INHIBITORY METABOLITES PRODUCTION BY THE CYANOBACTERIUM *Fischerella muscicola*

A thesis presented in partial fulfilment of
the requirements for the degree of
Doctor of Philosophy in Biotechnology
and Bioprocess Engineering at Massey University.

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Dedication

This study is dedicated to my Guru
Pandit Sri Ram Sharma Acharya and Vandaniya Mataji.

Without their blessings this study would have been impossible.

ABSTRACT

Studies have been carried out on the production and toxicity of inhibitory metabolites by cyanobacteria.

In the present study a detailed screening system was used to detect antimicrobial substances produced by the cyanobacteria. A range of cyanobacterial species were screened for the production of inhibitory metabolites. However, a majority of the cyanobacterial species screened showed no evidence of production of antimicrobial substances under the culture conditions used. However, *Fischerella muscicola* (UTEX 1829) produced antimicrobial metabolites and this cyanobacterium became the subject of further studies.

Preliminary characterization of both, intracellular and extracellular crude methanolic preparation from the cultures of cyanobacterium *Fischerella muscicola* (UTEX 1829) showed the inhibition of a range of cyanobacteria, eukaryotic algae and eubacteria, using an agar zone inhibition technique.

Using HPLC and GCMS techniques it was possible to demonstrate unique characteristics of these inhibitory metabolites in the crude methanolic cell extracts prepared from cultures of *F. muscicola.* The application of the GCMS technique confirmed the absence of fischerellin from the cultures of *F. muscicola* grown under various laboratory conditions used in these studies.

The effect of culture age on the production and leakage of inhibitory (toxic) metabolites was investigated in batch cultures. The total toxicity of *F. muscicola* was found to reach its maximum during the late exponential or early stationary phase of the growth. The higher toxicity was always found to be associated with the cell extracts rather than the cell-free culture broths indicating that a lower amount of leakage of the toxic material into the culture medium had occurred. However, there was a higher amount of toxicity found in the cell free culture broth extract when the culture was in a state of senescence.

Factorial experiments with the split plot design for the study of effects of various environmental factors such as light intensity, temperature, nitrogen and phosphorus on the production of inhibitory (toxic) metabolites, revealed that low light intensity (10 μmol m^{-2} s^{-1}) together with low temperature (10°C) significantly enhanced the toxicity of *F. muscicola.* Variations in the concentrations of the major nutrients, nitrogen and phosphorus did not have any significant effect on the toxicity of the cyanobacterium over the concentration ranges investigated.

The effect of various environmental factors on the release of inhibitory (toxic) metabolites from the cells of *F. muscicola* was investigated and it was found that the release of metabolites was enhanced under high light intensity (60 μmol m^{-2} s^{-1}) and higher temperature (30°C). However, at low light intensity (10 μmol m^{-2} s^{-1}) and low temperature (10°C) the release was minimal.

Under sets of conditions which generated the highest observed toxicity and the lowest observed toxicity in this cyanobacterium, it was found that growth of the organism was limited by the temperature.

This research contributes to an increased in the understanding of the physiology of *F. muscicola* in particular and to the toxic cyanobacteria in general. Furthermore, this new knowledge will contribute to a deeper understanding of the ecology of these important microorganisms.

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Chapter 1

Introduction

Eutrophication of water bodies often leads to the mass development of cyanobacterial populations which have characteristics of both bacteria and algae and create important problems in freshwater particularly if that water is needed as drinking water for animals and humans. Destabilization of the aquatic ecosystem can occur when metabolites which are inhibitory and/or toxic to other (micro)organisms are produced. Thus, a threat is posed to animals and to human public health. In recent years considerable research has been conducted on the detection and assay of these metabolites and from that research it has become possible to categorise both the metabolites and the microorganisms which produce them.

Based on the bioassay system used to screen for their activity cyanobacteria have been divided into two groups according to the type of toxins they produce. These toxins have been classified as either cytotoxins or biotoxins. Biotoxin producing cyanobacteria have been studied in more detail than the cytotoxin producers because of the serious effect biotoxins have on the organisms at higher trophic levels. The comparatively less studied, cytotoxin producers, which include species of the genus *Fischerella*, synthesise metabolites that are not highly lethal to animals but which do have a more selective bioactivity towards other cyanobacterial species, algae, bacteria, fungi and laboratory cultured mammalian cell lines. However, there is still much to be learned about these cytotoxin producers. It is not clear whether they produce the cytotoxins (inhibitory metabolites) only under particular environmental conditions, whether so called "toxic" strains become "non-toxic" under specific conditions or whether the cytotoxin producers vary in their toxicity at different times of their life cycle.

Given the broad biological significance of these cytotoxin producers, it is important to be able to screen for potentially toxic cyanobacterial species by detecting their bioactivity using appropriate bioassay methods. It would also be advantageous to isolate and characterize the toxic component(s) building, with time, a database of information specific to these cyanobacterial toxic and inhibitory substances. It is also necessary to acquire knowledge concerning the physiology of regulation of toxin production. These data would provide insights into how the toxicity of the organism varies during its growth cycle under the laboratory culture conditions and how the effects of various environmental factors contribute to the production or prevention of toxic metabolite synthesis by cells of cyanobacteria.

These speculations and questions, largely unaddressed in the literature, provided the starting point for the research described in this thesis. The objectives of the research were:

> firstly, to screen a range of cyanobacterial species to detect their bioactivity and to identify the potential cytotoxin producers; secondly, to isolate and identify the toxic component(s) synthesised by cytotoxin producing cultures; thirdly, to gain a better understanding of the importance of culture age not only on the synthesis of these inhibitory metabolites but also on their release into the surrounding medium, under laboratory culture conditions and finally, to investigate the role of key environmental factors, namely light, temperature and nutrients on the production of inhibitory metabolites using a statistical design

methodology.

The production of inhibitory metabolites by cyanobacterium *Fischerella muscicola* was a prime focus of the research to be described. In this thesis the terms inhibitor and toxin will be regarded as interchangeable words.

Chapter 2

Literature Review

2.1 CYANOBACTERIA

The cyanobacteria are gram-negative prokaryotes, with cell walls composed of peptidoglycan and lipopolysaccharide layers (Carmichael, 1992). They have no membrane enveloping their nuclear material and usually lack membrane-bound bodies within their interior (Figure 2.1). Although prokaryotic and having physiological and morphological similarities to eubacteria they can be differentiated from them by their ability to carry out oxygenic photosynthesis, mediated by the pigments chlorophyll-a and biliproteins by which they carry out photosynthesis with the (Carmichael, 1994; Stal, 1991).

2.1.1 Ecology

2.1.1.1 Factors influencing distribution

Cyanobacteria occur in the freshwater at almost every latitude. While some genera are cosmopolitan, others are apparently more restricted to cool, temperate or alternatively tropical and subtropical waters (Reynolds, 1987). Many cyanobacteria are benthic (Stal, 1991) and are therefore attached to surfaces or internal cavities of other organisms (Shilo, 1989). Cyanobacterial mats consisting of only one species are frequently reported in benthic habitats (Gross *et al.*, 1991). To some extent distribution is influenced by environmental factors. The major environmental factors that influence the growth of cyanobacteria are light, temperature, pH, and nutrient resources (Anon., 1990; Reynolds & Walsby, 1975). Growth in lakes is additionally subject to the direct effects of water movements and thermal stratification, which in turn affect the availability of the other factors such as nutrients (Reynolds & Walsby 1975).

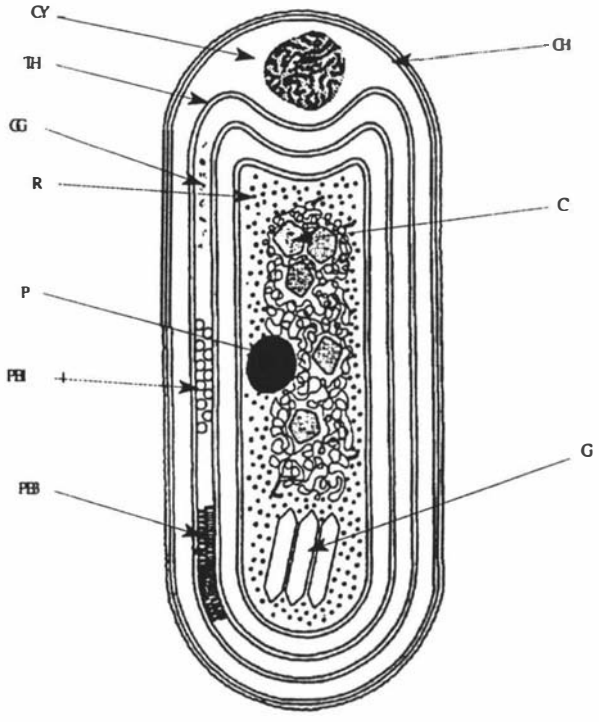


Figure 2.1. Diagrammatic representation of a cyanobacterial cell. CM, Cell membrane; TH, thylakoid; PB1 & PB2, front and side views, respectively, of two rows of phycobilisomes; GG, glycogen granules; CY, cyanophycin granule; P polyphosphate granule; R, 70S ribosomes; C, carboxysome; G, gas vesicle. (adapted from Stanier and Cohen-Bazire, 1977)

2.1.1.2 Light and its importance

Population growth is wholly or mainly supported by photoautotrophy (Bloor, 1989; Reynolds, 1987). One of the unique adaptive process found in attached cyanobacteria is their adaptation to either low light intensities of restricted spectral quality or to high light intensities. This adaptation involves firstly, phototactic gliding (Jorgensen, 1989) to the sediment surface to prevent the burying of the benthic mat by sedimentation and secondly, chromatic adaptation manifested as an enhanced or restricted synthesis of photosynthetic pigments with an increase or decrease in the numbers or sizes of reaction centres (Shilo, 1989). Cyanobacteria can utilize radiation over a wide-band of the visible spectrum due to the presence of accessory photosynthetic pigments, phycobilins (Carmichael, 1992). Light intensity is a factor which appears to exert differing effects on cyanobacteria according to whether they are in the field and/or growing in the laboratory. Some species of cyanobacteria appear to exist in the field at light intensities which they are unable to tolerate in culture (Hader,1987; Fogg *et al.*, 1973). Unlike the green algae, cyanobacteria do not appear to have an obligate requirement for alternating light and dark cycles. The maximum growth rate of most temperate freshwater cyanobacteria in laboratory cultures is reached at white light irradiance of approximately 50-60 $\mu E\ m^{-2}s^{-1}$. The minimum irradiance supporting the growth shows a significant dependence of light quality (Wayman & Fay, 1987).

2.1.1.3 Temperature and growth

Cyanobacteria are known to have preference for higher temperatures compared to other phytoplankton (Reynolds, 1975). However, the ranges suitable for growth are much

wider than for any other group of phytoplankton (Fogg *et al.*, 1973). Water temperatures below 20°C are unfavourable for mass development (Carmichael, 1992) and in general the temperature optima for most species are reported to be in the range of 25-35°C (Reynolds, 1975). Low temperature results in slower growth rates of cyanobacteria (Anon. 1990). The growth of cyanobacteria is temperature dependent with optima usually at 25°C or greater (Robarts & Zohary, 1987).

2.1.1.4 pH

Cyanobacteria are generally tolerant of low pH (ie <6.0) but grow best in a pH range of 7.5 - 9.0 (Reynolds, 1987). Shapiro (1973) observed a population shift from green algae to cyanobacteria at high pH values, indicating that these cyanobacteria are efficient in deriving carbon from very low concentrations of free carbon dioxide. However, Fogg *et al.* (1973) reported that at pH values much below 6 cyanobacteria show a generally negative growth response. In this respect it is significant that Reynolds & Walsby (1975) stated that the development of cyanobacterial populations in natural lake occurs after a rise in pH from 4.7 to 6.5. Harris (1986) reported that cyanobacteria tend to be replaced by green algae at lower pH values.

2.1.1.5 Nutrient preferences

Increasing eutrophication of lakes results in increasing biomass of cyanobacteria (Pick & Lean, 1987; Anon., 1990; Carmichael, 1992). Increasing concentrations of nitrogen and phosphorus promote the reproduction of toxigenic cyanobacteria and foster formation of blooms. Blooms (heavy growth) in nutrient-rich water usually consist of

a number of species of toxic cyanobacteria (Carmichael, 1994).

Nitrate-nitrite (NO_3^-/NO_2^-), ammonia (NH_3/NH_4^+) and orthophosphate (PO_4^{-3}) are the major soluble inorganic nutrients species of concern with respect to the promotion of growth of cyanobacteria (Carmichael, 1992). The long term availability of these nutrients are essential for their sustained growth (Simon, 1987). However, the absolute requirements for these nutrients by cyanobacteria are similar to requirements shown by other phytoplankton (Reynolds & Walsby, 1975). In most freshwater habitats physically amenable to cyanobacterial growth, phosphorus availability often dictates the magnitude of dominance and bloom formation (Pearl, 1988). Cyanobacteria are known to have the ability to store within the cell phosphorus in excess of growth requirements ie. "luxury consumption" (Gerber & Wickstrom, 1990) when it is freely available. Enough is stored to support up to two or three more doublings of cell numbers after phosphate exhaustion from the water (Anon., 1990; Reynolds, 1987).

The ratio of availability of nitrogen (N) to phosphorus (P) has been used to describe nutrients requirements (Carmichael, 1992). A lower ratio between 10 to 16 N:1 P favours the growth of cyanobacteria as compared with a ratio of 16 to 23 molecules N:1 of P which is required for the growth of other phytoplankton. However, these ratios would only be important if these nutrients were in short supply (Anon., 1990). In addition, temperature has also been suggested as having a role in the preferred N:P ratios (Christoffersen *et al.*, 1990).

Some filamentous cyanobacterial species possess the ability to fix atmospheric nitrogen (Carmichael, 1994) when the combined sources of this element are present in low concentrations (Reynolds, 1987). Nitrogen fixation is essentially an anaerobic process (Bloor, 1989). Under aerobic conditions when combined nitrogen (nitrate or ammonia) is absent, normal cells in the filament differentiate into metabolically specialized thick-walled cells called heterocysts for aerobic nitrogen fixation. However, nitrogen fixation by non-heterocystous cyanobacteria has also been reported under anaerobic or microaerobic conditions (Baalen, 1987).

2.1.2 The Toxic cyanobacteria

The relative abundance of cyanobacteria is also influenced by the production of toxic secondary metabolites (Keating, 1976). Cyanobacteria are poorly utilized by suspension-feeding zooplankton although they are primary producers in the food chain of the aquatic ecosystem. The reasons for this can be associated with their nutritional inadequacy and toxic effects which may be exerted by them (DeMott & Moxter, 1991). Carmichael (1994) stated that cyanobacteria producing toxic substances have been reported since 1878, when George Francis first published in Australia an account of the toxic effects of cyanobacteria *Nodularia spumegina* Mertens. Toxins of cyanobacteria have been implicated for mortality among wild and domestic animals (Lawton & Codd, 1991) and they constitute hazards to human health being implicated in human gastroenteritis, primary liver cancer in humans and skin irritation (Lahti *et al.*, 1995; Shi *et al.*, 1995). Therefore, the presence of cyanobacteria in water supplies poses a serious health problem (Rinehart *et al.*, 1994). The usual potable water-treatment process will

partially dilute cyanobacterial toxins (Carmichael, 1994) but will not remove them completely. The only possible way to remove these toxins from a drinking water supply is either by treatment employing activated carbon filtration or by oxidization using ozone. Both of these methods are expensive and are not usually practised (Kiviranta *et al.*, 1991).

The distribution of these toxigenic cyanobacteria is world-wide, including New Zealand. Skulberg *et al.* (1984) and Rinehart *et al.* (1994) reported that animal, fish and bird kills have been due to cyanobacterial poisoning in New Zealand. Table 2.1 shows their world-wide occurrence. They can be found in both marine and fresh water habitats.

Table 2.1. Countries in which toxigenic cyanobacteria have been reported in Fresh or Marine Waters (adapted from Carmichael, 1989).

ARGENTINA	EUROPE (continued)	U.S.A (continued)
AUSTRALIA	Portugal	Nevada
BANGLADESH	Sweden	New Hampshire
BERMUDA	INDIA	New Mexico
BRAZIL	ISRAEL	New York
CANADA	JAPAN	North Dakota
Alberta	NEW ZEALAND	Oregon
Manitoba	OKINAWA (MARINE)	Pennsylvania
Ontario	PEOPLES REPUBLIC OF CHINA	South Dakota
Saskatchewan	SOUTH AFRICA	Taxas
EUROPE	U.S.A	Washington
Czechoslovakia	California	Wisconsin
Denmark	Colorado	
Germany	Hawaii (marine)	UKRAINE
Finland	Idaho	
Great Britain	Illinois	
Hungary	Iowa	
Netherlands	Michigan	
Norway	Minnesota	
Poland	Montana	

About 12 genera and more than 25 species of freshwater cyanobacteria have been implicated in producing toxins (Carmichael *et al.*, 1990; Baker and Humpage, 1994). They include *Anabaena, Aphanizomenon, Microcystis, Nodularia, Nostoc,* and *Oscillatoria* species. Most produce more than one type of toxin (Carmichael, 1994). However, a second less studied group of toxic genera include *Coelosphaerium, Cylindrospermopsis, **Fischerella**, Gloeotrichia, Gomphosphaeria, Hapalosiphon, Microcoleus, Schizothrix, Scytonema, Spirulina, Symploca, Tolypothrix,* and *Trichodesmium*. No toxin has been isolated and characterized from these latter genera (Scott, 1991; Carmichael, 1992).

Two important categories of toxins, neuro-toxic alkaloids and hepatotoxic peptides (Carmichael, 1992; Sivonen *et al.*, 1992; Luukkainen *et al.*, 1993; Baker & Humpage, 1994; Lahti *et al.*, 1995; Lauren-Maatta *et al.*, 1995) can be produced by cyanobacteria; these are defined by the symptoms they produce in animals. Carmichael (1994) stated that neuro-toxins interfere with the functioning of the nervous system leading to paralysis of the respiratory muscles and often causing death within minutes. Hepatotoxins damage the liver and kill animals by causing blood to pool in the liver.

Cyanobacteria from the second less studied group above have been reported to produce a number of secondary metabolic chemicals that are not highly lethal to animals but instead show more selective bioactivity towards other cyanobacterial species, algae, bacteria and fungi and cultured mammalian cell lines (Carmichael *et al.*, 1990; Carmichael, 1992; Borowitzka, 1995).

Toxigenic cyanobacteria have been reported from freshwater, brackish water (Lahti *et al.*, 1995) and marine environments (Ikawa and Sanser, 1990). Because of the hazards created by these microorganisms they have received increasing attention in recent years (Hughes *et al.*, 1958; Carmichael, 1974, 1981, 1986, 1992, 1994; Carmichael and Gorham, 1978; Gorham and Carmichael, 1979, 1988; Carmichael *et al.*, 1985, 1990; Metting and Pyne, 1986; Gleason and Wood, 1987; Codd and Poon, 1988; Codd *et al.*, 1989; Anon., 1990; Ikawa and Sanser, 1990; Lawton and Codd, 1991; Rinehart *et al.*, 1994; Borowitzka, 1995).

2.1.2.1 Activity and structure of cyanobacterial toxins

Cyanobacteria represent a potentially rich source of chemically unique secondary metabolites. The classification of cyanobacterial toxins has been based mainly upon the type of bioassay used to detect activity. At present cyanobacterial toxins are divided into two major bioactivity related groups : cytotoxins and biotoxins. Cytotoxins are so named because the screening system is based upon a cell culture bioassay, whereas the toxins assayed using small animals are called biotoxins (Carmichael, 1992).

2.1.2.2 Cytotoxins

Reports on cyanobacteria exhibiting potentially useful biological activities (e.g. pharmacological) have increased. As described above cytotoxins are not highly lethal to animals but they do show a wide spectrum of bioactivity against algae, eubacteria, cyanobacteria, fungi and mammalian cell lines. Cyanobacterial extracts and/or extracellular products are known to be antibacterial (Metting & Pyne, 1986; Bloor &

England, 1991), antifungal (Metting & Pyne, 1986; Carmeli *et al.*, 1990; Park *et al.*, 1992; Patterson & Bolis, 1993), anticyanobacterial (Gleason & Paulson, 1984; Flores & Wolk, 1986; Gromov *et al.*, 1991 Gross *et al.*, 1991; Srivastava *et al.*, 1994), antialgal (Vincent & Silvester, 1979; Gleason & Paulson, 1984; Gromov *et al.*, 1991) and antiprotozoal (Metting & Pyne, 1986). However, for many of these cyanobacterial toxins the structure and identity of the active constituent is not yet known.

2.1.2.2.1 Cyanobacterin

The benthic filamentous cyanobacterium *Scytonema hofmanni* (UTEX 2349) produces a small, hydrophobic metabolite, called cyanobacterin which is toxic to most cyanobacteria and also to a variety of eukaryotic algae (Mason *et al.*, 1982). The concentration of cyanobacterin found in the cells of *Scytonema hofmanni* is reported to be 3 mg per gram dry weight (Pignatello *et al.*, 1983). Cyanobacterin is chemically characterized as a unique diaryl substituted γ-ylidene γ-butyro lactone with chlorine substituent on one of the aromatic rings (Gleason & Paulson, 1984). It has a molecular weight of 430 and a molecular formula of $C_{23}H_{23}ClO_6$ as deduced from high resolution mass spectroscopy (Gleason & Wood, 1987). Also, this toxin is the halogenated metabolite known to be produced by a freshwater cyanobacterium (Gleason & Wood, 1987). Figure 2.2 shows the structure of cyanobacterin.

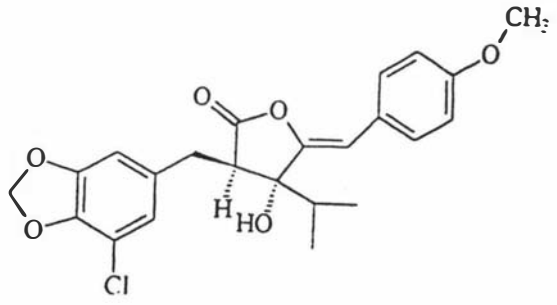


Figure 2.2. Molecular structure of the halogenated metabolite "cyanobacterin" produced by the freshwater cyanobacterium *Scytonema hofmanni* (Gleason & Wood, 1987).

Cyanobacterin inhibits the growth of many cyanobacteria at a minimum effective concentration of 2 µg/ml (Gleason & Paulson, 1984). It is also reported to be active against eukaryotic algae (Flores & Wolk, 1986). However, it is not toxic towards eubacteria, protozoans and fungi (Gleason & Wood, 1987). Similar effects of cyanobacterin LU-1 from another cyanobacterium *Nostoc linckia* CALU 892 have been reported by Gromov *et al.* (1991) but the minimum effective concentration was 1 µg/ml. Gleason & Paulson (1984) identified the site of action of cyanobacterin as the thylakoid

membranes. The compound has been shown to inhibit the photosynthetic electron transports' Hill reaction in photosystem II at a minimum concentration of 25 ng/ml at a unique site which is different from that for 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and atrazine (Gleason & Wood 1987) .

Limited work has been done concerning the biosynthesis of cyanobacterin. However, studies on the physiological properties of cyanobacterin have been extensively reviewed by Gleason & Wood (1987).

2.1.2.2.2 Tolytoxin

Tolytoxin (6-hydroxy-7-O-methyl-scytophycin b) is highly cytotoxic and is a potent antifungal antibiotic (Carmeli *et al*., 1990) although inactive towards eubacteria and viruses (Patterson & Carmeli, 1992). The minimum inhibitory concentration of tolytoxin is reported to be in the range of 0.25 to 8 nanomolar. The exact site of action for tolytoxin in eukaryotes is still obscure, although it has been postulated that tolytoxin inhibits those fundamental cell processes peculiar to eukaryotes (Patterson & Carmeli, 1992). Carmeli *et al.* (1990) elucidated the structure of tolytoxin. The cyanobacterial species producing tolytoxin are from the closely related genera *Scytonema* and *Tolypothrix* (Nostocales, Scytonemataceae) (Patterson & Carmeli, 1992).

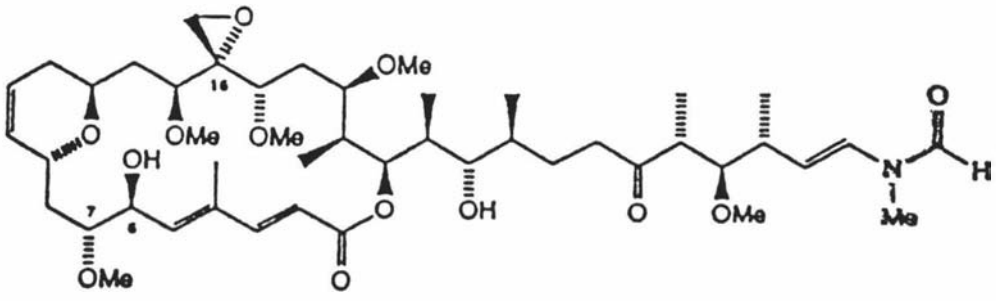


Figure 2.3. Molecular structure of tolytoxin produced by the cyanobacteria *Scytonema sp.* and *Tolypothrix sp.* (Patterson & Carmeli, 1992).

Tolytoxin has a structure that is closely related to that of scytophycin B, differing in its possession of methoxyl group on the C-6 (Ishibashi *et al.*, 1986).

2.1.2.2.3 Scytophycins

There are nine known scytophycins which are chemically well characterized (Patterson & Bolis, 1993). Species of *Scytonema* producing these scytophycines are *S. pseudohofmanni, S. ocellatum, S. mirabile* and *S. burmanicum.* Structures of some the

scytophycins are presented in Figure 2.4. These scytophycines are highly cytotoxic and antimycotic (Glombitza & Koch, 1989).

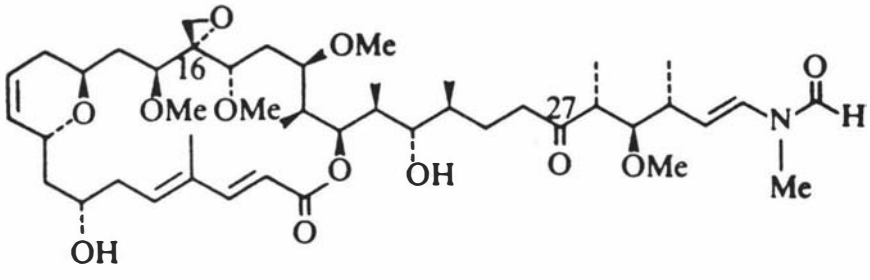
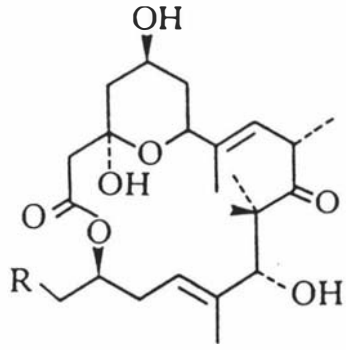


Figure 2.4 Molecular structure of scytophycin B.

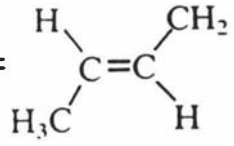
2.1.2.2.4 Acutiphycins

Two cytotoxins, acutiphycin and 20,21-didehydroacutiphycin has been reported from *Oscillatoria acutissima.* These toxins show a significant amount of antineoplastic activity against murine Lewis lung carcinoma cells (Barchi *et al.*, 1984). The molecular

structures for these compounds are shown in Figure 2.5.



R = $CH_3CH_2CH_2CH_2$ acutiphycin



didehydroacutiphycin

Figure 2.5. The cytotoxins of *Oscillatoria acutissima*, namely acutiphycin and didehydroacutiphycin (Barchi *et al.*, 1984).

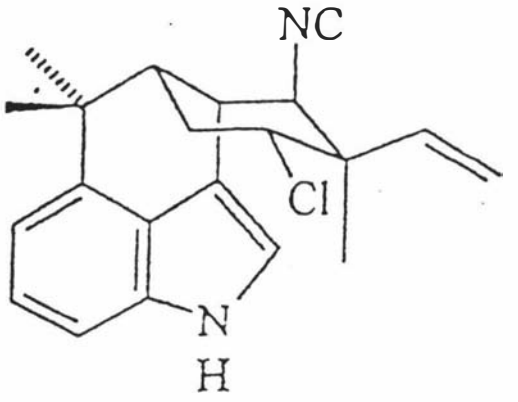
2.1.2.2.5 Hapalindole

Moore *et al.* (1984) isolated from the filamentous cyanobacterium *Hapalosiphon fontinalis* (Stigonemataceae) a cytotoxic alkaloid called hapalindole-a which was a chlorine- and isonitrile-containing indole alkaloid and present in cells at a concentration of 5.8 mg per gram dry weight. This lipophilic toxin has antialgal and antimycotic properties. Its molecular structure is given in Figure 2.6. Cyclopropane-containing hapalindolinones have been isolated from a cultured cyanobacterium belonging to the

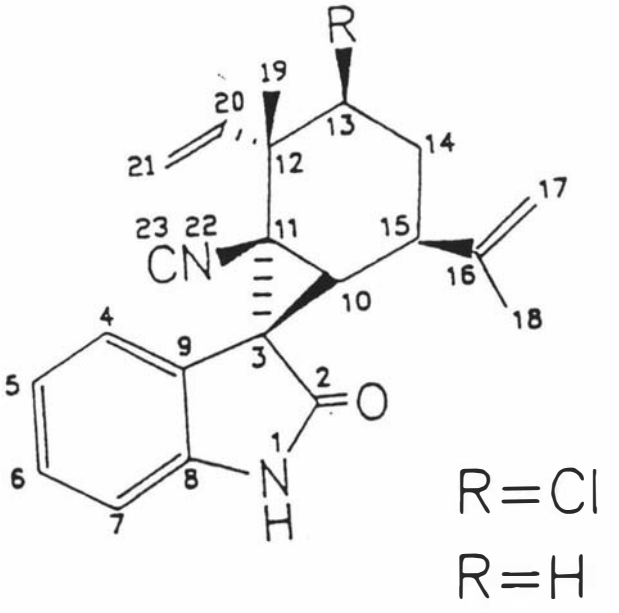
genus *Fischerella* (ATCC 53558) inhibited the binding of arginine vasopressin (Schwartz *et al.* 1987). Park *et al.* (1992) found an extract (70% ethanol) of cyanobacterium *Fischerella muscicola* to have antifungal properties when screened using agar-diffusion assay method. The compound has been isolated and chemically characterized as fischerindole L, a novel octahydroindenol[2,1-*b*]indole isonitrile reported to possess the same relative stereochemistry as hapalindole L.

Proteinaceous antibiotics that are active against bacterial strains closely related to the bacterium producing the substance are known as bacteriocins. Flores & Wolk (1986) first reported the production of a bacteriocin from a filamentous, nitrogen-fixing cyanobacterium and found that the antibiotic produced by the cyanobacterium *Nostoc* sp. 78-11A-E was a bacteriocin of low molecular weight.

Gross *et al.* (1991) observed allelochemical (fischerellin) production by the freshwater benthic cyanobacterium *Fischerella muscicola.* A partial chemical



hapalindolinone A



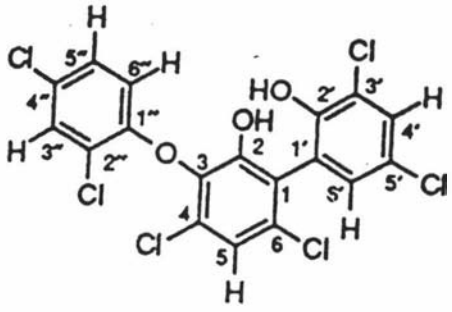
hapalindolinone B

Figure 2.6. Molecular structures of hapalindolinone A and B, the cytotoxins produced by the filamentous cyanobacteria *Hapalosiphon fontinalis* and *Fischerella* (ATCC 53558) (Moore *et al.*, 1984; Schwartz *et al.*, 1987).

characterization of this compound indicated a lipophilic substance which exhibited a molecular ion at *m/z* 408. Its site of action was located in photosystem II where it inhibited photosynthetic but not respiratory electron transport in both cyanobacteria and chlorophytes. The authors also reported the presence of fischerellin in *Fischerella ambigua*. Long-chain saturated fatty acids have been extracted from axenic cultures of *Fischerella* species and shown to have biocidal properties against other cyanobacteria and a green alga. Inhibition of the photosynthetic electron transport in photosystem II has been confirmed (Bagchi & Marwah, 1994). Falch *et al.* (1995) isolated three lipophilic compounds from *F. ambigua*; ambigol A (I) and B (II), and tjipanazole D (III). They found compounds I and II possessed antibacterial, antifungal, cytotoxic, molluscicidal, anti-inflammatory and anti-viral activities whereas compound III had antibacterial properties (see Figure 2.7). *Fischerella epiphytica* has been shown to produce antiprotozoan substance(s). However the chemical identification/characterization has not been done (Metting & Pyne, 1986).

2.1.2.3 Biotoxins

Many studies have been made on biotoxins in recent years (Carmichael & Gorham, 1978; Carmichael, 1981, 1986, 1992, 1994; Carmichael *et al.*, 1985; Gleason & Wood, 1987; Codd & Poon, 1988; Gorham & Carmichael, 1988; Glombitza & Koch, 1989; Ikawa & Sanser, 1990; Anon., 1990; Lawton & Codd, 1991; Rinehart *et al.*, 1994; Borowitzka, 1995). Therefore, the account of these toxins and their properties as presented here (and in Table 2.2) is to provide a background for the studies to be described in subsequent chapters.



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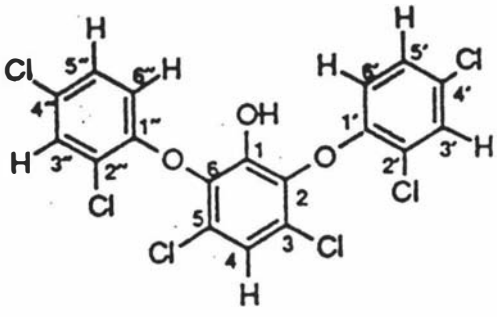

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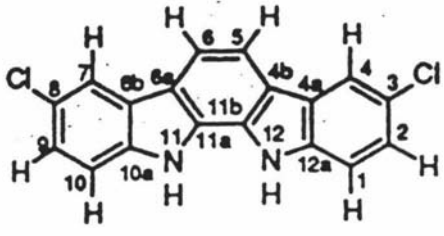


3

Figure 2.7. Molecular structures for the three lipophilic toxins from *Fischerella ambigua*: ambigol A (1); ambigol B (2) and tjipanazole D (3). All molecules are bioactive (Falch *et al.*, 1995).

These cyanotoxins (biotoxins) have produced intermittent but repeated cases of animal poisoning in many areas of the world (Ikawa & Sanser, 1990; Lawton & Codd, 1991; Carmichael, 1992). The two common chemical groups of cyanotoxins are the alkaloid neurotoxins and the cyclic peptide hepatotoxins and they are reported to occur more frequently than other toxins in water as a result of cyanobacterial blooms.

2.1.2.3.1 Neurotoxins

Freshwater filamentous cyanobacterial species producing neurotoxins are *Anabaena flos-aquae*, *Aphanizomenon flos-aquae*, *Oscillatoria agardhii* and *Trichodesmium* sp. (Anon., 1990; Carmichael, 1992 & 1994; Baker & Humpage 1994). Most of the research has been based on Canadian isolates of *Anabaena* (Ikawa & Sanser, 1990). According to Al-Layl (1989), *Anabaena flos-aquae* may produce at least six toxins; anatoxin-a, -b, -c, -d, -a(s) and -b(s). Four neurotoxins have been studied in detail by Carmichael (1994) including Antx-a and Antx-a (s). Anatoxin-a (antx-a) was the first toxin chemically and functionally defined from a unialgal clone of *Anabaena flos-aquae* designated as NRC-44h. It has a molecular weight of 165 Daltons, a molecular formula of $C_{10}H_{15}NO$, is water-soluble, basic and a cyclic amine (see Figure 2.8). Chemically it is referred to as the secondary amine 2-acetyl-9-azabicyclo(4-2-1) non-2-ene (Carmichael & Gorham, 1978; Carmichael, 1992).

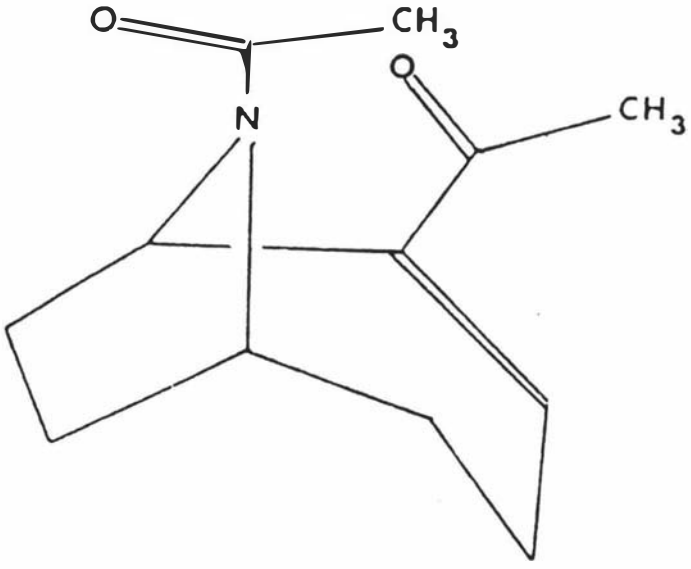


Figure 2.8. Molecular structure of Anatoxin-a produced by *Anabaena flos-aquae* (Carmichael, 1992).

The neurotoxic alkaloid Antx-a is a potent, postsynaptic, depolarising, neuromuscular blocking agent that mimics the effects of acetylcholine and acts as both a nicotinic and muscarinic receptor (Borowitzka, 1995; Carmichael, 1994; Codd & Poon, 1988).

The LD_{100} intraperitoneal (i.p.) mouse dose for pure antx-a is 250 $\mu g.kg^{-1}$ body weight, however susceptibility varies with different species. Symptoms produced include muscle fasciculation, loss of coordination, gasping, convulsions and death by respiratory arrest (Baker & Humpage, 1994; Ikawa & Sanser, 1990; Gorham & Carmichael, 1988).

