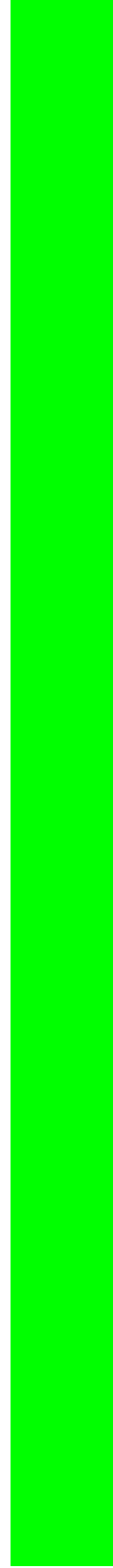# Chapter 2: Literature Review

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).
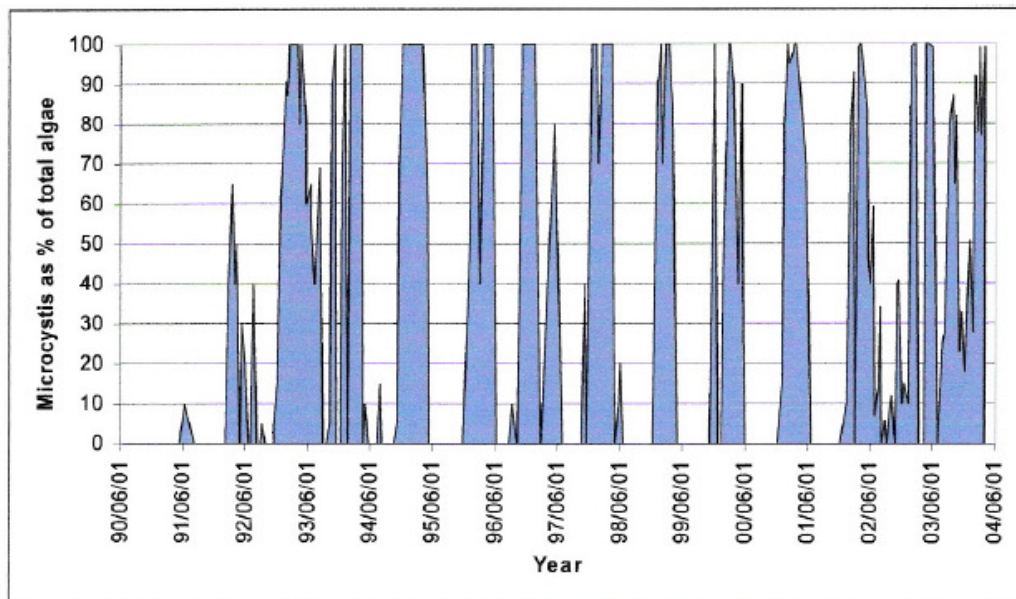

Figure 2.1: Occurrence of *Microcystis* in Hartbeespoort Dam from 1990 to 2004 (Harding et al., 2004).

