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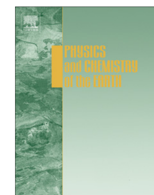

The viability assessment of *Microcystis aeruginosa* cells after co-culturing with *Bacillus mycoides* B16 using flow cytometry

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ABSTRACT

Microcystis aeruginosa is the dominate cyanobacteria in freshwater bodies causing proliferation of toxic harmful algal blooms (HABs), worldwide. Thus a biological control method based on predatory bacteria is an alternative environmental solution to the control of these HABs, A Flow cytometric technique was used to assess the viability of *Microcystis* spp. cells after deliberate co-culturing with a predatory bacterium, *Bacillus mycoides* B16. Under static conditions, *B. mycoides* had a lytic effect on *Microcystis* cells that resulted in a significant ($p = 0.0000$) population decline of 97% in six days. In contrast under turbulent conditions, *B. mycoides* had a lytic effect on *Microcystis* spp. cells resulting in a significant ($df = 5$; $t = -7.21$; $p = 0.0003$) population decrease of 85% in the same time period. The Levene test also showed a significant ($p = 0.0003$) decrease in *Microcystis* cell numbers, which also coincided with a significant ($t = 11.31$; $p = 0.0001$) increase in *B. mycoides* cell numbers. This suggested that *B. mycoides*, a heterotroph, was utilizing the *Microcystis* as a source of nutrition. The effect of agitation may have contributed to the delay in cell lysis as it disturbed the physical contact between the predator and prey. The control samples showed a significant ($df = 5$; $t = +6.86$; $p = 0.0010$) increase in *Microcystis* spp. cell numbers. *B. mycoides* was able to lyse *Microcystis* spp. cells under these conditions and may thus be considered as a potential biological control agent for the management of *Microcystis* spp. harmful algal blooms.

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1. Introduction

Most harmful algal blooms (HAB) in freshwater ecosystems are dominated by *Microcystis aeruginosa*, a cyanobacterium that produces potent microcystins that have been implicated in the deaths of humans, wild and domestic animals and microalgae and conventional water treatment processes are unable to remove the microcystins in the drinking waters (Sigee et al., 1999; de Figueiredo et al., 2004; Oberholster et al., 2009). Thus the control and management of HABs is important and may involve a number of measures each with its unique challenges (Hadjoudja et al., 2009; Chang et al., 2014). The control methods have been applied by water regulatory bodies in management of HABs with varying levels of success (Sigee et al., 1999). The use of biological control agents, bacteria, protozoa, fungi and virus have been attempted but are confined to laboratory studies (Burnham et al., 1981; Choi et al., 2005; Gumbo et al., 2010; Kang et al., 2012). Part of this challenge is to understand the actual dynamics between the microbial agent and cyanobacteria and to develop new tools to further elucidate the microbial agent/cyanobacteria interactions.

Other researchers such as Burnham et al. (1984), Nakamura et al. (2003) and Kang et al. (2012) have evaluated the lytic activity of predator bacteria on the reduction of *Microcystis* cells based on chlorophyll *a* (Chl-*a*) and cyanobacteria cell counts. Nakamura et al. (2003) initially used the criteria of Chl-*a* analysis with varying levels of success. The Chl-*a* method revealed that there were no differences in lytic activities between the bacteria treatments and controls. Closer inspection with light microscopy revealed that *Bacillus cereus* N14 lysed *Microcystis* spp. cells and that the bacteria did not degrade the Chl *a* moiety, hence the discrepancy in the Chl-*a* results (Nakamura et al., 2003). Daft and Stewart (1971) revealed a similar pattern of non-degradation of heterocysts by the bacterium CP-1. The heterocysts, a centre for oxygenated photosynthesis and nitrogen fixation, also contain Chl-*a* moiety. Thus an alternative method to assess to the reduction or death of *Microcystis* spp. cells based on cyanobacteria's metabolic and physiological

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status after exposure to a stressor is required. Flow cytometry is such a method and has been used to assess viability of *Microcystis* spp. 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; Hadjoudja et al., 2009), turbulence (Regel et al., 2004), acid mine drainage exposure (Regel et al., 2002), ultrasonic irradiation (Lee et al., 2000) and viral infection (Brussaard et al., 2001).

The viability of *Microcystis* spp. cells was assessed by flow cytometric analysis of two cellular functions, i.e. esterase activity and membrane integrity, after staining with fluorescent diacetate (FDA) and propidium iodide (PI) respectively. FDA is a cell-permeant molecule which diffuses in all cells, but is only cleaved in cells with intact esterase. 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 fluoresces green under blue light excitation (Joux and Lebaron, 2000). The intensity of the fluorescence will invariably increase over time depending on the metabolic status of active esterases. Propidium iodide (PI) is a polar substance that easily penetrates only damaged cell membranes. Once inside the cell, PI binds to double strand nucleic acids by intercalation and fluoresces bright red under blue light excitation (Yamaguchi and Nasu, 1997). The main objective of this study was to use flow cytometry for the assessment of *Microcystis* spp. viability after incubation with *Bacillus mycoides* B16 under static and turbulent conditions. The specific objectives were: to optimize the flow cytometric analysis; to determine the population heterogeneity of *Microcystis* spp. cells in an exponential phase; to determine the population changes of *Microcystis* spp. (live and dead cells counts) after co-culturing with *B. mycoides* B16 under static and turbulent conditions and to simultaneously determine the population changes of *B. mycoides* B16.

2. Materials and methods

2.1. Culture of organisms

A pure culture suspension *of M. aeruginosa* PCC7806 (prey) was kindly provided by Prof T Downing, Nelson Mandela Metropolitan University, South Africa. The *Microcystis* spp. was cultured in 500 ml sterilized and modified BG11 medium (Krüger and Eloff, 1977) in 1 l Erlenmeyer flasks under shaking incubation (Labcon shaker, South Africa) (78 rpm) for 8 d under continuous light at room temperature. Two 18 W cool white florescent lamps (Lohuis FT18W/T8 1200LM) that were suspended above the flasks provided continuous lighting (2000 lux). The Extech Instruments Datalogging light meter model 401036 measured the light intensity. A subsample of growing *Microcystis* spp. cells was sampled under aseptic conditions, stained simultaneous with FDA and PI fluorescent stains and then subjected to flow cytometric analysis.