Strains of *Anabaena* have also been reported to produce a less common neurotoxin, anatoxin-a (s) which exhibits somewhat different symptoms of toxicity and is also chemically different from anatoxin-a. The symptoms produced by this neurotoxin includes excessive salivation (s = salivation) and lachrymation. The LD_{50} intraperitoneal (i.p. mouse) dose for this pure toxin is reported to be 50 $\mu g\ kg^{-1}$ body weight . It has a molecular weight of 252 Daltons and molecular formula of $C_7H_{17}N_4O_4P$ (see Figure 2.9). Anatoxin-a (s) being an organophosphate could be used as pesticide. To date it is the only natural organophosphate discovered (Carmichael, 1992, 1994).

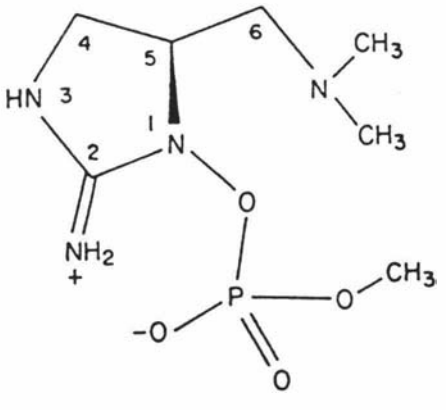


Figure 2.9. Molecular structure for Anatoxin-a(s) of *Anabaena flos-aquae* and other species of *Anabaena* (Carmichael, 1994).

Neurotoxins have also been found associated with the blooms of the filamentous cyanobacterium *Aphanizomenon flos-aquae* and have been referred to as "aphantoxins". Chemical and pharmacological evidence identified these "aphantoxins" as saxitoxin and neosaxitoxin, the primary toxins causing paralytic shellfish poisoning (PSP). The LD_{50} intraperitoneal (i.p. mouse) dose for these toxins was found to be about 5 mg kg^{-1} body weight. (Carmichael, 1992, 1994).

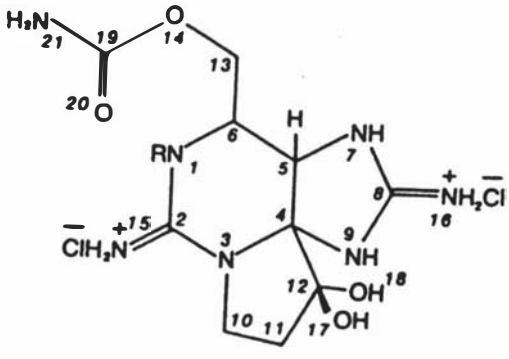


Figure 2.10. Molecular structure for Aphantoxins produced by *Aphanizomenon flos-aquae* (Carmichael, 1992, 1994).

2.1.2.3.2 Hepatotoxins

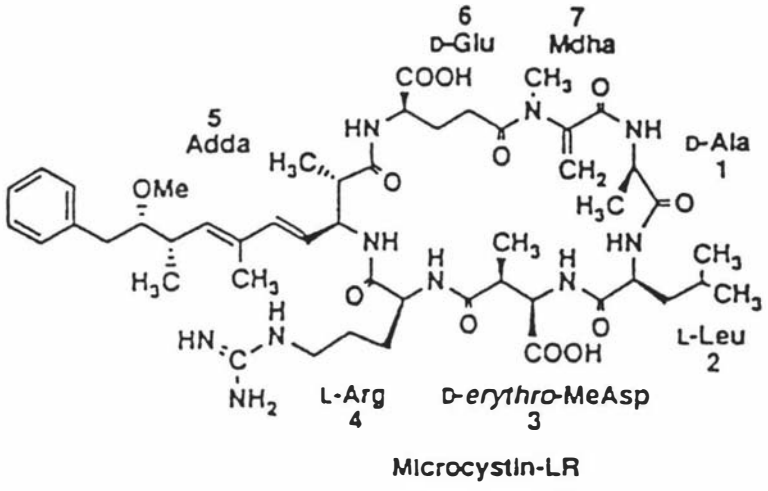
The most thoroughly investigated group of toxins are those regarded to as hepatotoxins. Strains of *Microcystis aeruginosa, M.wesenbergii, Nodularia spumigena, Oscillatoria agardhii/rubescens* group, *Anabaena flos-aquae, A. spiroides, Aphanizomenon flos-aquae*, various *Nostoc* species and *Gomphosphaeria lacustris* are suspected of producing hepatotoxins. These toxins act more slowly than alkaloid toxins (Gorham & Carmichael, 1988; Rinehart *et al.*, 1994). In terms of chemical structures there are two types, each being a low molecular weight peptide. One type called microcystins consist of seven amino acids. The second type called nodularins consist of five amino acids. These toxins target the liver where the peptides cause hepatocytes to shrink, resulting in these normally tightly packed cells to separate. Once separated other cells forming the so-called sinusoidal capillaries of the liver also separate. Capillary blood then seeps into liver tissue where its accumulation produces local tissue damage. This can often lead to shock (Carmichael, 1994). These toxins are potent inhibitors of protein phosphatases. Reversible phosphorylation of proteins of serine, threonine and tyrosine residues by protein kinases and phosphatases is the principal mechanism by which eukaryotic cells respond to extracellular signals (Borowitzka, 1995). The discovery of protein phosphatase inhibition by these toxins is of concern since nonlethal doses might contribute to the development of cancer in humans (Carmichael, 1994).

2.1.2.3.2.1 Microcystins

According to Al-Layl (1989) toxic peptides from *Microcystis aeruginosa* have been given various names: fast death factor (FDF), microcystin, cyanoginosin, cyanoviridin

and cyanogenosin. However, the term "microcystin" has found a general acceptance among contemporary specialists when describing heptapeptide hepatotoxins from various cyanobacterial genera (Sivonen *et al.*, 1992). About 47 molecular variants of the microcystins have been reported to date (Rinehart *et al.*, 1994) and the chemical structure of microcystins (see Figure 2.11) have been characterized as cyclo(D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), in which X and Z are variable L-amino acids, D-MeAsp is D-*erythro*-β-methylaspartic acid, Mdha is *N*-methyldehydroalanine, and Adda is (2*S*, 3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. The two acidic amino acids, D-MeAsp and D-Glu, are connected by an isolinkage. However, the toxicity of these compounds seems to be related with the presence of the unusual amino acid Adda (Carmichael, 1992; Luukkainen *et al.*, 1993; Rinehart *et al.*, 1994; Kotak *et al.*, 1995).

Most microcystins have an LD_{50} of 50-100 μg/kg in mice and changes in molecular structure appear to have little effect (Ikawa & Sanser, 1990; Rinehart *et al.*, 1994). Mutagenicity research has also been carried out on these toxins in short-term tests *in vitro* and in live animals. The conclusion reached from the results of Ames test and other bacterial mutagenicity assays was that these toxins were not mutagenic to bacteria (Anon., 1990).



Microcystin-LR

Figure 2.11. Molecular structure for the most commonly occurring microcystin. This is one of a number of toxic peptides produced by species of *Microcystis* (Kotak *et al.*, 1995).

2.1.2.3.2.2 Nodularins

Nodularia spumegina produces a cyclic pentapeptide toxin of a molecular weight 824 Daltons and which has been called nodularin (Carmicahel *et al.*, 1988; Jones *et al.*, 1994). It is similar in both structure (see Figure 2.12) and activity to other cyclic heptapeptides (Carmicahel *et al.*, 1988). Recently a detailed review on its structure and biosynthesis has been presented (Rinehart *et al.*, 1994). Nodularin is an inhibitor of protein phosphatases 1 and 2A and this is the proposed mechanism by which it exerts its hepatotoxicity (Borowtizka, 1995). Nodularin may act also as a tumour promoter (Rinehart *et al.*, 1994).

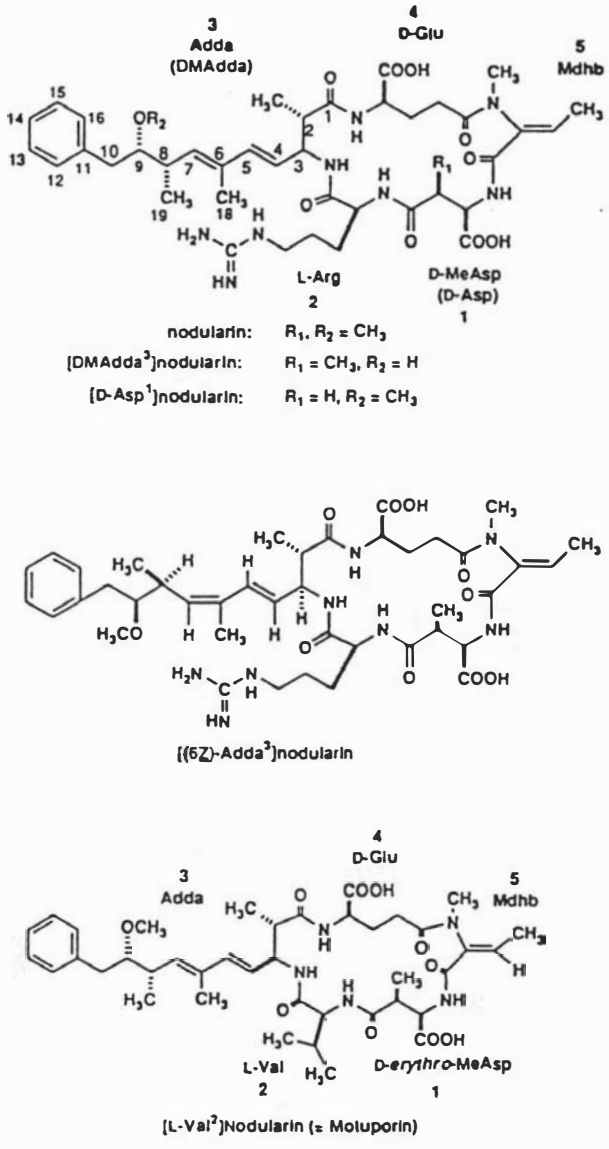
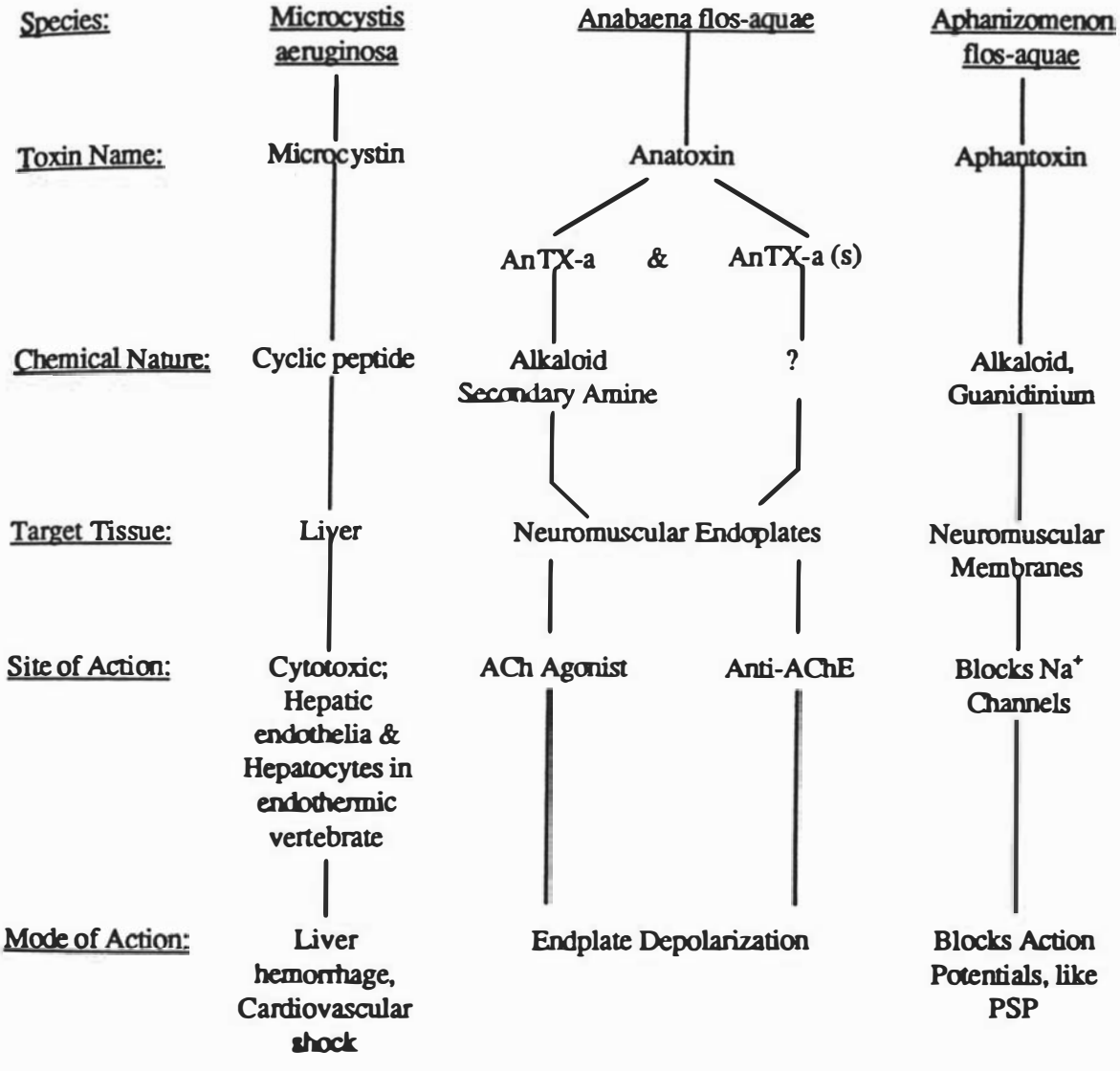


Figure 2.12. Three naturally occurring nodularins originally discovered in *Nodularia spumegina.*

Table 2.2. Characteristics of water-soluble toxins from cyanobacteria (Ikawa & Sanser, 1990).



2.1.2.4 Lipopolysaccharides (Endotoxins)

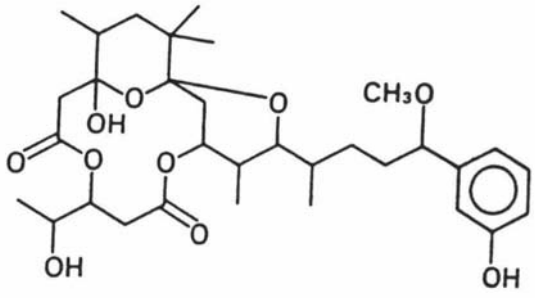
Lipopolysaccharides (LPS) are characteristic components of the cell wall of gram-negative bacteria and have been reported to exist in cyanobacterial species (Keleti *et al.*, 1981; Keleti & Sykora, 1981; Gorham & Carmichael, 1988; Codd *et al.*, 1989; Anon., 1990). The cyanobacteria from which LPS have been isolated and characterized include *Anacystis nidulans, Anabaena variabilis, A. flos-aquae, Agmenellum quadruplicatum, Phormidium africanum, P. laminosum, P. uncinatum, Schizothrix calcicola, Oscillatoria brevis, O. tenuis* and *Microcystis aeruginosa* (Al-Layl, 1989).

The cyanobacterial LPS vary in chemical composition and consist of varying combinations of fats and sugars. The LPS structural component, designated lipid A in some cyanobacterial species, is comparable to that of non-photosynthetic bacteria (Gleason & Wood, 1987). Lipid A is a complex molecule characterized by long-chain saturated and unsaturated fatty acids and hydroxy fatty acids. Unlike lipid A from heterotrophic gram-negative bacteria, they usually lack phosphates (Keleti & Sykora, 1982). These authors also reported that cyanobacterial LPS showed lower biological activity than did LPS derived from common heterotrophic gram-negative bacteria. However, the possibility of cyanobacteria being a significant source of endotoxins in water supplies can not be ruled out. These compounds may contribute to outbreaks of human gastroenteritis, diarrhoea, headache, cramps, nausea and dizziness (Metting & Pyne, 1986; Codd *et al.*, 1989; Anon., 1990).

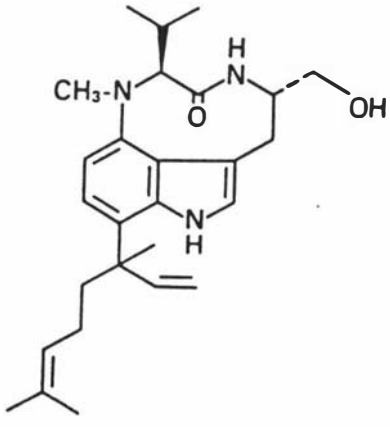
2.1.2.5 Marine cyanobacterial toxins

The problem posed by marine cyanobacteria is not as serious as the hazards created by their counterparts from the freshwater environment. The marine organisms have therefore received little attention (Moore, 1981). However, several species belonging to the family Oscillatoriaceae (unbranched filamentous, lacking heterocysts) have been implicated as producers of toxic compounds with tumour promoting activity in laboratory animals (Carmichael, 1986). Most commonly found species include *Lyngbya majuscula* (Moore, 1981; Carmichael, 1986; Ikawa & Sanser, 1990; Armstrong *et al.*, 1991), *Schizothrix calcicola* and *Oscillatoria nigroviridis* (Moore, 1981). A marine *Lyngbya* strain has been shown to produce cytotoxic compound(s) in two different phases of growth. Toxicity develops in early exponential phase but disappears as the exponential growth is established. However, toxicity re-develops in late exponential phase. The appearance of this pattern of toxicity during early and late exponential growth by this cyanobacterium was explained in terms of the production of two different compounds (Armstrong *et al.* 1991).

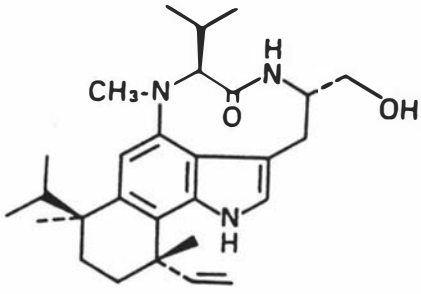
Lyngbya majuscula has been shown to cause "swimmers' itch", a severe form of contact dermatitis. The inflammatory agents have been identified as aplysiatoxin, debromoaplysiatoxin, and lyngbyatoxin A (Moore, 1981). The structures of these toxins are presented in Figure 2.13.



debromoaplysiatoxin



lyngbyatoxin A



teleocidin B

Figure 2.13. Structure of debromoaplysiatoxin, lyngbyatoxin A and teleocidin B, all produced by marine cyanobacterium *Lyngbya majuscula* (Moore, 1981).

Lyngbyatoxin A is identical to teleocidin A which is produced by cultures of the filamentous bacterium *Streptomyces* (Carmichael, 1986). It is an indole alkaloid, structurally and pharmacologically very similar to teleocidine B (Glombitza & Koch, 1989). Debromoaplysiatoxin and aplysiatoxin are polyacetates (Fujiki & Sugimura, 1983). The human dermatitis produced by lyngbyatoxin A is the same as that from debromoaplysiatoxin.

Two similar toxins (debromoaplysiatoxin and oscillatoxin A) have been isolated from two marine cyanobacteria *Schizothrix calcicola* and *Oscillatoria nigroviridis.* However these toxins lack tumour promoting activity (Moore, 1981; Fujiki & Sugimura, 1983). Oscillatoxin A has been reported to have a similar structure to aplysiatoxin (Moore *et al.*, 1984).

Armstrong *et al.* (1991) reported the production of two cytotoxic compounds from a marine *Lyngbya* species. Cytotoxicity assays using murine P-388 cells indicated that the semi-pure compound had an IC_{50} (Inhibitory Concentration) of $<0.25\ \mu g\ ml^{-1}$ to this cell line.

2.1.2.6 Regulation of toxin production

Only limited research has been carried out on the regulation of toxin production in cyanobacteria. The influence of environmental factors such as light, temperature, and nutrient supply is important and still needs further investigation for all principal cyanobacterial species in order to understand why there is a variability in toxicity and

to provide a rationale for predicting and perhaps restricting the formation of these toxic compounds (Anon., 1990; Carmichael, 1992; Busby, 1993; Kotak *et al.*, 1995).

Microcystis species and their toxins (microcystins) and to a lesser extent anatoxin-a have been the subject of a majority of both field and laboratory studies (Anon., 1990).

2.1.2.6.1 Field studies

Cyanobacterial toxin production per unit cyanobacterial biomass is highly variable in any particular natural water or reservoir (Codd *et al.*, 1989). In the field environment numerous studies have relied heavily on detection of cyanobacterial toxins by mouse bioassays. This is a useful initial toxicity screening method (Kotak *et al.*, 1995). However, a few studies involving high performance liquid chromatography, with its much higher detection limit for cyanobacterial toxins, have been attempted (Wicks & Thiel, 1990; Watanabe *et al.*, 1992; Shirai *et al.*, 1991; Kotak *et al.*, 1995). From field studies it has been deduced that environmental factors such as water temperature, pH, solar radiation and nutrients affect the toxicity of *Microcystis* species (Wicks & Thiel, 1990; Kotak *et al.*, 1995).

Wicks & Thiel (1990) reported that heptapeptide toxin production by the cyanobacterium *Microcystis aeruginosa* in Hartbeespoort Dam, South Africa were strongly and positively correlated with solar radiation, surface water temperature and pH. The toxins were either not detectable or in very low concentration during the winter (minimum water temperature of 13°C) and reached maximum concentration during the

summer when the surface water temperature reached 27°C. In contrast, Kotak *et al.* (1995) did not find any correlation of microcystin concentration with the water temperature even when their recorded water temperature was in the range of 7° to 24°C. Therefore, they concluded that water temperature was not a good *in situ* predictor of cyanobacterial toxin concentration.

Light also influences the toxicity of cyanobacteria. Correlation of cyanobacterial toxin concentration to the solar radiation in the field has suggested that light is a limiting factor for growth of cyanobacteria (Wick & Thiel, 1990). Kotak *et al.* (1995) found that over a 24-h period the toxin concentration decreased more than 6-fold at night relative to the daytime concentrations.

Progressive eutrophication of water-bodies either by natural or by anthropogenic activities may increase the cyanobacterial toxin production. A positive correlation of cyanobacterial toxin concentration with total and total dissolved phosphorus has been observed by Kotak *et al.* (1995) for a hypereutrophic lakes in Canada. They explained the negative correlation of the toxin production with inorganic nitrogen as a lack of heterocysts in the cyanobacterium *Microcystis aeruginosa* which can not carry out atmospheric nitrogen fixation in aerobic environment as do heterocystous genera. However, the lack of a significant correlation between cyanobacterial toxin concentration and inorganic nutrients (nitrate, ammonium, soluble reactive phosphorus), as found by Wicks & Thiel (1990), has been ascribed to the presence of excessive amounts of these nutrients in Hartbeespoort Dam, South Africa.

2.1.2.6.2 Laboratory studies

It has been shown that the toxicity produced per unit biomass of organism remained constant when cells of *Microcystis* were transferred from phosphate rich to phosphate-free growth media. However, the removal of nitrate and bicarbonate from the growth medium resulted in a decrease in toxicity per unit biomass (Codd & Poon, 1988; Anon., 1990). Nitrogen availability significantly affected the cellular toxin content in cyanobacteria (Jones *et al.*, 1994). High toxin production correlated with high nitrogen concentrations. In *Oscillatoria agardhii* strains toxin production depended on phosphorus concentration only at low concentrations of phosphorus and higher concentrations had no additional effect (Sivonen, 1990). Recently, Gross *et al.* (1994) found that nitrogen depletion from the growth medium resulted in a sharp and significant decline of fischerellin production from the cyanobacterium *Fischerella muscicola.* As fischerellin contains at least two nitrogen atoms in its molecule, they explained the decline in terms of a limitation in biosynthetically available nitrogen.

Temperature is also thought to be a key factor in regulating toxin formation. Many cyanobacteria grow best at temperatures greater than 20°C and often near 30°C (Al-Layl, 1989). The maximum toxicity of *Microcystis aeruginosa* cultures was reported to be achieved at temperatures between 18° and 25°C (Wicks & Thiel, 1990). Toxicity decreases after increases in the growth temperature have been reported for Japanese and South African strains of *M.aeruginosa* (van der Westhuizen & Eloff, 1985; Watanabe & Oishi, 1985). However, Sivonen (1990) reported that the optimum temperature for both growth and toxin production by a green strain of *Oscillatoria agardhii* was

identical, but that a red strain of *O. agardhii* produced almost similar amounts of toxin at temperatures from 15^0 to 25^0C. The lowest amount of toxin production by both the strains occurred at 30^0C.

Some laboratory studies have indicated that light intensity may have an effect on toxin production in cyanobacteria (Gorham, 1964; Watanabe & Oishi, 1985; van der Westhuizen & Eloff, 1985; van der Westhuizen *et al.*, 1986; Sivonen, 1990; Utkilen & Gjolme, 1992). Codd & Poon (1988) found no effect of light intensity (in the range of 5 to 50 $\mu E\ m^{-2}\ s^{-1}$) on toxin production by a *M. aeruginosa* strain. In contrast, van der Westhuizen & Eloff (1985) found lower toxin production at very low and high light intensities, with an optimum fluence rate of 145 μmol of photons $m^{-2}\ s^{-1}$ for cyanobacterium *M. aeruginosa* (UV-006). Sivonen (1990) found the highest toxin production in *Oscillatoria* strains occurred at the lowest light intensity of 12 $\mu E\ m^{-2}\ s^{-1}$, whereas Gross *et al.* (1994) reported that a light intensity of 10 $\mu E\ m^{-2}\ s^{-1}$ did not increase the production of fischerellin per unit biomass in the cyanobacterium *Fischerella muscicola.* These conflicting results may be due to spectral differences in light sources used, different behaviour of species and strains, different culture media used, and/or differences in toxin detection methods. Consequently, it becomes difficult to provide a definitive conclusion concerning the effect of light on toxin production.

2.1.2.7 Fate and biological significance of cyanobacterial toxins

2.1.2.7.1 Release of toxins into the water

It is necessary to understand the fate of cyanobacterial toxins released into the water, particularly if that water is to be used for drinking by animals or humans. If the toxins are largely inside the cells, then filtration or scum removal from water should effectively remove the toxins from a waterbody. If, however, the toxins are released from the algal cell into the surrounding water, then cell or scum removal is insufficient (Anon., 1990) and toxins will remain. Limited information is available on the stability and natural degradation in the water of the neuro- and hepato-toxins released from the cells (Kiviranta *et al.*, 1991; Stevens & Krieger, 1991).

Toxin release varies between species of cyanobacteria. Codd *et al.* (1989) observed that in batch cultures of *Microcystis* strains toxin was retained within the cells during the lag and growth phases. After maximum growth was attained, total toxin levels continued to increase but a major redistribution occurred, with the bulk of the toxin being present in the external medium. Similar findings were also reported by Watanabe *et al.* (1992) for *Microcystis* species in hypertrophic Japanese lake and Kiviranta *et al.* (1991) for the cyanobacteria *Oscillatoria agardhii* and *Anabaena circinalis*, from which release of toxins was associated with cell lysis during culture senescence. Nonetheless, a substantial release of microcystins from the actively growing cultures of *Oscillatoria* has been observed. A significant release of the toxins into lake water during the course of bloom development but before the bloom aged and degenerated has also been reported. Thus it is apparent that there is limited release of toxin from the cyanobacteria during

their growth phase, although the greatest release occurs upon cell death (Anon., 1990). These findings are of significance in terms of understanding the changes that occur in toxin concentrations during cyanobacterial bloom development in natural waters and reservoirs (Codd *et al.*, 1989).

Unfortunately, the effect of different environmental conditions on the release of toxins from the cells has not been studied in great detail. Penaloza *et al.* (1990) suggested that increasing temperature causes the release of toxins into the medium, whereas Sivonen (1990) reported that the leakage of toxin from the cells was higher at high light intensities.

2.1.2.7.2 Effect of toxins on aquatic life and food chains

The neurotoxins and hepatotoxins are certainly the most dangerous cyanobacterial compounds, but they are by no means the only bioactive chemicals synthesized by these microorganisms which produce an array of other cytotoxic substances that can harm cells but not necessary kill multicellular organisms (Carmichael, 1994). In addition, several cytotoxins show promise as useful algicides and bactericides.

In New Zealand, Vincent (1989) has reported that in two eutrophic lakes, Lake Okaro and Lake Rotongaio, zooplankton species were inhibited by substances that were dissolved within the water and which were thought to be released by cyanobacteria. Cyanobacterial neurotoxins and hepatotoxins can be extremely harmful not only to birds, cattle, horses and other large animals but also to minute animals (zooplankton) living

in lakes and ponds. These toxins may be directly lethal or they may reduce the number and size of offspring produced by zooplankton that feed on cyanobacteria (Carmichael, 1994). The toxicity of *Microcystis* to zooplankton has been studied extensively (Watanabe *et al.*, 1992). Laboratory experiments with purified microcystin have shown that isolated members of the aquatic zooplankton community are susceptible to this toxin. The toxin can be lethal to single-celled animals and *Daphnia* species that are exposed to the toxin (Anon., 1990). Carmichael (1994) reported that zooplankton species generally do not brouse on cyanobacteria capable of producing toxins unless there is no other food present. However, if feeding becomes necessary then, they often attempt to modulate their intake to ensure that they avoid a lethal dose.

The death of fish caused by cyanobacterial toxins has also been reported (Carmichael *et al.*, 1985; Phillips *et al.*, 1985; Flint, 1966). Penaloza *et al.* (1990) found a partially soluble component of the lethal fraction of toxin from *Microcystis* sp. which caused the death of the fish *Gambusia affinis*.

Lawton & Codd (1988) reported the adverse effects of hepatotoxins from *Microcystis aeruginosa* on the freshwater ciliate *Paramecium primaurelia.* The toxin has been reported to have inhibitory and lethal effects on the physiology, behaviour and the survival of the *Paramecium.*

2.1.2.7.3 The biological functions of toxins

Although the structure and basic toxicological properties of several toxins are known,

their biological functions are not. Some *Microcystis* hepatotoxins have been shown to be toxic to filter feeding water fleas (*Daphnia*), which are potential consumers of the microorganism and to some protozoan ciliates (Anon., 1990). DeMott *et al.* (1991) reported that the hepatotoxins microcystins and nodularin were potent inhibitors of invertebrate grazers in the aquatic environment. It is likely that cyanobacteria synthesize toxins as protection against other planktonic species. It is possible, though, that the protective effect is incidental. The toxins may once have had some critical function that is now lost. This likelihood is suggested by the fact that microcystins and nodularins act on the protein phosphatases that regulate the proliferation of eukaryotic cells. The hepatotoxins do not now seems to participate in cell function and cell division in cyanobacteria, but they may have played such a role as cell division regulators early in the evolution of these organisms (Carmichael, 1994).

Although hepatotoxins and neurotoxins produced by cyanobacteria do not have any affect on green algae (Foxall & Sanser, 1981; Chapter 4 of this thesis) there are however, allelopathic roles of some of other cyanobacterial toxins have been suggested (Maestrini & Bonin, 1981; Rice, 1984; Gross *et al.*, 1991 & 1994).

2.1.2.8 Procedures for detection of cyanobacterial toxins

It is not possible to determine the toxicity potential of cyanobacteria by their culture morphology (Lahti *et al.*, 1995). Methods of detection of cyanobacterial toxins must use bioassay, chemical assay and immunoassay techniques.

Detection of biotoxins from cyanobacteria has largely been dependent on the intra-peritoneal injection of toxic extracts into laboratory mice. The method's advantages are that it is easy to use, inexpensive and can detect, within few hours, the qualitative and quantitative characteristics of any toxin present. Based on the symptoms observed in the mouse, neuro- and hepato-toxins can be distinguished. However, using this technique the detection limit is quite high and it cannot detect low concentrations of toxin (Carmichael, 1992). Recently, Lahti *et al.* (1995) proposed a simple and easy-to-use bioassay employing *Artemia salina* (brine shrimp) larvae. This method can detect both the hepatotoxin and neurotoxin at concentration limits lower than is the case with the mouse bioassay.

Although the bioassay provides a general assessment of toxicity, it is necessary to determine the concentrations of the toxins more accurately by chemical assay. Chemical assay methods developed for neurotoxin and hepatotoxin include high performance liquid chromatography (Meriluoto & Eriksson, 1988; Harada *et al.*, 1988), thin layer chromatography (Ojanpera, 1991), high performance thin layer chromatography (Al-Layl *et al.*, 1988; Codd *et al.*, 1988), GC/ECD and fast atom bombardment mass spectroscopy (Stevens & Krieger, 1991). However, chemical assays *per se* can not predict the biological potencies of the active compound. Therefore, there remains a need to use a rapid bioassay in conjunction with accurate chemical analysis.

A further approach which may offer a rapid and simple screening test is that of immunoassay (Shi *et al.*, 1995; Carmichael, 1992; Anon., 1990). The technique is based

on the production of antibodies against the toxin and the conjugation of a specific enzyme to them (Chu *et al.*, 1990; Anon., 1990). When the antibodies attach to the toxin, the enzyme can be used to produce a secondary but non-related reaction with appropriate reagents to give an easily detectable end-point. This is the basis of the enzyme-linked immunosorbent assay (ELISA) (Anon., 1990).

Detection of cytotoxins (allelochemicals) from cyanobacteria can also be achieved using antimicrobial bioassays, liquid culture assays, agar zone diffusion assays, chromatographic techniques such as thin-layer chromatography, high-performance liquid chromatography or gas chromatography/mass spectrometry. Flores & Wolk (1986) used an agar zone diffusion bioassay system to detect the presence of bacteriocin and other antibiotic like substances from cyanobacteria. Antimicrobial activity produced by the cyanobacterium *Fischerella muscicola* has been detected under laboratory conditions using both liquid culture (Gross *et al.*, 1991) and agar zone diffusion assay methods (Srivastava *et al.*, 1994; Gross *et al.*, 1991).

An analytical problem limiting research has been the lack of well established and validated methods to enable the accurate quantification of toxin concentrations. Additionally, especially for cytotoxins having a wide spectrum of bioactivity against other algae and cyanobacteria, there is an insufficient data-base for these toxins as compared to other biotoxins of importance in the food chain. Nevertheless, it is important to study these toxins as bioactive substances because production of these allelochemicals (algicides) may disturb the aquatic ecosystem by causing the biotoxin

producer cells to burst and thus facilitating the sudden release of toxins into the water. However, the difficulties of developing and applying methods of detection and quantification are compounded by the complexity of the samples and the need to separate the toxins from other constituents (Anon., 1990).

2.1.3 Aims of this investigation

Cyanobacteria capable of producing cytotoxins (allelochemicals) can destabilise the aquatic ecosystem by exerting their adverse effects on other algae and cyanobacteria, the primary producers of the aquatic ecosystem (see Figure 2.14). Interest in these biotoxin producing cyanobacteria have attracted increasing attention over the last twenty five years. Given the importance of these cytotoxin (allelochemicals) producers, it was decided to screen available cyanobacterial species including those isolated and established in culture from New Zealand sources. The toxin preparation approach most commonly favoured and used here was that of solvent extraction after cell disruption then followed by toxin concentration. It was also decided to study the cyanobacterium *Fischerella muscicola.* This cyanobacterium was chosen because the initial work of Flores & Wolk (1986) indicated that the antibiotic production by this cyanobacterium kills all indicator strains tested to date. Furthermore, the active compound, named fischerellin has been isolated and partially chemically characterized (Gross *et al.*, 1991).

To gain a better understanding of the production and leakage of cyanobacterial cytotoxins (allelochemicals), *F. muscicola* was grown in pure batch culture to establish its growth characteristics. Subsequently, the toxin production and excretion by the

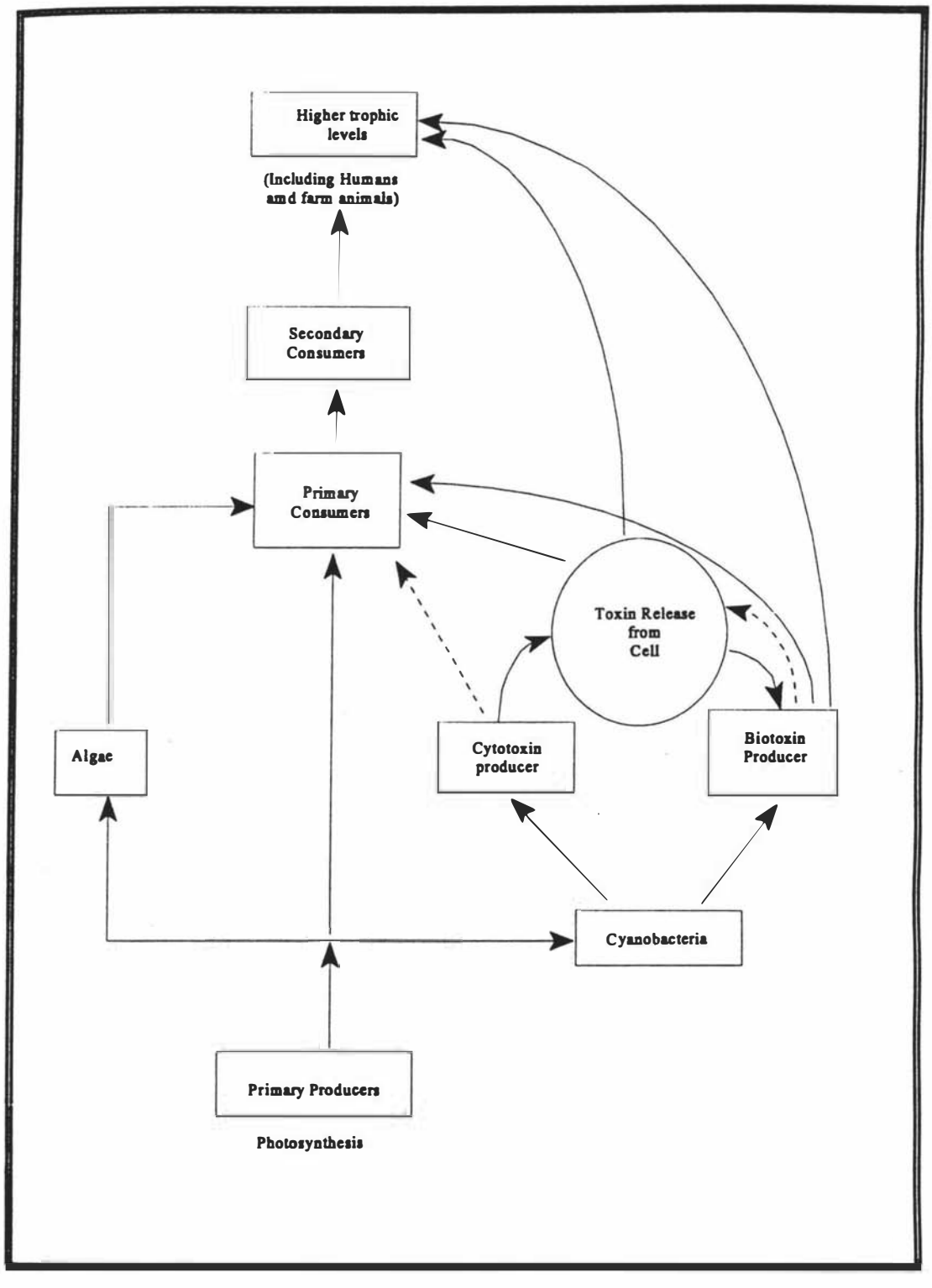


Figure 2.14. General scheme of toxin pathways through the food chain (adapted from Ikawa and Sanser, 1990).

culture was monitored. The study also provided some insight into the importance of culture age on toxin synthesis and its release.