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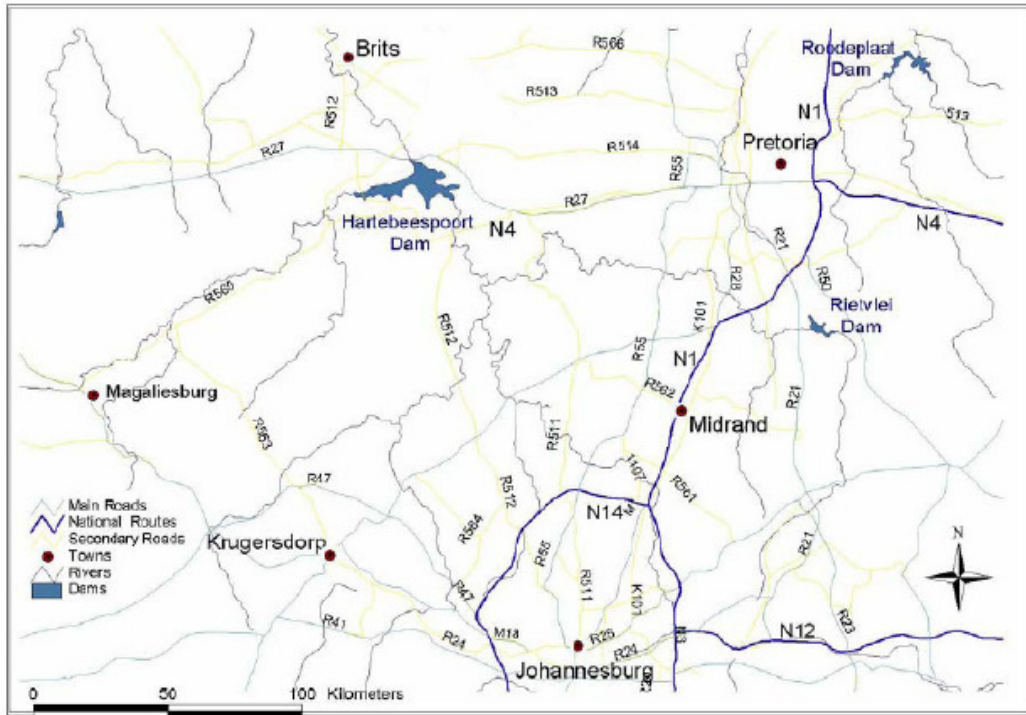
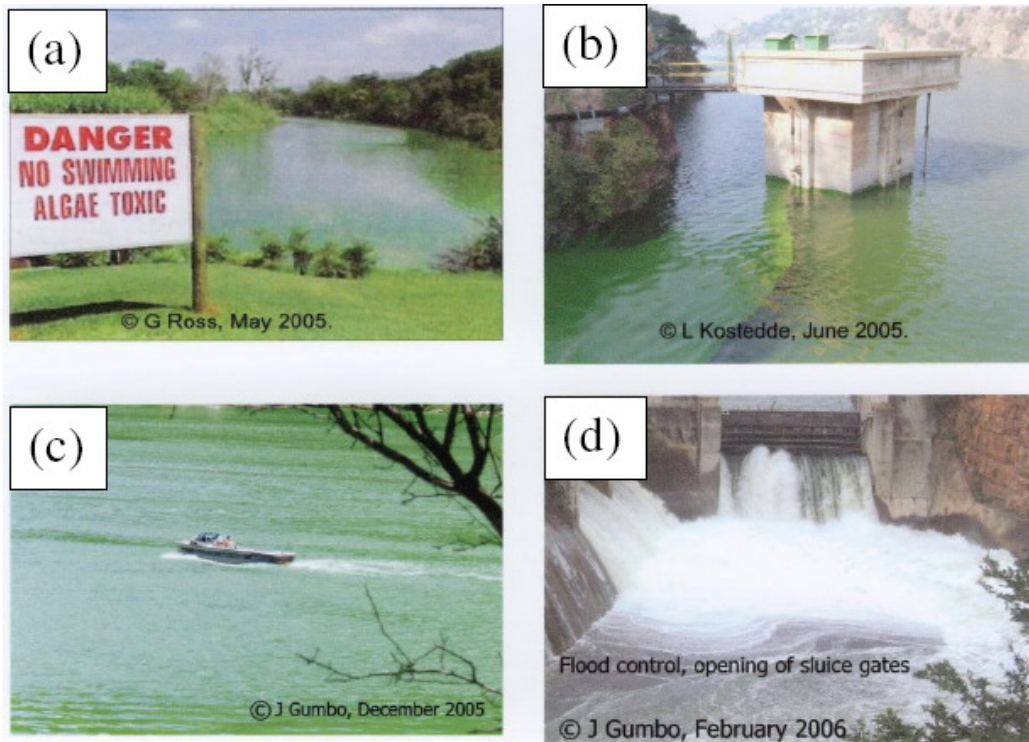Figure 2.2: Location of Hartbeespoort dam (Harding et al., 2004).

