ANTAGONISM OF *Bacillus* spp. TOWARDS *Microcystis aeruginosa*

Jabulani Ray Gumbo

Submitted in fulfilment of part of the requirements for the degree of

Philosophiae Doctor

In the Department of Microbiology & Plant Pathology,
Faculty of Natural and Agricultural Sciences,
University of Pretoria,
Pretoria

November 2006

Promoter: Prof T.E. Cloete

Table of Contents

DECLARATION

I declare that the thesis, which I hereby submit for the degree *Philosophiae Doctor* at the University of Pretoria, Pretoria has not been previously submitted by me for a degree at another university.

_____ _____

J. R. Gumbo Date

SUMMARY

Freshwater resources are threatened by the presence and increase of harmful algal blooms (HABs) all over the world. The HABs are sometimes a direct result of anthropogenic pollution entering water bodies, such as partially treated nutrient-rich effluents and the leaching of fertilisers and animal wastes. *Microcystis* species are the dominant cyanobacteria (algae) that proliferate in these eutrophic waters. The impact of HABs on aquatic ecosystems and water resources, as well as their human health implications are well documented. Countermeasures have been proposed and implemented to manage HABs with varying levels of success. These control measures include the use of flocculants, mechanical removal of hyperscums and chemical algicides. The use of flocculants such as Phoslock™ is effective in reducing the phosphates in a water body thus depriving nutrients that are available to cyanobacteria. The mechanical option entails the manual removal of hyperscums thus reducing the numbers of cyanobacteria cells that may be the inoculum of the next bloom. The major disadvantage of these two measures is cost. Copper algicides have been used successfully to control HABs in raw water supplies intended for potable purposes. The major disadvantages are copper toxicity and release of microcystins from lysed cyanobacteria cells. Algicides accumulate in the sediments at concentration that are toxic to other aquatic organisms and may also cause long-term damage to the lake ecology. In some studies, microcystins have been implicated in the deaths of patients undergoing haemodialysis. Therefore there is an increasing need to reduce the use of chemicals for environmental and safety reasons. Thus, the development of environmentally friendly; safe non-chemical control measures such as biological control is of great importance to the management of HABs. Some papers, describe bacteria, which were isolated from eutrophic waters, such as *Sphingomonas* species with abilities to degrade microcystins and *Saprospira albida* with abilities to degrade *Microcystis* cells. Further research is required to evaluate whether these bacteria are antagonistic towards cyanobacteria. Ideally, a combination of strategies should be introduced; that is, combine predatory bacteria that directly lyse the cyanobacteria with microcystin degrading bacteria that then 'mop up' the released microcystins.

The major objective of this study was to isolate organisms that have a similar antagonistic properties; determine their mechanism of action and then develop a model to account for the interaction between the predator and prey as the basis for the development of a biological control agent.

During the screening for lytic organisms from eutrophic waters of Hartbeespoort dam, seven bacterial isolates were obtained. Based on electron microscope observation, two of the isolates were found aggregated around unhealthy *Microcystis* cells. These were identified as *Pseudomonas stutzeri* strain designated B2 and *Bacillus mycoides* strain designated B16. Based

on efficiency and efficacy experiments *B. mycoides* B16 was a more effective antagonist than *P. stutzeri* B2. Furthermore the *B. mycoides* B16: *Microcystis* critical ratio was found to be 1:1 in 12 days. Thus altering the predator-prey ratio by increasing the predator bacteria numbers reduced the *Microcystis* lysis time to six days. The *B. mycoides* B16 managed to reduce the population of alive *Microcystis* cells by 85% under turbulent conditions and 97% under static conditions in six days. The increase in predator bacteria numbers coincided with a decrease in growth of *Microcystis*.

The study on the interactions of *Microcystis aeruginosa* and *Bacillus mycoides* B16 indicated a series of morphological and ultrastructural changes within the cyanobacteria cell leading to its death. These are summarised in a conceptual model that was developed. The predatory bacteria, *B. mycoides* B16 attached onto the *Microcystis* cell through the use of fimbriae and or exopolymers. During this attachment the bacteria released extracellular substances that dissolved the *Microcystis* cell membrane and interfered with the photosynthesis process. The presence of numerous bacterial cells that aggregated around *Microcystis* cell provided a 'shade' that reduced the amount of light (hv) that reached the *Microcystis* cell. In response to these adverse conditions, the *Microcystis* cell did the following: It expanded its thylakoid system, the light harvesting system, to capture as much light as possible to enable it to carry out photosynthesis and it accumulated storage granules such as phosphate bodies, glycogen and cyanophycin and swollen cells. Other researchers have also reported the swelling phenomenon prior to cell lysis but did not account for what might be the cause. During the course of the lysis process the *Microcystis* cell underwent a transition stage that involved changes from alive (with an intact membrane) to membrane compromised (selective permeability), to death (no membrane) and eventual cell debris. Due to the depleted *Microcystis* cells, the *B. mycoides* B16 (non-motile, non-spore former) formed chains, i.e., exhibited rhizoidal growth in search of new *Microcystis* cells to attack.

In conclusion, the present evidence in this study suggests that *B. mycoides* B16 is an ectoparasite (close contact is essential) in its lysis of *Microcystis aeruginosa* under laboratory conditions. These findings that *B. mycoides* B16 is a predatory bacterium towards *Microcystis aeruginosa* need to be further evaluated under field conditions in mesocosm experiments (secluded areas in a lake) to determine the possibility of using this organism as a biological control agent.

ACKNOWLEDGEMENTS

I would like to acknowledge the contributions of the various persons and organisations towards the successful completion of this study.

- My promoter, Prof T.E. Cloete for his guidance, support, encouragement throughout my study tenure. Many thanks for the final review of my thesis.
- National Research Fund for funding the project and financial support through the Grant Holder Linked fellowship.
- University of Pretoria for financial support through the Post-Graduate Bursary.
- Water Research Fund for Southern Africa for initial financial support.
- Mr Allan Hall, Laboratory of Microanalysis and Microscopy, University of Pretoria for assistance electron microscopy, constructive ideas and directions. 'Bugs do not think'.
- Prof GJJ van Zyl and Ms Jaqui Sommerville, Department of Statistics, University of Pretoria with assistance with analysis and statistics, research design and constructive ideas.
- Prof R. Anderson, Dr R. Cockeran, Dr H Steel, Department of Immunology, University of Pretoria with technical assistance with flow cytometry and constructive ideas.
- Dr Tim Downing, Nelson Mandela Metropolitan University for provision of *Microcystis aeruginosa* PCC7806.
- Ms Van Ginkel of DWAF for the provision of water samples from Hartbeespoort dam.
- Ms G. Ross carried out the identification of isolates B2 and B16 and predator prey ratio experiments as part of her BSc (Hons) degree in the Department of Microbiology and Plant Pathology. Many thanks.
- Fellow students and staff of the Department of Microbiology and Plant Pathology, for unselfish assistance rendered during this study.
- Ms Sandra van Wyngaardt of the Department of Biochemistry, for unselfish assistance rendered during this study
- Our parents for their unfailing support, inspiration and encouragement during my career.
- I am grateful for the love, trust and understanding of Tanatsa Gumbo, Tendai Gumbo and Farai Gumbo and Ms Prisca Mutsengi.
- I am indebted to special friends Dr Maryam Said and Dr Thantsha Mapitsi for endless constructive discussions, ideas and support.
- My heartfelt gratitude to friends and family. Thank you. God bless.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABSA	American Biological Safety Association
BCECF-AM	2',7',-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester
BCECF	2',7',-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein
Calcein-AM	Acetoxymethyl ester
CDC	Centre for Diseases Control
CFDA	Carboxyfluorescein diacetate
CFDA-AM	Carboxyfluorescein diacetate acetoxy methyl ester
CTC	5-cyano-2,3,-ditolyl tretazolium chloride
CSE	Chemunex, Maisons-Alfort, France
CYN	cylindrospermopsin
DiOC6	3,3′-dihexyloxacarbocyanine iodide
DiBAC4	bis-(1,3-dibutylbarbituric acid) trimethine oxonol
DEAT	Departments of Environmental Affairs and Tourism
DWAF	Department of Water Affairs and Forestry
DWAF, RQS	Department of Water Affairs and Forestry, Resource Quality Services
DWA	Department of Water Affairs
EA	ENVIRONMENTAL AUTHORISATION
EEC	European Economic Community
FDA	fluorescence diacetate
FITC	fluorescein isothiocyanate
FISH	fluorescent *in-situ* hybridisation
FSC	forward scatter
Geosmin	trans-1, 10-dimethyl-trans-9-decalol
GMOA	Genetically Modified Organisms Act (Act 15 of 1997)
HAB	Harmful algal blooms
HRE	Host range expansion
HS	Host switching
HWAG	Hartbeespoort Water Action Group
LPS	Lipopolysaccharides
Microcystins-LR	Microcystins- (L for leucine and R for arginine)
MC	microcystins
2-MIB	2-methyl isoborneol
MRC	South Africa Medical Research Council

NDA	NATIONAL DEPARTMENT OF AGRICULTURE
NH_4	ammonium
NO_x	nitrates/nitrites
NEMA	National Environmental Management Act (Act 107 of 1998)
NEMBA	National Environmental Management: Biodiversity Act (Act 10 of 2004)
NEMP	National Eutrophication Monitoring Program
NWA	National Water Act (Act 36 of 1998)
NIWR	National Institute of Water Research
NIH	National Institute of Health
NHMRZ/ ARMCANZ	National Health and Medical Research Council, Agriculture and Resource Management Council of Australia and New Zealand
PSP	Paralytic shellfish poisons
PO_4P	phosphates
P	Phosphate levels
PAR	photosynthetically available irradiance
PI	propidium iodide
PMT	photomultiplier tube
PS II	photosystem II
Reglone A	diquat, 1,1-ethylene-2, 2-dipyridilium dibromide
Rh123	rhodamine 123
SEM	scanning electron microscopy
Simazine	2-chloro-4,6-bis(ethylamino)-s-triazine
SRP	soluble reactive phosphorus
TEM	transmission electron microscopy
TP	Total phosphorus
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
WTP 1	WATER TREATMENT PLANT NUMBER 1
WTP 2	Water treatment plant number 2
WHO	World Health Organization

PUBLICATIONS AND PRESENTATIONS

Published articles

1. Gumbo JR, Cloete TE, and Hall AN, (2006). Elucidation of the mechanism of cyanobacteria lysis of *Microcystis* after exposure to *Bacillus mycoides.* Proceedings of the *Microscopy Society of Southern Africa.* 36: 38.

2. Gumbo JR, Cloete TE, and Hall AN, (2004). The Algicidal effect of predatory bacteria on *Microcystis aeruginosa*. Proceedings of the *Microscopy Society of Southern Africa. 34: 34.*

Peer-reviewed conference proceedings

Gumbo JR, and Cloete, TE, (2007). Preliminary assessment of *Bacillus mycoides* as a biological control agent for *Microcystis* blooms in Harmful Algae 2007. **Accepted** for publication in Proceedings of the XIIth International Society on the Study of Harmful Algae, Conference.

Articles submitted for publications

1. Gumbo JR, Ross G, and Cloete, TE, (xxxx). Biological Control of *Microcystis* dominated harmful Algal Blooms. **Submitted** to the Journal of Harmful Algae.

2. Gumbo JR, Ross G, and Cloete, TE, (xxxx). The isolation and identification of predatory bacteria from a *Microcystis* algal bloom. **Submitted** to the Journal of Water SA.

Articles in preparation

1. Gumbo JR, and Cloete, TE, (xxxx) Chapter 4: Electron Microscope Assessment of the lytic activity of bacteria on *Microcystis.* In preparation.

2. Gumbo JR, Cloete, TE, Van Zyl GJJ, Sommerville J, (xxxx) Chapter 5: Flow cytometry measurements on Microcystis cells after exposure to predatory bacteria. In preparation.

Published abstracts, oral and poster presentations at conferences

1. Gumbo JR, and Cloete TE, (2006). A flow cytometric technique to assess viable and membrane compromised cells of *Microcystis aeruginosa* upon predation by a biological control agent: *Bacillus mycoides*. (Oral presentation). International Conference and Exhibition on Water in the Environment. 20-22 February. Stellenbosch, South Africa.

2. Gumbo JR, and Cloete TE, (2006). A flow cytometry technique to assess viability of *Microcystis aeruginosa* cells following bacterial infection. (Oral presentation). The 14th Biennial Congress of the South African Society for Microbiology. 10-12 April. Pretoria, South Africa.

3. Gumbo JR, and Cloete TE, (2006). Flow cytometry in conjunction with dual staining assesses viability of *Microcystis* cells after exposure to bacteria. (Poster presentation). The 12th International Conference on Harmful Algae. 4-8 September. Copenhagen, Denmark.

4. Gumbo JR, Cloete TE, and Hall AN, (2006). Elucidation of the mechanism of cyanobacteria lysis of *Microcystis* after exposure to *Bacillus mycoides.* Proceedings of the *Microscopy Society of Southern Africa.* 36: 38. (Oral presentation). 29th November to 1st December. Port Elizabeth, South Africa.

5. Gumbo JR, and Cloete TE, (2004). Bacterial Predation on Harmful Algal Blooms: An Alternative Biological Control Option? XI Harmful Algal Bloom (HAB) Conference in Cape Town, South Africa. 14-19 November 2004.

6. Gumbo JR, Cloete TE, and Hall AN, (2004). The Algicidal effect of predatory bacteria on *Microcystis aeruginosa*. Proceedings of the *Microscopy Society of Southern Africa. 34: 34.* (Oral presentation). 30th November to 1st December. Pretoria, South Africa.

7. Gumbo JR, Emslie L, Cloete TE, (2003). Control of cyanobacteria through lytic bacterial/ cyanobacterial interaction. IWA Conference Water: Key to Sustainable Development in Africa. Cape Town, South Africa. 14 – 19 September 2003.
www.iwaconferences.co.za/abstracts/waterp/abstract%20Emslie%20Gumbo%20Cloete.doc

Awards

Second best student poster at The 12th International Conference on Harmful Algae. 4-8 September, 2006. Copenhagen, Denmark.

Chapter 1: Introduction

CHAPTER 1

INTRODUCTION

The freshwater resources in South Africa are a finite resource, which must be conserved for sustainable use and development. The country has an annual average rainfall of approximately 464 mm, which is half of the global average of 860 mm (Godden, 2005). The rainfall distribution is rather skewed with 85% of country receiving an annual rainfall of less than 500mm (Richard and Poccard, 1998) and 20% receiving less than 200mm (Godden, 2005). The water quality of some of the freshwater impoundments has continued to deteriorate over the years through pollution and nutrient enrichment (eutrophication) (Scott, 1991; Harding et al., 2001; Van Ginkel, 2002).

Eutrophication is a natural process or a human-induced activity that leads to the nutrient enrichment of water bodies with nitrates and phosphates, which in turn promote the excessive growth of aquatic weeds and cyanobacteria blooms (Codd, 2000). As a natural process the ageing of freshwater body may take thousands of years to occur. The natural process involves the following succession: from an oligotrophic (low in productivity and abundance in biodiversity of species) through to mesotrophic (moderate productivity and high species abundance) to eutrophic (high productivity and high species abundance but low in species diversity). The other extreme end of eutrophic conditions is known as hyper-eutrophic (Van Ginkel, 2002).

The Department of Water Affairs and Forestry (DWAF) as the legal custodian for the management of water resources in South Africa, as stipulated in the National Water Act, No. 36 of 1998 has established a National Eutrophication Monitoring Program (NEMP) to assess how spread is the problem of eutrophication in the country's freshwater resources (Figure 1.1). The Hartbeespoort dam is located in the North West Province of South Africa. It is one of the freshwater impoundments that are monitored as part of the NEMP. The dam has continued to receive large loads of urban runoffs and wastewater effluent from Johannesburg, Midrand and Krugersdorp. The effluents are rich in phosphates, ammonia and nitrates and have contributed to

eutrophication and are directly responsible for the proliferation of *Microcystis* algal blooms.

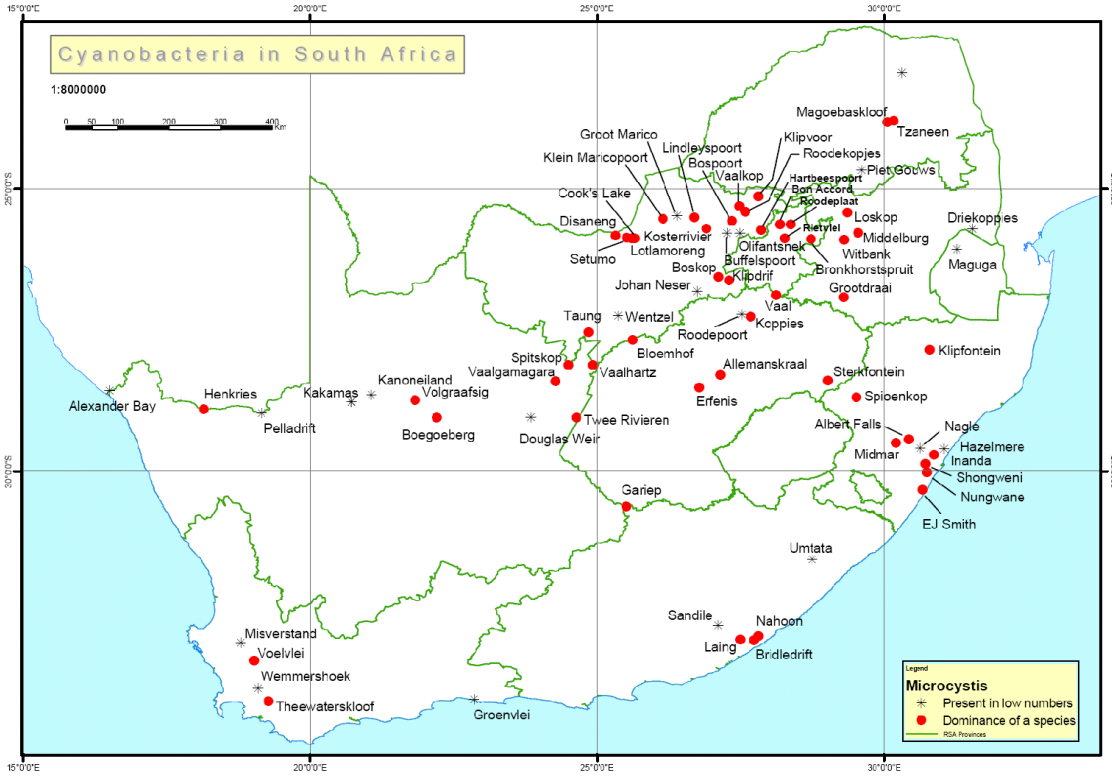


Figure 1.1: Distribution of *M. aeruginosa* algal blooms in South Africa (Van Ginkel, 2003).

The control measures such as mechanical harvesting (Harding et al., 2004) and use of chemical flocculants such as Phoslock™ (Greenop and Robb, 2001; Robb et al., 2003) have been attempted to manage the harmful algal blooms (HABs). These methods controlled the HABs through nutrient precipitation (depriving cyanobacteria of nutrients) and cell coagulation (removal of intact cells) but did not cause significant increase in microcystins (Lam et al., 1995). The major limitation for daily use has been their prohibitive cost.

The chemical use of copper algicides has been the first choice of managing *Microcystis* algal blooms that threaten raw water supplies that are intended for potable purposes (Lam et al., 1995; García-Villada et al., 2004). However, there are increasing demands to reduce the use of chemicals for environmental and safety

reasons (Mason, 1996). During the *Microcystis* lysis induced by copper there is release of microcystins into surrounding water. These microcystins presented health hazards to livestock and humans using the water for consumption (WHO, 1999). Thus the development of non-chemical control measures such as biological control is of great importance to the management of harmful algal blooms.

The biological control method is based on predatory bacteria, which are antagonistic towards *Microcystis*. These predatory bacteria have been isolated from the *Microcystis* algal blooms and are indigenous to the lake environment, thus providing an environmentally friendly solution. Other microbial agents such as fungi, virus and protozoa have been isolated from HABs (Shilo, 1970; Burnham et al., 1981; Ashton and Robarts, 1987; Yamamoto et al., 1998; Walker and Higginbotham, 2000; Bird and Rashidan, 2001; Nakamura et al., 2003a; Choi et al., 2005). In many cases these microbial agents are species- or genus-specific (Bird and Rashidan, 2001), while others attack a variety of cyanobacteria classes (Daft et al., 1975).

Other researchers have isolated and identified a *Sphingomonas* species and a strain of *Pseudomonas aeruginosa* that are capable of degrading microcystins. From the predatory bacteria, other studies have isolated and purified extracellular lysozyme that inhibited the growth of the cyanobacterium, *Oscillatoria williamsii* (Sallal, 1994).

Wright and Thompson (1985) isolated three *Bacillus* species from garden compost in Bath, Britain. Two of the strains were identified as *B. licheniformis* and *B. pumilis.* The *Bacillus* species produced volatile substances that inhibited the growth of the filamentous cyanobacterium, *Anabaena variabilis*. Nakamura et al. (2003a) isolated *Bacillus cereus* N14 from a eutrophic freshwater lake in Japan. *B. cereus* N14 released unidentified protease substance that inhibited the growth of *Microcystis* species. The bacterium *Saprospira albida* isolated from Hartbeespoort dam, a eutrophic freshwater reservoir, was observing lysing the cyanobacterium, *Microcystis aeruginosa* (Ashton and Robarts, 1987). However there was no further research carried out to evaluate its biological control potential.

In my doctoral studies, I hypothesized that there were predatory bacteria that are antagonistic to *Microcystis aeruginosa* that are naturally occurring in the Hartbeespoort dam. The major objectives of the research study were:

- To isolate and characterize the predatory bacteria that were antagonistic towards *Microcystis*;
- To determine the mechanism of lysis involved during the contact between the predator and prey and
- To assess the efficiency and efficacy of predatory bacteria against *Microcystis* under laboratory conditions.

Chapter 2: Literature Review

CHAPTER 2

LITERATURE REVIEW

Abstract

Freshwater resources are threatened by the presence and increase of harmful algal blooms (HAB) all over the world. The HABs are sometimes a direct result of anthropogenic pollution entering water bodies, such as partially treated nutrient-rich effluents and the leaching of fertilisers and animal wastes. The impact of HABs on aquatic ecosystems and water resources, as well as their human health implications are well documented. Countermeasures have been proposed and implemented to manage HABs with varying levels of success. The use of copper algicides, though effective in managing HABs, often results in negative impacts such as copper toxicity and release of microcystins into surrounding water after cyanobacterial lysis. Biological control of HABs presents a possible solution. Various mechanisms of cyanobacterial lysis have been proposed, including; physical contact between prey and predator, release of extracellular substances, entrapment of prey by the predator followed by antibiosis and endoparasitism or ectoparasitism of the host by the predator. Despite an increasing amount of work being done in this field, research is usually limited to laboratory cultures; assessment of microbial control agents is seldom extrapolated to field conditions.

Bacillus mycoides is closely related, with minor phenotypic differences, to *B. cereus*, *B. thuringiensis*, and *B. anthracis* based on the classified in the 16S rRNA/DNA group 1. The phenotypic differences are that *B. cereus* and *B. thuringiensis* are usually motile and whilst other species *B. cereus* (motile), *B. thuringiensis* (motile) and *B. mycoides* (non motile) are described as haemolytic and penicillin resistant. On the *Approved Lists of Bacterial Names* and WHO classification, *Bacillus mycoides* is classified under the genus *Bacillus*, in-group 1 together with *B. thuringiensis*, group 2 species (e.g. *B. cereus*) and highly pathogenic risk group 3 (*B. anthracis*). Therefore *B. mycoides* is classified in the lowest risk group 1 under the *Approved Lists of*

Bacterial Names and the bacterium is emerging as a biological control for a number of nuisance organisms.

Flow cytometry is now an established method for the direct numeration of *individual* cell numbers, cell size distribution and cell complexity (biochemical and physiological) in aquatic and environmental microbiology. To date the flow cytometry has been applied to phytoplankton and bacterioplankton studies but other organisms such as protozoa and viruses the studies are still in the infancy. Flow cytometry focuses on the use of this method in the viability analysis of phytoplankton, algae and cyanobacteria, in particular *Microcystis aeruginosa*, as it assesses the metabolic functions.

There are fluorescent dyes that are specific for cellular substances and are used to study a particular cellular function or process. The most common dyes are nucleic acid stains and have a wider application. These include the determination of cell viability; bacterial respiration activity using CTC; cell membrane potential using rhodamine 123 (Rh123); characterization of both polyclonal and monoclonal antibodies raised by toxic dinoflagellates; Also there are fluorescent dyes that evaluate cellular activity stains such as fluorescence diacetate (FDA); protein stains such as SYPRO; nutrient enrichment, copper toxicity, turbulence, acid mine drainage exposure and viral infection.

Keywords: Biological control, *Microcystis aeruginosa*, harmful algal blooms, *Bacillus*, Flow cytometry, fluorescent stains

2.1. INTRODUCTION

The enrichment of dams and lakes with nutrients is the major cause of eutrophication of freshwater sources. Nutrient enrichment is usually by nitrogen and phosphorus compounds, either from point sources such as the inflows of storm water drainage, industrial effluents, municipal wastewater and sewage effluents or non-point sources such as inorganic fertilisers and agricultural animal waste (WHO, 1999). Cyanobacteria thrive in eutrophic waters producing toxins and metabolite that reduces water quality with adverse effects on lake ecology, livestock, human water supply and recreational amenities (Sigee et al., 1999; Nakamura et al., 2003b).

Cyanobacteria are a diverse group of prokaryotes with over 1,000 species having been described (Kulik, 1995). They are now classified as a separate sub-class of Gram-negative prokaryotes (Kulik, 1995). Cyanobacteria are the scientific name for blue-green algae, or 'pond scum'. The cyanobacteria are classified into five orders namely Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematales (Skulberg et al., 1993).

Three types of intervention measures are utilized in cyanobacteria bloom control, namely: mechanical, physico-chemical and biological control. The mechanical approaches involve the use of filters, pumps and barriers such as curtains and floating booms (WHO, 1999) to take out the cyanobacteria scums, dead fish and other bloom related material. This is however, a short-term measure in the control of the blooms. The physico-chemical methods involve the application of chemical substances such as algicides to kill, lysed or inhibit growth of cyanobacteria cells (WHO, 1999; NSW, 2000). Though the chemical substances are able to damage and kill cyanobacteria cells, they lead to the release of cyanobacteria toxins into the surrounding water, thus exacerbating the problems (Lam et al., 1995). The chemical substances are also toxic to other aquatic microorganisms and may accumulate in sediments to harmful concentrations that may inevitably damage the lake environment in the long term (Mason, 1996; Sigee et al., 1999). The third alternative is the biological control method that involves the application of biological control organisms or pathogens

such as viruses, bacteria, protozoa and actinomycetes. These microbial herbicides are able to kill, lysed or inhibit the growth of cyanobacteria.

2.1.1. Eutrophication

Eutrophication is a natural process or a human-induced activity that leads to the enrichment of water bodies with inorganic nutrients such as nitrates and phosphates (Codd, 2000; Van Ginkel, 2002). The readily available nutrients promote the excessive growth of aquatic weeds and cyanobacterial blooms. As a natural process, the ageing of a lake, that occurs during the lifetime of an impoundment or a lake and may take thousands of years to occur. The natural process involves the following succession: from an oligotrophic (low in productivity and abundance in biodiversity of species) through to mesotrophic (moderate productivity and high species abundance) to eutrophic (high productivity and high species abundance but low in species diversity). The other extreme end of eutrophic conditions is known as hyper-eutrophic (Van Ginkel, 2002).

Cultural eutrophication is a human-induced activity that is caused by an increase in nutrient loading from point and non-point sources of pollution (Van Ginkel, 2002). The point sources of pollution include release of raw sewage or partially treated sewage and untreated industrial wastewater effluents. While non-point sources of pollution include agricultural and urban run-offs and septic tank leach fields. These sources of pollution may accelerate the eutrophication of impoundments (Van Ginkel, 2002).

Harding et al. (2004:vi) pointed out that 'eutrophication is the Number One ecological and water resource management threat to surface waters in many countries of the world today, as it should be in South Africa, a country… a single natural lake and a high level of dependence on impoundments, many of which receiving polluted runoff of one form or another as a bulk component of their annual inflow.' Thus the release of untreated or partially treated sewage water is the main contributor to eutrophication, which is further compounded by low rainfall and high levels of water abstraction (Joska and Bolton, 1994; Codd, 2000).

Eutrophication may result in an increase in cyanobacterial, algal and other plant biomass in water bodies. This may lead to the reduction of water quality for human water-uses due to increased turbidity and particulate matter. This consequently leads to blockage of water-filters and the production of taste and odour compounds in drinking water (Joska and Bolton, 1994; Klapper, 1999). Water quality is defined as the suitability of water to sustain various uses or a wide range of natural factors such as influences processes biological, geological, hydrological, meteorological and topographical (Meybeck et al., 1996). The Hartbeespoort dam water quality is high in nitrates, ammonia, phosphates and trihalogenated precursors and is suitable for irrigation but requires comprehensive water treatment if intended for human consumption (NIWR, 1985; Harding et al., 2004).

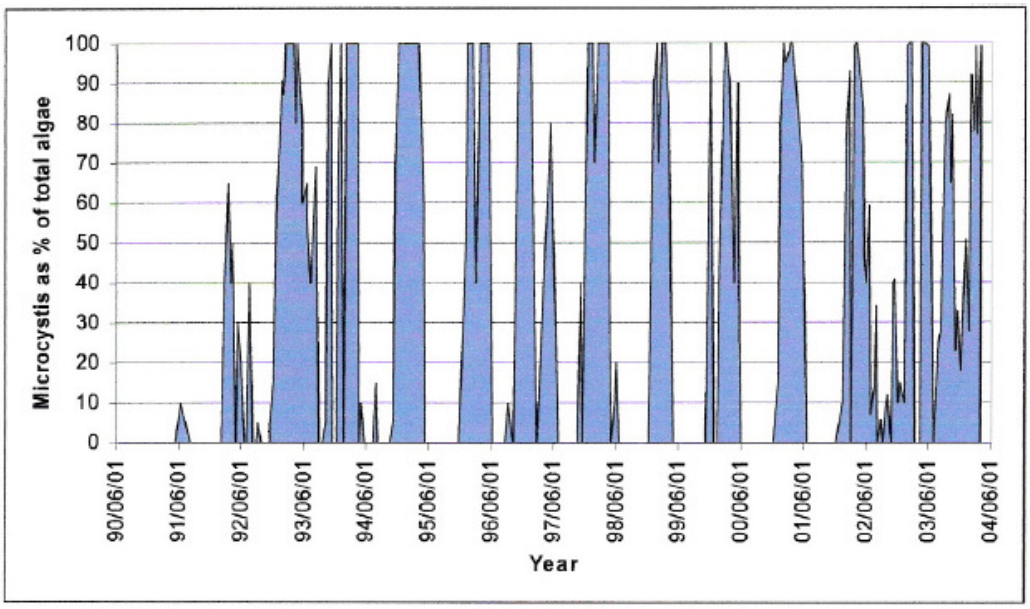


Figure 2.1: Occurrence of *Microcystis* in Hartbeespoort Dam from 1990 to 2004 (Harding et al., 2004).

The above quote illustrates that the problems of eutrophication are here to stay since the bulk of the dam's annual inflows are treated effluent rich in nutrients and *Microcystis* algal blooms will become almost an annual event (Figure 2.1). The question that springs to mind is what is being done to remedy the situation? The Department of Water Affairs and Forestry (DWAF) and Hartbeespoort Water Action Group (HWAG) have formed a partnership to seek short and long term solutions

bedeviling the dam (Harding et al., 2004). The HWAG is now a registered Section 21 'not-for-profit company'. The short-term solutions focused on the mechanical harvesting of dense algal blooms as a way of enhancing the aesthetic value of the lake. Earlier on the studies of Batchelor et al. (1992) showed that there was a commercial profitability for the utilization of algal hyperscums as potential sources of fine chemicals and animal feeds. But by then (1992) the starts up costs were rather prohibitive during the study period.

The long-term solutions were to address the causes of algal blooms: nutrients inflows and an unbalanced ecological system that is dominated by *Microcystis aeruginosa*. Further readings are recommended to obtain fuller details of the proposed strategies on the dam's restoration program (Harding et al., 2004).

2.1.2. The study area

The water bloom samples were obtained from Hartbeespoort Dam (25° 43´ S; 27° 51´ E), a freshwater body, located about 40 km west of Pretoria, South Africa (Table 2.1; Figure 2.2). The main inflowing rivers are the Crocodile River and its tributaries, which supply over 90% of the inflow into the reservoir, and the remainder is supplied by Magalies River. The upper reaches of the Crocodile River drains parts of Krugersdorp, Randfontein and Roodepoort while its Jukskei tributary drains the Johannesburg Northern suburbs and the Hennops tributary drains Kempton Park, Tembisa, Midrand and Verwoerdburg (Harding et al., 2004).

The Hartbeespoort Dam was constructed, on the Crocodile River, in the 1920s as an irrigation reservoir for the Government water scheme located near Brits. Over the years the freshwater resources have been developed further to include multiple uses such as flood control, ecological reserve, drinking, game fishing and recreational activities and the development of a waterfront residential settlement (Hartbeespoort Town) (Harding et al., 2004). The ecological reserve is a new concept designed to maintain minimum water flow in a riverine ecosystem and meet international obligations, as the Crocodile River is a sub-tributary of the Limpopo River system, as defined in the new National Water Act (NWA, 1998).

Table 2.1: Physical and hydrological characteristics of the Hartbeespoort dam (NIWR, 1985).

Parameter	**Information**
Geographical Location	25° 43´ S; 27° 51´ E
Catchment type	Urban and Industrial, Rural
Usage of reservoir	Irrigation, potable water and recreation
Catchment area (total)	4112 km^2
Main inflowing river	Crocodile River
Dam wall completed	1925 (modified 1971)
Volume	192.8 x 10^6 m^3
Area	2034 Hectare
Maximum depth	32.5 m
Annual runoff	163 x 10^6 m^3
Mean annual precipitation	703 mm
Annual evaporation	1684 mm

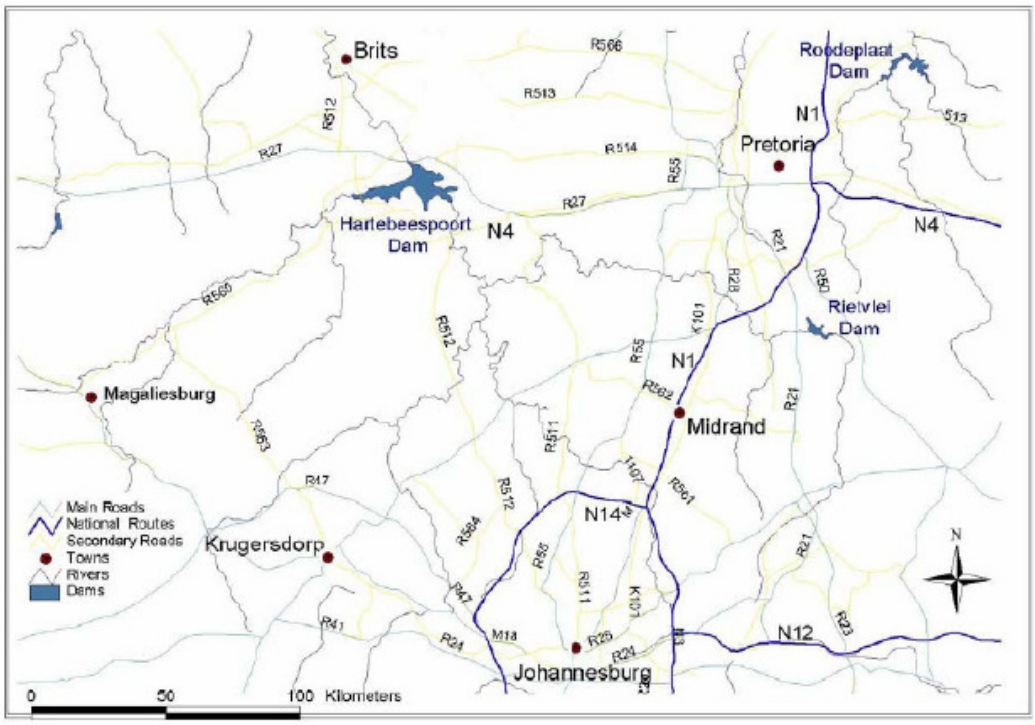


Figure 2.2: Location of Hartbeespoort dam (Harding et al., 2004).

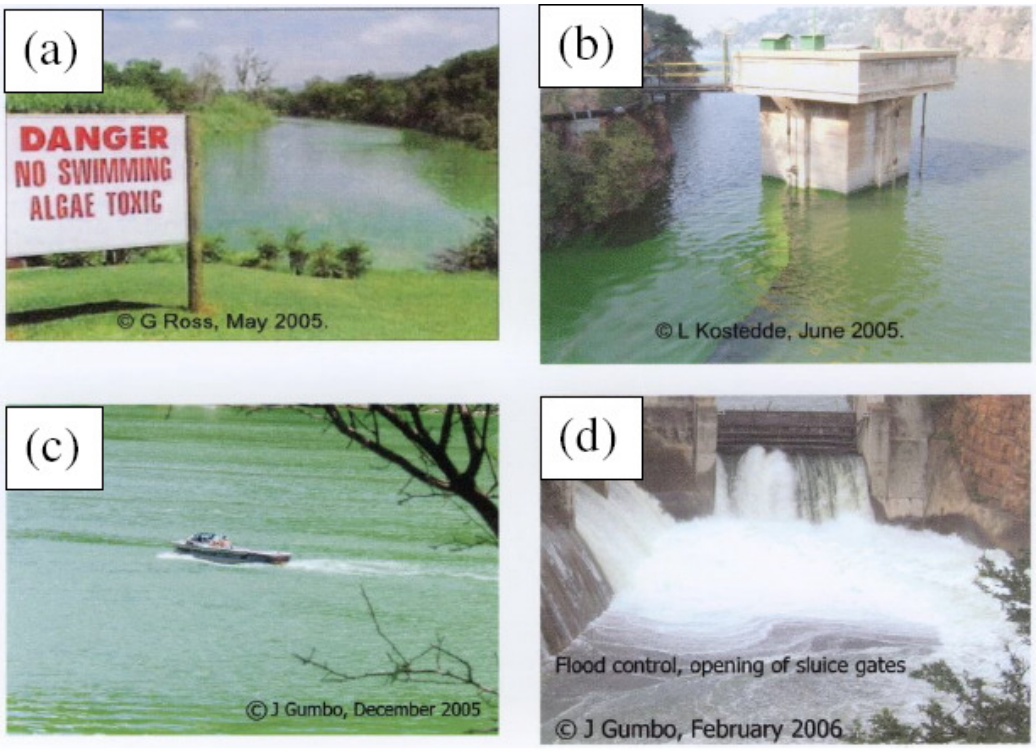


Figure 2.3: *Microcystis* algal blooms in winter of 2005 and summer of 2006. (a-b) winter of 2005 with (a) a warning sign that was erected at the Magalies Park Resort on the north-western shoreline of the Hartbeespoort dam. (b) Intake raw water tower drawing for potable purification and (c-d) summer of 2006 with (d) recreational

activities in the dam and (d) 'exporting' some of algae downstream Crocodile River after heavy rains in February, 2006.