Studies on the regulation of toxin(s) production have focused on the possible role of growth conditions on toxin production. It is possible that nutrient composition, temperature and light may independently or in combination influence toxin production. Thus, the role of environmental factors on the toxin production was also investigated.

Chapter 3

General Materials and Methods

3.1 Organisms

3.1.1 Cyanobacteria

The following cyanobacteria were used in the present study; *Fischerella muscicola* (UTEX 1829), *Anabaena flos-aquae* (Lyng.) Breb. (UTEX 1444), *Fischerella ambigua* (UTEX 1903), cultures were obtained from the culture collection of algae at the University of Texas (UTEX) at Austin, Austin, Texas.

New Zealand isolates of cyanobacteria were; *Nodularia spumigena* (strain M155, Lake Ellesmere, a hepatotoxin producer, *Oscillatoria* sp., *Oscillatoria* -2 M194 rt, *Oscillatoria* M193 rt, *Scytonema* sp. M53. All cultures were obtained from the Culture Collection of Plant Biology and Biotechnology Dept. of Massey University, Palmerston North.

3.1.2 Characteristics of genus *Fischerella*

Of special interest in this research was the genus *Fischerella.* Species of *Fischerella* are filamentous, heterocystous and nitrogen fixing. (see Figure 3.1). The true branches of this organism are uniseriate and composed of cells that are generally longer than broad, particularly those distal from the base. The axis (primary trichome) from which brances arise is mainly uniseriate as well but may become multiseriate in part, with divisions in more than one plane.

Heterocysts of *Fischerella* are elongate, spherical or even compressed (shorter than

broad) and are lateral, terminal or intercalary.

Fischerella forms can be found in hot springs and moist sub-aerial habitats. Marine forms are rare or lacking. Species of *Fischerella* reported from New Zealand environments are - *F. ambigua*, *F. moniliformis* and a thermophilic strain *Fischerella* as *Mastigocladus laminosus* PCC 7522 (ATCC 29539, Oregon NZ-86-m) isolated from a hot spring, Whakarewarewa (Castenholz, 1989).

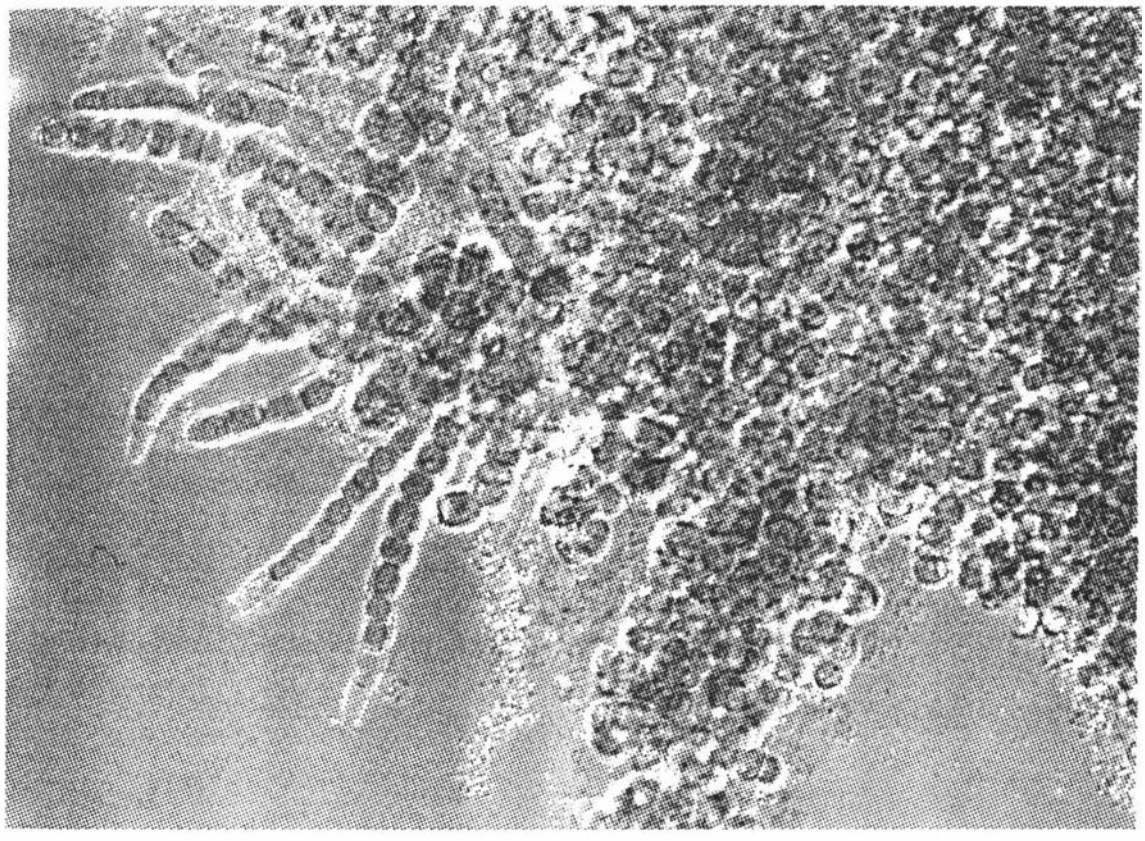


Figure 3.1. Light micrograph of filamentous cyanobacterium *Fischerella muscicola* (UTEX 1829), grown in Allen's medium (5 days old culture) at light intensity of 10 μmol m^{-2} s^{-1} and temperature 10°C (400 x magnification).

3.1.3 Chlorophyceae (Green algae)

The green algal cultures used in this study were all New Zealand isolates and maintained in the culture collection of Department of Plant Biology and Biotechnology, Massey University, Palmerston North.

The following species were used: *Scenedesmus* sp., *Chlorella* sp., *Pandorina* sp., *Oocystis* sp. and *Chlamydomonas* sp.

3.1.4 Xanthophycean alga

Heterococcus sp. (NZ isol.) was obtained from the culture collection of Department of Plant Biology and Biotechnology, Massey University.

3.1.5 Eubacteria

The following cultures were used and obtained from the culture collection of the Process and Environmental Technology Department, Massey University, Palmerston North: *Bacillus coagulans* ex FT N7 (45°C), *B. cereus*, *B. subtilis*, *Enterobacter aerogenes*, *Escherechia coli*, *Klebsiella pneumoniae* ex Vet. Sci. Dept., *Micrococcus luteus*, *Pseudomonas aeruginosa*, *P. florescence*, *Serratia marcescens*, *Staphylococcus aureus* 305, *Enterococcus faecalis*, *Lactococcus lactis* PL 183 4193 Fu^{n}.

3.2 Culture media

3.2.1 For cyanobacteria (fresh water)

The cyanobacterial cultures obtained from UTEX were grown (batch culture) and maintained in Allen's culture medium (Starr and Zeikus, 1987). The composition of Allen's culture medium is given in Table 3.1. The pH of the medium was adjusted with 1M HCl to 7.8.

Table 3.1. Composition of Allen's medium (Star & Zeikus, 1987).

Component	Weight/Volume	Stock: g/200 ml H_2O
$NaNO_3$	1.5 g	-
K_2HPO_4	5.0 ml	1.5
$MgSO_4.7H_2O$	5.0 ml	1.5
Na_2CO_3	5.0 ml	0.8
$CaCl_2.2H_2O$	10.0 ml	0.5
$Na_2SiO_3.9H_2O$	10.0 ml	1.16
citric acid	1.0 ml	1.2
PIV metal solution[1]	1.0 ml	

1- See Table 3.2 for the composition of PIV metal solution.

The above weight/volumes were added to 966 ml of glass distilled water to make up one litre of the culture medium.

PIV metal solution : 0.75 g of Na_2EDTA was added to 1000 ml of glass distilled water and dissolved fully. Then the following salts were added in the amounts indicated:

Table 3.2. PIV metal solution (Star & Zeikus, 1987).

Component	Weight (mg)
$FeCl_3.6H_2O$	97
$MnCl_2.4H_2O$	41
$ZnCl_2$	5
$CoCl_2.6H_2O$	2
$Na_2MoO_4.2H_2O$	4

New Zealand isolates of cyanobacterial cultures were grown (batch culture) and maintained in Bold's 3N Bristol's Solution. The recipe of Bold's 3N Bristol's solution is given in the Table 3.3.

Table 3.3. Composition of Bold's 3N Bristol's (Star & Zeikus, 1987).

Solution	Volume (ml)
Bristol's $NaNO_3$ stock soln[1]	20
PIV metal solution	6
vitamin B_{12}[2]	1
soilwater (GR +) supernatant[3]	40

1- Bristol's $NaNO_3$ stock solution: 10.0 g of $NaNO_3$ was dissolved into 400 ml of glass distilled water.
2- vitamin B_{12}: 15.0×10^{-6} g of vitamin B_{12} was dissolved into 100 ml of glass distilled water.
3- soilwater (GR+) supernatant: garden soil 1 tsp + 200 ml of glass distilled water + a pinch of $CaCO_3$. The medium container was covered and steamed for 2 h on each of 2 consecutive days.

3.2.2 For cyanobacteria (marine)

The *N. spumigena* was grown in an Enriched Sea Water medium (Starr and Zeikus, 1987). Sea water was collected from near Lyall Bay, Wellington, New Zealand. The formula for the medium is given in Table 3.4.

Table 3.4. ES-Enrichment for sea water medium (Star & Zeikus, 1987).

Component	Weight/ Volume
Glass distilled water	100 ml
$NaNO_3$	350 mg
Na_2 glycerophosphate	50 mg
Fe-solution*	25 ml
PII metals*	25 ml
Tris buffer (Sigma Co.)	500 mg
Vitamin B_{12}	10 µg
Thiamine	0.5 mg
Biotin	5 µg

* Description of special solutions are given in text.

The pH of ES-Enrichment solution was adjusted to 7.8 and then dispensed in tubes (20 ml/tube) and autoclaved at 121°C for 20 minutes. Sea water was filtered through Whatman No. 1 filter paper. A 20 ml volume of ES-Enrichment medium was added to 1000 ml of filtered sea water.

Fe-solution

In 500 ml of glass distilled water, 351 mg of $Fe(NH_4)_2(SO_4)_2.6H_2O$ and 300 mg of Na_2EDTA were dissolved.

PII metal solution

The formula for PII solution is given in Table 3.5.

Table 3.5. Composition of PII metal solution (Star & Zeikus, 1987).

Compound	Weight (mg)/Vol. (ml)
Na_2EDTA	100.0
H_3BO_3	114.0
$FeCl_3.6H_2O$	4.9
$MnSO_4.H_2O$	16.4
$ZnSO_4.7H_2O$	2.2
$CoSO_4.7H_2O$	0.48
Water, glass distilled	100 ml

3.2.3 For chlorophycean and xanthophycean algal cultures

Chlorophycean and xanthophycean algal cultures were also maintained in Bold's 3N Bristol's solution. See Table 3.3 for recipe.

3.2.4 For eubacterial cultures

The bacterial cultures were maintained in Brain Heart Infusion (BHI) agar purchased from GIBCO BRL. Life Technologies, UK.

Table 3.6. Composition of Brain Heart Infusion (BHI) medium.

Component	Weight (gm)
Calf brain infusion solids	12.5
Beef heart infusion solids	5
Proteose peptone	10
Glucose	2
NaCl	5
Di-sodium phosphate	2.5

To prepare the BHI agar medium, a 37 g amount was added to 1000 ml distilled water

and the pH of the medium was adjusted to 7.4 ± 0.2.

3.3 Chemicals

All chemicals used for media preparation and analytical work were of analytical grade. Those which were commonly used in the experiments and their sources, are listed below.

Ajax Chemicals (Sydney, Australia)

di-potassium hydrogen phosphate; citric acid.

BDH Chemicals Ltd (Poole, England)

calcium chloride; cobaltous chloride; ferric chloride; manganous chloride; magnesium sulphate; methanol; sodium carbonate; sodium nitrate; sodium sulphate; sodium silicate; *tert*-butylmethyl ether; zinc chloride.

May & Baker Ltd (Dagenham, England)

sodium molybdate.

3.4 Sterilization of culture media

Allen's culture medium and Bold's 3N Bristol's solution were sterilized at 121°C for 20 minutes. The pH of Allen's culture medium was adjusted to 7.8 before sterilization.

After cooling overnight at room temperature the medium pH was brought back to its original value of 7.8. Allen's or Bold's 3N Bristol's solution agar plates were prepared by adding to the liquid medium 10% (w/v) agar (Bacteriological agar, GIBCO BRL, Life Technologies, UK), dissolving the agar by boiling and then autoclaving as above. After cooling it was dispensed into presterilized plastic 9 cm diameter petri-dishes. The bacterial culture medium (Brain Heat Infusion) was also sterilized at 121°C for 20 min.

3.5 Cleaning of glassware

All culture flasks were cleaned with "Decon 90" (Decon Laboratories Ltd., England). A 2.5% (v/v) solution of Decon 90 was prepared and all glassware was fully immersed in the solution overnight. They were rinsed thoroughly with distilled water immediately upon removal from the cleaning solution and air dried carefully at 70 - 80°C. All the other glassware was washed in dishwasher (Mielabor, Miele, Automatic G 7733, Germany) with "dried decon" (Decon laboratories Ltd., England). Glassware used for the neuro-toxin experiment was treated with 0.1M NaOH for 24 hours prior to washing in the dishwasher. Heapto-toxin exposed glassware was treated with sodium hypochlorite for 24 hours at room temperature before washing in the dishwasher.

3.6 Growth conditions for cyanobacterial and algal culture

Cyanobacterial cultures were cultivated in 250 ml Erlenmyer's flasks at a constant

temperature of 25°C and at 100 rpm in an Environ Shaker - 3597-1234.10 (Labline Instruments Inc., Illinois, USA), illuminated with daylight tubes (Duro-Lite, TRUE-LITE, USA) at an intensity of 60 - 100 μmol m^{-2} s^{-1} as measured by a light meter (LI-189 Quantum/Radiometer/Photometer, LI-COR, inc., USA) fitted with LI-193SB Spherical Quantum Sensor. A 16 hour light and 8 hour darkness cycle (16:8 h LD) was provided. In all cases a 10% (v/v) inocula of exponential phase cultures were used.

Other algal cultures were cultivated in 250 ml Erlenmyer's flasks at a constant temperature of 20°C under a light bank with 16:8 LD cycle illumination provided by daylight tubes at ~100 μmol m^{-2} s^{-1}. No shaking was used for these cultures.

3.7 Inoculum preparation of cyanobacterial cultures for experimental flasks

Cyanobacterial cultures were subjected to "re-freshing" before growing them for inoculation into experimental flasks. Re-freshing of the culture was performed by first growing the cyanobacterial cultures in Allen's medium until they reached the mid - exponential phase (5 days old culture) and then subculturing them into a new flask of the same medium and growing them until they again reached the mid - exponential phase. This culture was then used as the inoculum. To increase the culture density the re-freshing and inoculum flasks' culture volumes were usually 1000 ml. Cell concentration was achieved by filtering the cultures through a presterilized 20 μm nylon net or 0.45 μm membrane filter (Millipore Corporation, Bedford, MA) in a filtration assembly under aseptic conditions. The cells were then resuspended in a minimal

volume of Allen's medium and the suspension used as inocula for experimental flasks.

3.8 Growth conditions for bacterial cultures

All the bacterial cultures were grown in Brain Heart Infusion (BHI) medium incubated at 30°C except for *Bacillus coagulans* ex FT N7 which was incubated at 45°C . Forty eight hours old cultures were used.

3.9 Growth measurement parameters

3.9.1 Determination of Biomass Concentration

Biomass of *F. muscicola* was determined by the following gravimetric method:

- A 0.45 µm pre-sterilized membrane filter (Millipore, cellulose nitrate, plain white, 47 mm diameter) was labelled and pre-dried overnight at 105°C (Contherm Digital Series Oven, Contherm Scientific Company, Lower Hutt, New Zealand).
- The filter was then cooled in a desiccator and weighed (Mettler AE 160 digital balance, Watson Victor, Wellington, New Zealand) after equilibration.
- The sample of up to 100 ml was filtered through the membrane filter under vacuum. The biomass was retained on the wet filter.
- The wet filter was then dried at 105°C overnight, and was subsequently

weighed after equilibration in a desiccator.

The biomass was calculated using the following formula:

$$\text{Dry Weight (mg/L)} = \frac{\text{Net weight on filter (mg)}}{\text{Volume of sample (ml)}} \times \frac{1000\ \text{(mg)}}{1\ \text{(l)}}$$

3.9.2 Optical density measurement of cyanobacterial growth

Growth was monitored by determining the optical density at 750nm, using a Phillips PU 8625 uv/vis spectrophotometer. This chosen wavelength avoided interference caused by photosynthetic pigments.

3.9.3 Cell counting for chlorophycean alga *Scenedesmus* sp.

For each sample at least six sub-samples were counted using a Fuchs-Rosenthahl haemocytometer. Samples homogeneity for cell counting were ensured by shaking the culture flasks before taking the sample out.

3.9.4 Protein determination

Protein concentrations were measured as an indicator of cyanobacterial cell growth. Bio-Rad Protein Assay (Bradford dye-binding procedure), was used.

- A 10 ml sample of *F. muscicola* culture was taken to be measured and cells spun down (Megafuge 1.0, Heraeus, Sepatech, W. Germany) at 5800 xg for 10 min at room temperature. Cells were resuspended in 5 ml of "milli-Q" water and disrupted at 0°C for 3 minutes using a SONIPREP MSE 150 ultrasonic disintegrator (MSE Scientific Instruments, England). Treated suspensions were then filtered through 0.45 µm (Millex-GS, Millipore Corporation, Bedford, MA filter. Triplicate samples of 800 µl were diluted with 200 µl dye reagent concentrate then mixed and measured at 595 nm against a reagent blank. Protein concentrations were read from a standard curve (see Appendix A) constructed with known concentrations of bovine serum albumin (BSA).

3.10 Other analytical methods

3.10.1 Measurement of Dissolved Oxygen

Dissolved oxygen concentrations of cyanobacterial cultures were measured directly from the culture flasks (Kiviranta *et al.*, 1991) using a dissolved oxygen analyzer (model 7932 Portable DO meter, Leeds & Northrup Company - D.B. 3572, North Wales, PA 19454, USA) equipped with an oxygen electrode (Nester Inst., N. Wales, PA 19454). The instrument was calibrated by using distilled water at a known temperature, chloride free, saturated with air water and an oxygen solubility table.

3.10.2 pH measurement

All pH measurements were performed using a PHM61 Laboratory pH meter (Radiometer, Copenhagen, Denmark) which was calibrated prior to use using pH 4.0 and pH 7.0 buffers, BDH Chemicals Ltd., Poole, England.

3.10.3 Microscopic examination

For cell counting an inverted microscope with phase contrast (Will, Wilovert, Wetzlar-21, GMBH D 6330, W. Germany) was used. A Namaski microscope (El- Einsatz 45 18 87, Zeiss, W. Germany) fitted with camera (Zeiss, 45, 60, 70, Germany) was used for photography of the cultures of the cyanobacterium *F. muscicola.*

3.11 Cyanobacterial toxicity testing

Cyanobacterial toxicity testing was carried out using an agar zone diffusion assay system (Cannell *et al.*, 1988). Fresh unbroken cells, cell free extracts and culture broths were all tested for their inhibitory activity towards the indicator organism.

Cyanobacterial (NZ isolates) toxicity testing was also carried out using liquid cultures of *Scenedesmus* sp.

3.11.1 Preparation of cyanobacterial samples

Cyanobacterial cells were harvested by centrifugation (Megafuge 1.0, Heraeus, Sepatech, W. Germany) at 5800 xg for 10 min at room temperature. They were then extracted twice with methanol (AR grade) in an ultrasonic bath (model ME 2.1, Mettler Electronics, USA). The combined extracts were rotary evaporated (Rotavapor R110, Buchi, Laboratoriums - Technik AG, CH 9230, Flawil/Schweiz, Switzerland) at 45°C to dryness and the residue re-dissolved in 2 ml methanol. This crude methanolic extract was then used for toxicity testing.

The culture supernatants (after centrifugation of cyanobacterial cells) were lyophilized (model 10-020, Virtis Company Inc., NY, USA) and re-dissolved in 2 ml methanol. This crude methanolic preparation of culture broths were also used for toxicity testing.

F. muscicola cultures were harvested by filtration onto 20µm nylon net (C.C.G. Industries, Auckland, New Zealand). Cells were extracted in an ultrasonic bath (model ME 2.1, Mettler Electronics, USA) three times with methanol each for 15 min. An equal amount of citric acid-citrate buffer (10mM HCl adjusted with 10mM sodium citrate solution [2.1 g of citric acid hydrate dissolved in 20 ml of 1 M sodium hydroxide solution and made up to 1 litre with distilled water] to pH 2.2) was added to the combined extracts. After filtration through a Whatman No.1 filter paper the extract was partitioned twice with half its volume of *tert*-butyl-methylether (HPLC grade). The two ether phase fractions were combined, dried over anhydrous sodium sulfate and

evaporated to dryness in a rotary evaporator at 45°C. The residue was redissolved in 1.5 ml of methanol. This methanolic crude extract was then either subjected to toxicity testing or HPLC analysis (see Chapter 5).

The toxic material from each culture broth was collected by passing it through a C18-cartridge - Waters tC18 environmental cartridge (Millipore Corporation, Milford, MA) and subsequently eluting the cartridge first with 5 ml of methanol and afterward with 5 ml of *tert*-butylmethylether. The eluate was evaporated under vacuum, redissolved in methanol and subjected to the agar zone diffusion assay and HPLC separation.

3.11.2 Agar zone diffusion method

The sample (methanolic extract) was placed into wells aseptically cut into agar plates seeded with indicator cells. Allen's culture medium containing 1% (w/v) agar (15 ml containing 1 ml of indicator organism) was poured into petri dishes (9 cm diameter). When set, wells were bored in these plates using a sterile cork borer (8 mm diameter) giving a well volume of 130 µl. These wells were filled with the sample (40 µl). The plates were incubated initially at +4°C for 2 hours to prevent the rapid evaporation of the solvent and thus allowing diffusion of the sample into the agar, then further incubated at 25°C, illuminated with daylight tubes 100 µmol m^{-2} s^{-1} (16:8 hour LD cycle) under the light bank. Zones of growth inhibition could be seen after 5-10 days of incubation as areas of clearing around the well. Zone diameters (in mm) were measured to provide a quantitative estimate of the inhibition. An average of four readings for each zone was taken.

For initial screening of inhibitory activity of the organism, a slightly different method of agar zone diffusion was adopted. Filaments of cyanobacterial cultures were toothpicked and spotted on to petri dish containing Allen's agar medium. This was then incubated under the light bank and allowed to grow for 4-5 days at 25°C. Then a suspension of agar media (10 ml) containing the indicator cells was overlaid. Growth inhibition zones could be seen after a further 5-10 days of incubation as areas of clearing around the well. Zone diameters were measured four times for each well.

To test inhibition of bacterial cultures, molten BHI agar (15 ml at 50°C) seeded with 100 µl bacterial culture (48 hours old) was poured into petri dishes. When set, wells were bored in these plates as described above and a 40 µl sample used. The plates were incubated initially at +4°C for 2 hours to prevent the rapid evaporation of the solvent and thus allowing diffusion of the sample into the agar, then further incubated at 30°C and 45°C, depending on the culture used. Plates were read after 36 hours as described above.

3.11.3 Toxicity testing of crude methanolic cell extracts using liquid culture

A liquid culture of *Scenedesmus* sp. was used for toxicity testing. Cultures were grown in 250 ml Erlenmyer flasks with a culture volume 50 ml. At the onset of exponential phase a 250 µl of methanolic crude extract was added under aseptic conditions. Control flasks received an equal amount of methanol solvent. Cell numbers were recorded to monitor growth using haemocytometer.

3.12 Toxicity testing of pure hepato- and neuro-toxin of cyanobacteria against green algae

Pure Microcystin *-LR & -RR* hepato-toxins were purchased from Calbiochem, Sydney, Australia. Neurotoxin (+)-Anatoxin-a.HCl was purchased from Biometric Systems Incorporated, Minnesota, USA.

The toxicity tests using these three known cyanobacterial toxins were carried out using liquid cultures of the green alga *Scenedesmus* sp. as follows :

Fifty ml cultures were grown in 250 ml Erlenmyer flasks. Growth was routinely monitored by taking samples aseptically every 12 hours for cell counts. Once cultures had entered the exponential phase the toxins were added under aseptic conditions. The concentrations tested for each of three toxins were as follows: Microcystin-LR; 0.1, 0.5, 2.0 & 5.0 µg/ml; Microcystin-RR; 0.1, 0.5, 2.0 & 5.0 µg/ml; Anatoxin-a; 0.1, 1.0, 10.0, & 20.0 µg/ml. These solutions were prepared as follows :

Because microcystin is not easily soluble in water it was first dissolved in 100 µl ethanol and then diluted with distilled water. This solution was then sterilized by passing through a Millipore filter (0.45 µm) obtained from Millipore Corporation, Bedford, Massachusetts, USA. Anatoxin-a stock solution was prepared in distilled water (pH adjusted to 4.0 with 0.1 N HCl) and then filter sterilized as described previously.

3.13 High Performance Liquid Chromatography (HPLC) analysis of methanolic crude extract of *F. muscicola*

The HPLC analysis of toxic material was carried out using the method developed by Gross, *et al.*, (1991). Further details of this method have been described in Chapter 5.

Chapter 4

Preliminary Screening for Toxin Producing Cyanobacteria

4.1 INTRODUCTION

In eutrophic fresh water lakes and ponds cyanobacteria often develop blooms. These bloom forming species are also known to produce a wide variety of chemically unique secondary metabolites including toxins that have harmful effect on other organisms. It is not clear why cyanobacteria produce toxins but a role in the seasonal succession of algae in a fresh water ecosystems have been proposed (Gross *et al.*, 1991). Also, Keating (1977, 1978) suggested that allelopathy is one reason for the rapid dominance of cyanobacteria in eutrophic lakes.

To study the possibility of an allelopathic role of these cyanobacterial toxins, a screening programme was conducted to detect those cyanobacteria which produce substances (bioactive material) that are inhibitory towards both algae and other cyanobacteria. Chemical or physicochemical assays of the bioactive compound alone can not give completely satisfactory results because the identification and confirmation of a compound's structure can not predict its biological activity. Therefore, it is necessary to conduct a biologically based screening test (bioassay) to provide the means by which novel bioactive compounds can be detected. The most important function of the bioassay is to detect the inhibitory activities of metabolites produced by the cyanobacteria under investigation. Often these biologically active compounds are present in minute quantities. Their detection, therefore requires the use of assay systems exhibiting maximum sensitivity. The two most commonly used antimicrobial assay methods are: (1) a liquid culture procedure and (2) an agar (zone diffusion) plate method. The basis

of both these techniques is a quantitative assessment of the effect of the test substance on the growth of an indicator microorganism in a nutrient medium. Both methods have advantages and disadvantages (see below).

4.1.1 Liquid culture method

Practical details concerning the crude methanolic extracts testing have been presented in Chapter 3. Initially this method was adopted in the current studies because it measures the total antibiotic activity the whereas agar plate assay method, which is dependent on the diffusion rate of the active substance, may not measure total activity. The inhibition of growth observed in flask cultures is the basis for calculating toxin potency. Although it is a frequently used assay method, it has disadvantages. Firstly, accessing the culture periodically for sampling increases the risk of contamination of the axenic culture, thus leading to false observations. This may contribute towards lack of reproducibility using this method. Secondly, a bias in the results may arise from the possible presence of nutrient substances in the sample which, by enriching the medium, could increase the growth rate of the target culture thus leading to a low bias. These disadvantageous factors necessitated the use of the agar diffusion screening method when conducting cyanobacterial bioassay.

4.1.2 Agar zone diffusion method

This is the most commonly used method for assaying antimicrobial activity (Bloor,

1989). Small wells were made in the seeded agar using a sterile cork borer. This well was then filled with the standard volume (to fill almost to the brim) of sample to be assayed. After incubation clear zones surrounding the well signified inhibition, whereas in other parts of the plate growth of indicator cells were seen. Zone boundaries were usually clearly defined, although the sharpness of definition depended upon the test organism, the density of the inoculum and the sample used for assay. The diffusion coefficient of the antibiotic is known to be a function of the concentration, the temperature and the viscosity of the solvent in which the antibiotic is suspended (Hewitt, 1977). This system is very simple and rapid. Test conditions can be controlled to ensure reproducibility in from day to day. Because of the procedure's ease of use, reproducibility and repeatability, it was adopted for all bioassay analyses throughout this project. The assay was standardized to give large and clear zones of inhibition with a minimal inoculum density of the test organism. The physiological state of the indicator cells were also standardized so as to decrease the potential for errors involved with the assay.

The purpose of the experimental work described in this chapter was to study the toxic effect exerted by cyanobacterial secondary metabolites on other algae and cyanobacteria and to locate a cyanobacterium capable of producing toxic metabolites. This work was carried out as follows. Firstly, to test the inhibitory effects of commercially available cyanobacterial neuro- and hepato-toxins on the New Zealand isolates of green algal species. Secondly, to test the inhibitory activity of New Zealand isolates of cyanobacterial species against both cyanobacteria and green algal species. The

filamentous nitrogen fixing genus *Fischerella* is known to produce toxic substances but as yet there are no detailed reports concerning this planktonic cyanobacterial genus (Carmichael, 1992). *F. muscicola* has been reported to synthesize substances which are inhibitory towards other cyanobacteria and algae (Flores & Wolk, 1986; Gross *et al.*, 1991). The third aspect of the present study was to test *Fischerella* for cytotoxin production.

4.2 MATERIAL AND METHODS

4.2.1 Pure toxins

Inhibitory effect of Anatoxin-a, a neuro-toxin, Microcystin-LR and -RR, both hepato-toxins, were tested against *Scenedesmus* sp. using shake flask cultures. The hepato-toxins were purchased from Calbiochem, Australia and stored at -18°C until ready for testing. The neuro-toxin was purchased from Biometric Systems Incorporated, USA and stored at room temperature until ready for screening. See chapter 3 for practical details concerning the toxicity test.

4.2.2 Organisms and growth conditions

The cyanobacteria used for preliminary screening are listed in Chapter 3 section 2.1.1. *Scenedesmus* sp. (a green alga) was isolated from Lake Taupo and an axenic culture was established in the laboratory using the method described by Keating (1976). The culture media and growth conditions were as described in Chapter 3.

4.2.3 Sample collection and preparation

The UTEX and NZ isolates of cyanobacterial cultures were harvested between 10 days and three weeks of growth according to the particular culture being maintained and prepared as described in Chapter 3. In addition, *Fischerella ambigua* (UTEX 1903) was

tested for the production of toxic substance(s). Colonies of *Fischerella ambigua* were transferred to Allen's culture medium containing 1% (w/v) agar in a petri plate. A bioassay was performed using the agar zone diffusion assay procedure (see Chapter 3 for method).

4.2.4 Antimicrobial assays

All indicator organisms used in the antimicrobial assays were cultured as described in Chapter 3. The bioassay procedures for both liquid culture and agar zone diffusion assay methods are described in Chapter 3.

For all liquid culture assays two separate controls (flasks) were set up; control one received no addition (neither solvent nor toxin) and the other control received solvent (methanol) only. The purpose of each control was to test for the growth of the organism without any addition in the first control and to test for, if there is any, an inhibitory effect of solvent (methanol) in the second control.

The agar zone diffusion assay method was devised to provide the widest zone of inhibition possible, using the smallest inoculum of the test organism that gave an easily visualizable zone boundary in visible growth after incubation. Preliminary experiments indicated that an inoculum of optical density (678 nm) of 0.4 units agar gave this required density of growth.

The indicator organisms used in the initial screening were an unidentified *Scenedesmus* sp. and *Anabaena flos-aquae*. Secondary antimicrobial screening organisms are listed in Tables 4.7 and 4.8.

4.2.5 Growth measurement parameters

Samples were taken aseptically at 12 hour intervals. At the onset of log phase crude methanolic cell extracts, lyophilised culture broths and methanol solvent only were added to the respective flasks. Readings (cell counts or optical density measurements) of duplicate flasks were used in this experiment.

4.2.5.1 Cell Counts

Samples for cell counting were taken after shaking the flask to ensure sample homogeneity. Cell counts were done using a Fuchs-Rosenthahl haemocytometer. For each sample at least six sub-samples were counted.

4.2.5.2 Optical density measurement

Aseptically harvested samples (3 ml in triplicate) were used to measure the optical density of the culture at 678nm. Sterile culture medium was used as a blank.

4.2.5.3 Calculation of specific growth rates

According to Hawker & Linton (1979), in a population in which all the cells are actively growing and dividing, the amount of growth (growth increment) during unit time is determined by two factors, the growth rate and the size of the population. During the exponential phase the growth rate remains constant, but in these circumstances the growth increment per unit time will change, since it is proportional to the size of the viable population, which is continuously growing. A thorough discussion of the concepts using eubacterial growth kinetics (binary fission) has been given in text books by Pirt (1975), Hawker & Linton (1979) and Brock *et al.* (1994). Hawker & Linton (1979) state that cells of filamentous cyanobacteria are "potentially capable of binary fission and hence the growth in length of the chain is merely the sum of the growth of the individual component cells". They also stated that during the exponential phase the average cell composition remains constant therefore the same relationship will apply if growth was measured in terms of either cell numbers, dry weight, optical density or nitrogen content. Because of a need for small samples, a direct estimates, and the need to compare the response of one culture to another, the use of optical density measurements seemed more appropriate. Furthermore, eubacterial growth kinetic models provided a means of quantifying growth responses in view of the binary fission potential of cyanobacteria. Eubacterial kinetic methods used for *F. muscicola* studies must be seen as an approximation which enables the experimenter to access these above advantages. Because of the steady increase in the cell mass during growth initial rates were measured. To enable the comparison of one culture to another in terms of growth

response, the specific growth rate (μ) as defined by Pirt (1975) as μ = 1/x.dx/dt was used. The $\log_{10}$ of cell count or optical density readings were plotted against time and the specific growth rate of the organism was calculated using following equation :

Specific growth rate per hour (μ) = 2.303 x slope for the exponential phase.

This is in accord with the mathematical growth defining procedures used by Blackburn *et al.* (1996) and Van Liere & Mur (1979).

4.3 **RESULTS**

4.3.1 Effect of commercial toxins on the target organism

To evaluate the response of the target organisms to the cyanobacterial toxins, tests were conducted using purified commercially obtained cyanobacterial toxins namely the neurotoxin, Anatoxin-a[1] and the hepatotoxins[1], Microcystin-lr and -rr. The concentrations used for anatoxin-a was 0.1, 1.0, 10.0 & 20.0 μg/ml. The concentrations for microcystin-lr and -rr were 0.1, 0.5, 2.0 & 5.0 μg/ml. These were tested on the liquid cultures of *Scenedesmus* sp. Samples were taken aseptically from the experimental flasks at regular 12 hour intervals and cell counts were performed to monitor the culture's response. These data are presented as specific growth rates (see Table 4.4) and have been computed from the slopes of the graphs shown in Figures 4.1a, b; 4.2a, b and 4.3 .

4.3.1.1 Effect of Anatoxin-a

[1] - The concentration ranges were adapted from DeMott *et al.* (1991).

The effects of anatoxin-a on the growth of *Scenedesmus* sp. are presented in Table 4.1 and in Figures 4.1a & b. Also, the specific growth rates were calculated and are shown in the table.

Table 4.1. The specific growth rates of *Scenedesmus* sp. in the presence of Anatoxin-a.

Toxin Concentration (µg/ml)	Time Interval* (hours)	(µ) specific growth rate (h^{-1})
20.00	24-96	0.039
Control (for 20 µg/ml)	24-96	0.037
10.00	24-96	0.04
Control (for 10 µg/ml)	24-96	0.04
1.00	24-96	0.039
Control (for 1 µg/ml)	24-96	0.04
0.10	24-96	0.038
Control (for 0.1 µg/ml)	24-96	0.037

* Time intervals refers to that period of growth identifiable as the exponential phase over which µ was calculated.

It is seen that no inhibitory effect of neuro-toxin Anatoxin-a was observed on the growth of green alga *Scenedesmus* sp. at the concentrations tested and that the specific growth rates for both control and test cultures were very similar.