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
forma aeruginosa	Irregular, net shaped and or ellipsoidal	(1987)

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 (μEm^{-2}s^{-1})	Minimum nutrient levels			Water Column	Low Wind speeds ms^{-1}	Water Temp. (ºC)
		$NO_2^{2-}N+ NO_3^{2-}$ μg ℓ^{-1}	SRP μg ℓ^{-1}	NH_4^+N μg ℓ^{-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 μEm^{-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.
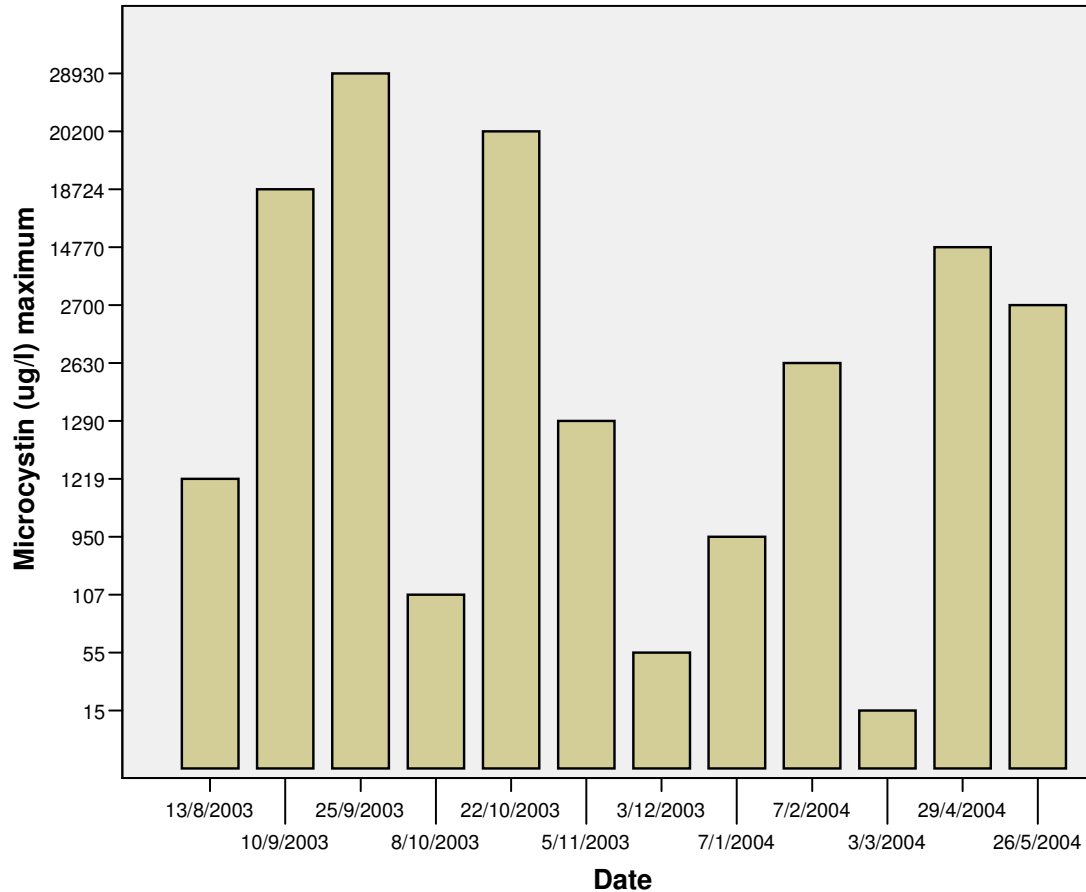
Figure 2.4: Maximum microcystin levels in raw water analysis for Hartbeespoort dam (Harding et al., 2004). The WHO microcystin guideline value is 1.0 µg ℓ^{-1}

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.

