Chapter 3: The isolation and identification of predatory bacteria from a *Microcystis* algal bloom

CHAPTER 3

THE ISOLATION AND IDENTIFICATION OF PREDATORY BACTERIA FROM A *MICROCYSTIS* ALGAL BLOOM

Abstract

Predatory bacteria were isolated from *Microcystis* algal blooms and were evaluated for lytic activity. The hypothesis that the plaque developments on *Microcystis* lawns were due to bacteria and not to cyanophage activity was tested with the chloroform test. The water samples that were treated with chloroform were negative for the presence of plaques. The samples that were positive for the presence of plaques was attributed to the presence of bacteria and not cyanophages. SEM images of the plaques showed the presence five distinct morphotypes of bacteria. The plumb rod-shaped bacilli were the most abundant and were found aggregated around unhealthy *Microcystis* cells and were probably the cause of distortion. In the control there were no plaques except green *Microcystis* lawn and the cyanobacteria cells were healthy and did not show any visible distortion of cell structure. Bacteria were scrapped from the plaque zones and Gram-stained results showed that the bacterial flora was composed of bacilli and coccoids. From the seven isolates, B2 and B16 were selected for further screening for their lytic activity on *Microcystis*. Isolates B16 was a more effective antagonist than B2 causing an 87% and 48% reduction in *Microcystis* biomass in six days respectively. Both bacterial isolates (B2 and B16) were found to be oxidase and catalase positive. This is important as it allows the bacteria to survive under limited oxygen conditions. Isolate B2 was identified as *Pseudomonas stutzeri* with 99.9% certainty and B16 as *Bacillus mycoides* with 99.7% certainty using the API system. The bacteria (1:1) lysed the cyanobacteria and increased in numbers in the absence of an external source of nutrients suggesting that *B. mycoides* B16 utilized *M. aeruginosa* as its sole nutrient source. Predator-prey ratios of 1:100, 1:1000 and 1:10000 did not inhibit the growth of *Microcystis*.

Key words: *Microcystis*, predator-prey ratio, *B. mycoides* B16, *Pseudomonas stutzeri*

3.1. INTRODUCTION

The Hartbeespoort Dam is classified as hypertrophic (WHO, 1999; Van Ginkel, 2002) due to high frequency of *Microcystis* algal blooms, throughout the year. The dam has continued to receive large loads of nutrients wastewater from metropolitan areas of Johannesburg, Midrand and Krugersdorp (NIWR, 1985; Harding et al., 2004). *Microcystis* have been implicated in the production of microcystins, methylisoborneol and geosmin (Codd et al., 1999). The immediate impact is the reduction in user potential, aesthetic value of the lake as a potential tourist destination and is a significant threat to animal and human health (Harding and Paxton, 2001).

Long-term solutions will have to address the causes of algal blooms. The current recommendations include developing strategies for: (1) reducing the external nutrient (phosphorus) inflows to the dam, (2) managing in-lake nutrient availability (both from the water column and from phosphorus-rich sediments); and (3) restructuring the impaired food web structures that no longer supported or provided a natural resilience to the eutrophication process (Harding et al., 2004).

In the natural environment, there are pathogenic or predatory microorganisms that are antagonistic towards particular these organisms (e.g. weeds, cyanobacteria) thus providing a natural means of controlling levels of nuisance organisms. Such antagonistic microbial populations are called microbial herbicides (Atlas and Bartha, 1998). Thus biological control of cyanobacteria provides a potential short-term measure to reduce the population of nuisance algal blooms. The microbial herbicides are often indigenous species of a particular lake environment and have not undergone any gene modification or enhancement (Sigee et al., 1999). For example Ashton and Robarts (1987) isolated a saprospira-like bacterium, *Saprospira albida*, which was indigenous to Hartbeespoort dam. Unfortunately there was no further research to evaluate the biological control potential of *Saprospira albida*. Microbial agents (bacteria, fungi, virus and protozoa) have been isolated from harmful algal blooms (Shilo, 1970; Burnham et al., 1981; Daft et al., 1985b; Ashton and Robarts, 1987; Bird and Rashidan, 2001; Nakamura et al., 2003b; Choi et al., 2005). This is not an exhaustive list of studies pertaining to microbial agents that predate on cyanobacteria.

The studies of Sigee et al. (1999) should be consulted for further information. These microbial agents may play a major role in the prevention, regulation and termination of harmful algal blooms. In many cases these bacterial agents are species- or genus-specific (Bird and Rashidan, 2001), while others attack a variety of cyanobacteria classes (Daft et al., 1975).

Biological control of cyanobacteria like other control measures for nuisance organisms (weeds, insect pests, plant pathogenic bacteria and fungi, etc) are often viewed with caution. This was attributed to the experiences of plant pathologists who observed the destruction of important crops such as chestnut blight in the United States and potato blight in Ireland after the accidental release of pathogens (Atlas and Bartha, 1998).

There are three types of biocontrol strategies, classical, neoclassical and augmentative. The neoclassical biocontrol is a controversial practice of introducing non-indigenous species to control a native pest (Secord, 2003). The classical biocontrol method is the introduction of a natural enemy of the pest in its new range, whereas the augmentative biological control is the practice of enhancing the populations of predators to help in regulating the populations of the pest in its natural habitat. The major goal is not to completely eradicate the pest but rather to keep it suppressed at socially or economically acceptable levels (Secord, 2003). The potential microbial pathogens must have the specific characteristics and attributes for it to be successful in managing harmful algal blooms (Table 3.1). Bacterial pathogens are more potent than viral pathogens in managing HABs (Table 3.1).

Table 3.1: Characteristics of selected microbial herbicides (Daft et al., 1985a)

Attributes	Predator					
	Amoebae	Cyanophages	Myxococci	Lysobacter	Lower Fungi Chytrids	Agents Excreting Extracellular products
1. Adaptability to variations in physical conditions	+++	--	+++	+	++	--
2. Ability to search or trap	+++	+	+++	+	++	++
3. Capacity and ability to multiply	+++	+++	+++	++	+++	+
4. Prey consumption	+++	+++	+++	+	++	+
5. Ability to survive low prey densities	+++	--	+++	+	--	--
6. Wide host range	+++	+	+++	+++	+	++
7. Ability to respond to changes in the host	+++	+	+++	--	--	--

+++ Good

++Fair

+Poor

-- Not known

The objectives of this study were the isolation, culturing and identification of microorganisms that formed plaques on *Microcystis* lawns.