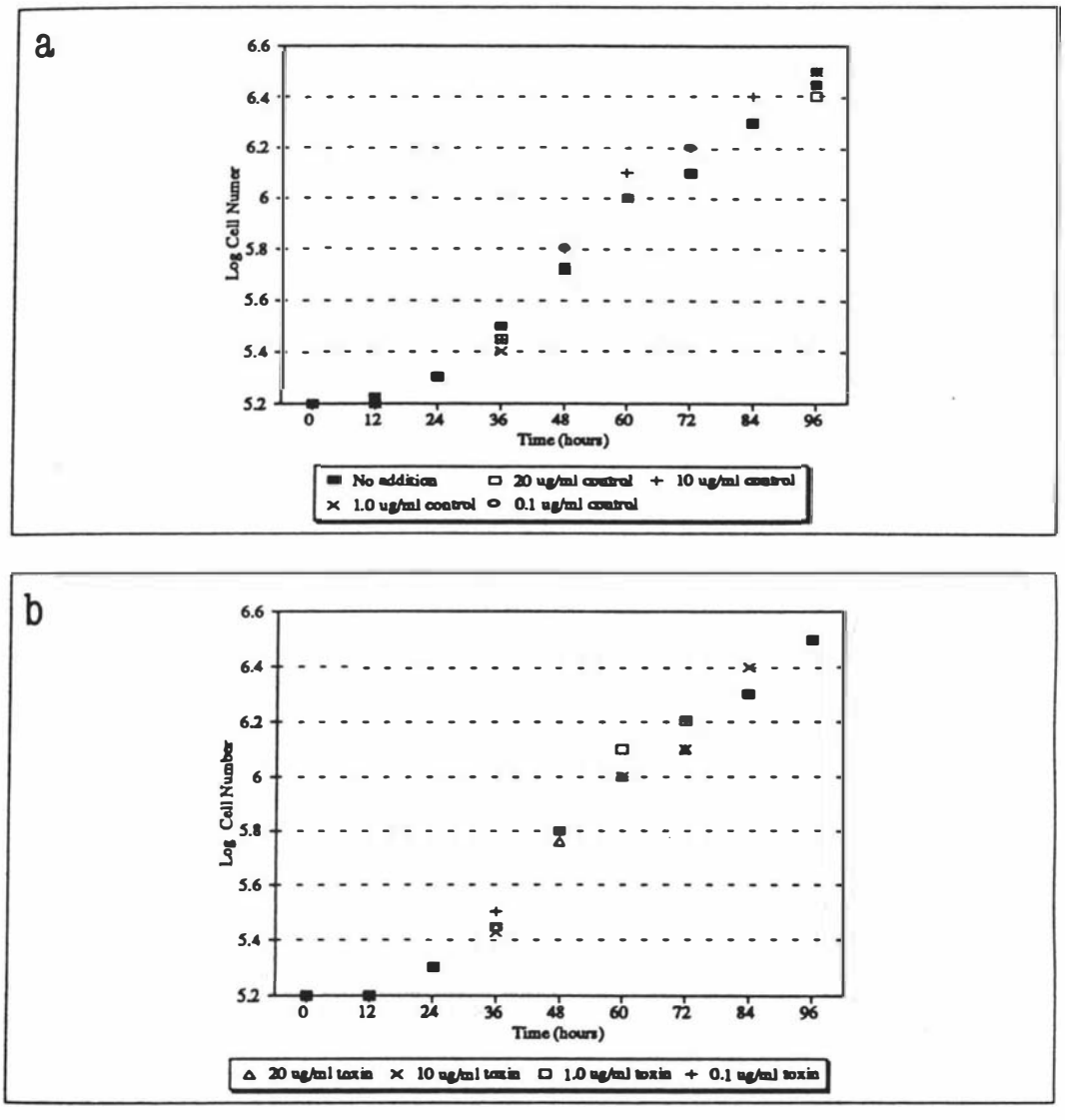


Figure 4.1. The growth of the target green alga *Scenedesmus* sp. in the presence of varying concentrations of anatoxin-a. (a) Control flasks for each experimental flasks, (b) tests flasks which contained upto 20 µg/ml of anatoxin-a.

4.3.1.2 Effect of Microcystin-lr and -rr on the growth of *Scenedesmus sp.*

The results illustrating the effect of the hepato-toxins microcystin-lr and microcystin-rr on the growth of *Scenedesmus* sp. are presented in Figures 4.2 a & b and 4.3. The specific growth rates of this alga in the presence of these toxins are shown in Table 4.2. When compared with the control flasks (with or without addition of solvent, see Table 4.2) there was no difference in the growth rates observed in test flasks which contained the toxins thus indicating a lack of inhibitory activity of these toxins against *Scenedesmus* sp. at the concentrations tested. Lag phases were of similar duration in each case (12 h) and the final population densities reached were similar (Log Cell Number 6.7) and had developed within comparable time intervals (96 h).

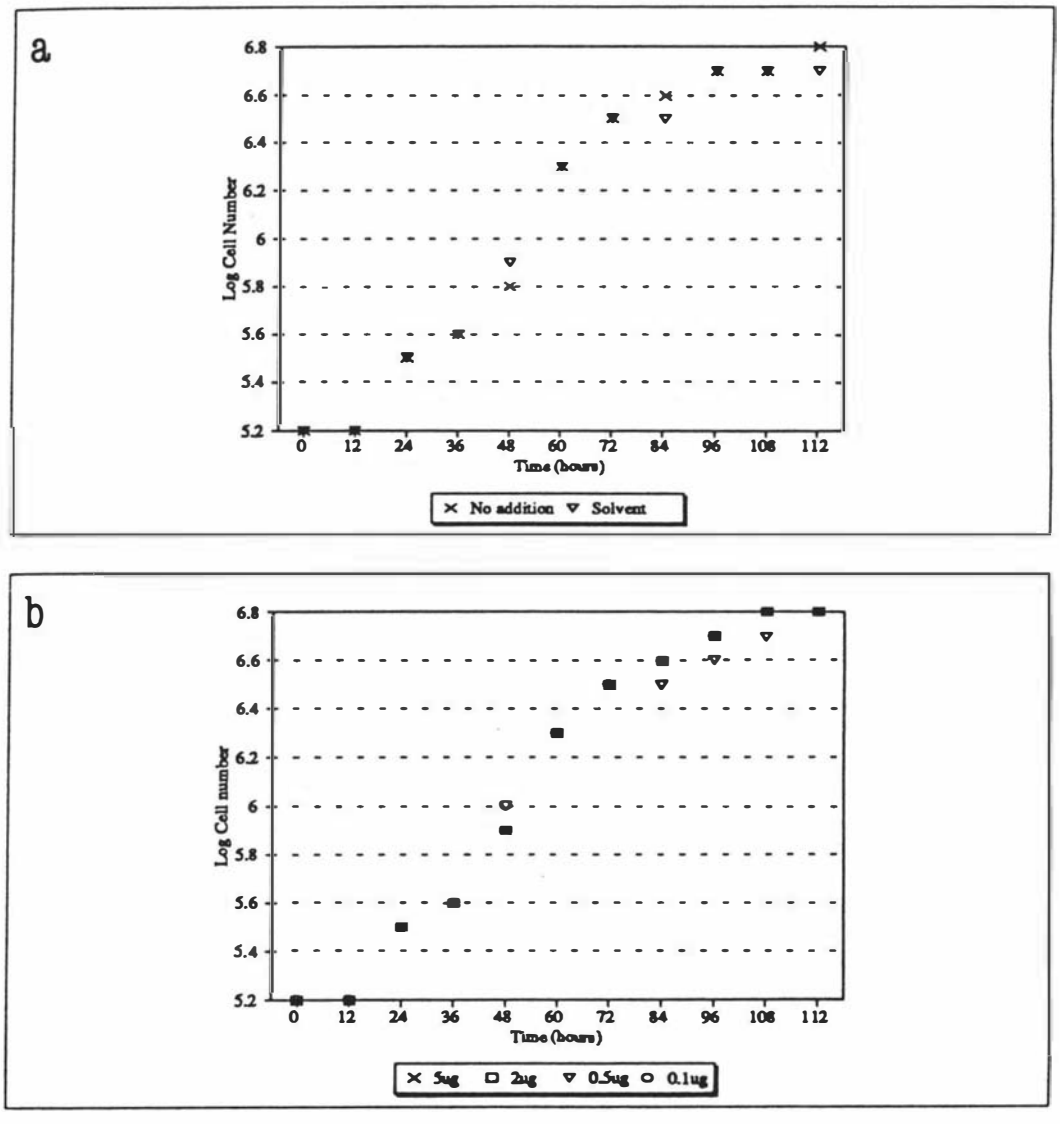


Figure 4.2. The growth of the target green alga *Scenedesmus* sp. in the presence of varying concentrations of the hepatotoxin microcystin-lr. (a) Control flasks, (b) tests flasks which contained the microcystin-lr.

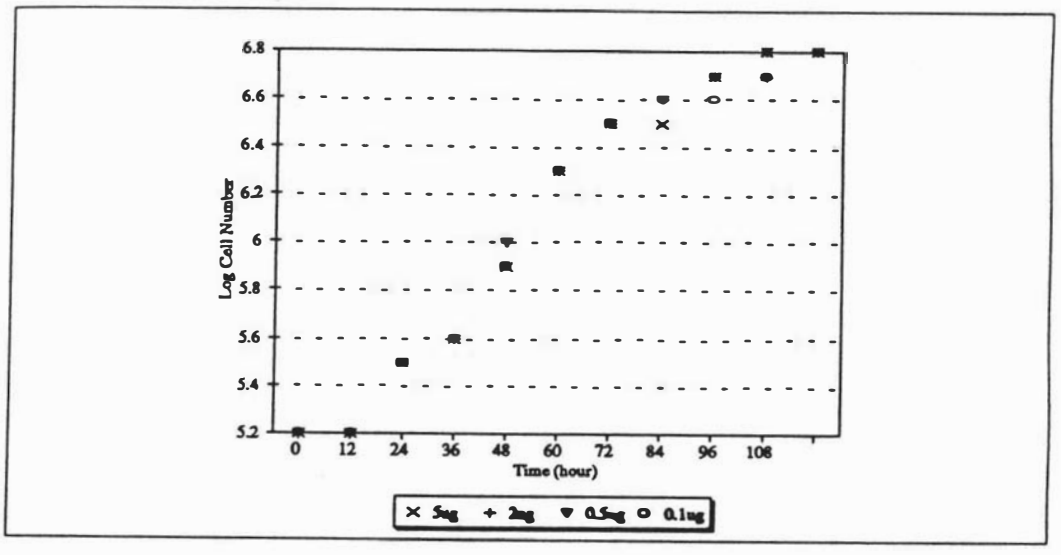


Figure 4.3. The growth of the target green alga *Scenedesmus* sp. in the presence of varying concentrations of the hepatotoxin microcystin-rr.

Table 4.2. The specific growth rates of *Scenedesmus* sp. in the presence of microcystins.

Toxin Concentration (μg/ml)	Time Intervals* (hours)	(μ) specific growth rate (h^{-1})
Control (no addition)	12-96	0.044
Control (solvent only)	12-96	0.043
MYCST-lr 5.0	12-96	0.044
MYCST-lr 2.0	12-96	0.044
MYCST-lr 0.5	12-96	0.041
MYCST-lr 0.1	12-96	0.043
MYCST-rr 5.0	12-96	0.043
MYCST-rr 2.0	12-96	0.043
MYCST-rr 0.5	12-96	0.043
MYCST-rr 0.1	12-96	0.042

* Time intervals refers to that period of growth identifiable as the exponential phase over which μ was calculated. Control (solvent) flask received 5 ml of distilled water containing 100 μl of ethanol.

4.3.2 The screening of NZ isolates of cyanobacteria for toxin production

4.3.2.1 Shake flask (liquid) culture detection of toxins

The toxicity of cyanobacterial cell extracts and culture broths were determined. Methanolic cell extracts and lyophilised broths of cultures were added to liquid cultures of *Scenedesmus* sp. Figures 4.4a & b, 4.5a & b and 4.6 show the growth responses of *Scenedesmus* sp. No difference in the specific growth rate was observed in the absence of cyanobacterial extract (Figure 4.4a), *Scytonema* extracts (Figure 4.4b), *Oscillatoria* sp. extracts (Figure 4.5a & b) and *N. spumigena* extracts (Figure 4.6). Calculated values for specific growth rates are presented in Table 4.3 and 4.4. In Allen's medium *Scenedesmus* sp. exhibited a specific growth rate of 0.024 and 0.028 h^{-1}. Addition of methanol diminished this value from 0.024 to 0.016 h^{-1}. Extracting cells or cell supernatants with methanol had no effect on the growth rates which varied between 0.018 and 0.023 h^{-1} for cultures other than *N. spumigena.* In this case cell extracts (in methanol) showed a 7% reduction in specific growth rate, presumably due to a trace of toxic material extracted from cells.

New Zealand isolates of cyanobacteria exhibited no cytotoxic activity in the liquid culture detection method, irrespective of whether cell extracts or lyophilized culture broths were assayed.

This suggested that the method was insufficiently sensitive or that the New Zealand

isolates of cyanbacteria were not producing cytotoxin under those conditions of growth employed. A second detection system was therefore investigated before the NZ isolates were eliminated as possible sources of toxins.

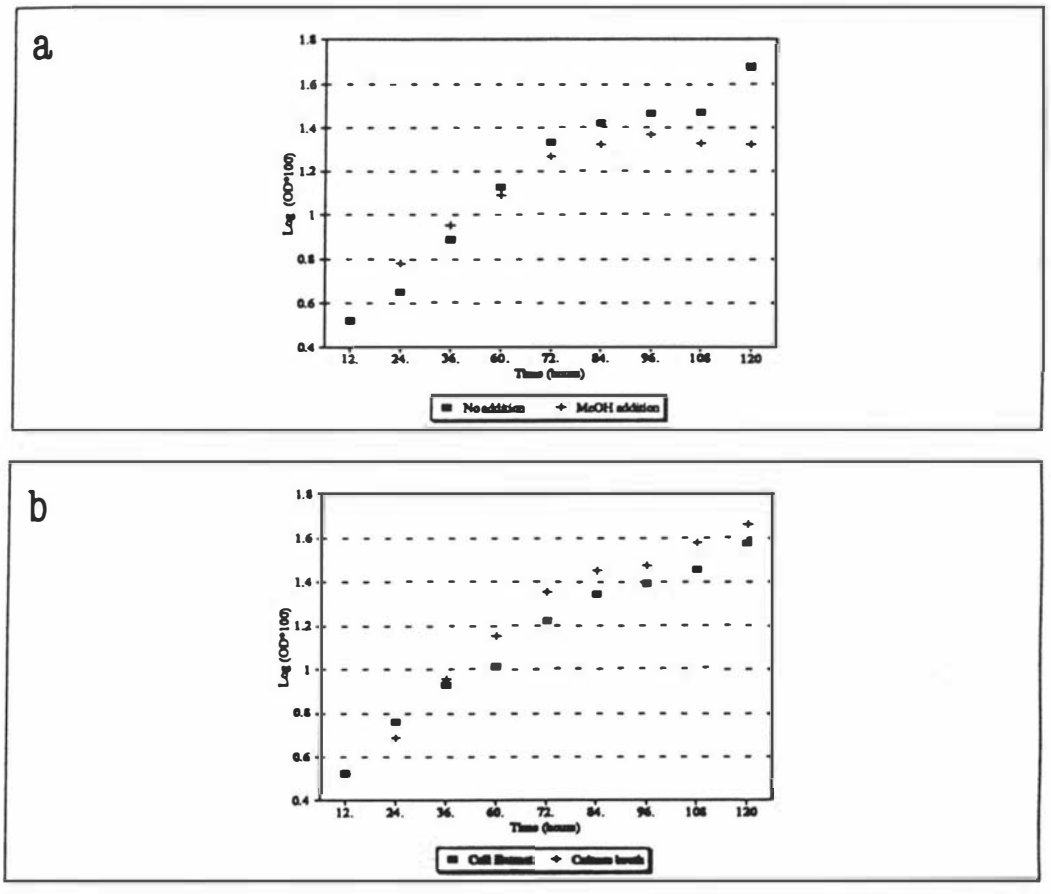


Figure 4.4. Growth of the indicator alga *Scenedesmus* sp. in Allen's medium containing material from cyanobacterium *Scytonema* prepared as methanol extracts. (a) No added cell extract, (b) cell extract present.

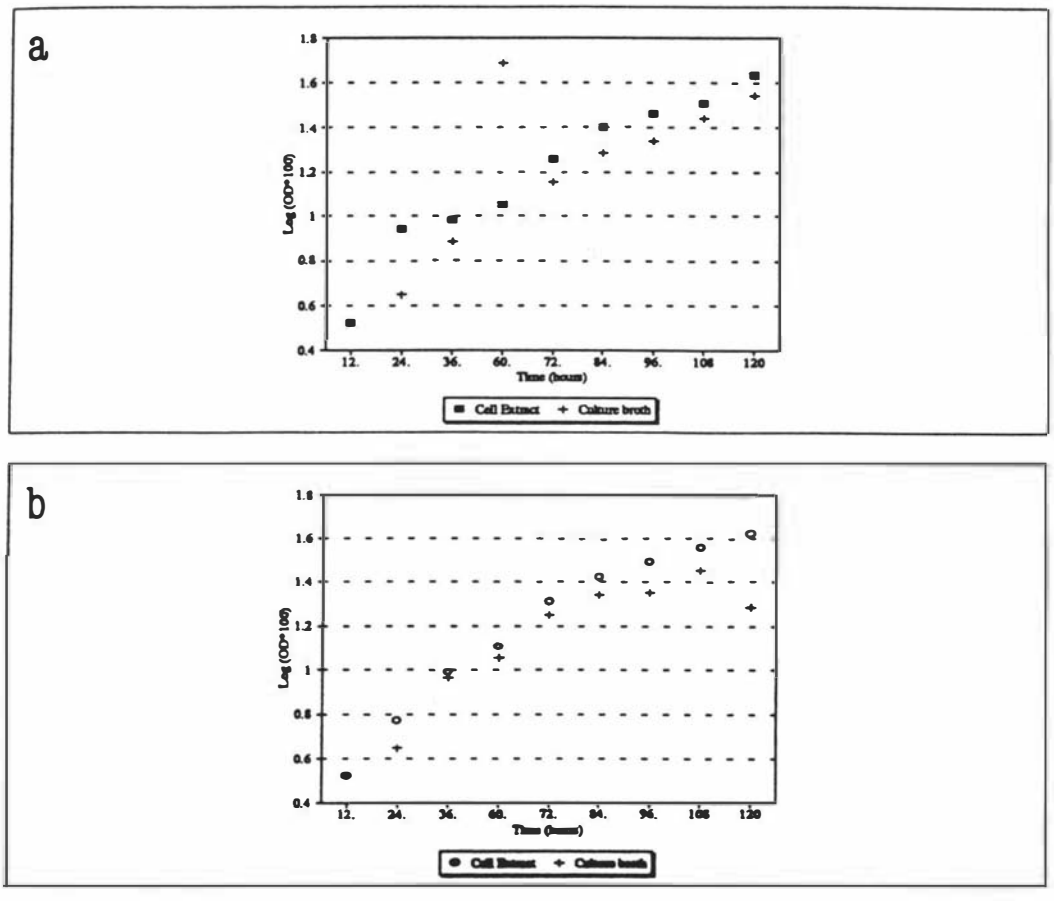


Figure 4.5. Growth of the indicator alga *Scenedesmus* sp. in the presence of methanolic crude extracts prepared from *Oscillatoria* species (a) M193rt and (b) M194rt.

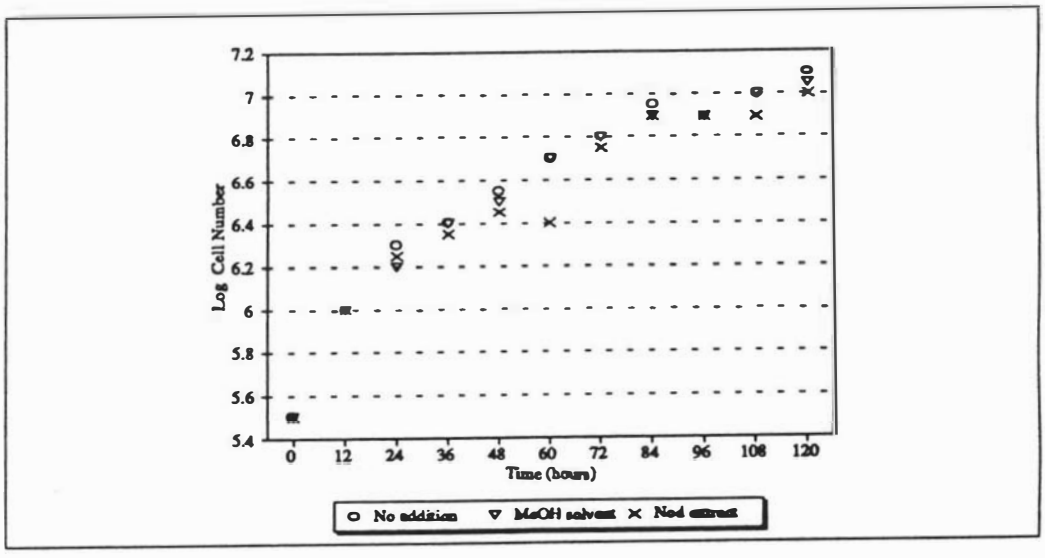


Figure 4.6. Growth of *Scenedesmus* sp. in Allen's medium, Allen's medium with methanol (+) and Allen's medium containing methanol extract of *Nodularia spumigena*.

Table 4.3. The specific growth rates of *Scenedesmus* sp. in the presence of cyanobacterial extracts.

Source	Time Intervals* (hours)	(μ) specific growth rate (h^{-1})
Control (Allen's medium)	12-120	0.024
Control (MeOH only)	24-120	0.016
Scytonema cell extract	24-120	0.019
Scytonema culture broth	24-120	0.023
Oscillatoria (M193rt) cell extract	24-120	0.018
Oscillatoria (M193rt) culture broth	24-120	0.018
Oscillatoria (M194rt) cell extract	24-120	0.021
Oscillatoria (M194rt) culture broth	24-120	0.020

* Time intervals refers to that period of growth identifiable as the exponential phase over which μ was calculated.

Table 4.4. The specific growth rates of a *Scenedesmus* sp. growing in the presence of *Nodularia* extracts.

Source	Time Intervals* (hours)	(μ) specific growth rate (h^{-1})
Control (no addition)	12-84	0.028
Control (MeOH only)	12-84	0.029
Nodularia cell extract	12-84	0.026

* Time interval refers to that period of growth identifiable as the exponential phase and over which μ was calculated.

4.3.2.2 An agar diffusion zone assay system for toxin detection

Crude methanolic cell extracts and lyophilized culture broths of the NZ isolates of cyanobacteria were tested for inhibitory activity against *Scenedesmus* sp. using an agar zone diffusion assay system. Results are presented in Table 4.5. Toxicity could not be demonstrated in the materials tested and the conclusion was drawn that these regional isolates of *Scytonema*, *Oscillatoria* and *Nodularia* did not produce toxins under the growth conditions used in these experiments. It became necessary to obtain and test further cultures in the search for a toxin producing strain.

Table 4.5 Antimicrobial activity of cyanobacterial cell extracts or culture broth tested against green alga *Scenedesmus* sp. using an agar diffusion method.

Cyanobacteria	Source	Inhibition
Scytonema M53	cell extract	NID
Scytonema M53	culture broth	NID
Oscillatoria M193rt	cell extract	NID
Oscillatoria M193rt	culture broth	NID
Oscillatoria M194rt	cell extract	NID
Oscillatoria M194rt	culture broth	NID
Nodularia M155	cell extract	NID

NID - No Inhibition Detected.

4.3.2.3 Screening of UTEX cultures for toxin production

Cyanobacterial cultures obtained from the University of Texas (Chapter 3 Section 3.1.1) were tested for their inhibitory activity towards green algae and other cyanobacteria. Screening tests were conducted using either the liquid culture method or the agar zone diffusion assay system. To Allen's medium containing the freshly inoculated green alga *Scenedesmus* sp. was added the methanolic crude extract or a lyophilized cell-free culture broth (re-dissolved in methanol) prepared from the UTEX cyanobacterial culture

under test. Cell counting at regular intervals was used to measure the specific growth rate of *Scenedesmus* sp. In the agar zone diffusion assay system either whole cells or a methanolic crude cell extract or the cell-free culture broth were applied.

4.3.2.3.1 *Anabaena flos-aquae* (UTEX 1444)

Figure 4.7 presents the results of an investigation of the inhibitory effect of *Anabaena flos-aquae* (UTEX 1444) on *Scenedesmus* sp.. The specific growth rates recorded for *Scenedesmus* sp. in the presence of methanol alone, methanolic extracts of *Anabaena flos-aquae* cells and the reconstituted lyophilised cell-free culture broth of this cyanobacterium is shown in Table 4.6. It was concluded from the graphical data and from a comparison of specific growth rates that *Anabaena flos-aquae* extract and culture filtrate had no effect on *Scenedesmus* sp. and that no toxin was produced under the growth conditions used.

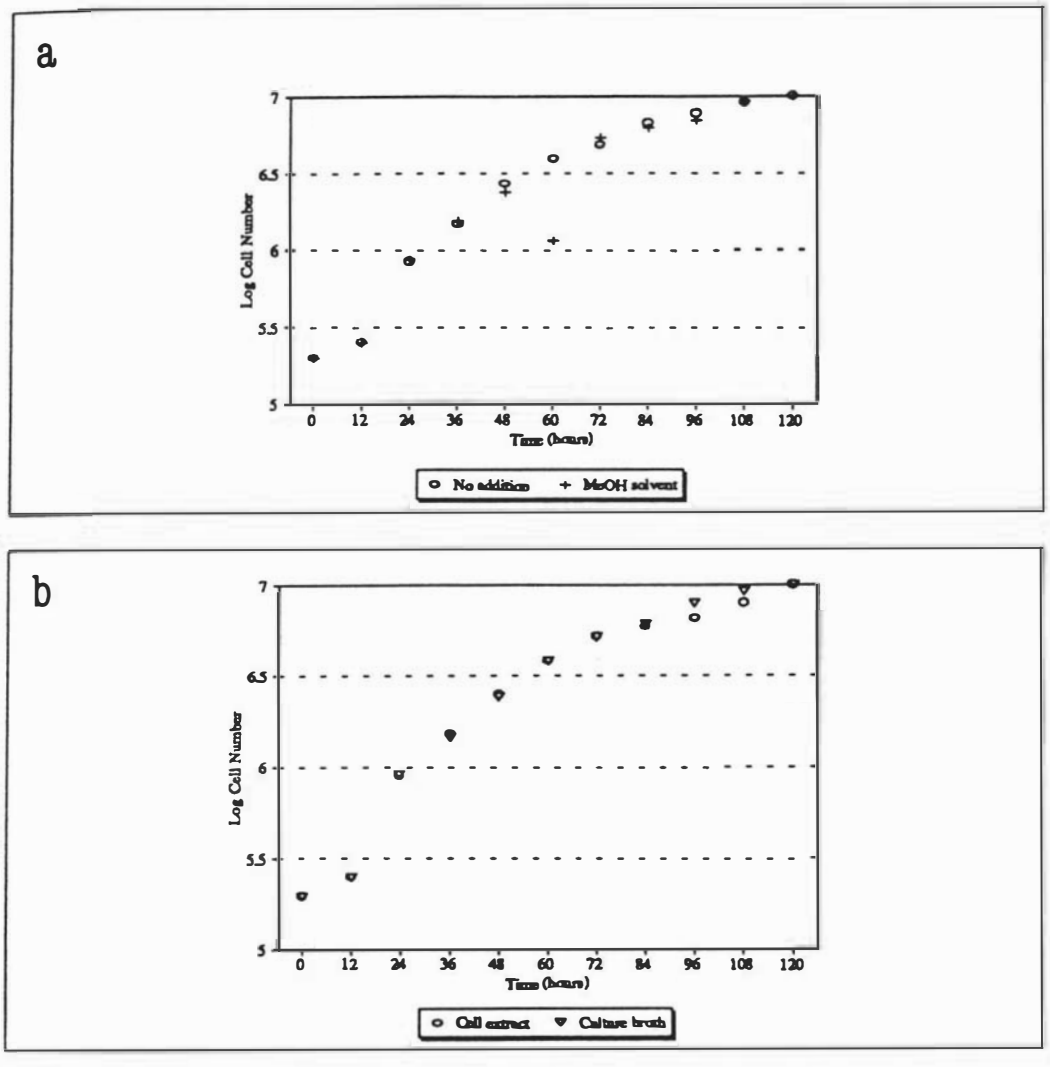


Figure 4.7. The growth of *Scenedesmus* sp. (a) control flasks, (b) methanolic crude extracts prepared from *Anabaena flos-aquae*.

Table 4.6. The specific growth rates of *Scenedesmus* sp. in the presence of extracted material from *Anabaena flos-aquae.*

Source	Time Interval* (h)	(μ) specific growth rate (h^{-1})
Control (no addition)	12-96	0.038
Control (solvent only)	12-96	0.036
Cell extract	12-96	0.036
Culture broth	12-96	0.038

* Time interval refers to that period of growth identifiable as the exponential phase and over which μ was calculated.

4.3.2.3.2 *Fischerella muscicola*(UTEX 1829)

The production of inhibitory substance(s) by *F. muscicola* was tested using the agar zone diffusion bioassay system. Two different genera of indicator organisms were used: *Scenedesmus* sp. and *Anabaena flos-aquae.* Fresh unbroken cells and methanolic crude cell extracts were prepared from *F. muscicola.*

There was no inhibitory effect on *Scenedesmus* sp. but *Anabaena flos-aquae* gave very clear positive inhibition results as presented in Table 4.7 and this is an important factor

when reproducible zone diameters measurements need to be taken. Thus it was decided to use this latter organism as the indicator organism for further work.

Because *Anabaena flos-aquae* showed strong inhibition of growth in the presence of *F. muscicola* extract, it was decided to assess more fully the spectrum of antimicrobial activity possessed by this cyanobacterium. This was considered a necessary step so that a suitable microorganism could be selected for use in subsequent assays of activity.

Tables 4.7 and 4.8 both show that the methanolic crude cell extract of *F.muscicola* possess observable antimicrobial activity against a wide range of test organisms. The auto-inhibitory activity of *F. muscicola* was also noted. However, the inhibition of cyanobacteria was stronger than that observed for eukaryotic algae and bacteria.

The conclusions drawn from these experiments were: firstly, that *Anabaena flos-aquae* was a useful indicator organism because it gave large and clear zones of inhibition and secondly, that because this culture was axenic and the results obtained were not complicated by the co-culture(s) likely to be present in non-axenic populations.

4.3.2.3.3 *Fischerella ambigua* (UTEX 1903)

The extent to which the genus *Fischerella* produces inhibitory substances was further investigated by examining the organism *F. ambigua* (UTEX 1903). An active culture of *F. ambigua* (UTEX 1903) was spot inoculated onto an Allen's medium agar plate

containing either *A. flos-aquae* or *F. muscicola* as the indicator organisms. After 10 days of incubation (under conditions described previously) no inhibition by *F. ambigua* of the two indicator species was observed. Thus, unlike *F. muscicola*, the related species *F. ambigua* was not toxic under the conditions tested. It was concluded from this that toxicity in the genus *Fischerella* may be restricted to *F. muscicola.*

Table 4.7. Inhibition of growth of different indicator organisms by *F.muscicola.*

Indicator strain	Inhibition
Cyanobacteria	
*Oscillatoria** sp	++
Anabaena flos-aquae	+++
Fischerella ambigua	-
Fischerella muscicola	++
Chlorophyceae	
*Pandorina** sp	++
*Chlamydomonas** sp	++
*Chlorella** sp	+++
*Scenedesmus** sp	-
*Oocystis** sp	-
Xanthophyceae	
*Heterococcus** sp	++

+++ very strong inhibition (34 - 39 mm), ++ strong inhibition (22 - 27 mm), - no inhibition. *- NZ isolates.

Table 4.8. Inhibition of growth of different indicator organisms by *F.muscicola.*

Indicator strain	Inhibition
Bacteria	
Bacillus coagulans	-
Bacillus cereus	-
Bacillus subtilis	-
Eneterobacter aerogenes	-
Eschrecia coli	-
Klebsiella pneumoniae	-
Micrococcus luteus	++
Pseudomonas aeruginosa	-
Pseudomonas fluorescence	-
Serratia marcescens	-
Staphylococcus aureus 305	-
Streptococcus faecalis	-
Streptococcus lactis	-

++ strong inhibition (22 - 27 mm), - no inhibition.

4.4 DISCUSSION

Although the harmful effects of neuro- and hepato-toxins of cyanobacteria on terrestrial animals, birds and humans following ingestion of water containing these compounds are well documented (Baker & Humpage, 1994; Falconer, 1991; Lawton & Codd, 1991; Turner *et al.*, 1990; Gorham & Carmichael, 1988; Mcinnes *et al.*, 1983; Flint, 1966), understanding the natural biological significance of these toxins in the context of aquatic organisms is largely lacking. The results of the bioassay tests conducted here using commercially available neuro- and hepato-toxins on the green alga *Scenedesmus* sp. showed no toxic effect of these toxins. Foxall and Sanser (1981) reported that crude cell extracts from hepato-toxin(s) producer *Microcystis aeruginosa* had no inhibitory effect on green alga *Chlorella.* In this study *Nodularia spumigena* methanolic cell extracts showed no effect on the green alga *Scenedesmus* sp., indicating that hepato-toxins may be active against animals only. However, in contrast to these results Kirpenko *et al.* (1982) found an inhibitory effect of *Microcystis* toxin extracts on the development of saprophytic microflora in artificial ponds, according to the review by Carmichael (1986). This probably reflects that these biotoxins (named because when bioassayed using animals eg. small animals and aquatic invertebrates gave positive results) (Carmichael, 1992) may not be toxic towards algae. Although this study has failed to demonstrate the inhibitory effect of neuro- and hepato-toxins on selected algae, the possibility that, at higher concentrations, these toxins will be inhibitory to the other suitable indicator organisms cannot be ruled out. Statistical validation of the data was not carried out because of the experiments were carried out in duplicate only in view

of the cost of toxins.

The initial screening of organic solvent extracts (cell and culture broth) of NZ isolates of cyanobacteria showed no toxic secondary metabolite production by these organisms when tested using either a liquid culture or an agar zone diffusion assay method. There could have been two reasons for the absence of toxins. One possibility is that each culture may have been harvested at sub-optimal times for toxin production and consequently toxin production would have been missed. Furthermore, if the toxic compounds are the products of secondary metabolism, then any variation in the parameters which affect cell physiology may result in the absence of the active product. An alternative possibility is that they do not produce any toxic antimicrobial compound. In the experiments described in this section of work, five genera of cyanobaceteria, i.e. *Anabaena flos-aquae, Fischerella* (*muscicola* and *ambigua*), *Nodularia spumigena, Oscillatoria* spp. and a *Scytonema* sp. were investigated for an ability to produce toxins active against algae, other cyanobacteria or eubacteria. Only *F. muscicola* was shown to produce a toxin. In these investigations care was taken to test both the cell free supernatants and cells themselves for toxin. These experiments were designed for preliminary screening and therefore duplicate experiments were deemed adequate.

Carmichael (1992) reported that the genus *Fischerella* was toxic but that no detailed study describing the toxicity of this organism had been documented. Of particular relevance to this current study is the research of Flores & Wolk (1986) and Gross *et al.* (1991). Flores & Wolk (1986) screened 65 filamentous nitrogen fixing cyanobacteria

for the production of bacteriocin. They found *F. muscicola* (UTEX 1829) highly toxic for all indicator organisms tested. Gross *et al.* (1991) isolated and purified a toxic component from *F. muscicola* and provided a partial chemical characterization. In this study described here *F. muscicola* has been shown to be toxic to *Anabaena flos-aquae* (cyanobacteria) but non-toxic for the green alga *Scenedesmus* sp. Thus the observations of Flores and Wolk (1986) and Gross *et al.* (1991) are confirmed. It was therefore decided to focus on this organism for further study.

Growth of selected eukaryotic algae, cyanobacteria and eubacteria found to be inhibited by toxin(s) from *F. muscicola.* Although there was no definite discrimination between eukaryotic algae and cyanobacteria, there was an observable increased toxicity towards cyanobacteria. Of the thirteen bacterial genera tested (gram positive and negative) for growth inhibition, only one gram positive bacterium, *Micrococcus luteus*, was inhibited by the methanolic crude cell extract of *F. muscicola.* Flores & Wolk(1986) reported that *F. muscicola* was also toxic towards other nitrogen fixing filamentous cyanobacteria. Gross *et al.* (1991) found that the toxicity of this organism towards the other cyanobacteria was greater than it was to eukaryotic algae. There was no toxicity to bacteria. Recently, Park *et al.* (1992) reported that an antifungal substance was produced by this organism.

Metting and Pyne (1986) described in some cases allelopathic algicidal activity to be auto-inhibitory. Rijstenbil (1989) found the toxic secondary metabolite of the diatom species *Ditylum brightwelli* and *Skeletonema costatum* to be auto-inhibitory. When the crude methanolic cell extract of *F. muscicola* was tested for auto-inhibition it also gave

positive result.

Earlier studies (Gross *et al.*, 1991) have indicated that *F. ambigua* may also produce toxic secondary metabolite(s). In the present study however, no toxic effect of this species was found towards *Anabaena flos-aquae* and *Fischerella muscicola.* However, it may be possible that the toxic secondary metabolite(s) are accumulated within the cytoplasm. Since most secondary metabolites are complex molecules, they probably do not diffuse readily through membranes. If this is the case then the methanolic cell extracts may show toxicity. Tests on cellular extracts were not carried out in the current series of experiments. It is also interesting to note that the *F. muscicola* culture's crude methanolic cell extract was auto-inhibitory but did not have any effect on *F. ambigua.*

Since an understanding of the toxicological effects of the genus *Fischerella* is still at an early stage, it is important to investigate more thoroughly the distribution of toxic secondary metabolite production by using some other indicator organisms. The decision to use the agar zone diffusion assays was based upon its ease of application and popularity. However, there are limitations governed by physicochemical factors (Hewitt, 1977) which include concentration, temperature and the viscosity of the solvent. Every effort was made to standardize the procedure so that the physiological responses observed above were a reflection of the organism's character.

Upon the identification of a cyanobacterium producing a strong antimicrobial activity of interest, the next stage of research was to determine the presence or absence of the allelochemical fischerellin, as reported by Gross *et al.* (1991) in the cultures of cyanobacterium *F. muscicola.*

Chapter 5

An Analysis of Toxic Material from *F. muscicola*

5.1 INTRODUCTION

Various kinds of toxic compounds can be produced by groups of photosynthetic microorganisms. Furthermore, Gross *et al.* (1991) have also noted that more than one type of toxin can be produced by the cyanobacterium *F. muscicola.* Methods for the detection and differentiation of cyanobacterial toxins include bioassays (Lahti *et al.*, 1995; Al-Layl, 1989), chemical assays which are invariably based upon chromatographic techniques (Al-layl, 1989; Watanabe *et al.*, 1989) and immunoassay techniques (Shi *et al.*, 1995; Brooks & Codd, 1988; Chu *et al.*, 1989).