A pure culture suspension of *Bacillus mycoides* B16 (predator) (isolated by us, Gumbo et al. (2010)) was prepared by inoculation into 100 ml sterilized one-tenth of Tryptic soy broth (TSB) in a 250 ml Erlenmeyer flask and shake incubated (128 rpm, 25 °C) for 24 h (Di Franco et al., 2002). Lyophilized cells were prepared by centrifugation of fresh cultures (10,000 rpm, 15 min, 25 °C) and freeze dried (Edwards freeze dryer: minus 50 °C, 2.8 mbar, 72 h). The lyophilized powder was stored at 4 °C until further use.

2.2. Experimental design

2.2.1. Predator-prey interactions under static conditions

Bacteria treated samples involved the use of six 250 ml cotton wool plugged Erlenmeyer flasks which were sterilized prior to addition of 100 ml *Microcystis* spp. suspension, in exponential growth phase (approximately 10^6 cells per ml) and then *Bacillus mycoides* B16 powder (approximately 0.4 g equivalent to 10^{12} cfu per ml) (Fig. 1). The control samples involved six 250 ml cotton wool plugged Erlenmeyer flasks were sterilized prior to addition of 100 ml *Microcystis* spp. suspension, in exponential growth phase (approximately 10^6 cells per ml).

The twelve Erlenmeyer flasks were then incubated under similar conditions as those for culturing of host cyanobacteria but without shaking or agitation of Erlenmeyer flasks for a period of 6 d. On a daily basis samples were withdrawn under aseptic conditions and subjected to bacterial plate counts; plating on modified BG11 agar plates; fluorescent staining and flow cytometric analysis.

2.2.2. Predator-prey interactions in a turbulent environment

The experimental set from above (Section 2.2.1) was followed with the following modification. The Erlenmeyer flasks were shaken (78 rpm) but incubated under similar conditions as those for culturing of host cyanobacteria for a period of 6 d. On a daily basis samples were withdrawn under aseptic conditions and subjected to bacterial plate counts; plating on modified BG11 agar plates; fluorescent staining and flow cytometric analysis.

2.3. Preparation of fluorescent dyes and the staining of Microcystis spp. samples

A fluorescent diacetate (FDA) (Sigma Chemicals F7378) stock solution was prepared by dissolving 50 mg FDA in 5 ml reagent grade acetone and stored in the dark at −20 °C until further use (Hadjoudja et al., 2009). The FDA staining technique for *M. aeruginosa* developed by Brookes et al. (2000) was followed in this study. A procedure similar to that of Ross et al. (1989) and Franklin et al. (2001) was followed in the development of a Propidium iodide (PI) staining technique for *M. aeruginosa*. A PI (Sigma Chemicals 81845) stock solution was prepared by dissolving 25 mg PI in 5 ml distilled water and was stored at 4 °C until further use.

From each of the twelve flasks (bacteria treated and control samples), 1 ml of sample was abstracted under aseptic conditions and then were homogenized for 20 s (Ultrasonic Homogenizer 4710 series, Cole-Palmer Instrument Co, Chicago, IL) to disrupt the clumps that were formed (Orr and Jones, 1998). Then after sonic homogenization of the bacteria treated and control samples, 100 µl of a *Microcystis* spp. was transferred to 10 ml centrifuge tube where 100 µl of FDA working solution (120 µg per ml) was added and incubated at room temperature for 7 min in the dark. Then after incubation, to the same centrifuge tube, 100 µl of PI working solution (60 µg per ml) was added followed by 100 µl of

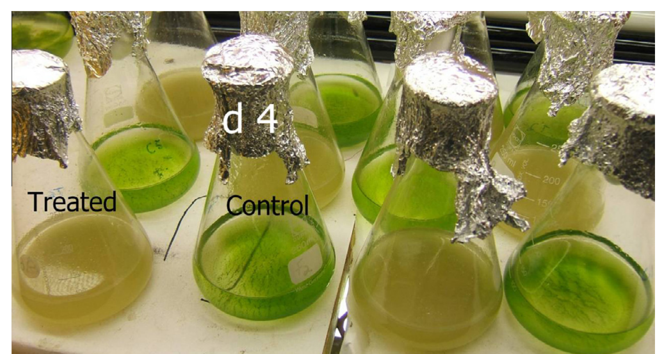

Fig. 1. The experimental layout of the twelve Erlenmeyer flasks, six control *Microcystis* spp. suspension samples and six with *B. mycoides* B16 treated and *Microcystis* spp. suspension samples on d4.

FLOW-COUNT Fluorospheres beads (Beckman Coulter, USA) were then added. The samples with the fluorescent dyes and Fluorospheres beads were then subjected to flow cytometric analysis.

2.4. Optimizing the flow cytometer

The Beckman Coulter Epics ALTRA flow cytometer was calibrated with flow check and UV beads to determine the range of particle sizes to reject or accept. A suspension of UV beads (particle range 1.7–2.2 μm), flow check beads (10 μm) and a pure culture suspension of *B. mycoides* B16 were analyzed on the forward scatter mode (FS). The UV beads and then a suspension of *B. mycoides* B16 was analyzed without any fluorescent staining to determine the region where the bacteria would lie in the dot plot diagram. A suspension of *Microcystis* cells (control) was stained with FDA to ascertain the region where the viable or live cells would lie. The PMT3 detector was used to analyze the fluorescein fluorescence. A suspension of *Microcystis* cells (control) was stained with PI to ascertain the region where the dead cells would lie. The PMT4 detector was used to analyze the PI red fluorescence.

2.5. Determining population changes of Microcystis spp. cells after co-culture with B. mycoides B16 with flow cytometric analysis

A Beckman Coulter Epics ALTRA flow cytometer (excitation: argon laser 15 mW, 488 nm) for the excitation of fluorescent stains, green fluorescein and propidium iodide (PI). But the green fluorescence interference of PI fluorescence was successfully resolved when the optical filters for PMT 2 and PMT 3 were interchanged so that the two fluorescent stains FDA and PI were simultaneous applied to the *Microcystis* samples in a single run. The green fluorescein fluorescence was measured in channel B (PMT 2 log, 553 voltage) after passing through the 550 nm dichroic long pass filter and then through a 525 nm band pass filter. The red PI fluorescence was measured in channel D (PMT 4 log, 740 voltage) after passing through the 640 nm dichroic long pass filter and then through a 610 nm band pass filter.

Thus the *Microcystis* spp. 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 (630 nm). Approximately 10,000 events or 300 voltages (whichever came first) were used in recording of flow cytometric data. The forward and side light scatter signals were used to derive 2-parameter cytograms.