2.2. *MICROCYSTIS* DOMINANCE DURING EUTROPHICATION

2.2.1. Introduction

Microcystis is a photoautotroph and colonial prokaryote of the order, Chroococcales. The colony cells are spherical, about 4-6µm in diameter embedded in a mucilaginous sheath of about 5-8µm wide and have many aerotopes (gas vacuoles) (Cronberg et al., 2003). Colony shape is highly variable and ranges from spherical colonies to irregular, net shaped colonies (Table 2.2). Oxygenic photoassimilation of carbon dioxide based on chlorophyll *a* (chl-*a*) is the predominant form of nutrition for the cyanobacterium (Zohary, 1987).

Table 2.2: Colony shapes for different types of *Microcystis aeruginosa*

Type of *Microcystis aeruginosa*	Colony shape	Reference
forma flos aquae	Spherical and or lens shaped	Zohary (1987)
forma aeruginosa	Irregular, net shaped and or ellipsoidal	

Microcystis aeruginosa Kütz. Emend. Elenkin, a bloom forming cyanobacterium, is a dominant primary producer in Hartbeespoort dam that thrives throughout the year (Table 2.3). The cyanobacterium easily proliferates due to the availability of nutrients and favourable climatic conditions (Table 2.3 and 2.4). During winter *Microcystis* cells sink to the bottom sediments and lay in dormancy. In addition these cells form the inoculum for the next bloom (Gibson et al. 1982; Zohary, 1987).

The formation of shallow diurnal mixed layers in winter or summer have led to the maintenance of *Microcystis* in the near surface illuminated zone as it lays in dormancy. The gas vacuoles are responsible for maintaining buoyancy thus giving it an advantage to move up or down in response to nutrient availability and light (Madison et al., 2003). The gas vacuole contents are high in winter thus contributing to the buoyancy of *Microcystis* (Zohary, 1987).

Table 2.3: Factors that favour dominance of *Microcystis* in Hartbeespoort dam. (Zohary, 1987).

Season	**Solar radiation** PAR ($\mu Em^{-2}s^{-1}$)	**Minimum nutrient levels**			**Water Column**	**Low Wind speeds** ms^{-1}	**Water Temp.** (ºC)
		$NO_2^{2-}N+ NO_3^{2-}$ $\mu g\ \ell^{-1}$	SRP $\mu g\ \ell^{-1}$	NH_4^+N $\mu g\ \ell^{-1}$			
Mid-winter (July)	1000	129	227	50	Mixed[1]	2.9	12-14
Mid-summer (Jan-Feb)	2000	129	227	50	Stratified[2]	2.9	22-25

PAR = photosynthetically available irradiance.

SRP = soluble reactive phosphorus in the upper 5m in the main basin of the dam.

Mixed[1] = low wind speeds coupled with solar radiation caused slight warming of water column and formation of shallow diurnally mixed layers.

Stratified[2] = low wind speeds coupled with high solar radiation caused the warming of upper 2 m of the column during the day and formation of shallow diurnally mixed layers.

Table 2.4: Presence of nutrients in Hartbeespoort dam sediments (Harding et al., 2004).

Sample	**Concentration, mg kg^{-1} dry mass of sample**			
	TP	**NH_4**	**NO_x**	**PO_4P**
Magalies	220	2.7	0.2	0.1
Crocodile	1230	7.9	0.2	0.44

TP= total phosphorus.

NH_4= ammonia

NO_x= nitrates

PO_4P= soluble phosphates

Microcystis has evolved adaptation strategies to survive high light intensities (1234 $\mu Em^{-2}s^{-1}$) by having low cellular chlorophyll *a* content (0.132 µg chl-*a* per 10^6 cells) (Zohary, 1987). At these light intensities, Wiedner et al. (2003) observed that there

was a positive correlation between high light irradiance (to a certain limit) with the production of microcystins. Thus besides adapting strategies to survive photo bleaching, the *Microcystis* is a cosmopolitan that uses the light intensities to produce microcystins in addition to its normal photosynthesis process.

2.2.2. Toxicity of cyanobacteria

The freshwater species that are often implicated with microcystin toxicity are: *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc*; and nodularin toxicity, from a marine cyanobacterium called *Nodularia spumigena* (Rapala et al., 1994; Cronberg et al., 2003) (Table 2.5). Cyanobacteria synthesize a variety of toxins that are defined by their chemical structure. These are classified into three groups: cyclic peptides, alkaloids and lipopolysaccharides (LPS). Cyanobacterial toxins are low molecular weight compounds, odourless, colourless and soluble in water.

These cyanobacterial toxins are harmful to humans, fish, birds and other animals. Illness and death may occur following oral ingestion of cells, or by contact with water that harbours toxin-releasing strains of cyanobacteria. Animal deaths may also occur following bioaccumulation of cyanobacterial toxins via food webs (Richard et al., 1983).

Table 2.5: Distribution of Cyanobacterial toxins and their genera (Codd, 1999).

Toxin	Number of structural Variants	Producer genera	Habitats
Neurotoxins: alkaloids			
Anatoxin-a (secondary alkaloidal amine)	2	*Anabaena, Oscillatoria*	Freshwater, Brackish water
		Microcystis, Phormidium	Freshwater
		Cylindrospermum, Aphanizomenon	Freshwater, Brackish water
Anatoxin-a(s)	1	*Anabaena*	Freshwater
Saxitoxins	~20	*Aphanizomenon, Anabaena, Lyngbya, Cylindrospermopsis*	Freshwater
Hepatotoxins			
Microcystin (cyclic peptide)	>60	*Microcystis, Anabaena*	Freshwater, Brackish water
		Oscillatoria, Nostoc	Freshwater, Brackish water
		Anabaenopsis, others	Freshwater
		Hapalosiphnon, others	Terrestrial
Nodularin (pentapeptide)	~6	*Nodularia*	Brackish water, Freshwater
Cylindrospermopsin (Cyclic guanine alkaloid)	1	*Cylindrospermopsis*	Freshwater
		Aphanizomenon	Freshwater
		Umezakia	Brackish water
***Endotoxins* and others**			
Lipopolysaccharides	>3	*Microcystis, Oscillatoria*	Freshwater
Lyngbyatoxin	>1	*Lyngbya*	Marine
Aplysiatoxin	2	*Lyngbya, Oscillatoria*	Marine
		Schizothrix	Marine

2.2.2.1. Cyanobacterial metabolites

Cyanobacteria also produce secondary metabolites: geosmin (trans-1, 10-dimethyl-trans-9-decalol) and 2-methyl isoborneol (2-MIB), that impact on taste of raw and drinking water (Brock et al., 1994). Geosmin and 2-MIB are low molecular weight compounds that are soluble in water. The substances often result in consumer

complaints regarding odour and taste of drinking water. The functional role of these secondary metabolites and toxins in nature is unclear (Herbert, 1989).

2.2.2.2. Neurotoxic alkaloids

Strains of *Anabaena, Aphanizomenon flos-aquae, Oscillatoria, Trichodesmium* (*Cylindrospermum* and *Microcystis aeruginosa* have been implicated in the production of anatoxin-a (Rapala et al., 1994; Carmichael, 1994). Anatoxin-a is a potent neurotoxin, which mimicked acetylcholine (Hitzfeld et al., 2000). It caused a depolarising neuromuscular blockade, which was not reversed by acetylcholinesterase. The end result was over stimulation of muscle followed by fatigue and paralysis (Oberholster et al., 2004). There are no known antidotes and death occurred within a few minutes as a result of respiratory failure.

Other potent neurotoxins are saxitoxin and neosaxitoxin, which are produced by species and strains of *Anabaena* and *Aphanizomenon*. These cyanobacterial species are often linked with paralytic shellfish poisons (PSP), which is a direct result of consumption of contaminated shellfish (Oberholster et al., 2004). These toxins are better known as products of dinoflagellates, a marine alga, which is responsible for red tides (Cronberg et al., 2003). These alkaloids inhibit nerve conduction by blocking sodium channels in axons preventing the release of acetylcholine at neuromuscular junctions.

2.2.2.3. Hepatotoxins

The cyclic peptide toxins (hepatotoxins) especially microcystins are the most wide spread in freshwater and therefore very important regarding treatment of drinking water (Rae et al., 1999). Oral consumption of water contaminated with microcystin was reported to cause intra-hepatic haemorrhage and hypovolaemic shock within a few hours leading to death (Rapala et al., 2002).

Microcystin-LR was reported to act as an inhibitor of protein phosphatase type 1 and 2A (Yoshizawa et al., 1990); an activator of phosphorylase *a* (Runnegar et al., 1987) and potent tumour promoter in humans and rodents (Rapala et al., 2002). The

phosphorylase on the other hand induced a depletion of glycogen in the liver (Oberholster et al., 2004). Cylindrospermopsin is another cyclic guanine alkaloid that is hepatotoxic. It is a protein synthesis inhibitor that caused damage to the kidneys, spleen, the heart, and thymus (Hawkins et al., 1997). As with other classes of cyanobacterial toxins, it is likely that several variants of cylindrospermopsin will emerge.

These hepatotoxins present a major problem in the management of public water supply utilities (Nakamura et al., 2003b). These cyanobacterial toxins and the metabolites are possible trihalomethane precursors (Lam et al., 1995). The microcystins were implicated in the deaths of patients undergoing haemodialysis in Brazil (Jochimsen et al., 1998). The toxins caused kidney and liver damage.

2.2.2.4. Irritant toxins -lipopolysaccharides

Many cyanobacteria contain lipopolysaccharides endotoxins (LPS) in their outer cell layers. The LPS of other bacteria are associated with gastroenteritis and inflammation problems. It is thought that cyanobacteria LPS may contribute to waterborne health incidents, although this possibility has not been adequately investigated (Sivonen and Jones, 1999).

2.2.3. The fate of cyanobacteria toxins in aqueous environment

Intracellular toxins are produced and contained within actively growing cyanobacteria cells. These become extracellular toxins when released to the external environment during cell senescence, lysis and death. Laboratory studies have demonstrated that healthy log phase cyanobacteria cultures have less than 10-20 per cent of total toxin pool as extracellular (Sivonen and Jones, 1999). However under field conditions the levels of dissolved extracellular toxins increased (0.1 to 10 µg ℓ^{-1}) in ageing and declining blooms (Sivonen and Jones, 1999). This has important implications for water treatment utilities, as it is preferably cheaper to remove intact cyanobacteria cells than ruptured or damaged cells. The conventional water treatment processes if operated in conjunction with dissolved air flotation are capable of removing intact cyanobacteria cells from raw water. Ruptured or damaged cells may release

extracellular toxins to surrounding water, necessitating the use of expensive chemical removal processes such as activated carbon and or oxidative ozone and chlorine. The use of algicides such as copper based or organic herbicides enhances the release of toxins from lysed cyanobacteria cells. The copper based algicides are effective in completely eradicating a bloom within three days (Falconer et al., 1983; Jones and Orr, 1994).

2.2.3.1. Challenges to drinking water utilities

In South Africa and other parts of the world, microcystins are a major concern to drinking water providers from a health and economic perspectives (Scott, 1991; Harding et al., 2001). The microcystins have been linked to liver damage that prompted the World Health Organization (WHO) to adopt a provisional guideline value for microcystins-LR (L for leucine and R for arginine) of 1.0 µg ℓ^{-1} drinking water (WHO, 1998; Hoeger et al., 2004). Earlier on Ueno et al. (1996) had proposed a more stringent guideline value of 0.01 µg ℓ^{-1} based on a possible correlation of primary liver cancer in certain locations in China. Consumers in these locations used potable water contaminated with microcystins (Oberholster et al., 2004). In Australia, the potable water standard for microcystins was set at 1.3 µg ℓ^{-1} (NHMRZ/ARMCANZ, 2001).

In South Africa, the Department of Water Affairs & Forestry (DWAF) detected high levels of microcystins in raw water samples taken from Hartbeespoort dam (Figure 2.4). The levels of microcystins greatly exceeded the WHO guideline value and the Australian water standard. The dam provides raw water supplies for Magalies Water, which operates the Schoemansville water treatment plant (NIWR, 1985). The Magalies Water supplies domestic water to the towns of Hartbeespoort and Brits with a population of 20,000. As a precautionary measure and to protect the residents from microcystin toxicity, the water utility had to temporarily close down its water treatment plant (SABC News, 2003). The residents had to resort to the use of bottled water and water tanks were trucked in from safer sources. The water utility relied on the use activated carbon to reduce the soluble microcystins.

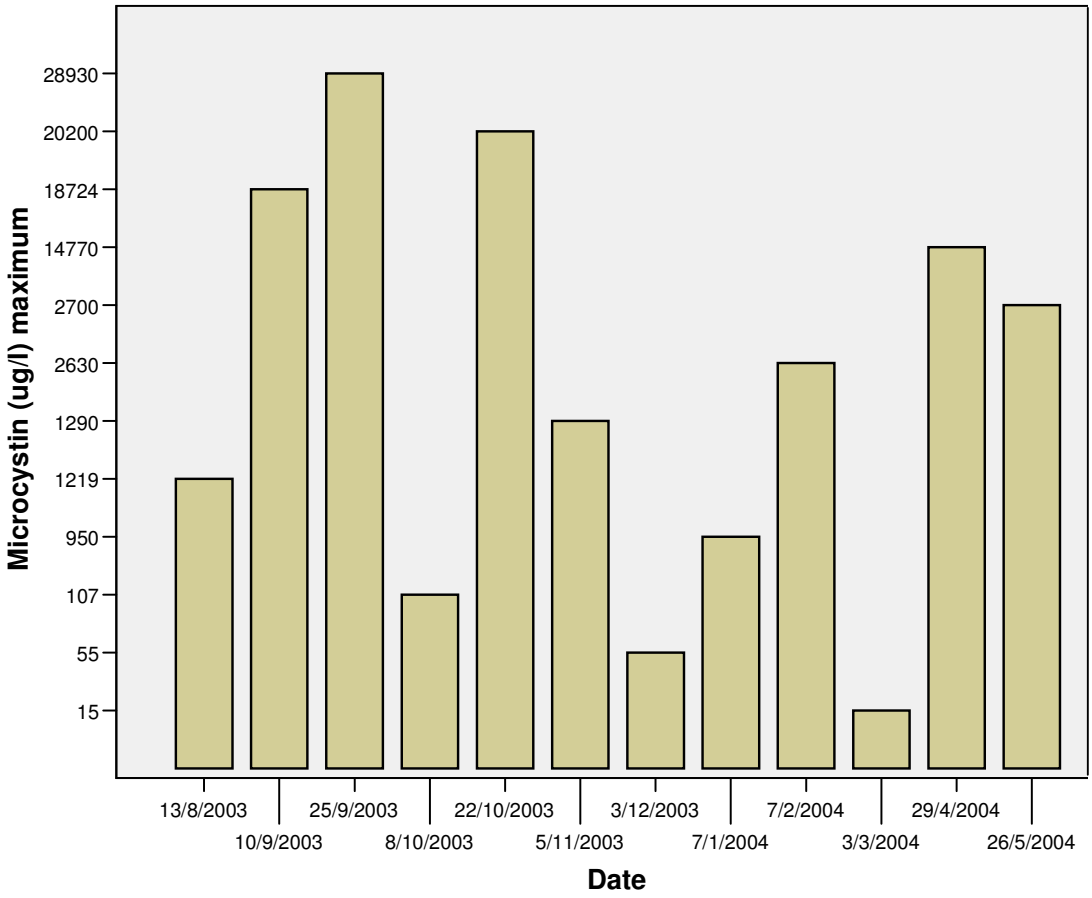


Figure 2.4: Maximum microcystin levels in raw water analysis for Hartbeespoort dam (Harding et al., 2004). The WHO microcystin guideline value is 1.0 µg ℓ^{-1}

Although humans do not consume cyanobacteria, they may be regularly exposed to sub-lethal dosages of cyanobacteria toxins in potable water derived from contaminated dams and reservoirs (Lam et al., 1995). In Australia, elevated concentrations of microcystins were linked epidemiologically to an outbreak of human hepatoenteritis (Falconer et al., 1983).

Ruptured or damaged cyanobacteria cells may release intracellular toxins to surrounding water, necessitating the use of expensive chemical removal processes such as activated carbon and or oxidative ozone and chlorine (Haider et al., 2003). A study of two water treatment plants in Australia with advanced water treatment methods (Table 2.6) relied on activated carbon and chlorination to remove soluble cyanobacteria toxins from potable water. The levels of microcystins in the potable water were within the Australian water standard and WHO guideline value.

Table 2.6: Reduction of cyanobacterial toxins with different water treatment process (Hoeger et al., 2004).

Water treatment plant	**Treatment process**	**Predominant cyanobacteria**	**Toxin**	**Maximum toxin in raw water (µg/ℓ)**	**Maximum toxin in treated water (µg/ℓ)**	**Reduction %**
WTP 1	Flocculation/ sedimentation, optional PAC, sand filtration, chlorination	*Microcystis aeruginosa, Anabaena circinalis*	MC	0.980	0.660	100[1]
			PSP	0.068	0.033	100[1]
WTP 2	Flocculation/ sedimentation, optional PAC, sand filtration, chlorination	*Cylindrospe-rmopsis raciborskii*	MC	ND	ND	---
			CYN	1.17	0.2	100[2]

After sand filtration and flocculation (possibly and chlorination?) [1]

After sand filtration and chlorination[2]

ND = Not detected

MC = microcystins

PSP = paralytic shellfish poison

CYN = cylindrospermopsin

The aim of the water treatment methods was to remove intact cyanobacteria cells and reduce cyanobacteria toxins. The use of activated carbon reduced the toxins through adsorption whilst chlorine oxidised toxins. The use of chlorine may lead to the formation of trihalomethanes (Lam et al., 1995). Sand filtration or flocculation techniques alone are not effective in the removal of soluble organics but are effective in removal of intact cyanobacteria cells. The study showed that the water treatment efficiency was a function of: type of cyanobacteria species and density; additional organic load; concentration and type of flocculants and activated carbon used; the pH

of water during flocculation and chlorination and lastly the regularity of filter back washing (Hoeger et al., 2004).

2.2.3.2. Bacterial degradation of microcystins

The microcystins are generally very stable compounds, are resistant to chemical breakdown and are persistent in natural waters for weeks to several months (Sivonen and Jones, 1999). The toxins on the other hand are susceptible to breakdown by aquatic bacteria found naturally in rivers and reservoirs. Other studies have failed to detect the presence of heterotrophic bacteria in eutrophic water bodies that have biodegradation abilities (Codd and Bell, 1996). Bourne et al. (1996) isolated a bacterial species identified as *Spingomonas* capable of degrading microcystin-LR and RR. The bacterium was reported to utilize the toxin as a sole carbon and nitrogen source for its growth. The bacterial degradation process removed 90 per cent of microcystin in 2 to 10 days under laboratory conditions. Of major interest is what is role-played by these bacteria in the actual lysis of cyanobacteria.

2.2.4. Current methods used to manage harmful algal blooms

2.2.4.1. Chemical Algicides

Mechanical and physico-chemical methods have been devised in attempts to manage cyanobacterial blooms, with limited success. The direct control method involves the use of chemical treatments such as algicides, including copper, Reglone A (diquat, 1,1-ethylene-2, 2-dipyridilium dibromide), potassium permanganate, chlorine and Simazine (2-chloro-4,6-bis(ethylamino)-s-triazine) (Lam et al., 1995; García-Villada et al., 2004). These chemicals induced cyanobacterial cell lysis, followed by the release of toxins into surrounding waters. An appropriate waiting period has to follow to allow for the degradation of the toxins (WHO, 1999). These algicides are toxic to other aquatic microorganisms, may accumulate in the sediment at harmful concentrations and cause long-term damage to the lake ecology (Mason, 1996). Copper sulphate or organo-copper compounds have been used to control harmful algal blooms in raw water supplies intended for potable purposes (Lam et al., 1995). However, there is an increasing need to reduce the use of chemicals for environmental

and safety reasons. Thus, the development of non-chemical control measures such as biological control is of great importance to the management of HABs.

2.2.4.2. Mechanical removal

Mechanical harvesting of cyanobacteria hyperscums have been attempted in Hartbeespoort dam as the hyperscums reached crisis proportions, causing obnoxious odours and fumes. This operation proved to be financially unsustainable as a mere 500 kg worth of hyperscums rich in phosphates (P) was removed at a cost of R1 million per ton (The Water Wheel, 2004). The phosphate levels in the dam have been estimated to be 25 tons (as P) when full with an additional annual inflow of 20 tons (Harding et al., 2004).

2.2.4.3. Nutrient limitation

Other water treatment chemicals such as Phoslock™, alum and lime (within pH 6-10) controlled cyanobacteria blooms through nutrient precipitation and cell coagulation but did not cause significant increase in extracellular toxins (Lam et al., 1995; Greenop and Robb, 2001; Robb et al., 2003). The major limitation for daily use of these chemical substances was their prohibitive cost.

In the mid-1980s the DWAF introduced a special phosphate standard of 1.0 mg ℓ^{-1} aimed at point source polluters (DWA, 1988; Chutter, 1989). Twenty years later still there was no improvement in the eutrophication problems as cyanobacteria blooms in Hartbeespoort dam continued to recur almost as a yearly event (Harding et al., 2004). However Hartbeespoort dam has not experienced hyperscums formation for many years, indicating the limited success of the phosphate standard as a control measure (Harding et al., 2004). In addition to the use of special phosphate standard some European countries such as Finland and the Netherlands adopting other control measures. These countries are currently in the process of introducing an integrated biological water management system, which aims at restructuring the aquatic food web (Harding et al., 2004).

2.2.4.4. Integrated biological water management

Based on the biogeochemical cycle, every organism has to cope with the natural limit of an essential mineral nutrient. Harding et al. (2004) proposed the following strategies for the restoration of Hartbeespoort dam: (1) reducing the external nutrient (phosphorus) inflows; (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. The first two proposed strategies were probably based on this premise to limit nutrients supply to *Microcystis* since the amount of available phosphorus in the water has a direct effect on its growth. The last strategy looks at possible ways of restructuring the food web and encourages other organisms that might feed directly or indirectly on *Microcystis*. The whole concept forms part of an integrated biological water management system. The strategy involved adjusting the dam's biodiversity by increasing the amount of zooplankton especially the *Daphnia* water flea and other zooplanktonic species, which feed on *Microcystis*. In the case of Hartbeespoort dam this meant the restructuring of phytoplankton-zooplankton-fish chain. However there are contradictions on *Microcystis* as a potential zooplanktonic nutritional source (Gliwicz, 1990). The factors that may explain the nutritional inadequacy of *Microcystis* are: its toxicity, concentration of colonies and its morphology and physiological state. *Daphnia*, planktonic herbivores, are selective feeders concentrating on non-toxic *Microcystis* strains but not on toxic ones. Other studies have indicated that the *Microcystis* may increase toxin production, as a defensive strategy, in response to the presence of zooplankton (Jang et al., 2003).

2.3. BIOLOGICAL CONTROL OF HARMFUL ALGAL BLOOMS

2.3.1. Introduction

The alternative approach of managing algal blooms involves application of biological control agents such as predatory bacteria, which are antagonistic towards the cyanobacterium *Microcystis*. These predatory bacteria have been isolated from the blooms and are indigenous to the lake environment, thus providing an environmentally friendly solution. The importance of predatory bacteria as biological

control agents, in the regulation and control of large harmful algal blooms (HAB) has largely been overlooked. Daft et al. (1985a) proposed the following seven attributes that defined a good predatory bacterial agent: adaptability to variations in physical conditions; ability to search or trap for prey; capacity and ability to multiply; prey consumption; ability to survive low prey densities (switch or adapt to other food sources); wide host range and ability to respond to changes in host. In addition to these, this work suggests an eighth attribute; i.e., the predatory bacteria should be indigenous to the particular water environment, thus providing an environmentally friendly solution. This is in agreement with Sigee et al. (1999), who suggested that the microbial antagonists must be indigenous species of that particular lake environment, having not undergone any gene modification or enhancement.

Biological control of cyanobacteria, like other control measures for nuisance organisms, is often viewed with caution. This may be 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). Further readings are recommended to obtain precise details of high profile cases of successful and catastrophic failures of biocontrol in the last century (Secord, 2003).

The practice of introduction of foreign microbial agents has raised some concern with regards to environmental safety due to the so-called host specificity paradigm involving host switching (HS) and host range expansion (HRE) (Secord, 2003). The foreign microbial agents are naturally reproductive and may exploit the opportunities that are available in the new environment by shifting their host affinities to other host species (set of species) and/or add another target species other than the original target. The change in direction of the microbial antagonist is difficult to anticipate, and there is the possibility that the organisms may affect other economically important crops or organisms. Secord (2003) has given an excellent treatise of this phenomenon with real world case studies with regards to the management of nuisance pests

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).

Viral pathogens would be ideal as biocontrol agents as they are target selective and specific for nuisance cyanobacteria. However, bacterial agents are considered more suitable than viruses as biological control agents because bacteria can survive on alternate food sources during non-bloom periods and the possibility of mutation within the host is not problematic, as bacterial predation is not reliant on unique attachment receptors (Rashidan and Bird, 2001).

2.3.2. The use of microorganisms to control cyanobacteria blooms

In the natural environment, there are predatory microorganisms that are antagonistic towards particular nuisance organisms (e.g. weeds, cyanobacteria) thus providing a natural means of controlling levels of nuisance organisms. Microbial agents (bacteria, fungi, virus and protozoa) have been isolated from harmful algal blooms (Shilo, 1970; Burnham et al., 1981; Ashton and Robarts, 1987; Yamamoto et al., 1998; Walker and Higginbotham, 2000; Bird and Rashidan, 2001; Nakamura et al., 2003a; Choi et al., 2005). This is not an exhaustive list of studies pertaining to microbial agents that predate on cyanobacteria but further information may be obtained (Sigee et al., 1999). 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). The bacterium *Saprospira albida* isolated from Hartbeespoort dam, was observing lysing the cyanobacterium *Microcystis aeruginosa* (Ashton and Robarts, 1987). There was no further research carried out to evaluate its biological control potential. The predatory bacteria are classified as members of the *Bacteroides-Cytophaga-Flavobacterium,* ranging from *Bacillus* spp to *Flexibacter* spp, *Cytophaga* and *Myxobacteria* (Table 2.7). Such microbial populations are called microbial herbicides (Atlas and Bartha, 1998). The biological

control of cyanobacteria provides a potential control measure to reduce the population of nuisance algal blooms to manageable levels.

Bacteria capable of causing or inducing cyanobacterial lysis have been isolated from different environments such as storm water drains (Burnham et al., 1984) and sewage works (Daft and Stewart, 1971; Stewart et al., 1973). In Kuwait, Sallal (1994) isolated *Flexibacter flexilis* and *F. sancti* from domestic sewage. The bacteria were found to lyse the cyanobacterium *Oscillatoria williamsii*. The bacteria produced extracellular lysozyme that caused growth inhibition of the cyanobacterium. Wright and Thompson (1985) isolated three *Bacillus* species from garden compost in Bath, Britain. Two of the strains were identified as *B. licheniformis* and *B. pumilis.* They produced volatile substances that inhibited the growth of the filamentous cyanobacterium, *Anabaena variabilis*.

Choi et al. (2005) isolated the bacterium, *Streptomyces neyagawaensis*, which had a *Microcystis*-killing ability, from the sediment of a eutrophic lake in Korea. Under natural conditions, *Cytophaga* spp. were implicated in the demise of marine red tides caused by the flagellate *Chatonella* spp. in the Seto Inland Sea of Japan. *Bacillus cereus* N14 was isolated by Nakamura et al. (2003a) from a eutrophic lake in Japan and caused lysis of the cyanobacteria *Microcystis aeruginosa* and *M. viridis.* The bacterium *Saprospira albida,* isolated from Hartbeespoort Dam, lysed *Microcystis aeruginosa* (Ashton and Robarts, 1987). There was no further research carried out to evaluate its biological control potential. Caiola and Pellegrini (1984) showed cells of *Microcystis aeruginosa* that were infected and lysed by *Bdellovibrio*-like bacteria in bloom containing water samples from Lake Varse, Italy.

Table 2.7: Lysis of cyanobacteria by different bacterial pathogens

Mechanism of cell lysis	**Predatory bacteria**	**Major host Cyanobacteria**	**Extra cellular Substances**	**Predator-Prey ratio**	**Flask shaking Conditions**	**Reference**
[1]Contact	*Streptomyces neyagawaensis*	*Microcystis*	Not identified	Not specified	Not specified	Choi et al. (2005).
	Bacillus cereus	*Microcystis*	Not identified	1:1	Not specified	Nakamura et al. (2003a).
	Cytophaga	*Microcystis*	Not identified	Not specified	Not specified	Rashidan and Bird (2001).
	Flexibacter flexilis, F. sancti	*Oscillatoria williamsii*	Identified	Not specified	Not specified	Sallal (1994).
[2]Entrapment	*Myxococcus fulvus* BGO2	*Phormidium luridum*	Not identified	1:6 x 10^7	100 rpm	Burnham et al. (1984).
	Myxococcus xanthus PCO2	*Phormidium luridum*	Not identified	1:10	100 rpm	Burnham et al. (1981).
[3]Endoparasitism	*Bdellovibrio-like bacteria*	*Microcystis aeruginosa*	Not identified	Not specified	Not specified	Caiola and Pellegrini (1984).
[4]Ectoparasitism	*Bdellovibrio bacteriovorus*	*Phormidium luridum*	Not identified	1:1	Shaker	Burnham et al. (1976).
Not specified	*Xanthomonas*	*Anabaena, Oscillatoria*	Not identified	Not specified	Shake flasks	Walker et al. (2000).
Not specified	*Saprospira albida*	*Microcystis aeruginosa*	Not identified	Not specified	Not specified	Ashton and Robarts (1987).
Not specified	*Bacillus spp*	*Anabaena variabilis*	Not identified	Not specified	Not specified	Wright and Thompson (1985).

[1]**Contact** = Initial physical contact between bacteria and cyanobacteria is established and leads to bacterial secretion of extracellular substances causing damage to cyanobacterial cell walls. Final result is cell lysis and death.

[2]**Entrapment** = Bacteria surround the cyanobacterial cell in 'wolf-like pack'; establish physical contact with the cyanobacteria, bacterial secretion of extracellular substances that cause damage to cyanobacterial cell wall. Final result is cell lysis and death.

[3]**Endoparasitism** = Bacteria penetrate the cyanobacterial cytoplasm, multiply inside cell using cyanobacterial nutrients. Final result is cell lysis and death.

[4]**Ectoparasitism** = Bacteria do not penetrate the cyanobacterial cytoplasm, associate closely with prey, deriving nutritional benefits that lead to prey death by starvation. Shaking conditions are designed to mimic the agitation of external environment.

Blakeman and Fokkerna (1982) observed that naturally occurring, resident microorganisms become adapted to survive and grow in their specific habitat. If these organisms were effective antagonists against a pathogen, they would be preferred for biological control purposes. Organisms from other habitats, which may be equally antagonistic to the pathogen, would be less likely to survive, and consequently would have to be reapplied more frequently. The same would be true in other habitats, such as where antagonists are used to control cyanobacterial blooms.

Augmentative biological control (deliberately enhancing the predator population through culturing in the laboratory) with resident predatory organisms is attractive as it offers certain advantages, such as being highly specific to the target organism, with no destruction of other organisms and no direct chemical pollution that might affect humans (Sigee et al., 1999). However, there are disadvantages, which include the limited destruction of the target organism, limited survival of the microbial agent or its removal by other organisms, problems of large scale production, storage and application, as well as reluctance to apply microbial agents in a field environment.

2.3.3. Predator-prey ratios

If these microbial agents are present in the natural ecosystem, why then are the harmful algal blooms so persistent in nature? This question was answered through the studies of Fraleigh and Burnham (1988). They showed 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. This is a fact why the predator bacteria population increases during the bloom period, is partly due to availability of algal carbon. The also showed that control of host prey was dependent on this threshold density of above 1 x 10^7 cells per mℓ in order to initiate cyanobacterial lysis.

Rashidan and Bird (2001) isolated *Cytophaga* bacteria from a temperate lake in Quebec, Canada. The bacteria were capable of lysing bloom-forming cyanobacteria. The population of *Cytophaga* strain C1 correlated well with the abundance of *Anabaena* in the natural lake environment. The bacterial population was at its peak when the cyanobacterial population was at its lowest. Daft and Stewart (1971) isolated

four bacterial pathogens of cyanobacteria of which three (CP-1, CP-2 and CP-3) were from a wastewater treatment plant (Forfar sewage works, Scotland) and the fourth (CP-4) was from a lysed *Oscillatoria* bloom (Lake Windermere, England). Under laboratory conditions, these bacterial pathogens were able to lyse bloom forming algae *Anabaena flos-aquae*, *A. circinalis*, *Aphanizomenon flos-aquae* and *Microcystis aeruginosa.* The bacterium CP-1 was found to be the most effective and underwent trials with field samples in an enclosed mesocosm, and a predator-prey ratio of approximately 10^5 cells.ml^{-1} was needed to cause rapid lysis of *Microcystis*. Nakamura et al. (2003a) found that a predator-prey ratio of 1:1 was needed for *Bacillus cereus* to lyse a *Microcystis* culture.

Burnham et al. (1981, 1984) isolated *Myxococcus xanthus* strain PCO2 and *M. fulvus* strain BGO2 and BGO3 from grab samples obtained from roadside ditches draining agricultural fields in Ohio, USA. The myxococcal strains effectively lysed agitated aqueous populations of *Phormidium luridium* and derived nutritional benefits from the cyanobacteria. *M. fulvus* strain BG02, at an initial predator density of 0.5 cells.ml^{-1}, was capable of lysing a *Phormidium* population of 3 x 10^7 cells per mℓ, a predator-prey ratio of 1:6 x 10^7. *Phormidium luridum* was lysed by *Myxococcus xanthus* PCO2 when the predator-prey ratio exceeded 1:10. *Phormidium luridum* was also lysed by *Bdellovibrio bacteriovorus*, at a predator-prey ratio of 1:1 (Burnham et al., 1976).

It is clear that the predator-prey ratio needed for cyanobacterial lysis is an important parameter to consider when using predatory organisms for biological control purposes. This ratio differs between species of prey and predator, and therefore needs to be determined for each relationship specifically. In a natural environment, it appears that the prey and predator are usually in contact with one another, but that the population of the predator is always lower. To be successful, the predator should preferably be able to colonize the cyanobacterial bloom, and multiple to numbers above the critical predator-prey ratio. Augmentative biological control may provide a means to increase the predator population to above the threshold needed to induce large-scale cyanobacterial lysis (Daft et al., 1973; Rashidan and Bird, 2001).

2.3.4. Mechanisms of cyanobacterial lysis

The mechanism of cyanobacterial lysis following exposure to a bacterial agent is poorly understood. Various mechanisms have been elucidated, including antibiosis, production of lytic enzymes, parasitism and competitive exclusion (Table 2.7). Cyanobacterial lysis by bacteria is caused by: contact lysis (Shilo, 1970; Daft and Stewart, 1973; Daft et al., 1985b; Nakamura et al., 2003a; Choi et al., 2005); production of lytic enzymes or extracellular products (Wolfe and Ensign, 1965 & 1966; Hart et al., 1966; Shilo, 1970; Christison et al., 1971; Wolfe et al., 1972; Dworkin et al., 1972; Gnosspelius, 1978; Burnham et al., 1981); antibiosis after entrapment of the host (Burnham et al., 1981 & 1984; Daft et al., 1985b; Sigee et al., 1999) and parasitism (Burnham et al., 1976; Caiola and Pellegrini, 1984; Rashidan and Bird, 2001).

2.3.4.1. Contact mechanism

The cyanobacterial cell wall resembles that of a Gram-negative bacterium, but is significantly thicker (Rapala et al., 2002). The cell wall consists of three or four outer layers between the plasma membrane (or plasmalemma) and the sheath (Holm-Hansen, 1968). The cell wall thickness may range from 10 to 20 nm and is coated with a relatively thick capsule of proteinaceous material (Skulberg et al., 1993). The outer membrane may be smooth or contain invaginations. It extends into the cell to form structures called mesosomes, which regulate substances entering and exiting the cell. In the cytoplasm, there are thylakoid membranes which are considered as sites for enzymatic reactions including photosynthesis, electron transport and ATP synthesis. The inner membrane consists of globular protein and mucopolymer molecules, with the mucopeptides being responsible for the additional structural strength of the cell. The cyanobacterial cell wall can be disrupted by the enzymatic actions of lysozyme and penicillin (Holm-Hansen, 1968).

Burnham et al. (1984) examined the degradation of cyanobacteria by bacteria and pointed out that the peptidoglycan component of the cyanobacterial cell wall was the ‘weak link’ against predatory bacteria. Cyanobacterial lipopolysaccharides (LPS)

differ to the LPS of other Gram-negative bacteria. They have a greater variety of long chain unsaturated fatty acids and hydroxy fatty acids with two or more double bonds, including the unusual fatty acid β-hydroxypalmitic acid which is found in the lipid A moiety. Other Gram-negative bacteria contain almost exclusively saturated and mono-unsaturated fatty acids with one double bond. Cyanobacterial LPS often lack ketodeoxyoctonate, a common LPS component of Gram-negative bacterial outer membranes, and contain only small amounts of bound phosphates when compared with other bacteria (Brock et al. 1994; Hoiczyk and Hansel, 2000).