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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 ($\mu g/\ell$)	Maximum toxin in treated water ($\mu g/\ell$)	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	$1:6 \times 10^7$	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 1×10^7 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°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).
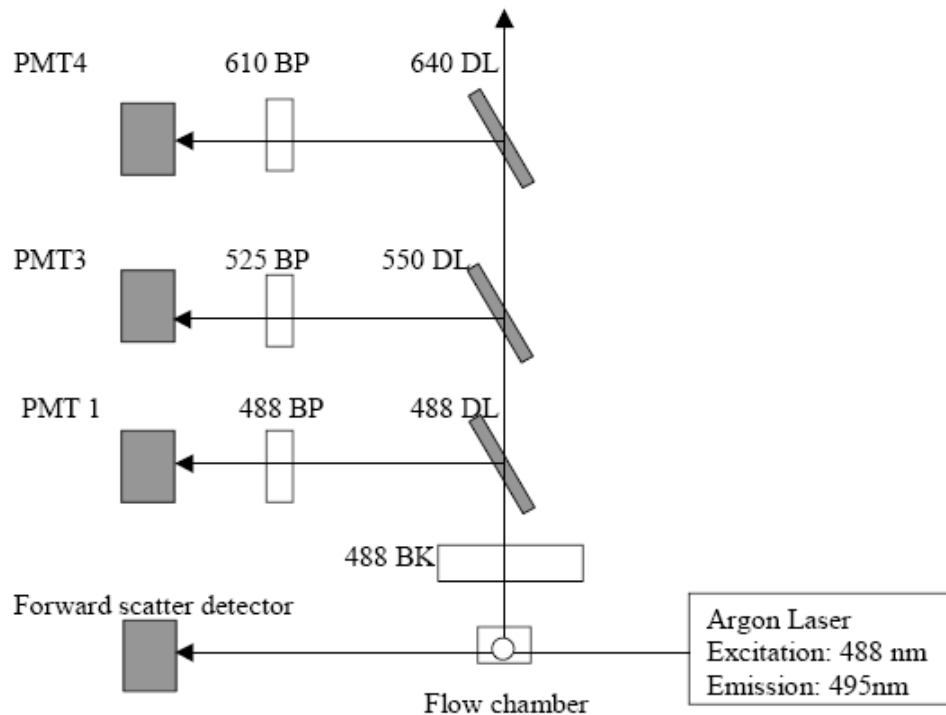

Figure 2.5: Schematic optical arrangement of the Beckmann Coulter Epics Alter® flow cytometer. The simultaneous measurement of forward scatter, side scatter, FDA fluorescence and PI fluorescence. DL=dichroic long pass filter, lets all wavelengths longer than specified through. BP= band pass filter, narrows 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).
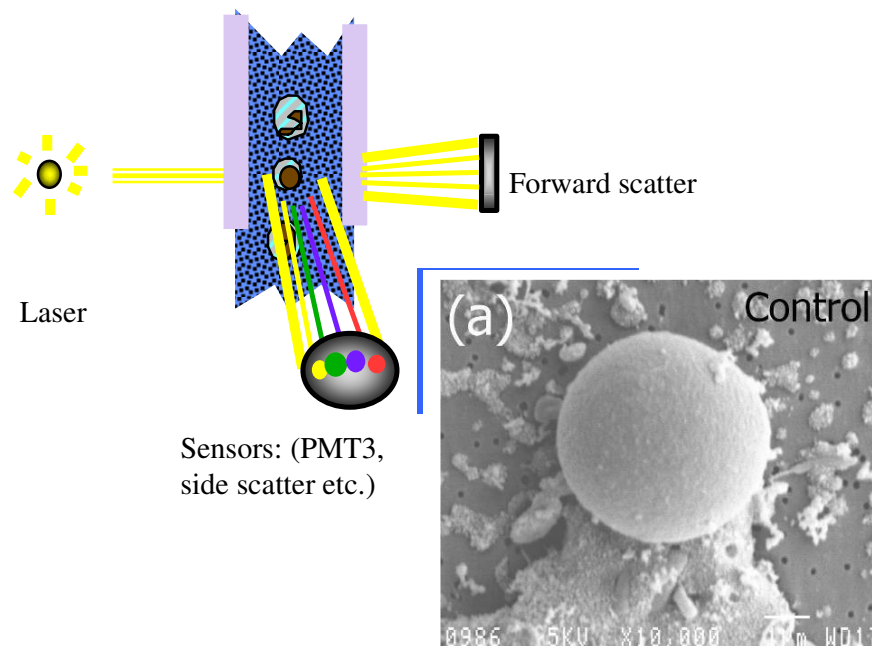

Figure 2.6: Forward and side scatter approximation (Murphy, 1996).