Detection of *F. muscicola* toxin(s) is based primarily upon the use of an antimicrobial bioassay system which depends on the toxins' antimicrobial effect on a target microorganism. The toxicity of *F. muscicola* has recently attracted attention (Srivastava *et al.*, 1994; Gross *et al.*, 1991; Flores & Wolk, 1986). Methods are available which allow purification and identification of the toxin(s) from this organism and high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) form the basis of these methodologies (Gross *et al.*, 1991).

The benthic cyanobacterium *F. muscicola* has been shown to produce a secondary metabolite, *fischerellin.* The procedures used for the detection of fischerellin involve organic solvent extraction of disrupted cell material followed by concentration and purification. The toxin is usually detected using HPLC and monitoring the absorbance at a wave length of 267 nm (Gross *et al.*, 1991).

The major aim of the work described in this Chapter was to confirm the presence of fischerellin in the culture of *F. muscicola*, in view of the fact that this organism has been shown to synthesise inhibitory substance(s) under different laboratory culture conditions (Chapter 3). The procedures followed in this study were very similar to those of Gross *et al.*, (1991). The results of HPLC fractionation and GC-MS analysis of the crude methanolic extracts will be described.

5.2 MATERIALS AND METHODS

5.2.1 Organisms and growth conditions

The cyanobacterium *F. muscicola* was cultured in Allen's culture medium and under the standard growth conditions described in Chapter 3.

5.2.2 Antimicrobial activity determination

The cultures were harvested and processed as detailed in Chapter 3. Fractions of crude methanolic eluted produced from HPLC were collected individually and tested for inhibitory activity using the agar diffusion bioassay procedure described in Chapter 3.

5.2.3 High Performance Liquid Chromatography (HPLC) of methanolic crude cell extract of *F. muscicola*

The HPLC analysis of toxic material was carried out using a method similar to that developed by Gross *et al.* (1991).

5.2.3.1 Isocratic run

The crude methanolic cell extracts prepared from a culture of *F. muscicola* were first subjected to reversed phase High Performance Liquid Chromatography (Millipore

Corporation, Milford, Massachusetts, U.S.A.). The chromatographic system consisted of a Waters 600E system controller equipped with a Waters U6K injector and Waters 486 Tunable Absorbance detector. An integrator (Waters 486, Alphatech, Auckland, New Zealand) was used to handle input signals from the detector and report the output in terms of peak area. Absorptions at 267nm and 415nm were monitored to detect active substances and contaminating photosynthetic pigments respectively. An RP18-column, 220 x 4.6-mm (Applied Biosystems, Brownlee columns, SPHERI-5 RP-18 5 micron, Serial No. 181428) was used for the analysis. Aliquots of 10-100 µl were separated by isocratic chromatography using a mobile phase of methanol:water (99:1 v/v) with a flow rate of 1 ml per min.

The samples (crude toxic cell extract) were centrifuged at room temperature for 5 min at 14926 xg in a Heraeus Christ Biofuge A (Heraeus-Christ GmbH, Gipsmuhlenweg, West Germany). Then 10-100 µl quantities of the toxic extracts were injected into the HPLC system. The appropriate peaks/fractions were collected manually in round bottom flasks and evaporated to dryness in a rotary evaporator (Rotavapor R110, Buchi, Laboratoriums - Technik AG, CH 9230, Flawil/Schweiz, Switzerland) at 50°C to remove the mobile phase. Each fraction was then redissolved in 1.0 ml of re-distilled methanol and stored at -20°C until ready for bioassay, usually within one week. Toxicity testing was carried out using the agar diffusion zone method (Chapter 3).

5.2.4 Gas Chromatography-Mass Spectrometric determination of *fischerellin*

5.2.4.1 Experimental 1.

Mass spectra of toxic fractions (No. 2 & 3) and *fischerellin* fraction collected from isocratic HPLC were obtained on a VG-70S double focusing magnetic sector mass spectrometer (VG Analytical, Manchester, U.K.). A 2 µl aliquot of each concentrated methanolic fraction was spotted onto a Direct Chemical Ionization (DCI) Probe and Electron Impact (EI) mode ionisation was used, with ion source temperature 280°C. Low resolution (1000 RP) EI spectra were obtained at electron energy 70 eV and 600-29 dalton mass range at 1 sec/decade scan rate.

5.2.4.2 Experimental 2.

Mass spectra was obtained by GC/MS on the above machine fitted with a Hewlett Packard 5890 series II GC and a 30 m x 0.25 mm i.d. (0.25 µm film thickness) SPB1 non-polar capillary column (Polydimethylsiloxane Column Phase). GC conditions were 150-300°C at 10°C/min and a head pressure of 5 p.s.i helium carrier gas. Source temperature was 300°C and interface and GC injector temperatures were 285°C respectively. A 2 µl sample of concentrated HPLC fractions was injected and a mass range of 600-29 daltons were scanned at 1 sec/decade (1 sec/10 mass unit) with a interscan time of 0.2 second. The analytical instrument was located at HortResearch, Palmerston North.

5.3 RESULTS

5.3.1 Results from high performance liquid chromatography (HPLC)

The results of the isocratic HPLC run (Table 5.1) indicate that upon fractionation, fractions of crude methanolic extracts collected from approximately 3 min. to 5 min. showed most of the bioactivity when bioassayed using the agar diffusion method. No bioactivity was detected in any of the later fractions. The fraction at approximately 3.8 min. was a single peak on the chromatogram and the fraction after that (up to 5 min.) was a mixture of at least three minor peaks at wavelength 267 nm. It was not possible to collect these peaks as individual fractions.

Table 5.1. Inhibition of growth of indicator strain *Anabaena flos-aquae* by HPLC fractions of methanolic crude extracts prepared from the culture of *F. muscicola.*

Fraction No.	Mean Inhibition Zone Diameter (mm)
1	NID
2	15.88 ± 0.09
3	25.25 ± 1.96
4	NID
5	NID

NID = No inhibition detected.
± = Standard Error.

The typical isocratic HPLC 267 and 415 nm absorption chromatograms of the fractionated crude methanolic cell extracts are shown in Figures 5.1 to 5.4. The retention times for significant components on the C18 column of the isocratic HPLC run are also shown. Each of these fractions was scanned for the presence or absence of an absorbance at 415 nm wavelength. Of the toxic fractions none was missing an absorbance peak at 415 nm, however, there was a 267 nm peak at approximately 7.8 min which showed no absorbance when the detector wave length was shifted from 267 to 415 nm. This gave a possible indication of being the "fischerellin" fraction since it was the only fraction which showed no absorbance at 415 nm. However, no bioactivity could be detected for this fraction when bioassayed against the indicator organism *Anabaena flos-aquae.*

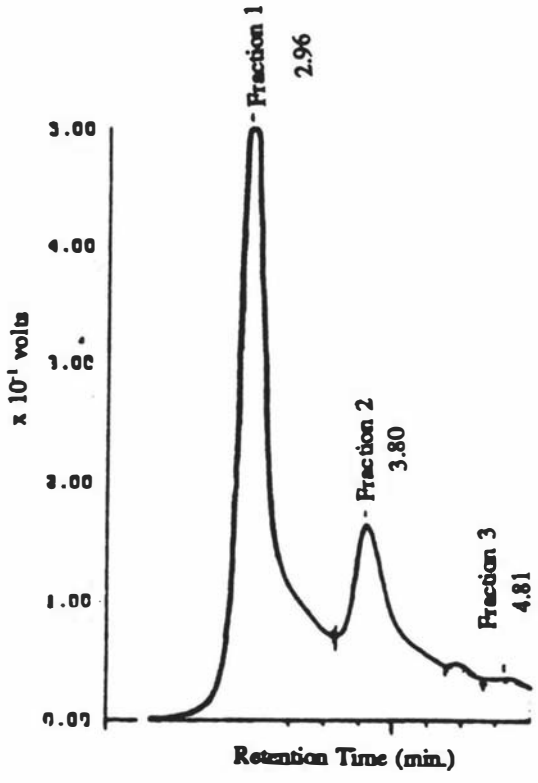


Figure 5.1. HPLC chromatogram (C_{18} isocratic) of the methanolic extract from a culture of *F. muscicola* showing toxic fractions No. 2 & No. 3 at λ 267nm; mobile phase MeOH:H_2O, 99:1 (v/v); flow rate 1 ml/min; 100 µl injection volume.

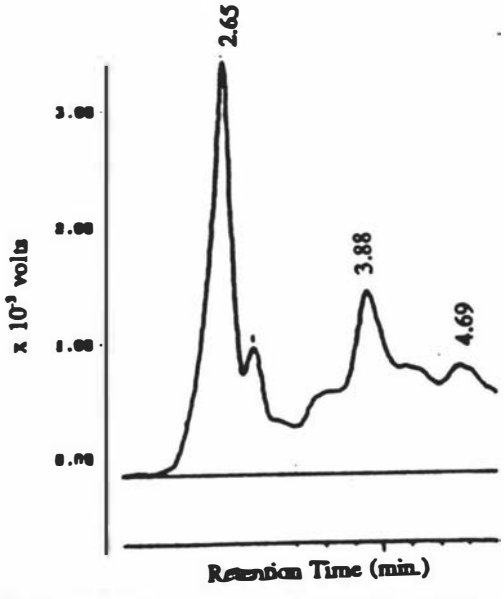


Figure 5.2. HPLC chromatogram (C_{18} isocratic) of the methanolic extract from a culture of *F. muscicola* showing the presence of absorbance at λ 415nm corresponding with toxic fraction No. 2 & 3. HPLC conditions as indicated in Fig. 5.1.

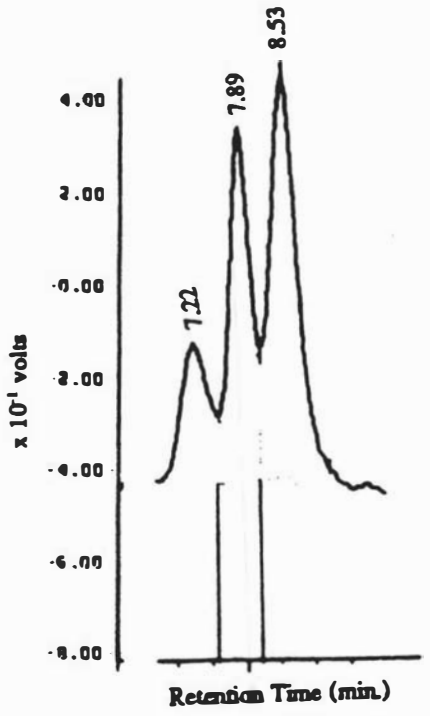


Figure 5.3. HPLC chromatogram of fraction No. 5 (collected between 7 to 9 min) at λ 267nm and tested for absorbance at λ 267nm. HPLC conditions as indicated in Fig. 5.1. Injection volume 10 µl.

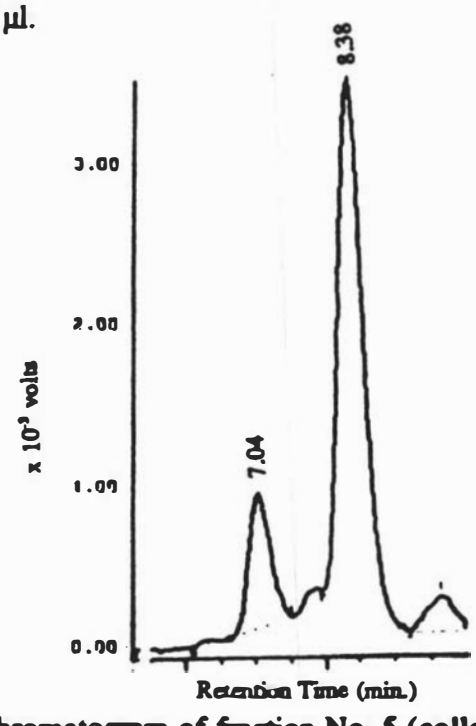


Figure 5.4. HPLC chromatogram of fraction No. 5 (collected between 7 to 9 min) at λ 267nm and tested for absorbance at λ 415nm. The peak at 7.89 min is absent. HPLC conditions as indicated in Fig. 5.1. Injection volume 10 µl.

In this study the HPLC results were parallel to the findings of Gross *et al.* (1991) in terms of detecting the presence or absence of the "fischerellin" molecule in the crude methanolic cell extracts. However, the results from the bioassays did not corroborate these HPLC results. Toxicity occurred at different retention times. To detect the presence of *fischerellin* in the fractions, samples were further analyzed by gas chromatography/mass spectrometry. The presence of *Fischerellin* would be detected by the signal of a molecular ion of mass-to-charge ratio (*m/z*) of 408 (Gross *et al.*, 1991).

5.3.2 Results from gas chromatography/mass spectrometry (GC-MS)

Detection and confirmation for *fischerellin* produced by laboratory cultures of *F. muscicola* were achieved more reliably by using the GC/MS system (Rowan, personal comm.) as described in the Materials and Methods section of this Chapter. Three fractions, two toxic and one non-toxic, were subjected to GC/MS analysis. The results of these analyses indicated that none of the three fractions showed significant components with a molecular ion of mass-to-charge ratio (*m/z*) of 408.

GC/MS results for toxic fraction No. 3 showed a component with molecular ion of mass-to charge ratio (*m/z*) of 410. The chromatogram for toxic fraction No. 3 is presented in Figure 5.5.

A further examination of these fractions was conducted using a DCI probe on the mass

spectrometer. In this method the whole sample is introduced directly into the ion source. A slight advantage is gained in terms of detecting *fischerellin* as a compared to sample passing first through the gas chromatography where the fraction of interest may not elute from the column thus giving a false negative result.

Using the Direct Chemical Insertion Probe (DCI Probe) method no molecular ion of *m/z* 408 was detected in toxic and non-toxic fractions. Figure 5.6 illustrates the non-toxic *fischerellin* fraction, in which the absorbance at 415 nm was missing. However, a molecular ion of *m/z* 412 was present. Figure 5.7 shows the GC/MS chromatogram for the "fischerellin" fraction. Similarly for toxic fraction No. 3 (Figure 5.8) it also showed a molecular ion of *m/z* 412. However, this *m/z* 412 molecular ion could not be detected in toxic fraction No. 2, which exhibited slightly less toxicity as compared to that of fraction No. 3, Table 5.2. Fraction No. 2 contained metabolites with unusual polychlorinated structure(s) (Figure 5.9). Figures 5.10 & 5.11 present the GC/MS spectra and DCI probe spectra (full scan).

From the DCI probe method it appeared that in all of the samples tested (both toxic and non-toxic) if any molecular ion of *m/z* 408 is present ie. fischerellin, it was a very minor component.

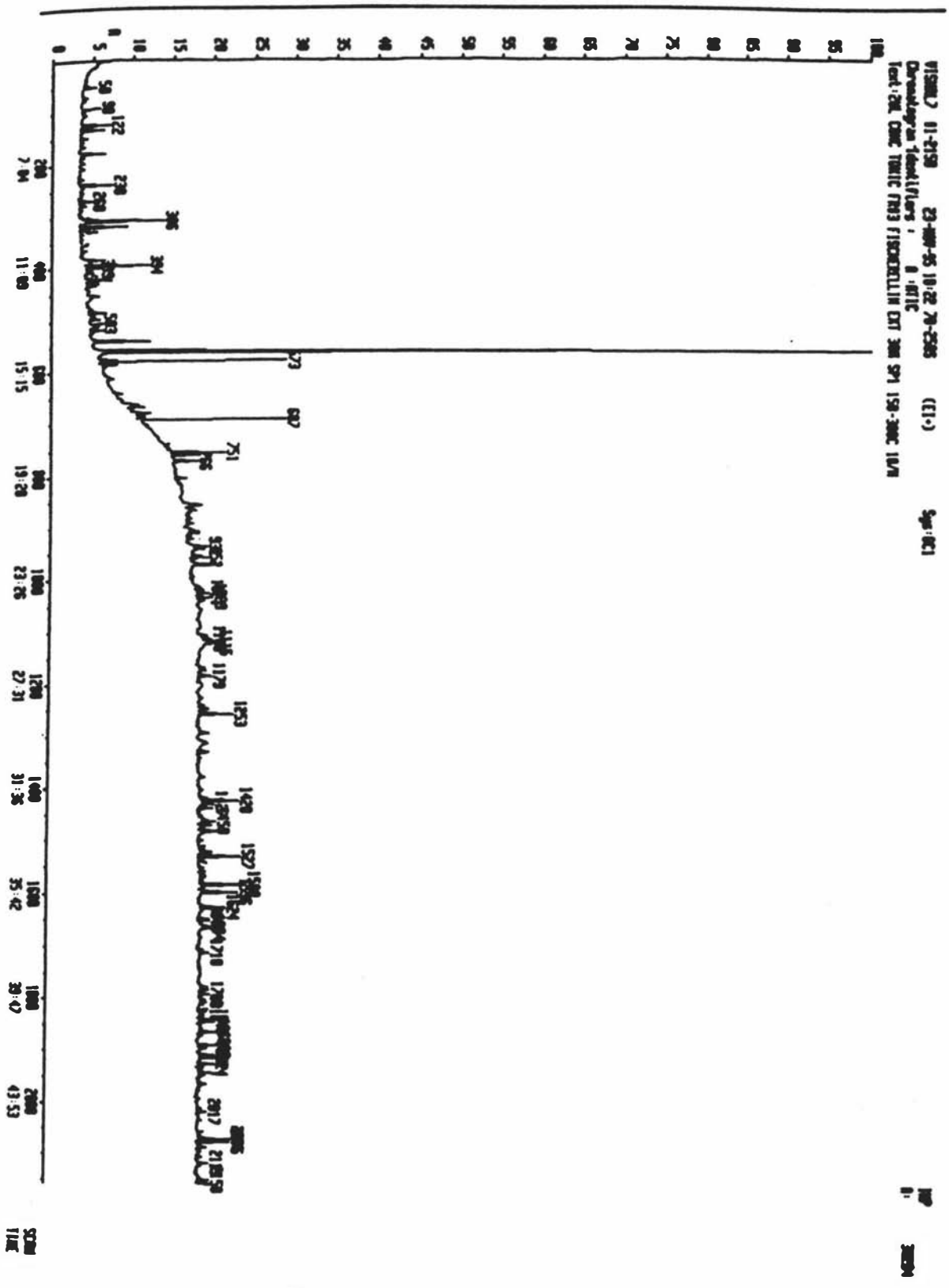


Figure 5.5. GC-MS chromatogram of toxic fraction No. 3.

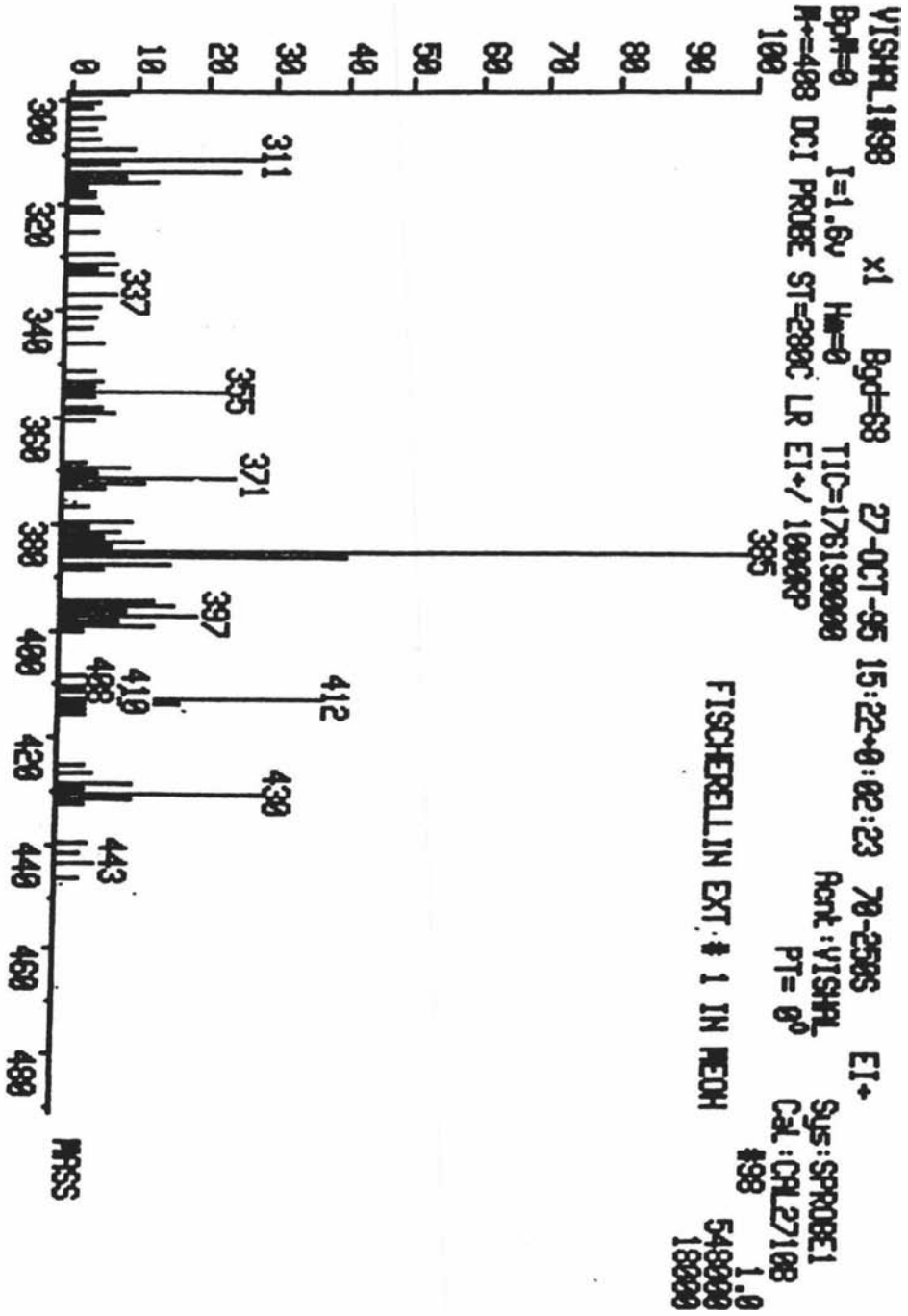


Figure 5.6. Molecular ion scan of fraction No. 5, using DCI probe method for the detection of a signal at *m/z* 408.

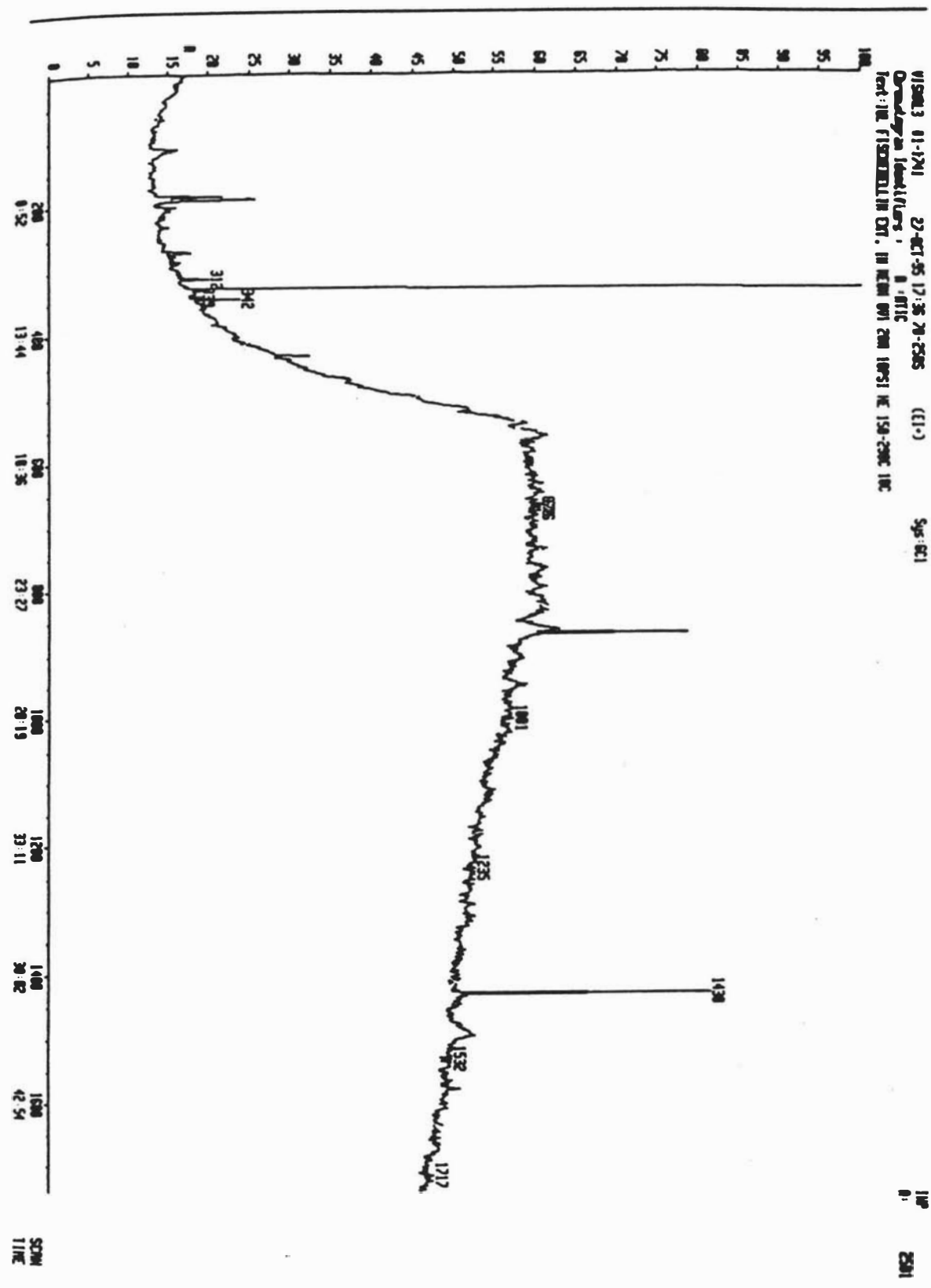


Figure 5.7. GC-MS chromatogram of fraction No. 5.

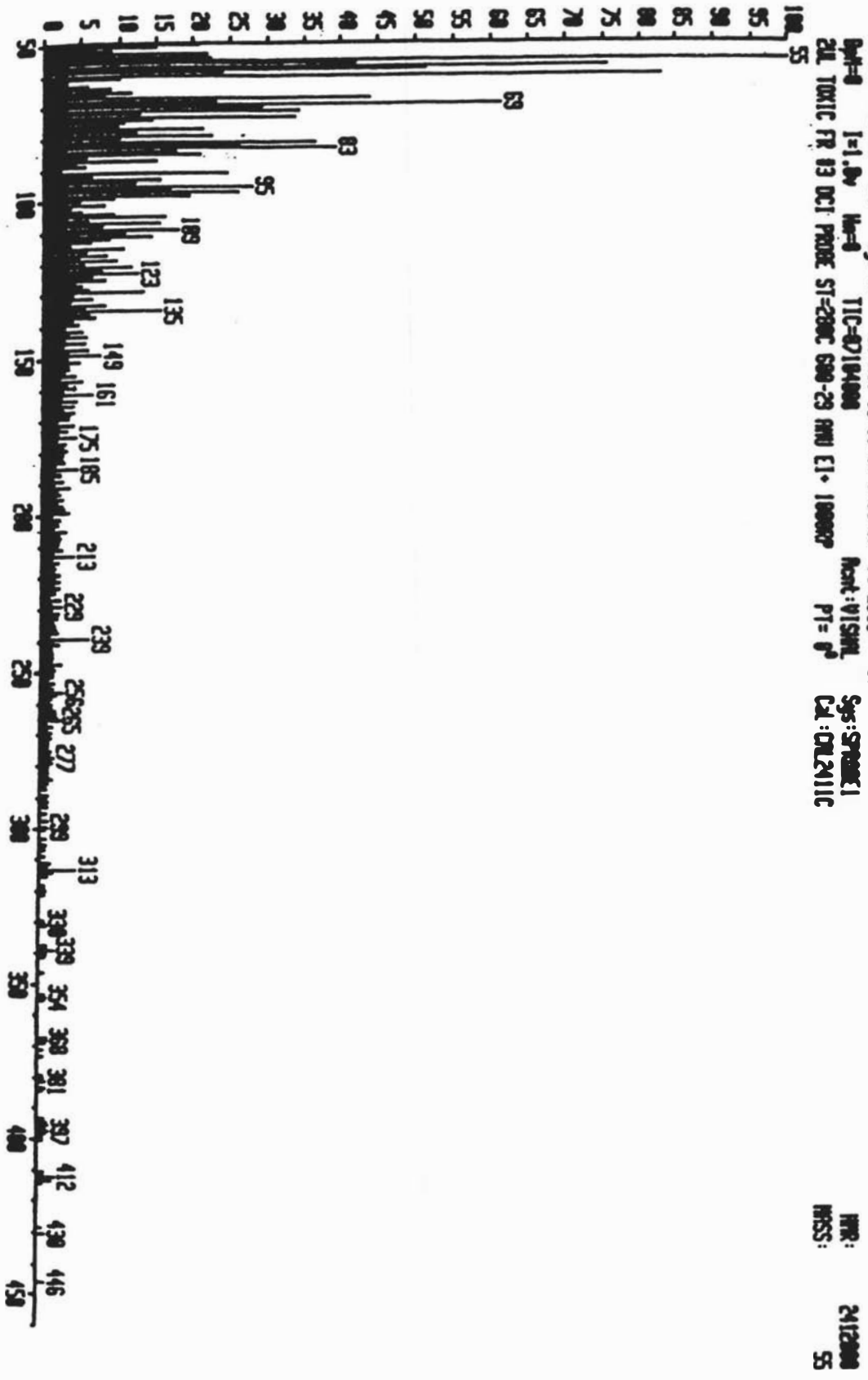


Figure 5.8. Molecular ion scan of toxic fraction No. 3, using DCI probe method for detection of a signal at *m/z* 408.

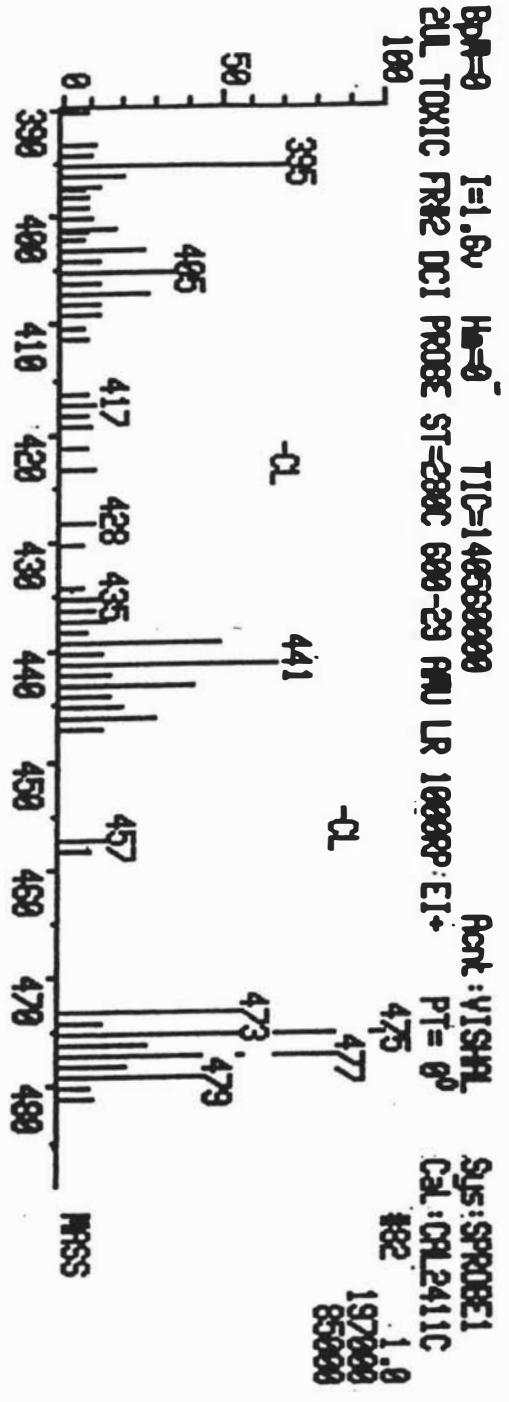


Figure 5.9. Molecular ion scan of toxic fraction No. 2, using DCI probe method for detection of a signal at *m/z* 408.

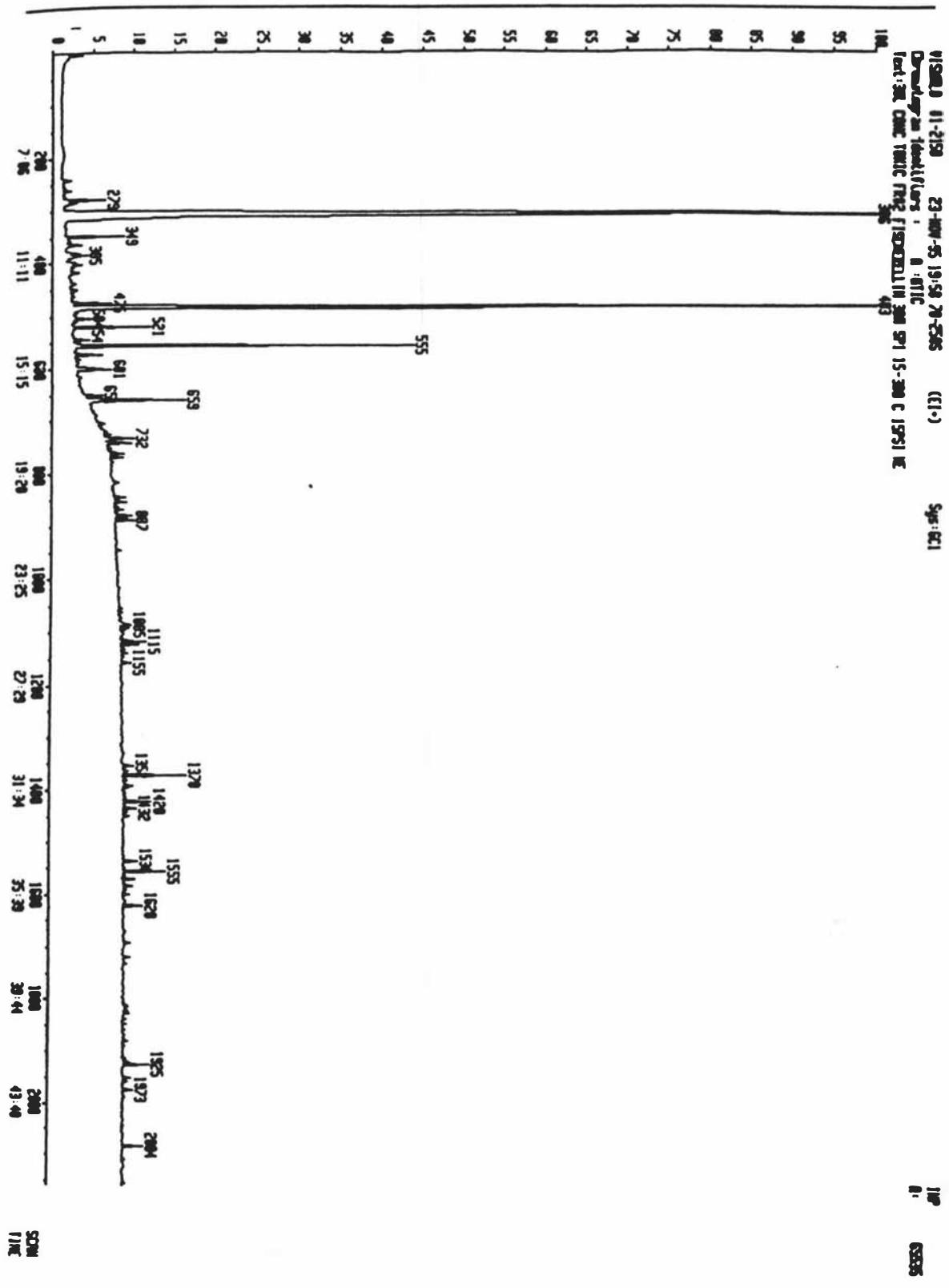


Figure 5.10. GC-MS chromatogram of toxic fraction No. 2.