Contact between the predatory bacterium and the cyanobacterium is a pre-requisite for effective lysis to take place. Shilo (1970) and Daft and Stewart (1971) observed that during this contact, the predatory bacteria released lytic enzymes or extracellular substances that resulted in the dissolution of the cyanobacterial cell membrane. Agitation or turbulence disturbed this physical contact, and no cyanobacterial cell lysis was observed in the absence of contact. This indicated that the lysing enzyme was not excreted into the medium. Cyanobacterial lysis of *Lysobacter* by bacterium CP isolates again illustrated that contact was necessary for lysis (Daft et al., 1985a; Rashidan and Bird, 2001). Although no extracellular lytic enzymes were produced by CP isolates, within 20 minutes after establishing contact with the cyanobacteria, the host cell was disrupted, presumably due to the transfer of enzymes across the adjacent cell walls. This type of predation involved the production of extracellular chemicals or enzymes by the prey during contact with the host. Daft and Stewart (1971) showed that extracellular products alone are insufficient for lysis to occur, and that the bacterial cells themselves must be present. Bacteria caused lysis of *Nostoc ellipsosporum* by inhibiting algal metabolic activity (nitrogenase activity and photosynthesis). There was no evidence of extracellular enzymes but the enzymes responsible for causing cyanobacterial cell lysis appeared to be on the bacterial surface, provided that there was contact between the organisms. Myxobacter lysis of vegetative cells of *Nostoc ellipsosporum* was observed whereas heterocysts were unaffected. As the cell walls of heterocysts contain cellulose and those of vegetative cells do not, this suggested that the bacteria were unable to degrade cellulose. Adams and Duggan (1999) again demonstrated the greater resistance of heterocysts and akinetes to predatory bacteria when compared with vegetative cells. During the differentiation of a vegetative cell into a heterocyst, major structural and biochemical changes occurred that affected

nitrogen fixation. The cell wall was thickened by the decomposition of three extra layers external to the normal cell structure. The inner layer consisted of glycolipid; the centre layer was a homogeneous layer consisting of polysaccharide, and the outer layer was a fibrous layer

The culture supernatant of *Bacillus cereus* was effective in the lysis of *Microcystis aeruginosa* and *M. viridis* (Nakamura et al., 2003a). Based on microscopic observation, the *B. cereus* cells were observed to attach to the surface of the cyanobacteria cell thereby inducing cell aggregation. The extracellular substances that were released lysed the cyanobacterial cell wall, leaving the chlorophyll a intact. The extracellular substances effectively lysed the cyanobacterial cells within 24 hours under alkaline conditions, which are most prevalent during a bloom. The unidentified extracellular substances were non-proteinaceous, hydrophilic, heat stable and had a molecular weight of less than 2kDa.

The studies of Choi et al. (2005) showed that the unidentified anti-algal substances originated in the bacterial periplasm and were secreted when the bacterium, *S. neyagawaensis*, was in physical contact with *M. aeruginosa*. Although the growth of *M. aeruginosa* was suppressed, there was no increase in bacterial biomass.

2.3.4.2. The release of lytic enzymes and extracellular substances

There are numerous studies on the isolation and characterization of lytic enzymes for a member of the lytic gliding bacteria, mainly members of the *Myxobacteria* and *Cytophaga* groups (Wolfe and Ensign 1965, & 1966; Hart et al., 1966; Shilo, 1970; Christison et al., 1971; Wolfe et al., 1972; Dworkin et al., 1972; Gnosspelius, 1978; Burnham et al., 1981). The lytic action of the numerous strains of the *Myxobacteria* and *Cytophaga* groups has been attributed to the presence of a variety of extracellular enzymes. Extracellular enzymes were capable of hydrolyzing the bacterial cell wall by targeting the peptidoglycan (Haska, 1974; Gnosspelius, 1978). Proteolytic enzymes were responsible for the primary lysis of eubacterial cell walls (Gnosspelius, 1978).

Wolfe and Ensign (1965, 1966) isolated and characterized enzymes protease II and I from a *Myxobacter* species. Protease I caused lysis of the bacterial cell wall, whereas

protease II did not cause any cell wall lysis, although it displayed specificity for lysine residues. Proteases I and II are relatively small, and are stable under alkaline conditions and high temperatures up to a maximum of 60^{o}C (Wolfe et al., 1972). Protease I lysed some Gram-positive bacteria, and to a lesser extent Gram-negative bacteria with the exception of *Spirillum serpens* and *Rhodospirilllum rubrum*. These organisms were lysed instantaneously by the protease I enzyme (Wolfe and Ensign, 1965). The Protease I, is an amidase and is capable of splitting the peptidoglycan into an oligosaccharide and a peptide (Dworkin, 1966). The enzyme cleaved the pentaglycine bridge in the cell wall of *Staphylococcus* and removed the peptide moieties from the peptidoglycan. These studies involved a *Cytophaga* species and the host *Arthrobacter crystallopoietes.* The degree of cross-linking between the peptidoglycan chains within the cell wall of cyanobacteria is higher than the 20 to 33% found in most Gram-negative bacterial peptidoglycan, with the extent of cross-linking (56 to 63%) being more similar to the values reported for Gram-positive bacteria (Hoiczyk and Hansel, 2000). Protease I has not been tested for the cyanobacterial lysis, but it is possible these lytic enzymes may cleave the pentaglycine bridge in the cell wall of cyanobacteria in the same manner as in Gram-positive organisms (Wolfe et al., 1972).

2.3.4.3. Antibiosis after entrapment of host

Burnham et al. (1981, 1984) indicated that the entrapment of cyanobacteria and release of enzymes, possibly antibiotics appeared to be an efficient system for cyanobacterial cell lysis. The predatory bacteria *Myxococcus xanthus* PCO2 and *M. fulvus* BGO2 were capable of inducing lysis of both agar- and liquid-grown cultures of the filamentous cyanobacterium *Phormidium luridum, var. olivacea*. The predatory bacteria caused rapid cyanobacterial lysis in agitated liquid grown cultures of *Phormidium,* which indicated that a mechanism other than the contact lysis was operating.

It appeared that *Myxococcus* formed colonial spherules, which entrapped the cyanobacteria prey in a ‘wolf-like manner’. The formation of these spherules was dependent on the number of myxococci per ml in an aqueous environment. It took about an hour to form mature spherules with 10^{7} myxococci per ml, followed by rapid

lysis of 10^7 *Phormidium* cells per ml (a predator-prey ratio of 1:1). The cyanobacterial prey cultures were inoculated with myxococci (predator-prey ratios of 1:10 and 1:100) and were lysed within 48 h. The earliest sign of cyanobacteria degradation was shown by light microscopy and involved the separation of a trichome into shorter filaments and single cells. The progressive formation of surface 'spikes' was due to the motile nature of *Myxococcus*, which gradually shifted the cyanobacteria to the centre of the core of the spherule. Once the cyanobacteria reached the core, there was physical contact between the predator and prey leading to the release of enzymes that acted on the cyanobacterial cell wall. Transmission electron microscopy studies showed that the *Phormidium* skeletal remains lacked the peptidoglycan layer.

Myxococcus strains appeared to be effective predators, especially *M. fulvus* BGO2, which lysed a *Phormidium* culture with a density of 10^7 cells per ml, reducing it to 10^3 in 2 days (Fraleigh and Burnham, 1988). The standard reference strain *M. xanthus* ATCC 25232 caused very little cyanobacteria lysis. *Myxococcus* strains lysed cyanobacteria cells of *Phormidium* growing in an agitated autotrophic aqueous environment. This is important for biological control of cyanobacteria. In nature, the aqueous environment is never 'still' but in continuous flux, causing mixing of water columns and layers.

2.3.4.4. Parasitism

There are few published reports on *Bdellovibrio* (Burnham et al., 1976) and *Bdellovibrio*-like bacteria (Wilkinson, 1979; Caiola and Pellegrini, 1984) that caused cyanobacteria lysis. In a separate but unrelated study, Burnham et al. (1968) demonstrated that *Bdellovibrio bacteriovorus* penetrated a Gram-negative *Escherichia coli,* causing its lysis and death. *Bdellovibrio bacteriovorus* behaved as an endoparasite occupying the cytoplasmic section. The *Bdellovibrio's* actively and violently stroke the host, *Escherichia coli,* with the end of the cell opposite the sheathed flagellum. During this initial period of irreversible attachment to host, *Bdellovibrio* commenced a grating motion which lasted for several minutes as observed by phase contrast microscopy. During attachment the *Bdellovibrio* developed unique receptors that bound tightly to the host. Attempts to separate the *Bdellovibrio* and hosts using violent shaking or vortex mixing at maximum speed had no visible

effect. The *Bdellovibrio* continued to push into the host cytoplasm space while the host was constricting in an attempt to prevent entrance by the predator. At the penetration pore, there was no visible damage to the host cell wall. Once inside the prey, *Bdellovibrio* commenced to inactivate host metabolism and feed off its nutrients (Yair et al., 2003). The exhaustion of cytoplasm contents triggered the *Bdellovibrio* to undergo multiple fission replications to produce progeny called bdelloplast. The bdelloplast, now flagellated, emerged after breaking the prey cell wall leaving behind ghost prey remnants.

In another study, the bacterium *Bdellovibrio bacteriovorus* behaved as an ectoparasite. When the bacterium was added to an aqueous culture of *Phormidium luridum* it caused lysis of the cyanobacteria through contact mechanism (Burnham et al., 1976). The bacterium released extracellular substances that dissolved the cyanobacteria cell wall. The bacterium was then able to gain nutrients from the cyanobacterium.

2.3.5. Field applications of biological control agents

Although there are non-indigenous bacterial agents that have been isolated and characterised, it appears that the studies on application of biocontrol agents are rather limited. Most of the studies have been limited to lysis of laboratory-cultured cyanobacteria. Before application of bacterial biocontrol agents to freshwater systems, information must be available on: the anti-algal activity against target alga, the effects of bacteria on other organisms in the freshwater ecosystem, and the prediction of the algal dynamics after removal of target alga (Choi et al., 2005). Another aspect of importance is agitation. Shilo (1970) and Daft and Stewart (1971) found that cyanobacterial lysis was ineffective if there was agitation, especially where contact lysis was involved. Under natural conditions, rapid mixing may favour the proliferation of cyanobacteria and discourage attachment of predatory bacteria.

During a field trial performed by Wilkinson (1979) and Caiola and Pellegrini (1984) a *Bdellovibrio*-like bacterium caused lysis of *Neofibularia irata, Jaspis stellifera* and *Microcystis* cells respectively. The bdelloplast were localised within the cell wall and cyanobacteria cytoplasm membrane. The infecting bacterium was similar in size and appearance to previously described *Bdellovibrio's*. These observations, though not

replicated under controlled laboratory conditions, indicated the possibility of endoparasitism of the cyanobacteria by *Bdellovibrio*-like bacteria. The *Bdellovibrio*-like bacteria are an attractive biological control agent because they penetrate the host cells specifically, exhaust host cell contents and replicate to form bdelloplasts, which attack further cells.

Under laboratory conditions, Choi et al. (2005) showed that *S. neyagawaensis*, had an anti-algal effect on a range of algae including green alga *Chlorella* spp., diatoms *Aulacoseira granulate* and *Stephanodicuss hantzschii* and four cyanobacteria: *Microcystis aeruginosa* NIES-44, *Anabaena cylindrica, A. flos-aquae* and *Oscillatoria sancta*. The bacterium had no effect on some species of *Anabaena macrospora* and *A. affinis.*

Nakamura et al. (2003b) immobilised *Bacillus cereus* N-14 in floating biodegradable plastic carriers, at a cell concentration of 3 x 10^7 cells per mℓ per 1 g-dry weight of starch-carrier float. This was used as an effective *in situ* control of natural floating *Microcystis* blooms, eliminating 99% of floating cyanobacteria in 4 days. The bacteria utilized the starch as a nutrient source and amino acids were derived from the lysis of *Microcystis*. The floating carrier enabled immobilized bacteria to be directed to floating cyanobacteria blooms.

Asaeda et al. (2001) installed two vertical curtains having depths that covered the epilimnion thickness of Terauchi dam in Japan. The purpose of the curtains was to curtail the nutrient supply from nutrient rich inflows to the downstream epilimnion of the reservoir. There was a marked reduction in cyanobacterial blooms downstream from the curtain in spring and summer. The curtain prevented the direct intrusion of nutrients into the downstream zone. Epilimnion algal concentrations were higher in the upstream zones. Thus, within the upstream zone the algae consume large amounts of the inflow nutrients, reducing the nutrient supply to the downstream zone of the reservoir. Floating curtains such as these may be used to segregate *Microcystis* algal blooms, minimising turbulence. This would allow the introduction of microbial antagonists, and afford the predator ample time to attach to the prey and initiate the lytic process.

2.4. *BACILLUS MYCOIDES* AS AN EMERGING BIOLOGICAL CONTROL AGENT

Taxonomy and characterisation of *Bacillus*

Bacillus mycoides B16 is classified in the 16S rRNA/DNA group 1 together with *B. cereus*, *B. thuringiensis*, and *B. anthracis* with noticeable phenotypic differences (Fritze, 2004). Within this group phenotypic differentiation exists *B. cereus* and *B. thuringiensis* are usually motile and three other species *B. cereus, B. thuringiensis* and *B. mycoides* are described as haemolytic and penicillin resistant. *B. anthracis* is exclusively lysed by gamma phage.

On the *Approved Lists of Bacterial Names*, *Bacillus mycoides* is classified under the genus *Bacillus*, in-group 1 (Fritze, 2004). *B. mycoides* B16 is a rod shaped gram-positive bacteria, is non-motile but other bacilli species are motile, aerobic and grows in a long chain forming a rhizoidal colony shape in 1.2% Tryptone soy plates (Di Franco et al., 2002). The genus comprises of the following: highly pathogenic risk group 3 (*B. anthracis*); group 2 species (e.g. *B. cereus*) which causes diarrhoea, emesis or mastis causing and lowest risk group 1: (e.g. *B. mycoides*) a harmless saprophytic soil inhabitants and (*B. thuringiensis*) a well know plant pest control microbial agent. 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.

The World Health Organisation (WHO) defines risk groups (or hazard groups) for classification purposes. The microorganisms are classified in four hazard groups (groups 1, 2, 3 and 4) applicable to work with in a laboratory (Fritze, 2004). Organisms are allocated to these groups according to the increasing risk they pose for human beings or animals. Allocation of species to risk groups is decided upon on a regional or national level with European including German legislation consistent with the classifications of the United States, Canada and Australia (Fritze, 2004).

The American Biological Safety Association (ABSA) website, accessed on 23/04/06, has different definitions for risk group 1. The EEC (Directive 93/88/EEC, Oct, 1993) defines Group 1 as 'biological agent means one that is unlikely to cause human

disease'. The NIH guidelines on Recombinant DNA (April 2002) defines risk group (RG1), as 'agents are not associated with disease in health adult humans'. The Canadian laboratory Biosafety Guidelines (2nd ed. 1996) defines risk group 1 as 'low individual and community risk). This group includes those microorganisms, bacteria, fungi, viruses and parasites, which are unlikely to cause disease in healthy workers or animals. The CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (4th ed. 1999) defines Biosafety 1 as 'is suitable for work involving well characterised agents not known to cause disease in healthy adult humans, and of minimal potential hazard to laboratory personnel and the environment. The South African Medical Research Council (MRC) website accessed on 23/04/06, defines hazard group 1 as ' an organism, i.e., any infective agent, that is most unlikely to cause human disease'.

Therefore *B. mycoides* B16 is classified in the lowest risk group 1 under the *Approved Lists of Bacterial Names* and the bacterium is emerging as a biological control for a number of nuisance organisms (Table 2.8).

Table 2.8: Biological control involving *B. mycoides* species

***B. mycoides* isolate**	**Source of isolation**	**Host prey**	**Country**	**Reference**
BmJ	Phyllosphere of sugar beet leaves	*Cercospora* leaf spot in sugar beet	Montana, USA.	Jacobsen et al. (2004).
JC192 & K184	Rhizosphere of winter wheat	*Fusarium culmorum* on winter wheat	Poland.	Czaban et al. (2004).
MW27	Pea rhizosphere soil sample	*Aphanomyces* root rot and ooospore formation in peas	New Zealand.	Wakelin et al. (2002).
B16	Tomato leaves	*Botrytis cinerea* on strawberry	Israel.	Guetsky et al. (2002).

2.5. FLOW CYTOMETRY FOR THE MEASUREMENT OF VIABLE *MICROCYSTIS* CELLS

2.5.1. Introduction

Flow cytometry is now an established method for the direct numeration of *individual* cell numbers, cell size distribution and cell complexity (biochemical and physiological) in aquatic and environmental microbiology (Vives-Rego et al., 2000). To date the flow cytometry has been applied to phytoplankton and bacterioplankton studies but other organisms such as protozoa and viruses the studies are still in the infancy (Vives-Rego et al., 2000). The most important feature of flow cytometry is that it enables measurements to be made on *individual* cells at high speeds after staining with a fluorescent marker or through the excitation of a naturally occuring fluorescent substance. This allows one to quantify the heterogeneity of the population of interest rather than merely to obtain average values for a population.

There are some naturally occurring cellular substances such as pyridine, flavin nucleotides and chlorophyll *a*, are capable of fluorescing when excited with a light of a suitable wavelength (Davey, 1994). Sometimes these cellular substances may interfer with an artificial fluorescent dye but are easily resolved through use of optical filters and colour compensation. There are fluorescent dyes that are specific for cellular substances and are used to study a particular cellular function or process. The most common dyes are nucleic acid stains and have a wider application. These include the determination of cell viability (Brussaard et al., 2001); bacterial respiration activity using CTC (Sieracki et al., 1999); cell membrane potential using rhodamine 123 (Rh123) (Kaprelyants and Kell, 1993); characterization of both polyclonal and monoclonal antibodies raised by toxic dinoflagellates (Collier, 2000); Also there are fluorescent dyes that evaluate cellular activity stains such as fluorescence diacetate (FDA) (Brookes et al., 2000); protein stains such as SYPRO (Zubkov et al., 1999); 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) and ultrasonic irradiation (Lee et al., 2000).

Thus the review on flow cytometry focuses on the use of this method in the viability analysis of phytoplankton, algae and cyanobacteria, in particular *Microcystis aeruginosa*, after exposure to different environmental factors. A working knowledge of the basics of flow cytometry is assumed; thus the technical aspects of instrumentation, methods of data analysis are not included but have been reviewed (Shapiro, 1998 in Davey, 1994).

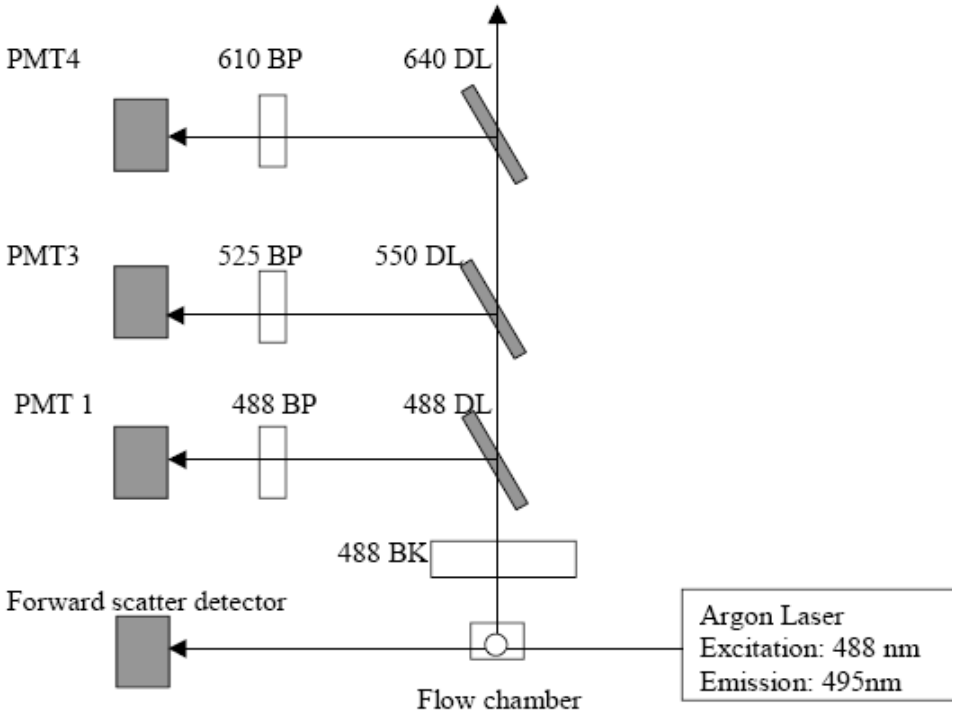


Figure 2.5: Schematic optical arrangement of the Beckmann Coulter Epics Alter® flow cytometer. The simultaneous measurement of forward scatter, side scatter, FDA fluorescence and PI fluorescence. DL=dichroic long pass filter, lets all wavelengths longer than specified through. BP= band pass filter, narrows down wavelength ±10nm. BK= block bar (blocks specific wavelength). PMT1: side scatter (cell granularity and complexity); PMT2: measures forward scatter; PMT3 measures FDA fluorescence; PMT4 measures PI fluorescence.

In a typical flow cytometer (Figure 2.5), a suspension of cells passes through a beam of light (source: laser or arc lamp). The incident light interacts with biological components of the cell and some of the light is either absorbed (forms the basis of

fluorescence measurements) or scattered (forms the basis for light scatter measurements) out of the incident light and is captured by an array of detectors positioned at different angles. For the scattered light there is no change in its wavelength and is later used for cell sorting, forward and side scatter measurements.

2.5.2. Light scattering measurements

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. Thus light that is scattered at small angles as demonstrated by Figure 2.6; that is forward scatter, could be used in the determination of relative cell size and cell volume based on homogenous model spheres that tend to approximate biological cells (Davey, 1994).

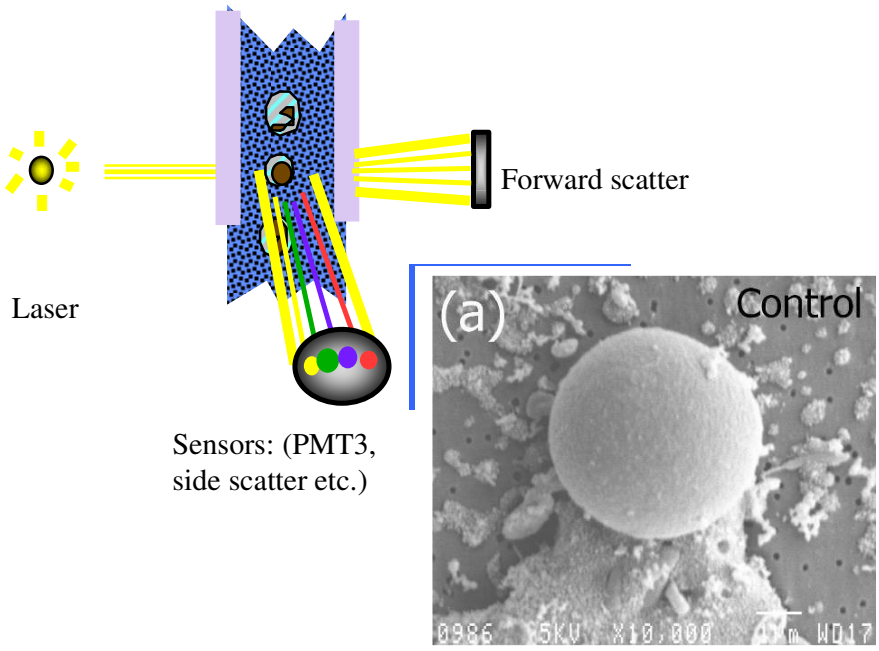


Figure 2.6: Forward and side scatter approximation (Murphy, 1996).

The *Microcystis aeruginosa* cell in its spherical structure (Figure 2.6) 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.

2.5.3. Fluorescence measurements

2.5.3.1. Principles of Fluorescence

When a compound absorbs light, electrons are raised from the ground state to an excited state. The excited electrons may thereafter return to the ground state via a number of routes (Figure 2.7): namely non-radiative process (energy is lost as heat) and radiative process (fluorescence).

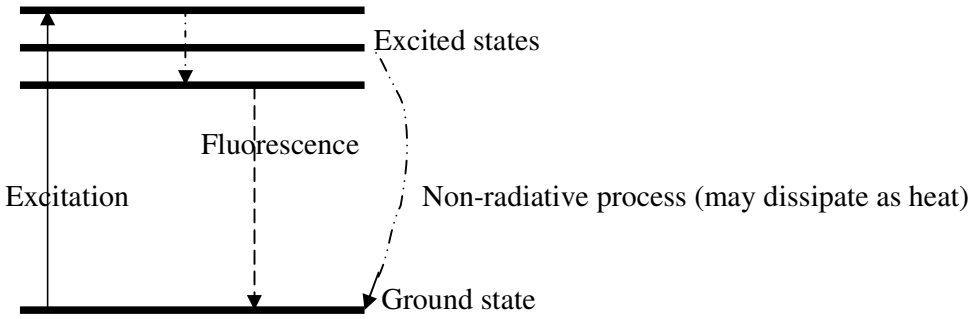


Figure 2.7: The absorption and emission of light during fluorescence

Phosphorescence is similar to fluorescence except that fluorescence stops immediately once the source of stimulation is stopped while with phosphorescence it continues for a while.

The excitation wavelength of a fluorescent stain results in fluorescence of a lower energy (emission) and hence a longer wavelength. The difference between the absorption and emission maximum is referred to as the Stoke's shift (Figure 2.8). It can be deduced that a difference of at least 20 nm (Table 2.9) may be required to correctly resolve incident from emitted light by optical filters (Petit et al., 1993).

It is possible to simultaneously excite different fluorescent stains such as propidium iodide (PI) and fluorescence diacetate (FDA) with a single light source such as a laser when tuned at 488 nm. The result is emission of PI fluorescence (Figure 2.8) and

fluorescein fluorescence (from FDA) (Figure 2.9). The emission of fluorescein is centred on 514 nm, while that of PI is centred at 625 nm. This difference in Stoke's shift between the two fluorophores enables their fluorescence emissions to be separated by the use of optical filters, and so permits multiple fluorescence parameters to be measured on individual cells. The simultaneous measurements of several fluorescent stains (two or more) in conjunction with light scattering measurements (at one or more angles) on individual cells demonstrate the potential of flow cytometry for multi-parameter data acquisition (Davey, 1994).

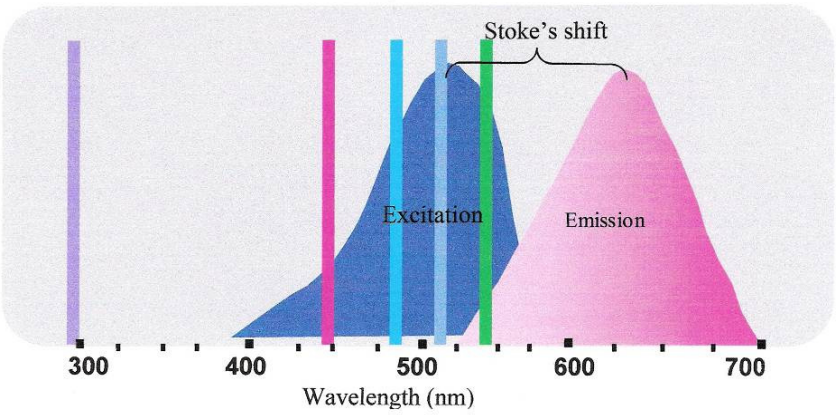


Figure 2.8: The absorption wavelength of propidium iodide (PI) is at 535 nm (Murphy, 1996). The the closest excitation wavelength is at 488 nm with an argon laser. The PI fluorescence emission (when electrons return to ground state) results in a longer wavelength at 617 nm. The difference in wavelengths (excitation and emission) is known as Stoke's shift. If a cell is labelled with PI, this results in PI fluorescence at wavelengths 550 and 750nm.

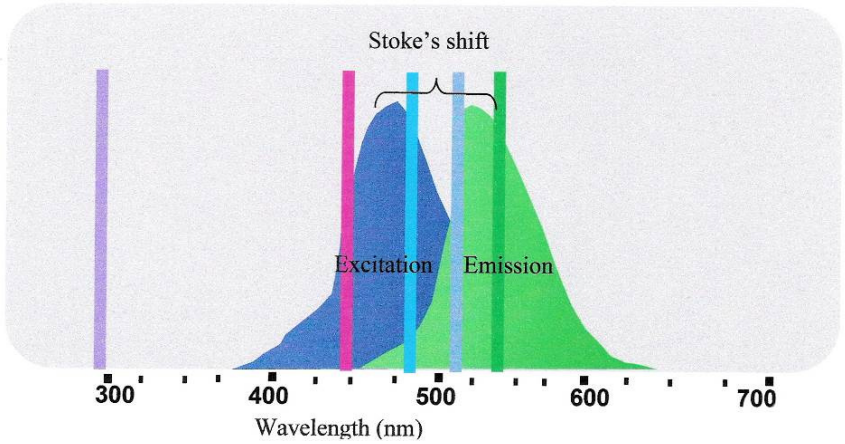


Figure 2.9: The absorption wavelength of fluorescein fluorescence is at 473 nm (Murphy, 1996). The emission is at 514 nm. FDA can both be efficiently excited by the same light source (e.g. an argon ion laser tuned to 488 nm). The fluorescein fluorescence emission (when electrons return to ground state) results in a longer wavelength at 514 nm. The difference in wavelengths (excitation and emission) is known as Stoke's shift.

2.5.3.2. Natural autofluorescence

Some naturally occurring cellular substances such as pyridine, flavin nucleotides and chlorophyll *a*, are capable of fluorescing when excited with a light of a suitable wavelength (Davey, 1994). Pyridine impacts UV-excited blue fluorescence while flavin nucleotides when blue-excited impacts a green fluorescence. In the field of aquatic biology, flow cytometry is applied in the study of autofluorescence of pigments that are used in the identification of algae and cyanobacteria. The light reaction site responsible for photosysnthesis occurs on a series of parrallel membranes within the cyanobacteria cytoplasm. These membranes contain chlorophyll *a* and several accessory pigments (phycobilisomes). Chlorophyll *a* absorbs when excited at both the UV-blue (< 450 nm) and in the far red (~ 680 nm). The chlorophyll *a* fluorescence is collected in the near infrared. The other chlorophylls *b* and *c* together with the carotenoids, capture photons and pass them to chlorophyll *a* (Glazer, 1989; Davey, 1994).

The accessory pigments, phycoerythrin, phycocyanin and allophycocyanin, which absorb blue-green, yellow-orange and red light are used for classification of mixed algal samples (Glazer, 1989). A job was made easier with multi-parametric flow cytometry analysis. Based on the chlorophyll *a* fluorescence (used as a gating parameter) it is then possible to discriminate *Microcystis* cells from other organisms including bacteria from further analysis.

2.5.4. Fluorescent stains

Fluorescent dyes that are specific for cellular substances and in conjunction with flow cytometry have been used for a variety of applications (Table 2.8). The most common dyes are nucleic acid stains that have a wider application. These include the determination of cell viability (Brussaard et al., 2001); bacterial respiration activity using CTC (Sieracki et al., 1999); cell membrane potential using rhodamine 123 (Rh123) (Kaprelyants and Kell, 1993); characterization of both polyclonal and monoclonal antibodies raised by toxic dinoflagellates (Collier, 2000); FITC-labelled oligonucleotides directed against 18rRNA for discrimination of chlorophytes from non-chlorophytes (Collier, 2000); a rRNA-directed oligonucleotides for detection of toxic dinoflagellates *Alexandrium fundyense* (Collier, 2000) and analyses involving interactions between algae and viruses (Brussaard et al., 2001). The combination of taxonomy (rRNA probes) and different fluorescent stains has extended flow cytometry to the study of *individual* cells that may be responsible for particular event such as bacterial pathogens in water and food.

Other dyes that have been used include protein stains such as SYPRO (Zubkov et al., 1999); cellular activity stains such as FDA (Brookes et al., 2000); to quantify viability in phytoplankton, in particular *Microcystis* following exposure to different environmental stress factors. These include 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) and ultrasonic irradiation (Lee et al., 2000). The review focuses on two fluorescent probes that evaluate cellular viability and membrane integrity, namely propidium iodide (PI)

and fluorescence diacetate (FDA). Joux and Lebaron (2000) gave a detailed review of the other fluorescent probes and their applications.

2.5.4.1. Determination of dual cell activity

Defining cell death and cell viability is philosophically and experimentally difficult (Joux and Lebaron, 2000). However for the purpose of this study, cells in which metabolic activity can be detected are called active cells (live) and those with damaged membranes are considered dead cells, with the loss of nucleiod material (Joux and Lebaron, 2000).

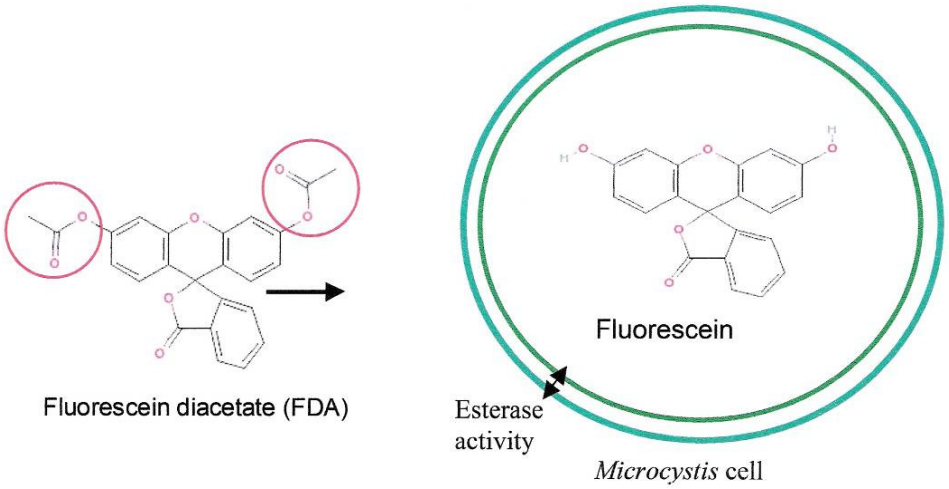
Esterases are present in all living organisms and these enzymes can provide useful information on the status of bacterial metabolism including that of cyanobacteria. The synthesis of enzymes is energy dependent but enzyme-substrate reaction is energy independent (Joux and Lebaron, 2000). Dead or dying cells with damaged membranes (even with residual esterase activity) rapidly leak the fluorescent dye. Thus the fluorescent dyes are both indicators for metabolic activity and cell integrity. Therefore enzyme activity is required to activate their fluorescence and cell membrane integrity is then required for intracellular retention of the fluorescent substance. Some of the more commonly used fluorescent stains that target enzyme activities as a measure of cell metabolism and cell viability are shown in Table 2.9.

Table 2.9: [a]Characteristics of different fluorescent stains and their applications in flow cytometry (Joux and Lebaron, 2000).

Characteristic	**Fluorescent stain**	**Absorption (λ_A) (nm)**	**Fluorescence Emission (λ_F)(nm)**	**[b]Stoke's shift $\lambda_F - \lambda_A$**	**Staining properties**	**Applications**
Dehydrogenase activity	CTC (CTC formazan, CTF)	450	580-660	70- 110		
Esterase activity	FDA (fluorescein)	473	514	39	Cytoplasm	Cell viability
	CFDA (carboxyfluoroscein)	492	517	25		
	CFDA-AM (carboxyfluoroscein)	492	517	25		
	BCECF-AM	482	520	38		
	Calcein-AM	494	517	13		
	ChemChrome	488	520	32		
Membrane potential	Rh123	507	529	22		
	DiOC6(3)	484	501	17		
	DiBAC4(3)	493	516	23		
	Oxonol VI	599	634	35		
Probe efflux	Ethidum bromide	518	605	87		
Membrane integrity	Syto-9 (membrane permeant stain)	*blue	*green			
	Syto-13 (membrane permeant stain)	488	509	21		
	Propidium iodide	535	617	82	Nuclear DNA binding	Membrane integrity
	Sytox Green	502	523	21		
	PO-PRO-3	539	567	28		
	CSE	*blue	*orange			

[a]This is not an exhaustive lists and refers mainly to two applications that are discussed in this review.

[b]Fluorescence Stoke's shift (nm) may be correctly resolved with optical filters.



Source of molecular structures: PubChem substance

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=pcsubstan

Figure 2.10: A diagrammatic model of a *Microcystis* cell illustrating the enzymatic deacetylation of acetate molecules (red circle) of FDA.
The acetate molecules are non-toxic and do not interfere with normal cell functions. The non-specific esterases are located within the cell wall (↔). The resultant product: fluorescein is a polar substance, which is strongly retained inside a cell with an intact cell membrane. It is the fluorescein molecule which gives rise to green fluorescence when excited by a laser.

Fluorescence diacetate (FDA), a lipophilic substance, enters freely into bacteria cells including cyanobacteria and in particular the gram-negative *Microcystis* cells (Brookes et al., 2000). Most cells including mammalian cells, yeast cells, gram-positive or gram-negative bacteria can hydrolyze FDA (Breeuwer et al., 1995). Once inside the active cell, the substrate (FDA) is cleaved by non-specific esterases releasing a polar fluorescent product, fluorescein (Figure 2.10). The fluorescein is retained inside cells with an intact membrane but leaks out if the membrane is damaged. The fluorescein is then excited with a laser tuned @ 488 nm, which results in emission of a green fluorescence (514 nm).

In general, gram-negative bacteria are impermeable to lipophilic fluorescent probes such that a permeabilisation procedure for the outer membrane is required (Joux and Lebaron, 2000). The purpose of permeabilisation is to sensitise the outer membrane

such that it is easier to introduce fluorescent stains. Though in other studies it was shown that FDA was known to give weak fluorescence signals due to poor retention of fluorescein inside cells with an intact membrane (Petit et al., 1993). To improve fluorescein fluorescence signals and strong retention, derivatives of FDA has been synthesized. Example of some of the derivatives are: acetoxymethyl easter (Calcein-AM), carboxyfluorescein diacetate (CFDA), carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM) and 2',7',-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM).

