

Review

***Microcystis aeruginosa*: source of toxic microcystins in drinking water**

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Cyanobacteria are one of the earth's most ancient life forms. Evidence of their existence on earth, derived from fossil records, encompasses a period of some 3.5 billion years in the late Precambrian era. Cyanobacteria are the dominant phytoplankton group in eutrophic freshwater bodies worldwide. They have caused animal poisoning in many parts of the world and may present risks to human health through drinking and recreational activity. Cyanobacteria produce two main groups of toxin namely neurotoxins and peptide hepatotoxins. They were first characterized from the unicellular species, *Microcystis aeruginosa*, which is the most common toxic cyanobacterium in eutrophic freshwater. The association of environmental parameters with cyanobacterial blooms and the toxicity of microcystin are discussed. Also, the synthesis of the microcystins, as well as the mode of action, control and analysis methods for quantitation of the toxin is reviewed.

Key words: Cyanobacteria, microcystins, *mcyB* gene, PCR-RFLP.

INTRODUCTION

Cyanobacteria are the dominant phytoplankton group in eutrophic freshwater bodies (Davidson, 1959; Negri et al., 1995). They are prokaryotes possessing a cell wall composed of peptidoglycon and lipopolysaccharide layers instead of the cellulose of green algae (Skulberg et al., 1993). All Cyanobacteria are photosynthetic and possess chl_a. Morphological diversity ranges from unicells; to small colonies of cells to simple and branched filamentous forms (Weier et al., 1982).

The cytoplasm contains many ribosomes and appears granular. In filamentous forms, fine plasmodesmata connect adjacent cells. The plasmalemma may form invaginations but in addition, there are a series of parallel membranes within the cytoplasm that are separate from the plasmalemma. The process of photosynthesis occurs on these membranes, which contain chl_a and a few

other accessory pigments are grouped together in rods and discs that are called phycobilisomes that are attached to the outside of the membranes (Weier et al., 1982). These pigments capture light of wavelengths 550 to 650 nm, and pass their light energy on to chl_a.

Other cytoplasmic inclusions are gas vesicles, granules of glycogen, lipid droplets, granules of arginine and aspartic acid polymers and polyhedral carboxysomes. Gas vesicles are especially prominent in floating aquatic species and it is likely that they contribute to buoyancy. The nucleoplasm is sharply delimited from the cytoplasm, even though there is no nuclear membrane as in bacterial cells and is composed of a circular, double-stranded molecule of DNA. Cell volume ranges from 5 to 50 μm³, in contrast to 0.01 to 5 μm³ for bacteria. They have about twice as much DNA as does *E. coli*, with one chromosome (Weier et al., 1982).

About one third of all cyanobacteria species are able to fix nitrogen. In most of the cases, nitrogen fixation occurs in specialized cells called heterocysts. These are enlarged cells with an envelope. The internal membranes

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Figure 1. Cyanobacterial bloom visible as green scum on the water of the Hartbeespoort Dam, South Africa (December 2002).

no longer lie in parallel arrays, and these cells may have lost photosystem II, hence do not generate O₂. A plasmodesmata connect the heterocysts to adjacent cells within a filament. It is possible that the thick wall maintain an anaerobic condition in the cytoplasm (Weier et al., 1982).

Cyanobacteria are especially abundant in shallow, warm, nutrient rich or polluted water low in oxygen and can grow to form thick scums that could colour the water, creating blooms (Figure 1) (Stotts et al., 1993). Most blooms disappear in a few days, but the cells can release toxins lethal to animals and humans that swim in or drink the water (Weier et al., 1982).

ASSOCIATION OF ENVIRONMENTAL PARAMETERS WITH CYANOBACTERIAL BLOOMS AND TOXICITY OF MICROCYSTIN

Field studies in South Africa (Wicks and Thiel, 1990) have shown that certain environmental factors are associated with the amount of toxins found in cyanobacterial blooms. The effects of environmental factors on toxin production by cyanobacteria have also been shown by laboratory studies (Sivonen, 1990; Utkilen and Gjolme, 1992).

PHYSICAL FACTORS

Temperature

In general, cyanobacteria prefer warm conditions, and low temperatures are one of the major factors that ends cyanobacterial blooms. Robarts and Zohary (1987) found that *Microcystis* was severely limited at temperatures below 15°C and were optimal at temperatures around

25°C. Temperature alone may only partly determine bloom formation and it is accepted that a combination of factors are responsible for a bloom to develop. These are increasing temperatures, decreasing nutrients and increased water column stability. This also explains why succession of algae usually follow typically patterns in freshwater bodies from diatoms through chlorophytes to cyanobacteria.