3.2. MATERIALS AND METHODS

3.2.1. Plaque formation on *Microcystis* lawns

Water samples were collected from Hartbeespoort dam in sterile 1ℓ Schott bottles and transported to the laboratory in a cooler box packed with ice. An aliquot (100 µℓ) was spread plated onto modified BG 11 agar plates (Table 3.2) (Krüger and Eloff, 1977). The plates were incubated for 30 d at ambient temperatures (24 – 26°C) under continuous lighting and monitored for plaque development. For continuous lighting, two 18W cool white fluorescent lamps (Lohuis FT18W/T8 1200LM) were suspended above the plates. The light intensity (2000 lux) was measured with an Extech Instruments Datalogging light meter model 401036.

Table 3.2: Mineral composition of modified BG 11 (Krüger and Eloff, 1977).

Component	Final concentration ℓ^{-1}
$NaNO_3$	1.500g
K_2HPO_4	0.040g
$MgSO_4.7H_2O$	0.075g
$CaCl_2.2H_2O$	0.036g
Na_2CO_3	0.020g
$FeSO_4.7H_2O$	0.006g
$EDTA.Na_2H_2O$	0.001g
Citric acid	0.012g
*Agar	12.000g
A5 trace metal solution	1 mℓ
Component (A5 trace metal solution)	**Final concentration g ℓ^{-1}**
H_3BO_3	2.8600
$MnCl_2.4H_2O$	1.8100
$ZnSO_4.7H_2O$	0.2220
$Na_2MoO_4.5H_2O$	0.3900
$Co(NO_3)_2.6H_2O$	0.0494
$CuSO_4.5H_2O$	0.0790

*For liquid media, this was omitted.

3.2.2. Cyanophage check

A chloroform test was carried out to test whether plaque formation was caused by bacteria or cyanophages (Daft et al., 1975; Tucker and Pollard, 2004). 10 mℓ of *Microcystis* sample was mixed with 0.5 mℓ of chloroform and vortex mixed for 5 min. From this mixture, 100 µℓ was spread onto modified BG11 agar plates and incubated at room temperature for 30 d under continuous lightning (2000 lux) and monitored for plaque development. A control sample, lacking chloroform was used and the same procedure was followed. Scanning and transmission electron microscopy were also used to confirm that bacteria were responsible for plaque developments (Chapter 4).

3.2.3. Isolation of predatory bacteria

A sterile loop was used to pick bacteria from the plaque zones and then streaked onto nutrient agar plates (Biolab Merck). The nutrient agar plates were incubated at 37^oC for 24 h and visually inspected for the development of colonies. Colonies were streaked onto nutrient agar until pure cultures were obtained. A total of seven bacterial isolates were obtained and were further subjected to screening for their lytic activity on *Microcystis*.

3.2.4. Lytic activity of bacterial isolates on *Microcystis*

3.2.4.1. Culturing host cyanobacteria

Microcystis aeruginosa PCC7806 was cultured in 500 mℓ Erlenmeyer flasks using modified BG11 medium (Krüger and Eloff, 1977) under shaking incubation (78rpm, 25^oC) for 8 d under continuous light. Two 18W cool white florescent lamps (Lohuis FT18W/T8 1200LM) that were suspended above the flasks provided continuous lighting (2000 lux), being measured by an Extech Instruments Datalogging light meter model 401036. After cultivation the cyanobacteria cell suspensons were used as prey.

3.2.4.2. Culture of bacterial isolates

An inoculum of the *B. mycoides* B16 was cultured in a 250 mℓ Erlenmeyer flask using 100 mℓ of nutrient broth under shaking incubation (128rpm, 37^oC) for 24 h. The process was repeated for other bacterial isolates. After cultivation the bacterial cell suspensons were used as predator bacteria.

3.2.4.3. Culture of *Bacillus mycoides* B16

An inoculum of the *B. mycoides* B16 was cultured in nutrient agar for 12 h. The bacterial colonies were washed off the plate into 10 mℓ Ringer's solution. The cell count was then determined by serial dilution in Ringer's and plating on nutrient agar plates (24 h, 28^oC). The bacterial cell suspensons were used as predator bacteria.

3.2.4.4. Bacterial viable plate count

Samples were homogenized for 20 s to break clumps that formed (Joyce et al., 2003) and then serially diluted in quarter Ringer's solution and were plated on solid media consisting of 10% TSB and with 10% of Agar. The petri dishes (duplicate) were incubated at ambient temperature to simulate experimental conditions for 24 h.

3.2.4.5. Experimental set up

Culture suspensions of bacteria (20 mℓ) and cyanobacteria (20 mℓ) were mixed in a 250 mℓ Erlenmeyer flask. The BG11 control was composed of: 20 mℓ of BG11 medium and 20 mℓ of cyanobacteria suspension whereby no bacteria suspension was added. The flasks were then incubated, without shaking, at room temperature for 10 d under continuous light (2000 lux). On a daily basis, samples (5 mℓ) were removed for cyanobacteria cell counting. On 4 d samples were taken for microscopy analysis. All the experiments and controls were done in duplicate.

3.2.4.6. Cyanobacteria cell counting

The estimation of *Microcystis* biomass was achieved through cell counting (Burnham et al., 1973; Guilard, 1973 & 1978; Smayda, 1978). A Nikon labophot-2 microscope, with a standard bright field 40X objective and a Petroff-Hauser counting chamber were used. The cyanobacteria suspension was diluted with PBS. Phosphate-buffered saline (PBS) was composed of 0.01M Na_2HPO_4:0. 15M NaCl: pH 7.35. The counting of cells was carried out in duplicate.

3.2.5. Identification of predatory bacteria

Gram staining was performed on the bacterial isolates to confirm the purity of cultures. The Gram stains were examined on a Nikon optiphot light microscope with standard brightfield and 100x objective (oil immersion).

For identification and characterization of the bacterial isolates, different approaches were used including: morphology of the colonies, pigmentation, and biochemical properties of bacteria and properties such as sensitivity to different antibiotics. The API 20E, 20NE and API 50CH tests (bioMérieux) were used to identify the bacterial isolates. Hugh-Liefson's O-F, catalase and oxidase tests were performed on the two bacterial isolates to determine which API test to use.

3.2.6. Different predator: prey ratios and their effect on *Microcystis* survival

Treated samples: Serial dilutions (10^{-1} to 10^{-4}) of predator bacteria (Section 3.2.4.3) were made and added to cyanobacteria suspensions (Section 3.2.4.1). For control cyanobacteria samples, no bacteria were added. Cyanobacteria cell counts (in duplicate) were performed after 24, 48 and 72 h followed by counts every three days up to 15 d (Section 3.2.4.6). Duplicate bacterial cell counts were made for the same period (Section 3.2.4.4).

