Chapter 5: Flow cytometry measurements on *Microcystis* cells after exposure to predatory bacteria

CHAPTER 5

FLOW CYTOMETRY MEASUREMENTS ON *MICROCYSTIS* CELLS AFTER EXPOSURE TO PREDATORY BACTERIA

Abstract

Flow cytometry (FCM) was used to assess the viability of *Microcystis* cells after exposure to *Bacillus mycoides* B16. Two methods of fluorescent staining were used: (1) use of separate staining and (2) dual staining of *Microcystis* cells. The method that was eventually adopted for routine analysis was dual staining that revealed the population heterogeneity (living, membrane compromised and dead cells) of *Microcystis*. In the copper and *B. mycoides* treated samples; the majority of *Microcystis* cells were dead in comparison with the control samples. The use of gating percentages gave a qualitative expression of alive or dead *Microcystis* cells, i.e., the majority was either alive or dead. It was then resolved to incorporate flow count beads to allow for a quantitative analysis of alive or dead *Microcystis* cells. Under static conditions, the flow cytometric counts revealed that *B. mycoides* B16 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* B16 had a lytic effect on *Microcystis* 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* B16 cell numbers. This may suggest that *B. mycoides* B16, 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* cell numbers in six days. *B. mycoides* B16 was able to lyse *Microcystis* cells under static and turbulent conditions and may thus be considered as a potential biological control agent for the management of *Microcystis* algal blooms.

Key words: *Microcystis*, flow cytometry, biological control, algicides, copper, *Bacillus mycoides*.

5.1. INTRODUCTION

The technique of flow cytometry coupled with the use of fluorogenic probes is now well developed and is applied to the counting and viability assessment of aquatic microorganisms and cyanobacteria in particular (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 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) and viral infection (Brussaard et al., (2001).

Other researchers such as Burnham et al. (1984) and Nakamura et al. (2003a) evaluated the lytic activity of predator bacteria on the viability of cyanobacteria based on cell counts. Nakamura et al. (2003a) initially used the criteria of chlorophyll *a* analysis with varying levels of success. The chlorophyll *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* cells and that the bacteria did not degrade the chlorophyll *a* moiety, hence the discrepancy in the chlorophyll *a* results. Daft and Stewart (1971) revealed a similar pattern of non-degradation of heterocysts by the bacterium CP-1. Heterocysts, contain chlorophyll *a*, are used for oxygenated photosynthesis and nitrogen fixation. Thus a much simpler and more

accurate method was required to assess the viability of *Microcystis* based on its metabolic and physiological status after exposure to a bacterial agent.

Flow cytometry, a technique widely applied in the medical sciences (Rieseberg et al., 2001) and later applied to phytoplankton, including *Microcystis* (Dubelaar et al., 1995; Marie et al., 2005). It was used to assess the viability of *Microcystis* after exposure to an external environmental stress such as nutrient limitation and regeneration of gas vacuoles after ultrasonication. In the study of Brookes et al. (2000) they found that there was a correlation between the recovery of the nutrient starved *Microcystis* and availability of nutrients (nitrates and phosphates) over a five-day incubation period. They also noted that stressed cells had a greater membrane permeability thus increasing the efflux of fluorescein diacetate (FDA). Lee et al. (2000) applied ultrasonication on *Microcystis* cells. The control and ultrasonicated samples had two subpopulations that were different. The ultrasonication caused the *Microcystis* cells to collapse the gas vacuoles and render them non-buoyant. The control samples had intact gas vacuoles. Combined with dual staining (LIVE/DEAD BacLight bacterial viability kit) their results showed that the fluorescent intensity of PI stained dead *Microcystis* cells was about 4-fold different from the chlorophyll *a* fluorescence. The sonicated *Microcystis* cells were able to regenerate their gas vacuole if provided with sufficient light illumination, i.e., the cells were viable with an active metabolism as assessed by the FDA fluorescence. *Microcystis* cells that were dead with an inactive metabolism were assessed by propidium iodide (PI) fluorescence following ultrasonication. To the best of our knowledge there are no reports on flow cytometric assessment of *Microcystis* viability following bacterial infection.

Brussaard et al. (2001) went a step further by using flow cytometry to assess viability of phytoplankton following viral infection. The results revealed that the optimal viability assay depended on phytoplankton species. *Phaeocystis pouchetii* and *Micromonas pusilla* were studied and revealed insights into the population heterogeneity following viral infection.

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 FDA and 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 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 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).

The main objective of this study was to determine the potential of *Bacillus mycoides* B16 as a biological control agent for *Microcystis aeruginosa.* The specific objective was to use flow cytometry for the assessment of *Microcystis* viability after exposure to *B. mycoides* B16.

5.2. MATERIALS AND METHODS

5.2.1. The determination of particle size range

A suspension of flow checks beads, UV beads and a pure culture suspension of *B. mycoides* B16 (Section 3.2.4.3) and a pure culture suspension of *Microcystis* (Section 3.2.4.1) were analyzed on the Beckman Coulter Epics ALTRA flow cytometer (Section 5.2.2.2.).

5.2.2. Optimizing the staining of *Microcystis* cells

5.2.2.1. Preparation of fluorescent dyes

Prior to fluorescent staining, *Microcystis* colonies were disrupted with ultrasonication for 20 s (Ultrasonic Homogenizer 4710 series, Cole-Palmer Instrument Co, Chicago, IL) (Orr and Jones, 1998). 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^{o}C until further use. 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 5 mℓ distilled water and was stored at 4^{o}C until further use.

5.2.2.2. Flow cytometric analysis

Prior to cytometric analysis, unstained *Microcystis* colonies were disrupted with ultrasonication for 20 s (Ultrasonic Homogenizer 4710 series, Cole-Palmer Instrument Co, Chicago, IL) (Orr and Jones, 1998). A Beckman Coulter Epics ALTRA 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).

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

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.

5.2.2.3. Separate staining of *Microcystis* samples

A 50 mℓ suspension of *Microcystis* (Section 3.2.4.1) was added to a 200 mℓ suspension of *B. mycoides* B16 (3.2.4.1) and incubated under continuous light (2000 lux).

The samples (treated and control) were homogenized for 20 s to disrupt the clumps that may form. Esterase activity and absolute *Microcystis* cell counts were determined by adding 100µℓ of FDA working solution (120 µg per mℓ) to 100µℓ of a *Microcystis* sub sample in a 10 mℓ centrifuge tube and incubated at room temperature for 7 min in the dark. Cell viability and absolute *Microcystis* cell counts were determined by adding 100 µℓ of PI working solution (60 µg per mℓ) to 100 µℓ of *Microcystis* sub sample in a 10 mℓ centrifuge tube and incubated at room temperature for 1 min.

5.2.2.4. Simultaneous staining of *Microcystis* samples

Experimental set up in Section 5.2.2.3 was followed with the following modification. For colour compensation, the optical filters for PMT 2 and PMT 3 were interchanged.

The samples (control and treated) were homogenized for 20 s to disrupt the clumps that may form. Esterase activity and absolute *Microcystis* cell counts were determined by adding 100µℓ of FDA working solution (120 µg per mℓ) to 100µℓ of a *Microcystis* sub sample in a 10 mℓ centrifuge tube and incubated at room temperature for 7 min in the dark. To the **same** centrifuge tube, 100 µℓ of PI working solution (60 µg per mℓ) was added followed by 100 µℓ of FLOW-COUNT Fluorospheres (Beckman Coulter, USA) were then added.

Cyanobacteria cell counts were carried out (Section 3.2.4.6).

5.2.2.5. Effect of copper and *B. mycoides* B16 on *Microcystis* cells

An aliquot (1 mℓ) of *B. mycoides* B16 suspension (Section 3.2.4.3) was added to 2 mℓ of *Microcystis* suspension (3.2.4.1) and incubated under the same conditions (Section 3.2.4.1). For the evaluation of the copper (Section 4.2.3) procedure was followed. The aliquots were stained (Section 5.2.2.1.) and analyzed on the Beckman Coulter Epics ALTRA flow cytometer (Section 5.2.2.2.).