Van der Westhuizen and Eloff (1985) determined that temperature has the most pronounced effect on toxicity. The highest growth rate in laboratory cultures was obtained at 32°C, while the highest toxicity was found at 20°C, but reduced at temperatures in excess of 28°C. At temperatures of 32 °C and 36°C cell cultures toxicity was 1.6 and 4 times less than cells cultured at 28°C, suggesting that highest growth rate is not correlated with highest toxicity. Van der Westhuizen and Eloff (1985) considered the decreased toxin production to be possibly related to decreased stress levels at temperatures above 20°C.

Temperature changes were found to induce variations in both the concentration and peptide composition of the toxin (Yokoyama and Park, 2003). Of the 3 toxic peptides, C was discovered at a higher concentration than either peptides A or B at 16°C. The percentage content of peptide A increased between 16° C and 36° C, while overall toxicity decreased sharply. This being due to a decrease in the concentration of peptides A and B. Peptide C disappeared gradually at higher temperatures as a consequence of reduced synthesis or increased decomposition, rather than leaching, since cells were still growing after the growth phase (Van der Westhuizen and Eloff, 1985).

Light and buoyancy

The effect of light intensity on the fine structure of *M. aeruginosa* under laboratory conditions has been investigated. The optimal growth rate for *M. aeruginosa* cells was at 3 600-18 000 lux (Abelovich and Shilo, 1972). The lag phases lasted approximately 5 days, followed by an 11-day period of exponential growth. At light levels in the excess of 18 000 lux the growth rate declined rapidly. Pigment ratios and visual pigmentation were found to change considerably at different light intensities. At 3 600 lux and lower, cultures were green for the duration of the experiment period of 28 days. At 5700 lux, cultures were yellow, and at 18 000 lux they were orange. The ratio of chl_a to carotenoids, plotted against light intensity showed that as light intensity increased, carotenoid pigments increased relative to chl_a. A reduction in this ratio occurred with ageing. Carotenoid pigments shield cells from high light intensity, preventing the destruction of chl_a and the photo-oxidation of photosynthetic pigments (Abelovich and Shilo, 1972). In a recent publication the quality of light i.e. 16 μmol photons/ms in the red light spectrum, increase toxin

production in a *M. aeruginosa* strain (Kaebnick et al., 2000).

It was also found in laboratory cultures that the effect of light intensity affected the gasvacuole content and thylakoid configuration. The gasvacuole content increased as light intensity increased to 6 000 lux, thereafter decreasing between 6 000 and 8 000 lux (Waaland et al., 1971), suggesting that the vesicles could act as light shields in addition to their possible buoyancy functions. The absence of gasvacuoles at low light intensities of 400 lux supported this observation.

Buoyancy is regulated by a number of mechanisms, such as the form of stored carbohydrates and turgor pressure regulation. Compositional changes in the protein:carbohydrate ratios during buoyancy reversals suggest a complex relationship between light and nutrients (N:P) (Villareal and Carpenter, 2003). It however, seems that the regulation of gasvacuole synthesis is the most important. This almost unique feature of cyanobacteria gives these organisms a significant advantage over other phytoplankton. In turbulent waters cyanobacteria lose this advantage and often this characteristic is used to control their blooms (Grobbelaar, 2002).

CHEMICAL FACTORS

Nitrogen and phosphorus ratios

Much has been made of the relationship between prevailing ratios of nitrogen and phosphorus and the composition and density of phytoplankton assemblages that may occur. While certain broad categories generally and accurately support prediction of which algal division that may predominate, other biophysical features and attributes should not be excluded from the equation. It is becoming increasingly apparent that, notwithstanding the prevailing nitrogen and phosphorus ratio, the phytoplankton assemblage may be significantly altered through biomanipulation, and without any changes whatsoever to the ambient availability of nitrogen and phosphorus (Harding and Wright, 1999). In 1986, Carmichael demonstrated that the omission of nitrogen causes approximately tenfold decrease in toxicity.