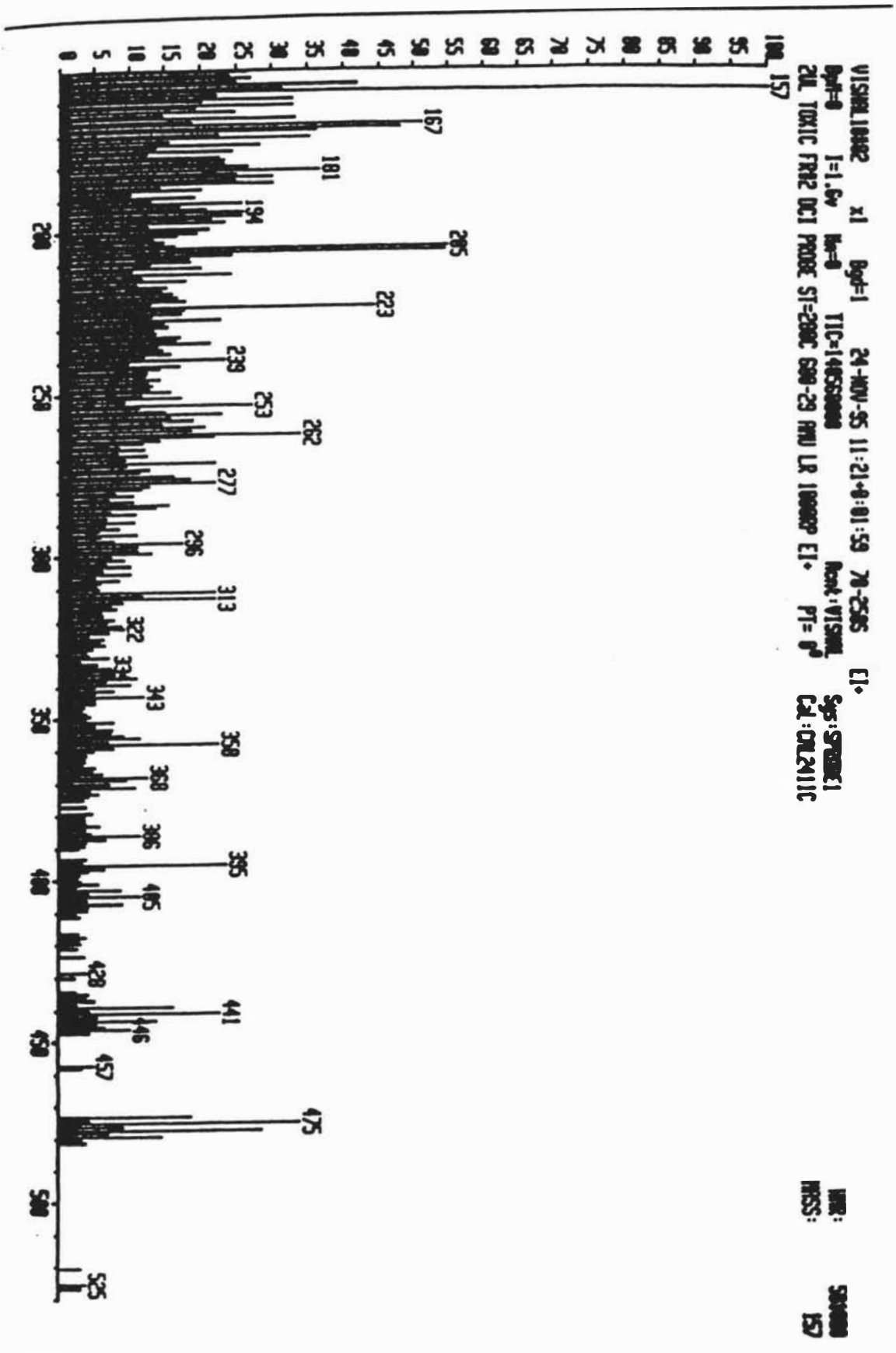


Figure 5.11. Full scan of toxic fraction No. 2, using DCI probe method for the detection of a signal associated with *m/z* 408.

5.4 DISCUSSION

The significance of toxins of individual cyanobacteria will depend on their population density in the aquatic ecosystem and the nature and potency of the toxicity towards other (micro)organisms. As indicated in Chapter 2, the toxic effects caused by cyanobacteria are prevalent in New Zealand. Several species of the genus *Fischerella* have been reported to occur in the New Zealand environment (Chapter 1). Therefore an attempt to detect and characterize the toxin molecule was justified in the context of the current research.

In the present investigation an initial screening for *F. muscicola* toxin(s) using an agar zone diffusion method detected the production of bioactive material from this microorganism. Further attempts were made to isolate the allelochemical (*fischerellin*) known to be produced by this microorganism and identified and characterized by Gross *et al.* (1991). The preliminary work using isocratic high-performance liquid chromatography (HPLC) and similar analytical conditions as described by Gross *et al.* (1991) indicated that there was a peak (at approximately 7.8 min) with similar characteristics of absorption at two different wavelengths (ie. 267 & 415 nm). However, when screened for bioactivity this peak was not toxic to the indicator organism. Nevertheless, toxicity was detected in two other fractions which eluted earlier from the column (see Figure 5.1). These were the only two fractions with which toxicity was associated. These results were confirmed several times by repetition of the experiment. Thus, in this study the GC/MS technique was included to confirm whether firstly, the

fischerellin molecule was present in the toxic fraction and secondly, whether it was absent in the non-toxic (no absorbance at λ 415 nm) fractions. With this information the results of HPLC analyses could be re-assessed in terms of fischerellin being present in those fractions exhibiting inhibitory activity.

However, the GC/MS (and/or DCI probe) results confirmed that the HPLC peak at approx. 7.8 min. (non-toxic fraction) was not fischerellin because of the absence (ie. not present to a significant degree) of molecular ion with a *m/z* of 408. When the other two toxic fractions were subjected to GC/MS analysis they also exhibited an absence of molecular ion, *m/z* 408. The possibility exists that fischerellin may be present in the sample but that it was in too dilute a concentration to detect. However, Gross *et al.*, (1991) have reported both chemical and biological characteristics of this molecule which they call fischerellin. Therefore it was concluded that during this present study the cyanobacterium *F. muscicola* (UTEX 1829) synthesized toxic metabolites although the production of fischerellin with the characteristics of as published by Gross *et al.*, (1991) could not be confirmed using the GC/MS procedure.

Chapter 6

Growth and toxin production by *Fischerella muscicola* in batch culture

6.1 INTRODUCTION

The products of secondary metabolism are usually defined as those compounds which are synthesized by the cell and which have no obvious role in the primary metabolism of the organism. Secondary metabolites are chemically very heterogenous in structure and their distribution in nature is usually so restricted that a particular compound will be synthesized by only few strains within a species. The toxins (inhibitory metabolites) of the cyanobacteria are good examples of secondary metabolites which are distributed irregularly throughout this group of microorganisms (Carmichael, 1989; Al-layl, 1989). Little is known about the physiology underlying secondary metabolite (toxin) production by cyanobacteria and for various reasons the subject has received little attention in the past. Understanding the regulatory mechanism of toxin production may help in the prediction of toxin production in aquatic environments. Only few growth studies have been conducted to investigate possible regulation of toxin production and most of them were either on *Microcystis* sp. or *Anabaena* sp. Growth of organism has been found to be related to toxin (secondary metabolite) production in cyanobacteria (Codd and Poon, 1988; Carmichael, 1986). Production of inhibitory metabolites varies not only with the nutrient growth medium used but also with the stage of culture development. Secondary metabolites production in the microorganisms is generally said to be biphasic i.e. they are formed only when the growth rate of the organism has declined (Vining, 1986). Furthermore, during the growth in batch culture cyanobacterial isolates have been shown to vary in their toxicity (Al-Layl, 1989). The accumulation of secondary metabolites (cyanobacterial toxin) in batch culture after rapid growth has been reported (Kiviranta

et al., 1991; Watanabe *et al.*, 1989). Uniphasic production of inhibitory metabolites in cyanobacteria has been reported also. Armstrong *et al.* (1991) reported that bioactive metabolites may be either constitutively produced throughout the period of exponential growth phase in batch culture or, alternatively, synthesized during the pre-stationary or stationary phases.

Another important aspect is the release of toxins from the cells into the surrounding medium during the growth of the cyanobacteria. Toxin release varies between the species of cyanobacteria, but this subject has not been extensively studied. Laboratory studies with toxic *Microcystis* strains collected from lakes have shown that a substantial release of toxins from the cells into the waters occurs when the blooms die, because of cells breaking down. A substantial release of microcystins from healthy growing cultures of *Oscillatoria* has, however, been observed. It is, therefore, conceivable that there is some release of toxin from the blooms during their growth phase, with a greater release upon cell death (Anon., 1990). Table 6.1 shows the distribution of the hepatotoxin microcystin during the laboratory culture of toxic cyanobacteria.

Table 6.1. Distribution of microcystin during laboratory culture of *Microcystis aeruginosa* (from Anon., 1990).

Age of Culture	% Distribution of Toxins	
	Cells	Water
Young, slowly-growing cells	100	0
Young, rapidly-growing cells	75-90	10-25
Old slowly-growing intact cells	70-80	20-30
Old decaying cells (leaking cell contents)	30-40	60-70

There has been little work on the effects of the growth and growth conditions on toxin (inhibitory metabolites) production for any of the toxic phytoplankton group. Most of the findings reported were on maintaining a few strains in culture in order to isolate, purify, and characterize the toxin present (Carmichael, 1986).

To facilitate our understanding of the role of these inhibitory metabolites in the broad ecology of the cyanobacteria, studies linking growth and production is a pre-requisite. In Chapter Four, it was shown that *Fischerella muscicola* exhibited inhibitory activity, thus confirming reports in the literature. However, as there have been no reports of studies which characterize the growth and toxin production by this cyanobacterium under laboratory conditions, it was decided to examine the growth characteristics and toxin production in batch culture of *Fischerella muscicola* as part of a general investigation of this organism's ecology.

6.2 MATERIAL AND METHODS

6.2.1 Organisms and growth conditions

6.2.1.1 Culture source

Axenic cultures of the cyanobacteria *Fischerella muscicola* (UTEX 1829) and *Anabaena flos-aquae* (UTEX 1444) were obtained from UTEX culture collection. See Chapter 3 for detail. Cultures were grown at 25°C, illuminated with daylight tubes at 60 µmol m^{-2} s^{-1} with 16:8 hour of light/dark cycle. Allen's culture medium (see Chapter 3) was used for both organisms. Growth was conducted in Erlenmyer's flasks (100 rpm shaking in environ shaker) using medium volumes ranging from 50 to 200 ml.

6.2.1.2 Culture maintenance

The cyanobacterial cultures were maintained on Allen's culture medium (Chapter 3).

6.2.1.3 Inoculum preparation

The procedure for inoculum preparation was as outlined in Chapter 3.

6.2.2 Measurement of growth parameters

Duplicate flasks containing 50 ml Allen's culture medium was used for dry weight

determinations. Optical density measurements were on 3 ml samples taken in triplicate measuring the absorbance at an optical density of 750 nm. Dissolved oxygen concentrations were measured directly from the experimental flasks 30 minutes precisely before the end of the light phase. Details for these procedures are provided in Chapter 3.

6.2.3 pH measurement

The pH of the culture medium was measured after filtration through Whatman GF/C glass fibre filters. Chapter 3 provides details.

6.2.4 Toxicity measurement

Toxicity was measured using an agar zone diffusion assay system (Chapter 3). *Anabaena flos-aquae* was used as indicator organism.

6.2.5 Presentation of Data

The experiments in Chapter 6 are presented in three sections. The first section describes toxicity measurements performed on a weekly basis using 200 ml culture volumes. These experiments provided a perception as to how the toxin is produced during growth. During the four week cultivation period as required by *Fischerella muscicola*, samples were removed at the end of each week for analysis, i.e. for toxicity measurements. For

growth measurements 250 ml Erlenmyer's flasks were set up and contained 50 ml culture volumes. For logistic reasons sampling for growth measurement was done every alternate day.

In the second section of the Chapter only the growth measurement and selection of growth measurement parameters are presented for an experimental period of three weeks. Growth measurements were made in terms of Optical Density which was then used to calculate the specific growth rate. A linear relationship existed between Optical Density and Dry Weight (see Fig. 6.5). Normally the specific growth rates are calculated by Dry Weight but in view of this linear relationship and the convenience of Optical Density, this later procedure was used throughout. This Optical Density method has been recommended by Stein (1973), who provides a thorough discussion of the concepts. The growth characteristics of *F. muscicola* is presented in Section 3.1.2, and during growth filaments fragment and re-grow, creating the Optical Density that can be measured (see Fig. 6.5). The Chapter's third section describes the production and leakage of toxin(s) into the culture media during the batch culture period of two weeks. This period of study was established from the results obtained from the first section describing month-long studies. The toxicity measurement in these latter experiments were conducted more frequently, once every day for 15 days and also every 8^{th} hour each 3^{rd} day. These shorter sampling intervals were chosen to provide a more detailed account of toxin production during the growth.

6.3 RESULTS

6.3.1 Preliminary determination of growth and toxicity of *F. muscicola*

Table 6.2 and Figure 6.1 show the changes in toxicity (measured as zones of inhibition) during the growth of *F. muscicola.* Although the growth curve (Figure 6.2) and the toxicity data (Figure 6.1) cannot be interpreted together as the samples for toxicity measurement and the samples for growth measurement were taken from different flasks, the results, however, do give an indication of toxin(s) production at different stages of growth. The gradually decreasing values for the zone of inhibition suggest that the toxin is produced only by the actively growing cells. The toxicity of the culture cell extract starts decreasing after one week and by the end of the fourth week it had diminished by two thirds of that observed initially.

Table 6.2. Cytotoxicity of cell extracts of *F. muscicola* grown in shake flask and tested against the indicator organism *A. flos-aquae.*

Culture Age	Inhibition zone Diameter (mm) of cell extract
1 week	31.3 ± 1.2
2 week	25.0 ± 1.5
3 week	22.6 ± 1.7
4 week	22.2 ± 1.1

± = Standard Error.

F. muscicola grown in Allen's medium exhibited a specific growth rate of 0.26 day^{-1}.

The biomass, measured increased until the stationary phase of the growth.

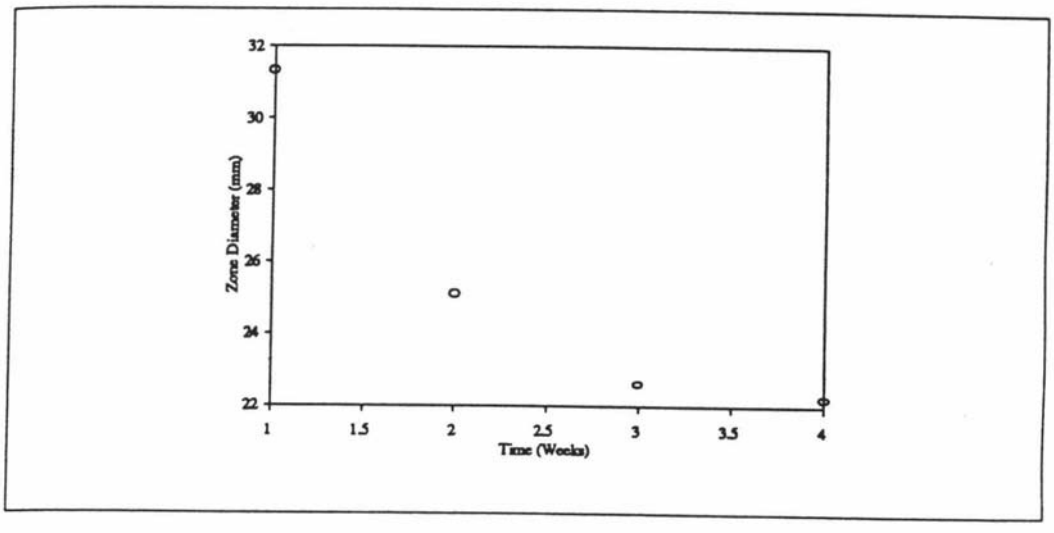


Figure 6.1. Toxicity of *F. muscicola* towards *Anabaena flos-aquae*.

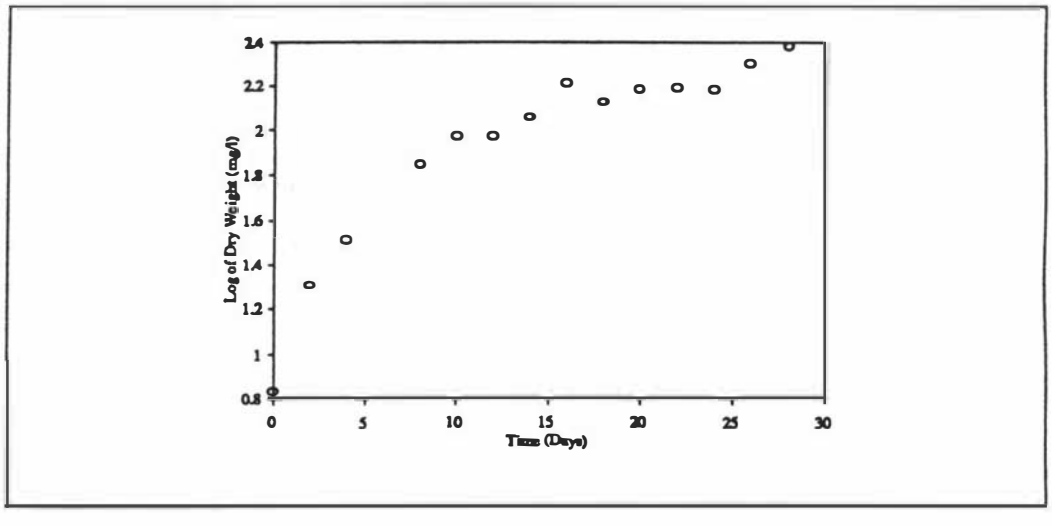


Figure 6.2. Growth curve of *F. muscicola*.

Figure 6.3 Agar plates demonstrating the agar zone diffusion method for toxin production. Growth of *Anabaena flos-aquae* UTEX 1444 is drawn on Allen's Agar. (a) No cytotoxin in central well, (b), (c), (d), (e) cytotoxin in central well producing a qualifyable zone on inhibition.

Figure 6.3a

Figure 6.3b

Figure 6.3c

Figure 6.3d

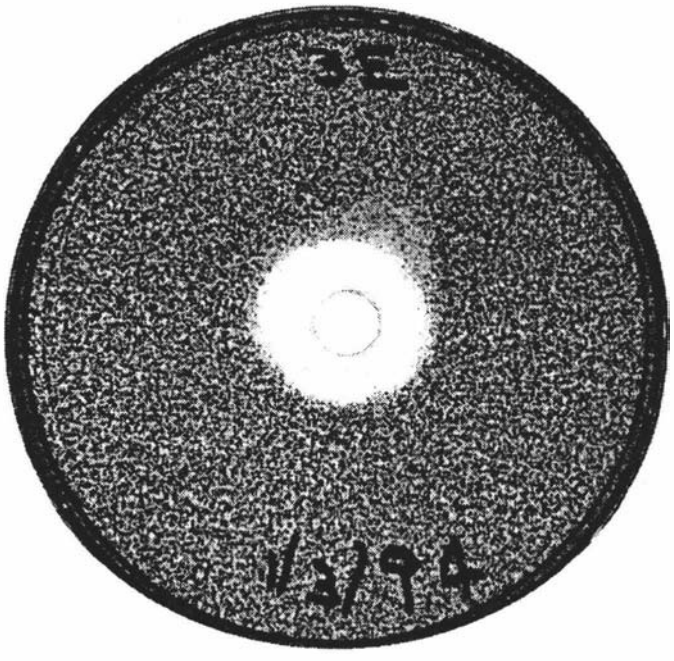


Figure 6.3e

6.3.2 Quantifying Growth of *F. muscicola* (Choice of Method)

The procedures examined were those of optical density at 750 nm; dry weight and cellular protein concentration. The results of the investigations are presented in Figure 6.4 (a, b) which shows the growth curve of *F.muscicola* in batch cultures, illustrated as dry weight (Figure 6.4a) and protein concentration (Figure 6.4b). There was a good correlation found between the dry weight of the culture and the optical density measurement (Figure 6.5). However, the correlation of either of these parameters with the protein measurement was poor (not shown). The protein-dye binding method (Bio-rad Bradford's method) determines only water soluble proteins, and soluble protein concentration did not necessarily correlate with the growth of the organism as estimated by dry weight or optical density. Therefore, this protein-dye binding procedure was not used in subsequent experiments. Because of the convenience of the optical density method all subsequent growth experiments used this procedure. Conversion of optical density to dry weight was achieved using a standard curve (Figure 6.5). The specific growth rate of *F.muscicola* under the culture conditions described in the "Materials & Methods" section, was 0.26 day^{-1} (Figure 6.4a).

It was necessary to establish an appropriate procedure for quantifying the growth of the cyanobacterium *F. muscicola* particularly in view of its mode of growth and cellular form (see Figure 6.6).

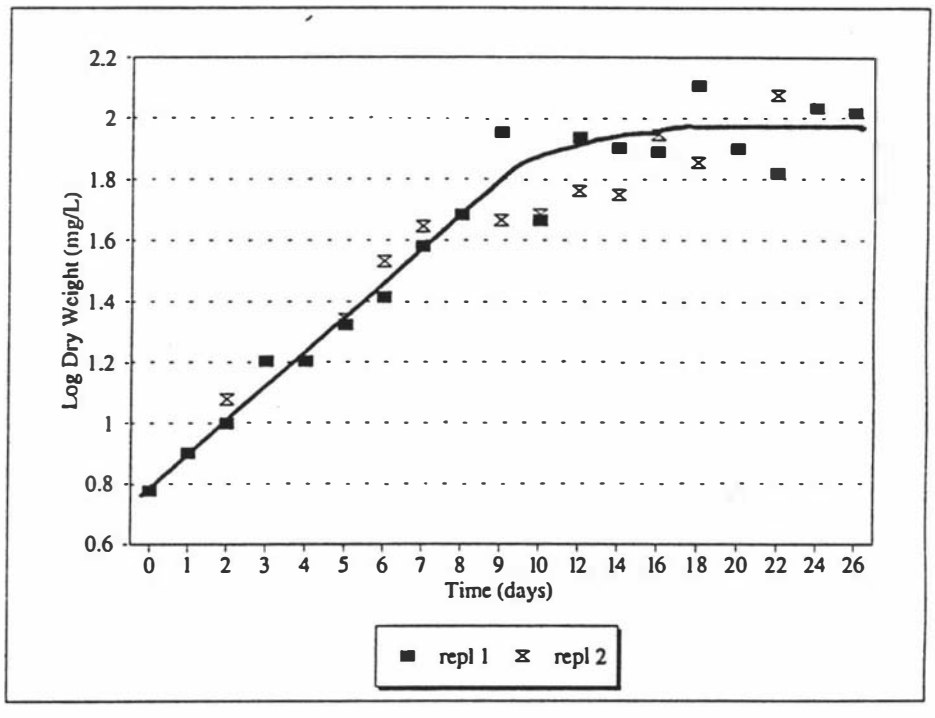


Figure 6.4. Growth of *F. muscicola* as measured by dry weight (mg/l).

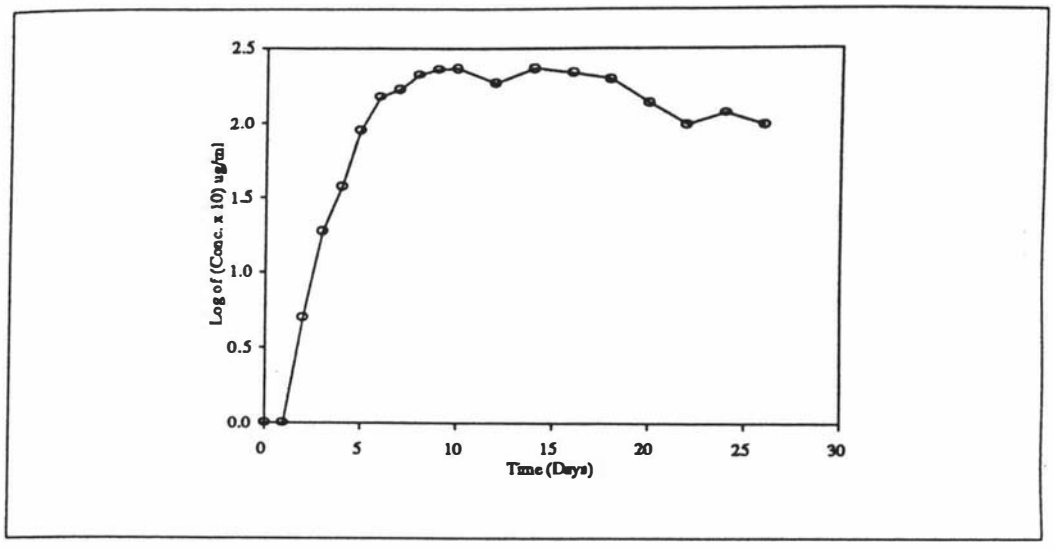


Figure 6.4b. Growth of *F. muscicola* followed by Protein measurements.

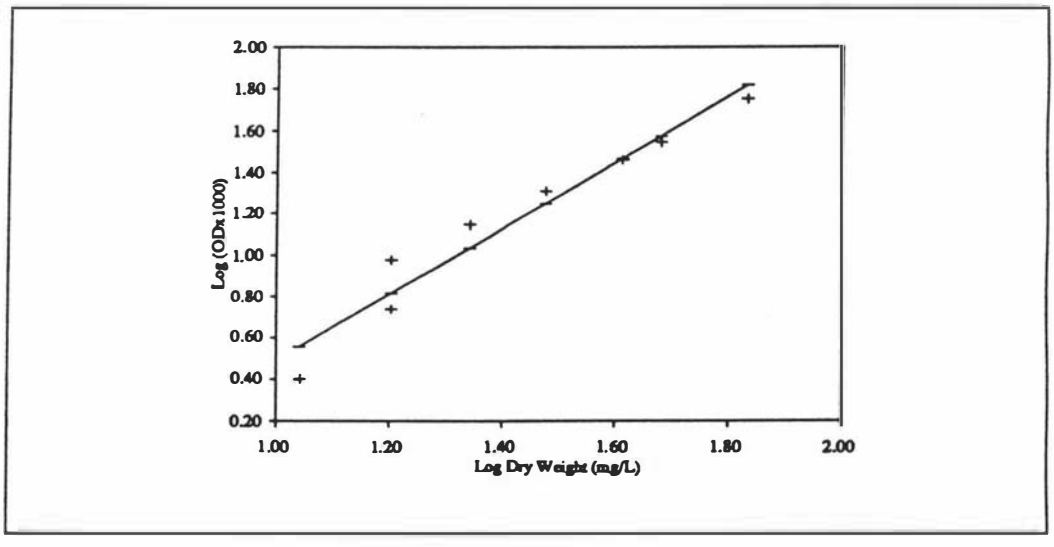


Figure 6.5. Standard curve of Optical Density vs Dry weight. Correlation Coefficient (r) = 0.94

6.3.3 Growth and toxicity of *F.muscicola* in batch culture

Investigations were made for the production and leakage of *F. muscicola* toxin(s) into the batch culture medium. The organism was grown over a period of 15 days. A sample was taken every day and the optical density reading was recorded.

6.3.3.1 Extractable Intracellular Toxicity

Constantly low but consistent toxicity in the cell extracts of *F. muscicola* was found up to 5th day of growth (Figure 6.6b). Toxicity started to increase however after this time. When no further growth was occurring (days 9-12) the highest amount of toxicity was found in cell extracts. Toxicity was expressed as inhibition zone diameters (mm). After the 11th day there was a gradual decrease in the toxicity of cell extracts of *F. muscicola.* At the beginning of stationary phase it was expected that high numbers of intact cells would be present. However, as the stationary phase proceeded the presumption was that the cells may begin to die allowing the toxins to be released into the culture medium, therefore resulting in a decreased toxicity from the cell extracts.

Equivalent of day 0 reading for toxicity and biomass based on the dilution underwent on addition of a 6 day inoculum was 5.4mm and 4.9 mg/L dry weight respectively. A strong increase in activity over the first 24 hours of steady culture growth was observed

(see Table 6.3). After this initial period of high activity the amount of toxins in the cells increased slightly[1] to the 9th day and the amount of toxicity per unit cell mass was approximately constant after the 5th day. The complete release of toxin into the supernatant was not observed suggesting that the toxin did not leach from non-growing cells and may have been degraded therein as is suggested from the measurements at 196th day i.e. 12.7±0.6 mm zone (Table 6.4).

Table 6.3. Ratio of Extractable Toxicity (inhibition zone diameter) versus biomass of *F. muscicola.*

Culture Age (Days)	Inhibition Zone Diameter (mm)	DW (mg/l)	Ratio (Tox/DW)
1	46.84 ±1.51	13.48	3.47
3	47.45 ± 1.66	28.18	1.68
5	47.19 ± 1.21	38.46	1.23
6	53.07 ± 4.79	48.53	1.14
8	53.66 ± 2.08	58.48	0.92
9	60.21 ± 3.23	46.88	1.29

6.3.3.2 Specific Toxicity

A calculation of specific toxicity (Table 6.3) enabled an understanding of how toxicity changed throughout the nine days of the study in relation to the amount of cells present. It was shown that the ratio of toxicity per unit biomass remained approximately at 1.15 ± 0.07 (SE) for days 5 to 9 having diminished from initial high values (3.47 at day 1 and 1.68 at day 3).

[1] For weighted non-linear regression, p=0.007

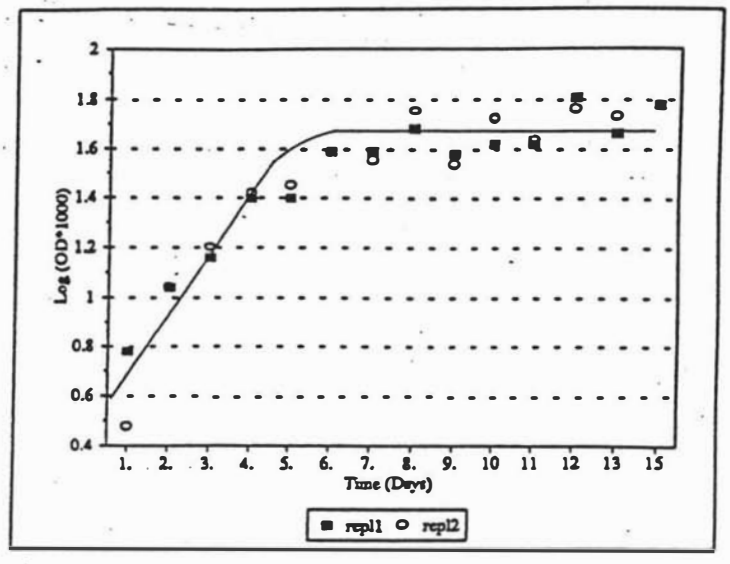


Figure 6.6a. Growth of *F. muscicola* as measured by OD changes at wavelength 750 nm.

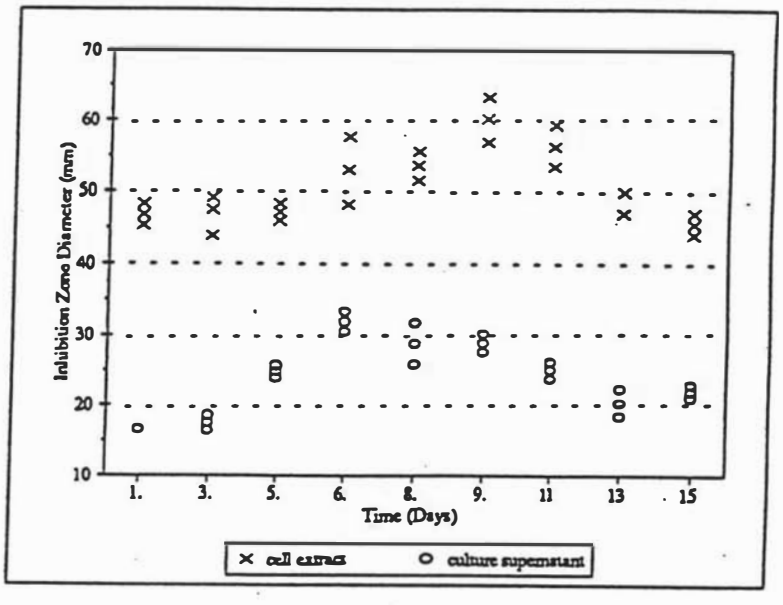


Figure 6.6b. Toxicity of *F. muscicola* culture towards *A. flos-aquae*.

6.3.3.3 Diffusible (Supernatant) Toxicity

The leakage of toxic material in the culture medium was detected from the day one and throughout growth. This toxicity was found to be consistently low throughout the growth period compared with intracellular toxicity ie. extractable toxicity. The pattern of leakage of the toxic material into the culture medium was consistent too. Since the toxicity of the supernatants was approximately 50% of that observed for extractable toxicity, it seemed that most of the toxicity was retained within the cell. The highest amount of toxicity in the cells was found on the ninth day when the population had stabilized indicating the start of stationary phase. By contrast, the highest amount of toxicity in the culture medium was found on the sixth day when the population was in its late logarithmic growth phase.

6.3.3.4 Supernatant pH and Dissolved Oxygen Concentrations

The pH value of the culture medium (initially 7.8) started to increase after day 3 as the population progressed and reached pH 10.0 by 15th day of growth (Figure 6.7). The higher limit of this pH range (7.8-10.0) is indicative of a weak base, possibly caused by the formation of various classes of compounds e.g. combined nitrogen-ammonia, amino acids and or peptides. Also, the removal of nitrate by metabolism can also force up the pH. Nitrate (supplied as $NaNO_3$) in the culture medium is utilized by the cells for amino acids and peptide synthesis. Therefore, NO_3^- concentration reduces in the culture medium. However, the requirement for Na^+ by the cells is not so much, therefore, the cation anion balance shifts towards alkali side. Cyanobacteria are known to produce

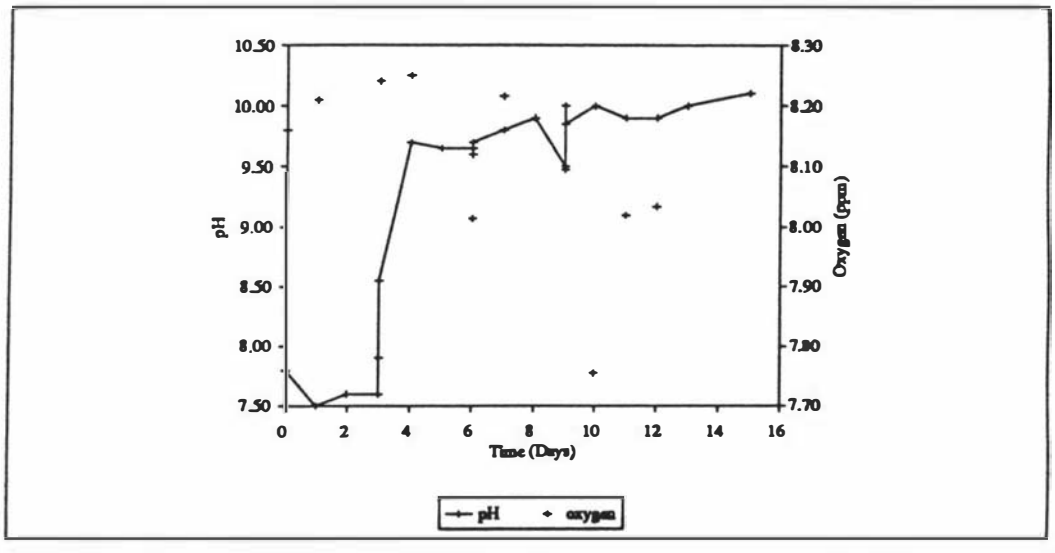


Figure 6.7. pH and Oxygen curve of *F. muscicola* culture.

extracellular substances during the course of their growth (Fogg *et al.* 1973). However, in the present study no attempt has been made to confirm the exact identity of the chemical compounds released and which may be alkaline in their reaction. Also this pH range is unlikely to affect the growth as cyanobacteria will grow well at higher pH values (Fogg *et al.* 1973). The increase in pH (Fig. 6.7) of the medium has no effect on the toxicity of the organism (Fig. 6.6b). Toxicity of this cyanobacterium seems to be independent of the pH values between 7.8 - 10.0. However, the pH of 9.5 has been reported to increase the production of hepatotoxin in cultures of *Microcystis aeruginosa* (van der Westhuizen & Eloff, 1985, Wicks & Thiel, 1990). In the present study, highest toxicity was noted (day 9) when the pH was approximately 9.8.

Photosynthetic activity appeared to be maximal between the second and sixth days which coincided with the exponential growth activity after which there was a slight decrease in dissolved oxygen concentration and hence photosynthetic activity.

The cytotoxicity of *F. muscicola* culture after 196 days, when the culture was in death phase, was also determined by antimicrobial assay. Although the toxicity of the culture was low there was more activity detected in the culture medium than the cell extract. Results are presented in Table 6.4.

Table 6.4. Cytotoxicity of extracts of *F. muscicola* grown in shake flask and tested against the indicator organism *A. flos-aquae*.

Culture Age 196 days	Inhibition Zone Diameter (mm)
cell extract	12.7 ± 0.6
culture broth	16.8 ± 0.4
control (solvent only)	NID

NID = No Inhibition Detected.
± = Standard error.

6.4 DISCUSSION

The purpose of the experiments this chapter describes was to define the toxicity of *F. muscicola* in relation to its growth. The initial week long experiment gave an indication that the production of toxic metabolite(s) (extractable or diffusible) reached its highest in the late logarithmic phase of growth and then gradually decreased as the culture aged. Concurrent growth measurements carried out in parallel with toxicity detection experiments indicated that in a period of one week the population reached the late exponential phase of growth.

In this experiment, with the culture conditions similar to those used by Gross *et al.*, (1991), the specific growth rate was 0.26 day^{-1}. Scott & Barlow (1981) recorded growth rates for the toxic cyanobacterium *Microcystis aeruginosa* (South African strain) of 0.37, 0.395, & 0.385 day^{-1} under various light intensities. Armstrong *et al.* (1991) found a growth rate of 0.23 day^{-1} for the cytotoxin producing cyanobacterium *Lyngbya* strain.

The growth characteristics of *Microcystis* and *Anabaena flos-aquae* in nature and in culture has been established by a number of investigators (Kiviranta *et al.*, 1991; Al-Layl, 1989; Priestly, 1989; Watanabe *et al.*, 1985, 1989). Bloor and England (1989) found that *Nostoc muscorum* produces an antimicrobial antibiotic in its post-exponential phase of growth. At 24 hours after inoculation in the present study the toxicity value was 77% of the maximum value obtained on day 9. By calculation, initial activity at (associated with inoculum) was 9% of that noted on day 9. The inference that can be drawn is that there has been a strong increase in the activity over the first 24 hours. In transferring a culture from its previous stationary growth phase into a new culture medium, rapid metabolic changes can be induced involving biosynthesis, which has been described as a "shift-up" phenomenon by Mandelstam & McQuillen (1973). One may hypothesise that in *F. muscicola*, a metabolic shift-up may lead to the rapid biosynthesis of inhibitory metabolites detectable at the 24^{th} hour. It is expected that in the first 24 hours the physiological state of the culture was passing through the lag and into the early exponential phases of growth. It may not be unusual for a culture to produce inhibitory metabolites during these early growth phases. In *Lyngbya* during its late lag and early exponential phases, 30% of the total toxicity was produced (Armstrong *et al.*, 1991).

During growth from day 1 to day 15 the culture exhibited toxicity changes which require some consideration. The data obtained would suggest that one of possibly two

explanations may describe these toxicity changes. Either the toxicity is increasing and then decreasing or there has been no absolute change in the toxicity over the observed period. Since extreme care was taken to ensure the elimination and or minimization of all possible procedural errors involved in the technique, the observed increase then decrease in the bioactivity from day 1 to day 15 may suggest an increasing and decreasing pattern of toxicity. Armstrong *et al.* (1991) also has reported an increase in the toxicity during the late exponential phase of growth of a marine *Lyngbya* strain.