5.2.3. Preliminary assessment of *Microcystis* after exposure to *B. mycoides* B16 predator bacteria

Experimental set in Section 5.2.2.3. was followed for 48 h. After 2 and 48 hours of incubation an aliquot (1 mℓ) of the treated sample was sampled, simultaneously stained (Section 5.2.2.4.) and analyzed on the Beckman Coulter Epics ALTRA flow cytometer (Section 5.2.2.2.).

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

A known mass of lyophilized antagonist powder (approximately 0.4g equivalent to 10^{12} cfu/mℓ) (Section 5.2.4.1.) was added separately to each of six 250-mℓ cotton plugged Erlenmeyer flasks containing 100 mℓ cyanobacterial suspension (Section 3.2.4.1.).

Six 250 mℓ cotton plugged Erlenmeyer flasks containing 100 mℓ cyanobacterial suspension (approximately 10^6 cells per mℓ) but not inoculated with lyophilized antagonist powders, served as controls.

The Erlenmeyer flasks were incubated under similar conditions as those for culturing of host cyanobacteria but without shaking or agitation of flasks as this may disturb the lysis process (Shilo, 1970). On a daily basis samples were withdrawn under aseptic conditions

and subjected to staining (Section 5.2.2.3) and analyzed on the Beckman Coulter Epics ALTRA flow cytometer (Section 5.2.2.2.) for 6 days. The data was statistically analyzed (5.2.5.1.).

5.2.4.1. Preparation of lyophilized predator bacteria

The mass cell production for *B. mycoides* B16 procedure that was developed by Korsten et al. (1996) was followed. A 50-mℓ Erlenmeyer flask containing 20 mℓ of 10% TSB was inoculated with *B. mycoides* B16. After 24 h of shake incubation (Labcon Shaking Incubator, 128 rpm) at 25°C, the contents (starter culture) of 50-mℓ Erlenmeyer flasks were added in its entirety to a 500-mℓ Erlenmeyer flask containing 200 mℓ of 10% TSB. After a further 24 h, the contents of the flask culture were added in its entirety to a 2-litre Erlenmeyer flask containing 1 litre of 10% TSB. The 2-litre Erlenmeyer flask was incubated as before but for 48 h. The above procedure was then repeated with seven other Erlenmeyer flasks.

The bacterial suspension was harvested by centrifugation (10,000 rpm; 15 min; 25°C). The pellets were combined and frozen overnight before being lyophilized (Edwards freeze dryer: -50°C, 2.8mbar, 72 h). The lyophilized antagonist powder was stored at 4°C until further use.

5.2.5. The effect of *B. mycoides* B16 on *Microcystis* in a turbulent environment

Experimental set in Section 5.2.4. was followed with the following modification. The Erlenmeyer flasks were shake incubated under similar conditions as those for culturing of host cyanobacteria. On a daily basis samples were withdrawn under aseptic conditions and subjected to bacterial plate counts (Section 3.2.4.4.), plating on BG11 agar plates, simultaneously stained (Section 5.2.2.4) and analyzed on the Beckman Coulter Epics ALTRA flow cytometer (Section 5.2.2.2.) for 6 days. The data was statistically analyzed (5.2.5.1.).

5.2.5.1. Statistical 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 untreated *Microcystis* 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) in the *B. mycoides* B16 numbers related to the decreases (negative) in *Microcystis* cell counts?
- In untreated *Microcystis* samples indicate an increase (positive). How large is the direction of the increase?

5.3. RESULTS AND DISCUSSION

5.3.1. Determining particle size range

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 (10µm) and a pure culture suspension of *B. mycoides* B16 were analyzed on the forward scatter mode (FS) (Figures 5.1). The amount of light that is scattered by a cell is a complex function of its size, shape and refractive index whilst the light intensity is directly related to the angle of light collection. The forward scatter is the light that is scattered at small angles and is used in the determination of relative cell size and cell volume. This is based on homogenous model spheres that tend to approximate biological cells (Davey, 1994).

The *Microcystis* cell in its spherical structure is an ideal candidate for a homogenous model sphere. Based on the cell size, the forward scatter (used as a gating parameter) is then used to exclude cell aggregates, debris and other microorganisms such as bacteria from further analysis. The side scatter light is collected or refractive index of the cell (its complexity and granularity) at 90^{o} from the incident light. This side scatter light may be used to reveal internal structure.

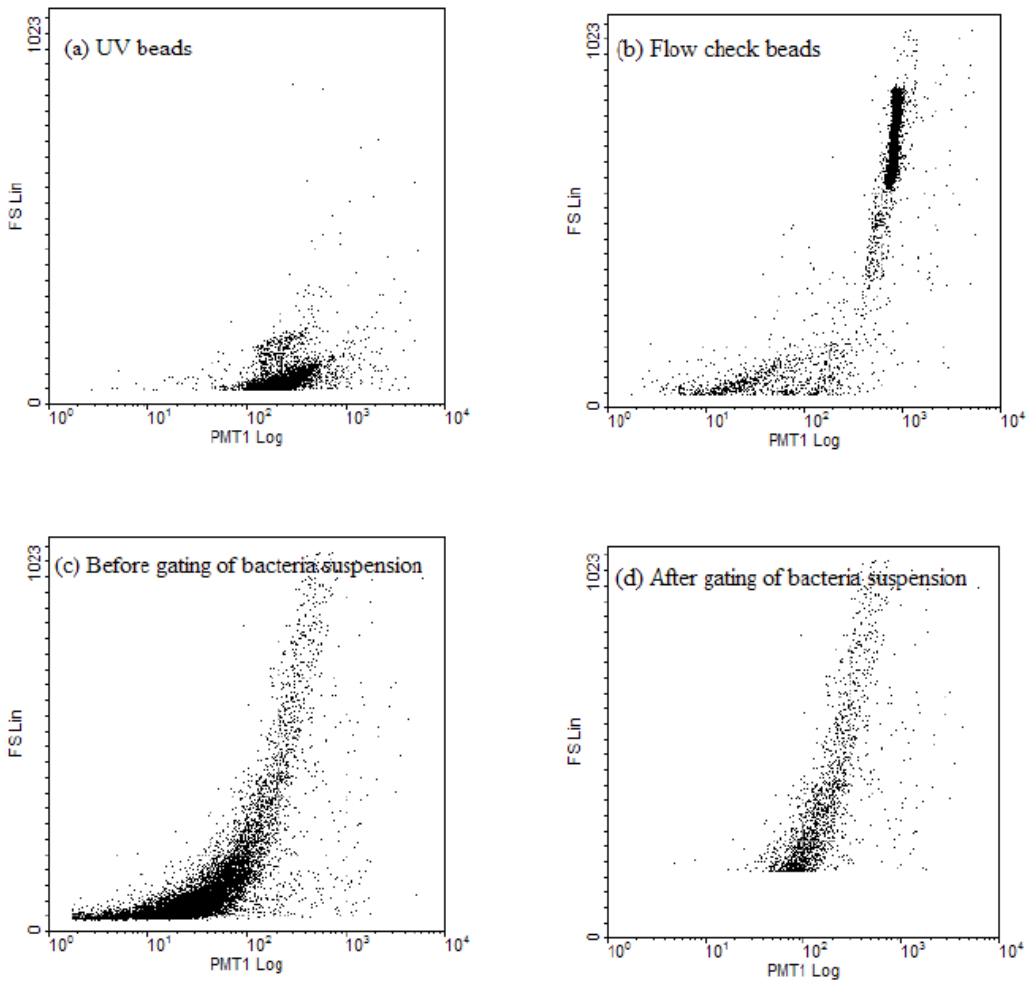

Figure 5.1: Calibration of instrument- particle size exclusion: (a) UV beads, range 1.7 – 2.2 µm, (b) Flow check beads (10 µm in size), (c) Before and (d) after gating to exclude particles less than 1µm such as *B. mycoides* B16 from subsequent FCM analysis. FSLin = measures the size of a particle, forward scatter mode. PMT1 log= measures side scatter (cell granularity and complexity).