Iron and zinc

Certain metal ions such as Zn^{2+} and Fe^{2+} significantly influence toxin yield. Zn^{2+} is involved in the hydrolysis of phosphate esters, the replication and transcription of nucleic acids, and the hydration and dehydration of CO_2 (Sunda, 1991). All cyanobacteria require Fe^{2+} for important physiological functions such as photosynthesis, nitrogen assimilation, respiration and chlorophyll synthesis (Boyer et al., 1987). It is not yet clear how Fe^{2+}

deficiency modulates microcystin production, but it has been noted that as cyanobacteria experiences iron stress, they appear to compensate for some of the effects of iron loss by synthesizing new polypeptides (Lukač and Aegerter, 1993).

THE TOXICOLOGY OF MICROCYSTINS IN CYANOBACTERIA

Cyanobacteria are capable of producing two kinds of toxin, the cyclic peptide hepatotoxin and the alkaloid neurotoxin. Serious illness such as hepatoenteritis, a symptomatic pneumonia and dermatitis may result from consumption of, or contact with water contaminated with toxin producing cyanobacteria (Hawkins et al., 1985; Turner et al., 1990). The neurotoxins include anatoxin-a, a depolarizing neuromuscular blocking agent; anatoxin-a [s], an anti-cholinesterase; and saxitoxin and neosaxitoxin that inhibit nerve conduction by blocking sodium channels (Carmichael, 1994).

Microcystins are a family of toxins produced by different species of freshwater Cyanobacteria, namely *Microcystis* [order Chroococcales], *Anabaena* [order Nostocales], and *Oscillatoria* [order Oscillatoriales]. The microcystin are monocyclic heptapeptides composed of D-alanine at position 1, two variable L-amino acids at positions 2 and 4, γ -linked D-glutamic acid at position 6, and 3 unusual amino acids; β -linked D-erythro- β -methylaspartic acid (MeAsp) at position 3; (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8 trimethyl-10-phenyldeca-4, 6-dienoic acid (Adda) at position 5 and N-methyl dehydroalanine (MDha) at position 7. There are over 50 different microcystins that differ primarily in the two L-amino acids at positions 2 and 4, and methylation/demethylation on MeAsp and MDha. The unusual amino acid Adda is essential for the expression of biological activity. Other microcystins are characterized largely by variation in the degree of methylation; amino acid 3 has been found to be D-aspartic acid, replacing β -methylaspartic acid and amino acid 7 to be dehydroalanine, replacing N-methyldehydroalanine (An and Carmichael, 1994; Trogen et al., 1996). The most common microcystin, is microcystin-LR, where the variable L-amino acids are leucine (L) and arginine (R). Its structure is shown in Figure 2 (An and Carmichael, 1994). Some esters of glutamic acid have been observed for amino acid 6 replacing γ -linked glutamic acid itself and N-methylserine sometimes replaces amino acid 7. Variations in the Adda subunit (amino acid 5) include 0-acetyl-0-demethyl-Adda and (6Z)-Adda (Rinehart et al., 1988).

The adda and D-glutamic acid portions of the microcystin-LR molecule play highly important roles in the hepatotoxicity of microcystins. Esterification of the free carboxyl group of glutamic acid results essentially in inactive compounds. Some of the Adda subunits assert little effect, especially the 0-dimethyl-0-acetyl analogs.

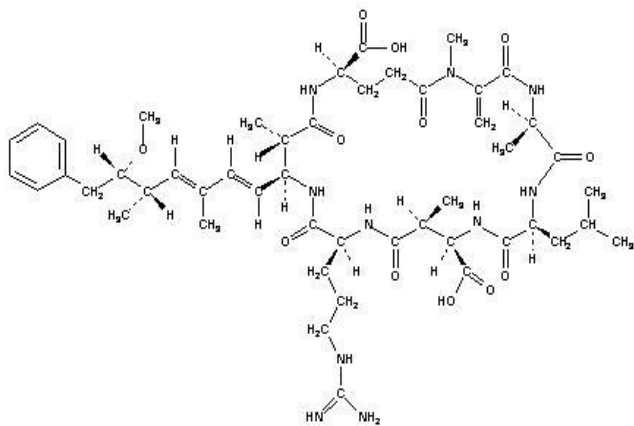


Figure 2. Chemical structure of microcystin-LR (An and Carmichael, 1994).

However, the Adda molecule's overall shape seems to be critical since the (6Z0-Adda)(*cis*) isomer is inactive (Rinehart et al., 1988).

SYNTHESIS OF MICROCYSTINS

As stated before, *M. aeruginosa* is an organism that produces a vast number of peptides, some of which are highly toxic (Carmichael, 1986). The most commonly occurring toxin is microcystin and to synthesize this complex peptide there obviously has to be genetic material present in the organism. Different possible localities of this genetic material have been investigated.