The flow cytometric (FCM) graphs were drawn with the WinMDI 2.8 (Joseph Trotter 1993–1998) free software that was made available on the Internet. <http://facs.scripps.edu/software.html>.

2.6. Determining the population changes of B. mycoides B16 through bacteria plate counts

From each of the twelve flasks (bacteria treated and control samples), 1 μl of sample was abstracted under aseptic conditions and then plated on 10% Tryptic Soy agar and BG 11 agar (Krüger and Eloff, 1977) respectively and the plates were incubated at room temperature.

2.7. Data analysis

Data from FCM were analyzed using the BMDP Statistical Software Inc (1993). An independent sample *t*-test compared the means of two independent groups, i.e. bacteria treated and control (bacteria untreated) *Microcystis* spp. samples. The Levene's Test for Equality of Variances tests whether the variances of the two groups are different. The null hypothesis would state that the means are the same. A $p < 0.05$ (indicating a sufficiently large difference between groups) would suggest that the null hypothesis is rejected and conclude that the two groups are significantly different.

A one-sample *t*-test compares the mean of one sample to a fixed estimate, usually zero (0). A significant result indicates that the group's mean differs from the fixed value. Hypothesis testing can help answer questions such as:

- Are the increases (positive sign) in the *B. mycoides* B16 population numbers related to the decreases (negative sign) in *Microcystis* (population) cell counts?
- In controls (bacteria untreated) *Microcystis* spp. samples indicate an increase (positive sign). How large is the direction of the increase?

3. Results and discussion

3.1. Optimizing the flow cytometric analysis

The Beckman Coulter Epics ALTRA flow cytometer was calibrated with flow check and UV beads to determine the range of particle sizes. The fluorescence intensity of UV beads and flow check beads formed the basis on which particles (>1 μm) were excluded and inclusion of particles (<10 μm) in subsequent flow cytometric analysis (Fig. 2a and b). The suspension of *B. mycoides* B16 was analyzed without any fluorescent staining to determine the region where the bacteria would lie in the dot plot diagram (Fig. 2c). Therefore the gating process was carried to exclude particles including cell debris, bacteria from subsequent flow cytometric analysis (Fig. 2d).

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. A suspension of *Microcystis* cells (control) was stained with FDA to ascertain the region (R1) where the viable or live cells would lie (Fig. 3a). The PMT3 detector was used to analyze the fluorescein fluorescence. The other particles such as cell debris, *B. mycoides* B16 bacteria were located in region (R2). *Microcystis* cells have the green photosynthetic pigment, chlorophyll *a* that was excited by the blue laser causing it to fluoresce red that contributed to inference with PI red fluorescence. Thus the FDA gating parameter was set to exclude the analysis of chlorophyll *a* autofluorescence (Fig. 3b and c).

A suspension of *Microcystis* cells (control) was stained with PI to ascertain the region (R1) where the dead cells would lie (Fig. 3d). The other particles such as cell debris and *B. mycoides* B16 bacteria were located in region (R2). *Microcystis* cells have the green photosynthetic pigment, chlorophyll *a* that was excited by the blue laser causing it to fluoresce red and contributed to interference with PI fluorescence. Thus the PI gating parameter was set to exclude the analysis of chlorophyll *a* autofluorescence (Fig. 3e). A major point was the observation that the 'live cell' region (Fig. 3a) was similar to the 'dead cell' region (Fig. 3d) and as such it was difficult to distinguish the two regions. The green fluorescence was the main cause of this interference (Fig. 3f). The green fluorescence and PI red fluorescence were well resolved from chlorophyll *a* red fluorescence (Fig. 3b and e). The green fluorescence interference of PI fluorescence was successfully resolved when the optical filters for PMT 2 and PMT 3 were interchanged; hence the two fluorescent stains were then simultaneous applied to the *Microcystis* samples in a single run.

3.2. The Simultaneous staining of Microcystis spp. samples in an exponential phase

The simultaneous and dual staining of *Microcystis* spp. revealed the presence of four sub-populations (population heterogeneity)

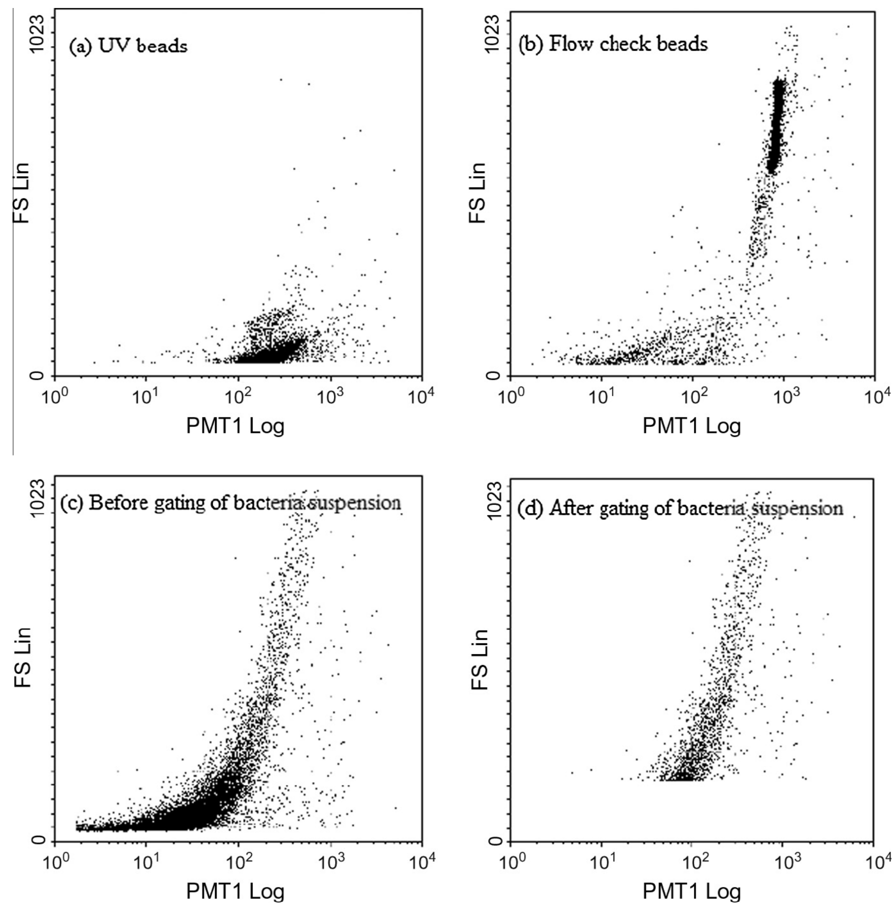

Fig. 2. Calibration of instrument-particle size exclusion: (a) UV beads, range 1.7–2.2 μm, (b) Flow check beads (10 μm in size), (c) Before and (d) after gating to exclude particles less than 1 μm such as *B. mycoides* B16 from subsequent FCM analysis. FSLin = measures the size of a particle, forward scatter mode. PMT1 log = measures side scatter (cell granularity and complexity).