3.3. RESULTS AND DISCUSSION

3.3.1. Cyanophage check

It was assumed that the plaques originated from a single bacterium (Daft et al., 1975; Bird and Rashidan, 2001). Nevertheless a cyanophage activity test was done to confirm that the plaques were not caused by viruses.

After 10 d of incubation a green *Microcystis* lawn was observed in both treated and control samples (Figure 3.1). In the water samples treated with chloroform there were no plaques. In the control samples (no chloroform added) there were plaques present. Chloroform is known to destroy bacteria but not cyanophages (Daft et al., 1975; Tucker and Pollard, 2004). Thus in the treated samples, there were no bacteria or protozoa as these had been destroyed by chloroform that was added. The control samples indicated that the plaque development was due to the presence of bacteria and not cyanophages.
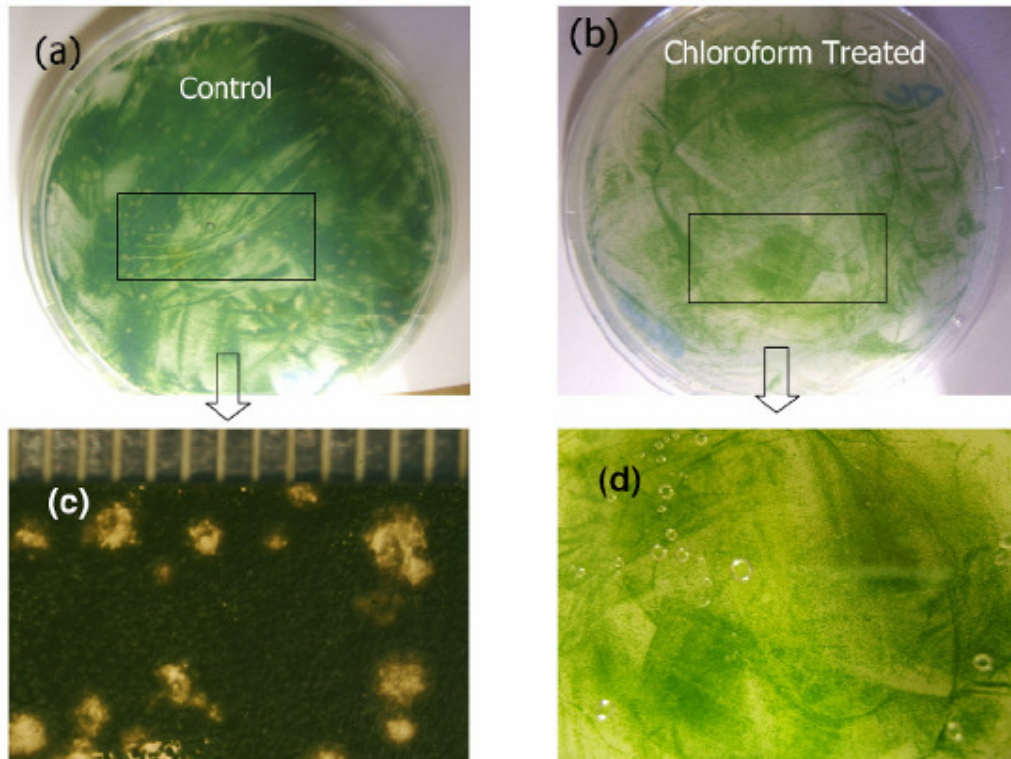
Figure 3.1: Analysis for cyanophage activity on *Microcystis* lawns. (a) Control sample (no chloroform added) showing the development of plaques indicating that bacteria were probably responsible for plaque development. (b) Chloroform treated sample showing the absence of plaque development. (c) Magnification of plaques in (a) and (d) magnification of *Microcystis* lawn in (b).

3.3.2. Plaque formation on *Microcystis* lawns

Plaques appeared on *Microcystis* lawns after 25 to 30 d of incubation (Figure 3.2). The plaque zones were irregular shapes with width ranging from 2 to 8 mm. Using a sterile nichrome wire, bacteria were scrapped from the plaque zones and streaked onto nutrient agar plates. Nutrient agar was the first choice media to use since it's a general-purpose medium for the cultivation of a wide range of bacteria, which are not fastidious in their nutritional requirements.

Bacteria were also scrapped from the plaque zones and Gram-stained to identify what types of bacteria were present. From these staining results it was observed that the bacterial flora was composed of rods and coccoids.
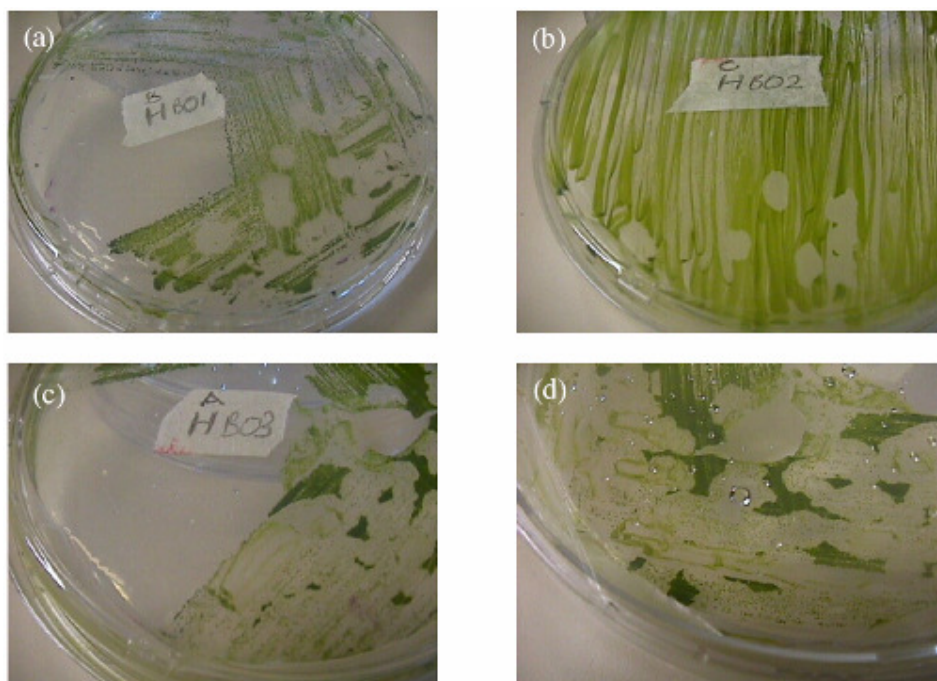
Figure 3.2: Appearance of plaques on *Microcystis* lawns after 30 days of incubation
The samples were obtained different locations at Hartbeespoort dam: from boat pier
(a) HB01; (b) HB02; (c) HB03 and (d) DWAF 2 dam wall.