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° 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).
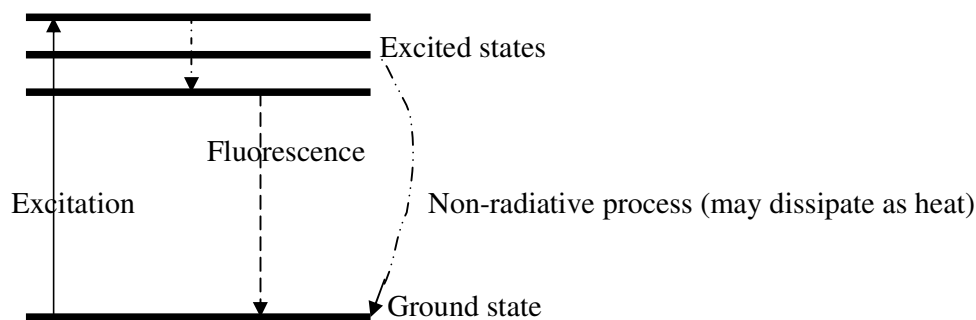

Figure 2.7: The absorption and emission of light during fluorescence

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).
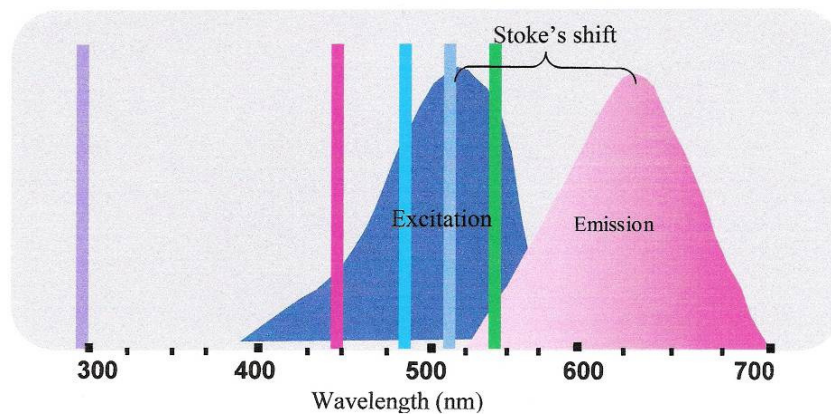