2.5.4.2. Determination of membrane integrity

The cell membrane has multiple functions including permeability barrier, transport and respiration, such that loss of membrane integrity represents a significant damage to a cell (Joux and Lebaron, 2000). The loss of membrane integrity is an indicator of cell death (Vives-Rego et al., 2000). The cells with membranes displaying selective permeability (as result of permeabilisation steps, or caused by other means) are classified as dead since their cellular structures are exposed to the environment and will eventually decompose (Vives-Rego et al., 2000). However such cells may have the potential to give rise to active metabolism or proliferation should favourable conditions exist or due to removal of the external stimulus.

The existence of DNA in all bacteria including cyanobacteria makes this an ideal staining target though accessibility to it may be problematic. As it were the membrane of bacteria is a complex structure. For gram-negative bacteria the membrane consists of three interacting layers: the outer membrane, the rigid peptidoglycan layer and the inner membrane (plasma membrane). For gram-positive bacteria, the outer membrane is absent. Some gram-positive and gram-negative bacteria have an additional protective structure called the capsule.

The *Microcystis* nucleoplasmic region consists of DNA fibrils which are organised in a complex helical and folded configuration distributed throughout the centroplasm. The genomic size is variable for different cyanobacterial species with a molecular weight range of 1.6×10^9 to 8.6×10^9 daltons. Ribosomes are widely distributed

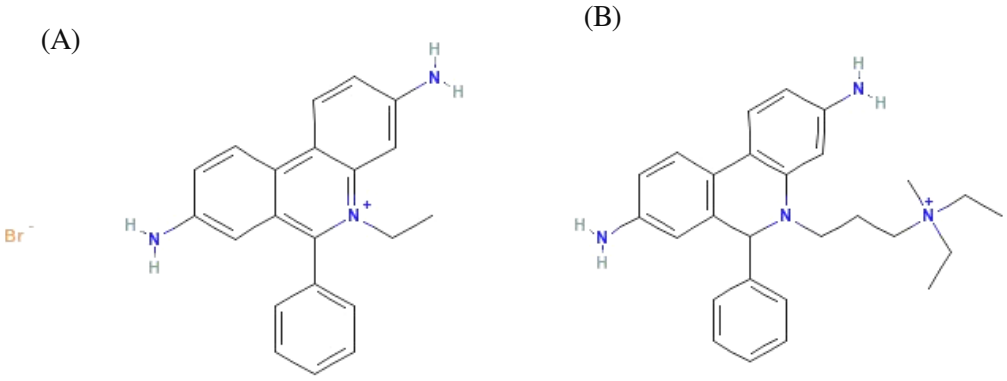
throughout the cytoplasm and are concentrated in the nucleoplasmic region (Fray, 1993).

The cyanobacteria frequently contain unsaturated fatty acids with two or more double bonds whereas other bacteria contain almost exclusively saturated and mono-unsaturated fatty acids with one double bond (Brock et al., 1994). Although the cyanobacterial cell wall is significantly thicker, it resembles that of a gram-negative bacterium (Rapala et al., 2002). The cell wall consists of three or four outer layers between the plasma membrane (or plasmalemma) and the sheath (Holm-Hasen, 1968). The cell wall thickness may range from 10 to 20 nanometers and is coated with a relatively thick, jellylike capsule or slime of proteinaceous material (Skulberg et al., 1993). The outer membrane which may be smooth or possess infoldings that extend into the cell form structures called mesosomes. The membrane regulates what enters and leaves the cells. In the cytoplasm are thylakoid membranes which are sites for enzymatic reactions including photosynthesis, electron transport and ATP synthesis. The cell walls of *Anabaena nidulans* contain 24% sugar, 28% protein and 36% lipid (Holm-Hansen, 1968). The main components of the carbohydrate fractions are mannose, glucose, galactose and fructose.

Membrane integrity assessments are based on the exclusion of fluorescent stains which when used at low concentrations do not pass across intact membranes. Thus most nucleic acid stains target DNA located within the cell nucleus and is accessible once the membrane is damaged to some degree. The loss of membrane integrity is measured by uptake of membrane-impermeant stains and the reaction is considered irreversible. Some of the more commonly used nucleic acid stains are shown in Table 1.8. The phenanthridinium stains (Figure 2.11); ethidium iodide and propidium can bind to both DNA and RNA. Propidium iodide is the stain of choice since its emission spectrum is about 10 – 15 nm further towards the red ethidium bromide and is easily separated from that of fluorescein (Davey, 1994). Though there is the problem of fluorescence quenching if both stains are used in combination, opportunities do arise where this quenching is applied in other assays (Nebe-von-Caron et al., 1998).

Propidium iodide (PI) is a polar substance that easily penetrates only inactive or damaged cell membranes. Once inside the cell, PI intercalates between base pairs of

both double-stranded DNA and RNA and gives a bright red fluorescence under blue light excitation (Yamaguchi and Nasu, 1997). The staining of nuclei with PI showed that fluorescence intensity correlated with DNA degradation, which is a hallmark of apoptosis (Petit et al., 1993).



Source of molecular structures: PubChem substance

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=pcsubstan

Figure 2.11: The structures of RNA/DNA fluorescent stains: (a) ethidium bromide and (b) propidium iodide. The stains intercalates between base pairs of both double stranded DNA and RNA in an irreversible reaction which results in an enhancement of fluorescence over that of the free stain.

2.5.4.3 Multi-staining assays: combination of fluorescent stains

The use of multi-staining assays provides confident tools and some of these are actually under validation for industrial applications such as water quality assessment in the pharmaceutical industry (Joux and Lebaron, 2000).

By combining different fluorescent stains targeting different cellular functions, a more accurate picture of cell activity may be realised. Assays in which both membrane and metabolism based probes are used simultaneously provide information on whether the multi-staining assays accurately reflect cell activity (Joux and Lebaron, 2000). The fluorescent stains should be selected with contrasting wavelengths, excitation and

emission, which allow discrimination of each stain in the presence of the other. The selection of fluorescent stains must take into account molecular interactions, which can result in reduction in fluorescence through quenching. This is a phenomenon whereby fluorescence emission of one stain is used to excite the other stain and or loss in energy dissipation by non-radiative processes. Nebe-von-Caron et al. (1998) used this concept of quenching studies involving ethidium bromide and propidium iodide in the viability assessment of *Salmonella typhimurium* cells.

When the fluorescent stains such as FDA and PI are used in multi-staining assay, cells with intact membranes showed a green fluorescein fluorescence while damaged cells showed a red PI fluorescence. The problems that may arise are due to membranes with selective permeability that allow both stains to permeate and fluorescence; such cells are classified as dead even if there is residual esterase activity (Vives-Rego et al., 2000).

The use of combined FDA and PI in flow cytometry has been successfully applied in a wide variety of microbiological work. Hickey et al. (2004) applied the multi-staining assay to the study of viability of fungal cells in a cell population. Because of the poor fluorescein retention in fungal cells they recommended that the assay should be immediately performed after adding the stains and incubation. As an alternative to using FDA, the researchers suggested the use of BCECF, which was better retained by cells due to cross-linking of fluorescein derivative. Earlier on, Lee et al. (2000) used the LIVE/DEAD BacLight viability kit (L7007, Molecular Probes) to study the viability of *Microcystis* after ultrasonication treatments. The researchers assessed the ability of *Microcystis* cells to regenerate their gas vacuole, which had collapsed after sonic treatments. The study showed that *Microcystis* cells were able to regenerate their gas vacuole once the external stimulus was removed and growth conditions were favourable. Thus flow cytometry managed to distinguish live and dead cells of *Microcystis* cells.

The development of multi-staining techniques and flow cytometry has enabled the accurate evaluation of cell activity especially when determining *individual* cell heterogeneity at either population levels or community levels.

2.6. CONCLUSIONS

There are increasing demands to reduce the use of chemicals such as copper algicides for HAB management for environmental and safety reasons. Thus, the development of a non-chemical control measure such as biological control is of great importance.

Predatory bacteria are the more potent biological control agent when compared with viral pathogens as a result of their ability to survive on low prey availability and are adaptive to variations in physical conditions. These bacterial agents have been isolated from a variety of sources such as the terminal stages of harmful algal blooms. Some papers, describe bacteria, which were isolated from eutrophic waters, such as *Sphingomonas* species with abilities to degrade microcystins and *Saprospira albida* with abilities to degrade *Microcystis* cells. Further research is required to evaluate whether these bacteria are antagonistic towards cyanobacteria. Ideally, a combination of strategies should be introduced; i.e., combine predatory bacteria that directly lyse the cyanobacteria with microcystin degrading bacteria that then 'mop up' the released microcystins.

Although the mechanisms of cyanobacterial lysis have been proposed, which include antibiosis, production of lytic enzymes, parasitism and competition for nutrients and space; it is often difficult to ascribe cyanobacterial lyses to one mechanism only. The predatory bacteria that cause cyanobacterial lysis appear to act in four major ways: contact lysis, production of lytic enzymes or extracellular products, antibiosis after entrapment of host and endoparasitism or ectoparasitism of host. Most of these studies were based on laboratory cultures, and need to be extended to field trials to determine which mechanisms may be applicable to large-scale applications. However, care should be taken when extrapolating laboratory- based observations to field conditions.

Very little information is available on the successful use of predatory bacteria under natural conditions. The predator-prey ratio needed for cyanobacterial lysis is an important parameter to consider when using predatory organisms for biological control purposes. It is clear that the critical predator-prey ratio needs to be met or exceeded if successful cyanobacterial lysis is to occur. Augmentative biological

control may provide a means to increase the predator population to above the threshold required to induce large-scale cyanobacterial lysis.

Further studies are required in the development of anti-algal chemicals such as protease I that may cleave pentaglycine bridge in the cell wall of cyanobacteria (Wolfe et al., 1972; Nakamura et al., 2003b; Choi et al., 2005). These anti-algal substances may be less toxic to the environment when compared to copper algicides.

Some authors have described the use of *Bacillus* species in the management of *Microcystis* blooms. Recent studies have also shown that *Bacillus mycoides* is an emerging biological control agent. It has been applied to control a variety of nuisance organisms except *Microcystis*. One researcher found out that *Bacillus cereus*, a close relative of *B. mycoides* was antagonistic towards *Microcystis*. Thus further studies are required to explore the possibility that *B. mycoides* that might show similar attributes.

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^{o}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^{o}C 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^{o}C) 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 suspensions 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^{o}C) for 24 h. The process was repeated for other bacterial isolates. After cultivation the bacterial cell suspensions 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^{o}C). The bacterial cell suspensions 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.

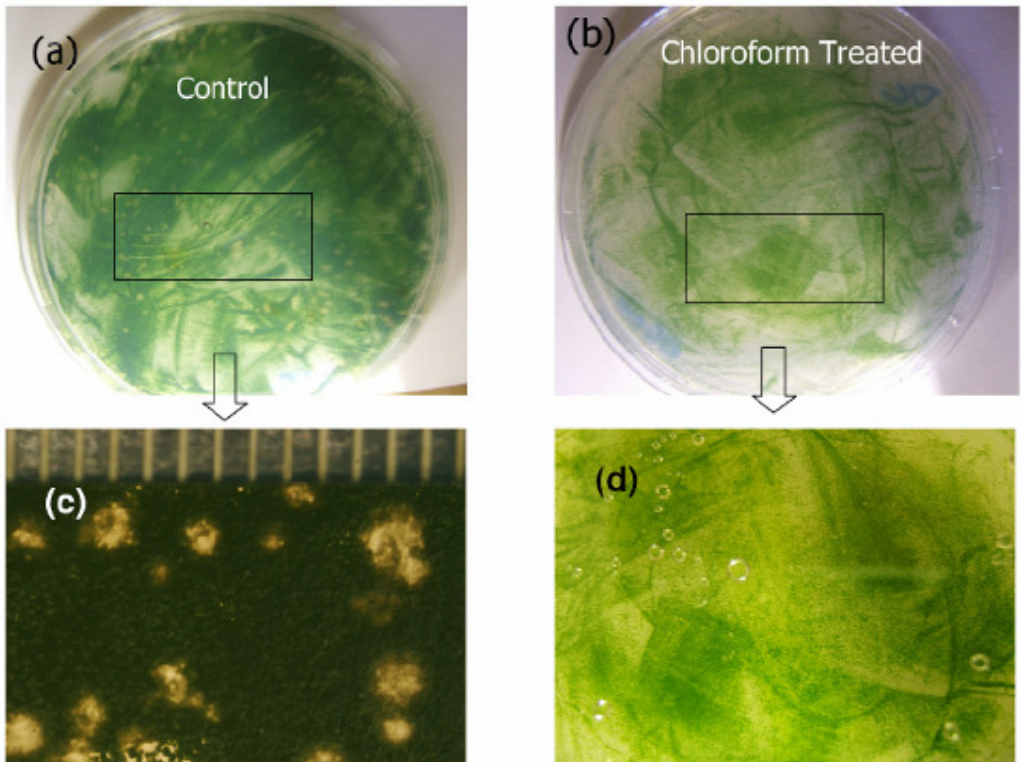


Figure 3.1: Analysis for cyanophage activity on *Microcystis* lawns. (a) Control sample (no chloroform added) showing the development of plaques indicating that bacteria were probably responsible for plaque development. (b) Chloroform treated sample showing the absence of plaque development. (c) Magnification 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.

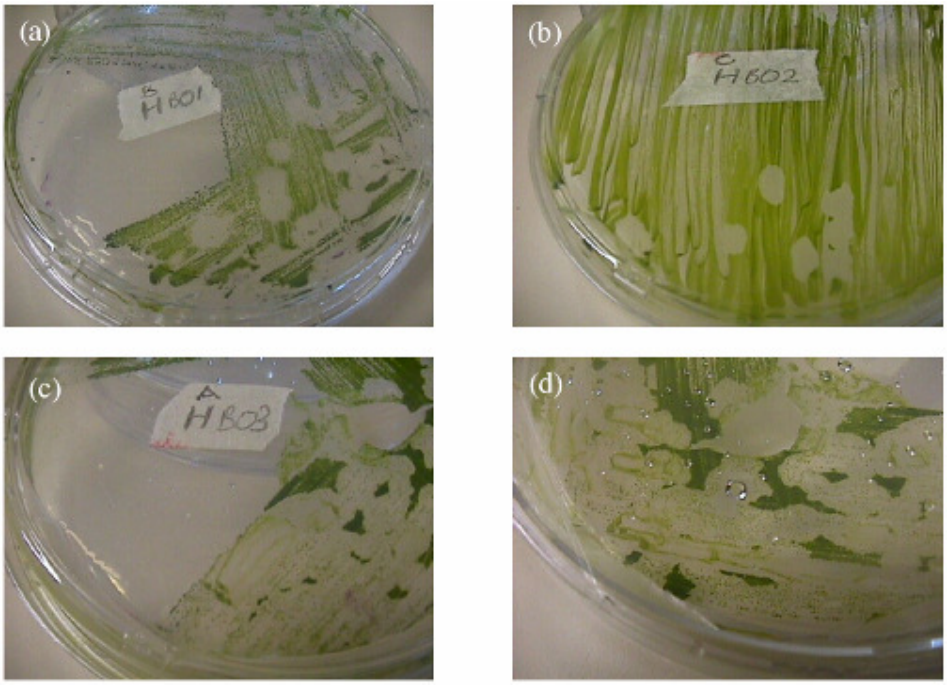


Figure 3.2: Appearance of plaques on *Microcystis* lawns after 30 days of incubation The samples were obtained different locations at Hartbeespoort dam: from boat pier (a) HB01; (b) HB02; (c) HB03 and (d) DWAF 2 dam wall.

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.

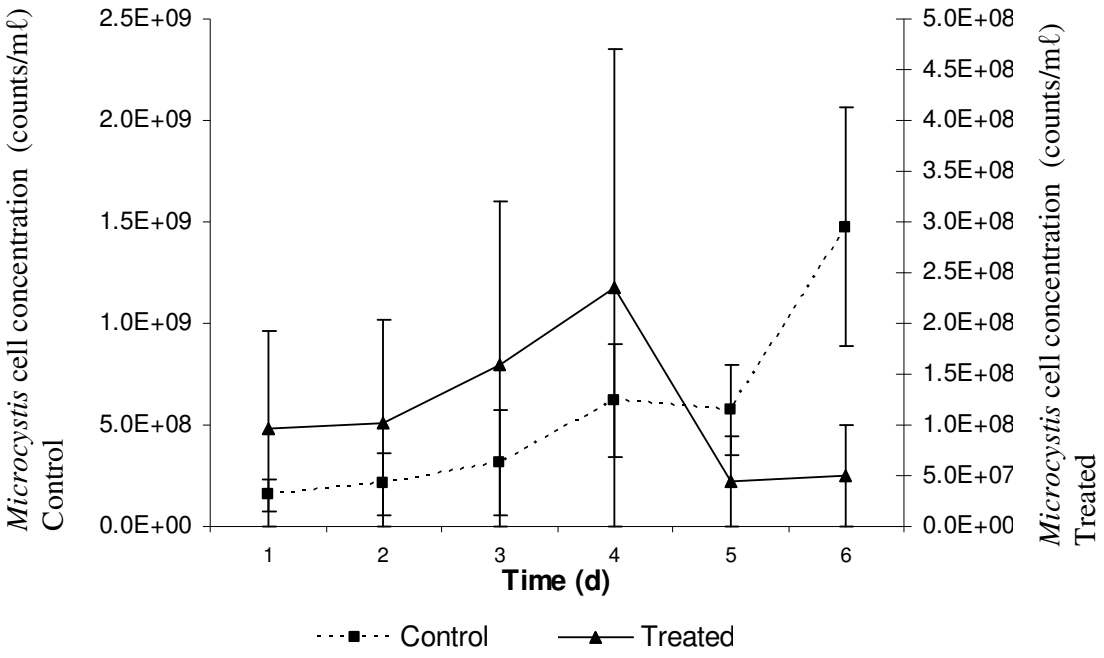


Figure 3.3: *Microcystis aeruginosa* PCC7806 cell counts after exposure to isolate B2. In control samples, changes in cell density of *Microcystis* without bacterial treatment. Bars indicate the standard deviation.

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

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).

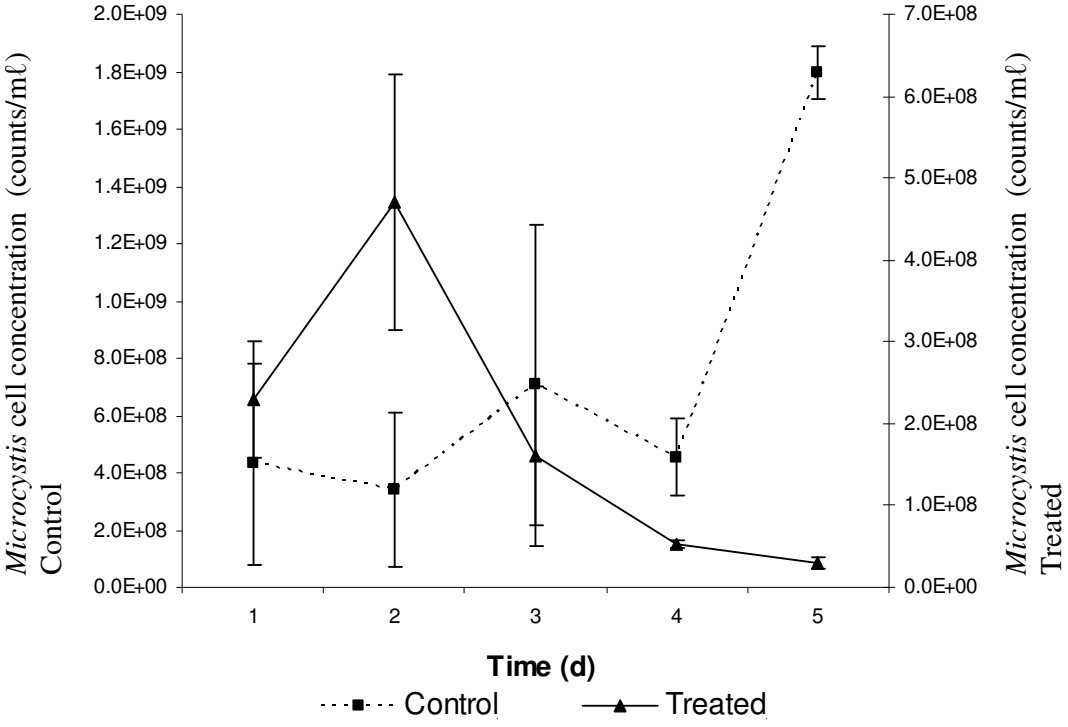


Figure 3.4: *Microcystis aeruginosa* PCC7806 cell counts after exposure to isolate B16. In control samples, changes in cell density of *Microcystis* without bacterial treatment. Bars indicate the standard deviation.

An initial inoculum of 1.00 x 10^8 cfu per mℓ predator cells caused lysis of 4.3 x 10^8 cells per mℓ *Microcystis.* The predator-prey ratio was 1:4.3 ≈ 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.

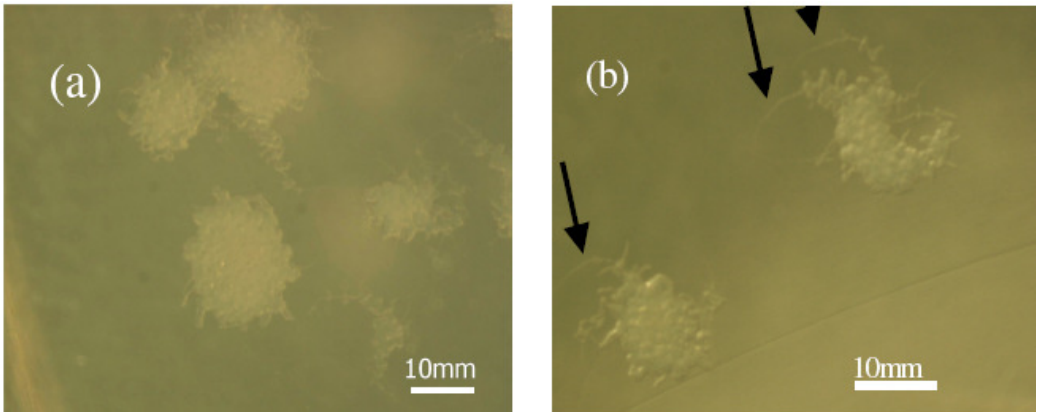


Figure 3.5: (a) Cotton-like spread colonies and (b) *B. mycoides* B16 SIN type. Note the filament projections curve anti-clockwise (black arrow), as observed from the bottom of a petri dish and is classified as SIN.

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

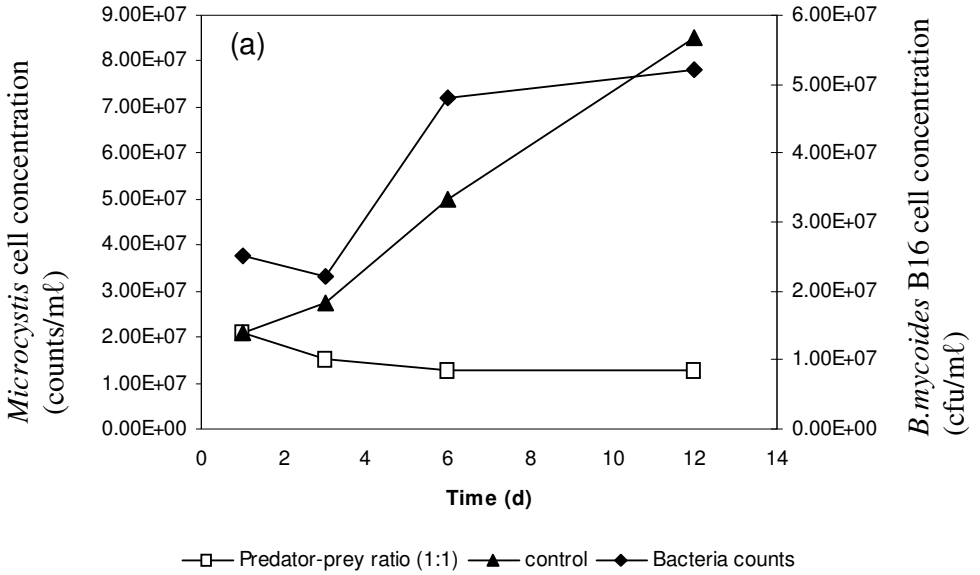
Table 3.5: Different predator: prey ratios

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.



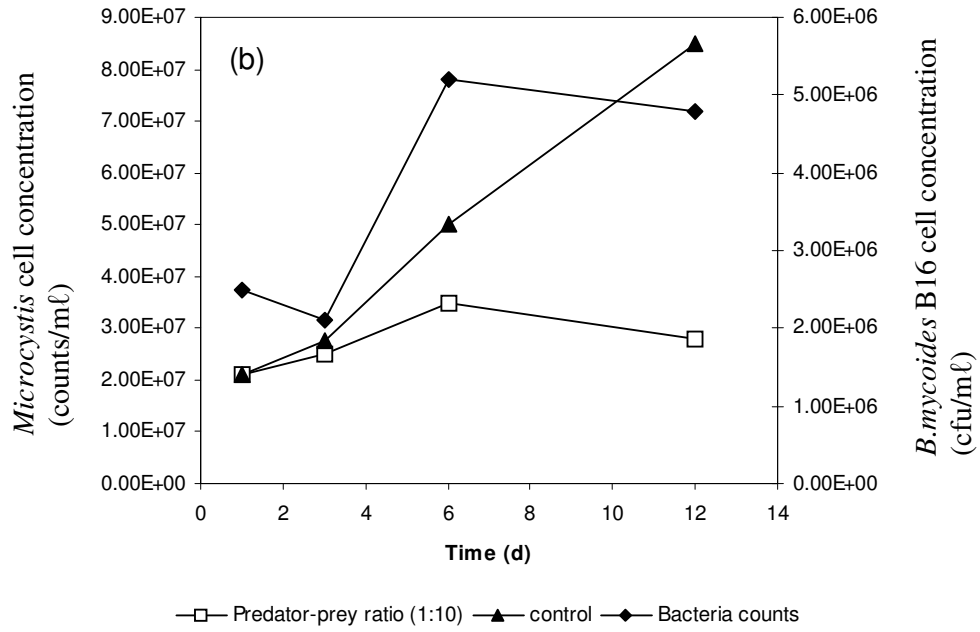


Figure 3.6: The effect of predator-prey ratio on *Microcystis* viability and changes in predator numbers: (a) 1:1 ratio and (b) 1:10 ratio

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).

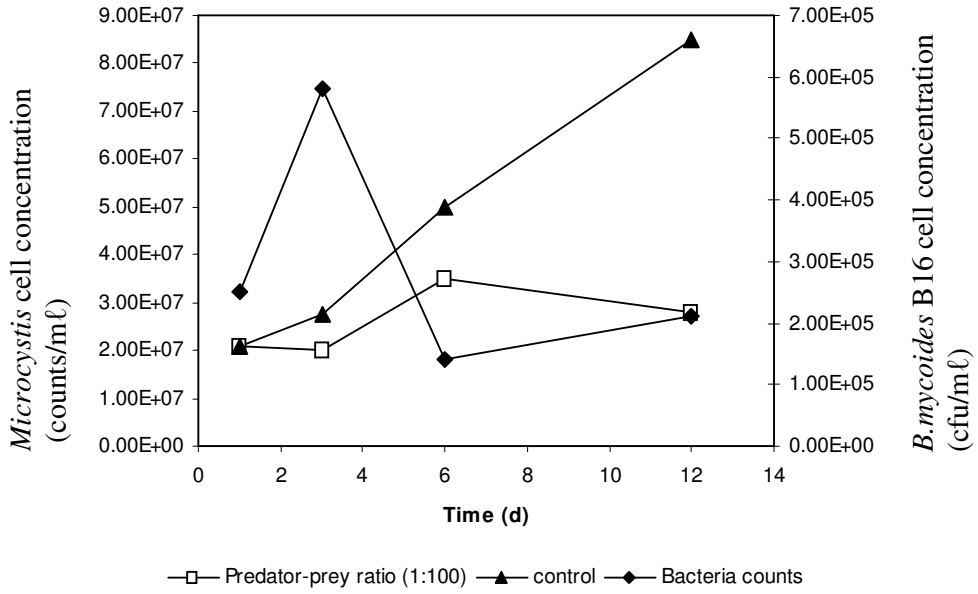


Figure 3.7: The effect of predator-prey ratio (1:100) on *Microcystis* viability and changes in predator numbers

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.

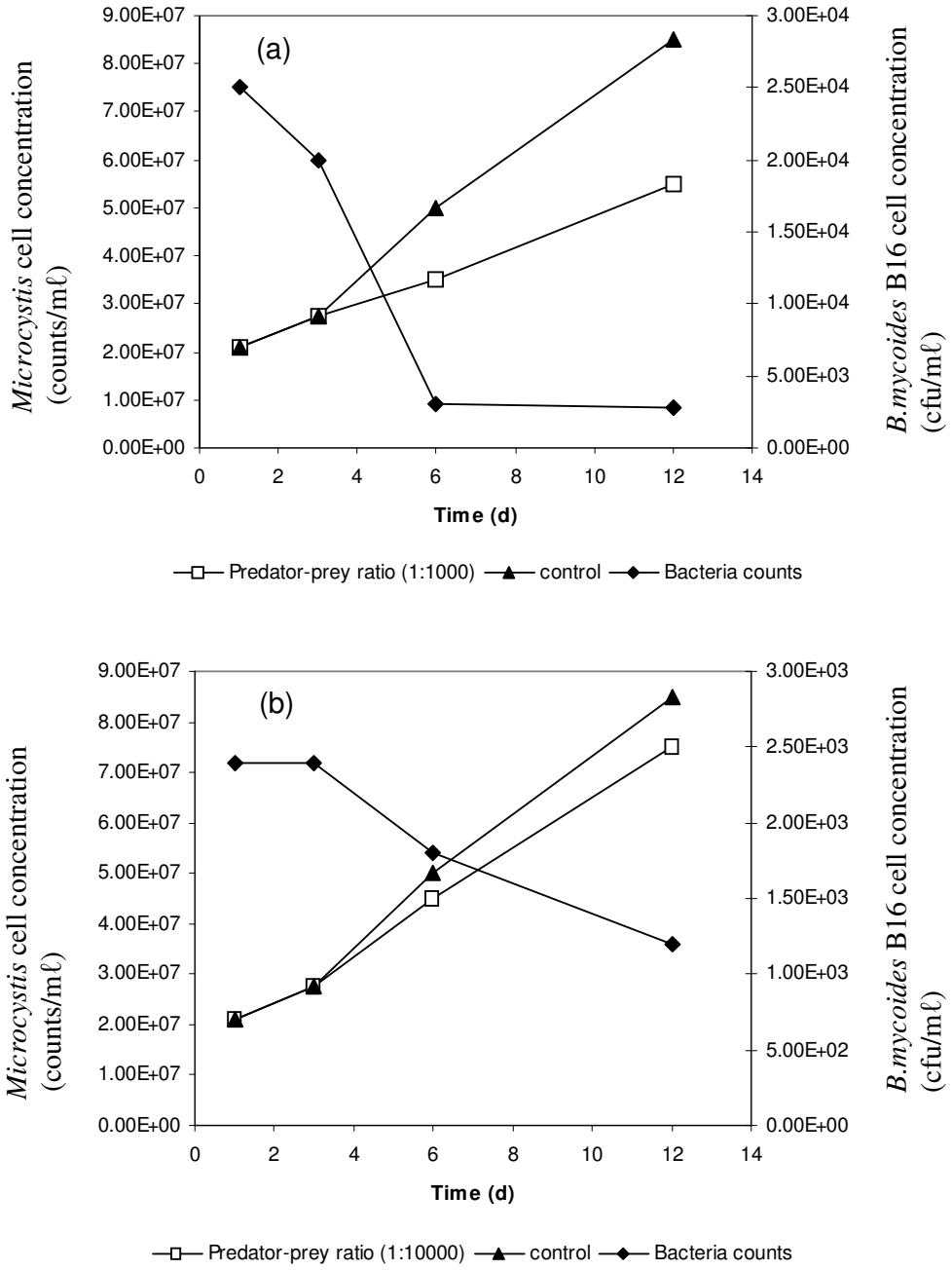


Figure 3.8: The effect of predator-prey ratio on *Microcystis* viability and changes in predator numbers: (a) 1:1000 ratio and (b) 1:10000 ratio

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*.

Chapter 4: Light and electron microscope assessment of the lytic activity of bacteria on *Microcystis*

Parts of this chapter have been published in:

1. Proceedings of the *Microscopy Society of Southern Africa.* 36: 38 (2006).
2. Proceedings of the *Microscopy Society of Southern Africa.* 34: 34 (2005).
3. Proceedings of the 12th International Conference on Harmful Algae. xx:x (2007).

CHAPTER 4

LIGHT AND ELECTRON MICROSCOPE ASSESSMENT OF THE LYTIC ACTIVITY OF BACTERIA ON *MICROCYSTIS*

Abstract

During the screening of lytic bacteria, plaques were obtained on *Microcystis* lawns. In the plaques, at least five distinct morphotypes of bacteria were found. The plumb rod-shaped bacilli were the most abundant and were found aggregated around unhealthy *Microcystis* cells and were the probable cause of deflation and lysis. Maybe these bacteria utilized the cyanobacteria cell contents as their nutrient source. In contrast the control areas the cyanobacteria cells were healthy and did not show any visible distortion of cell structure. The presence and possible role of free-bacteria, i.e., bacteria that was not attached or associated with the cyanobacteria in the plaque is not clear. Maybe their function is to scavenge the skeletal remains of *Microcystis* cells. Isolates B2 and B16 were found to have a lytic effect on *Microcystis* cells. SEM images of isolate B2 and B16 did not reveal any unique attachments that may allow them to adhere to *Microcystis* cells. The *Microcystis* cells were exposed to copper, *B. mycoides* B16 and Triton X-100 in order to ascertain the level of cell membrane damage. The membrane cell damage was most severe with copper, stripping the entire *Microcystis* cell membrane leaving a honeycomb skeletal structure and *B. mycoides* B16 leaving perforations on the cell membrane. The electron microscopy observations appear to reveal at least two mechanisms of *Microcystis* lysis i.e. contact and parasitism. The TEM observations did not reveal any endoparasitism of *B. mycoides* B16 or *Bdellovibrio*-like behaviour. There were ultrastructural changes that occurred in bacteria treated *Microcystis* cells. SEM images showed swollen *Microcystis* cells, either due to osmosis and or the presence and multiplication of *Bdellovibrio*-like bacteria inside the *Microcystis* cell. The *Bdellovibrio*-like behaviour of *B. mycoides* B16 was indirectly shown by the presence of bdelloplasts that were present during the last stages of *Microcystis* lysis.

Key words: *Microcystis*, mechanism of lysis, *B. mycoides* B16, photosynthesis, copper

4.1. INTRODUCTION

Harmful algal blooms (HABs) in freshwater resources, are often dominated by *Microcystis* species, are on the increase worldwide including South Africa and can cause a wide range of social, economic and environmental problems. The HABs are associated with the production of microcystins that affect water quality with adverse effects on lake ecology, livestock, human water supply and recreational amenities (Codd et al., 1997; Nakamura et al., 2003b; Choi et al., 2005). During the bloom period, there are microbial agents such as bacteria and viruses that have been found to have commensalistic and antagonistic relationships with the cyanobacteria (Shilo, 1970; Burnham et al., 1981; Ashton and Robarts, 1987; Bird and Rashidan, 2001; Nakamura et al., 2003a; Choi et al., 2005).

The interactions of bacteria and cyanobacteria in aquatic environments are numerous, ranging from: (a) competition for available organic matter; (b) provision of extracellular substances by cyanobacteria which are beneficial to bacteria and vice versa; (d) antagonistic behaviour whereby the bacteria feeds on cyanobacteria and (d) production of cyanobacteria exudates which inhibit growth of bacteria and vice versa (Bates et al., 2004).

The relationships between these microbial agents and cyanobacteria are becoming increasingly important for the better understanding harmful algal bloom dynamics (Bates et al., 2004). There is a close spatial and temporal coupling of microbial agents and cyanobacteria and both groups tend to synthesise metabolites that can be beneficial (Brunberg, 1999) or even harmful to one another (Grossart, 1999 in Bates et al., 2004). Different types of bacteria with specialized extracellular substances are associated with the initiation, maintenance and termination phases of algal blooms (Riemann et al., 2000). Specific bacteria may also be attracted to the phycosphere, the region surrounding the algae cells, where their growth may be stimulated by algal exudates (Bates et al., 2004). Some bacteria are found attached to live or dead algal cells indicating the possibility of an antagonistic relationship, which may be explored for biological control (Maruyama et al., 2003). Scanning electron micrographs revealed the attachment of *Flexibacter flexilis* to the sheaths of filamentous

Oscillatoria willimasii, which resulted in excretion of lysozyme and then lysed the cyanobacterium (Sallal, 1994). The *F. flexilis* bacterium benefits from the cyanobacterium nutrients after lysis and helps regulate population levels of *O. willimasii* in raw sewage aeration tanks. The cyanobacterium *O. willimasii* is known to produce exudates that contribute to the biodeterioration of raw sewage settling tanks. Thus the management control of this cyanobacterium is important.

Microcystis cells were lysed by a *Bdellovibrio*-like bacterium (BLB) after penetration (Caiola and Pellegrini, 1984). Once the host was penetrated the BLB was localised between the cell wall and cytoplasm membrane, which appeared thickened on TEM negative staining. The cell wall appeared broken at many sites and this was attributed to the breakdown of the cell wall leading to cell lysis and death. The BLB adhered to the *Microcystis* cell plasmalema by means of tubular structures. These membranous extensions may possibly represent recognition sites to allow for interactions between bacteria and cyanobacteria. In the natural environment, the BLB are selective only invading *M. aeruginosa* but not *M. wesenbergii* (Caiola and Pellegrini, 1984). This was attributed to the fibrous glycocalyx, that function as a recognition site between the bacteria and its prey. In an earlier study involving a BLB, *Bdellovibrio bacteriovorus,* lysed the cyanobacterium *Phormidium luridum* without penetrating the prey, which indicates a close physical relationship (Burnham et al., 1976). These studies indicate that there are two types of BLB, which are parasitic towards cyanobacteria in the natural environment.