that was composed of 91.6% live cells; 7.6% cells with selective permeability; 0.5% cell debris and 0.3% dead cells (Fig. 4a). The population heterogeneity was as a result of the following. The FDA stain was transported across the cell wall into the cell and in the process the esterase enzymes decoupled the FDA to produce fluorescein, which was retained within the cell (Breeuwer et al., 1995). The fluorescein was then excited by blue laser and fluoresced green (Fig. 4b). These *Microcystis* spp. cells with active metabolism and no leaking membrane) were designated as viable cells (live) (R3). Some *Microcystis* spp. cells had damaged cell membranes such that these cells allowed both stains (FDA and PI) to diffuse through. As a result of residual esterase activity, the cells were able to produce fluorescein, which fluoresced green upon blue laser excitation (Fig. 4b). The PI intercalated with the nuclear acid material to fluoresce red upon excitation (Fig. 4c). The cell debris category was classified as R4 and therefore this R4 data was discarded. The fluorescent stains, FDA and PI, were simultaneous applied to a culture of *Microcystis* spp. cells that was in an exponential growth phase (Fig. 4d). The transmission electron microscope study of Gumbo and Cloete (2011) confirmed that these *Microcystis* spp. cells had intact cell membranes and cell debris.

The other category of *Microcystis* spp. cells without a cell membrane the PI easily diffused through and reacted with the nuclear acid material to fluoresce red. Thus the *Microcystis* spp. cells without a cell wall and or damaged cell membrane were designated as dead cells (R1 and R2) for the purposes of this study. This supports the observation of Joux and Lebaron (2000) that cells with damaged and or with selective membranes would eventually lose their nucleoid material to the external environmental must be considered as dead cells. Thus the flow cytometric counts confirmed that the majority of *Microcystis* cells were live and were in exponential phase on 5 d.

3.3. Predator-prey interactions as determined by FDA/PI staining under static conditions

The *B. mycoides* B16, the predatory bacterium was added to each of the six Erlenmeyer flasks, of approximately 1×10^{12} colony forming units (CFU) per ml was added to *Microcystis* spp. cell suspensions (1×10^6 cells per ml) to give a predator: prey ratio of 10^6:1 in order to achieve lysis of *Microcystis* spp. in a shorter time.

Under static conditions, i.e., daily hand shaking before sampling, the flow cytograms (FCM) counts revealed that during the first two days, the treated and control samples showed a 49% increase (a positive t value of 0.59) in numbers of live *Microcystis* spp. cells (Fig. 5; Table 1). The independent Levene *t*-test analysis of the live *Microcystis* numbers means (treated and control samples) showed no significant difference ($p > 0.05$). Thus, the bacteria that were added had no effect on the growth of *Microcystis* spp. The results of earlier study of Gumbo and Cloete (2010), suggested that the predator bacteria were adjusting to their new environment, during the 'lag phase' and hence did not cause lysis of the *Microcystis* spp. cells.

From d 3 to 6 the predatory bacteria, *Bacillus mycoides* B16 had a lytic effect on *Microcystis* spp. cells and resulted in a significant 4-log decrease ($p < 0.05$; negative t values) after in five days (Fig. 5). In another related study by Gumbo and Cloete (2010) showed ultrastructural damage that was inflicted upon the *Microcystis*