Before gating 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 (Figure 5.1c). This region coincided with the region for the UV beads with particle sizes of less than 2.2µm (Figure 5.1a). Therefore the gating process was carried to exclude particles including cell debris, bacteria from subsequent flow cytometric analysis (Figure 5.1d).

The purpose of flow check beads was to verify that the cytometer was capable of analyzing particles of similar sizes to those of *Microcystis* cells. The fluorescence intensity of UV beads and flow check beads formed the basis on which particles (> 1µm) were excluded and inclusion of particles (< 10 µm) in subsequent flow cytometric analysis.

5.3.2. Optimizing the staining of *Microcystis* cells

5.3.2.1. Separate staining of *Microcystis* cells with FDA and PI

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 is a lipophilic substance that easily diffuses into cells with intact membranes. But once inside the active cell, the FDA substrate was cleaved by non-specific esterases releasing a polar fluorescein product that was strongly retained. On excitation with the blue laser, the fluorescein become fluorescent and stained the cells green (Joux and Lebaron, 2000). The intensity of the fluorescence may invariably increase over time depending on the metabolic status of active esterases. Hence it was imperative to carryout the FDA analysis within 1 min after the 7 min incubation.

A suspension of *Microcystis* cells (control) was stained with FDA to ascertain the region (R1) where the viable or live cells would lie (Figure 5.2a). The PMT3 detector was used

to analyze the fluorescein fluorescence. The other particles such as cell debris, 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 may contribute to inference. Thus the FDA gating parameter was set to exclude the analysis of chlorophyll *a* autofluorescence (Figures 5.2b and c).

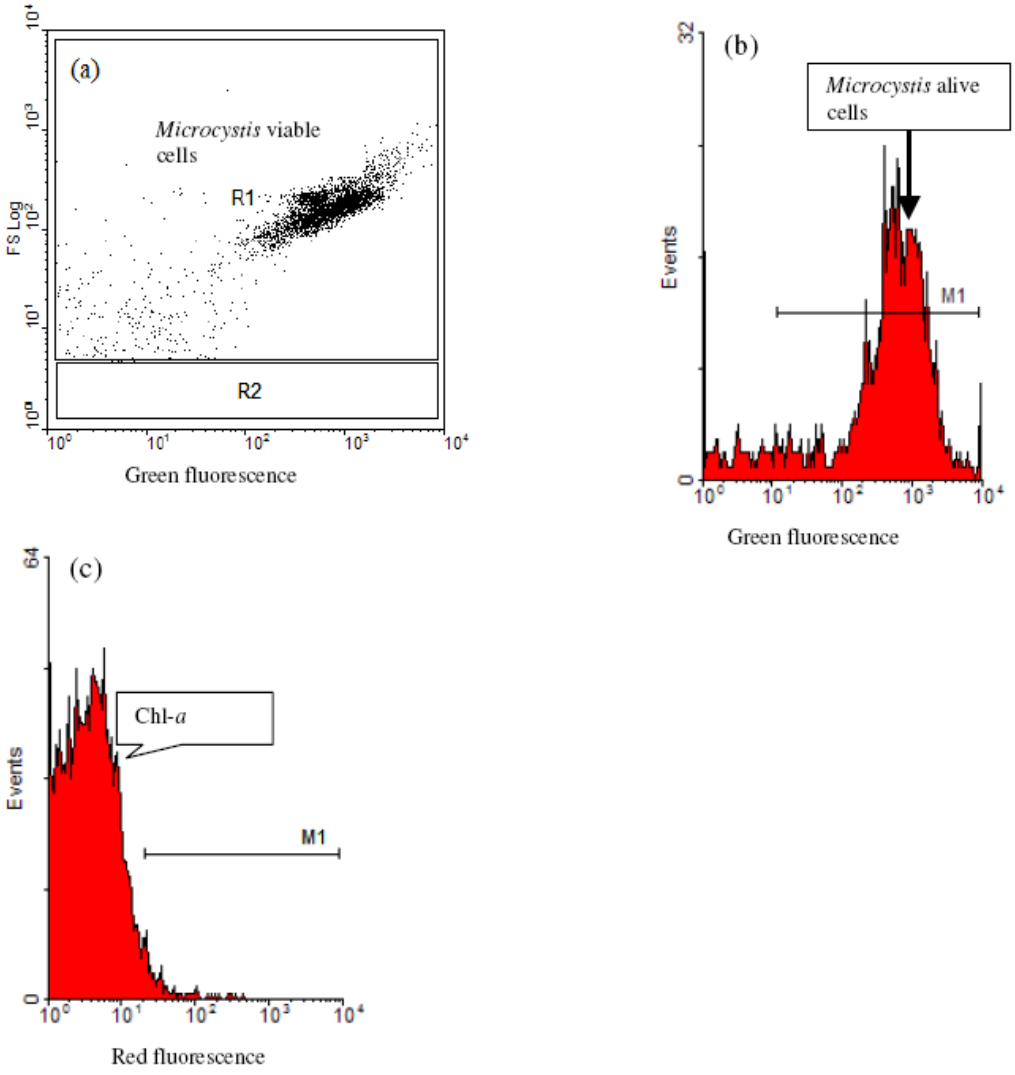

Figure 5.2: *Microcystis* control sample after staining with FDA: (a) dot plot representing region of alive cells (R1) and (R2) region for bacteria and cell debris on a 4-log scale; (b) histogram measures green fluorescence intensity of alive *Microcystis* cells. This gating parameter (M1) allows for 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. See text for further interpretation.

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

A suspension of *Microcystis* cells (control) was stained with PI to ascertain the region (R1) where the dead cells would lie (Figure 5.3a). The PMT4 detector was used to analyze the PI red fluorescence. The other particles such as cell debris and 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 may contribute to inference. Thus the PI gating parameter was set to exclude the analysis of chlorophyll *a* autofluorescence (Figures 5.2b).