Chloroplast DNA

Shi and coworkers (1995) localized microcystins in a toxin-producing strain [PCC 7820] and non-toxin-producing strain [UTEX 2063] of *Microcystis aeruginosa* by using a polyclonal antibody against microcystins in conjunction with immuno-gold labelling. In the non-toxin-producing strain no specific labeling was found. In the toxin-producing strain specific labelling occurred in the region of the nucleoid, in the thylakoid and to a lesser extent in the cell wall and sheath area. No specific labelling was found in cellular inclusions with storage functions. The reasons for this could not be determined, but Shi and coworkers (1995) suggested that microcystins are not compounds that the cell stores, but that they may be involved in specific cell activities.

Plasmids

Vakeria and coworkers (1985) investigated genetic control of toxin production by plasmids commonly found in some strains of *M. aeruginosa*. Plasmid-curing agents were applied to toxin-producing strains, but no significant decrease in toxicity were observed. Schwabe and coworkers (1988) also supported this argument that

toxin-producing strains do not contain plasmids. Apart from the reports of Vakeria and coworkers (1985) evidence has been presented of a South African strain [WR 70] that shows a decrease in toxicity after treatment with plasmid-curing agents (Hauman, 1982).

Thiotemplate Mechanism

Lipmann (1954) predicted a poly- or multienzymatic pathway of peptide synthesis and this mechanism has been verified for various types of peptides (Laland and Zimmer, 1973). The first authors to propose the term thiotemplate mechanism and to distinguish this mechanism from other mechanisms of non-ribosomal peptide synthesis were Laland and Zimmer (1973). Many similarities are apparent when comparing ribosome-mediated protein synthesis with the thiotemplate mechanism. The most notable similarities are [1] the amino acids are activated through the formation of an amino acid adenylate; and [2] the activated amino acyl residue is transferred to a receptor molecule and the peptide chain grows from the N-terminal end by insertion of the next amino acid at the activated C-terminal.

TOXICITY

There have been many reports of the intoxication of birds, fish and other animals by cyanobacteria toxins (Vasconceles et al., 2001; Alonso-Andicoberry et al., 2002; Best et al., 2002; Romanowska-Duda et al., 2002; Krienitz et al., 2003). As stated before, blooms of cyanobacteria usually follow enrichment by nutrients such as phosphates and nitrates in the water. Most of these nutrients are derived from human wastes such as sewage and detergents, industrial pollution, run-off of fertilizers from agricultural land, and the input of animal or bird wastes from intensive farming (Bell and Codd, 1994; Baker, 2002). Illnesses caused by cyanobacterial toxins to humans fall into three categories; gastroenteritis and related diseases, allergic and irritation reaction, and liver diseases (Bell and Codd, 1994). Microcystins have also been implicated as tumour-promoting substances (An and Carmichael, 1994; Bell and Codd, 1994; Rudolph-Böhner et al., 1994; Trogen et al., 1996; Zegura et al., 2003).

The LD₅₀ of microcystin-LR i.p or i.v. in mice and rats is in the range 36-122 µg/kg, while the inhalation toxicity in mice is similar; LCT₅₀=180 mg/min/m³ or LD₅₀=43 µg/kg (Stoner et al., 1991). Therefore microcystin-LR has comparable toxicity to chemical organophosphate nerve agents. Symptoms associated with microcystin intoxication are diarrhea, vomiting, piloerection, weakness and pallor (Bell and Codd, 1994). Microcystin targets the liver, causing cytoskeletal damage, necrosis and pooling of blood in the liver, with a consequent large increase in liver weight. Membrane blebbing and blistering of hepatocytes *in vitro* has been observed

(Runnegar et al., 1991; Romanowska-Duda et al., 2002). High chromatin condensation and apoptotic bodies were observed in 90% of the cells of *Sirodela oligorrhizza* and rat hepatocytes after a treatment with microcystin-LR (MC-LR=500µg/dm) (Romanowska-Duda et al., 2002). Death appears to be the result of haemorrhagic shock (Hermansky et al., 1990) and can occur within a few hours after a high dose of microcystin-LR (Falconer et al., 1981; Bell and Codd, 1994). The concentration of microcystin-LR in drinking water for humans as prescribed by the world health organization (WHO) is 1 µg/L (WHO, 1998), however, Ueno and coworkers (1996) proposed a value of 0.01 µg/L, based on a possible correlation of primary liver cancer in certain areas of China with the presence of microcystins in water of ponds, rivers and shallow wells.