Microbial agents such as bacteria and viruses may play a major role in the prevention, regulation, and termination of harmful algal blooms (Shilo, 1970; Burnham et al., 1981; Ashton and Robarts, 1987; Bird and Rashidan, 2001; Nakamura et al., 2003a; Choi et al., 2005). Some of these microorganisms have been isolated from eutrophic waters and have a lytic effect on the growth of cyanobacterial species (Nakamura et al., 2003a). Often, predatory bacteria are in abundance during the decline of a harmful algal bloom (HAB), and may be involved in the collapse of blooms in nature (Bird and Rashidan, 2001).

In previous studies (Chapter 3), it was observed that bacterial agents were responsible for plaque developments on *Microcystis* lawns. The main objectives of this study were

to assess which bacterial morphotypes were present in the plaque zones and to explore the relationships between the bacterial agents and *Microcystis* cells during the lytic process. Scanning electron microscopy (SEM) was used to assess the morphological changes of the *Microcystis* cells. Transmission electron microscopy (TEM) was used to determine ultrastructural changes that occurred during the interactions between *Microcystis* and predatory bacteria. Light microscopy was used to observe the interactions between predator and prey (wet mounts). The light and electron microscopy studies formed the basis for the development of a model describing the mechanism of *Microcystis* lysis.

4.2. MATERIALS AND METHODS

4.2.1. Evaluations of cyanobacteria-bacteria interactions in solid media/phases (plaques)

Agar sections were cut from the plaques (Chapter 3: 3.3.2.) on BG11 agar plates and were examined with SEM and TEM. Areas with green *Microcystis* lawns indicating the absence of plaques served as controls.

4.2.1.1. Scanning Electron Microscopy

The agar sections were fixed with 2.5% v/v gluteraldehyde in 0.075M-phosphate buffer (30 min) and then filtered through a 0.22µm membrane. The membrane filter was washed three times with 0.075M phosphate buffer (15 min); dehydrated with 50% ethanol (15 min); 70% ethanol (15 min); 90% ethanol (15 min) and three times with 100% ethanol (15 min). This was followed by critical point drying (Bio-Rad E3000) and gold coating process (Polaron E5200C). The material was then examined in a Joel JSM 840 scanning electron microscope operating at 5.0kV.

4.2.1.2. Transmission Electron Microscopy

Thin sections of agar were made with a stainless blade and then immersed in gluteraldehyde solution for 30 minutes. This was washed three times with 0.075M phosphate buffer (15 min) and fixed with osmium tetroxide (1 hour). The osmium tetroxide was removed through repeated washings (three times with 0.075M phosphate buffer (15 min)) and embedded overnight in Quetol resin. Sections were

cut on an ultramicrotome (Reicher-Jung Ultracut E), stained in uranyl acetate and lead acetate before being examined and photographed using a transmission electron microscope (Philips EM301).

4.2.2. Evaluations of cyanobacteria-bacteria interactions in liquid phases

4.2.2.1. Experimental set up

Culture suspensions of cyanobacteria (20 mℓ) (Section 3.2.4.1) and bacteria (20 mℓ) (Section 3.2.4.3) were mixed in a 250 mℓ Erlenmeyer flask. The incubation procedure in Section 3.2.4.1 was followed. On d 4 samples were taken for microscopy analysis. All the experiments and controls were done in duplicate.

4.2.2.2. Light microscopy

One drop of the suspension (control and treated samples) was placed onto a microscopic slide and then covered with a cover slip. The material was examined using a Nikon optiphot light microscope fitted with appropriate illumination sources and filters and pictures were captured with a Nikon digital camera DXM1200.

4.2.2.3. Scanning electron microscopy

A suspension (control and treated) was filtered through a 0.22µm membrane filter and fixed with 2.5% v/v gluteraldehyde in 0.075M phosphate buffer (30 min). The same procedure in Section 4.2.1.1 was followed.

4.2.3. Algicide disruption of *Microcystis* cell membranes

Aliquots of copper sulphate (10 mg/mℓ) and (0.01%) Triton X-100 were added to *Microcystis* suspension (Section 3.2.4.1). The experimental design is shown in Figure 4.1 was incubated as in Section 4.2.2.1 for 24 h. SEM was performed as described above.

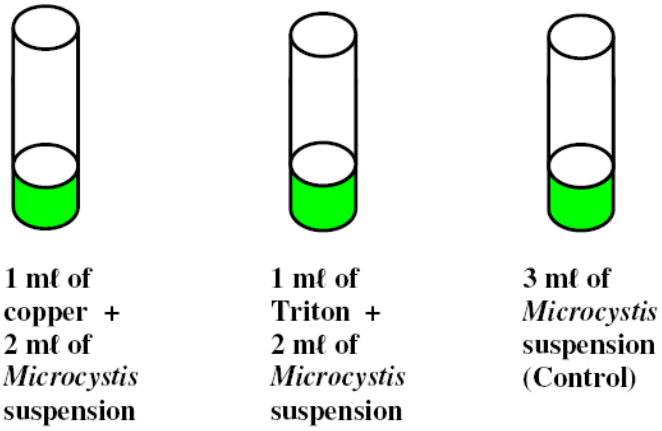


Figure 4.1: Experimental design for testing of algicides

4.2.4. Ultrastructural changes in *Microcystis* cells during lysis after exposure to *B. mycoides* B16

4.2.4.1 Preparation of freeze dried *B. mycoides* B16

An inoculum of the *B. mycoides* B16 was cultured in 100 mℓ of one-tenth of tryptic soy broth (TSB) in a 250 mℓ Erlenmeyer flask under shaking incubation (128 rpm, 25^{o}C) for 24 h (Di Franco et al., 2002). After cultivation the bacterial cell suspensions were harvested by centrifugation (10,000rpm, 15 min, 25^{o}C) and freeze dried (Edwards freeze dryer: minus 50^{o}C, 2.8mbar, 72 h). The lyophilized powder was stored at 4^{o}C until further use.

4.2.4.2. Experimental set up

To a 20 mℓ *Microcystis* suspension (Section 3.2.4.1), 198.3 mg of lyophilized powder (Section 4.2.4.1) was added and subjected to shake incubation (78 rpm; 25^{o}C; 2000 lux continuous light illumination) for 20 h. For a control, 20 mℓ *Microcystis* suspension was used and incubated as above. At hourly intervals 1 mℓ of treated and control samples were sampled and centrifuged (10,000 rpm, 10 min, 25^{o}C). The pellet was re-suspended and fixed in 1 mℓ of 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer at pH 6.65 and stored at 4^{o}C until further use for TEM (Section 4.2.4.3) and SEM (Section 4.2.2.3).

4.2.4.3. Transmission electron microscopy

The samples were then fixed with 1mℓ of 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer supplemented with 1% alcian blue (previously filtered through a Cameo acetate 0.22 µm syringe filter) for 2 h at 4°C. After primary fixation, the samples were centrifuged (10,000 rpm, 15 min, 25°C) and the pellet re-suspended in 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer. This process was repeated three times and the samples were then post-fixed for 1 h in 1% OsO_4. The samples were then rinsed in 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer and dehydrated in an ascending ethanol series [50, 70, 90 and 100% (thrice)].

4.3. RESULTS AND DISCUSSION

4.3.1. Evaluations of cyanobacteria-bacteria interactions in solid media/phases (plaques)

After incubation of an aliquot of the eutrophic waters on BG11 agar, plaques were formed. Epiphytic and free-living bacteria were observed in the plaque areas. At least five distinct morphotypes of bacteria were found in the plaque zones: (1) plumb rod-shaped bacillus that was attached (1 to 1.5 µm) (Figures 4.2 and 4.3) and free-living (Figure 4.4c); (2) a long rod-shaped bacillus with one end sharpened, not attached (3 to 6µm) (Figure 4.4b); (3) a plumb rod-shaped bacillus with fimbriae, not attached (1.5 to 3 µm)(Figure 4.4d); (4) vibrio shaped rods, not attached (Figure 4.4a) and (5) coccoid shaped bacteria (0.6 µm) (Figure 4.4a & c). SEM micrographs showed the presence of plumb shaped bacillus aggregated around unhealthy *Microcystis* cells (Figure 4.2). The *Microcystis* cells appeared distorted or deflated wherever these bacillus rods were present.

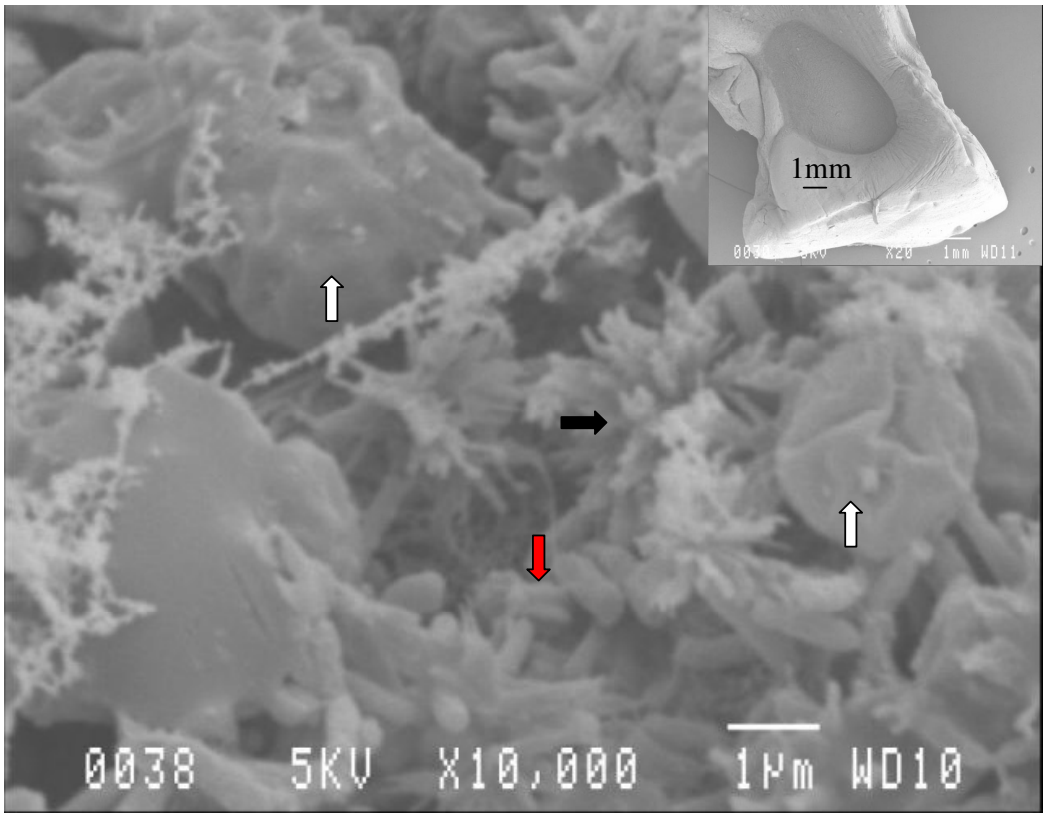


Figure 4.2: SEM micrograph of plaque zone (insert) showing interactions of plumb rod-shaped bacillus (red arrow) and *Microcystis* cells. In the background some of the *Microcystis* cells are 'deflated' (white arrow). The 'star-like' items (black arrow) are sections of agar material.

These results show similar bacteria flora that were observed and consisted of extremely small cocci (0.1 to 0.2 µm), large rods (~1 µm), presumably bacilli, that were mostly attached to *Microcystis* cells (in hyperscums) and filamentous bacteria (Robarts and Zohary, 1986). In the same study, the researchers observed that when hyperscum reached its peak mass it was accompanied by an increased bacterial heterotrophic activity that was followed by a breakdown (decline) of the hyperscum. These findings may suggest that the bacteria were responsible for the termination of the hyperscums.

The direct examination of the plaques did not reveal a clear association between bacteria and cyanobacteria due to interference of agar material (Figure 4.2). To eliminate the interference, material from plaque zones was scrapped and suspended in minimum Ringer's solution and then processed for SEM. The SEM micrographs

showed that the bacteria flora was mainly composed of plumb rod-shaped bacillus (Figure 4.2) that were closely associated with unhealthy *Microcystis* cells (Figure 4.3a).

In the control areas the cyanobacteria cells were healthy and did not show any visible distortion of cell structure (Figure 4.3b). At this stage the unhealthy *Microcystis* cells appeared to be associated with plumb rod-shaped bacillus that were probably the cause of deflation. Maybe these bacteria caused the cyanobacteria cells to leak out their cell contents, and the bacteria benefited nutritionally. This supports Stewart et al. (1973) and Burnham et al. (1984) who also concluded that the plaque formation was attributed to a single predatory bacterium that had multiplied and caused cyanobacterial lysis.

During bloom conditions, bacteria are known to exist embedded within the *Microcystis* mucilage with their abundance and community structure composition differing according to *Microcystis* species (Maruyama et al., 2003). The researchers illustrated the possible role of free and attached bacteria found in the mucilages of *Microcystis* colonies as degrading the microcystins.

TEM micrographs of the plaque showed intermingled bacteria and *Microcystis* cells in various stages of degradation (Figures 4.5). Possibly these bacteria were scavenging the skeletal remains of *Microcystis* (Figures 4.5 e-f).

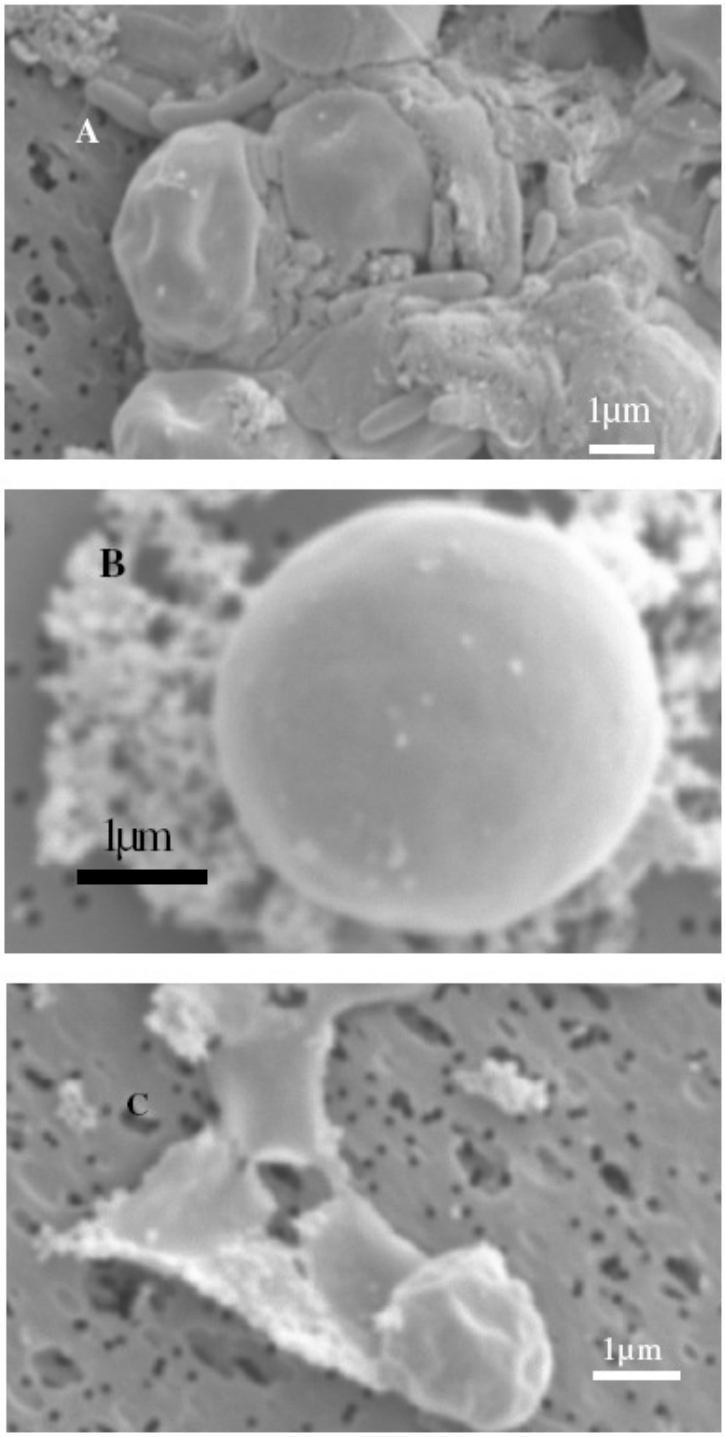


Figure 4.3: (A) Plumb rod-shaped bacillus bacteria were abundant and were found aggregated around *Microcystis* cells, which were deflated and unhealthy. (B) A healthy looking *Microcystis* cell from the control area. Note the absence of any distortion on the cell structure or 'deflation'. (C) Disintegration of scrolls from a *Microcystis* cell wall.

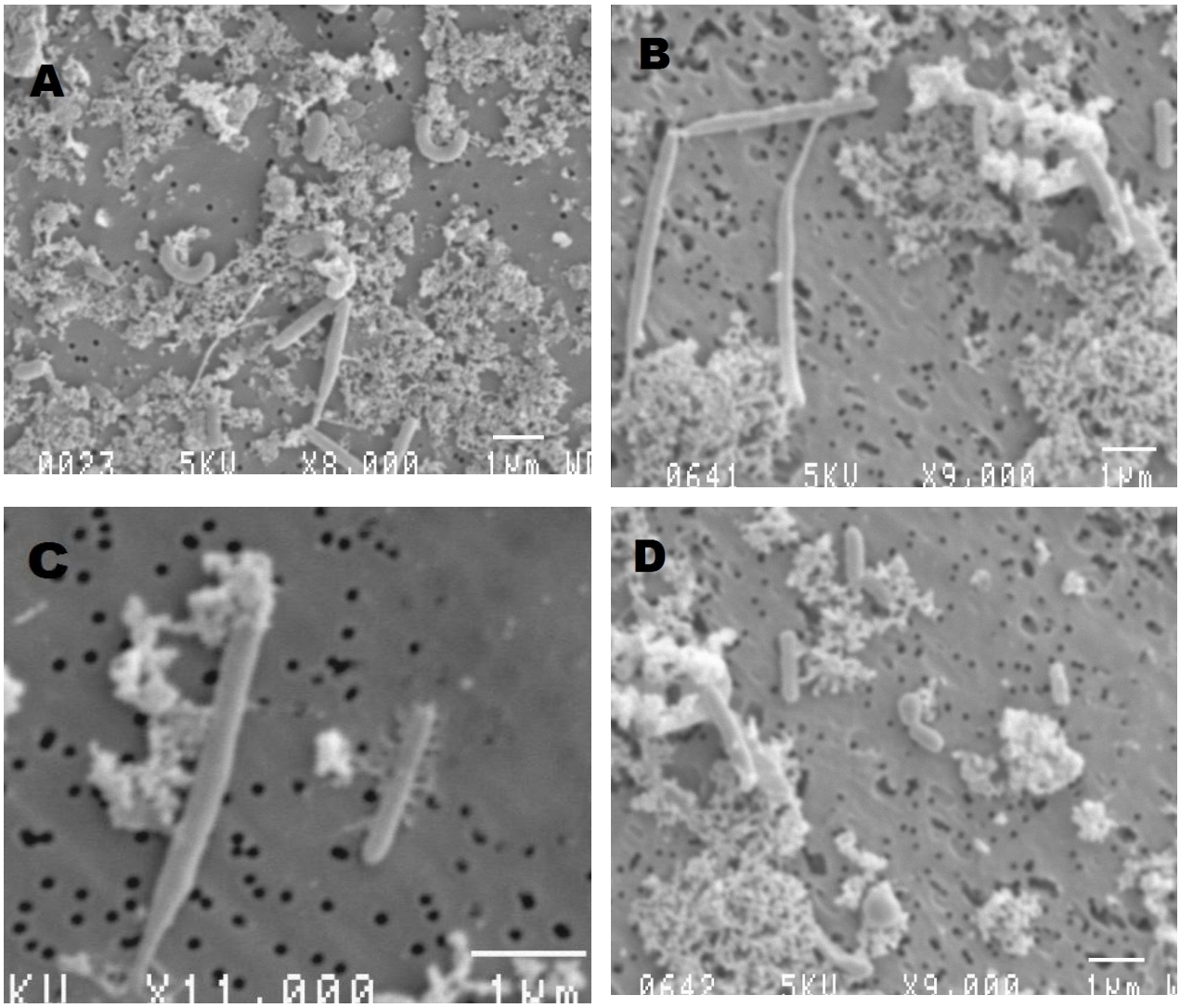


Figure 4.4: (A) Vibrio shaped, long and short rod-shaped bacillus, coccoid shaped bacteria; (B) Long rod-shaped bacillus with sharp ends (C) Long rod-shaped bacillus with prominent fimbriae and (D) Short rod-shaped bacillus, coccoid shaped bacteria.

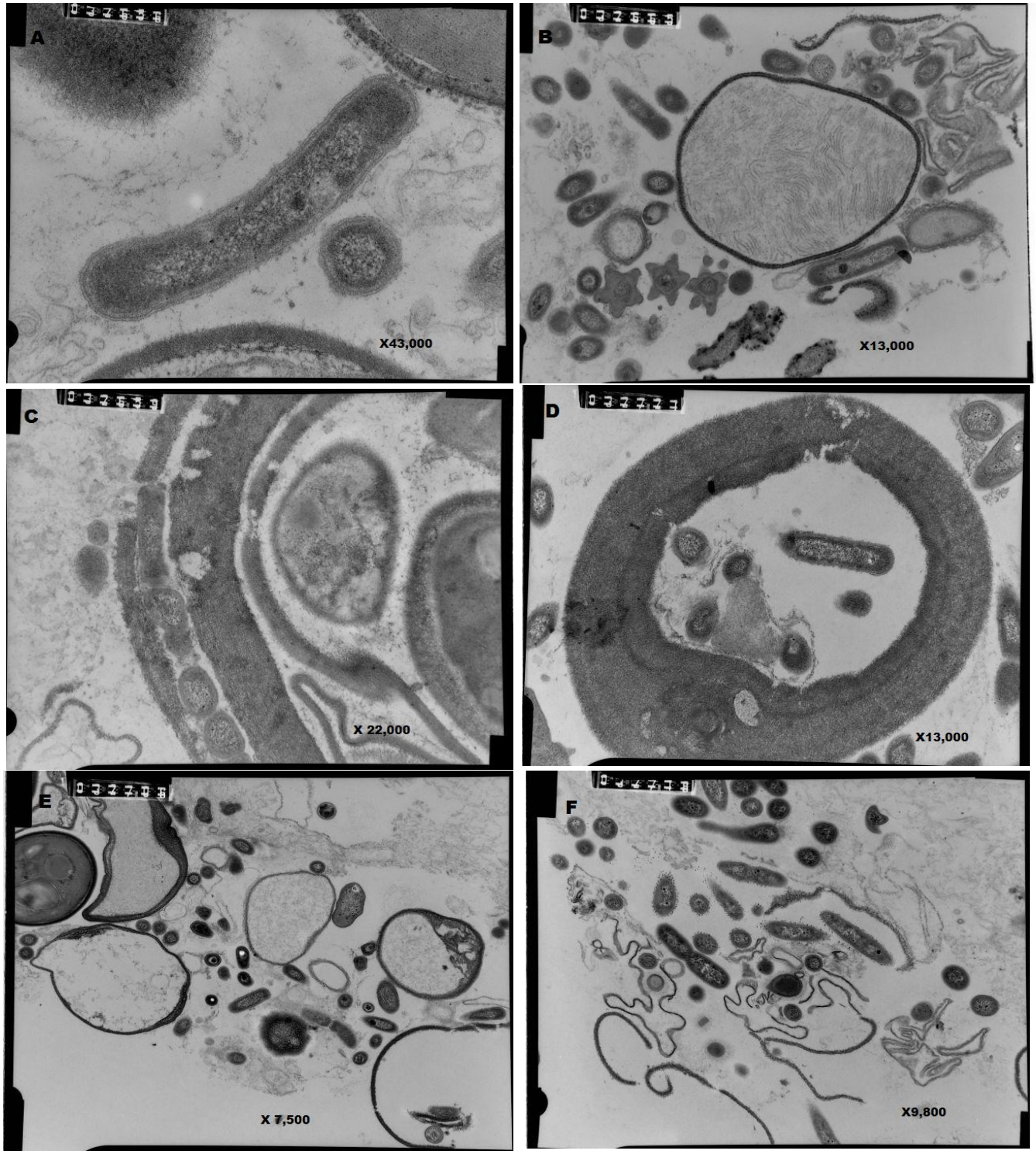


Figure 4.5: TEM micrographs showing interactions between bacteria and *Microcystis* cells. (A) Physical contact between bacteria and *Microcystis* cell; (B) Bacillus rod shaped bacteria around a skeleton *Microcystis* cell; (C) damage on *Microcystis* cell membrane may be entry point for bacteria; (D) some of the bacteria are inside a *Microcystis* cell and or skeleton. (E and F) Bacteria amongst ghost *Microcystis* cells and cell debris.

Burnham et al. (1981) showed that the colonial spherule of *Myxococcus xanthus* PCO2 entrapped the filamentous cyanobacterium *Phormidium luridum* which then proceeded to degrade the cyanobacterium. Their studies indicated that *M. xanthus* PCO2 released an extracellular substance that dissolved the cyanobacteria cell wall at the point of contact. It was therefore speculated based on the Burnham studies (1981) that there is a possibility of release of exoenzymes during the physical contact between bacteria and *Microcystis* cells used in this study (Figure 4.5a). The result is damage to the cyanobacteria cell wall, indicated by a number of sites that had ruptured (Figures 4.5c-d). The lysed *Microcystis* cells are shown at various stages of degradation, some are deflated and some with damaged outer membranes (scroll like structures) (Figures 4.4d and 4.5f). These findings agree with the research work of Daft et al. (1973), who pointed out that the 'scroll –like structures' originate from a cyanobacteria cell wall layer. Bacteria were also observed inside the *Microcystis* skeletal remains (Figure 4.5d). There are a number of theories that may be advanced. Maybe the bacteria behaved like a *Bdellovibrio* and entered the cyanobacteria, or other bacteria came in at later stage to scavenge the remains.

4.3.2. Evaluations of cyanobacteria-bacteria interactions in liquid phases

Light and electron microscopy were used to assess the morphological changes that occurred on the *Microcystis* cell membrane after exposure to *B. mycoides* B16. The micrographs revealed that the morphological details of *Microcystis* cells (treated with *B. mycoides* B16) were different from the control. The results of the control were normal and healthy *Microcystis* cells (Figures 4.6a-b) and bacterial presence resulted in swollen *Microcystis* cells (Figures 4.6c-d).

SEM images of swollen *Microcystis* cells were presumably due to osmosis or the presence and multiplication of *Bdellovibrio*-like bacteria inside the *Microcystis* cell. The later is more plausible since bacterial movements were observed (wet mounts) inside swollen *Microcystis* cells. Reim et al. (1974) and Burnham et al. (1981) also reported the existence of swollen cyanobacteria cells prior to cell lysis, but did not account for what may have caused the swelling phenomenon. These findings suggest that the bacteria penetrated the *Microcystis* cell and replicated producing progeny that

caused the *Microcystis* cells to swell. The bdelloplasts then (*Bdellovibrio* progeny) feed on the host nutrients such that the end result was distorted *Microcystis* cells. These progeny became part of the normal bacterial population. *Bdellovibrio*-like bacteria have been observed in field water samples of *Microcystis* cells and were localised within the cell wall and cytoplasm membrane (Caiola and Pellegrini, 1984). However the studies did not indicate the life cycle of the *Bdellovibrio*-like bacteria or the presence of bdelloplast and these results were therefore difficult to compare with our studies.

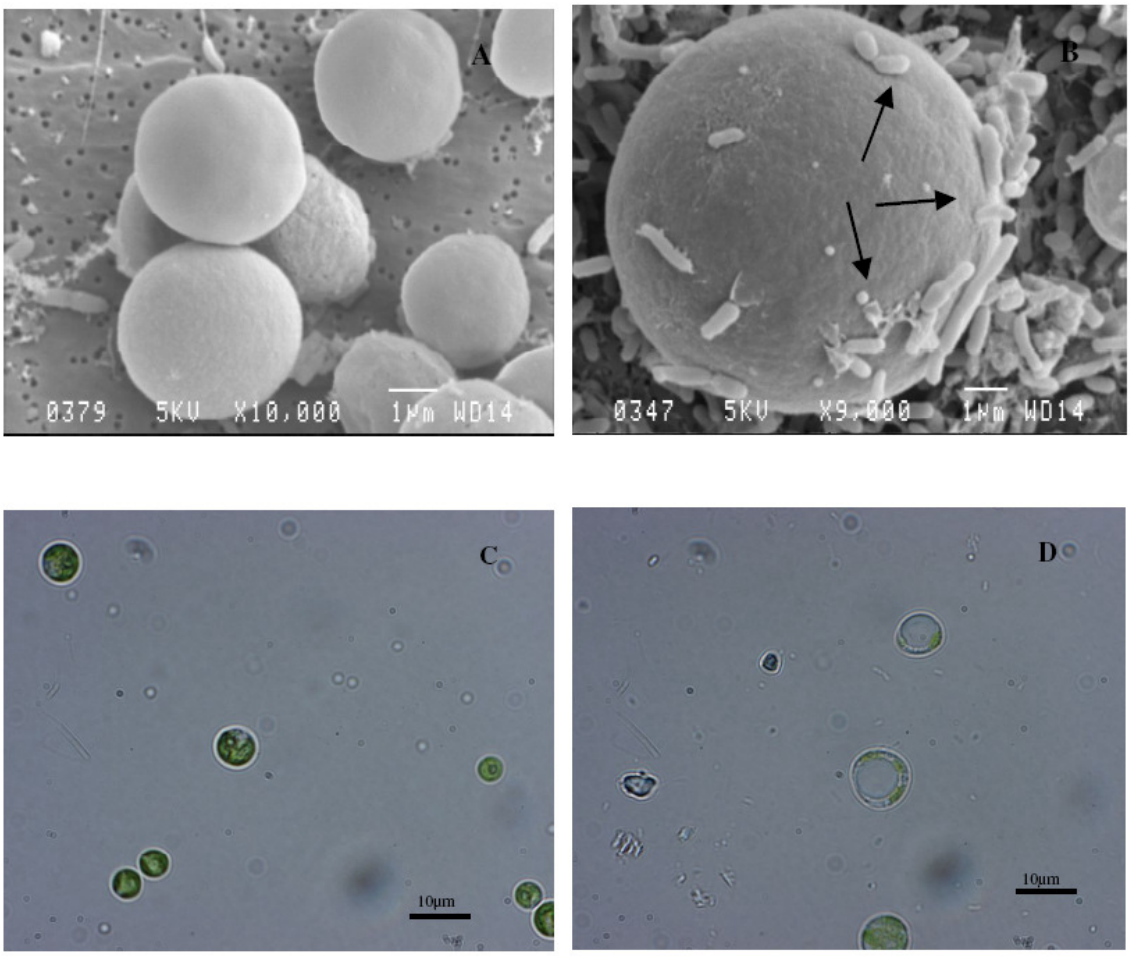


Figure 4.6: (A & C) Control *Microcystis* cells which are normal and healthy cells and (B & D) *B. mycoides* B16 treated *Microcystis* cells showing the size of the swollen cells. Note the presence of plumb rod-shaped bacillus bacteria attached to the *Microcystis* cell (arrows).

Scanning electron microscopy observations showed bacteria that were attached to *Microcystis* cells (Figure 4.7). The bacterial rods appear to bind onto the surface of the *Microcystis* cell. The bacterial attachment appears to be related to either fimbriae (Dobson and McCurdy, 1979) and or through the use of exopolymers (Cloete and Oosthuizen, 2001). The use of fimbriae as an attachment may either be temporary or irreversible. If it is temporary then any agitation of liquid cultures is bound to disrupt the attachment. This in turn delays or even disrupts the *Microcystis* lysis process. Earlier on Shilo (1970) and Daft and Stewart (1971) pointed out that agitation of samples disturbed the physical contact process between the cyanobacteria and bacteria.

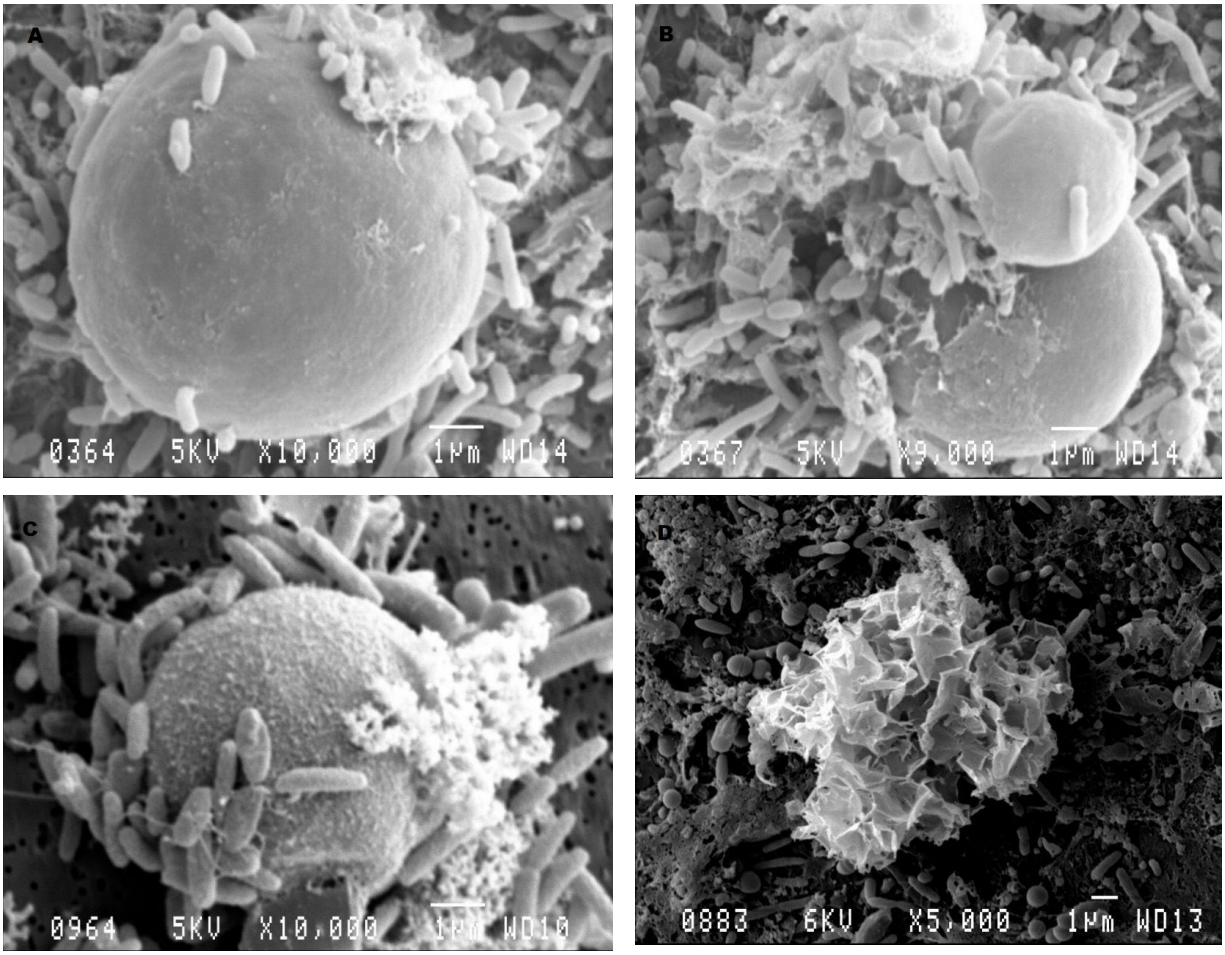


Figure 4.7: SEM micrographs showing the *Microcystis* interaction with *B. mycoides* B16; (a) bacterial attachment on cell; (b) damage on cell membrane; (c) perforations on cell membrane (c) and (d) skeletal remains.

Thus the exposure of *Microcystis* cells to *B. mycoides* B16 resulted in complete lysis as indicated by the skeletal remains (Figure 4.7d). These findings indicate the potential use of *B. mycoides* B16 in the management of *Microcystis* algal blooms.

4.3.3. Algicide disruption of *Microcystis* cell membranes

Microcystis cells were exposed to copper and Triton X-100 to ascertain the level of damage to the cell membranes. Copper sulphate is a well-known algicide that is used to treat *Microcystis* algal blooms (Liam et al., 1995; García-Villada et al., 2004). Triton X-100 is used a permeabilising agent that causes damage of the cell membrane such that fluorescent dyes are able enter to cell and stain a specific cell function during flow cytometric analysis (Hayden et al., 1988).

SEM images showed variations in the degree of damage on the *Microcystis* cell membrane (Figure 4.8). A normal and healthy *Microcystis* cell has a spherical shape with a smooth exterior surface and showed no visible damage (Figure 4.8a). *Microcystis* cells that were treated with copper were stripped of their entire cell membrane leaving behind a skeleton structure (Figure 4.8b).

Triton X-100 caused lesions on the *Microcystis* cell membrane structure (Figure 4.8c). Copper in the form of cupric ions (Cu^{2+}) lysed the *Microcystis* cell in the following ways: inhibition of carbon dioxide fixation and PSII activity, inhibition of nitrate uptake and synthesis of nitrate reductase and changes in cell volume (García-Villada et al., 2004). The consequences of copper use results in stripping of the *Microcystis* cell membrane and the release of intracellular contents including microcystins into the water.