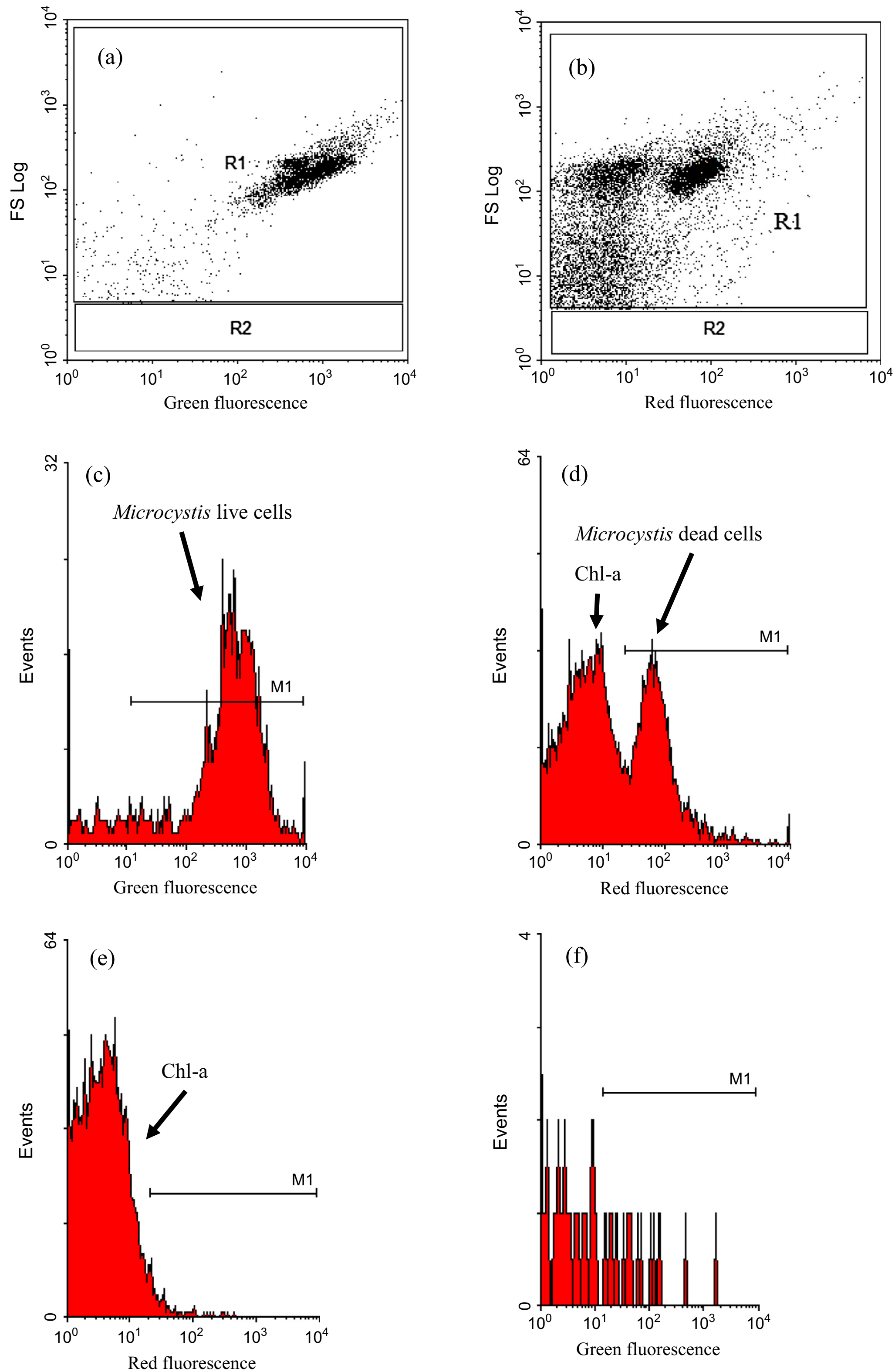

Fig. 3. *Microcystis* control sample after staining with FDA: (a) dot plot representing region of live cells (R1) and (R2) region for *B. mycoides* B16 bacteria and cell debris on a 4-log scale; (b) histogram measures green fluorescence intensity of live *Microcystis* cells. This gating parameter (M1) allows for measurement of green fluorescence on a 4-log scale and (c) histogram measures chlorophyll *a* (chl-*a*) red autofluorescence. The gating parameter (M1) allows for measurement of red PI fluorescence on a 4-log scale. Note there is no interference of PI red fluorescence from chl-*a* autofluorescence. *Microcystis* control sample after staining with PI: (d) dot plot representing region of dead cells (R1) and (R2) bacteria, cell debris on a 4-log scale; (e) histogram measures PI red fluorescence on a 4-log scale. The gating parameter (M1) discriminates the red fluorescence of PI instead the chlorophyll *a* (chl-*a*) red fluorescence. The chl-*a* fluorescence is resolved from PI red fluorescence. (f) The green fluorescence with gating parameter (M1) coincides with the PI red fluorescence. The result is interference PI fluorescence from green fluorescence.

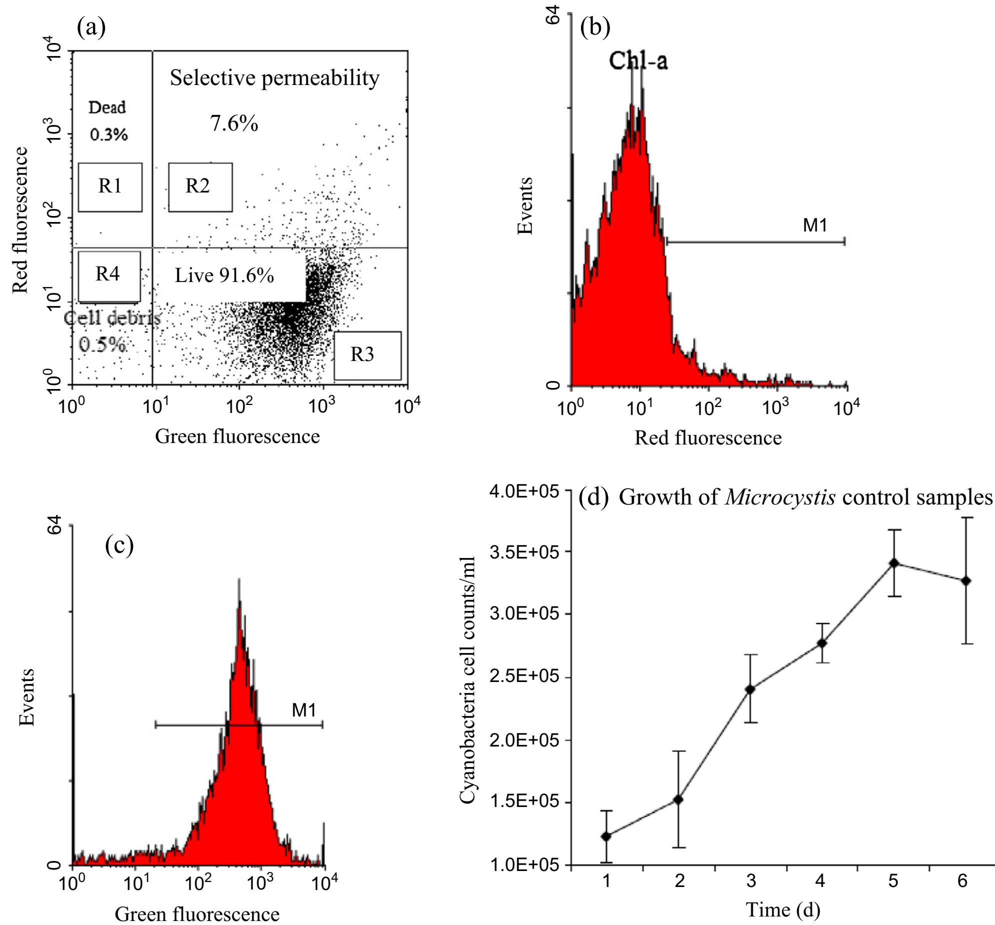

Fig. 4. *Microcystis* control sample dual stained with FDA and PI: (a) dot plot representing regions of dead cells (R1); selective permeability cells (R2); live cells (R3) and cell debris (R4) on a 4-log scale; (b) histogram measures green fluorescence of live *Microcystis* spp. cells. The gating parameter (M1) allows for measurement of green fluorescence on a 4-log scale. Note there is no interference of PI red fluorescence from Chl-*a* autofluorescence and (c) histogram measures red PI fluorescence intensity of dead *Microcystis* spp. cells. This gating parameter (M1) allows for measurement of read PI fluorescence and NOT Chl-*a* autofluorescence on a 4-log scale (d) Independent confirmation of growth of *Microcystis* control samples. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation).