Mechanism of action of microcystins

It is known that microcystins mediate their toxicity by uptake into hepatocytes *via* a carrier-mediated transport system, followed by the inhibition of serine protein phosphatases 1 and 2A. The protein phosphorylation imbalance causes disruption of the liver cytoskeleton, which leads to massive hepatic haemorrhage that can cause death (Honkanen et al., 1990; Eriksson et al., 1990a, b; Romanowska-Duda et al., 2002). The entry of toxin into the hepatocytes of the liver and other targeted tissues is accomplished by the broad specificity anion transport bile acid carrier (Runnegar et al., 1991). In both cultured and *in vitro* hepatocytes, a rise in the amount of phosphorylated protein as a consequence of phosphatase inhibition was observed (Yoshizawa et al., 1990). The action of microcystin as a phosphatase inhibitor is not limited to mammalian cells, but also applies to plant phosphatases (MacKintosh et al., 1990; Siegl et al., 1990). It is, therefore, likely that the microcystins are general inhibitors of eukaryotic phosphatases of types 1 and 2A, limited only by the ability of the toxins to enter cells.

Phosphatase inhibition

The National Cancer Center Research Institute, Tokyo did discover the potency of microcystin-LR as an inhibitor of protein phosphatases types 1 and 2A (Yoshizawa et al., 1990; Matsushima et al., 1990) and this was also confirmed in other studies (MacKintosh et al., 1990; Honkanen et al., 1990; Eriksson et al., 1990a, b). The toxin-phosphatase interaction is extremely strong, and binding is essentially stoichiometric. Constant accurate inhibition can, therefore, only be obtained by extrapolation of the phosphatase concentration to zero. The value of k_i for protein phosphatase types 1 and 2A has been reported to be between 0.06–6 nM and <0.01-2

nM respectively, with microcystin-LR showing up to a 40 fold higher affinity of microcystin-LR for protein phosphatase type 2B. This is at least 1 000 fold lower than that for phosphatase type 1, while no interaction of microcystin-LR was observed with protein phosphatase type 2C or with a variety of other phosphatase or protein kinases (MacKintosh et al., 1990; Honkanen et al., 1990; Suganuma et al., 1992).

The correlation between inhibition of phosphatase activity and toxicity is indicated by the results of Runnegar and coworkers (1993), who administered microcystin-YM or LR to mice and observed that inhibition of liver protein phosphatase 1 and 2A activity preceded or accompanied clinical changes due to microcystin intoxication in all cases. Inhibition of protein phosphatases leads to phosphorylation of cytoskeletal protein and cytoskeletal associated protein and consequent redistribution of these proteins. Ghosh and coworkers (1995) showed that the collapse of cytoskeletal actin microfilaments occurs in rat hepatocytes prior to the dislocation of the associated proteins, α -actinin and talin rather than being caused by their dislocation.

Other effects of microcystins

Hermansky and coworkers (1991) observed a decrease in hepatic microsomal membrane fluidity, when they administered mice with microcystin-LR. These changes involve an indirect and secondary effect of the toxin, as no changes in membrane fluidity were observed when microcystin was incubated with control microsomes *in vitro*.

LeClaire and coworkers (1995) suggested a potential cardiogenic component in the pathogenesis of shock, in addition to the effects on the liver. The authors observed a sustained, rapid decline in cardiac output and stroke volume in rats intoxicated with microcystin-LR. The acute hypotension was responsive to volume expansion with the whole blood, and the acute drop in heart rate responded to both isoproterenol and dopamine. A peripheral vasoconstriction appeared to occur in response to hypotension.

CONTROL AND DEGRADATION OF CYANOBACTERIAL BLOOMS

Cousins and coworkers (1996) found in laboratory experiments with reservoir water using low levels of microcystin-LR [10mg/L], that degradation of the toxin occurred in less than one week. The toxin was stable for over 27 days in deionized water, and over 12 days in sterilized reservoir water, indicating that in normal reservoir water instability is due to biodegradation. Purified microcystins are also stable under irradiation by

sunlight. However, significant decomposition of toxins by isomerization of a double bond in the Adda-side chain, occurs during sunlight irradiation in the presence of the pigments contained in cyanobacteria, the half-life for the whole process was about ten days. Microcystin-LR and RR degraded much more rapidly when the toxins were exposed to UV light at wavelengths around their absorption maxima [238-254nm] (Tsuji et al., 1995).