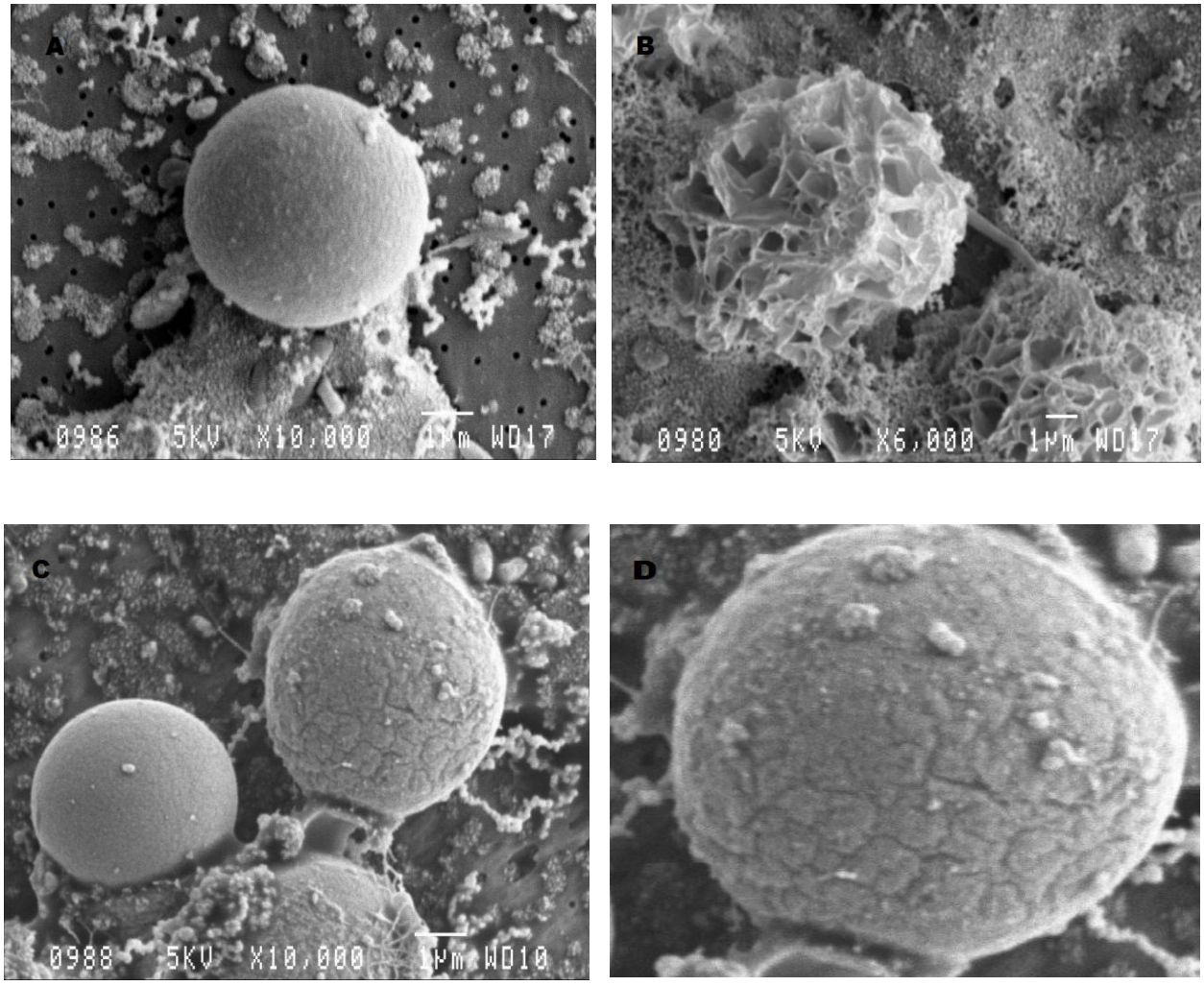


Figure 4.8: SEM indicating the morphological changes to *Microcystis* cell membrane (A) control sample showing smooth cell structure, no visible damage; (B) copper treated *Microcystis* showing the remains of a skeleton; (C) Triton X showing damage to cell membrane and (D) enlargement of (C) showing the 'cracks' on the cell membrane.

The results showed that the exposure of *Microcystis* to copper and Triton X-100 caused cell membrane damage with copper stripping the entire cell. These findings also confirm that the extensive use of copper in the management of *Microcystis* algal blooms.

4.3.4. Ultrastructural changes in *Microcystis* cells during lysis after exposure to *B. mycoides* B16

Transmission electron microscopy observations revealed that the ultrastructural details of *Microcystis* cells treated with *B. mycoides* B16 were different from the control. The ultrastructural characteristics of a healthy and normal *Microcystis* cell are shown in Figure 4.9(a) and confirmed that of previous studies (Reynolds et al., 1981; Zohary, 1987). The internal structure of a healthy *Microcystis* consisted of a multilayered cell wall (CW), plasmalemma (P) and storage granules made up of cyanophycin (C) and polyphosphate bodies (P.B.) and parallel arrays of thylakoid membranes (TH).

After 2 h of incubation with *B. mycoides* B16, the internal structure of *Microcystis* was changed with a distorted cell wall (CW) enclosing the distorted plasmalemma (P) surrounding large glycogen granules (G), large polyphosphate bodies (P.B.) and large thylakoid membranes (TH) (Figure 4.9b). The *Microcystis* cells (bacteria treated) shrunk in size in comparison to the controls and scattered intracellular material presumably polyphosphate bodies were found in the external media (evidence of lysed *Microcystis* cells) (Figure 4.9c). Between 4 h to 8 h a similar lytic action was observed, i.e., distorted *Microcystis* cell walls and damage of the cell wall membrane and disoriented thylakoid membranes (Figure 4.10b). After 23 h, the *Microcystis* control showed evidence of growth as indicated by cell division (Figures 4.10e), whilst the *Microcystis* (bacteria treated) showed a distorted cell wall with bacteria attached (Figure 4.10d) and lysed *Microcystis* cell debris (Figure 4.10f).

These findings suggest that during physical contact the bacteria released extracellular substances (Daft and Stewart, 1971; Burnham et al., 1981) that damaged the *Microcystis* cell membrane. Nakamura et al. (2003a) isolated an extracellular substance from *B. cereus* N14, closely related to *B. mycoides* B16 (von Wintzingerode et al., 1997) that showed lytic activity towards *Microcystis*. It is possible that a similar extracellular substance produced by *B. mycoides* B16 may have resulted in cell lysis during this study.

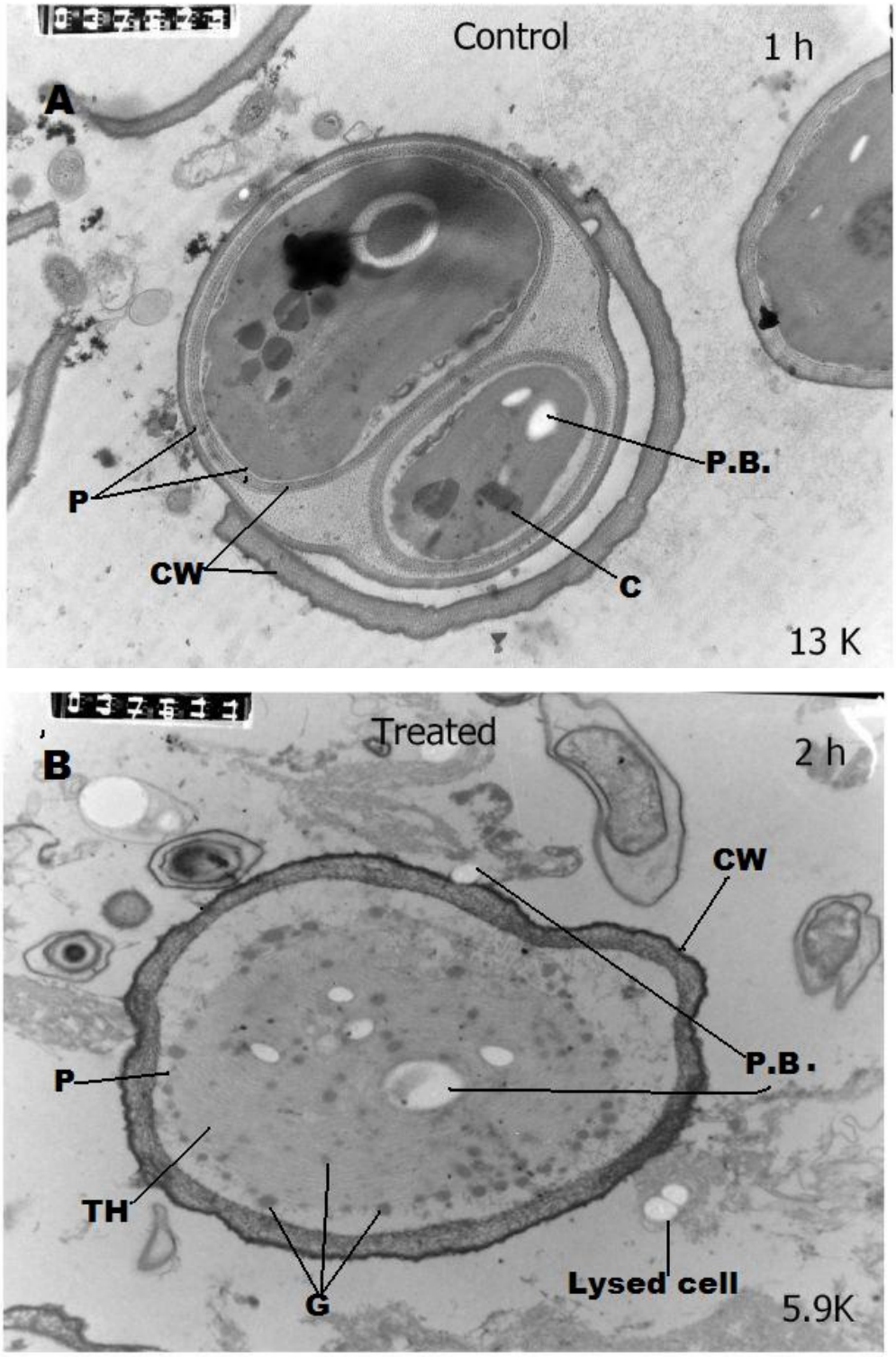


Figure 4.9: (A) Control: Healthy *Microcystis* cell in the process of division, cell wall (CW) and plasmalemma (P), storage granules cyanophycin (C) and polyphosphate bodies (P.B.); (B) *Microcystis* (bacteria treated); large glycogen granules (G); polyphosphate bodies (P.B.), large thylakoids (TH); (C) After 4 h incubation, *Microcystis* cell was smaller due to loss of internal contents and lysis.

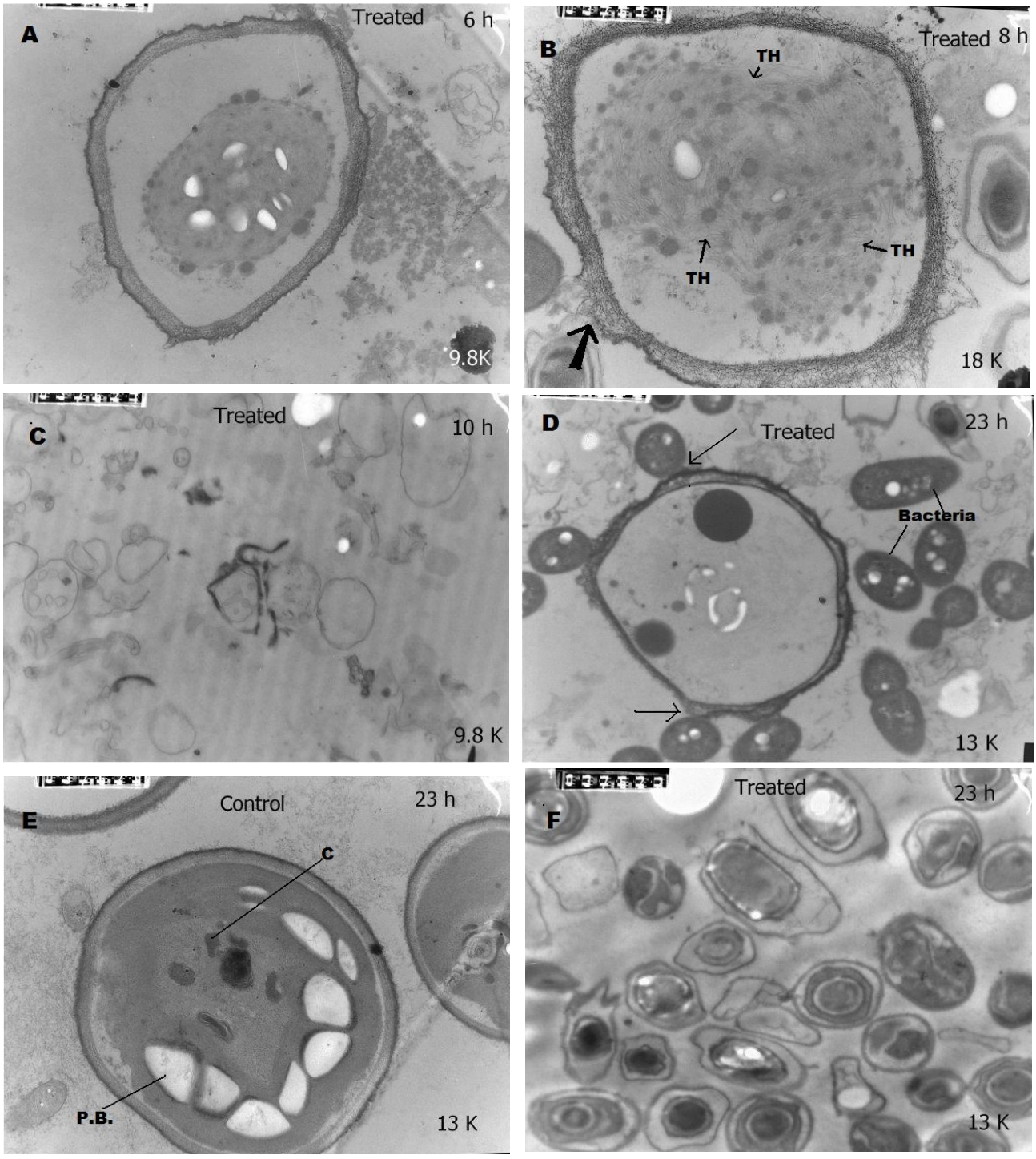


Figure 4.10: (A-B) *Microcystis* cell showing signs of distortion after 6h and 8h in the presence of bacteria. Note damage on cell wall membrane (arrow) and distorted thylakoids (arrow). (C) Lysed *Microcystis* cells. (D) Bacteria surrounding the *Microcystis* cell and attacking the cell (arrow); (E) Normal and healthy *Microcystis* cell after 24 h and (F) Lysed *Microcystis* cells after 24 h after exposure to bacteria.

Previous studies have shown that under experimental conditions of low light irradiance, *Microcystis* cells had large cyanophycin granules, numerous thylakoid membranes and a large nucleoplasmic area (Canini et al., 2001). On switching to high light conditions, the *Microcystis* produced superoxides to minimise damage to the cell and thylakoid systems and internal organelles were normal. Borbéy et al. (1990) reported similar results, except that the cyanobacteria, when subjected to an adverse external environment such as the presence of predatory bacteria, the cyanobacteria responded by accumulating of storage granules. Polyphosphate bodies, function as a store of phosphorus and appear as spherical inclusions of varying diameters located in the centre of the cell (Jensen, 1968). In our study numerous *B. mycoides* B16 numbers formed a 'cover or shade' over the *Microcystis* cells creating conditions of low light irradiance. It was hypothesized that the cyanobacteria then resorted to the production of storage granules like polyphosphates and glycogen. These are for the maintenance of essential processes instead of growth. The other evidence is the expansion of the thylakoid system to capture as much light as possible (Stewart and Alexander, 1971; Schneegurt et al., 1994; Moezelaar and Stal, 1997). The bacteria surrounding to the *Microcystis*, presumably also released extracellular substances that interfered with the *Microcystis* photosynthesis system as indicated by the collapse in the plasmalemma and thylakoid membranes that was more evident after 24 h of bacteria treatment (Figures 4.10b and f).

Veldhuis et al. (2001) noted that during automortality the last lysis stage involved the degradation of photosynthetic pigments and the reduction in photosynthesis of the phytoplankton. Nakamura et al. (2003a) also observed that during the lytic action by *B. cereus* N14 on *Microcystis* led to the exudation of photosynthetic pigments to the external media. At the same time the *B. mycoides* B16 may have derived nutrition from cell contents that leaked out of the *Microcystis* cells due to the damage of cell wall making it permeable (Figure 4.10d).

4.3.5. Behavioural changes in *B. mycoides* B16 during the lysis of *Microcystis*

Burnham et al. (1984) and Daft et al. (1985b) showed that *Myxococcus fulvus* BG02 exhibited polymorphism: spherules and myxospores, which showed lytic activity against *Phormidium luridum* and only resorted to the vegetative state when the

external environment was nutrient limited. Their mechanism of cyanobacteria lysis was based on the process of spore formation that led to the successful entrapment of cyanobacteria and release of extracellular substances (antibiotics) that targeted the peptidoglycan layer thus enabling *M. fulvus* BG02 to obtain nutrients. *B. mycoides* B16 displayed similar characteristics changing from single cells to chains during the lysis of *Microcystis* (Figure 4.11).

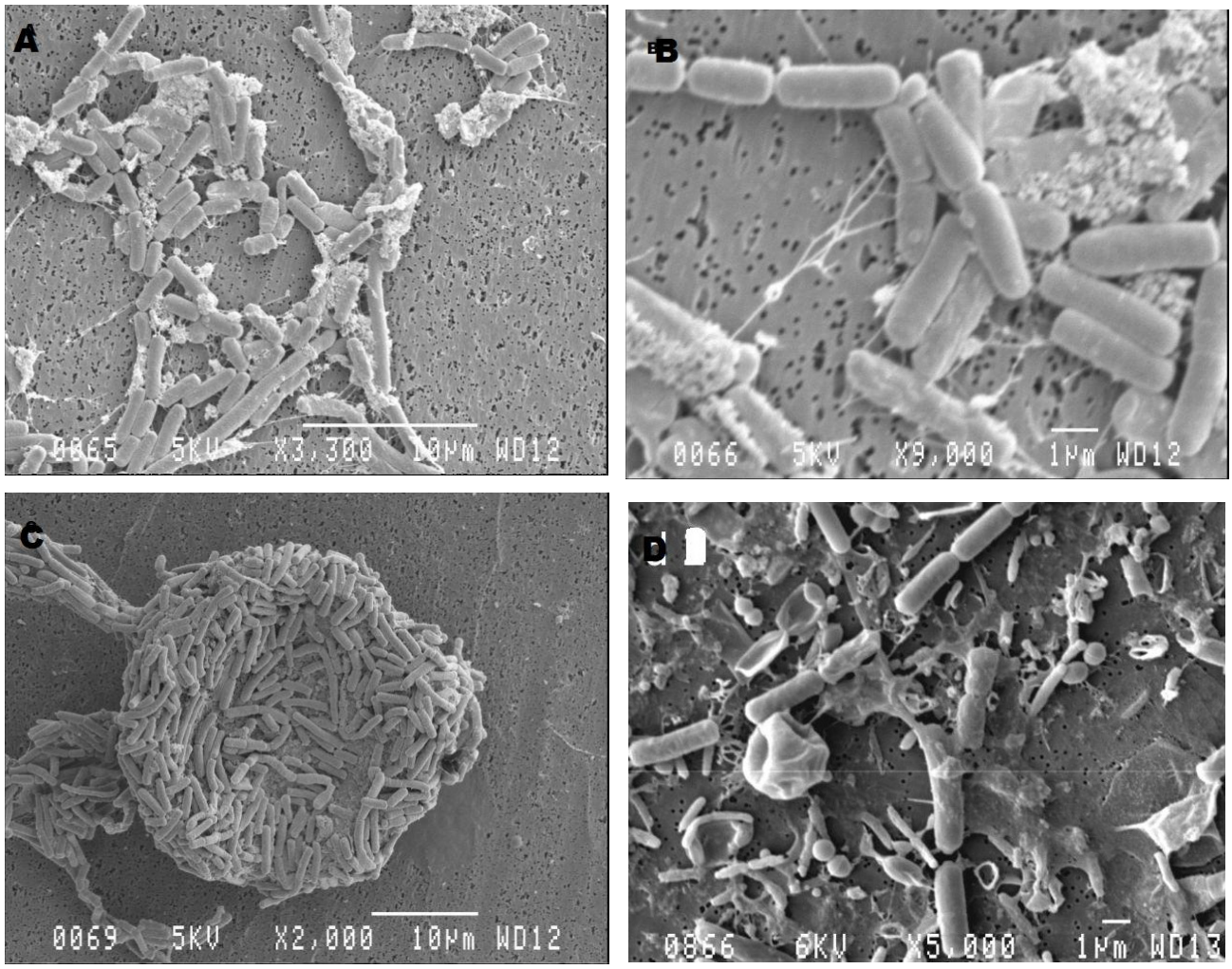


Figure 4.11**:** SEM images of *Bacillus mycoides* B16: (A) individual cells and chains; (B) in chains; (C) multicellular forms and (D) in chains during the *Microcystis* lysis stage.

B. mycoides B16 switched from single cell to a multicellularity phenotypes which is a characteristics of rhizodal phenotypes of *B. mycoides* species (Villain et al., 2006). The rhizoidal growth of *B. mycoides* through chain elongation over long distance may

be viewed as translocation through the *Microcystis* algal blooms. Thus the bacteria were able to derive nutrients from the lysis of *Microcystis* cells during the translocation process. Also the *B. mycoides* species formed clumps, possible as a survival strategy in the event of nutrient limitations (Villain et al., 2006). It was very difficult to break these clumps except through ultrasonication. Some *Bacillus* species were reported to revert to spore formation, only germinating and becoming active once the external environment was conducive for growth (Villain et al., 2006). The spore formation state was not observed with *B. mycoides* B16 through the malachite test.

4.3.6. The mechanism of lytic action of *B. mycoides* B16 on *Microcystis*

The results of this study on the interactions of *Microcystis aeruginosa* and *Bacillus mycoides* B16 indicated a series of morphological and ultrastructural changes with the cyanobacteria cell leading to its death. These are summarised in a conceptual model that was developed (Figure 4.12).

We have hypothesised that the first phase in the lysis process is the bacterial attachment onto the *Microcystis*. At this encounter the large bacteria numbers create conditions of 'shading' over the *Microcystis* cells, thus creating an environment of low light irradiance. Within 2 h with insufficient light the following features were observed: accumulation of glycogen, polybetahydroxybutyrate, cyanophycin and expansion of parallel thylakoids membranes. The controls showed normal and healthy *Microcystis* cells. After 6 h the cyanobacteria cell wall was distorted, in some cases broken. The thylakoids, light gathering apparatus, was in disarray and probably not functioning as well. The cyanobacteria resorted to use of accumulated granules for survival and not for growth.

The second and third phases are the bacterial release of lytic substances that damage the cyanobacteria cell membrane. In a separate study of *Bacillus cereus*, closely related to *Bacillus mycoides*, was found to release an unidentified lytic substance that lysed *Microcystis* cells (Nakamura et al., 2003b). The study of algicides showed that this was possible as the evidence of *Microcystis* cell membrane damage was similar to that of the bacterial attack. Swollen cyanobacterial cells were observed during this

stage. This was presumably due to osmosis or the presence of *Bdellovibrio*-like bacteria that multiplied within the cyanobacteria cell. The ultrastructural evidence presented does not support the later hypothesis despite light microscopy studies (wet mounts) detected bacterial movements within the cyanobacteria cell.

The last phase involved the leaking of cyanobacteria cell contents to the external environment indicating cell death. This aspect is the basis for the next study (Chapter 5) on the assessment of cell viability using flow cytometry. The *B. mycoides* B16 derived nutrition from the cell contents that leaked out of the *Microcystis*. An interesting point was the formation of chains by *B. mycoides* B16 cells. It was suggested that the rhizoidal growth of *B. mycoides* through chain elongation over a long distance could be viewed as a translocation through lysed *Microcystis* algal blooms in search of new prey.

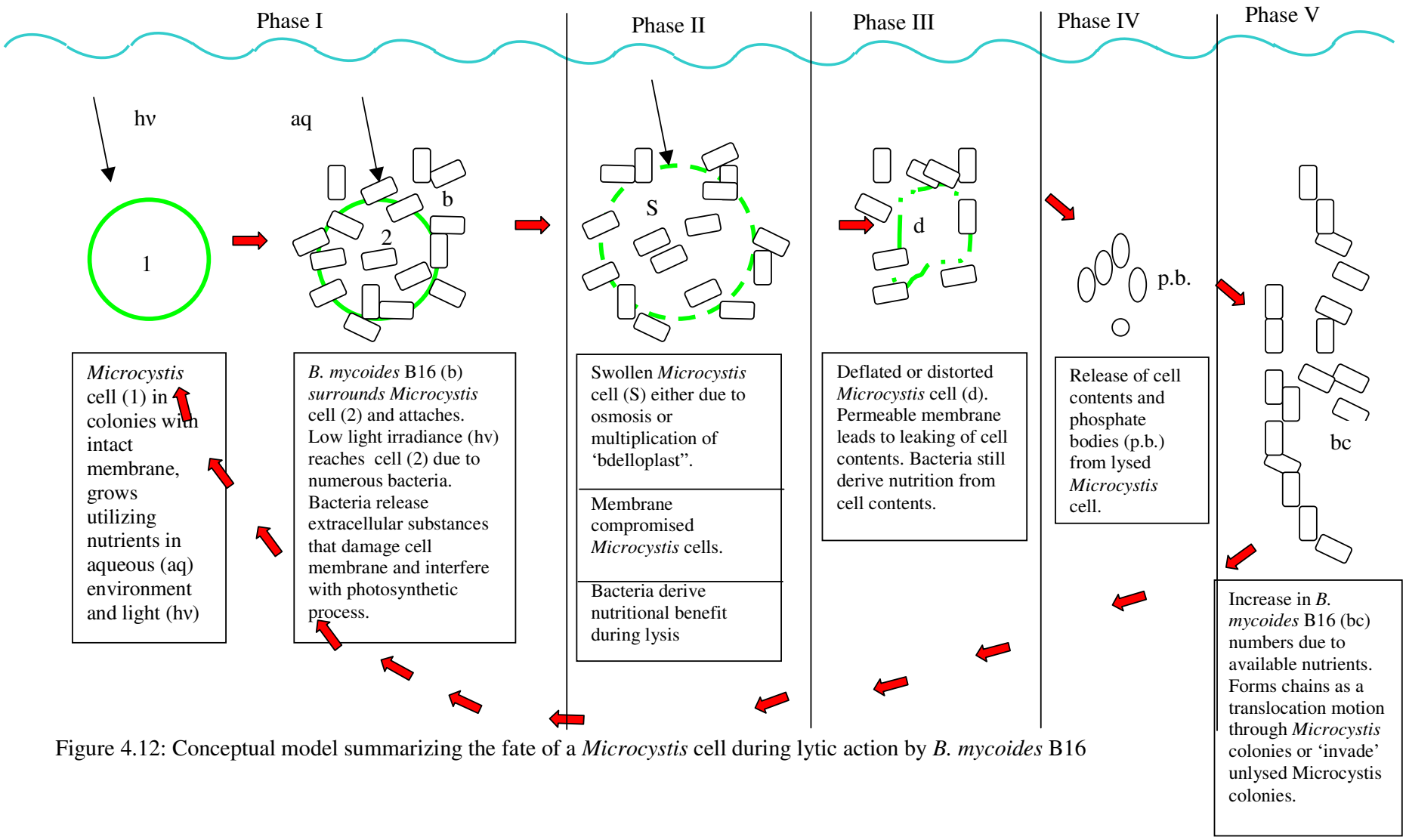


Figure 4.12: Conceptual model summarizing the fate of a *Microcystis* cell during lytic action by *B. mycoides* B16

4.4. CONCLUSIONS

- Electron microscope studies confirmed that there were at least five distinct morphotypes of bacteria found in the plaques: (1) plumb rod-shaped bacillus that was attached and free-living; (2) a long rod-shaped bacillus with one end sharpened, not attached; (3) a plumb rod-shaped bacillus with fimbriae, not attached; (4) vibrio shaped rods, not attached and (5) coccoid shaped bacteria.
- The plumb rod-shaped bacilli were abundant and were found aggregated around unhealthy *Microcystis* cells and were the probably the cause of deflation and lysis of the algae.
- Isolates B2 and B16 were capable of causing damage of the *Microcystis* cell membrane.
- Electron microscope studies showed the extent of *Microcystis* membrane damage, most severe with copper, followed by *B. mycoides* B16 and Triton X-100.
- There is evidence, which showed that *Bacillus mycoides* B16 might be an ectoparasite during the lysis of *Microcystis* cells and exhibit multicellularity forms that are *Bdellovibrio*-like bacteria during the last stages lysis of *Microcystis* cells in order to survive an adverse external environment that was nutrient limited.
- The mechanism of cyanobacterial lysis may involve changes in ultrastructure of *Microcystis aeruginosa*, possibly affecting energy sources and the photosynthetic system after exposure to bacteria. This may lead to the death of the cyanobacteria after exhaustion of energy sources and loss of nutrients to the predator bacteria, *B. mycoides* B16.

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.

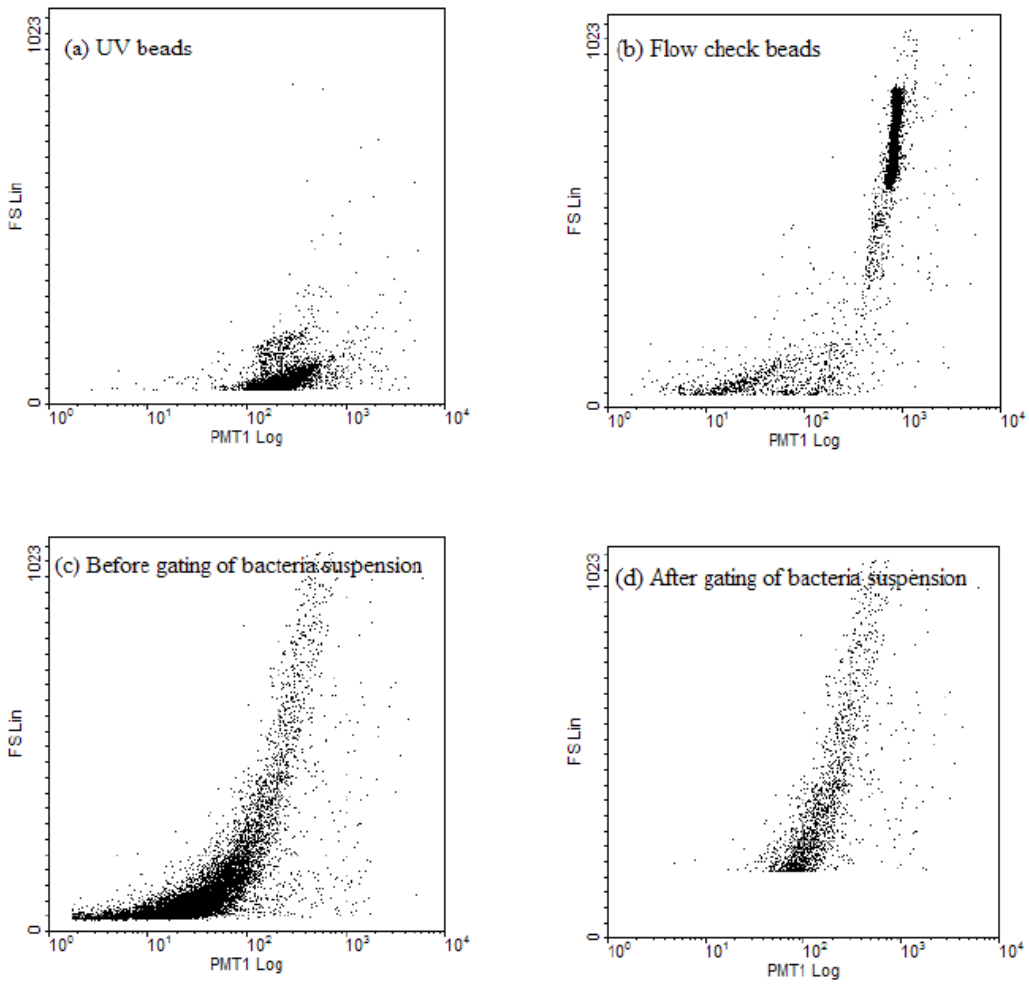


Figure 5.1: Calibration of instrument- particle size exclusion: (a) UV beads, range 1.7 – 2.2 µm, (b) Flow check beads (10 µm in size), (c) Before and (d) after gating to exclude particles less than 1µm such as *B. mycoides* B16 from subsequent FCM analysis. FSLin = measures the size of a particle, forward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

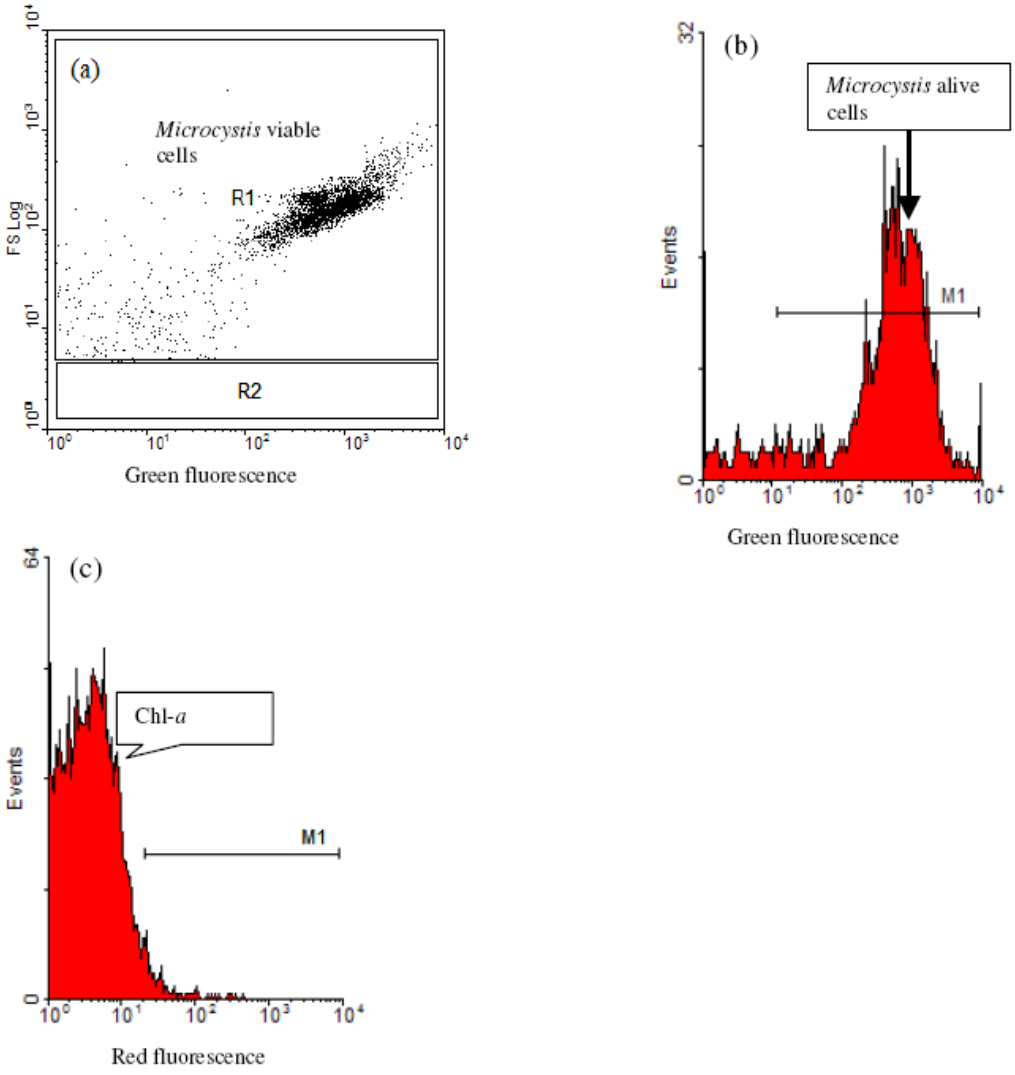


Figure 5.2: *Microcystis* control sample after staining with FDA: (a) dot plot representing region of alive cells (R1) and (R2) region for bacteria and cell debris on a 4-log scale; (b) histogram measures green fluorescence intensity of alive *Microcystis* cells. This gating parameter (M1) allows for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

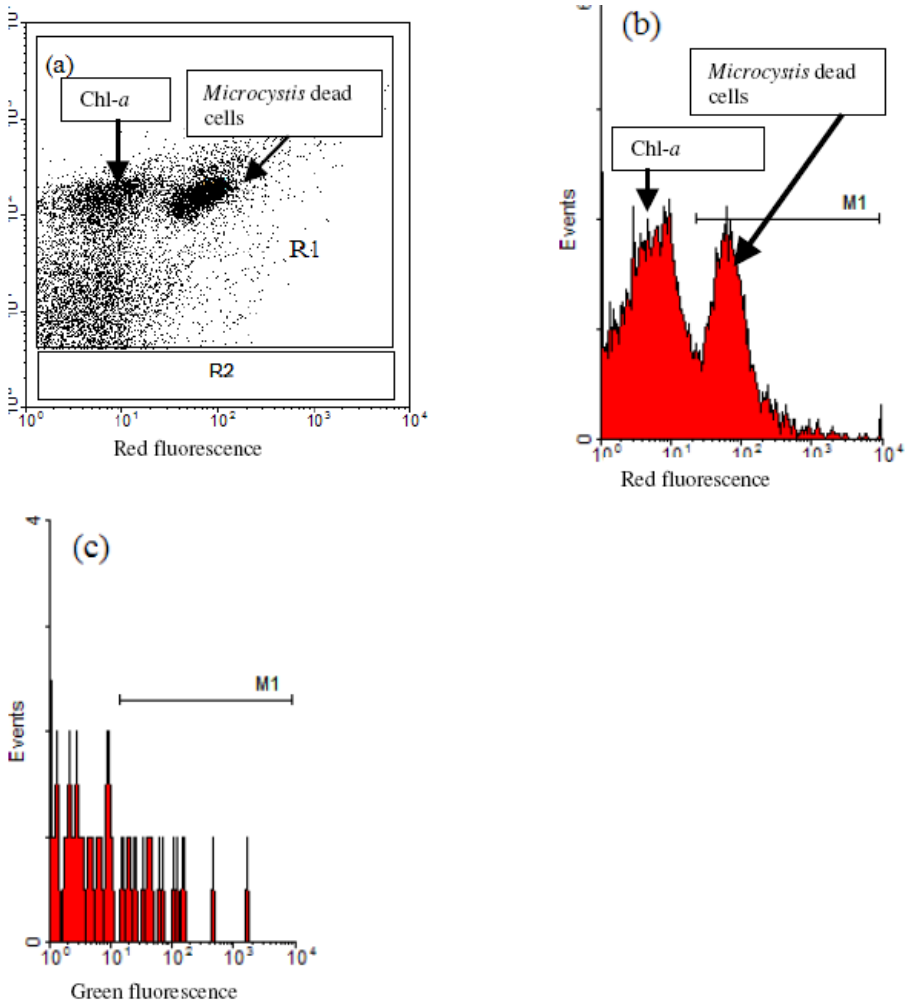


Figure 5.3: *Microcystis* control sample after staining with PI: (a) dot plot representing region of dead cells (R1) and (R2) bacteria, cell debris on a 4-log scale; (b) histogram measures PI red fluorescence on a 4-log scale. The gating parameter (M1) discriminates the red fluorescence of PI instead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

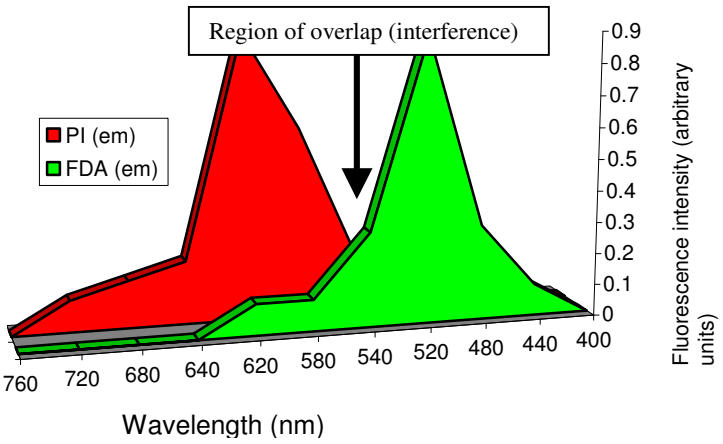


Figure 5.4: Colour compensation in resolving the PI (emission) and FDA (emission) interference (Davey, 1994).