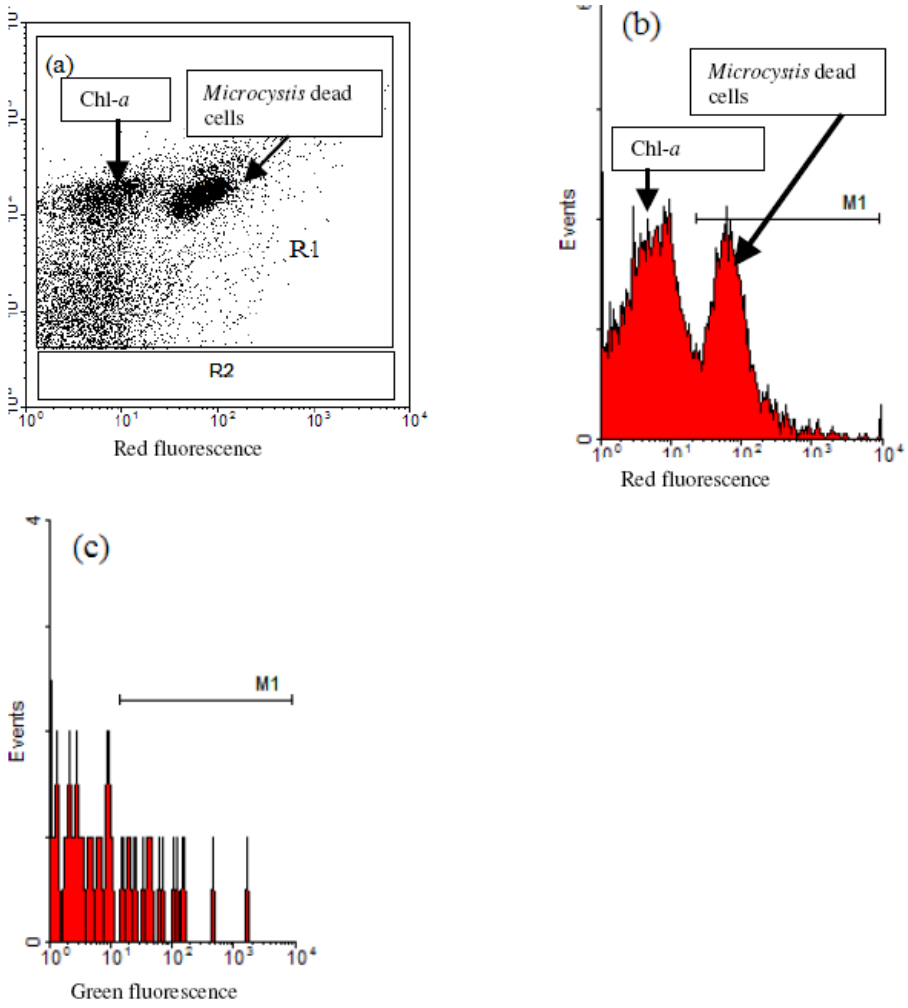

Figure 5.3: *Microcystis* control sample after staining with PI: (a) dot plot representing region of dead cells (R1) and (R2) bacteria, cell debris on a 4-log scale; (b) histogram measures PI red fluorescence on a 4-log scale. The gating parameter (M1) discriminates the red fluorescence of PI instead the chlorophyll *a* (chl-*a*) red fluorescence. The chl-*a* fluorescence is resolved from PI red fluorescence. (c) The green fluorescence with gating parameter (M1) coincides with the PI red fluorescence. The result is interference PI fluorescence from green fluorescence.

A major point was the observation that the 'alive cell' region (Figure 5.2a) was similar to the 'dead cell' region (Figure 5.3a) and as such it was difficult to distinguish the two regions. The green fluorescence was the main cause of this interference (Figure 5.3c). It

was then resolved to separately stain the *Microcystis* cells and analyze separately the data generated. The green fluorescence and PI red fluorescence were well resolved from chlorophyll *a* red fluorescence (Figures 5.2b and 5.3b).

5.3.2.2. Simultaneous staining of *Microcystis* samples

The green fluorescence interference of PI fluorescence was successfully resolved when the optical filters for PMT 2 and PMT 3 were interchanged (see Literature Review on Flow Cytometry; Figure 2.5). Hence the two fluorescent stains were simultaneous applied to the *Microcystis* samples in a single run.

The fluorescence diacetate (FDA) and propidium iodide (PI) are both excited at 488nm with an argon laser. There is interference between the PI fluorescence emission at 617nm and the FDA fluorescence emission at 514nm (Figure 5.4). This is resolved through the use of optical filters and appropriate colour compensation software to enable dual staining of a cell and then measuring resultant fluorescence.

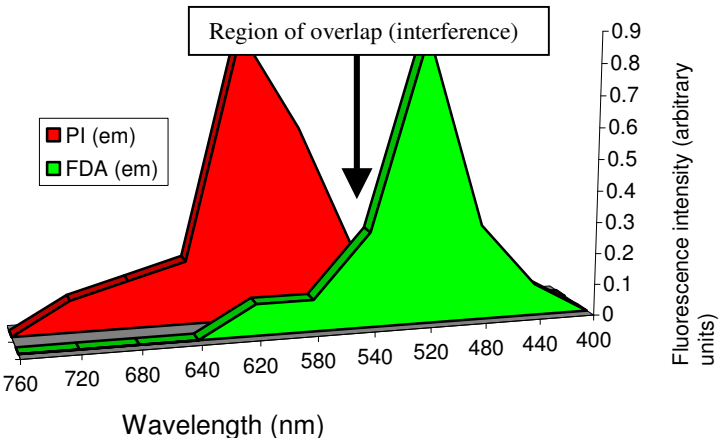

Figure 5.4: Colour compensation in resolving the PI (emission) and FDA (emission) interference (Davey, 1994).

Both FDA and PI stains were applied to a culture of *Microcystis* cells that was in an exponential growth phase (Figure 5.5d). The dual staining revealed the presence of four

sub-populations (population heterogeneity) that was composed of 91.6 % alive; 7.6% cells with selective permeability; 0.5% cell debris and 0.3% dead cells (Figure 5.5a). 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. The fluorescein was then excited by blue laser and fluoresced green (Figure 5.5c). These cells with active metabolism and no leaking membrane were designated as active cells (alive) (R3) (see Electron microscopy studies; Figures 4.6a and 4.10e).

Some cells with damaged and or selective permeability allowed both stains (FDA and PI) to diffuse through (see Electron microscopy studies; Figures 4.5d and 4.7d). As a result of residual esterase activity, the cells were able to produce fluorescein, which fluoresced green upon blue laser excitation (Figure 5.5c). The PI intercalated with the nuclear acid material to fluoresce red upon excitation (Figure 5.5b). The cell debris category (R4) were not stained by FDA or PI and therefore will not be considered. The other category of cells without a cell membrane the PI easily diffused through and reacted with the nuclear acid material to fluoresce red (see Electron microscopy studies; Figures 4.7d and 4.10c). Thus the 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 will 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 alive, 91.6% alive and 7.9% dead cells, and were growing on 5 d (Figure 5.5d). A major point of interest that may be inferred from the dual staining results is that in the separate staining, FDA results has a component of cells with selective permeability such that the sum of live and dead cells did not add up to 100%.