It was found by Lam and coworkers (1995) that most of the microcystin-LR present in cells remains inside the cell until the cell is lysed. To control cyanobacteria blooms, cells are usually lysed in the presence of chemicals [e. g. Reglone A, NaOCl, KMnO₄, Simazine and CuSO₄] that inhibit new cell wall synthesis, enzymatic reactions or photosynthesis (Kenefick et al., 1993, Lam et al., 1995). A sudden release of microcystins into the surrounding waterbody can present a hazard to animals and humans using the water (Lam et al., 1995), especially when used as potable water source.

Chemical control

Verhoeven and Eloff (1979) reported that copper is an effective algicide in natural waters for the control of cyanobacteria. *M. aeruginosa* isolated from the Hartbeespoort Dam [UV-006] as well as *M. aeruginosa* Berkeley strain 7005 [UV-007] were used to test the effects of copper on the ultrastructure of cells. Once cultures had been grown, copper sulphate was added at different concentrations. It was found that toxicity of the copper depends on cell concentration. At cell concentrations of 1.8x10⁶ cells/ml [148 Klett units], 0.3 and 0.4 ppm Cu²⁺ decreased growth rates temporarily, whereas 0.5 ppm Cu²⁺ cause cell death. It was found that copper decreases the electron-density of the nucleoplasm, as well as cause aggregation of the DNA fibrils. Thylakoids were present as short membrane structures and membrane-bounded inclusions, while polyphosphate bodies disappeared.

Hoeger and coworkers (2002) tested the efficacy of ozonation coupled with various filtration steps to remove toxic cyanobacteria from raw water. They found that ozone concentrations of at least 1.5 mg/L were required to provide enough oxidation potential to destroy the toxin present in 5 x 10⁵ *M. aeruginosa* cells/ml (total organic carbon (TOC), 1.56 mg/L). Raw water with high cyanobacterial cell densities reduces the efficiency of the process, resulting in cell lysis and the liberation of intracellular toxins.

Biological control

Microcystins can be biodegraded by complex natural populations of micro-organisms from diverse ecosystems, such as sewage sludge (Lam et al., 1995), lake sediment,

natural waters (Jones and Orr, 1994; Jones, 1990) and biofilms (Saitou et al., 2002). Jones (1990) demonstrated that microcystins extracted from *M. aeruginosa* blooms are biodegraded in natural water bodies within 2-3 weeks. This time is reduced to a few days if the water body has been previously exposed to microcystins.

Scott and Chutter (1981) suggest that viruses may be an important factor in controlling cyanobacteria. The first virus that was capable of lysing a filamentous cyanobacteria *Plectonema* sp. was isolated from an oxidation pond. It was assumed by the authors that viruses were not important in controlling eukaryotic algae in large cultures, this on the basis of there being no apparent evidence to the contrary (e.g. reviews by Lemke, 1976; Hoffman and Stanker, 1976; Dodds, 1979). Recently it was demonstrated that aqueous and methanolic extracts of cultured cyanobacteria of several genera, including *Microcystis*, expressed antiviral activity against the influenza virus (Zainuddin et al., 2002).

A myxobacterium capable of lysing freshwater algae was first reported by Stewart and Brown (1969, 1971). Scott and Chutter (1981) suggest that myxobacteria are a more important biological agent than viruses in controlling algae populations, since they are less host specific. Pioneering work was conducted by Canter (1950, 1951, 1957) on fungal parasites of freshwater algae in the English Lake District. Up to 70% of the individuals in an algae population could be infected by fungal parasites. A large proportion of fungal parasites were found to be host-specific, suggesting that in some cases, they may prevent cyanobacteria species from growing while allowing environmental friendly species to proliferate.

Certain Pyrrophyta and Chrysophyta are capable of phagotrophic nutrition. In some instances, smaller algae such as *Chlorella* may be ingested. Cole and Wynne (1974) noted that when the chrysophyte *Ochromonas danica* was mixed into a culture with *M. aeruginosa*, they declined 30-fold in 10 min as a result of ingestion by *Ochromonas*.