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%.

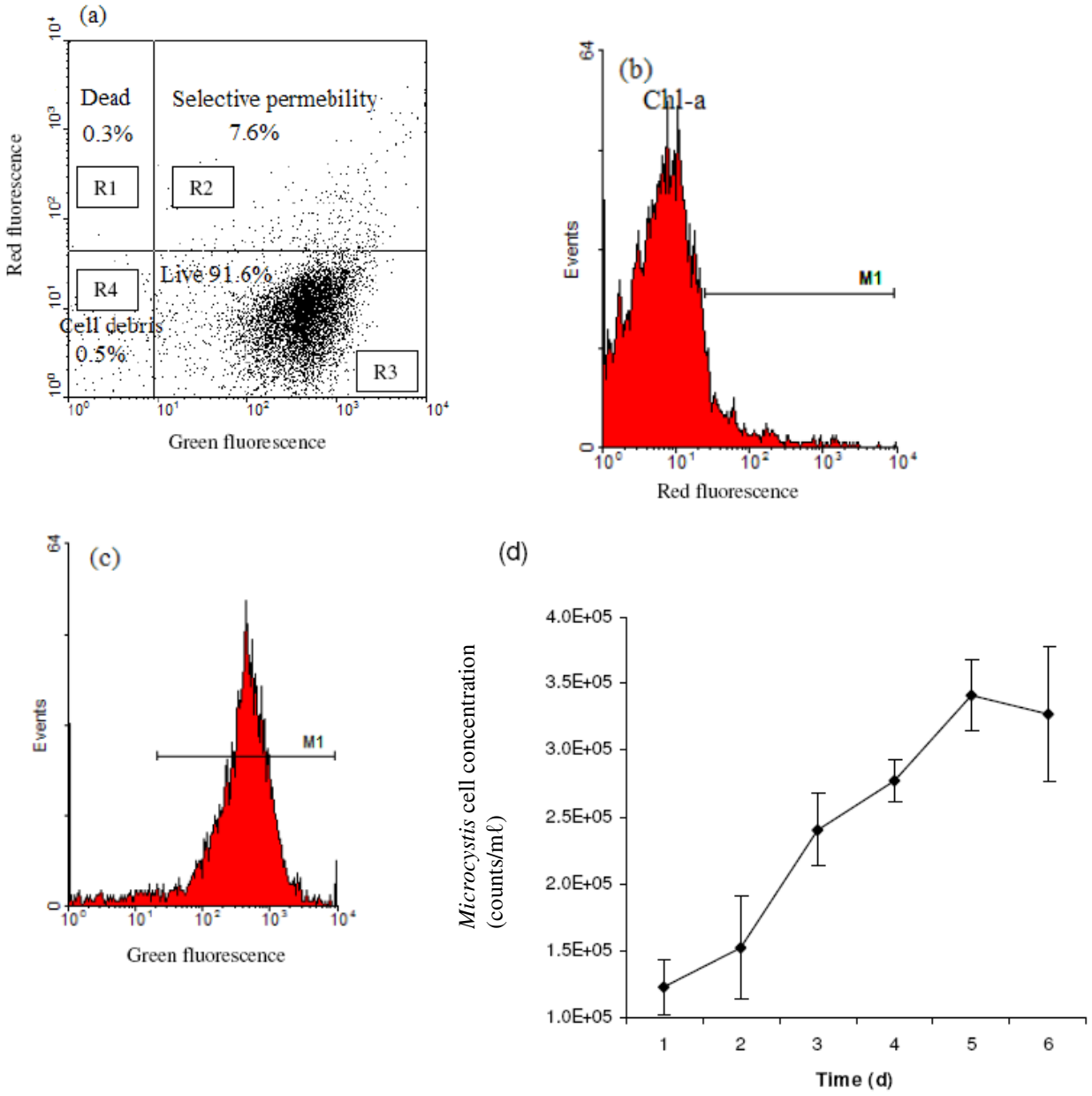


Figure 5.5: *Microcystis* control sample dual stained with FDA and PI: (a) dot plot representing regions of dead cells (R1); selective permeability cells (R2); alive cells (R3) and bacteria and cell debris (R4) on a 4-log scale; (b) histogram measures red PI fluorescence intensity of dead *Microcystis* 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).

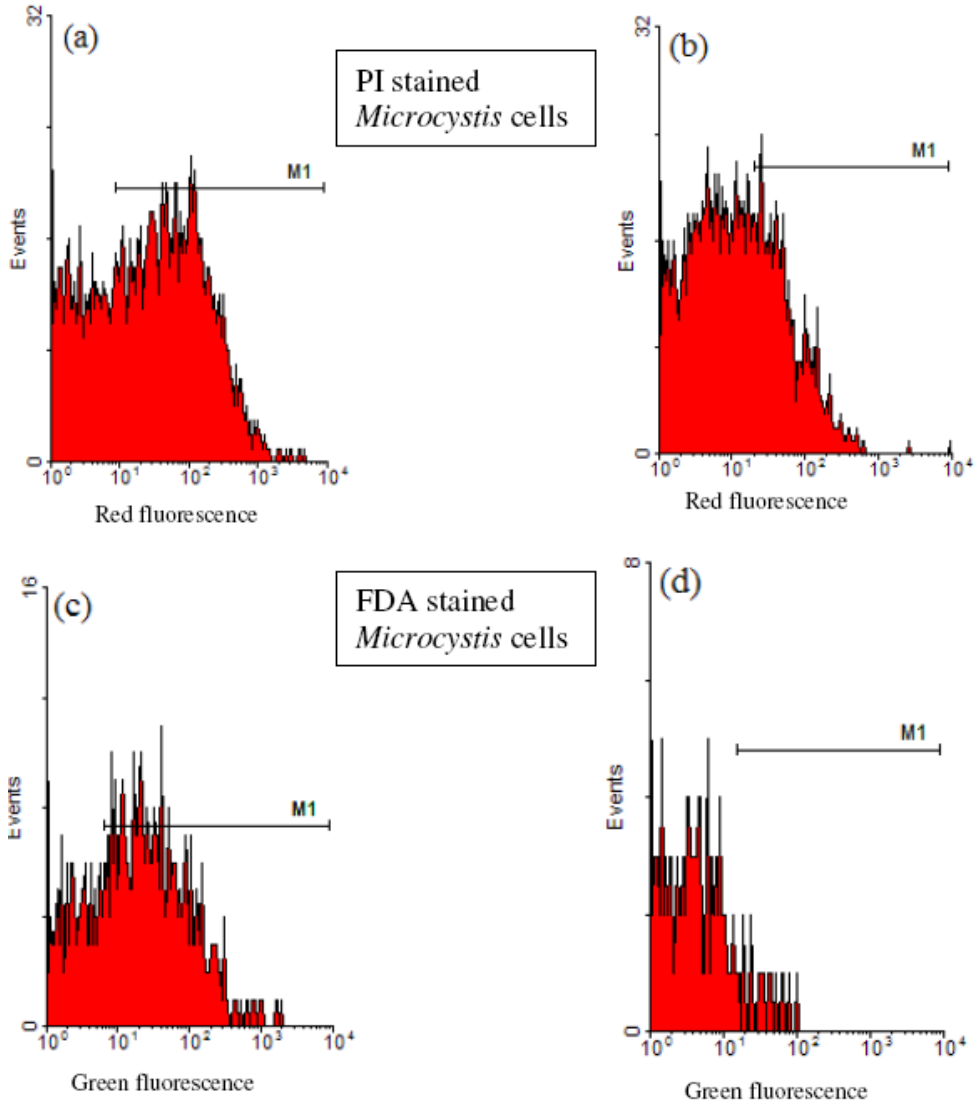


Figure 5.6: Evaluation of copper algicide and predator bacteria on *Microcystis* cells: (a-b) after PI staining: (a) treated with *B. mycoides* B16 and (b) treated with copper solution; (c-d) after FDA staining: (c) treated with *B. mycoides* B16 and (d) treated with copper solution.

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.

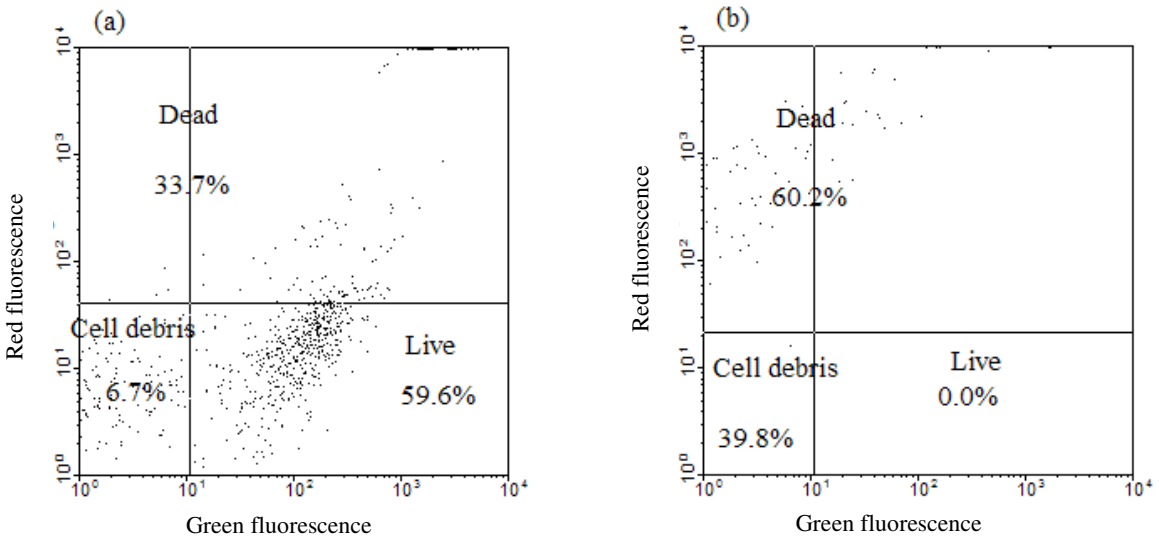


Figure 5.7: Dual stained *Microcystis* sample after exposure to *B. mycoides* B16 (a) after 2 h and (b) after 48 h showed *Microcystis* population heterogeneity: dead cells and alive cells.

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*.

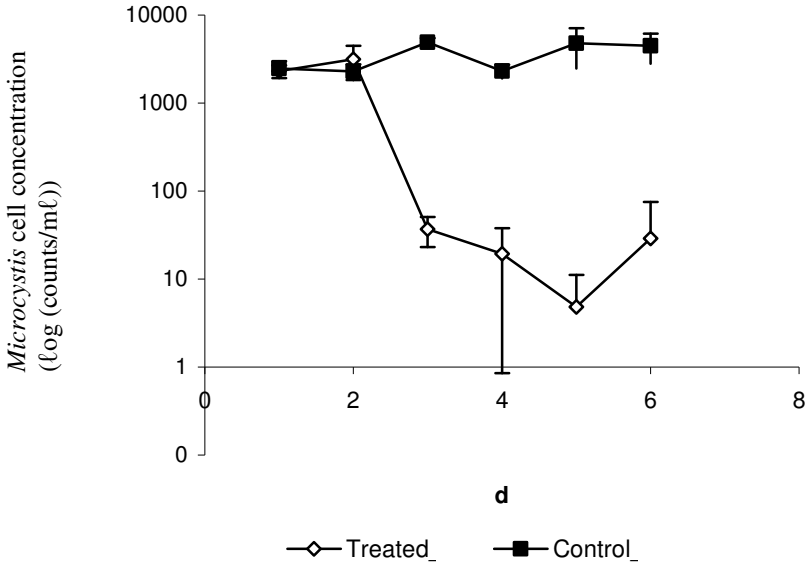


Figure 5.8: Changes in *Microcystis* cell numbers after exposure to *B. mycoides* B16 and controls under static conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation).

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*.

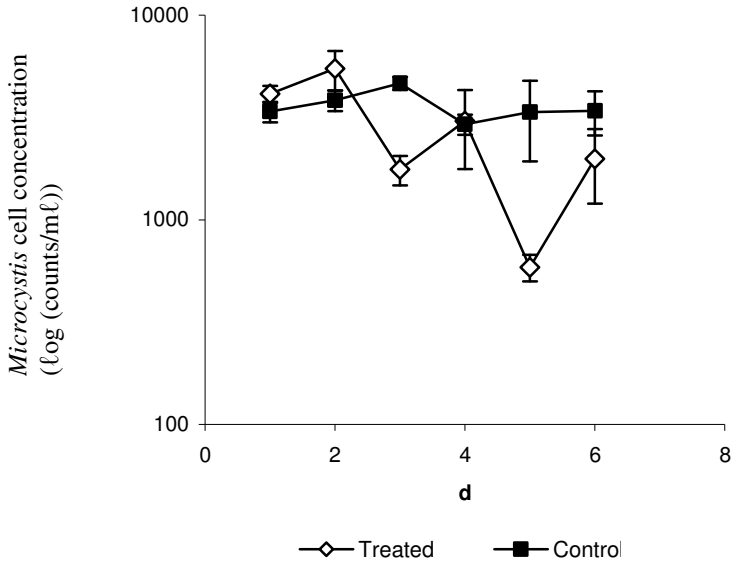


Figure 5.9: PI fluorescence illustrating changes in *Microcystis* cell numbers after exposure to *B. mycoides* B16 and control samples under static conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation).

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.

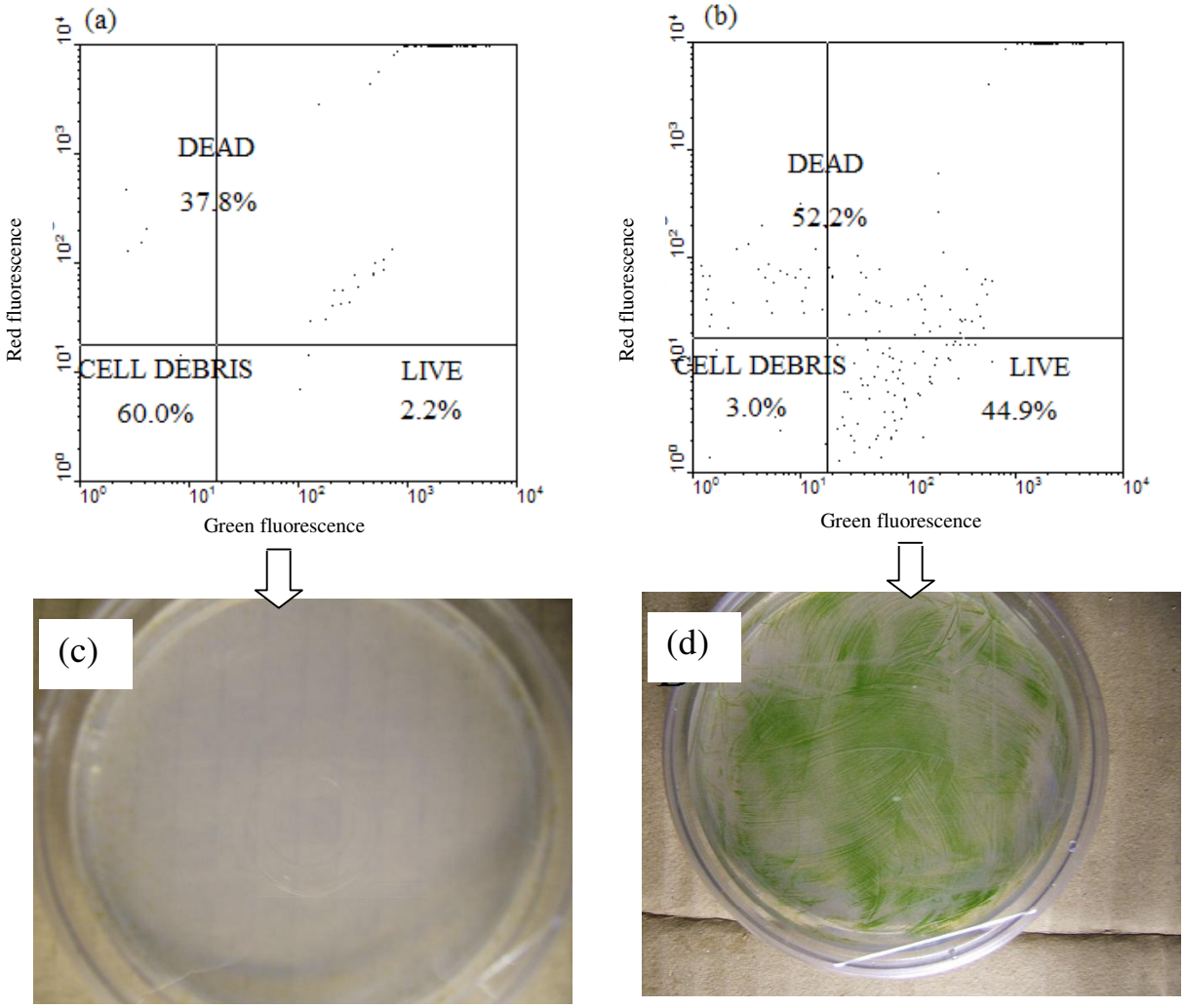


Figure 5.10: A typical two parametric plot illustration of *Microcystis* population heterogeneity on 6 d: (a) *B. mycoides* B16 treated sample and (b) Untreated (control) sample. BG 11 agar plates with (c) No growth of *Microcystis* cells after exposure to *B. mycoides* B16; (d) Control sample showing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

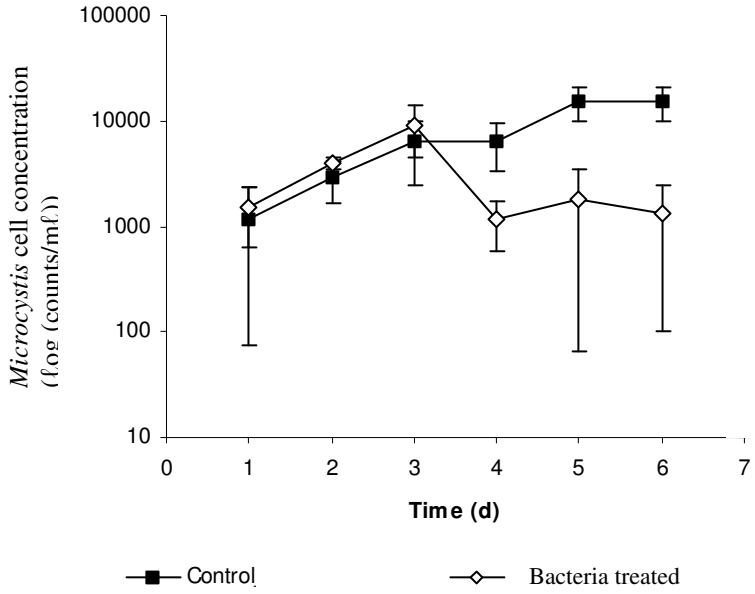


Figure 5.11: Changes in population levels of live *Microcystis* cells in *B. mycoides* B16 treated and control samples under turbulent conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation).

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

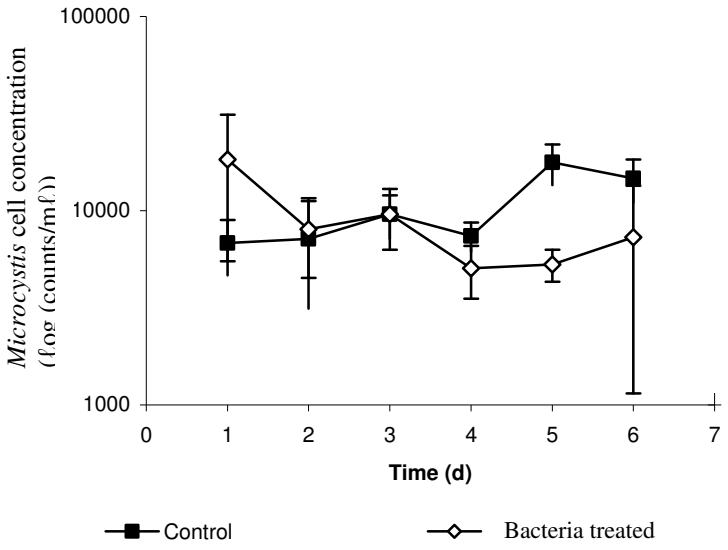


Figure 5.12: Changes in population levels of dead *Microcystis* cells in *B. mycoides* B16 treated and control samples under turbulent conditions. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation).

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.

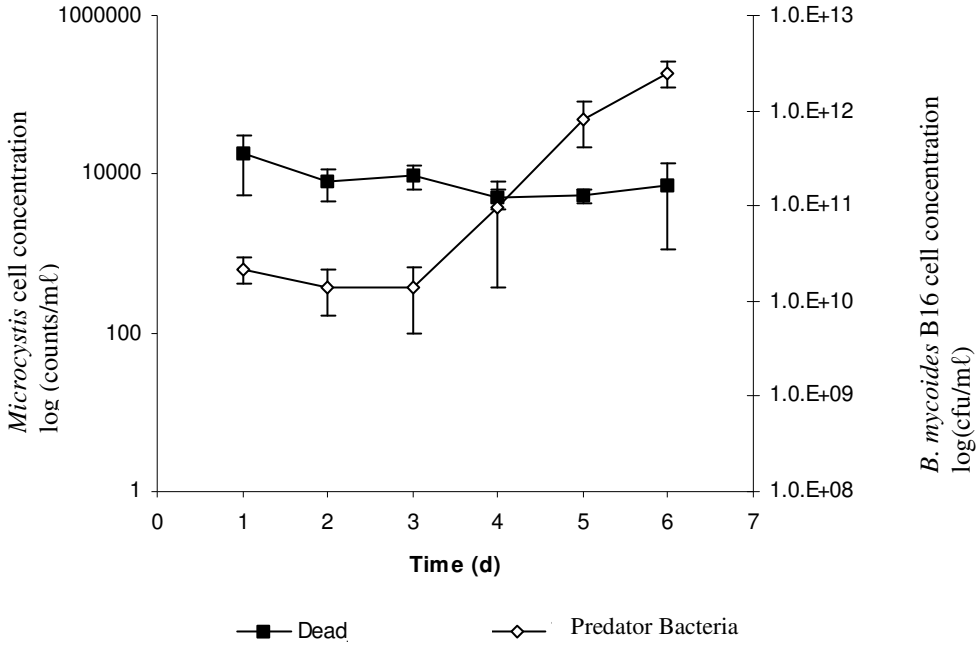


Figure 5.13: Increase in Predator bacteria numbers (colony forming units/mℓ) coincided with the decrease in *Microcystis* cells as indicated by the decrease in PI flow cytometric counts. (Mean values of six replicates ± standard deviation. Bars indicate standard deviation).

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.

Chapter 6: Conclusions and Perspectives

CHAPTER 6

CONCLUSIONS AND PERSPECTIVES

6.1. Isolation of predatory bacteria and its identification

6.1.1. Isolation and identification of predator bacteria

Water samples collected from Hartbeespoort dam (25° 43´ S; 27° 51´ E) were the source of predatory bacteria isolation. The plaques appeared on *Microcystis* lawns after 25 to 30 d of incubation. The plaque zones were irregular in shape with a width size ranging from 2 to 8 mm. They were scrapped from the plaque zones using a sterile nichrome wire and streaked onto nutrient agar plates. The hypothesis that the plaque developments on *Microcystis* lawns were due to cyanophage activity and not bacteria was tested with the chloroform test (Daft et al., 1975; Tucker and Pollard, 2004). The water samples that were treated with chloroform were negative for the presence of plaques. In contrast with control samples there were positive for the presence of plaques and this was attributed to the presence of bacteria and not cyanophages. This result agreed with Daft et al. (1975) observations that the presence of plaques was due to a single bacterium that had multiplied and caused cyanobacterial lysis.

SEM images of the plaques showed the presence five distinct morphotypes of bacteria. These morphotypes were: (1) plumb rod-shaped bacillus that was attached and free-living; (2) a long rod-shaped bacillus with one end sharpened, not attached; (3) a plumb rod-shaped bacillus with fimbriae; (4) vibrio shaped rods and (5) coccoid 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. Maybe these bacteria utilized the cyanobacteria cell contents as their nutrient source. In contrast with control areas (there were no plaques except a green *Microcystis* lawn), the cyanobacteria cells were healthy and did not show any visible distortion of cell structure. In the hyperscums of Hartbeespoort dam, similar bacteria flora were observed that consisted of extremely small cocci, large rods, presumably bacilli, that were mostly attached to *Microcystis* cells (in hyperscums) and filamentous bacteria (Robarts and Zohary, 1986). In the same study, the researchers observed that when the hyperscum reached its peak mass it was accompanied by an increased bacterial heterotrophic activity that was followed by a breakdown (decline) of the hyperscum. These findings may suggest that the bacteria were responsible for the termination of the hyperscums.

Isolates B2 and B16 were selected from the seven isolates for further screening of their lytic activity on *Microcystis.* The selection criteria were based on the presence of these isolates in the plaques and association with unhealthy *Microcystis* as per SEM observations. Isolates B2 and B16 had a lytic effect on the growth of *Microcystis* causing a 48% and 87% reduction in *Microcystis* biomass in six days. These results contrasted with the control samples, which showed an increase in the same period. Thus isolate B16 was a more effective than isolate B2 and was therefore, selected for further evaluation tests such as determining the critical predator-prey ratio; mechanism of cyanobacterial lysis and viability assessments of *Microcystis* under static and turbulent conditions. Isolates B2 and B16 were oxidase and catalase positive. This is an important characteristic because it allows the bacteria to survive under anaerobic conditions such as those found in *Microcystis* hyperscums that were formed in Hartbeespoort dam (Zohary, 1987). The dam has not experienced hyperscum formation for many years. The scums no longer reach 0.75m thickness. In the scums there is limited oxygen availability (Zohary and Breen, 1989). The limitations in oxygen availability 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.

Isolate B2 was identified as *Pseudomonas stutzeri* with 99.9% certainty using the API system. *Pseudomonas stutzeri* B2 and had not been reported to have lytic activity towards *Microcystis.* Thus, detailed research is required to develop a better understanding of the lytic mechanisms. Isolate B16 was identified as *Bacillus mycoides,* with 99.7% certainty using the API system and has not previously been reported to have lytic activity towards *Microcystis aeruginosa*. Isolate B16 formed a cotton-like spread colony that was characteristic of wild type *B. mycoides* SIN, filament projections curved anticlockwise (Di Franco et al., 2002). There were other wild types of *B. mycoides* DIX where the filament projections curved clockwise. *B. mycoides* joins other *Bacillus* species (*B. pumilis, B. megaterium, B. subtilis,* B. *licheniformes*, *B. brevis* and *B. cereus*) that were found to be antagonistic towards *Microcystis* species (Reim et al., 1974; Wright and Thompson, 1985; Wright et al., 1991; Nakamura et al., 2003b).

6.1.2. A simple predator prey model and ratio

According to the simple Lotka-Volterra predator-prey model, the prey population (*Microcystis*) increases whilst the predator population (*B. mycoides* B16) 'follows, overtakes and overcomes it' thus, producing a decline in the prey population (Atlas and Bartha, 1998:88). This model does fit into the predator-prey ratio results but to certain degree as governed by nutrient limitation for the prey (batch cultures). The predator prey relationship was expected to be an exponential relationship i.e., a decrease in predator bacteria may result in an increase in prey population or an increase in predator bacteria may result in a decrease in prey population. A high predator bacteria population, 5.2 x 10^7 cfu per mℓ, was a prerequisite for the effective lysis of *Microcystis*, which corresponded to a 1:1 ratio with a 3 d lag phase. There are a number of explanations as to what may be happening during the lag phase. Presumably during the 'lag phase' the predator bacteria population was adjusting to the new environment and increasing in numbers 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., low population of predators resulted in a longer lag phase. Shilo (1970) and Daft and Stewart (1971) pointed out that agitation of samples may disrupt or disturb the physical contact process between the cyanobacteria and bacteria thus may have caused a delay in the lytic process. May be the *Microcystis* adopted a defensive mechanism to ward off the predators by releasing microcystins. Choi et al. (2005) speculated that microcystins inhibit growth of organisms such as cladocerans, copepods, and mosquito larvae and have been shown to be allelopathetic towards green alga, *Chlamydomonas neglecta.* However, there are no published reports about microcystin toxicity with regards to bacteria (Choi et al., 2005). It is therefore speculated that a combination of initial low predator population and agitation of culture suspensions may be the main reasons for the delay in the lytic process.

Other ratios such as 1:10000, the low predator bacteria numbers had minimal lytic effect on the growth of *Microcystis.* A predator-prey plot assumed a sigmoid relationship, which was different from that of ratio 1:1. The low numbers of predator bacteria may explain insignificant biological control of nuisance algal blooms in the natural environment. Fraleigh and Burnham (1988) earlier suggested that low predator numbers could not survive and increase to a threshold density while feeding on inorganic nutrients in the lake alone but also

required algal carbon. Threshold density is the number of predator bacteria that is required to initiate cyanobacteria lysis. This may account for the reason why the numbers of predator bacteria increased during the bloom period (Bird and Rashidan, 2001). Nakamura et al. (2003a) investigated this further by providing a carbon source (starch) to stimulate bacterial population growth in order to achieve the required threshold densities that may initiate cyanobacteria lysis and it resulted in a 99% elimination of *Microcystis* algal blooms in four days.

6.1.3. Adaptation of predator bacteria to different environments

The hypothesis that *B. mycoides* B16 was capable of inducing lysis of *Microcystis* under static and turbulent conditions was tested by flow cytometric (FCM) viability assessment of individual *Microcystis* cells. The FCM analysis involved assessment of two cellular functions, esterase activity and membrane integrity, after dual staining with fluorescein diacetate (FDA) and propidium iodide (PI). In this procedure *Microcystis* cells were stained with FDA, a non-polar substrate that easily diffused through intact cell membranes. Once inside an active *Microcystis* cell, the FDA was cleaved by non-specific esterase releasing a polar fluorescent product, fluorescein, which was trapped inside cells with intact membranes and the cells were stained green (Joux and Lebaron, 2000). PI is a polar substance that easily penetrated only inactive or damaged cell membranes. Inside the cell, PI was bound to nucleic acids with intercalation and gave a bright red fluorescent under blue light excitation (Yamaguchi and Nasu, 1997). Two methods of fluorescent staining were tested: use of separate staining and dual staining of *Microcystis* cells. The method that was eventually adopted for routine analysis was the dual staining as indicated later.

Separate fluorescent staining was initially used as a result of the FDA fluorescence signal that interfered with the PI fluorescence signal due to overlap. The flow cytometric analysis in conjunction with separate fluorescence of *Microcystis* cells revealed two different cell populations: alive and dead cells. 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 alive *Microcystis* cell numbers. The independent Levene t-test analysis of the alive *Microcystis* cell numbers showed no significant difference ($p > 0.05$). Thus, the bacteria that were added had no effect on the growth of cyanobacteria. As indicated above, it was suggested that the bacteria was adjusting

to their new environment, during the 'lag phase'. Thereafter from 3 d to 6 d, *B. mycoides* B16 caused a significant decrease ($p < 0.05$) in alive *Microcystis* cell numbers by 97% in six days. Under turbulent conditions *B. mycoides* B16 caused a significant decrease ($p < 0.05$) in alive *Microcystis* cell numbers by 85% in six days. Burnham et al. (1981) reported similar results in the rapid *Myxococcus xanthus* PCO2 lysis of *Phormidium luridum*.

The predator bacteria numbers (from *Microcystis* bacteria treated) showed a significant increase ($p < 0.05$; $t = +11.31$; $df = 5$) and coincided with a decrease in growth of *Microcystis* (bacteria treated). These findings may suggest that the *Bacillus mycoides* B16, a heterotroph, was utilizing the *Microcystis* as a source of nutrition. Nakamura et al. (2003a) reported similar findings that *Bacillus cereus* N14, closely related to *B. mycoides* B16 (von Wintzingerode et al., 1997), was able to drive nutrition solely on *Microcystis aeruginosa* and *M. viridis*.

Thus *B. mycoides* B16 has the potential of a good biological agent; it must adapt to extreme conditions (turbulent and static) and survive on *Microcystis* (sole food source), as no external food could be provided.

6.2. The mechanism of lytic action of *B. mycoides* B16 on *Microcystis*

The results of this study on the interactions of *Microcystis aeruginosa* and *Bacillus mycoides* B16 indicated a series of morphological and ultrastructural changes with the cyanobacteria cell leading to its death. These are summarised in a conceptual model that was developed.

We hypothesized that the predatory bacteria, *B. mycoides* B16 attached onto the *Microcystis* cell through the use of fimbriae and or exopolymers. The ultrastructural characteristics of a healthy and normal *Microcystis* cell are shown and were confirmed with previous studies (Reynolds et al., 1981; Zohary, 1987). The ultrastucture of *Microcystis* cells (bacteria treated) showed large glycogen granules, numerous thylakoid membranes and polyphosphate bodies. Previous studies have shown that under experimental conditions of low light irradiance, *Microcystis* cells possess large cyanophycin granules, numerous thylakoid membranes and a large nucleoplasmic area (Canini et al., 2001). Borbéy et al. (1990) reported similar results, except that the cyanobacteria when subjected to an adverse external environment such as the presence of predatory bacteria, the cyanobacteria responded by accumulation of storage granules. Polyphosphate bodies, function as a store of phosphorus, appeared as spherical

inclusions of varying diameters located in the centre of the cell (Jensen, 1968). These findings suggest that the presence of numerous bacterial cells that aggregated around *Microcystis* cells provided a 'shade' that reduced the amount of light (hv) that reached the *Microcystis* cell to enable it to carry out photosynthesis. The bacteria surrounding to the *Microcystis*, presumably released extracellular substances that interfered with the *Microcystis* photosynthesis system (Schneegurt et al., 1994; Moezelaar and Stal, 1997). In response to these adverse conditions, the *Microcystis* cell did the following. It expanded its thylakoid system, the light harvesting system, to capture as much light as possible. It accumulated storage granules such as phosphate bodies, glycogen and cyanophycin. We hypothesise that this expansion and accumulation state resulted in 'swollen' *Microcystis* cells. Reim et al. (1974) and Burnham et al. (1981) also reported the swelling phenomenon prior to cell lysis but did not account for what might be the cause. Similar to the automortality model of Veldhuis et al. (2001) the last lysis stage involved the degradation of photosynthetic pigments and reduction in photosynthesis of the phytoplankton. Nakamura et al. (2003b) further reported that the interaction between *B. cereus* and *Microcystis* led to the release of photosynthetic pigments from *Microcystis* cells, presumably during the last lysis state to the external media.

In the absence of viable *Microcystis* cells the *B. mycoides* B16 (non-motile, non-spore former) formed chains, i.e., exhibited rhizoidal growth. This was a translocation exercise for the bacteria to 'move' in search of nutrients or new *Microcystis* cells to invade. Some *Bacillus* species were reported to revert to spore formation during nutrient poor periods (Villain et al., 2006).

In conclusion, the present evidence based on this study suggests that *B. mycoides* B16 is an ectoparasite (close contact is essential) in its lysis of *Microcystis aeruginosa* under laboratory conditions. The next step is to evaluate the efficiency and efficacy of *B. mycoides* B16 on *Microcystis* algal blooms under field conditions.

6.3. Future research

It was clear that *B. mycoides* B16 SIN induced the *Microcystis* cell lysis resulted in leaking of its cell contents including microcystins to the external environment. The following questions may provide a basis for future research. What is the significant of DIX form in the lysis of *Microcystis*? The SIN form curved anticlockwise during its growth on TSB agar. DIX form curved clockwise. Other researchers have isolated an unidentified protease substance from

Bacillus cereus that lysed *Microcystis* cells. *B. mycoides* B16, is closely related to *B. cereus*, may have released a similar protease substance (during this study) that caused perforations on the *Microcystis* cell membrane but this requires further investigations.

What is the impact and fate of microcystins that were released during *Microcystis* lysis? Other researchers have isolated and identified a *Sphingomonas* species and a strain of *Pseudomonas aeruginosa* that are capable of degrading microcystins. Thus the six isolates including *Pseudomonas stutzeri* B2 must be investigated for microcystin degrading abilities.

Laboratory studies were carried out to evaluate the potential of *B. mycoides* B16 as a biological control agent towards *Microcystis aeruginosa*. The issue of application of biological agent against a nuisance organism in the field is fraught with danger thus it is important to address the following. Studies are required to assess the efficiency and efficacy of *B. mycoides* B16 against a wide range of *Microcystis* species (host specificity) and non-target species before field trials are made. Lastly the issue of laboratory scale up may pose considerable challenges. The preparation of lyophilized powder still caused problems in the area of purity, culturing conditions, health hazardous and availability of media. Thus the economics and practicalities of this scale up require a through investigation.