In this study it was found that *F. muscicola*, however, synthesizes most of its inhibitory metabolite(s) during first 24 hours (as noted above) and which perhaps then remained at the same level. A possible explanation for this apparent steady toxicity is provided by Hewitt (1977) who has stated that "most of the observed variations (in agar zone diffusion assay) are caused by neglect of the physicochemical aspects and not by biological variation". Therefore, the observed increase in the Figure (6.6 b) may be only the variations due to the technique used (agar zone diffusion) for measuring bioactivity. The diffusion coefficient of an antimicrobial substance is known to be a function of the concentration, temperature and the viscosity of the solvent in which antimicrobial substance is dissolved. A dose response curve is characterized by a linear relationship between zone diameter and concentration after which saturation occurs and linearity is lost. Although all precautions were taken to standardize the procedure it was not known whether the toxin concentration used was in the linear or saturation part of the dose response curve. Alternatively, in the present study 40 µl of methanolic crude extract was applied in an 8 mm agar well. Another possibility exists that the solvent used diffused only to a maximum distance of 63 mm which is the maximum inhibition zone observed on day 9 (Figure 6.6 b). These procedural limitations may have led to significant errors in toxicity values observed after 24 hours.

The possiblity exists that *F. muscicola* may synthesize two distinct toxic components. The increase in activity at the beginning of stationary phase suggested that the toxic compound(s) may be different from that detected in the early log phase. Armstrong *et al.* (1991) also suggested that the cytotoxic principles produced during early and late exponential growth of *Lyngbya* may be different compounds. However, in their observation early exponential phase toxicity was undetectable during exponential phase and then re-appeared at late exponential phase. Whereas in this study the inhibitory metabolite(s) produced during early exponential phase did not disappear. Toxicity remained at approximately the same level until the culture entered in the early stationary phase when toxicity rose to a peak and suggesting the possibility of more than one inhibitory metabolite.

This is the first report in which the production of toxin has been monitored throughout the growth of a population of *F. muscicola*. The pattern of production was found to be similar to that of other cyanobacteria. Also, other investigators (Gross *et al.*, 1991) have noted that other species of *Anabaena* are inhibited by the toxin(s) of *F. muscicola*. Thus it is not surprising that the toxicity also exists for *A. flos-aquae*.

These studies have also shown that a uniphasic pattern of toxic inhibitory metabolite(s) production has been demonstrated by *F. muscicola* in batch culture. Both diffusible and extractable toxicity of *F. muscicola* was detected from the day one and persisted until day 196 when the experiment was ended. However, it was found that the cell extracts were more toxic than the culture broth throughout the period of study. Carmichael (1992) has observed that toxins of bloom forming species of cyanobacteria are produced at all stages of the organisms' growth and generally remain inside the cell until age or stress causes their release into the surrounding water. Mason *et al.* (1982) reported that the toxin cyanobacterin is constitutively produced by *Scytonema hofmanni* (UTEX B1581).

The detection of the toxins in the culture broth at the end of growth phase and during the senescence of the culture contributes to our understanding of the fate of cyanobacterial toxins in natural blooms. All toxic species can form water blooms (Carmichael, 1992) and, once the bloom has developed, those toxins will be present and may remain in the natural environment even though the bloom itself is in a state of senescence. Kiviranta *et al.* (1991) observed that both the hepato- and neuro-toxins are retained in the cells during the exponential and declining logarithmic phase of growth and that cell lysis caused the leakage of the toxin into the water. Rijstenbil (1989) found the lysis of the diatom *Ditylum brightwellii*, if induced by osmotic shock, caused the release of toxic metabolites. Results from the 196 days old culture, when *F. muscicola* was in its death phase, showed that the culture broth was more toxic than the cell extract, indicating that the cell lysis due to dying cells may have caused the release of the toxic material(s).

This work has established that toxic substance(s) both diffusible and extractable, are produced by *F. muscicola* during all phases of the growth cycle and reach their highest toxicity once the culture reaches late exponential and early stationary phase. The next Chapter describes an investigation of the environmental factors which affect the production of toxic substance(s) in *F. muscicola.*

Chapter 7

Effect of Environmental Factors on Toxins Production.

7.1 INTRODUCTION

It is imperative to acquire in depth knowledge of the regulation of toxin production to enable predictions concerning the formation of toxic metabolites by cyanobacteria. Environmental conditions favouring growth also favour toxicity of the organism (Sivonen, 1990). However, it has been suggested that the conditions which stimulate growth are not necessarily those which promote toxin production in cyanobacteria (van der Westhuizen *et al.*, 1986; Kotak *et al.*, 1995). Since bloom formation of cyanobacteria is dependent upon a number of environmental factors such, as light intensity, temperature, nutrient conditions and pH, then it is also possible that these factors also influence the toxicity of cyanobacteria (Runnegar *et al.*, 1983; Watanabe and Oishi, 1985; Codd and Poon, 1988; Codd *et al.*, 1989; Sivonen, 1990; Anon., 1990; Borowitzka, 1995; Kotak *et al.*, 1995).

However, there is only limited knowledge concerning qualitative and quantitative aspects of the actual factors that induce the toxicity of cyanobacteria. Moreover, there is no information available on the bio-regulation of the toxicity of *Fischerella muscicola.* Consequently, more information is needed on how environmental factors influence the dynamics of toxic blooms in nature. Light intensity, temperature and nutrients are examples of environmental factors that may be of particular significance in this respect.

Light intensity is a factor which appears to exert different effects on cyanobacteria. These effects can vary depending on whether the observations are made in the field or in the laboratory (Fogg *et al.* 1973). The cyanobacteria are known to have preference for low light environments and can respond to low light intensities over a wide band of the visible spectrum (Carmichael, 1992). van der Westhuizen & Eloff (1985) and Gorham (1964b) have reported that light intensity has a distinctive effect on the growth but not on the toxicity of *Microcystis aeruginosa* (NRC-1). Wayman & Fay (1987) noted that 50-60 $\mu E\ m^{-2}\ s^{-1}$ of white light irradiance is enough to attain the maximum growth rate for most temperate fresh water cyanobacteria. Literature (Chapter 2) indicates that light intensity may be one of the primary factors which influences the toxicity in cyanobacteria, and therefore it was decided to examine its importance in the present study.

Temperature is also known to be a key factor in regulating toxin formation (Carmichael *et al.*, 1985; van der Westhuizen & Eloff, 1985; Watanabe & Oishi, 1985; Codd *et al.*, 1989; Sivonen, 1990; Wicks & Thiel, 1990; Patterson & Bolis, 1993; Borowitzka, 1995). It has been reported, however, that water temperatures below 20°C are unfavourable for the mass development of bloom forming genera (Carmichael, 1992). The above literature describes the temperature as an important factor in toxin formation for other cyanobacterial genera. In the present study this factor was included to explore its role in toxin formation in the cyanobacterium *F. muscicola.*

Major nutrients such as nitrogen and phosphorus are heavily implicated in eutrophic waters where cyanobacterial blooms are quite common and are the main factors which limit the cyanobacterial biomass development in many lakes. Carmichael (1986) stated that toxin production in cyanobacteria is stimulated as growth decreases due to a shortage of nitrogen and phosphorus. As indicated earlier in this Chapter, the conditions for growth may be different from the conditions promoting the toxin formation, therefore it was decided to study the effect of two major nutrients, nitrogen and phosphorus on the toxicity of *F. muscicola* grown under laboratory condition. The usual procedure when deciding what concentrations of these nutrients that are growth limiting for the organism, it is necessary to conduct preliminary experiments for each nutrient in the chosen medium over a range of concentrations that cover both limiting and sufficient conditions. This was not possible in the present study because of time constraints and therefore it was decided to study nutrient concentration diminished and increased by 75% below and above (respectively) of the concentrations of nitrogen and phosphorus. The conditions were subjected to a factorial experimental design. The advantages of this approach has been discussed further in the Introduction. A 50% (+ or -) variation in the concentration of nitrogen and phosphorus from an original concentration of 1.5 g/l in the culture medium (similar to the one used in the present study) resulted in a significant effect on the production of antibiotic by a filamentous, nitrogen fixing cyanobacterium *Nostoc muscorum* (Bloor & England, 1991). Therefore, for the present study a concentration range variation of 75% (+ and -) was chosen to give a wider range than that recommended by Bloor & England (1991). The mid-point concentration of nutrients for the experimental design was 1.5 g/l for nitrogen and 0.038

g/l for phosphorus as recommended by Allen (1968). The upper and lower limit (i.e. ± 75%) was arbitrarily chosen. At the upper value the expectation was that this concentration would provide either an ongoing nutrient sufficiency or possibly an inhibition whereas at lower value the expectation was that the culture of *F. muscicola* would experience a nutrient deficiency (Bloor & England, 1991, observed nutrient deficiencies with a 50% reduction in the nutrient concentrations). Under nutrient limiting situations the culture experiences a slowing of metabolic activity which is reflected in lower growth rates, lower yields of cell mass and possibly diminished toxin synthesis. These experiments were conducted to test whether changes in nutrient concentrations would lead to changes in toxicity of the cultures of *F. muscicola.*

Do variations in environmental factors influence the synthesis and subsequent release of toxins by cyanobacteria? It is still too early to answer this question. However, in Chapter 6 it was shown that the release of toxic material(s) into the culture medium increased as the growth of the *F. muscicola* proceeded. Earlier studies with *Oscillatoria* sp. (Kiviranta *et al.*, 1991) and *Microcystis* strains (Anon., 1990) found a substantial release of toxins from the cells into the water due to cell lysis caused by culture ageing under the laboratory conditions, while experiments with *F. muscicola* have shown a constant release of toxic material from the cell into the culture medium (Chapter 6). There is a need then to study toxin release from the cyanobacteria under various environmental conditions because it is essential to know whether changes in any of the environmental conditions (factors) will cause a change in the release of toxic material(s) from the cell into the culture medium. Any proposed study in this area is, however,

likely to be confounded by many interacting factors. Therefore it would appear important to design with care the experimental work.

According to Mead *et al.* (1993) it is important to distinguish two components of an experimental design, the structure of the experimental units and the structure of the treatments. A proper understanding of experimental design requires that each component is first understood separately. Understanding the structure of the experimental units requires an identification of the units and a description of likely patterns of variation between them if a single treatment were applied to all the units. Treatment structure is concerned with the choice of different treatments to be included in the set of experimental treatments. The choice must be determined by the objectives of the experiment.

Since it takes several weeks to grow cyanobacteria under laboratory conditions, a sequential study of each factor is laborious and time consuming. Therefore it was decided to use a factorial experimental design which offered several advantages when studying the effect of two or more factors, as was required. The method used all treatment combinations simultaneously in the same experiment and partitioned the various degrees of freedom into components for main effects and interactions. Without this factorial structure it would have been difficult or perhaps impossible to investigate the interactions and to draw accurate interpretations of a factor's effects. For example, a study of the effect of light might rightfully entail observations on the interactions with temperature. More generally, factorial experiments provide information on whether

increasing or decreasing the level of a factor causes an increase or decrease in the toxicity of the cyanobacterium under study.

The other advantage of factorial experiments is that when interactions are believed to be negligible, the factorial experiment is more economical than sequential single factor experiments. It seemed appropriate, therefore, to employ an interaction model and to determine that a particular interaction was not important, rather than to find that it was important but was not detectable in a sequential experiment.

STATISTICAL AIM: In order to examine the effect of various physical and chemical environmental factors separately and their interaction on the toxicity of *Fischerella muscicola*, a split plot experiment was adopted.

7.2 MATERIAL AND METHODS

The basic experimental units in this study were 250ml Erlenmyer culture flasks which contained the cyanobacterial culture.

7.2.1 Inoculum preparation

Cultures of *F. muscicola* (UTEX 1829) were grown at 25°C in Erlenmyer flask (1000-ml) with a culture volume of 500 ml Allen's medium (light intensity 60 μmol m^{-2}

s^{-1}, 100 rpm shaking) until they reached mid exponential phase (Chapter 3).

To increase the cell density, flask cultures were concentrated by filtration in a pre-sterilized filtration unit assembled and used in laminar flow cabinet. The harvested cells were re-suspended in an appropriate volume of the sterile filtered medium to give the final culture concentration of 10% (v/v) inoculum in two flasks of 500 ml culture volume.

These flasks were then incubated under the same conditions as above and the cultures were allowed to grow again until they had reached their mid exponential phase. Once the cultures had reached mid-exponential phase, cells were concentrated again by filtration under aseptic conditions and washed with freshly prepared (pH adjusted to 7.8 before autoclaving) pre-sterilized Allen's medium containing neither nitrogen nor phosphorus. The washing solution's volume was similar to the culture volume in which cells were grown. After washing, cells were re-suspended in a pre-sterilized Erlenmyer flask containing Allen's medium (300 ml) containing neither nitrogen nor phosphorus (pH 7.8) . This cell suspension was used as the **inoculum flask** for inoculating aseptically (10% v/v) into Erlenmyer flasks containing 100 ml Allen's medium with varying combinations of nitrogen and phosphorus.

7.2.2 Sample collection and culture preparation

Cells of *F. muscicola* were harvested on the ninth day of incubation under experimental

conditions appropriate to the particular treatment-combination-experiment. The culture was processed for the extraction of toxins as described in Chapter 3.

7.2.3 Toxicity measurement

Toxicity of *F. muscicola* (crude cell extract and culture broth preparation) was measured using an agar diffusion assay system, with *Anabaena flos-aquae* as the indicator organism (Chapter 3). Because of the large number of samples and lengthy procedure for toxin(s) extraction from *F. muscicola*, the timing of the harvesting of cultures of *Anabaena flos-aquae* at the exponential phase of the growth was so adjusted that as soon as the toxin(s) extraction from *F. muscicola* was finished the bioassay was commenced within 24 hours.

7.2.4 Measurement of growth parameters

Optical density and dry weight of the *F. muscicola* culture (duplicate flasks) was measured (Chapter 3) to monitor the growth of the organism.

7.2.5 Experimental design

This study considers the effect of light, temperature, nitrogen and phosphorus on the production of toxin(s) by *F. muscicola*. As light and temperature (physical factors) could not be varied widely for each experimental flask, they were controlled according

to the split plot procedure statistical experimental design (Mead *et al.*, 1993). In this design the main plot factors were light and temperature, both at two levels each and the split plot factors were nitrogen and phosphorus, both at three levels. Sometimes in factorial experiments many investigations in which two or more treatment factors are being investigated, the size of experimental unit which is appropriate for one treatment factor is different from that appropriate to another treatment factor. This form of design is called a split plot design, the large plots being termed **main plots** and the smaller plots, within main plots, **split plots.** The factors are grouped into those to be applied to the main plots and those for to the split plots. The randomization of treatment proceeds in two parts, the experimental treatments consisting of all combinations of levels of factors in the main plot group and are allocated randomly to the main plots in each block and then all combinations of the split plot factors are allocated randomly to the split plots in each main plot.

Batch culture flasks were incubated in an Environ Shaker (see Chapter 3) where each run was conducted under one combination of light and temperature values. Nine different combinations of nitrogen and phosphorus were studied, each in triplicate flasks (a total of 27 flasks). In this experiment a total of two blocks were considered, representing an exact replication of treatment combinations conducted at separate times (see Figure 7.1 and below). Blocking was chosen to reduce the amount of random variation. Variation between blocks was removed by comparing errors involved in treatments. Within each block the experimental treatments were allocated to flasks in a random manner. In the experiment, each flask in each block was treated as one of

four "sequences" in which treatment combinations were evaluated, thus, all four "sequences" together formed the set of main plots (Figure 7.1). In the first block the choice of sequence was as follows: Ist run - low light:low temp, IInd run - high light:low temp, IIIrd run - low light:high temp and IVth run - high light:high temp. For second block the run sequencing was as follows: Ist run - low light:high temp, IInd run - low light:low temp, IIIrd run - high light:high temp and IVth run - high light:low temp. The low and high values for the main plot factors are presented in Table 7.1.

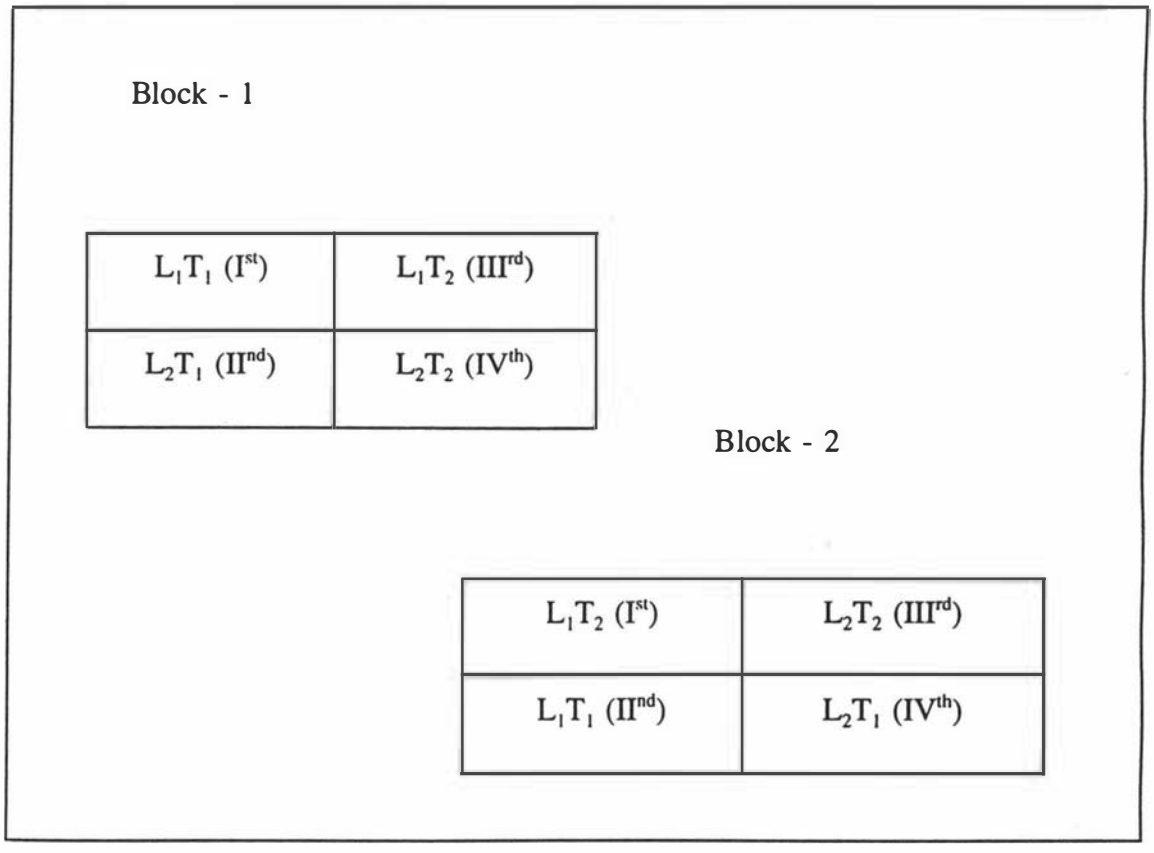


Figure 7.1 Diagrammatic representation of experimental runs showing the main plot factors Light (L) and Temperature (T).

Table 7.1 Values of light and temperature used.

Physical Factor	Values	
	Low	High
Light Intensity ($\mu mol\ m^{-2}\ s^{-1}$)	10	60
Temperature (°C)	10	30

Two levels of light and temperature (low and high) in combination were employed to determine their effect on the toxicity of the organism. The values for low and high levels of light were 10 and 60 $\mu mol\ m^{-2}\ s^{-1}$ and values for temperature were 10°C as low and 30°C as high. There were four combinations of light and temperature together.

The values for various levels of split plot factors Nitrogen (N) and Phosphorus (P) are shown in Table 7.2.

Table 7.2 Concentration of each nutrient factor at their low, original and high levels.

Nutrient at their low and high levels	Concentration g/l		
	1	2	3
N	0.375	1.5	2.625
P	0.009	0.038	0.066

1 75% low concentration from original concentration of nutrients of the Allen's medium.
2 original concentration of nutrients of the Allen's medium.
3 75% high concentration from original concentration of nutrients of the Allen's medium.
N nitrogen as $NaNO_3$.
P phosphorus as K_2HPO_4.

Within the Environ-shaker itself the positions of the culture flasks were also completely randomized. This constituted the split plot structure of the experiment. Table 7.3 shows all the possible permutations of split plot factors Nitrogen (N) and Phosphorus (P).

Table 7.3 Arrangement of all possible combinations of nitrogen and phosphorus.

Treatment combinations for nitrogen and phosphorus with three levels		
N_1P_1	N_3P_3	N_2P_1
N_2P_3	N_3P_1	N_1P_2
N_3P_2	N_1P_3	N_2P_2

7.2.6 Analysis of a split plot experiment

In a split plot design, the analysis of variance is divided into two parts, one is concerned with variation between main plots and the other with variation between split plots within main plots. The experimental data was analyzed using the computing software package "The SAS System" (1990). SAS programming is given in appendix B.

The toxicity of *F. muscicola* was measured using crude methanolic cell extract, hereafter it will be referred as toxicity only.

7.3 **RESULTS**

The main effects and interaction effects were examined by means of two Anovas and the output data from the SAS analysis is presented in Table 7.4.

7.3.1 Effect of light and Temperature

From the data shown in the Anova table (Table 7.4) it was concluded that there was no significant difference (*p*- value 0.53) resulted in the toxicity from the cultures of different experimental runs divided in two blocks. The main plot factors, light and temperature do not show any significant effect (*p*- value 0.21 & 0.66 respectively) on the toxicity of *F.muscicola* on their own whereas their interaction had a significant effect (*p*- value 0.06). The *f*-ratio for treatment was significant at 10% and therefore it was necessary to examine particular comparisons between treatments using (LS Means) *least squares means* (Mead *et al.*, 1993), in order to observe which treatment combinations differed. Pair-wise comparison of LS Means for the main plot factors are presented in Table 7.5.

Table 7.4. ANOVA Table showing the overall effect of various factors and their combinations on the toxicity of *F. muscicola*.

Source	DF	SS	Mean Square	F-ratio	p-value
Main Plot					
BLOCK	1	403.79	403.79	0.50	0.53
LIT	1	2062.54	2062.54	2.56	0.21
TMP	1	193.50	193.50	0.24	0.66
LIT*TMP	1	7077.37	7077.37	8.79	0.06
Error (1)	3	2415.66	805.22	66.52	0.0001
Split Plot					
NG	2	1.6	0.80	0.07	0.94
PH	2	11.91	5.95	0.49	0.61
NG*PH	4	40.58	10.15	0.84	0.50
LIT*NG	2	36.31	18.15	1.50	0.23
TMP*NG	2	36.32	18.16	1.50	0.23
LIT*TMP*NG	2	18.56	9.28	0.77	0.47
LIT*PH	2	4.48	2.24	0.18	0.83
TMP*PH	2	4.99	2.50	0.21	0.81
LIT*TMP*PH	2	61.59	30.79	2.54	0.08
LIT*TMP*NG*PH	12	181.23	15.10	1.25	0.25
Error (2)	172	2082.14	12.11		
Total	211	14632.80			

LIT - light (L)
TMP - temperature (T)
NG - nitrogen (N)
PH - phosphorus (P)

DF - Degree of Freedom
SS - Sums of Square

Table 7.5. Pair-wise comparison of LS Means of Light and Temperature combinations.

LIT	TMP	TOX	Pr > |T| HO: LSMEAN(i)=LSMEAN(j)			
		LSMEAN	i/j	1	2	3	4
1	1	49.72	1	.	0.09	0.05	0.23
1	2	36.24	2	0.09	.	0.48	0.42
2	1	31.84	3	0.05	0.48	.	0.18
2	2	41.34	4	0.23	0.42	0.18	.

where, LIT 1 = light at low levels (10 μmol m^{-2} s^{-1}),
LIT 2 = light at high levels(60 μmol m^{-2} s^{-1}),
TMP 1 = temperature at low levels (10°C),
TMP 2 = temperature at high levels (30°C),
TOX = toxicity (Inhibition zone diameters, mm),
Pr = Probability,
HO = Hypothesis,
LSMEAN = least squares mean,
i = i^{th} combination,
j = j^{th} combination.

7.3.1.1 The main effect of temperature on the toxicity

The main effect of temperature on its own was not as important as the main effect of light level variation on the toxicity of the organism. Low temperature in combination with low light however resulted a statistically significantly higher amount of toxic activity by the organism (see Table 7.5 and Figure 7.2). The toxicity was significantly reduced (at 10%) when the temperature was increased from 10°C to 30°C at low light level.

At high light intensities the effect of temperature variation on the toxicity was not significant (*p*- value 0.18), although on plotting the response data points (Figure 7.2) there is evidence for an increase in the toxicity of the organism as temperature was increased, but only at high light intensities.

7.3.1.2 The main effect of light on the toxicity

There was a significant (at 5%) effect of light intensity at low temperatures (Figure 7.2), with a decrease in the toxicity occurring when light intensity was increased from 10 μmol m^{-2} s^{-1} to 30 μmol m^{-2} s^{-1} at 10°C. The contrasting effects of high light levels at two different temperatures (low & high) in the graph of mean toxicity plots indicated that the high light level at the higher temperature increased the amount of toxic activity in the organism. From the graph it is evident that by reducing the light levels at 30°C reduces the toxicity of the organism. Although this difference is statistically not significant (*p*- value 0.42) for the higher temperature.

Also from the graph of mean toxicity (Figure 7.2) it can be seen that the culture's respective behaviours at two light levels were exactly opposite to each other; at low temperatures, low light produced a higher toxicity compared to that found at the higher light level whereas at the higher temperature this situation is reversed and there was a higher toxicity at the high light level and low toxicity at the low light level.

It was inferred from these studies that low light and low temperature induced higher

toxicity in the organism, and it was further inferred that the involvement of light in the development of cyanobacterial toxicity was a factor of primary importance.

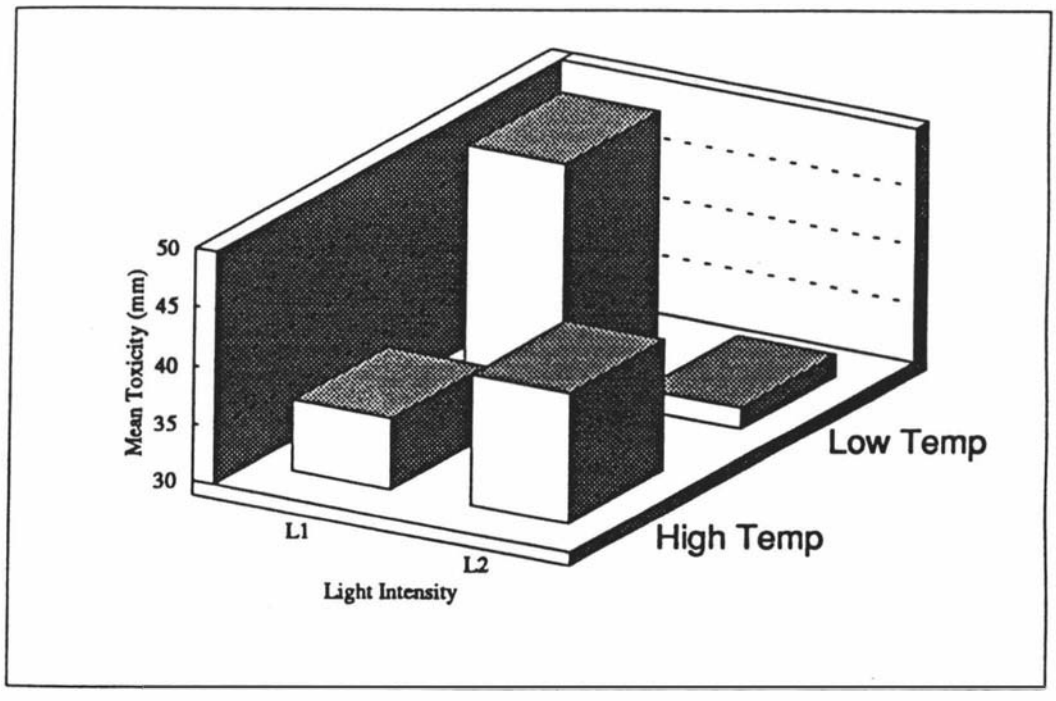


Figure 7.2. Toxicity variation at different levels of light and temperature. L1 = 10 μmol $m^{-2}s^{-1}$; L2 = 60 μmol $m^{-2}s^{-1}$; Low Temp = 10°C; High Temp = 30°C.

7.3.2 The main effect of nitrogen on the toxicity

From the data shown in the Anova table (Table 7.4) it was deduced that, although the nitrogen concentration was varied above and below the prescribed concentration given in Allen's medium there was no significant effect (p- value 0.94) on the toxicity of *F.muscicola.* However, some of the interactions between factors were investigated and that data is presented in Section 7.3.4.

7.3.3 The main effect of phosphorus on the toxicity

There was no significant effect (p- value 0.61) on the toxicity when the phosphorus concentration in the culture media was varied above and below the prescribed concentration given in Allen's medium. However, interactions of phosphorus with light and temperature affecting toxicity production was possible (p- value 0.085) and these aspects are further described in section 7.3.4.

7.3.4 Effect of various physical and chemical factor interaction terms on the toxicity

It can be seen from the Table 7.4 that most of the interaction terms were statistically not significant except for light*temperature*phosphorus (LIT*TMP*PH). The interaction terms which have low p- values are described in Sections 7.3.4.1, 7.3.4.2 and 7.3.4.3 where empirical evidence is presented indicating whether or not variations in the toxicity of *F. muscicola* occurred when these various factors interacted with each other

at various levels.

7.3.4.1 The interaction effects of nitrogen and phosphorus on culture toxicity

No statistically significant differences (*p*- value 0.5) in the toxicity of cultures was observed when the interaction of various combinations' effects of nitrogen and phosphorus in the culture media were analyzed.

7.3.4.2 Interaction effects of light and nitrogen on culture toxicity

The effect of nitrogen on toxicity gave the highest toxic values at low light levels, as evident in the graph mean toxicity plot (Figure 7.3). A highly significant reduction in the toxicity was observed when light intensity was increased from 10 μmol m^{-1} s^{-1} to 30 μmol m^{-1} s^{-1}. However, in Figure 7.3, data shows empirical evidence for an opposite behaviour of cultures in the presence of different nitrogen levels from low light to high light intensities. At low light intensity, a 75% (w/v) increase in the concentration of nitrogen from its normal concentration found in Allen's medium produced a higher toxicity, but this toxicity decreased when the nitrogen concentration was decreased to a value that was 75% (w/v) below the normal amount. This behaviour was exactly opposite to that observed at high light intensities where reducing concentration gives higher toxicity and increasing concentration gives lower toxicity.

No statistically significant differences in the toxicity arose that were attributable to

variations in nitrogen concentration at the light intensities used.

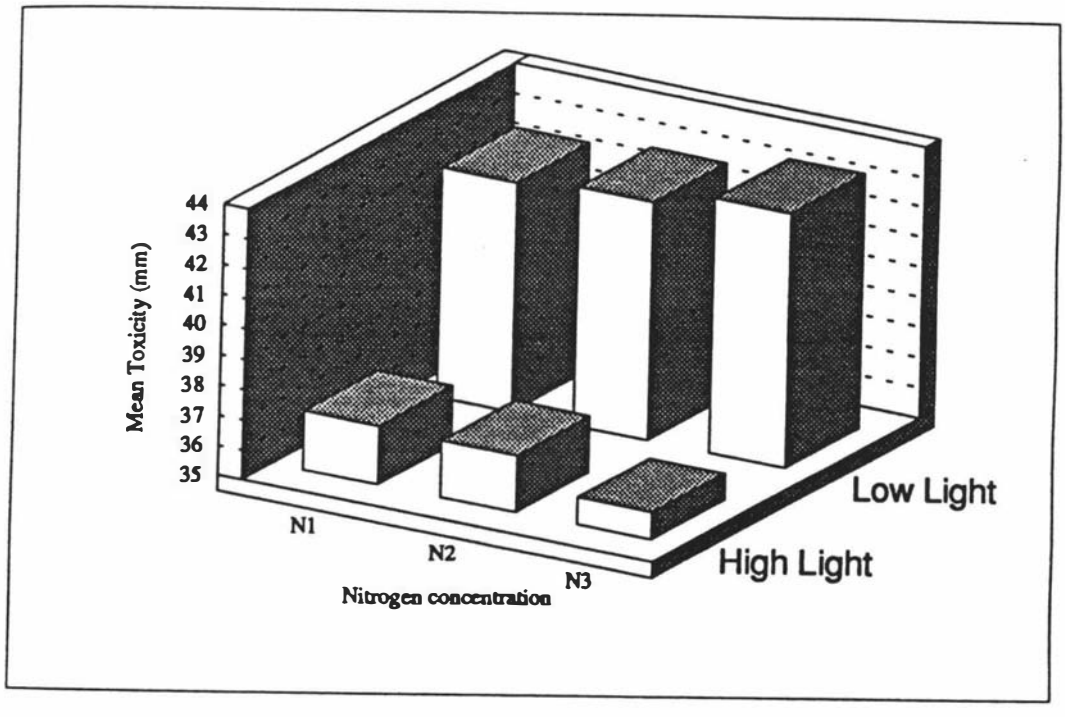


Figure 7.3 Effect of nitrogen on the toxicity at two levels of light. N1 = 75% (w/v) low concentration ie. 0.375 g/l; N2 = Formula concentration ie. 1.5 g/l; N3 = 75% high concentration ie. 2.625 g/l; Low light = 10 μmol $m^{-2}s^{-1}$; High light = 60 μmol $m^{-2}s^{-1}$.

7.3.4.3 The interaction effects of temperature and nitrogen on culture toxicity

At low (10°C) temperature higher toxicity was observed than at high (30°C) temperature (Figure 7.4). When the temperature was increased to higher levels (30°C) a lower mean toxicity was recorded at all three levels of nitrogen. However, the decrease in the mean toxicity at normal concentration of nitrogen was not statistically significant (*p*- value 0.245) whereas the decrease in mean toxicity of cultures containing the other two levels of nitrogen (*p*- value 0.0176 for 75% (w/v) low concentration and *p*- value 0.0004 for 75%

high concentration) were statistically significant.

The highest mean culture toxicity was recorded at 10°C at a concentration of nitrogen in the medium that was 75% (w/v) above the prescribed amount. This same concentration (2.625 g/l) induced the lowest mean toxicity when the temperature was increased to 30°C. Therefore, it is apparent that the decrease in the toxicity of the organism is associated with high values of medium nitrogen (2.625 g/l). The inhibitory effects were more pronounced than those observed at lower concentrations (1.5 & 0.375 g/l) even at the higher temperature of 30°C.

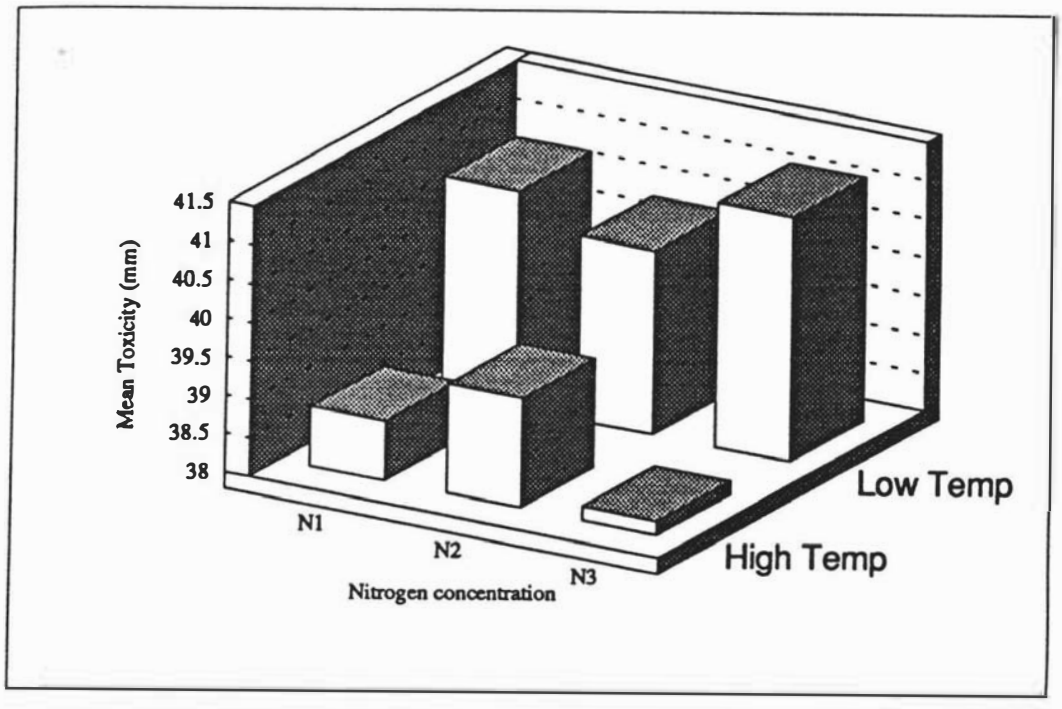


Figure 7.4. Effect of nitrogen on the toxicity at two levels of temperature. N1 = 75% (w/v) low concentration ie. 0.375 g/l; N2 = Formula concentration ie. 1.5 g/l; N3 = 75% high concentration ie. 2.625 g/l; Low Temp = 10°C; High light = 60°C.

7.3.4.4 The interaction effects of light, temperature and phosphorus

From the data of Table 7.4, it was noted that phosphorus in combination with light and temperature does exert a statistically significant (*p*- value 0.08) effect on the toxicity of the organism. Therefore a comparison of the mean toxicities of cultures exposed to all combinations of light, temperature and phosphorus is presented in Table 7.6. The graph presenting culture toxicities (Figures 7.5 & 7.6) shows that at low light and low temperature the overall effect of phosphorus on the toxicity was significantly higher than at any other combination of light and temperature. The lowest toxicity in *F. muscicola* cultures was observed when the phosphorus concentration was low (0.009 g/l) in the culture medium and at the low temperature (10°C) and high the light intensity (60 µmol m^{-2} s^{-1}). There was a significant increase in the toxicity compared to high light and low temperature (Figure 7.5) when light levels were reduced (low light) and temperature was increased to its highest level (Figure 7.6). A further increase in the toxicity was observed when both light and temperature levels were increased to their respective higher levels. The toxicities were significantly different at different phosphorus concentrations and light and temperature combinations. See appendix C for pairwise comparison of data presented in Table 7.6.