3.3.3. Isolation of predatory bacteria

An initial twenty-one bacterial isolates, designated B1 to B21, were obtained upon
streaking on nutrient agar. Repeated streaking on nutrient agar and PY agar (10g
peptone, 1g yeast extract and 15g agar in 1ℓ of distilled water, pH 7.0) was carried out
until seven pure colonies were obtained (Table 3.3).

Table 3.3: Basic characteristics of seven bacterial isolates

Sample location	Bacterial isolate	Colony colour	Gram staining	Morphology
HB03	B13	Peach orange	Negative	Rods
HB01	B2.2	Yellow	Negative	Coccoids
HB01	B4.1	Cream/off white	Negative	Rods
HB02	B5	Off-white	Negative	Coccoids
HB02	B9	Light brown	Negative	Rods
*Dwaf 2	B16	White	Positive	Rods
HB01	B2	Gold	Negative	Rods

*Dwaf 2 was Hartbeespoort dam wall.

Other samples were obtained from surface waters off the boat pier.

Daft et al. (1975) showed that lytic bacteria were abundant in surface waters and algal scums of eutrophic freshwaters of Scottish lochs, reservoirs and water treatment works. The present results (Table 3.3) confirmed these earlier findings that algal scum could be the source of lytic bacteria (Daft et al., 1975). The bacterial isolates were re-tested for purity (Gram stained) and the results indicated that the bacterial flora was similar to that found on staining of plaque zones. These staining results showed that either one and or a combination of the bacteria were responsible for plaque development. Consequently the isolates were subjected to screening to evaluate their lytic activity on liquid cultures of *Microcystis*.

3.3.4. Lytic activity of bacterial isolates on *Microcystis*
Bacterial isolates B2 and B16 were used in the screening tests.

3.3.4.1. Effect of isolate B2 on *Microcystis* cells

Isolate B2 caused a 48% reduction in *Microcystis* biomass whereas the control samples showed an 853% increase over a period of 6 d (Figure 3.3). The results showed that there was an increase in *Microcystis* biomass for both samples (control and treated) for the first 4 d. For the control sample there was a slight increase up to d 5 after which the cell numbers increased on d 6.
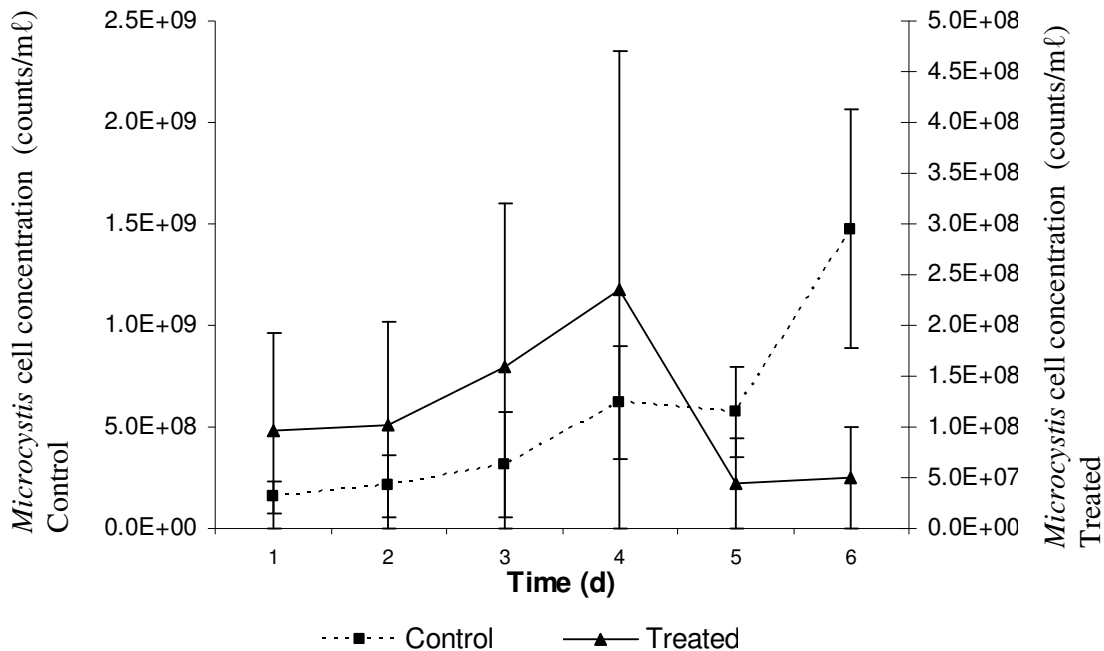
Figure 3.3: *Microcystis aeruginosa* PCC7806 cell counts after exposure to isolate B2. In control samples, changes in cell density of *Microcystis* without bacterial treatment. Bars indicate the standard deviation.

In the treated samples, after d 4, there was a rapid decrease in *Microcystis* biomass (Figure 3.3). The results showed that an initial 2.45 x 10^8 cfu per mℓ isolate B2 was capable of initiating lysis for 1.5 x 10^8 cells per mℓ *Microcystis* cells thus giving a predator to prey ratio of (1.6:1 ≈ 2: 1). This implies that there were slightly more predator cells than prey cells. The question is then why the delay in the lysis of *Microcystis* cells? Presumably during the 'lag phase' the predator bacteria population was adjusting to the new environment before initiating cyanobacterial lysis. Fraleigh and Burnham (1988) observed that the length of the lag phase was inversely proportional to population of predator bacteria, i.e., a low population of predators resulted in a longer lag phase. Perhaps the *Microcystis* adopted a defensive mechanism to ward off the predator by releasing cyanotoxins. Choi et al. (2005) speculated that microcystins are known to inhibit growth of organisms such as cladocerans, copepods, and mosquito larvae and have been shown to be allelopathetic

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towards green alga, *Chlamydomonas neglecta.* However there are no published reports about microcystin toxicity with regards to bacteria (Choi et al., 2005).

The purpose of the daily hand shaking (agitation) was to ensure uniformity of the suspension and prevent the *Microcystis* cells from adhering to the bottom of the flask. This procedure was discontinued for the duration of the experiment. Shilo (1970) and Daft and Stewart (1971) pointed out that agitation of samples might disrupt or disturb the physical contact process between the cyanobacteria and bacteria. This results in a delay in the lytic process. It is therefore speculated that a combination of initial low predator numbers and agitation of culture suspensions were the main reasons for the delay in the lytic process.