6.3.1. *In situ* biological control of *Microcystis* algal blooms

Another important issue that require investigation is applying for an environmental authorisation (EA) from the Departments of Environmental Affairs and Tourisms (DEAT) and Water Affairs, Forestry (DWAF) and National Department of Agriculture (NDA) to carry out field trials. It is a complex process. The release of an organism into the environment for the purpose of biological control is a listed activity 21 and 22 under National Environmental Management Act (Act 107 of 1998) (NEMA) regulations. DEAT manages the NEMA and National Environmental Management: Biodiversity Act (Act 10 of 2004), DWAF manages the National Water Act (Act 36 of 1998) and NDA manages the Genetically Modified Organisms Act (Act 15 of 1997). This may take time and the EA may or may not be granted.

The alternative is to develop fluorescent *in-situ* hybridisation (FISH) probes that target the *B. mycoides* in the *Microcystis* algal blooms. From this study we have seen that the predator bacteria are present in algal blooms but in low numbers to cause any noticeable impact on the *Microcystis* colonies. Once the predator bacteria are detected in algal blooms and their

numbers must be quantified. The next step is to add a carbon source/nutrients to the algal bloom. This encourages the predator bacteria to multiply such that they can then predate on the *Microcystis* and cause the bloom to collapse.

BIBLIOGRAPHY

Adams, D.G., and Duggan, P.S., 1999. Transley Review No. 107 Heterocyst and Akinetes differentiation in cyanobacteria. **New Phytol**. 144, 3-33.

Asaeda, T., Pham, H.S., Nimal P.D.G., Manatunge, J., Hocking, G.C., 2001. Control of algal blooms in reservoirs with a curtain: a numerical analysis. **Ecological engineering**. 16, 395-404.

Ashton, P.J. and Robarts, R.D., 1987. Apparent predation of *Microcystis aeruginosa kutz emend elenkin* by a saprospira-like bacterium in a hypertrophic lake (Hartbeespoort dam, South Africa). **Journal of Limnological Society of. South Africa.** 13, 44-47.

Atlas, R.M. and Bartha, R., 1998. Microbial ecology: fundamentals and applications. 4th edition. Benjamin/Cummings Science Publishers, 2725 sand Hill Road, Menlo Park, California 94025. 698pp.

Batchelor, A.L., Scott, W.E., Wood, A., 1992. *Microcystis* scums from Hartbeespoort dam as a source of fine chemicals. Report No. 264/1/92 to the Water Research Commission.

Bates, S.S., Gaudet, J., Kaczmarska, I., Ehrman, J.M., 2004. Interactions between bacteria and the domoic-acid-producing diatom *Pseudo-nitzschia multiseries* (Hasle) Hasle: can bacteria produce domoic acid autonomously? **Harmful Algae**. 3, 11-20.

Bird, D.F. and Rashidan. K.K. 2001. Role of predatory bacteria in the termination of a cyanobacterial bloom. **Microbial Ecology**. 41, 97-105.

Blakeman, J.P. and Fokkerna, N.J. 1982. Potential for biological Control of plant diseases on the phylloplane. **Ann Rev Phyopatho**. 20, 167-192.

Bourne, D.G., Jones, G.J., Blakeley, R.L., Jones, A., Negri, A.P., Riddles, P., 1996. Enzymatic pathway for the bacterial degradation of the cyanobacterial peptide toxin microcystins LR. **Applied and Environmental Microbiology**. 62, 4086-4094.

Borbéy, G., Surrnyi, G., Kós, P., 1990. Stress responses of cyanobacteria and the pleiotropic effects of light deprivation. **FEMS Microbiology Ecology**. 74, 141-152.

Brock, T.D., Madigan, M.T., Martinko, J.M., Parker, J., 1994. Biology of Microorganisms, 7th Edn., 909 pp. Prentice-Hall International, Englewood Cliffs, NJ.

Breeuwer, P., Drocourt, J.L., Bunschoten, N., Zwietering, M.H., Rombouts, F.M., Abee, T., 1995. Characterization of Uptake and Hydrolysis of Fluorescein Diacetate and Carboxyfluorescein Diacetate by Intracellular Esterases in *Saccharomyces cerevisiae*, Which Result in Accumulation of Fluorescent Product. **Applied and Environmental Microbiology**. 61, 1614–1619.

Brookes, J.D., Geary, S.M., Ganf, G.G., Burch, M.D., 2000. Use of FDA and flow cytometry to assess metabolic activity as an indicator of nutrient status in phytoplankton. **Marine & Freshwater Research**. 51, 817 – 823.

Brunberg A-K., 1999. Contribution of bacteria in the mucilage of *Microcystis* spp.(Cyanobacteria) to benthic and pelagic bacterial production in a hypereutrophic lake. **FEMS Microbiology Ecology**. 29, 13-22.

Brussaard, C.P.D., Marie, D., Thyrhaug, R., Bratbak, G., 2001. Flow cytometric analysis of phytoplankton viability following viral infection. **Aquatic Microbial Ecology.** 26, 157-166.

Brussaard, C.P.D.,Thyrhaug, R., Marie, D., Bratbak, G., 1999. Flow cytometric analyses of viral infection in two marine phytoplankton species, *Micromonas pusila* (Prasinophyceae) and *Phaeocystis pouchetii* (Prymnesiophyceae). **J. Phycol.** 35**,** 941–948.

Burnham, J.C., Hashimoto, T., Conti, S.F., 1968. Electron microscopic observations on the penetration of *Bdellovibrio bacteriovorus* into gram-negative bacterial hosts. **Journal of Bacteriology**. 96, 1366-1381.

Burnham, J.C., Stetak, T., Boulger, J., 1973. An improved method of cell enumeration for filamentous algae and bacteria. **Journal of Phycology**. 9, 346-349.

Burnham, J.C., Stetak, T., Gregory, L., 1976. Extracellular lysis of the blue-green alga *Phormidium luridum* by *Bdellovibrio bacteriovorus*. **Journal of Phycology**. 12, 306-313.

Burnham, J.C., Collart, S.A., Highison, B.W., 1981. Entrapment and lysis of the cyanobacterium *Phormidum luridum* by aqueous colonies of *Myxococcus xanthus* PCO2. **Archives in Microbiology**. 129, 285-294.

Burnham, J.C., Susan, A.C., Daft, M.J., 1984. *Myxococcal* predation of the cyanobacterium *Phormidium luridum* in aqueous environment. **Archives in Microbiology**. 137, 220-225.

BMDP Statistical Software Inc, (1993). 12121 Wilshire Blvd, Suite 300 Los Angeles, CA 90025 USA.

Canini, A., Leonardi, D., and Caiola, M. G., (2001). Superoxide dismutase activity in the cyanobacterium *Microcystis aeruginosa* after surface bloom formation. **New Phytologist**. 152, 107-116.

Carmichael, W.W., 1994. The toxins of cyanobacteria. **Scientific American**. 64-67.

Carmichael, W.W., 1997. The cyanotoxins *IN*: Callow, J.A(Ed), Advances in Botanical Research vol 27. Academic press, London pp211 256.

Caiola, M.G., and Pellegrini, S., 1984. Lysis of *Microcystis aeruginosa (Kutz)* by *Bdellovibro*-like bacteria. **Journal of Phycology**. 20, 471-475.

Caron, N-v., Stephens, P., Badlyey, R.A., 1998. Assessment of bacterial viability status by flow cytometry and single cell sorting. **Journal of Applied Microbiology.** 84, 988-998.

Choi, H-j., Kim, B-h., Kim, J-d., Han, M-s., 2005. *Streptomyces neyagawaensis* as a control for the hazardous biomass of *Microcystis aeruginosa* (Cyanobacteria) in eutrophic freshwaters. **Biological Control**. 33, 335-343.

Chutter, F.M., 1989. Evaluation of the impact of the 1mg/L phosphate-P standard on the water quality and trophic state of Hartebeespoort Dam. Contract Report to the Water Research Commission, Pretoria.

Christison, J., and Martin, S.M., 1971. Isolation and preliminary characterization of an extracellular protease of Cytophaga sp. **Can J Microbiol**. 17, 1207-1216.

Cloete, T.E., and Oosthuizen, D.J., 2001. The role of extracellular exopolymers in the removal of phosphorus from activated sludge. **Water Research**. 35, 3595 - 3598.

Codd, G.A., and Bell, S.G., 1996. The occurrence and Fate of Blue-Green Algal Toxins in Freshwaters. R&D Report 29 for National Rivers Authority. Department of Biological Sciences, University of Dundee, Dundee, United Kingdom. Pp 30.

Codd, G.A., Ward, C.J., Bell, S.G., 1997. Cyanobacterial toxins: occurrence modes of action, health effects and exposure routes. **Archives Toxicology Supplement**. 19, 399-411.

Codd, G. A., Bell, S. G., Kaya, K., Ward C. J.,Beattie, K., A., Metcalf, J. S. 1999. Cyanobacterial toxins, exposure routes and human health. **Eur. J. Phycol**., 34, 405-415.

Codd, G.A., 2000. Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. **Ecological Engineering**. 16, 51-60.

Collier, J.L., 2000. Flow cytometry and single cell in phycology. **A Review. Journal of Phycology.** 36, 628-644.

Cronberg, G., Carpenter, E.J., Carmichael, W.W., 2003. Taxonomy of harmful cyanobacteria. In: G.M. Hallegraeff, D.M. Anderson and A.D. Cembella (**eds**.), Manual on Harmful Marine Microalgae. Unesco Publishing pp. 523-562.

Czaban, J., Księżniak, A., Perzyński, A., 2004. An attempt to protect winter wheat against *Fusarium culmorum* by the use of Rhizobacteria *Pseudomonas fluorescens* and *Bacillus mycoides*. **Polish Journal of Microbiology.** 53, 175-182.

Daft, M.J., and Stewart, W.D.P., 1971. Bacterial pathogens of freshwater blue green algae. **New Phytologist**. 70, 819-829.

Daft, M.J., and Stewart, W.D.P., 1973. Light and electron microscope observations on algal lysis by bacterium CP-1. **New Phytologist**. 72, 799-808.

Daft, M.J., McCord, S.B., Stewart, W.D.P., 1973. The occurrence of blue-green algae and lytic bacteria at a waterworks in Scotland. **Water Treatment & Examination**. 22, 114–124.

Daft, M.J., McCord, S.B., Stewart, W.D.P., 1975. Ecological studies on algal lysing bacteria in fresh waters. **Freshwater Biology**. 5, 577-596.

Daft, M.J., Burnham, J.C., Yamamato, Y., 1985a. Algal blooms: consequences and potential cures. **Journal of Applied Bacteriology symposium Supplement**. 175S-186S.

Daft, M.J., Burnham, J.C., Yamamato, Y., 1985b. Lysis of *Phormidium luridum* by *Myxococcus fulvus* in continuous flow cultures. **Journal of Applied Bacteriology**. 59, 73-80.

Davey, M.H., 1994. Flow cytometry of micro-organisms. PhD Thesis. Institute of Biological Sciences, University of Wales, Aberystwyth. http://qbab.aber.ac.uk/thesis/topen.html (accessed 21/05/06).

Di Franco, Beccari, E., Santini, T., Pisaneschi, G., Tecce, G., 2002. Colony shape as a genetic trait in the pattern-forming *Bacillus Mycoides.* Tecce, Università La Sapienza Roma, Italy, November 2002. http:// bmc.ub.uni - potsdam.de /1471-2180-2-33/ text.htm (accessed 07/03/2006).

Dobson, M.J., and McCurdy, H.D., 1979. The function of fimbriae in *Myxococcus xanthus*. 1. Purifications and properties of *M. xanthus* fimbriae. **Canadian Journal of Microbiology**. 25, 1152-2260.

Dubelaar, G.B.J., and Reijden, van der C.S., 1995. Size distribution of *Microcystis aeruginosa* colonies: A flow cytometric approach. **Water Science Technology**. 32, 171 – 176.

DWA (1988). Important announcement on implementation of Special Phosphate standard in sensitive catchments. **IMIESA (Johannesburg).** 13(9), 35.

Dworkin, M., 1966. Biology of the myxobacteria. **Annu. Rev. Microbiol**. 20,75-106.

Dworkin, M., and Sudo, S., 1972. Bacteriolytic enzymes produced by *Myxococcus xanthus*. **J Bacteriol**. 110, 236-245.

Falconer, I.R., Beresford, A.M., Runnegar, M.T.C., 1983. Evidence of liver damage by toxin from a bloom of the blue green alga, *Microcystis aeruginosa*. **Med. J. Aust.** 1, 511-514.

Fraleigh, P.C., and Burnham, J.C., 1988. Myxococcal predation on Cyanobacterial Populations: Nutrient Effects. **Limnology and Oceanography**. 33, 476-483.

Franklin, N.M., Adams, M.S., Stauber, J.L., Lim, R.P., 2001. Development of a improved Rapid Enzyme Inhibition Bioassay with Marine & Freshwater Microalgae Using Flow Cytometry. **Archives of Environmental Contamination and Toxicology.** 40, 469 – 480.

Franklin, N.M., Stauber, J.L., Lim, R.P., 2004. Development of multispecies algal bioassays using flow cytometry. **Environmental Toxicology and Chemistry**. 23, 1452-1462.

Fray, P., 1993. The blue-greens (Cyanophyta-Cyanobacteria).-Studies in biology/Institute of Biology: no. 160. (1st ed). Edward Arnold Ltd, London. Pp 5-83.

Fritze, D., 2004. Taxonomy of the genus *Bacillus* and related genera: The aerobic endospore-forming bacteria. **Phytopathology.** 94, 1245-1248.

García-Villada, L., Rico, M., Altamirano, M., Sánchez-Martín L., López-Rodas, V., Costas, E., 2004. Occurrence of copper resistant mutants in the toxic cyanobacteria *Microcystis aeruginosa*: characterisation and future implications in the use of copper sulphate as algaecides. **Water Research**. 38, 2207-2213.

Gestsky, R., Shtienberg, D., Elad, Y., Dinoor, A., 2002. Improved Biocontrol of *Botrytis cinerea* on Detached Strawberry Leaves by Adding Nutritional Supplements to a Mixture of *Pichia guilermondii* and *Bacillus mycoides.* **Biocontrol Science and Technology**. 12, 625-630.

Gibson, C.E., and Smith, R.V., 1982. Freshwater plankton. In: Carr N.G and Whitton B.A. (**eds**.), The Biology of cyanobacteria, Botanical monographs, vol 19. University of California Press. Pp 463-489.

Glazer, A.N., 1989. Light guides. Directional energy transfer in a photosynthetic antenna. **Journal of Biological Chemistry**. 264, 1-4.

Gliwicz, Z.M., 1990. Why do cladocerans fail to control algal blooms? Hydrobiologia 200/201: 83-97. In R.D Gulati, E.H.R.R. Lammens, M.-L. Meijer & E. van Donk (**eds**), Biomanipulation – Tool for Water Management. Kluwer academic Publishers, Belgium.

Gnosspelius, G., 1978. Purification and properties of an extracellular protease from *Myxococcus virescens*. **J Bacteriol**. 133, 17-25.

Godden, L.,2005, Water Law Reform in Australia and South Africa: Sustainability, Efficiency and Social Justice, **Journal of Environmental Law**. 17 (2), 181–205.

Greenop, B., and Robb, M., 2001. Phosphorus in the Canning: 1999-2000 Phoslock™ trials. **River Science**. 17, 1-7.

Guilard, R.R.I., 1973. Division rates. In: J.R. Stein (**ed**.), Handbook of phycological methods, Culture methods and growth measurements, pp 289-311. London, Cambridge Univ. Press.

Guilard, R.R.I., 1978. Using the standard microscope – counting slides. In: Sournia, A. (**ed**.), Phytoplankton Manual. United Nations Educational, Scientific and Cultural Organization, Paris, pp. 182–189.

Haider, S., Naithani, V., Viswanathan, P.N., Kakkar P., 2003. Review Cyanobacterial toxins: a growing environmental concern. **Chemosphere**. 52, 1-21.

Harding, W.R., and Paxton, B., 2001. Cyanobacteria in South Africa: A Review. WRC Report No. TT 153/01, July 2001.

Harding, W.R., Thornton, J.A., Steyn, G., Panuska, J., Morrison, I.R., 2004. Hartbeespoort dam Remediation Project (Phase 1). Final Report (Volume 1). Project Number 58/2003. Completed October 2004. Department of Agriculture, Conservation, Environment and Tourism (DACET) of the Provincial Government of North West Province (NWPG), South Africa. 166pp.

Hart, B.A., and Zahler, S.A., 1966. Lytic enzyme produced by *Myxococcus xanthus*. **J Bacteriol.** 92, 1632-1637.

Haska, G., 1974. Extracellular lytic enzymes of *Myxococcus virescens*. IV. Purification and characterization of a D-alanyl-e-N-lysine and endopeptidase. **Physiol Plant**. 31, 252-256.

Hawkins, P.R., Chandrasena, N.R., Jones, G.J., Humpage, A.R., Falconer, I.R., 1997. Isolation and toxicity of *Cylindrospermopsis raciborskii* from ornamental lake. **Toxicon.** 35, 341-346.

Hayden, G.E., Walker, K.Z., Miller, J.F.A.P., Wotherspoon, Raison, R.L., 1988. Simultaneous Cytometric Analysis for the expression of cytoplasmic and surface antigens in Activated T Cells. **Cytometry**, 9, 44-51.

Herbert, R.B., 1989. The biosynthesis of secondary metabolites. 2nd edn, Chapman and Hall, London.

Hickey, P.C., Swift, S.R., Roca, M.G., Read, N.D., 2004. Live-cell Imaging of Filamentous Fungi Using Vital Fluorescent Dyes and Confocal Microscopy. **Methods in Microbiology**. 34, 63-87.

Hoeger, S.J., Shaw, G., Hitzfeld, B.C., Dietrich, D.R., 2004. Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants. **Toxicon**. 43, 639-649.

Hoiczyk, E., and Hansel, A., 2000. Cyanobacterial cell walls: news from an unusual prokaryotic envelope. **J. Bacteriol**. 182:1191-1199.

Holm-Hasen, O., 1968. Ecology, physiology and biochemistry of blue-green algae. **Ann Rev Microbiol**. 22, 47-70.

Hitzfeld, B.C., Höger, S.J., Dietrich, D.R., 2000. Cyanobacterial Toxins: Removal during Drinking Water Treatment, and Human Risk Assessment. Environmental Health Perspectives Supplements, **A Review in Environmental Health.** 108 (1), 113-122. (online edition accessed 25/08/2004). http://ehpnet1.niehs.nih.gov/docs/2000/suppl-1/113-122hitzfeld/abstract.html

Jacobsen, B. J., Zidack, N. K., and Larson, B. J. 2004. The role of *Bacillus*-based biological control agents in integrated pest management systems: Plant diseases. **Phytopathology** 94,1272-1275.

Jang, M-H., Ha K., Joo, G-J., Takamura, N., 2003. Toxin production of cyanobacteria is increased by exposure to zooplankton. **Freshwater Biology**. 48, 1540-1550.

Jensen, T.E., 1968. Electron microscopy of polyphosphate bodies in blue green alga, *Nostoc pruniforme*. **Archive fur Mikorobiologie**. 62, 144-152.

Jochimsen, E.M., Carmichael, W.W., An, J., Cardo, D.M., J.M., Cookson, S.T., Holmes, C.E.M., Antunes, M.B., de Melo Filho, D.A., Lyra,D.M., Barreto,VS.T., Azevedo, S., Jarvis, W.R. 1998. Liver Failure and Death after Exposure to Microcystins at a Haemodialysis Centre in Brazil. **New England Journal of Medicine**. 338, 873-878.

Jones, G.J., Bourne, D.G., Blakeley, R.L., Jones, A., Negri, A.P., Riddles, P., 1996. Enzymatic pathway for the bacterial degradation of the cyanobacterial peptide toxin microcystins LR. **Applied and Environmental Microbiology**. 62, 4086-4094.

Jones, G.J., and Orr, P.T., 1994. Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. **Water Research**. 28, 871-876.

Joska M.A.P., and Bolton J.J, (1994). Preliminary investigation into algal weeds in inland waters. *Water Research Commission*, *WRC Report No. 426/1/94*.

Joux, F., and Lebaron, P., 2000. Use of fluorescent probes to assess functions of bacteria at single cell-level. **Microbes and Infection**. 2, 1523-1535.

Joyce, E., Phull, S.S., Lorimer, J.P., Mason, T.J., 2003. The development and evaluation Of ultrasound for the treatment of bacterial suspensions. A study of frequency, power and sonication time on cultured Bacillus species. **Ultrasonics Sonochemistry.**10, 315–318.

Kaprelyants, A.S., and Kell, D.B., 1993. Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. **Applied and Environmental Microbiology**. 59, 3187-3196.

Kirchman, D.L., 2002. The ecology of *Cytophaga* flavobacteria in aquatic environments. **FEMS Microbiology Ecology**. 39, 91-100.

Klapper H, (1999). Control of eutrophication in inland waters Ellis Horwood, Chichester, UK 337 pp97 100.

Korsten, L., and Cook, N., 1996. Optimizing culturing conditions for *Bacillus subtilis*. **South African Avocado Growers' Association Yearbook.** 19, 54-58.

Krüger, G.H.J., and Eloff, J.N., 1977. The influence of light intensity on the growth of different *Microcystis* isolates. **Journal of Limnology Society of South Africa**. 3, 21-25.

Kulik, M.M., 1995. The potential for using cyanobacteria (blue green algae) and algae in the biological control of pathogenic bacteria and fungi. **European Journal of Plant Pathology.** 101, 585-599.

Lam, A.K.-Y., Prepas, E.E., David, S., Hrudey, S.E., 1995. Chemical control of heptatotoxic phytoplankton Blooms: Implications for human health. **Water Research**. 29, 184-554.

Latour, D., Sabido, O., Salencon, M.J., Giraudet, H., 2004. Dyanmics and metabolic activity of the benthic cyanobacterium *Microcystis aeruginosa* in the Grangent reservoir (France). **Journal of Plankton Research**. 26, 719-726.

Lee, T.J., Nakano, K., Matsumura, M., 2000. A new method for the rapid evaluation of gas vacuoles regeneration and viability of cyanobacteria by flow cytometry. **Biotechnology Letters**. 22, 1833-1838.

Madison, M.T., Martinko, J.M. and Parker, J., 2003. Biology of Microorganisms. 10th edition.

Marie, D., Simon, N., Vaulot, D., 2005. Chapter 17: Phytoplankton cell counting by flow cytometry. In Algal Culturing Techniques. Academic Press. online edition accessed: 2005/02/11.

Maruyama, T., Kato, K., Yokoyama, A., Tanaka, T., Hiraishi, A., Park, H.-D., 2003. Dynamics of Microcystin-Degrading Bacteria in Mucilage of *Microcystis*. **Microb Ecol**. 46:279-288.

Mason, C.F., 1996. Biology of freshwater pollution, 3rd edition. Longman, Essex.

Meybeck M, Kuusisto E, Mäkelä A, Mälkki E, (1996). *Water Quality*, In: Bartram, J, Balance, R (**eds**), *Water Quality Monitoring*. E and FN Spon, London 9-33.

Moezelaar R., and Stal, L.J., 1997. A comparison of fermentation in the cyanobacterium *Microcystis* PCC7806 grown under a light/dark cycle and continuous light. **Eur. J. Phycol**. 32, 373-378.

Murphy, R., 1996. Basic Theory 1 - PowerPoint lecture slides, *Carnegie Mellon University, Pittsburgh, PA* www.cyto.purdue.edu/flowcyt/educate/theory/theory.htm (accessed 14/05/06)

NHMRZ/ARMCANZ, 2001. Australian drinking water guidelines, micro-organism 3: toxic algae, Fact Sheets No. 17a-17d, National Health and Medical Research Council, Agriculture Resource Management Council of Australia and New Zealand, Canberra.

Nakamura, N., Nakano, K., Sungira, N., Matsumura, M., 2003a. A novel control process of cyanobacterial bloom using cyanobacteriolytic bacteria immobilized in floating biodegradable plastic carriers. **Environmental Technology**. 24, 1569-1576.

Nakamura, N., Nakano, K., Sugiura, N., Matsumura, M., 2003b. A novel cyanobacteriolytic bacterium, *Bacillus cereus*, Isolated from a Eutrophic Lake. **Journal of Bioscience and Bioengineering**. 95, 179-184.

National Institute for Water Research (NIWR) (1985). The Limnology of Hartbeespoort Dam, South African National Scientific Programs Report No. 110, Foundation for Research Development, Council for Scientific and Industrial Research, Pretoria, 269 pp.

NSW, 2000. Preventing and managing blue-green algal blooms . NSW Department of Land and Water Conservation**.** www.dlwc.nsw.gov.au (accessed 16/10/2005)

National Water Act (NWA), 1998. Act 36 of 1998. Government Gazette, vol 398 number19182, Cape Town, 26 August, 1998. Republic of South Africa.

Oberholster, P.J., Botha, A.M., Grobbelaar, J.U., 2004. Review *Microcystis aeruginosa*: source of toxic microcystins in drinking water. **African Journal of Biotechnology.** 3, 159-168.

Orr, P.T., and Jones, G.J., 1998. Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. **Limnology and Oceanography**. 43, 1604-1614.

Petit, J.M., Denis-Gay, M., Ratinaud, M.H., 1993. Assessment of fluorochromes for cellular structure and function studies by flow cytometry. **Biol Cell**. 78, 1-13.

Phinney, D.A., and Cucci, T.L., 1989. Flow cytometry and phytoplankton. **Cytometry.** 10, 51 – 521.

Rae, B., Moolman, R.M., Clark, R.C., 1999. Algal toxins in drinking water supply. WRC Report No. 549/1/99.

Rapala, J., Lahti, K., Räsänen, L.A., Anna-Liisa, E., Niemelä, S.I., Sivonen, K., 2002. Endotoxins associated with cyanobacteria and their removal during drinking water treatment, **Water Research**. 36, 2627-2635.

Rapala, J., Lahti, K., Niemelä, S.I., Sivonen, K., 1994. Biodegradability and adsorption on lake sediments of cyanobacterial hepatoxins and anatoxin-a. **Letters in Applied Microbiology**. 19, 423-428.

Regel, R.H., Ferris, J.M., Ganf, G.G., Griffiths, R.W., 2002. Algal esterase activity as a biomeasure of environmental degradation in a freshwater creek. **Aquatic Toxicology.** 59, 209-223.

Regel, R.H., Brookes, J.D., Ganf, G.G., Griffiths, R.W., 2004. The influence of experimentally generated turbulence as the Mash01 unicellular *Microcystis aeruginosa* strain. **Hydrobiologia.** 517, 107-120.

Reim, R.L., Shane, M.S., Cannon, R.E., 1974. The characterization of *Bacillus* capable of blue green bactericidal activity. **Canadian Journal of Microbiology**. 20, 981-986.

Reynolds, C.S., Jaworski, G.H.M., Cmiech, H.A., Leedale, G.F., 1981. On the annual cycle of the blue green alga *Microcystis aeruginosa Kutz. Emend. Elenkin*. **Philosophical Transaction of the Royal Society of London. Series B, Biological Sciences**. 293, 419-477.

Richard, D.S., Beatiie, K.A., Codd, G.A., 1983. Toxicity of cyanobacterial blooms from Scottish freshwaters. **Environ. Technol. Letters**. 4, 377-382.

Richard, Y., and Poccard, I., 1998. A statistical study of NDVI sensitivity to seasonal and interannual rainfall variations in Southern Africa. **International Journal of Remote Sensing.** 19, 2907-2920.

Rieseberg, M., Kasper, C., Reardon, K.F., Scheper, T., 2001. Flow cytometry in Biotechnology. **Appl Microbiol Biotechnol**. 56:350–360

Riemann, L., Steward, G.F., Azam, F., 2000. Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. **Appl. Environ. Microbiol.** 66, 578-587.

Robarts, R.D., Ashton, P.J., Thornton, J.A., Taussig, H.J., Sephton, L.M., 1982. Overturn in a hypertrophic, warm, monomictic impoundment (Hartbeespoort dam, South Africa). **Hydrobiologia**. 97, 209-224.

Robarts, R.D., and Zohary, T., 1986. Influence of cyanobacterial hyperscum on heterotrophic activity of planktonic bacteria in a hypertrophic lake. **Applied and Environmental Microbiology.** 51, 609-613.

Robb, M., Greenop, B., Goss, Z., Douglas, G., Adeney, J., 2003. Application of Phoslock an innovative phosphorus binding clay, to two Western Australian waterways: preliminary findings. **Hydrobiologia**. 494, 237-243.

Rositano, J., Newcombe, G., Nicholson, B., Sztajnbok, P., 2001. Ozonation of NOM and algal toxins in four treated waters. **Water Research**. 35, 23-32.

Ross, D.D., Joneckis, C.C., Ordőńez, J.V., Sisk, Am., Wu, R.K., Hamburger, A.W., Nora, R.E., 1989. Estimate of cell survival by flow cytometric Quantification of Fluorescein Diacetate/Propidium Iodide viable Cell Number. **Cancer Research.** 49, 3776 – 3782.

Runnegar, M.T.C., Andrews, J., Gerdes, R.G., Falconer, I.R., 1987. Injury to hepatocytes by a peptide toxin from the cyanobacterium *Microcystis aeruginosa*. **Toxicon**. 25, 1235-1239.

SABC News, 2003. Contaminated water affects thousands in Brits. October 23, 2003. www.sabcnews.com/article/printwholestory/0,2160,67894,00.html (accessed 24/10/03)

Sallal, A-K.J., 1994. Lysis of cyanobacteria with Flexibacter spp isolated from domestic sewage. **Microbios.** 77, 57-67.

Scott, W.E., 1991. Occurrence and significance of toxic cyanobacteria in Southern Africa. **Water Research Technology**. 23, 175-180.

Schneegurt, M.A., Sherman, D.M., Nayar, S., Sherman, L.A., 1994. Oscillating Behavior of Carbohydrate Granule Formation and Dinitrogen Fixation in the Cyanobacterium Cyanothece sp. Strain ATCC 51142. **Journal of Bacteriology**. 176, 1586-1597.

Secord, D., 2003. Biological control of marine invasive species: cautionary tales and land-based lesions. **Biological Invasions**. 5, 117-131.

Shilo, M., 1970. Lysis of Blue Green Algae by *Myxobacter*. **Journal of Bacteriology**. 104, 453-461.

Sieracki, M.E., Cucci, T.L., Nicinski, J., 1999. Flow cytometric analysis of the 5-cyano-2,3-ditolyl tetrazolium chloride activity of marine bacterioplankton in dilution cultures. **Applied and environmental Microbiology**. 65, 2409-2411.

Sigee, D.C., Glenn, R., Andrews, M.J., Bellinger, E.G., Butler, R.D., Epton, H.A.S., Hendry, R.D., 1999. Biological control of cyanobacteria: principles and possibilities. Hydrobiologia 395/396:161-172, *In* The Ecological Bases for Lake and Reservoir Management, Harper DM, Brierley, Ferguson AJD, Philips G (**eds**), Kluwer Academic Publishers, Netherlands.

Sivonen, K., and Jones, G., 1999. Cyanobacterial toxins. In: I Chorus and J Bartram (**eds**.), Toxic Cyanobacteria in water, E & FN Spon, London, pp 41-111.

Skulberg, O.M., Carmichael, W.W., Codd, G.A., Skulberg, R., 1993. Taxonomy of toxic Cyanophyceae (cyanobacteria). In: Falconer R. (**ed**.). Algal toxins in seafood and drinking water. Academic Press Ltd., London. Pp 145-164.

Smayda, T.J., 1978. Estimating cell numbers. In: Sournia, A. (**ed**.), *Phytoplankton Manual*. United Nations Educational, Scientific and Cultural Organization, Paris, pp. 165–166.

Stewart, W.D.P., and Alexander, G., 1971. Phosphorus availability and nitrogenase activity in aquatic blue-green algae. **Freshwater Biology**. 1, 389-404.

Stewart, W.D.P., Daft, M.J., McCord, S., 1973. The occurrence of blue green algae and lytic bacteria at a waterworks in Scotland. **Water Treatment and Examination**. 22, 114-124.

The Water Wheel, 2004. New Hope for Troubled waters: The Hartbeespoort dam, Eutrophication management. January/February 2004 3(1):19

Tucker, S., and Pollard, P., 2004. Identification of Cyanophage Ma-LBP and Infection of the Cyanobacterium *Microcystis aeruginosa* from an Australian Subtropical Lake by the Virus. **Applied and Environmental Microbiology**. 71, 629-635.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa, A., Watanabe, M.F., Park, H.D., Chen, G.C., Yu, S.Z., 1996. Detection of microcystins, a blue green algal hepatotoxin, in drinking water sampled in Hauimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. **Carcinogenesis**. 17, 1317-1321.

Van Ginkel, C.E., 2003. A National Survey of the incidence of cyanobacterial blooms and toxin production in major impoundments. Internal Report No. N/0000/00/DEQ/0503. Resource Quality Services, Department of Water Affairs and Forestry. Pretoria.

Van Ginkel, C.E., 2002. Trophic Status Assessment, Executive Summary. Institute for Water Quality Studies, Department of Water Affairs and Forestry, Pretoria. June 2002.

Veal, D.A., Deere, D., Ferrari, B., Piper, J., Attfield, P.V., 2000. Fluorescence staining and flow cytometry for monitoring microbial cells. **Journal of Immunological Methods**. 243, 191–210.

Veldhuis, M.J.W., Kraay, G.W., Timmermans, K.R., 2001. Cell death in phytoplankton: correlation between changes in membrane permeability, photosynthetic activity, pigmentation and growth. **European Journal of Phycology.** 36: 167-177

Villain, S., Luo, Y., Hildreth, M.B., Brözel, V.S., 2006. Analysis of the Life Cycle of the Soil Saprophyte *Bacillus cereus* in Liquid Soil Extract and in Soil. **Applied and Environmental Microbiology**. 72, 4970-4977.

Vives-Rego, J., Lebaron, P., Nebe-von Caron G, 2000. Current and future applications of flow cytometry in aquatic microbiology. **FEMS Microbiology Reviews**. 24, 429 – 448.

Wintzingerode, von F., Rainey, F.A., Kroppenstedt, R.M., Stackebrandt, E., 1997. Identification of environmental strains of *Bacillus mycoides* by fatty acid analysis and specific 16S rDNA oligonucleotides probe. **FEMS Microbiology Ecology**. 24, 201-209.

Wakelin, S.A., Walter, M., Jasper, M., Stewart, A., 2002. Biological control of *Aphanomyces euteiches* root-rot of pea with spore-forming bacteria. **Australasian Plant pathology**. 31, 401-407.

Walker, H.L., Higginbotham, L.R., 2000. An aquatic bacterium that lyses cyanobacteria associated with off-flavor of channel catfish (*Ictalurus punctatus*). **Biological control**. 18, 71-78.

Wiedner, C., Visser, P.M., Fastner, J., Metcalf, J.S., Codd, G.A., Mur, L.R., 2003. Effects of Light on the Microcystin Content of *Microcystis* Strain PCC 7806. **Applied and Environmental Microbiology**. 69. 1475-1481.

Wilkinson, C.R., 1979. *Bdellovibrio*-Like Parasite of Cyanobacteria Symbiotic in Marine Sponges. **Archives in Microbiology**. 123, 101-103.

Wolfe, R.S., and Ensign, J.C., 1965. Lysis of bacterial cell walls by an enzyme isolated from a *Myxobacter*. **J Bacteriol**. 90, 395-402.

Wolfe, R.S., and Ensign, J.C., 1966. Characterisation of a small proteolytic enzyme which lyses bacterial cell walls. **J Bacteriol**. 91, 524-534.

Wolfe, R.S., Wingard, M., Matsueda, G., 1972. *Myxobacter* AL-1 Protease II: Specific Peptide Bond Cleavage on the Amino Side of Lysine. **J Bacteriol**. 112, 940-949.

World Health Organization (WHO), 1997. Report of the Working Group on Chemical Substances in Drinking Water, Geneva, 22-26 April 1997. Section 5.2, Microcystin-LR. World Health Organization, Geneva, 1pp.

World Health Organization (WHO), 1998. Guidelines for Drinking Water Quality. Second Edition, addendum to Volume 2, Health Criteria and Other Supporting Information, World Health Organization, Geneva, pp 95-110

World Health Organization (WHO), 1999. Toxic cyanobacteria in water, Chorus I, Bartram J, (**eds**), E&FN Spon, Routledge, London.

Wright, S.J.L., and Thompson, R.J., 1985. *Bacillus* volatiles antagonize cyanobacteria. **FEMS Microbiology Letters**. 30, 263-267.

Wright, S.J.L., Linton, C.J., Edwards, R.A., Drury, E., 1991. Iso-amyl alcohol (3-methyl-1-butanol), a volatile anticyanobacterial and phytotoxic product of some *Bacillus* spp. **Lett Appl. Microbiol**. 13, 130-132.

Yair, S., Yaacov, D., Susan, K., Jurkevitch, E., 2003. Small eats big: ecology and diversity of *Bdellovibrio* and like organism, and their dynamics in predator-prey interactions. **Agronomie**. 23, 433-439.

Yamaguchi, N., and Nasu, M., 1997. Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment. **Journal of Applied Microbiology.** 83, 43-52.

Yamamoto, Y., Kouchiwa, T., Hodoki, Y., Hotta, K., Uchida, H., Harada, K., 1998. Distribution and identification of actinomycetes lysing cyanobacteria in a eutrophic lake. **J. Appl. Phycol**. 10, 391-397.

Yoshizawa, S., Matsushima, R., Watanabe, M.F., Harada, K., Ichihara, A., Carmichael, W.W., Fujiki, H., 1990. Inhibition of protein phosphatases by microcystins and nodularin associated with hepatoxicity. **J. Cancer Res. Clin. Oncol.** 116, 609-614.

Zohary, T., Breen, C.M., 1989. Environmental factors favouring the formation of *Microcystis aeruginosa* hyperscums in a hypertrophic lake. **Hydrobiologia.** 178, 179-192.

Zohary, T., 1987. On the ecology of hyperscum-forming *Microcystis aeruginosa* in a hypertrophic African lake. Unpublished PhD Thesis, University of Natal, Pietermaritzburg.

Zubkov, M.V., Fuchs, B.M., Eilers, H., Burkill, P.H., Amann, R., 1999. Determination of total protein content of bacterial cells by SYPRO staining and flow cytometry. **Applied Environmental Microbiology**. 65, 3251-3257.