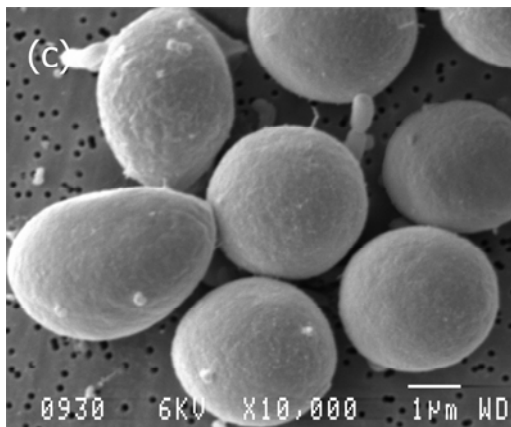
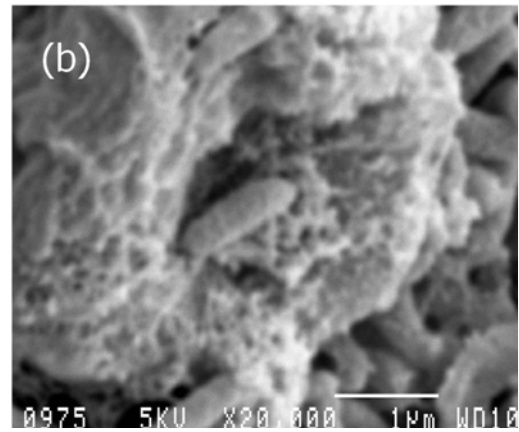
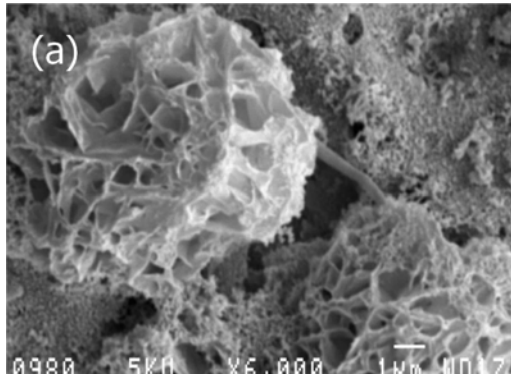


Figure 4: SEM indicating the morphological changes to *Microcystis* cell membrane (a) treated with copper solution, (b) treated with *B. mycooides* and (c) control samples showing no visible damage.

Conclusions

Flow cytometry was used to successfully to assess viable and membrane compromised *Microcystis* cells. The biological control agent *B. mycooides* (suspended in Ringers' solution) caused *Microcystis* cell lysis resulting in cells with compromised membranes with a FCM cytogram signature equivalent to a copper generated cytogram. The study highlights the potential of *B. mycooides* as a biological control agent for the control of harmful algal blooms dominated by *Microcystis*.

Acknowledgements

We are indebted to National Research Fund of South Africa for funding this research program and provision of scholarship through the Grant Holder Linked scheme to Mr JR Gumbo. We also thank Mr A. Hall for assistance with electron microscopy, Prof R. Anderson, Dr R. Cockeran and Dr H. Steel for technical assistance with flow cytometry and Mr T Downing of Nelson Mandela Metropolitan University (formerly University of Port Elizabeth) for the provision of *Microcystis aeruginosa* PCC7806.

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