3.3.4.2. Effect of isolate B16 on *Microcystis* cells

Isolate B16 caused a 87% reduction in *Microcystis* biomass whereas the control samples showed a 317% increase in *Microcystis* biomass during six days (Figure 3.4). For treated samples, there was an increase in *Microcystis* biomass, as measured from cell counts, after 2 d. For control samples (no bacteria was added), thereafter there was a variable increase in *Microcystis* biomass up to 4 d. After 2 d there was a gradual decline in *Microcystis* biomass for the treated samples. On 5 d the isolate B16 had reduced growth of cyanobacteria cells by approximately 87% (Figure 3.4).
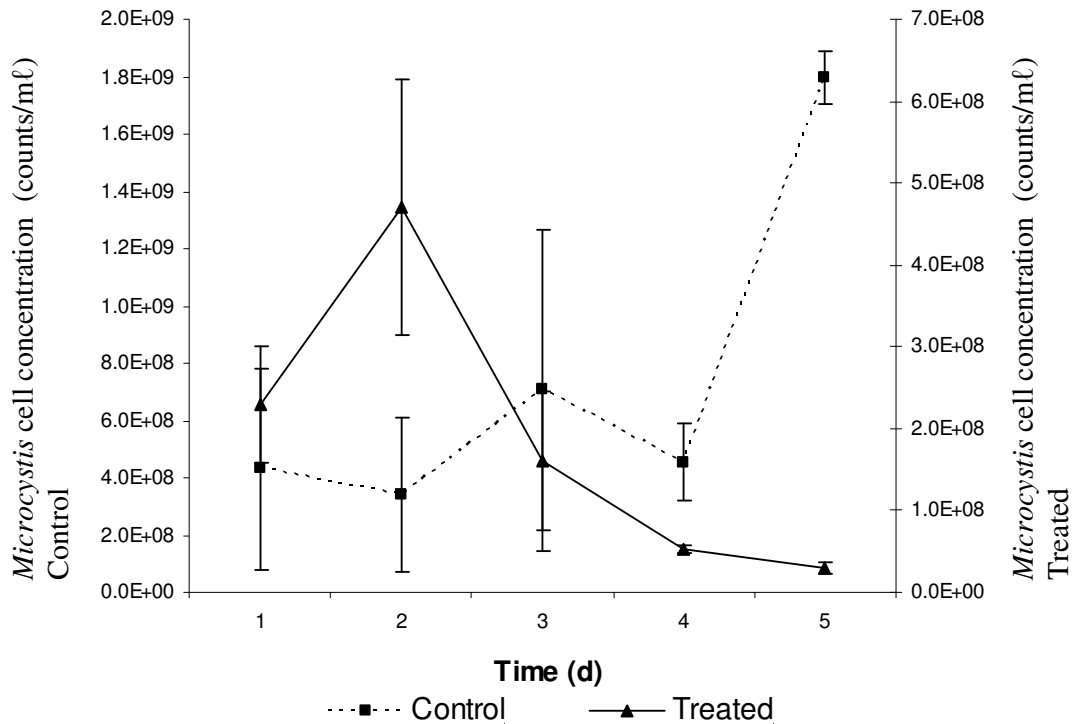
Figure 3.4: *Microcystis aeruginosa* PCC7806 cell counts after exposure to isolate B16. In control samples, changes in cell density of *Microcystis* without bacterial treatment. Bars indicate the standard deviation.

An initial inoculum of 1.00×10^8 cfu per mℓ predator cells caused lysis of 4.3×10^8 cells per mℓ *Microcystis*. The predator-prey ratio was $1:4.3 \approx 1:4$. This implied that there were more prey cells than predator cells. Nevertheless 87% of the prey cells were lysed in 5 days. The daily agitation contributed to the rapid cyanobacteria lysis. Burnham et al. (1981) observed a similar increase in lysis of *Phormidium luridum* by *Myxococcus xanthus* PCO2. The rapid agitation of liquid samples caused a complete lysis of 10^7 cells per mℓ *P. luridum* in 48 h.

Therefore the two bacterial isolates had a lytic effect on the *Microcystis* cells with isolate B16 having a greater effect than isolate B2. The control samples showed an exponential increase in *Microcystis* biomass. Also the mechanism of cyanobacteria cell lysis appears to be different between the two isolates. With isolate B16 the daily hand shaking (agitation) did not result in delayed cyanobacteria cell lysis as with isolate B2. The agitation of the samples resulted in rapid *Microcystis* cell lysis. This

fact is of great importance as in the real world, the water environment is never 'still' but there is continuous mixing (agitation) such that a bacterium that is able to operate under such adverse conditions has the potential for a good biological control product. On the basis of predator-prey ratio isolate B16 had a considerable more lytic effect on *Microcystis* biomass than isolate B2.

3.3.5. Identification of predatory bacteria

Isolates B2 and B16 were cultivated on nutrient agar and the stock cultures were maintained on nutrient agar slants and stored at 4°C. Isolate B2 colonies were golden in colour, compact, small, convex with smooth edges, whereas isolate B16 colonies were white, spreading, and large with irregular edges. Isolate B2 was a Gram-negative rod whilst isolate B16 was a Gram-positive rod (Table 3.4).

Table 3.4: Characteristics of bacterial isolates B2 and B16

Characteristics	Bacterial isolate	
	B2	**B16**
Gram test	Negative	Positive
Rod ends	One end rounded, other is sharp	Both ends rounded
Colony colour	Golden	White
Hugh-Liefson's oxidation/fermentation	+	+
Catalase	+	+
Oxidase	+	+
Glucose as sole carbon source	+	+
Growth on peptone and yeast	+	+
Growth on one tenth tryptone soy	Poor growth	Rapid, spreading
Inhibitory action of antibiotics		
Doxycycline (30 µg/mℓ)	S	S
Gentamicin (40 µg/mℓ)	S	S
Ampicillin (25 µg/mℓ)	R	R

R: resistance, S: sensitive

Both bacterial isolates were oxidase and catalase positive. This aspect is important as it allows the bacteria to survive under aerobic and also under anaerobic conditions as found in *Microcystis* blooms (Zohary, 1987; Zohary and Breen, 1989). This is an advantageous condition for the development of a biological control product, since the

bacteria used for biological control must be able to adapt to all conditions whether aerobic or anaerobic, as there is no external supply of oxygen.