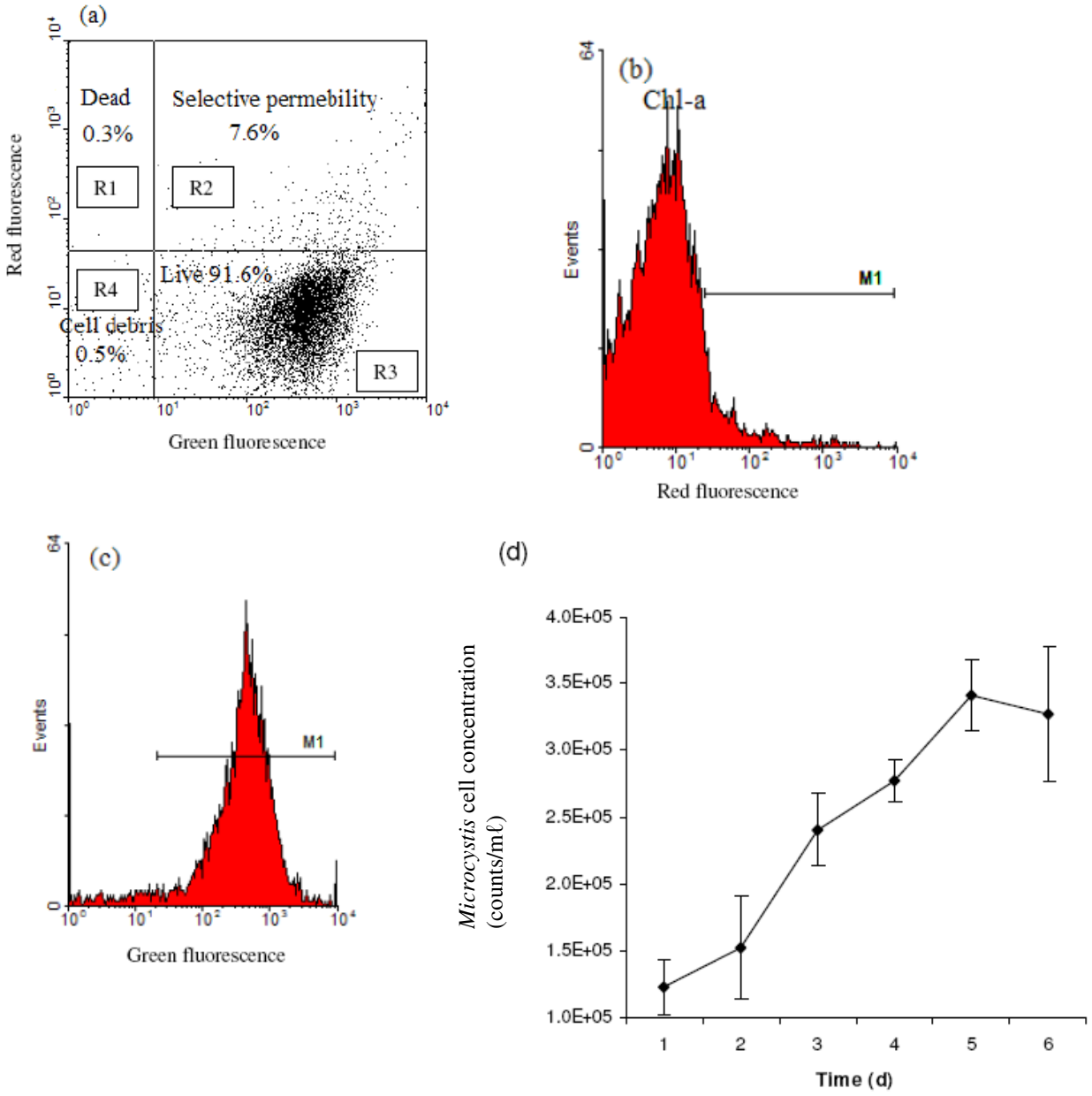

Figure 5.5: *Microcystis* control sample dual stained with FDA and PI: (a) dot plot representing regions of dead cells (R1); selective permeability cells (R2); alive cells (R3) and bacteria and cell debris (R4) on a 4-log scale; (b) histogram measures red PI fluorescence intensity of dead *Microcystis* cells. This gating parameter (M1) allows for measurement of read PI fluorescence and NOT chl-*a* autofluorescence on a 4-log scale. Note there is no interference of PI red fluorescence from chl-*a* autofluorescence. and (c) histogram measures green fluorescence of alive *Microcystis* cells. The gating parameter (M1) allows for measurement of green fluorescence 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).

5.3.2.3. Effect of copper and *B. mycoides* B16 on *Microcystis* cells

We have observed through electron microscopy studies that the application of copper and *B. mycoides* B16 on *Microcystis* cells leads to the damage on cell membrane (Sections 4.3.2. and 4.3.3.). This study was carried out to determine the region where dead *Microcystis* cells lie after exposure to these agents (Figure 5.6).

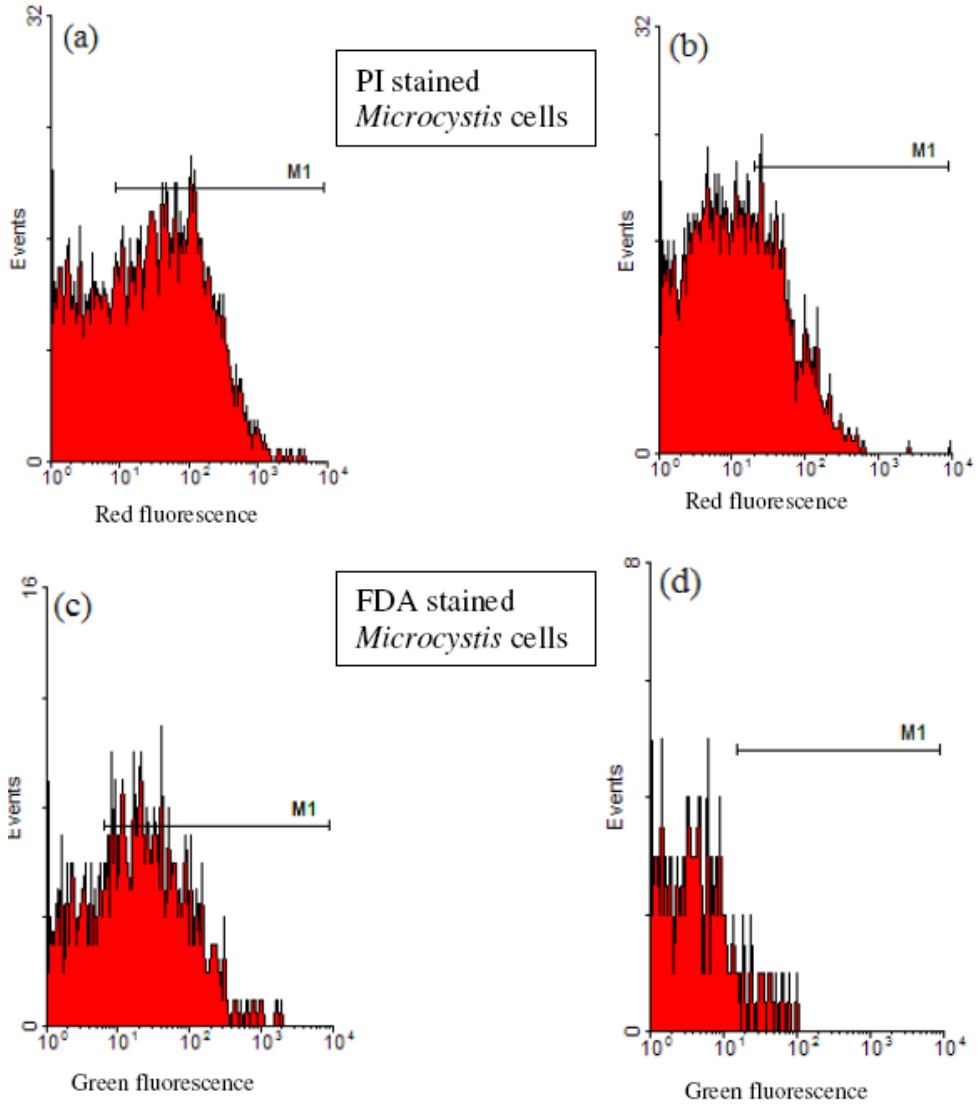

Figure 5.6: Evaluation of copper algicide and predator bacteria on *Microcystis* cells: (a-b) after PI staining: (a) treated with *B. mycoides* B16 and (b) treated with copper solution; (c-d) after FDA staining: (c) treated with *B. mycoides* B16 and (d) treated with copper solution.

In bacteria treated samples, the percentage of gated dead *Microcystis* cells was 81% and that of copper treated was 58% (Figures 5.6a and b). For the bacteria treated samples, the percentage of live *Microcystis* cells was 27% and that of copper treated was 2% (Figures 5.6c and d). The control samples showed that there were 44% of live *Microcystis* cells and 1.3% dead cells.

In the copper and *B. mycoides* treated samples, the majority of *Microcystis* cells were dead in comparison with the control samples. *B. mycoides* B16 had a profound effect on the viability of *Microcystis* and showed its potential as a biological control agent. The use of gating percentages gave a qualitative expression of live or dead *Microcystis* cells, i.e., the majority was either live or dead. It was then resolved to incorporate flow count beads to allow for a quantitative analysis of live or dead *Microcystis* cells.

5.3.3. Preliminary assessment of *Microcystis* after exposure to *B. mycoides* B16 predator bacteria

A preliminary trial was carried to assess the viability of *Microcystis* cells after incubation with a culture suspension of *B. mycoides* B16 for 2 and 48 h. The results showed that the population heterogeneity of *Microcystis* was subdivided into two distinct groups namely dead and alive (Figure 5.7). After 2 h of incubation with *B. mycoides* B16 33.7% of *Microcystis* cells were dead (including membrane compromised cells) and 59.6% were alive. After 48 h of incubation, 60.2% of *Microcystis* cells were dead and 0.0% alive.