Numerous reports exist in the literature documenting the success of using barley straw for the control of cyanobacteria. Newman and Barret (1993) demonstrated that decomposing barley straw effectively inhibits the growth rate of *M. aeruginosa* to a sixth of that achieved in control experiments. This inhibitory effect is presumably caused by the release of a chemical during aerobic microbial decomposition of the straw. This chemical, or chemicals, are so far unidentified, but there are several probabilities; firstly, antibiotics may be produced by fungal flora active in the decomposition of the barley straw; secondly, during decomposition the release of modified cell wall components may have an effect on cyanobacteria growth; and thirdly, certain phenolic and aromatic compounds produced during cell wall biodegradation may also contribute to the declining of cyanobacteria numbers. It seems that the inhibitory effect is rather algistatic than algicidal; therefore, the presence

of decomposing barley straw can help prevent the development of cyanobacterial blooms.

Another report on the application of hay by a local municipality, to two small farm dams in Linfield Park near Pietermaritzburg, South Africa, suggested that hay may be useful in cyanobacterial control. The farm dams receive the bulk of their nutrient rich flow from a small sewage works which caused the development of cyanobacterial scums. Reduction of algae populations in the upper of the two dams, closest to the sewage works, was total, with zero algae being detected within a few weeks of application of small quantities of hay in the water bodies (Harding and Plaxton, 2001).

Water that had been treated with chlorine may have killed the algae, but the result will be the release of the toxins into the water. Very high concentrations of chlorine could, however, inactivate the microcystins. Conventional water treatment processes do not completely remove microcystins from raw water, even when activated carbon is included in the treatment (Lambert et al., 1996).

Blooms have been controlled with the treatment of lime without any significant increase in microcystin concentration in the surrounding water (Kenefick et al., 1993). Chemical control of *Microcystis* blooms appears to be the best solution, thus removing the source of the microcystins. It has been found that microcystins persist in the dried crust of lakes formed as water levels recede during dry seasons. Large quantities of microcystins leach from the dry materials upon re-wetting within 48 hours (Jones et al., 1995; Brunberg and Blomqvist, 2002). This could present a significant problem with coagulation and sedimentation treatment as the water would not be suitable for consumption for up to three weeks before biodegradation commences (Jones, 1990).

ANALYSIS OF MICROCYSTINS

Several methods for the assessment of microcystin are available, including HPLC, MS, ELISA, or direct DNA test to detect genetic sequences unique for the multigene cluster required for toxin synthesis (Nishizawa et al., 2000).

Biochemical analysis

There are five basic methods to analyze microcystins namely; those based on reactions with a fluorescent probe; enzyme-linked immunosorbent assays [ELISA]; inhibition of protein phosphatase and mass spectrometry (Dawson, 1998); and by polymerase chain reaction (PCR, Baker et al., 2002, Pan et al., 2002). Shimizu and colleagues (1995, as cited by Dawson, 1998) targeted conjugated dienes using a synthesized fluorogenic reagent called DMEQ-TAD. This reagent reacted with

vitamin D metabolites and synthetic analogues, and the fluorescent products could be quantified linearly down to fmol quantities by HPLC. The reagent also reacted well with microcystin-LR, YR and RR at the conjugated diene moiety (Adda). An and Carmichael (1994) has used a direct competitive ELISA to examine the specificity of the rabbit anti-microcystin-LR polyclonal antibodies. Cross reactivity with some, but not all microcystin variants studied was observed and it became clear that Adda and arginine are essential for expressing the antibodies specificity. The inhibitor IC₅₀ for microcystin-LR of the binding of microcystin-LR-horseradish peroxidase conjugate to the antibodies was 3 ng/ml. McDermott and coworkers (1995) described an ELISA potentially able to detect microcystins in water at a concentration as low as 100 pg/ml water.

Microcystins are inhibitors of protein phosphatase (Honkanen et al., 1996). An and Carmichael (1994) had reported an IC₅₀ of 6 ng/ml for microcystin-LR in their direct competitive ELISA. Whilst ELISA microcystin-LR/YR/RR detection limits of 0.10, 0.12, 0.14 and 0.20 ng/ml was reported by Yu and coworkers (2002). A screening method for microcystins in cyanobacteria has been developed based on the formation of 3-methoxy-2-methyl-4-phenylbutyric acid by ozonolysis (Harada et al., 1996). The acid was detected by electron ionization-gas chromatography/mass spectrometry, using selected ion monitoring in a procedure that detected nanogram levels of microcystin in only 30 min.