Bacterial isolate B2 was identified as *Pseudomonas stutzeri* with 99.9% certainty and B16 as *Bacillus mycoides* with 99.7% certainty using the API system. Further colony forming tests were carried on bacterial isolates by culturing them on 1.2% agar tryptone soy. The growth of B2 was restricted whereas that of B16 was rapid and spreading covering the petri dish in 10 d. Isolate B16 formed a cotton-like spread colony that was characteristic of wild type *B. mycoides* SIN (Figure 3.5) (Di Franco et al., 2002). There are other wild types of *B. mycoides* DIX where the filament projections curve clockwise. The significance of these filament projects (SIN or DIX) in the lysis of cyanobacteria is unknown at this stage.
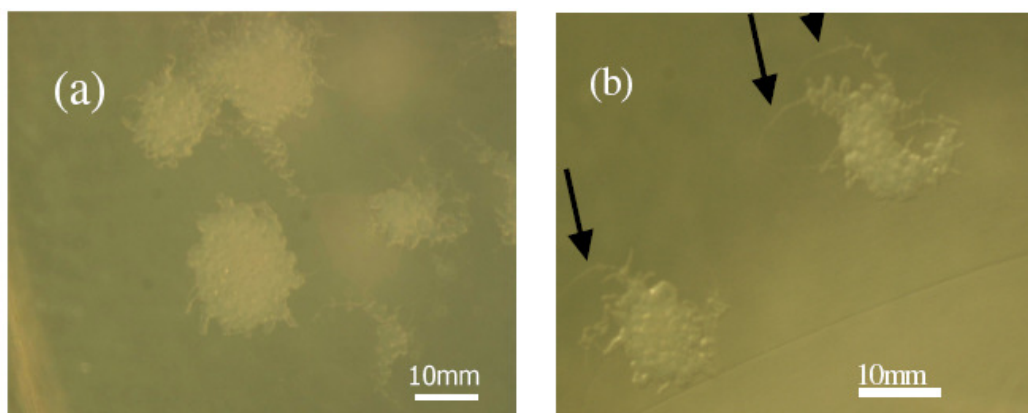

Figure 3.5: (a) Cotton-like spread colonies and (b) *B. mycoides* B16 SIN type. Note the filament projections curve anti-clockwise (black arrow), as observed from the bottom of a petri dish and is classified as SIN.

3.3.6. The effect of different predator-prey ratios on *Microcystis* viability

Various predator-prey ratios were prepared from initial *B. mycoides* B16 and *Microcystis* cell (Table 3.5). The predator bacteria counts were performed on d 3, 6 and 12. For the predator: prey ratios of 1:1 and 1:10 there was an initial

Table 3.5: Different predator: prey ratios

Predator: prey ratio	1:1	1:10	1:100	1:1000	1:10000	Control
Predator cells x 10^7 cfu per mℓ	2.5	2.5	2.5	2.5	2.5	No bacteria
Prey cells x10^7 per mℓ	2.5	250	2500	25000	250000	2.5

Colony forming units (cfu)

lag phase in the first three days, which was followed by an exponential increase in bacteria numbers for the next three days (Figure 3.6). The delay in the lytic activities was due to initial low number of predator bacteria, which had to increase to a certain threshold before the onset of lytic action (Fraleigh and Burnham, 1988). A predator-prey ratio of 1:1 caused a gradual decline in *Microcystis* biomass by almost 50% from 2.09 x 10^7 cells per mℓ to 1.25 x 10^7 cells per mℓ, on d 12 (Figure 3.6a). The 1:10 ratio the initial bacteria population increased from 2 x 10^6 to 5 x 10^6 cfu per mℓ (Figure 3.6b). This ratio managed to inhibit the growth of *Microcystis* cells in comparison with untreated sample.

These results demonstrate that there must be physical contact (with minimum agitation) between predator bacteria and cyanobacteria, as bacteria numbers only started increasing in the 1:1 flask after 3 d. The daily sampling was discontinued in favour of a 3 d sampling because the hand shaking (agitation) may disturb the lytic action thus causing its delay (Shilo, 1970). There must a certain threshold density, probably above 5.2 x 10^7 cfu per mℓ, where the bacteria population must be able to initiate cyanobacteria lysis. The figure of 5.2 x 10^7 cfu per mℓ was arrived at after noticing that it is the bacteria numbers that were able initiate lysis of *Microcystis* (Figure 3.6a). The bacterial numbers increased while cyanobacteria numbers decreased may indicate that the bacteria are utilizing *Microcystis* cells as their only nutrient source as no nutrients were added to the medium. This is of great importance in terms of the development of a biological control product, as no addition of nutrients will be supplied to the bacteria.
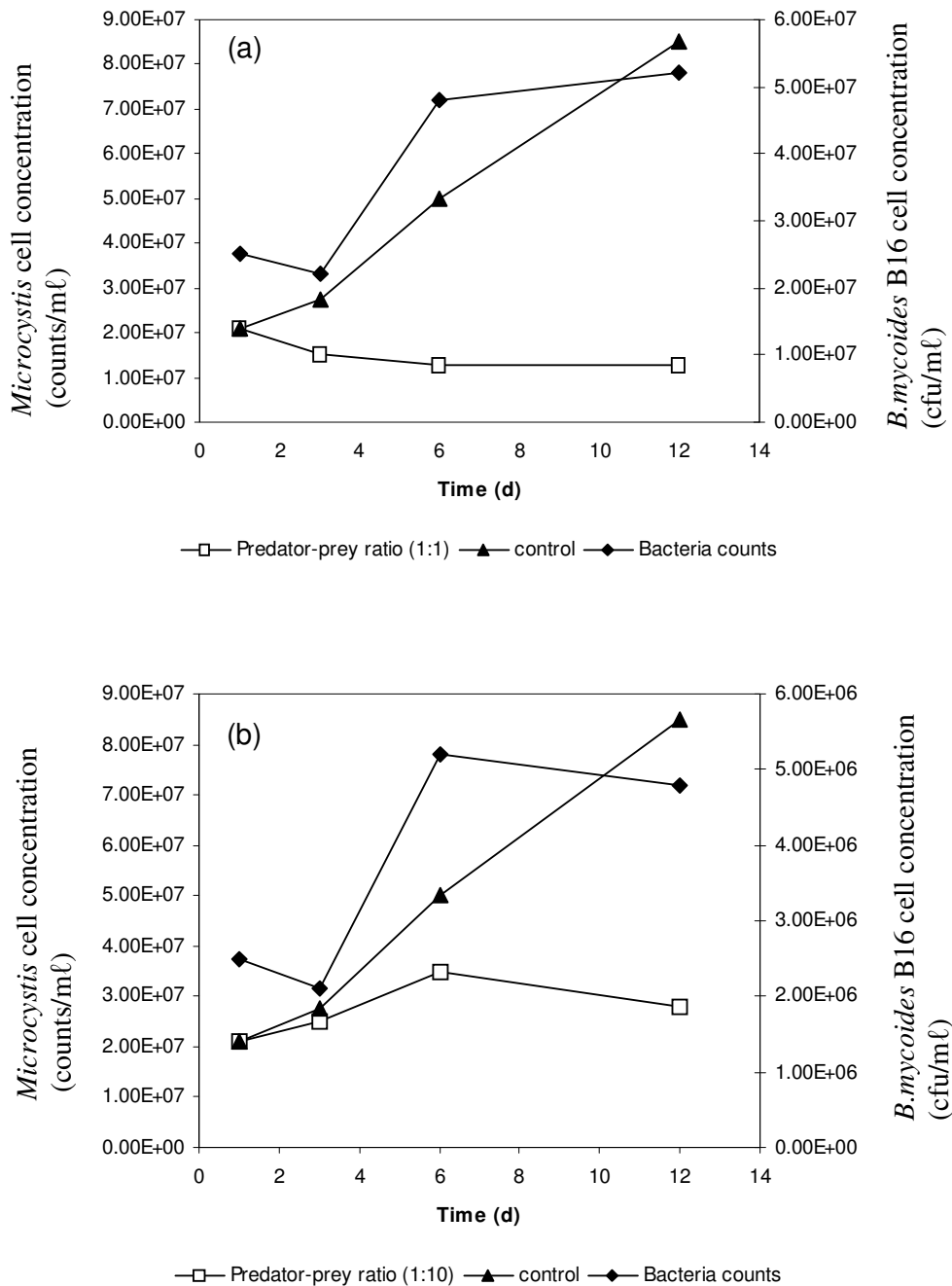
Figure 3.6: The effect of predator-prey ratio on *Microcystis* viability and changes in predator numbers: (a) 1:1 ratio and (b) 1:10 ratio