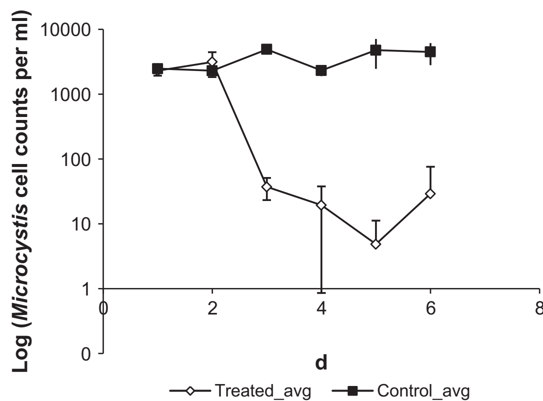

Fig. 5. Changes in *Microcystis* spp. cell numbers after exposure to *B. mycoides* B16 and controls under static conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation.)

spp. cells and the disruption and damage of the cell membranes by the predatory bacterium, *Bacillus mycoides* B16. The control samples showed an increase of 65% of live *Microcystis* spp. cells over the same period (Fig. 5). For the controls samples, the light microscopy and scanning electron microscopy study have showed healthy and normal *Microcystis* cells in colonial formations (Gumbo and Cloete, 2010). Some of bacteria treated flasks showed a slight

Table 1
Independent Levene *t*-test analysis of *Microcystis* spp. numbers mean (treated and control samples) under static conditions. Pooled *t*-test values and associated probabilities.

Time (d)	df Degrees of freedom	Live		Dead	
		t test	*p* value	*t* test	*p* value
1	10	−0.27	0.7915*	+1.98	0.0754*
2	10	+0.59[a]	0.5701*	+1.49	0.1659*
3	10	−15.98[b]	0.0000**	−7.73	0.0000**
4	10	−7.68	0.0000**	−2.68	0.0230**
5	10	−14.50	0.0000**	−12.15	0.0000**
6	10	−7.19	0.0000**	−3.85	0.0032**

* No significant difference, i.e., bacteria had no effect.
** Significance different, i.e., bacteria had an effect.
a An increase (+ positive sign).
b A decrease (− negative sign).

increase in live *Microcystis* spp. cells on d 6. This may be explained by aggregating damaged *Microcystis* cells as seen on region R2 of the flow cytograms (Fig. 5a). These *Microcystis* spp. cells though indicating that they are live, but plating an aliquot sample from the bacteria treated samples on BG11 agar did not reveal any viable cells that were able to grow. This observation was also supported by the study of Joux and Lebaron (2000) who showed that cells with damaged and or with selective membranes would eventually die as because their nuclear material was compromised. However

the light microscopy and scanning electron microscopy study did not show any intact *Microcystis* cells on d 6 (Gumbo and Cloete, 2010).

The independent Levene *t*-test analysis of the population of dead *Microcystis* spp. cell counts was not similar to that of live *Microcystis* spp. cell counts (Table 1). The dead *Microcystis* spp. cells were assessed using propidium iodide (PI) fluorescence. From day 1 to 2, there was an insignificant increase in the numbers of dead *Microcystis* spp. cells in the treated and control samples ($p > 0.5$; Fig. 6; Table 1). The results of earlier study of Gumbo and Cloete (2010), suggested that the predator bacteria were adjusting to their new environment, during the 'lag phase' and hence did not cause lysis of the *Microcystis* spp.

There was a variable decline in the numbers of dead *Microcystis* spp. cells, for the bacteria treated samples (Fig. 6). In the same period there was a slight increase and decrease in the numbers of dead *Microcystis* spp. cells, in the control samples. In the absence of predator bacteria, the decrease in the control samples may be due to natural aging and death. The independent Levene *t*-test analysis of dead *Microcystis* spp. cells (bacteria treated and control) showed a significant decrease (t values negative; $p < 0.05$), i.e. *B. mycoides* B16 was responsible for lysing the *Microcystis* spp. cells resulting in dead cells.

The number of dead *Microcystis* spp. cells was expected to increase in the bacteria treated samples. However the opposite was observed. The PI stained the nucleic acids (RNA and DNA). The study of Nakamura et al. (2003) has indicated that *B. cereus* N14 was found feeding exclusively on *M. aeruginosa* and *M. viridis* as its sole nutritional source. The study of von Wintzingerode et al. (1997) also confirmed the close relationship between *B. mycoides* and *B. cereus.* Thus we can speculate that the predatory bacteria, *B. mycoides* B16 was feeding on the nucleic acids indicating a decrease in the PI value hence a fewer 'dead' cells. Alternatively there was a natural degradation of the nucleic acids in the aqueous environment. Veldhuis et al. (2001) reported that the last stages of automortality of phytoplankton involved fragmentation (degradation) of genomic DNA. Another possibility that may contribute to the erratic PI results is the interspecies variation of RNA and DNA per cyanobacteria cell. Brussaard et al. (1999) reported that prior to flow cytometric analysis, the phytoplankton cells were incubated with RNase to remove the RNA component in order to report only the DNA component.

The findings of our research suggest that *B. mycoides* B16 had effect on the growth of *Microcystis* spp. by disrupting the plasmalemma and thylakoid membranes (Gumbo and Cloete, 2011) resulting in reduced photosynthetic activity. The *Microcystis* spp. responded to these adverse conditions by utilizing stored energy reserves for maintenance of essential processes instead of growth and therefore there was a decline in RNA activity (Borbéy et al., 1990).

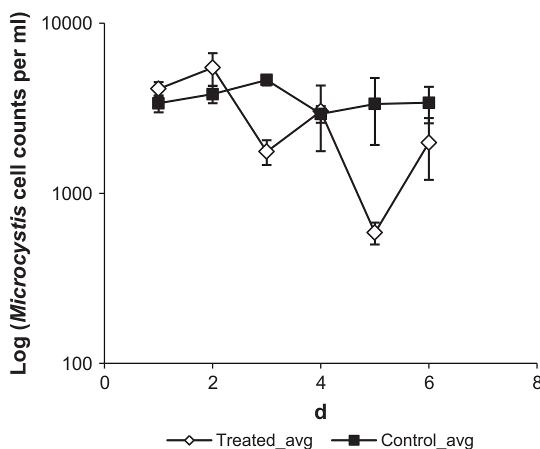

Fig. 6. PI fluorescence illustrating changes in *Microcystis* spp. cell numbers after exposure to *B. mycoides* B16 and control samples under static conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation.)

3.4. Predator–prey interactions in a turbulent environment

The effect of *B. mycoides* B16 on the growth of *Microcystis* spp. was investigated under turbulent conditions, i.e., shaking of flasks. The cyanobacteria growth was monitored through flow cytometric counts of *Microcystis* spp. cells after dual staining with fluorescence diacetate (FDA) which stained only live cells followed by propidium iodide (PI) which stained only dead cells. The dual staining of *Microcystis* cells revealed two different cell populations: live and dead cells (Fig. 7). Both fluorescent stains (FDA and PI) were able to stain *Microcystis* cells with compromised membranes and these cells were classified as dead.