The exposure of *Microcystis* cells to *B. mycoides* B16 resulted in a lytic process involving a transition phase from live *Microcystis* cells to membrane compromised cells, the death stage and finally the last stage, composed of cell debris which retained autofluorescence.

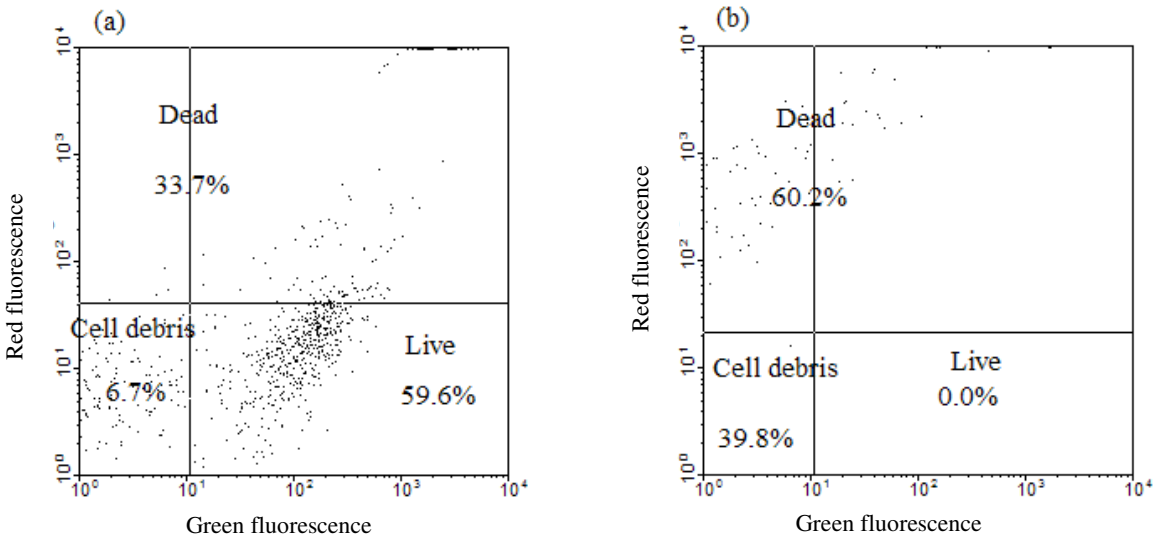

Figure 5.7: Dual stained *Microcystis* sample after exposure to *B. mycoides* B16 (a) after 2 h and (b) after 48 h showed *Microcystis* population heterogeneity: dead cells and alive cells.

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

Working with fresh *B. mycoides* B16 liquid cultures presented a number of challenges such that we decided to use lyophilized powder. The main challenges were to keep the cultures alive and carry out bacteria cell counts before the experiments. The lyophilized powder was the attractive option for the following reasons. It was easier to manipulate (weigh the powder), carry out bacterial cell counts, and prepare the predator-prey ratios once the powder was available. Secondly it was anticipated that the preparation of lyophilized powder might be a prelude the commercial manufacture of the bacteria in future. Lastly it was anticipated that it might give direction on the method of application of lyophilized powder to a surface *Microcystis* algal bloom.

5.3.4.1. Predator-prey interactions as determined by FDA staining

The *B. mycoides* B16 numbers were increased to achieve a predator: prey ratio of 100:1 in order to achieve lysis of *Microcystis* in a shorter time. To each of the six flasks, lyophilized powder (400mg ≡ 1. x 10^8 colony forming units per mℓ) was added to

Microcystis cell suspensions (1. x 10^6 cells/ml). No powder was added to the other six flasks and these served as control samples.

Under static conditions, i.e., daily hand shaking before sampling, the 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* cells (Figure 5.8; Table 5.1). The independent Levene t-test analysis of the live *Microcystis* numbers means (treated and control samples) showed no significant difference ($p > 0.05$; Table 5.1). Thus, the bacteria that were added had no effect on the growth of *Microcystis*. The results of earlier studies (Chapter 3: 3.3.4), suggest that the bacteria were adjusting to their new environment, during the 'lag phase' and hence did not cause lysis of the *Microcystis*.

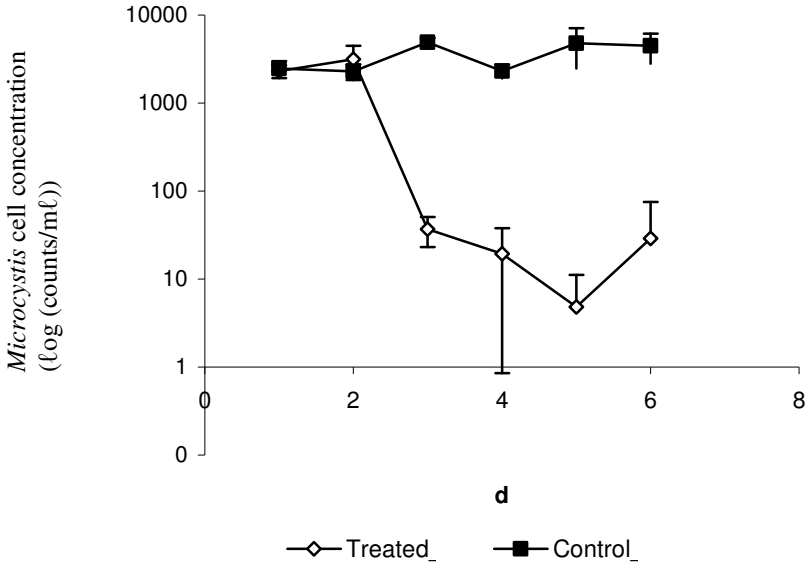

Figure 5.8: Changes in *Microcystis* cell numbers after exposure to *B. mycoides* B16 and controls under static conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation).

Table 5.1: Independent Levene t-test analysis of *Microcystis* 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	t value
1	10	-0.27	0.7915*	+1.98	0.0754*
2	10	+0.59[1]	0.5701*	+1.49	0.1659*
3	10	-15.98[2]	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.
[1]an increase (positive)
[2]a decrease (negative)

From d 3 to 6 *Bacillus mycoides* B16 had a lytic effect on *Microcystis* cells and resulted in a significant ($p < 0.05$; Table 5.1), 4-log decrease (negative t values) after in four days (Figure 5.8). SEM showed *Microcystis* cells that had damaged cell membranes, cell debris for the treated samples (Chapter 4: 4.3.4). The control samples showed an increase of 65% of live *Microcystis* cells over the same period (Figure 5.8). For the controls samples, SEM showed healthy and normal *Microcystis* cells in colonies (Chapter 4: 4.3.4).

Some of treated flasks showed an increase in live *Microcystis* cells on d 6. However SEM and light microscopy did not show any intact *Microcystis* cells on d 6.

5.3.4.2. Predator-prey interactions as determined by PI staining

The independent Levene t-test analysis of the population of dead *Microcystis* was similar to that of live *Microcystis* cells (Table 5.1). The dead *Microcystis* cells were assessed using PI fluorescence. For d 1 and 2, there was an insignificant increase in the numbers of dead *Microcystis* cells in the treated and control samples ($p > 0.5$; Figure 5.9; Table 5.1). The results of earlier studies (Chapter 3: 3.3.4), suggest that the bacteria were adjusting to their new environment, during the 'lag phase' and hence did not cause lysis of the *Microcystis*.