The predator-prey ratio of 1:100 showed an increase in *Microcystis* biomass up to d 6 that was followed by a decline in d 12 (Figure 3.7). The bacteria numbers increased

up to d 3 before decline on d 6 and then an increase on d 12. At this point it is difficult to explain what caused the erratic changes in the bacteria numbers. With other ratios (1:1000 and 1:10000) the bacteria numbers decreased considerably (Figure 3.8).
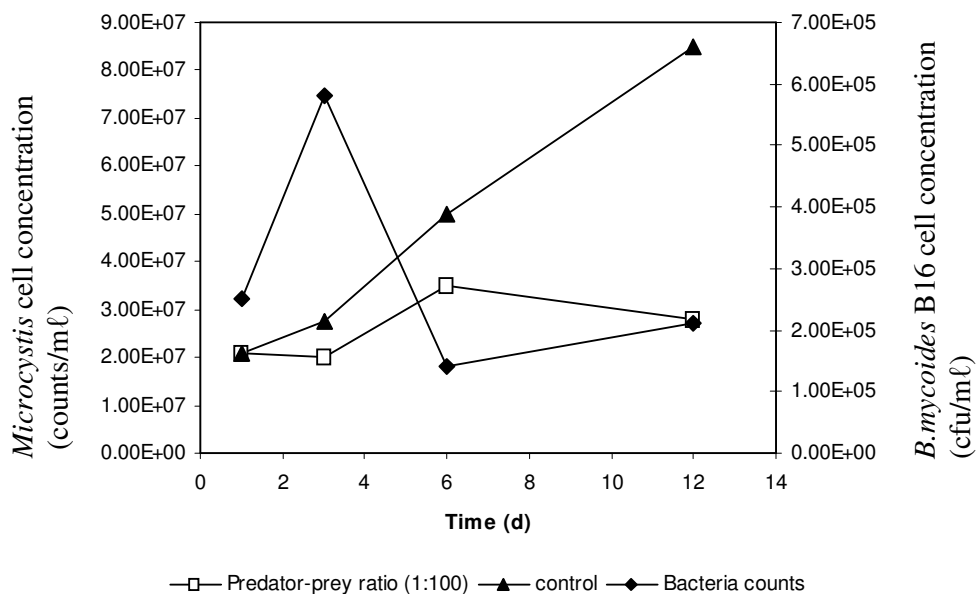

Figure 3.7: The effect of predator-prey ratio (1:100) on *Microcystis* viability and changes in predator numbers

The other predator-prey ratios (1:1000 and 1:10000) showed a gradual increase in *Microcystis* biomass up to d 12 (Figure 3.8). The predator bacteria numbers were very low, 2.5 x 10^3 cfu per mℓ, below the threshold density of 5.2 x 10^7 cfu per mℓ such that the bacteria did not inhibit the growth of *Microcystis* cells.
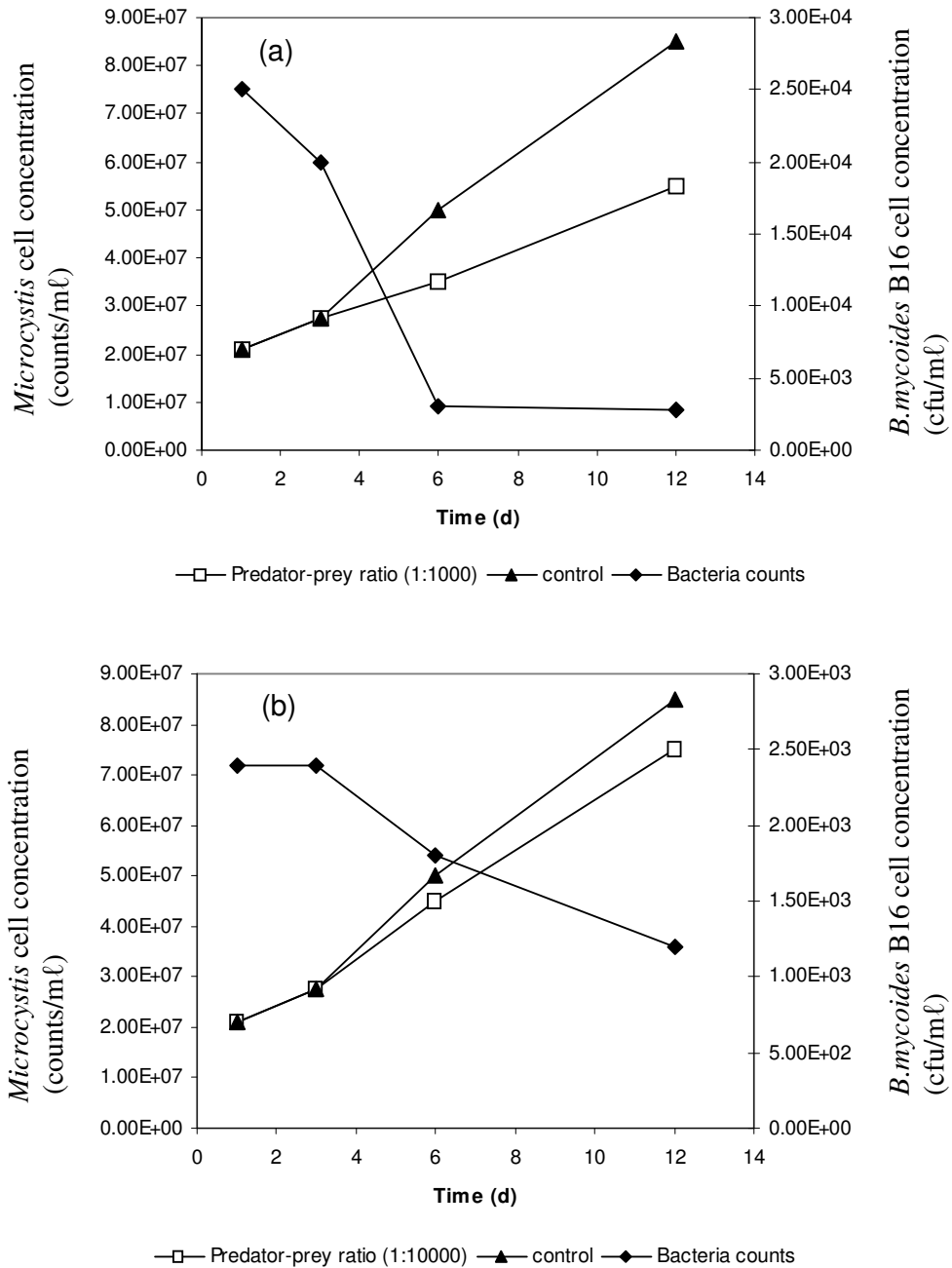
Figure 3.8: The effect of predator-prey ratio on *Microcystis* viability and changes in predator numbers: (a) 1:1000 ratio and (b) 1:10000 ratio