The aged *Microcystis* spp. batch culture had an addition of fresh modified BG11 media before subdividing them into bacteria treated and control samples. The purpose of introducing fresh nutrients was to stimulate growth of cyanobacteria cells. Under turbulent conditions, the flow cytometric counts revealed that during the first three days, the treated and control samples showed a 10× increase (a positive *t* value of +1.27; Table 2; Fig. 8) in numbers of live *Microcystis* spp. cells. The statistical comparisons of live *Microcystis* spp. cells mean (treated and control) were not significantly affected ($p > 0.05$; Table 2), i.e., the bacteria did not contribute to the death of *Microcystis* spp. cells.

During the same time period, d 1–d 3, the one sample *t*-test analysis showed that there was a significant increase in *Microcystis* spp. cell numbers (bacteria treated) ($t = +7.77$; df = 5; $p < 0.05$; Table 3) (Fig. 8). Where the predator bacteria numbers in the bacteria treated *Microcystis* samples were assessed the bacteria cell numbers increased significantly ($t = +3.30$; df = 5; $p > 0.05$; Table 3) (Fig. 9). Between 4 d and 6 d there was a decrease of almost 1-log in the population of live *Microcystis* cell numbers in the bacteria treated samples (Fig. 9). This contrasted with the control samples, which showed an increase of 1-log in live *Microcystis* cell numbers (Fig. 8). The independent Levene *t*-test, comparisons of live *Microcystis* numbers (treated and control) showed significant decrease ($p < 0.05$; Table 2), i.e., *B. mycoides* B16 was responsible for lysing the *Microcystis* cells resulting into dead cyanobacteria cells.

The one sample *t*-test confirmed the reduced growth in *Microcystis* spp. cell numbers (bacteria treated) was significant ($t = -7.21$; df = 5, $p < 0.05$; Table 3). This result was in contrast with the control samples, where a 60% increase in *Microcystis* spp. cell numbers was observed in six days (Table 3; Fig. 8). The predator bacteria numbers showed a significant increase ($t = +11.31$: df = 5; $p < 0.05$; Table 3: Fig. 10) and coincided with a decrease in *Microcystis* cell numbers. These findings suggest that the *B. mycoides* B16, a heterotroph, was probable utilizing the *Microcystis* as a source of nutrition as supported by the study of Nakamura et al. (2003). The flow cytometric counts showed that *B. mycoides* B16 had a lytic effect on the growth of *Microcystis* cells resulting in a population decline of 85% in six days under turbulent conditions. Burnham et al. (1981) reported similar results in the lysis of *Phormidium luridum* by the predator, *Myxococcus xanthus* PCO2 under turbulent conditions.

The dead *Microcystis* cell numbers increased by 30% in bacteria treated samples (Fig. 9). The independent Levene *t*-test of dead *Microcystis* cell numbers (treated and control) showed a significant decrease in treated samples ($p < 0.05$; Table 2; Fig. 9), indicating that *B. mycoides* B16 was responsible for lysing the *Microcystis* cells resulting in dead cells. In the control samples, the population of dead cyanobacteria cells increased by 49% after 6 d. This may be

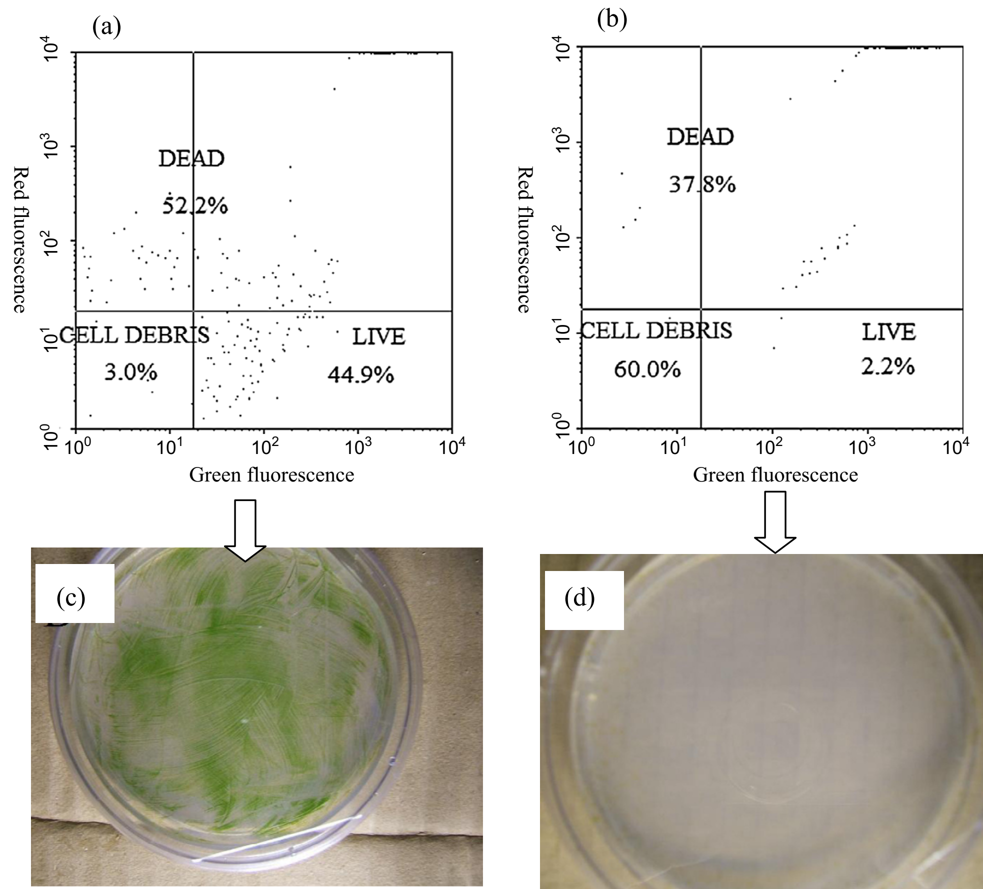

Fig. 7. A typical two parametric plot illustration of *Microcystis* spp. population heterogeneity on 6 d: (a) *B. mycoides* B16 treated sample and (b) Untreated (control) sample. BG 11 agar plates with (c) No growth of *Microcystis* spp. cells after exposure to *B. mycoides* B16; (d) Control sample showing growth of *Microcystis* spp. cells indicating viability.