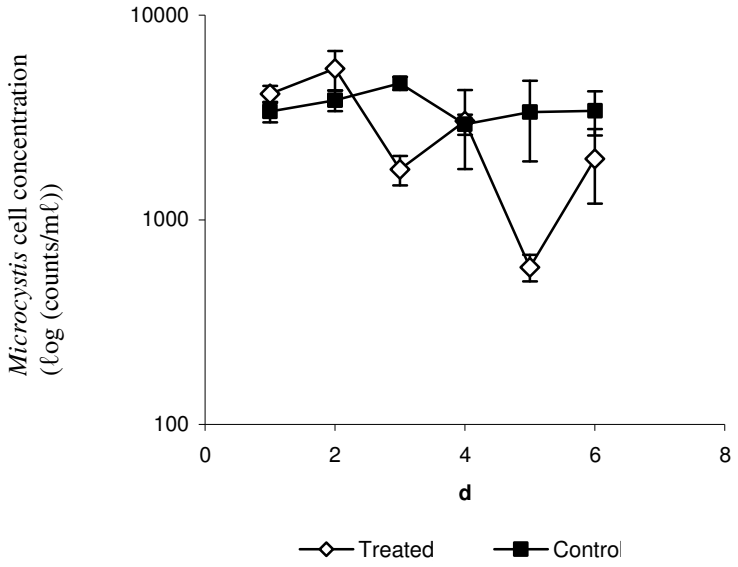

Figure 5.9: PI fluorescence illustrating changes in *Microcystis* cell numbers after exposure to *B. mycoides* B16 and control samples under static conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation).

There was a variable decline in the numbers of dead *Microcystis* cells, for the treated samples (Figure 5.9). In the same period there was a slight increase and decrease in the numbers of dead *Microcystis* 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* cells (treated and control) showed a significant decrease (t values negative; $p < 0.05$; Table 5.1), i.e. *B. mycoides* B16 was responsible for lysing the *Microcystis* cells resulting in dead cells.

The number of dead *Microcystis* cells was expected to increase in the treated samples. However the opposite was observed. The PI stained the nucleic acids (RNA and DNA). It was therefore speculated that the bacteria were 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.

The findings of our research suggest that *B. mycoides* B16 had effect on the growth of *Microcystis* by disrupting the plasmalemma and thylakoid membranes (Chapter 4: 4.3.4) resulting in reduced photosynthetic activity. The *Microcystis* responded to these adverse conditions by utilizing stored energy reserves for maintenance of essential processes instead of growth and there was a decline in RNA activity (Borbély et al., 1990).

5.3.5. The effect of *B. mycoides* B16 on *Microcystis* in a turbulent environment

The effect of *B. mycoides* B16 on the growth of *Microcystis* was investigated under turbulent conditions, i.e., shaking of flasks. The cyanobacteria growth was monitored through flow cytometric counts of *Microcystis* 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 (Figure 5.10). Both fluorescent stains (FDA and PI) were

able to stain *Microcystis* cells with compromised membranes and these cells were classified as dead.

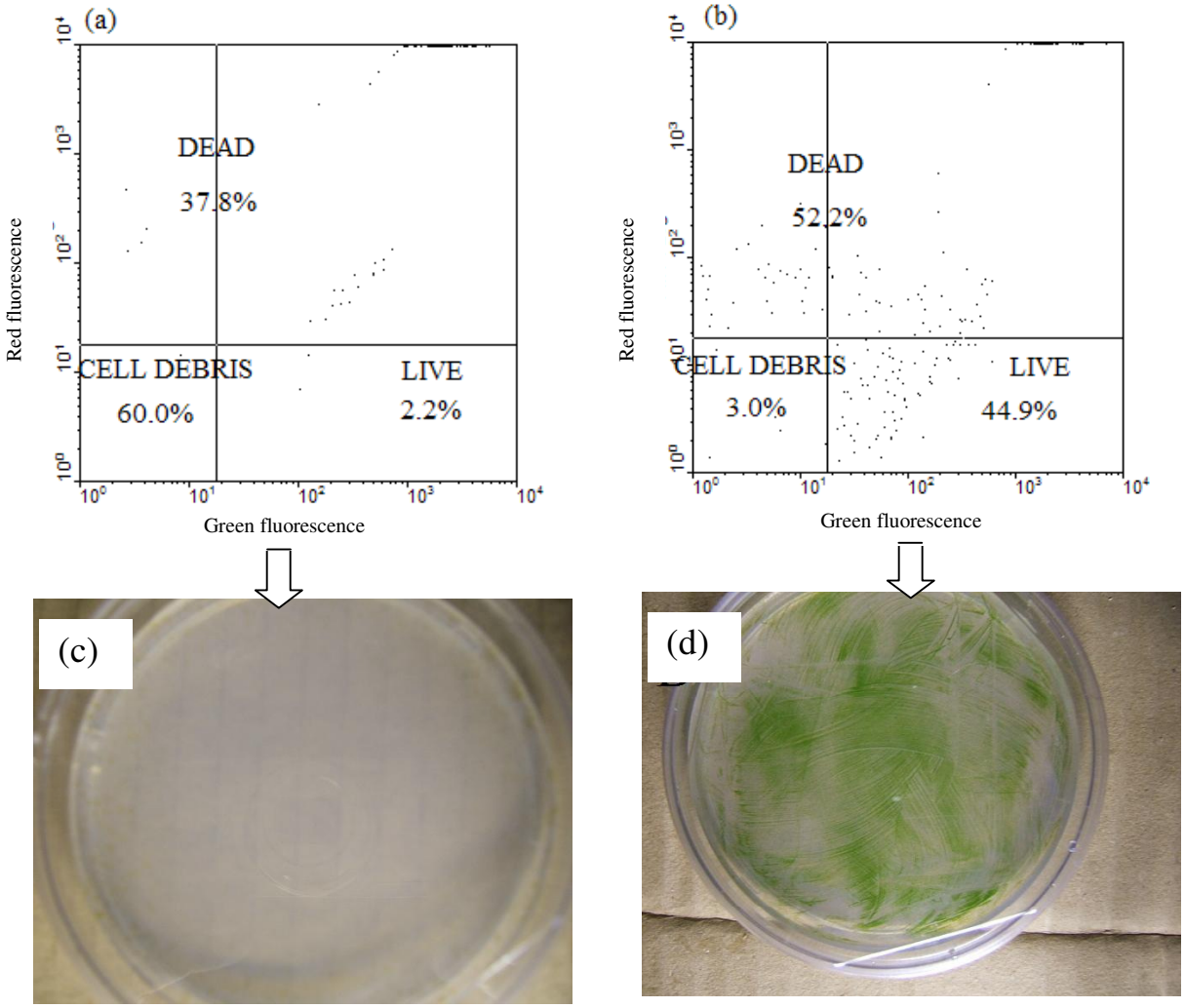

Figure 5.10: A typical two parametric plot illustration of *Microcystis* population heterogeneity on 6 d: (a) *B. mycoides* B16 treated sample and (b) Untreated (control) sample. BG 11 agar plates with (c) No growth of *Microcystis* cells after exposure to *B. mycoides* B16; (d) Control sample showing growth of *Microcystis* cells indicating viability.

The aged *Microcystis* 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 FCM counts revealed that during the first three days, the treated and control samples showed a 10X increase (a positive t value of +1.27; Table 5.2; Figure 5.11) in numbers of live *Microcystis* cells. The statistical comparisons of live *Microcystis* cells mean (treated and control) were not significantly affected ($p > 0.05$; Table 5.2), i.e., the bacteria did not contribute to the death of *Microcystis* cells.

