Chapter 6: Conclusions and Perspectives

CHAPTER 6

CONCLUSIONS AND PERSPECTIVES

6.1. Isolation of predatory bacteria and its identification

6.1.1. Isolation and identification of predator bacteria

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

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

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

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

6.1.2. A simple predator prey model and ratio

According to the simple Lotka-Volterra predator-prey model, the prey population (*Microcystis*) increases whilst the predator population (*B. mycoides* B16) 'follows, overtakes and overcomes it' thus, producing a decline in the prey population (Atlas and Bartha, 1998:88). This model does fit into the predator-prey ratio results but to certain degree as governed by nutrient limitation for the prey (batch cultures). The predator prey relationship was expected to be an exponential relationship i.e., a decrease in predator bacteria may result in an increase in prey population or an increase in predator bacteria may result in a decrease in prey population. A high predator bacteria population, 5.2×10^7 cfu per mℓ, was a prerequisite for the effective lysis of *Microcystis*, which corresponded to a 1:1 ratio with a 3 d lag phase. There are a number of explanations as to what may be happening during the lag phase. Presumably during the 'lag phase' the predator bacteria population was adjusting to the new environment and increasing in numbers before initiating cyanobacterial lysis. Fraleigh and Burnham (1988) observed that the length of the lag phase was inversely proportional to population of predator bacteria, i.e., low population of predators resulted in a longer lag phase. Shilo (1970) and Daft and Stewart (1971) pointed out that agitation of samples may disrupt or disturb the physical contact process between the cyanobacteria and bacteria thus may have caused a delay in the lytic process. May be the *Microcystis* adopted a defensive mechanism to ward off the predators by releasing microcystins. Choi et al. (2005) speculated that microcystins inhibit growth of organisms such as cladocerans, copepods, and mosquito larvae and have been shown to be allelopathetic towards green alga, *Chlamydomonas neglecta.* However, there are no published reports about microcystin toxicity with regards to bacteria (Choi et al., 2005). It is therefore speculated that a combination of initial low predator population and agitation of culture suspensions may be the main reasons for the delay in the lytic process.

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

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

6.1.3. Adaptation of predator bacteria to different environments

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

Separate fluorescent staining was initially used as a result of the FDA fluorescence signal that interfered with the PI fluorescence signal due to overlap. The flow cytometric analysis in conjunction with separate fluorescence of *Microcystis* cells revealed two different cell populations: alive and dead cells. Under static conditions, i.e., daily hand shaking before sampling, the FCM counts revealed that during the first two days, the treated and control samples showed a 49% increase (a positive t value of 0.59) in alive *Microcystis* cell numbers. The independent Levene t-test analysis of the alive *Microcystis* cell numbers showed no significant difference ($p > 0.05$). Thus, the bacteria that were added had no effect on the growth of cyanobacteria. As indicated above, it was suggested that the bacteria was adjusting

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

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

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

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

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

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

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

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

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

6.3. Future research

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

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

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

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

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

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

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

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