The low population of predator bacteria may help to account for the insignificant biological control of nuisance algal blooms in the natural environment. Fraleigh and Burnham (1988) earlier confirmed this fact that the low predator population could not

survive and increase to a threshold density while feeding on lake inorganic nutrients alone but also required algal carbon (Figure 3.8). This may help to account why the predator bacteria population increases during the bloom period, is partly due to availability of algal carbon. Robarts and Zohary (1986) supported this observation with their studies involving the Hartbeespoort dam hyperscum community, a cyanobacteria-bacteria interactions that reached 10^9 cells per mℓ *Microcystis* cells and bacteria levels of 8 x 10^9 cells per mℓ. The bacteria had more than sufficient inorganic nutrients (phosphates 0.5 mg per ℓ and nitrates, range 1-2 mg per ℓ) but limiting substrate was organic nutrients that only available during the breakdown of the hyperscum (lysis of *Microcystis* cells). Thus the bacteria heterotrophic activity increased sharply after the organic nutrients were available as a result of the breakdown of the hyperscum and then decline thereafter.

In the *Microcystis* (bacteria treated) flasks the adherence to the flasks' bottom was reduced, most noticeable and this coincided with the increase in bacteria population (Figure 3.6). There was *Microcystis* adherence in the control flasks and other predator-prey ratios (1:1000; 1:10000) and this coincided with the decrease in bacteria population (Figure 3.8). Nakamura et al. (2003b) observed that *B. cereus* N14 attached to the surface of *Microcystis* to cause aggregation of the cyanobacteria cells before lysis with extracellular products. In contrast to the observations of Jang et al. (2003) who reported an increase in *Microcystis* colony formation (accompanied by release in microcystins) as a defensive measure against herbivorous zooplanktonic *Daphnia* species. These findings may suggest that there are separate modes of lytic action against *Microcystis* by *Daphnia* species and between *B. mycoides* B16 and *B. cereus* N14.

To our current knowledge this is the first reported case where *Bacillus mycoides* B16 showed lytic activity towards *Microcystis aeruginosa*. A number of *Bacillus* species (*B. pumilis, B. megaterium, B. subtilis,* B. *licheniformes*, *B. brevis* and *B. cereus*) were found to be antagonistic towards *Microcystis aeruginosa* (Reim et al., 1974; Wright et al., 1991; Wright and Thompson, 1985; Nakamura et al., 2003b). These *Bacillus* species, namely *B. pumilis*, *B. megaterium*, *B. subtilis* and *B. licheniformes* have been shown to produce lytic volatile substances (Wright et al., 1991; Wright and Thompson, 1985) that resulted in lysis of the cyanobacteria. In the same manner *B.*

cereus N14 showed a high degree of lytic activity towards *Microcystis aeruginosa and M. viridis* (Nakamura et al., 2003b). In the stationary growth phases of *B. brevis* and *B. cereus* N14 coinciding with sporulation were known to produce unidentified non-proteinaceous, hydrophilic, heat stable substances that were responsible for the *Microcystis* lysis (Reim et al. 1974; Nakamura et al., 2003b).

B. mycoides and *B. cereus* are genetically very closely related with the latter is known to produce an enterotoxin, causing diarrheal-type syndrome and an emetic toxin called cereulide that cause vomiting type syndrome (Nakamura et al. 2003b; Vilain et al. 2006). On the *Approved Lists of Bacterial Names*, *Bacillus mycoides* is classified in the lowest risk group 1 and other species included in this group are *B. thuringiensis*, a well know plant pest control microbial agent (Fritze, 1994). Of interest is that certain strains of *B. cereus* are non-toxigenic and have proven success as animal probiotics and these have been downgraded to risk group 1.

3.4. CONCLUSIONS

- The plaques that appeared on *Microcystis* lawns were attributed to the presence of bacteria and not cyanophages.
- The two bacterial isolates B2 and B16 had a lytic effect on the *Microcystis* cells with isolate B16 having a greater effect than isolate B2.
- Bacterial isolate B2 was identified as *Pseudomonas stutzeri* with 99.9% certainty and B16 as *Bacillus mycoides* with 99.7% certainty. Isolate B16 had characteristics of wild type *B. mycoides* SIN.
- The critical *Bacillus mycoides* B16: *Microcystis aeruginosa*, predator-prey ratio of 1:1 inhibited the growth of the cyanobacteria.
- The other predator-prey ratios (1:10; 1:100; 1:1000; 1:10000) did not inhibit the growth of *Microcystis*.