Molecular DNA tools for microcystin identification

The *mcy* gene cluster assembly consists of 10 bidirectionally arranged genes that reside in two operons (*mcy A-C* and *mcy D-J*) of *M. aeruginosa*. The activities of these chromosomal gene products are primarily peptide synthetases [*mcy A-C*, *E,G*], polyketide synthases [*mcy D*, parts of *E* and *G*], and methylation [*mcy J*], epimerization [*mcy F*], dehydration [*mcy I*], and localization [*mcy H*], resulting in nonribosomal toxin synthesis. Disruption of some of these genes [*mcy A,B,D*, or *E*] resulted in no detectable toxin production (Nishiwaza et al., 2000).

Baker and coworkers (2002) determined the potential of microcystin detection by PCR amplification of a gene in the microcystin biosynthetic pathway and the 16S rRNA gene of *Anabaena circinalis* strains. Pan et al. (2002) used primers deduced from the *mcy* gene to discriminate between toxic microcystin-producing and non-toxic strains. Cyanobacterial cells enriched from cultures, field samples, and sediment samples could successfully be used in the PCR assay.

In a recent study by Oberholster (2004), PCR-restriction fragment length polymorphism analysis (Figure 3) and the sequence of *mcyB* were used to identify different strains of *M. aeruginosa*. After analysis of *mcyB*

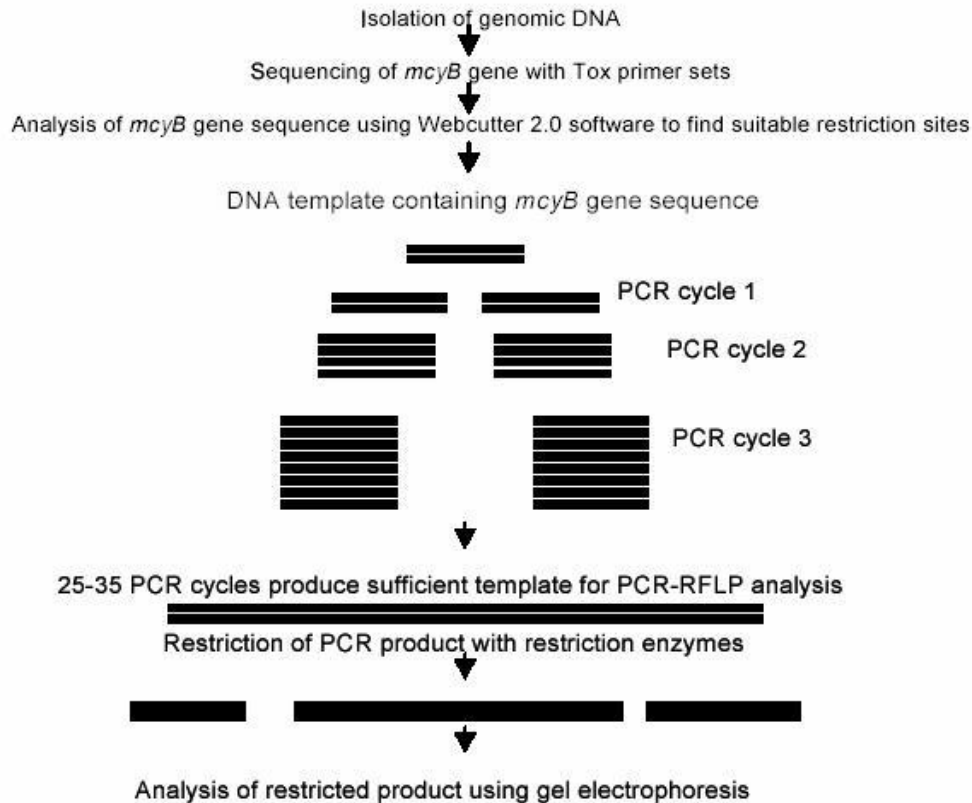


Figure 3. Schematic representation of PCR-RFLP analysis of the *mcyB* gene after amplification with selected TOX primer sets.

gene sequences obtained from strains PCC7813 and UV027 (GenBank accessions AY034601 for PCC7813 and AY034602 for UV027; e-value = 0.0), differences in endo-nuclease restriction sites were obtained. A selected number of these insertions/deletions were used to analyse four strains and differences were obtained, indicating that changes in insertions/deletions of the *mcyB* gene sequence may be a feasible way to differentiate between different strains of *M. aeruginosa* (unpublished). The question that remains, however, is whether these nucleotide changes may be indicative of different levels of toxicity in different strains of *M. aeruginosa*, since it is known that the presence of the *mcyB* gene coincide with toxin production.

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