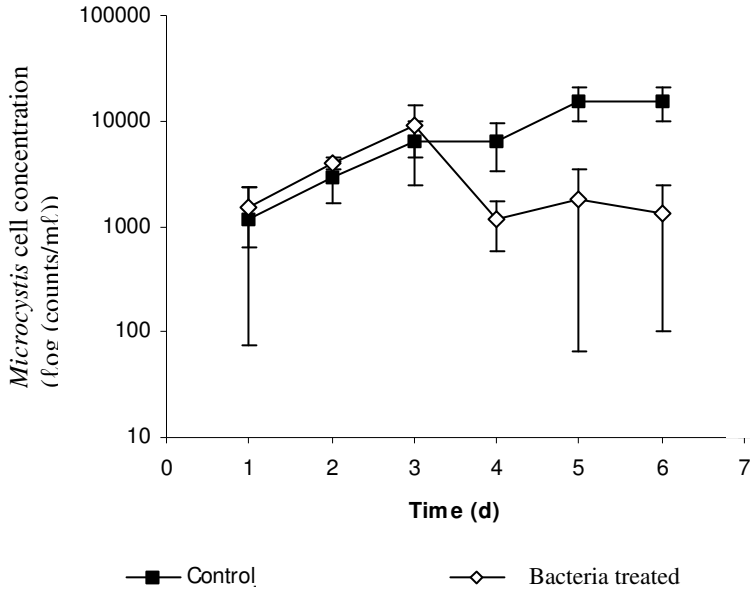

Figure 5.11: Changes in population levels of live *Microcystis* cells in *B. mycoides* B16 treated and control samples under turbulent conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation).

During the same time period, d 1 to d 3, the one sample t-test analysis showed that there was a significant increase in *Microcystis* cell numbers (bacteria treated) ($t = +7.77$; $df = 5$; $p < 0.05$; Table 5.3)(Figure 5.11). 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 5.3)(Figure 5.12).

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 (Figure 5.11). This contrasted with the control samples, which showed an increase of 1-log in live *Microcystis* cell numbers (Figure 5.11). The independent Levene t-test, comparisons of live *Microcystis*

numbers (treated and control) showed significant decrease ($p < 0.05$; Table 5.2), i.e., *B. mycoides* B16 was responsible for lysing the *Microcystis* cells resulting into dead cells.

Table 5.2: 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	0.2342*	-0.16	0.8749*
4	10	-5.30	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.
[1]an increase (positive)
[2]a decrease (negative)

The one sample t-test confirmed the reduced growth in *Microcystis* cell numbers (bacteria treated) was significant ($t = -7.21$; $df = 5$, $p < 0.05$; Table 5.3). This result was in contrast with the control samples, where a 60% increase in *Microcystis* cell numbers was observed in six days (Table 5.3; Figure 5.11). The predator bacteria numbers showed a significant increase ($t = +11.31$: $df = 5$; $p < 0.05$; Table 5.3: Figure 5.13) and coincided with a decrease in *Microcystis* cell numbers. These findings suggest that the *B. mycoides* B16, a heterotroph, was utilizing the *Microcystis* as a source of nutrition. Nakamura et al. (2003a) reported similar findings where *B. cereus* N14, closely related to *B. mycoides* B16 (von Wintzingerode et al., 1997), was able to obtain nutrition solely from *Microcystis aeruginosa* and *M. viridis*. The FCM 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.

Table 5.3: One sample t-test, showing t values and associated (p) probabilities.

Time (d)	df	*Microcystis* (*B. mycoides* B16 treated)			*B. mycoides* B16			*Microcystis* (control)		
		Numbers	t	P	Numbers	t	P	Numbers	t	P
1 to 3	5	Increase	+7.77[1]	0.0006	Increase	+3.30	0.0215	Increase	+7.13	0.0008
4 to 6	5	Decrease	-7.21[2]	0.0008	Increase	+11.31	0.0001	Increase	+3.91	0.0113

[1]an increase (positive)
[2]a decrease (negative)
df = degrees of freedom

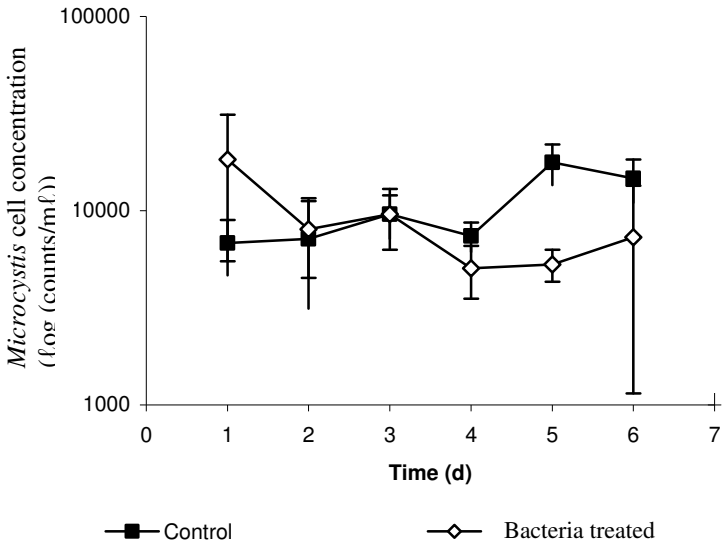

Figure 5.12: Changes in population levels of dead *Microcystis* cells in *B. mycoides* B16 treated and control samples under turbulent conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation).

The dead *Microcystis* cell numbers increased by 30% in bacteria treated samples (Figure 5.12). The independent Levene t-test of dead *Microcystis* cell numbers (treated and

control) showed a significant decrease in treated samples ($p < 0.05$; Table 5.2; Figure 5.12), 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.

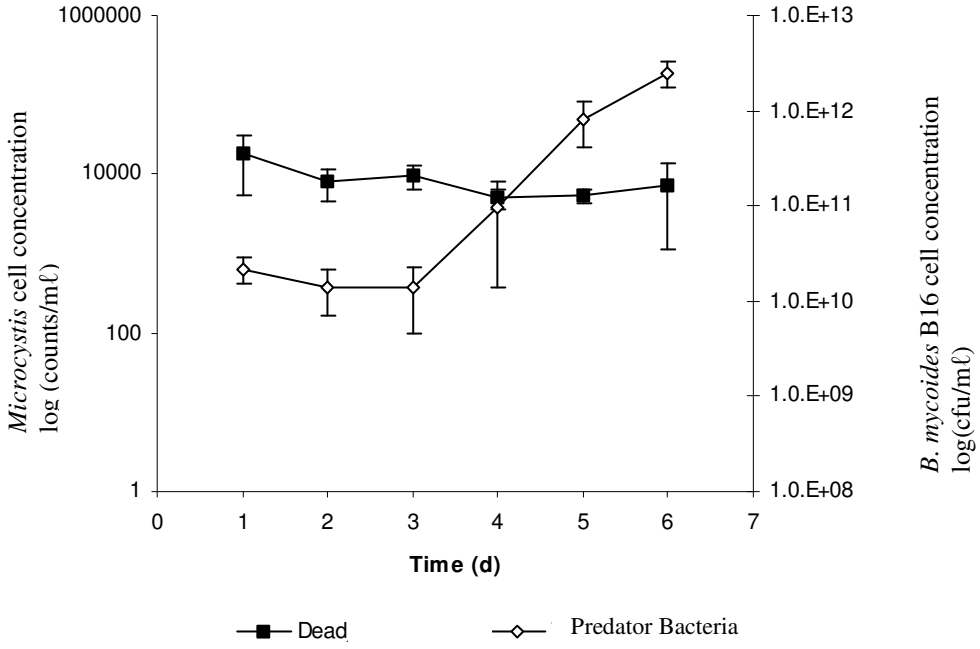

Figure 5.13: Increase in Predator bacteria numbers (colony forming units/mℓ) coincided with the decrease in *Microcystis* cells as indicated by the decrease in PI flow cytometric counts. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation).

The *B. mycoides* B16 managed to reduce the numbers of live *Microcystis* cells by 85% under turbulent conditions (Table 5.2; Figure 5.11) and 97% under static conditions (Table 5.1; Figure 5.8) in six days. These results suggest that *Microcystis* lysis is 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. FCM 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.

5.4. CONCLUSIONS

- FCM was able to successfully assess viable and membrane compromised *Microcystis* cells after 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 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 85% under turbulent conditions and 97% under static 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.