Figure 2.8: The absorption wavelength of propidium iodide (PI) is at 535 nm (Murphy, 1996). The the closest excitation wavelength is at 488 nm with an argon laser. The PI fluorescence emission (when electrons return to ground state) results in a longer wavelength at 617 nm. The difference in wavelengths (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.
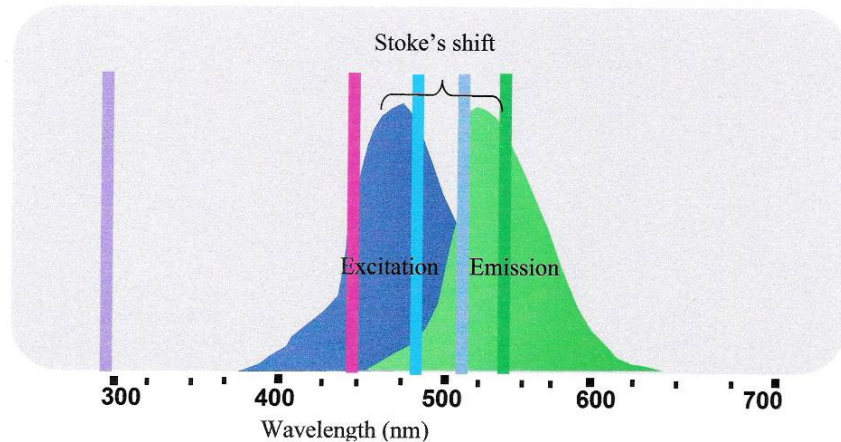
Figure 2.9: The absorption wavelength of fluorescein fluorescence is at 473 nm (Murphy, 1996). The emission is at 514 nm. FDA can both be efficiently excited by the same light source (e.g. an argon ion laser tuned to 488 nm). The fluorescein fluorescence emission (when electrons return to ground state) 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).
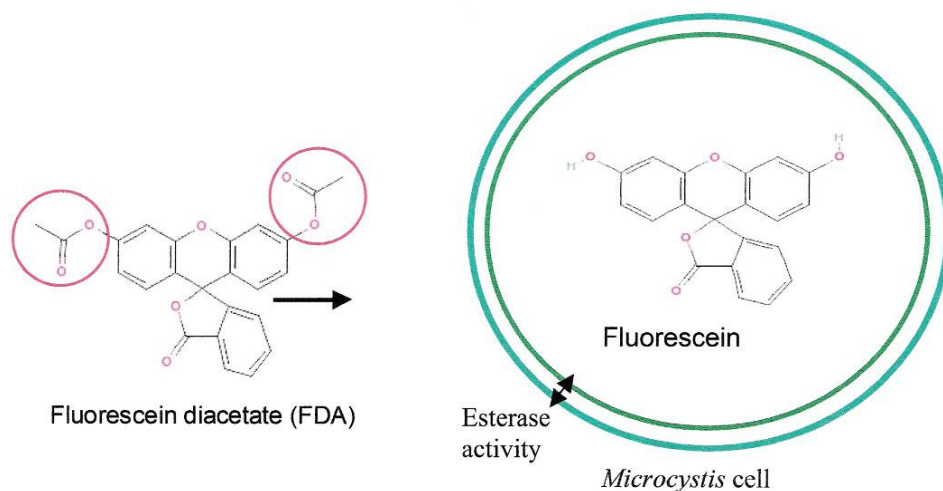
Esterases are present in all living organisms and these enzymes can provide useful information on the status of bacterial metabolism including that of cyanobacteria. The synthesis of enzymes is energy dependent but enzyme-substrate reaction is energy independent (Joux and Lebaron, 2000). Dead or dying 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.

Fluorescein diacetate (FDA)

Esterase activity

Fluorescein

Microcystis cell

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 x 10^9 to 8.6 x 10^9 daltons. Ribosomes are widely distributed
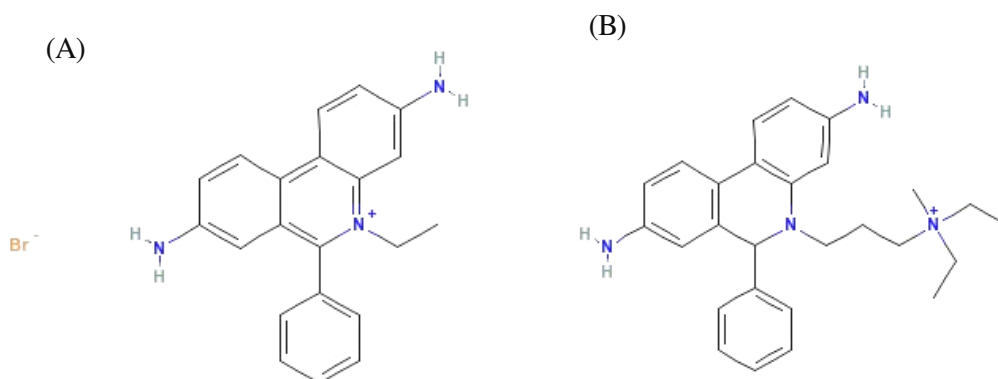
throughout the cytoplasm and are concentrated in the nucleoplasmic region (Fray, 1993).

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).

(A)

(B)

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.