Table 2
Independent Levene *t*-test analysis of *Microcystis* cell numbers (treated and control samples) under turbulent conditions. (Pooled *t*-test values and associated probabilities).

Time (d)	df Degrees of freedom	Live		Dead	
		t test	*p* value	*t* test	*p* value
1	10	+0.77	0.4611*	+2.77	0.0198*
2	10	+1.85	0.0937*	+0.59	0.5693*
3	10	+1.27[a]	0.2342*	−0.16	0.8749*
4	10	−5.30[b]	0.0003**	−2.54	0.0293**
5	10	−4.72	0.0008**	−10.19	0.0000**
6	10	−6.19	0.0001**	−2.87	0.0165**

* No significant difference, i.e., bacteria had no effect.
** Significance different, i.e., bacteria had an effect.
[a] An increase (+ positive sign).
[b] A decrease (− negative sign).

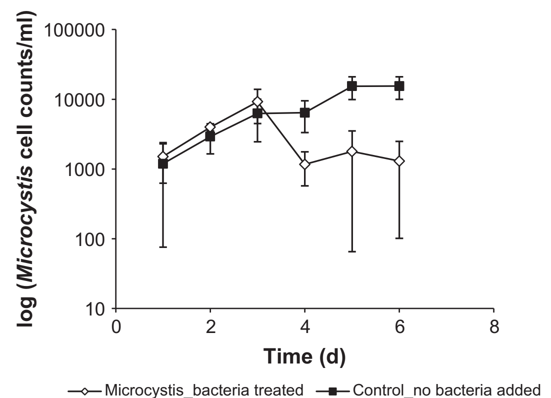

Fig. 8. Changes in population levels of live *Microcystis* spp. cells in *B. mycoides* B16 treated and control samples under turbulent conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation.)

due to natural aging and death due to limited food source in the Erlenmeyer flasks.

The *B. mycoides* B16 managed to reduce the numbers of live *Microcystis* spp. cells by 97% under static conditions (Table 1) and 85% under turbulent conditions (Table 2) in six days. These results suggest that *Microcystis* spp. lysis is probable dependent on physical contact efficiency. Shilo (1970) and expanded by Burnham et al. (1984) also found that the agitation of flasks might disturb the bacteria from establishing contact with cyanobacteria thus slowing the lysis process. Nevertheless *B. mycoides* B16 was able to lyse *Microcystis* under static and turbulent conditions. This suggests that *B. mycoides* B16 has potential as a good biological control agent since it might encounter such conditions in the real world. Other researchers such as Ashton and Robarts (1987) and Bird and Rashidan (2001) have implicated predatory bacteria in the termination of harmful algal blooms. The flow cytometry technique proved to be convenient, fast, reliable and accurate method for the determination of large numbers of live (viable) and dead (non-viable) *Microcystis* cells after exposure to the predator bacteria, *B. mycoides* B16.

Table 3
One sample *t*-test, showing t values and associated (p) probabilities showing changes in organism populations.

Time (d)	df	*Microcystis* (*B. mycoides* B16 treated)			*B. mycoides* B16			*Microcystis* (control)		
		Numbers	*t*	*p*	Numbers	*t*	*p*	Numbers	*t*	*p*
1–3	5	Increase	+7.77[a]	0.0006	Increase	+3.30	0.0215	Increase	+7.13	0.0008
4–6	5	Decrease	−7.21[b]	0.0008	Increase	+11.31	0.0001	Increase	+3.91	0.0113

df = degrees of freedom.
[a] An increase (+ positive sign).
[b] A decrease (− negative sign).

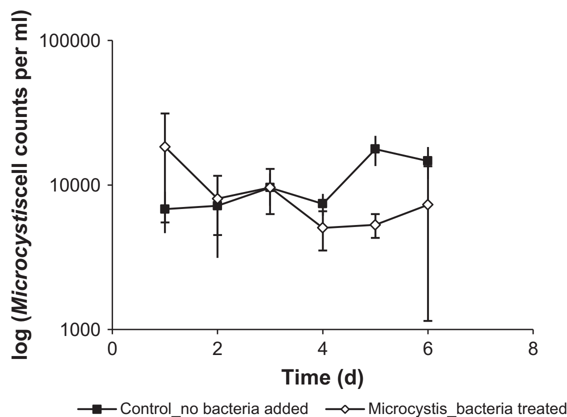

Fig. 9. Changes in population levels of dead *Microcystis* cells in *B. mycoides* B16 treated and control samples under turbulent conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation.)

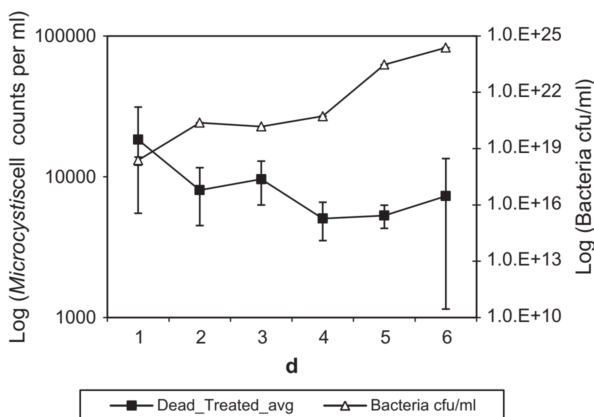

Fig. 10. Increase in Predator bacteria numbers (colony forming units/ml) coincided with the decrease in *Microcystis* cells as indicated by the decrease in PI flow cytometric counts. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation.)

4. Conclusion

- The flow cytometry technique was able to successfully assess viable and membrane compromised *Microcystis* cells after simultaneous staining with fluorescein diacetate and propidium iodide.
- The dual staining of *Microcystis* cells revealed the presence four-population groups (heterogeneity). During the progress of *B. mycoides* B16 induced lysis of *Microcystis* cells, a transition phase from live cells through membrane compromised state, death phase and lastly cell debris was observed.
- The control samples of *Microcystis* cells resulted in a 65% growth under static and turbulent conditions in six days
- The predator bacteria numbers showed a significant increase and coincided with a decrease in *Microcystis* cell numbers. These findings suggest that the *B. mycoides* B16 was utilizing the *Microcystis* as a source of nutrition.
- FCM showed that *B. mycoides* B16 had a lytic effect on *Microcystis* cells that resulted in a population decline of over 97% under static conditions and 85% under turbulent conditions in six days. This suggests that *B. mycoides* B16 has potential as a good biological control agent since it might encounter such conditions in the real world.

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