No statistically significant changes in the toxicity of *F. muscicola* were observed when the concentration of phosphorus was varied above and below the "actual concentration" as specified for Allen's medium and at each different combination of light and temperature. Inspection of Figures 7.5 and 7.6 suggest that the concentrations of

phosphorus used show some empirical evidence of producing differences in the observed mean toxicity of the cultures. That is, at low light and low temperature there was no difference in the mean toxicity when the concentration of phosphorus was decreased or increased by 75% (w/v) ie. 0.009 g/l or 0.066 g/l of the original concentration.

At high light intensity and low temperature combination (Figure 7.5), higher mean toxicity was observed by increasing the phosphorus concentration to 75%, and similarly, lower mean toxicity values were recorded when the concentration was decreased to 75% lower from its original value. There was an increase in toxicity when the concentration of phosphorus was increased from 0.009 g/l to 0.066 g/l.

Further increases in toxicity were observed at the low light and high temperature combination (Figure 7.6). The phosphorus concentration of 0.009 g/l gave the lowest toxicity whereas the phosphorus concentration of 0.066 g/l gave the highest toxicity in *F. muscicola.*

A decrease in toxicity during growth under conditions of high light intensity and temperature (Figure 7.6). Reducing the phosphorus concentration to 0.009 g/l in the medium resulted in higher mean toxicity whereas increasing the concentration of phosphorus to 0.066 g/l resulted in lowest mean toxicity values.

Further observations on the response of toxicity production to environmental growth

conditions were possible by considering the data obtained from high light and low temperature and the other at low light and high temperature conditions. Diminishing concentrations of phosphorus produced lowest mean toxicity and in contrast, increasing concentrations of this nutrient resulted in the highest mean toxicity.

Table 7.6. LS Means of light, temperature and phosphorus combinations.

LIT	TMP	PH	TOX LSMEAN	LSMEAN Number
1	1	1	50.06	1
1	1	2	49.51	2
1	1	3	49.58	3
1	2	1	35.70	4
1	2	2	35.89	5
1	2	3	37.13	6
2	1	1	31.27	7
2	1	2	31.54	8
2	1	3	32.70	9
2	2	1	42.53	10
2	2	2	40.80	11
2	2	3	40.70	12

where,
LIT = Light intensity in μmol m^{-2} s^{-1} (L),
TMP = Temperature in °C (T),
PH = Phosphorus concentration in g/l,
TOX LSMEAN = Least square (LS) mean of inhibition zone diameters (mm),
LSMEAN Number = Serial number, ie. computer generated code.

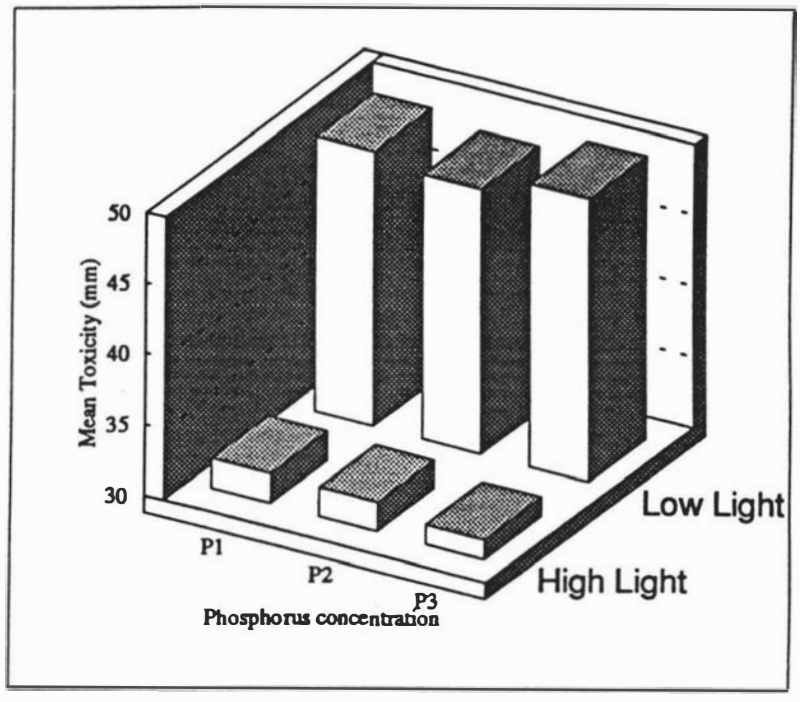


Figure 7.5. Light intensity variation at low temperature (10°C).

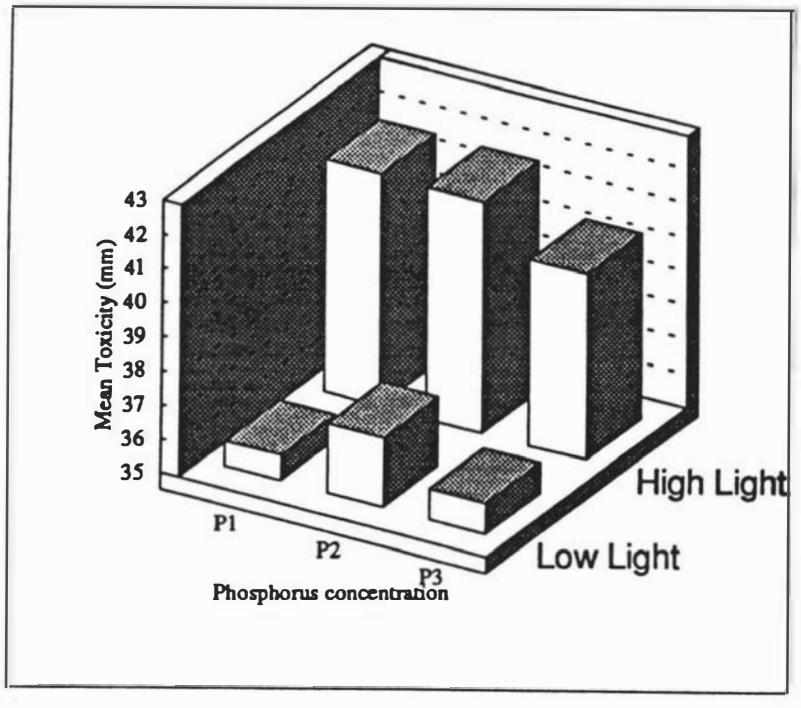


Figure 7.6. Light intensity variation at high temperature (30°C).

P1 = 75% (w/v) low concentration ie. 0.009 g/l
P2 = Formula concentration ie. 0.038 g/l
P3 = 75% (w/v) high concentration ie. 0.066 g/l

Table 7.7. LSMeans for LIT*TMP*NG*PH showing all possible treatment combinations and their mean toxicity values. (For legend see next page).

LIT	TMP	NG	PH	TOX LSMEAN	LSMEAN Number
1	1	1	1	49.63	1
1	1	1	2	48.10	2
1	1	1	3	50.65	3
1	1	2	1	49.83	4
1	1	2	2	48.90	5
1	1	2	3	47.41	6
1	1	3	1	50.73	7
1	1	3	2	51.53	8
1	1	3	3	50.69	9
1	2	1	1	34.25	10
1	2	1	2	36.43	11
1	2	1	3	36.56	12
1	2	2	1	36.23	13
1	2	2	2	36.10	14
1	2	2	3	38.86	15
1	2	3	1	36.62	16
1	2	3	2	35.14	17
1	2	3	3	35.98	18
2	1	1	1	32.48	19
2	1	1	2	31.67	20
2	1	1	3	32.23	21
2	1	2	1	32.00	22
2	1	2	2	29.10	23
2	1	2	3	35.02	24
2	1	3	1	29.33	25
2	1	3	2	33.88	26
2	1	3	3	30.84	27
2	2	1	1	42.77	28
2	2	1	2	41.45	29
2	2	1	3	41.20	30
2	2	2	1	43.32	31
2	2	2	2	41.24	32
2	2	2	3	40.79	33
2	2	3	1	41.50	34
2	2	3	2	39.71	35
2	2	3	3	40.11	36

where,
LIT = Light intensity in μmol m^{-2} s^{-1} (L),
TMP = Temperature in °C (T),
NG = Nitrogen concentration in g/l,
PH = Phosphorus concentration in g/l,
TOX LSMEAN = Least square mean of inhibition zone diameters (mm),
LSMEAN Number = Serial number.

It was deduced from the data for light and temperature in Table 7.4 that these factors were statistically significant because the *p*- value was 0.06 (significant just under 10%). The combinations which gives the highest toxicity and the combinations which gives the lowest toxicity are shown in the Table 7.7 and are indicated below.

Growth conditions leading to highest toxicity

Light = Low Intensity (10 μmol m^{-2} s^{-1}).

Temperature = Low (10°C).

Nitrogen = High Concentration[1] (2.625 g/l ie. 75% (w/v) higher than the formula Concentration).

Phosphorus = Medium concentration ie. 0.038 g/l.

Growth conditions leading to lowest toxicity

Light = High Intensity (60 μmol m^{-2} s^{-1}).

Temperature = Low (10°C).

Nitrogen = Formula concentration, 1.5 g/l.

Phosphorus = Formula concentration, 0.038 g/l.

* Statistically there is no significant difference in the cyanobacterium's response when growing in the nitrogen concentration as given in Allen's medium and a concentration that is 75% (w/v) high. The reason for choosing the high concentration is that the actual value (empirical) for toxicity is high at higher concentration.

7.3.5 The leakage of *F. muscicola* toxins into the culture medium

The experimental work of this section primarily confirms the findings of the previous Chapter 6 namely, Studies on the growth and toxin production by *Fischerella muscicola* in batch culture, that the toxicity of the cells is higher than the toxicity of the cell free culture broth. Methanolic extracts of the culture supernatants were used throughout and the inhibition zone diameters obtained are presented in Figure 7.7. It has been shown above that there was no statistically significant difference in the toxicity produced when the nitrogen and phosphorus concentrations in the medium varied around the formula concentration by 75% (w/v) for nitrogen and phosphorus Therefore, the bioassays were carried out using only crude methanolic preparations of supernatants of cultures grown in Allen's medium. The mean inhibition zone diameters are presented in Table 7.8.

These experiments have indicated that irrespective of the growth conditions used higher toxicity was always obtained from cell extracts and lower toxicity from cell free supernatants. These results are in accord with those observed in Chapter 6.

It was obvious from Figure 7.7 is that in a low light and low temperature environment the leakage of toxins into the culture medium was minimal, whereas at high light and high temperature there was a pronounced leakage of toxic materials into the culture medium.

Table 7.8 Mean inhibition zone diameters (Toxicity) of methanol extracted cell free culture broths from *F. muscicola* grown under different environmental conditions.

Combinations	Mean Inhibition Zone Diameters (mm)
L_1T_1	19.25
L_2T_1	24.5 ± 0.6
L_1T_2	25.5 ± 1.4
L_2T_2	26.6 ± 1.9

L_1 = 10 µmol m^{-2} s^{-1}; L_2 = 60 µmol m^{-2} s^{-1}.
T_1 = 10°C; T_2 = 30°C.
± SE of three replicates.

The target organism was *Anabaena flos-aquae.*

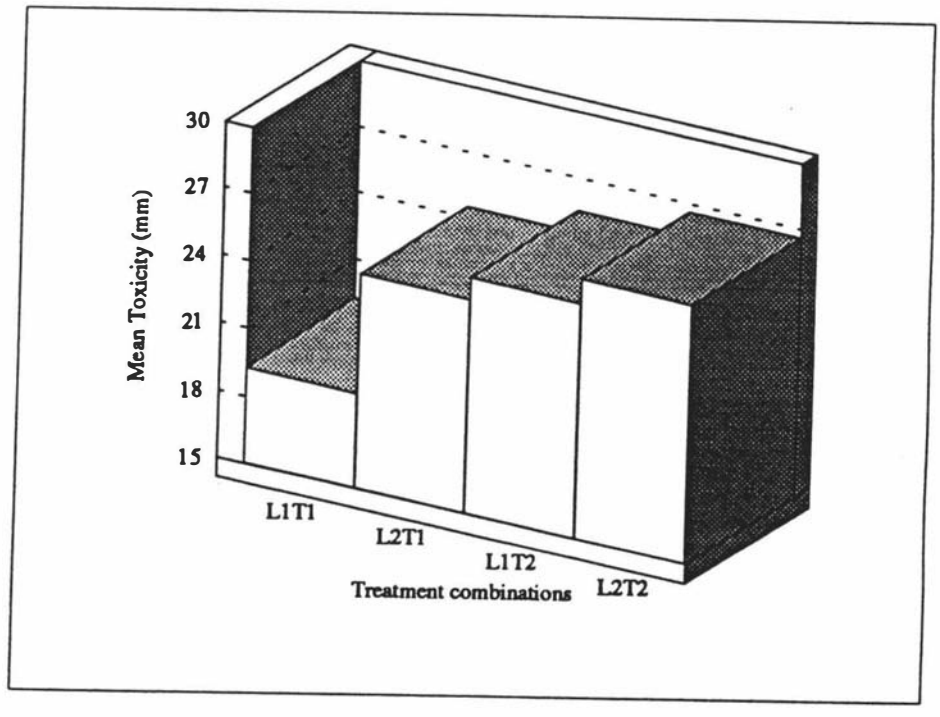


Figure 7.7. Toxicity (Inhibition zone diameters) results of *F. muscicola* culture broths as a response to different environmental growth conditions. L1 = 10 μmol $m^{-2}s^{-1}$; L2 = 60 μmol $m^{-2}s^{-1}$; T1 = 10°C; T2 = 30°C. Toxicity (Inhibition zone diameters) were measured against the indicator organism *Anabaena flos-aquae*.

7.3.6 Growth curve of *F. muscicola* under conditions leading to highest and lowest toxicity in the cyanobacterium

Table 7.7 showed the growth conditions which lead to high and low toxicity conditions for *F. muscicola.* Since the literature indicates (see Introduction for this Chapter) that the conditions for growth may be different from the conditions for toxin(s) production in the cyanobacteria, therefore, it was decided to investigate the growth response of this cyanobacterium under the conditions which induce higher and lower toxicities in the organism.

Both, the highest and the lowest toxicity conditions resulted in the poor growth of the cyanobacterium *F. muscicola.* In Figure 7.8 temperature was the limiting factor since the variation in the light intensity from its low level to high level at low temperature (10°C) had a minimal effect. Even by increasing the light intensity to its higher level the growth of the *F. muscicola* did not improve. The specific growth rates were 0.005 per day (for L1T1) and 0.008 per day (for L2T1), for two conditions of light intensity.

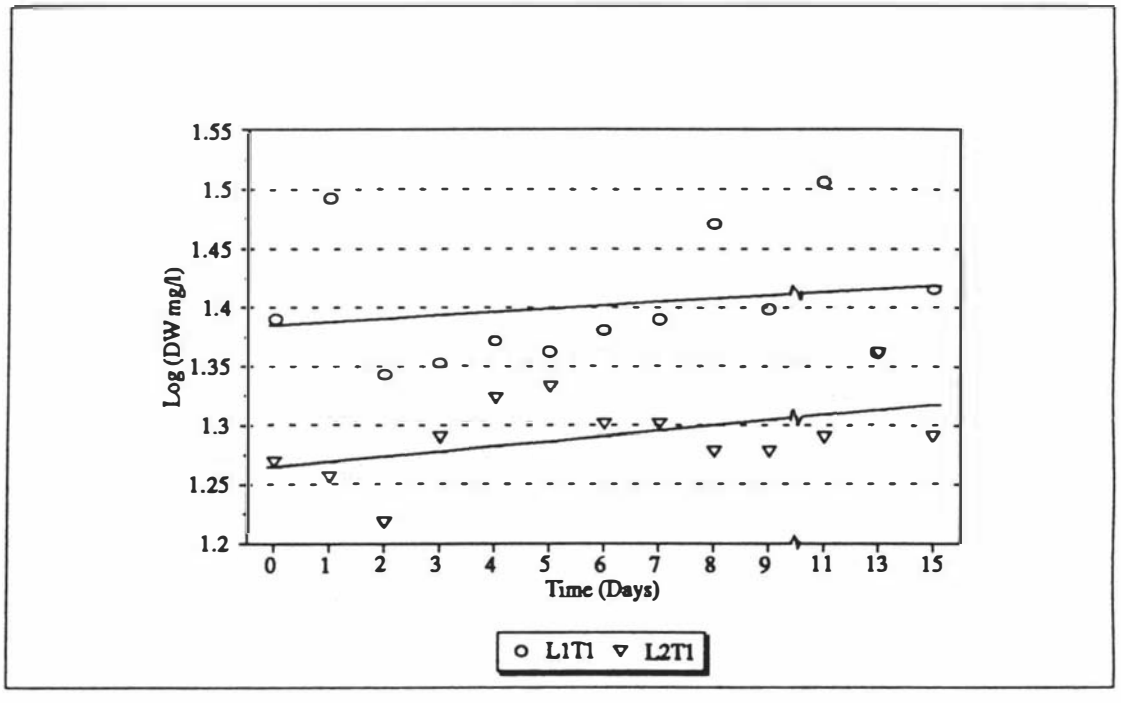


Figure 7.8. Growth curve of *Fischerella muscicola* under different environmental conditions.

7.4 DISCUSSION

In this study the effect of a number of environmental factors on toxin(s) production by *F. muscicola* in modified Allen's culture medium under batch culture were studied. A split plot design was the method chosen to study both the primary effects of the environmental factors as well as interactions. Most of the previous research on the effect of environmental factors on the toxicity of cyanobacteria investigate one factor at a time (Watanabe & Oishi, 1985; van der Westhuizen & Eloff, 1985; Priestley, 1989; Sivonen, 1990; Patterson & Bolis, 1993) which is time consuming and, more importantly, does not allow the identification of the interactions. However, some media optimization studies for toxin production by cyanobacteria have used the factorial approach (Bloor and England, 1991).

The results generated by SAS analysis of split plot experimental data gave information concerning the primary effects of factors and their interactions affecting the production of toxins in *F. muscicola*. It was possible to show whether increasing or decreasing the level of factor caused any increase or decrease in toxins production.

From the ANOVA table (Table 7.4) generated by SAS it was found that only the interactions of light and temperature from the main plot and the interaction of light, temperature and phosphorus from the split plot, was statistically significant at the 10% level. The major nutrients, nitrogen and phosphorus did not show any statistically significant effect on the toxins production by *F. muscicola* at the concentrations tested,

nor did their interactions. Furthermore, by changing the environmental conditions the organism did not lose its ability to produce toxins. Sivonen (1990) also said that the toxin production in cyanobacteria is not a response to environmental stress conditions.

The results of these experiments showed that temperature on its own did not have a statistically significant effect on the toxicity of the organism. However, low temperature (10°C) in combination with low light intensities (10 μmol m^{-2} s^{-1}) induced higher toxicity in the organism. This result is in broad agreement with the findings of Watanabe & Oishi (1985). They found in *Microcystis aeruginosa* a slight increase in the toxicity at low temperatures. Borowitzka (1995) stated that low temperatures enhance antibiotic production in cyanobacteria. However, conflicting observations were found in which some researchers (Codd & Poon, 1988; Bloor, 1989; Priestley, 1989; Sivonen, 1990; Wicks & Thiel, 1990) reported that low temperatures decrease the toxicity in cyanobacteria. The reasons for these conflicting results may be due to the differences in the strains of the organisms, or the method used for detecting toxicity, or the use of different culture media and culture conditions (ie. different light sources). Therefore, a direct and quantifiable comparison is not possible. In this experiment the higher temperature (30°C) increased the toxicity in *F. muscicola* at high light (60 μmol m^{-2} s^{-1}) intensities, but this toxicity was still lower than the toxicity values obtained at low temperatures. Conversely, a decrease in the toxicity of cyanobacteria at 30°C has also been previously reported (Runnegar *et al.*, 1983; Sivonen, 1990; Gromov *et al.*, 1991; Patterson & Bolis, 1993).

Light intensity is the factor of primary importance in governing the production of the toxin in all cyanobacteria. High toxin production can occur at relatively low light intensity (Watanabe & Oishi, 1985; Utklin & Gjolme, 1992). In the present study the variation of light intensities induced significant effects on the toxicity of *F. muscicola.* Low light intensities at low temperatures produced higher toxicity in the organism. This result is similar to the findings of Priestley (1989) who used *Microcystis aeruginosa* and Sivonen (1990) who also found the highest toxin production occurred in *Oscillatoria agardhii* at low light levels. However, contrary to this, Gross *et al.* (1994) reported that light limitation (10 $\mu E\ m^{-2}\ s^{-1}$) does not increase the fischerellin production in *F. muscicola.*

At higher temperatures, light intensity also influenced toxicity in cyanobacteria (Eloff & van der Westhuizen, 1981). The toxicity of *F. muscicola* grown at higher temperatures was lower than the toxicity at lower temperatures although the lowest toxicity recorded for *F. muscicola* was at high light and low temperature combination. Utkilen and Gjolme (1992) proposed that a decrease in the toxicity of *Microcystis aeruginosa* at light intensities of more than 40 $\mu E\ m^{-2}\ s^{-1}$ may be associated with the accumulation of carbohydrates at high light intensities. Codd and Poon (1988) reported that light intensities between 5 to 50 $\mu E\ m^{-2}\ s^{-1}$ had no effect on the toxicity of *Microcystis aeruginosa* when maintained at its optimum growth temperature of 25°C. In the present study, low light and high temperature produced slightly lower toxicity than that found at high light levels at high temperature, but this difference was statistically not significant.

The SAS analysis of the data showed that for the major nutrients (nitrogen and phosphorus), concentration variation in the culture medium did not produce any statistically significant influence on the toxicity of *F. muscicola.* Phosphorus removal does not influence the toxicity whereas nitrogen removal decreases the toxicity in *Microcystis aeruginosa* (Codd & Poon, 1988). A decrease in the toxicity in cyanobacteria induced by nitrogen limitation has been observed by many other workers (Gross *et al.*, 1994; Al-Layl, 1989; Priestley, 1989 and Watanabe & Oishi, 1985). Borowitzka (1995) stated that variation in nitrogen and phosphorus concentrations affect toxin production in cyanobacteria. In the present study which extended over the range of 0.375 g/l to 2.625 g/l of nitrogen and 0.009 g/l to 0.066 g/l of phosphorus, nutrient variation resulted in no significant effect on the toxicity of the culture. Of particular relevance to this study was the work of Bloor & England (1991). They found that a 50% increase in the nitrogen concentration from the original concentration (ie. 1.5 g/l) in the culture medium increased the antibiotic production by the cyanobacterium. They used BG-11 culture medium which is similar to the culture medium used in the present study. However, Gross *et al.* (1994) reported that nitrate limitation does not increase fischerellin production in *F. muscicola* and Kotak *et al.* (1995) reported a negative correlation of toxin from *Microcystis aeruginosa* with nitrogen.

In the present studies a statistically significant effect of an interaction term "light*temperature*phosphorus" at 10% level was detected. An in-depth analysis of this particular phenomenon revealed that the major effect on the toxicity was coming from a "light*temperature" term independently of the phosphorus concentration in the culture

medium. Studies on *F. muscicola* showed that phosphorus limitation did not increase the production of fischerellin by this cyanobacterium (Gross *et al.*, 1994). However, a positive correlation between the *microcystin-lr*, a hepatotoxin from the cyanobacterium *Microcystis aeruginosa* and total dissolved phosphorus concentration has been reported by Kotak *et al.* (1995), whereas Wicks and Thiel (1990) did not find any significant correlation of the toxin with either nitrogen or phosphorus.[2]

Many studies have reported the effect of growth limiting concentrations of various nutrients on toxicity and/or toxin production (Gross *et al.*, 1994; Al-Layl, 1989; Priestley, 1989; Codd & Poon, 1988; Codd *et al.*, 1988). In this study the aim was to observe the effect of variation in concentrations of inorganic nutrients on the toxicity of *F. muscicola.* While there is no previous literature indicating the use of split plot design where an interaction between both physical and chemical factors was studied the mathematical technique has proved useful since it offers insight into an interaction concerning the responses of cyanobacteria to interacting growth factors and the physical environment.

Sivonen (1990) found that the toxin concentrations in the cells were usually highest under conditions which also favoured growth whereas, and in contradistinction van der Westhuizen & Eloff (1985) reported that optimum conditions for growth did not coincide with those for toxin production in cyanobacteria. Poor growth of *F. muscicola*

2 Nevertheless, the fact it could be argued that, the concentrations of nitrogen and phosphorus (- or + value) in the present study may still be too high to observe any effect can not be ignored.

was observed under the conditions generating both the highest toxicity and the lowest toxicity in the organism. It was found that temperature was the growth limiting factor under both the toxicity conditions because even the increase in the light intensity to its highest level did not improve the growth of the organism. Therefore the observations of this study were similar to those made by van der Westhuizen & Eloff (1985). The extent of toxin release from the cells varies between species of cyanobacteria, but this subject has not been extensively studied under different environmental conditions (Anon., 1990). Penaloza *et al.* (1990) reported that increasing temperature caused the release of toxins into the medium. Similar observations were made by Sivonen (1990) who found that high light intensities and high temperatures promoted toxin leakage from the cells. This was also the case in the present study where the leakage of toxins from *F. muscicola* was minimal under low light and low temperature environments but as the light and temperature was increased to their upper experimental limits there were increases in the amount of toxicity found in the cell free culture supernatants. However, this subject area requires further exploration as in the present study there were insufficient replicates to confirm statistically this result.

It is obvious that further work is required and which could include other factors such as carbon and micro-nutrients for a fuller understanding of the influence of environmental conditions on the toxins production by this cyanobacterium. Studies are also needed to determine the mechanisms of genetic regulation of toxins production in order to have a more comprehensive understanding of the cellular biology of toxins production.

Furthermore, the toxins from *F. muscicola* have not been thoroughly identified and structurally defined. Therefore another dimension which needs immediate attention is the elucidation of the chemical characteristics of toxins. This will allow a more precise and accurate definition and determination of toxicity of the organism. To this end, Gross *et al.* (1991) have presented some initial chemical characteristics of a toxin from this cyanobacterium.

Chapter 8

General Discussion

There have been a number of reports on the production of toxic (inhibitory) substances (both biotoxins and cytotoxins) by cyanobacteria (Carmichael, 1994; Borowitzka, 1995). For example, *F. muscicola* has recently been shown to produce extensive antimicrobial activity (Flores and Wolk, 1986; Gross *et al.*, 1991; Park *et al.*, 1992). The initial work of Flores and Wolk (1986) and then later work of Gross *et al.* (1991) on the production of an allelopathic substance, fischerellin, was of particular relevance to this study. Flores and Wolk (1986) initially discovered, during a preliminary screening programme for the production of bacteriocins by nitrogen-fixing cyanobacteria, that *Fischerella muscicola* was the only one which inhibited all of the selected indicator strains. Later, Gross *et al.* (1991) isolated and partially characterized the bacteriocin-like substance and named it fischerellin. It showed inhibitory activity against other cyanobacterial species and eukaryotic algae but notably no activity against eubacteria. In the present study *F. muscicola* has been shown to synthesise substances which show inhibitory activity against a range of eukaryotic algae and cyanobacteria, thus confirming the findings of Flores and Wolk (1986) and Gross *et al.* (1991). However, this inhibitory activity exhibited by the substances produced by *F. muscicola* was not only active against algae and cyanobacteria but also extended to the inhibition of the growth of eubacteria. This suggests that the substances produced in culture in these present studies differ to those present in the cultures of Gross *et al.* (1991). The reasons for this are unclear and require further investigation. Recently, Park *et al.* (1992) have noted the production of an anti-fungal substance from this cyanobacterium. Attempts were, however, made to confirm chemically that the metabolite which Gross *et al.* (1991) named fischerellin was present in the cultures used in this study. The chromatographic data indicated that a

pure sample of the compound may have been prepared but mass spectroscopy revealed that the sample did not contain a single compound. Gross *et al.* (1991) isolated and partially characterized an algicide "fischerellin" from this cyanobacterium. However, in the present study, fischerellin production by this cyanobacterium could not be confirmed despite growth under optimal conditions for toxin production. A preliminary conclusion is that toxins other than fischerellin are being produced by this cyanobacterium.

The fact that a particular cyanobacterium produces toxic metabolites is important. But it is equally important to know how that production changes during the life-cycle of the organism. The toxicity of cyanobacterial blooms can vary widely from one week to another, in terms of biomass, and to a lesser extent from one day to another during the week in natural environments (Codd & Bell, 1985). Watanabe & Oishi (1985) described changes in both cyanobacterial populations and environmental factor(s) as the two possible causes for such variation. In this study the growth characteristics were established for this organism under laboratory growth conditions (batch culture study was used) and also the effect of culture age on the production and leakage of inhibitory substances in the culture medium was monitored. This latter aspect was considered to be important in determining the fate of toxins. The characteristics of production and leakage of the toxic metabolites at various growth stages of this cyanobacterium were revealed for the first time in this present study. Simultaneous growth and toxicity measurements (by means of an agar zone diffusion assay procedure) were carried out and demonstrated that the maximum inhibitory activity of cell extracts of the organism

occurred when cells reached their late exponential phase or early stationary phase. The ratio of toxicity versus biomass (Table 6.3) was found to vary over the range 0.92 to 3.47 units. This indicated a limited increase in toxicity with increase in biomass of *F. muscicola.* This is contrary to the findings of Watanabe *et al.* (1989) who reported a strong increase in the toxin content of the cells of *Microcystis* species during the growth of this cyanobacterium in batch culture. It is unclear at this stage as to why these two genera, *Fischerella* and *Microcystis* might vary in their respective behaviours towards toxin production.

The phenomena of both uniphasic and biphasic production of toxic Secondary metabolites by cyanobacteria have been reported previously in the literature (Armstrong *et al.*, 1991; Kiviranta *et al.*, 1991). However, *F. muscicola* showed a uniphasic production pattern for toxic metabolites in batch culture and attained its maximum inhibitory activity towards the end of the exponential phase. Other researchers (Utkilen & Gjølme, 1992; Watanabe *et al.*, 1989; Codd *et al.*, 1989; Watanabe & Oishi, 1983; Gorham, 1964) have also observed higher toxicity in cyanobacteria such as *Microcystis* species when it is in late exponential phase.

The most frequently studied groups of cyanobacterial toxins, namely, hepato- and neuro-toxins are retained within the cells during the lag and growth phases and the leakage of these toxins into the environment occurs only as a result of cell lysis due to senescence.

Inhibitory activity of the cell free culture-supernatants taken from cultures of *F. muscicola* has been detected over a period from day one throughout growth to stationary phase of the culture, with the increases in inhibitory activity occurring during the senescence of the culture. Similar observations have been recorded for other biotoxin producers such as *Oscillatoria agardhii* strains, *Anabaena circinalis* and *Microcystis aeruginosa* (Kiviranta *et al.*, 1991; Sivonen, 1990; Codd *et al.*, 1989). These findings are significant in terms of the fate of toxins during cyanobacterial development in natural waters and reservoirs. All toxic species of cyanobacteria in natural water bodies are capable of forming heavy growths (Carmichael, 1992) and mats in cases of the benthic cyanobacteria (Cohen & Rosenberg, 1989). Once the bloom of these cyanobacteria has developed, their toxic secondary metabolites will be present in the water and may remain in the environment even though the heavy growth itself is in a state of senescence. This phenomenon poses a threat to human and animal health if blooms are present in water bodies used for drinking water supply. Furthermore, the toxins will pose a serious problem to the stability of the ecosystem because these inhibitory metabolites of *F. muscicola* inhibit the growth of a wide range of microorganisms which form the basis of the food chain ie. primary producers (Figure 2.14). However, the direct toxic effects of these inhibitory metabolites on the other organisms of higher trophic levels was not confirmed in the present study. It is known that biotoxins are released from the cells during cell lysis. The hypothesis is that, if the inhibitory metabolites of other cyanobacteria have an algicidal property (cytotoxin producers) and which may kill the biotoxin producing cyanobacteria by causing their cells to burst then this sudden population death may also cause a sudden release of

biotoxins into the water, thus imposing an even greater threat in the ecosystem. This cascade of events may be initiated by a sudden blooming of cyanobacterial populations, induced often by an environmental factor.

It has been postulated (Anon.,1990) that environmental factors will play an indirect role in regulating the formation of toxic secondary (inhibitory) metabolites through their primary regulatory effect on the population development of cyanobacteria. The present work has confirmed this although the results differ from those of Gross *et al.* (1994). Data are still lacking which would indicate that environmental factors have a more direct effect on the formation of toxic secondary metabolites by cyanobacteria (Watanabe & Oishi, 1985; Sivonen, 1990). At the time of commencement of this experimental program of study there was little information available concerning the interactive influence of the environmental factor(s) on the formation of toxic metabolites.

The method employed to explore the interaction of environmental factors and toxin production was a factorial experiment with a split plot design (Mead *et al.*, 1993). The procedure allowed the elucidation of the factors responsible for controlling the production of inhibitory metabolites as well as providing information on the various factors and their interactions affecting the antimicrobial production. The analysis of experimental data showed that only the interaction of physical factors, ie., light and temperature, was affecting the (cell extract) toxicity of the cyanobacterium at a statistically significant level (<10%). However, the major nutrients, nitrogen and phosphorus, did not have any statistically significant effect. *F. muscicola* is a nitrogen-

fixing cyanobacterium (Gross *et al.*, 1994) and therefore variations in nitrogen (as $NaNO_3$) concentration in the culture medium was not expected to have any effect. However, Gross *et al.* (1994) observed that nitrate depletion from the culture medium resulted in a sharp and significant decline in the production of fischerellin from the cultures of *F. muscicola* whereas phosphorus limitation increased the production of fischerellin. This may be explained as follows. Firstly, their study was confined to the production of fischerellin only, a molecule which contains at least two nitrogen atoms in its molecule. Therefore, the synthesis of this metabolite may be affected by nitrate depletion in that nitrogen fixation was unable to supply completely the cells requirement. Secondly, the present study deals with the crude methanolic cell extract's inhibitory activity. There is evidence also that the substances responsible for inhibitory activity were different from fischerellin. The synthesis of these substances were possibly maintained by nitrogen fixation. Thirdly, Gross *et al.* (1994) used different culture conditions, ie., a different culture system and culture media along with the use of different concentration of the nutrients. Nutrients concentrations used in the present study, although varied 75% up or down from the formula concentration (ie. 1.5 g/l for nitrogen and 0.038 g/l for phosphorus) of the medium. As such, they may still be too high to significantly interfere with the antimicrobial substance's production . Interestingly, Bloor & England (1991), found a 50% increase of nitrogen concentration (as $NaNO_3$) above normal strength in the culture medium increased the production of an antimicrobial compound by nitrogen-fixing cyanobacterium *Nostoc muscorum.*

The growth response of the cyanobacterium under those conditions which allowed the

highest and the lowest toxicity was also investigated. It was found that under both sets of conditions growth of the cyanobacterium was limited by temperature. That is, the best conditions for growth are not the best condition for toxin production. van der Westhiuzen *et al.* (1985 & 1986) also reported that the conditions for growth did not coincide with the conditions for toxin production by the cyanobacterium *Microcystis aeruginosa.*

In the present study experiments were conducted to observe the effect of different environmental conditions on the release of inhibitory metabolites from the cells into the culture medium. Results showed that high light intensity and high temperature caused an increase in the release of inhibitory metabolites from the cells into the medium. Similar observations have been made for the biotoxin producing cyanobacterium *Oscillatoria agardhii* in which high light intensity and high temperature promoted the leakage of the toxin from the cells (Sivonen, 1990).

Hazards created by cyanobacteria in the environment have attracted the attention of many researchers world-wide. Despite a substantial increase in the recent literature, there is still lacking a complete awareness of the effects of cyanobacterial toxic secondary metabolites (Lawton and Codd, 1991). Kotak *et al.* (1995) stated that, adequate prediction of the occurrence of dangerous concentrations of toxic cyanobacteria has been hampered by a lack of information on the natural variability of the production of toxins and relationship to environmental parameters. This present study has attempted to observe and analyze the natural variability in production of the toxic

substances produced by one particular cyanobacterium. However, before the insights gained from these laboratory experiments and others are tested under field conditions there is still much work that can be done not only for this particular cyanobacterium but also for all principal toxin producing cyanobacterial species. This thesis contributes to the overall improvement in the understanding of the physiological character of *F. muscicola* in particular and the toxic cyanobacteria in general. The work carried out provides some insight into the pattern of variation of toxicity of the cyanobacterium during its growth stages. Another important question that has been addressed in this study is whether there are environmental factors which forces an organism that produces toxins into one that does not has also been explored in this study. In this study an attempt has been made to understand the differences in toxicity of the cyanobacterium grown under different environmental conditions.

Chapter 9

Conclusions

The conclusions that arose from the body of research described in this present study revealed that:

- Inhibitory activity of cyanobacterium *F. muscicola* was detected readily by an antimicrobial agar zone diffusion assay system.

- Cyanobacterium *F. muscicola* synthesizes inhibitory substances which were active against a wide range of microorganisms namely, eukaryotic algae, cyanobacteria and eubacteria and which were different in their chemical characteristics to fischerellin.

- Production of an algicide fischerellin by *F. muscicola* in the cultures could not be confirmed under any of the conditions investigated ie. neither under the conditions which stimulate the toxicity of the cyanobacterium nor under conditions which promote the growth of the cyanobacterium.

- The pattern of synthesis of inhibitory metabolite(s) production allowed an early detection of high toxicity at day 1 which was maintained throughout growth until late stationary phase. This pattern may have been superimposed by a second pattern of production whereby activity rose to a peak at day 9 then subsided to its day 1 value.

- The leakage of inhibitory metabolites into the culture medium was low

during the actual growth period. However, cell lysis as the culture aged caused leakage of intracellular material into the medium.

- Interaction of environmental factors - light and temperature affected the biological activity of the cyanobacterium. Low light intensity (10 μmol m^{-2} s^{-1}) together with low temperature (10°C) induces higher toxicity in the organism.

- The major nutrients nitrogen and phosphorus did not seem to have any major influence on the toxicity of the organism at the concentrations tested. These concentrations were in the range of 0.375 to 2.625 g/l for nitrogen and 0.009 to 0.066 g/l for phosphorus.

- Conditions which promoted growth (light intensity of 60 μmol m^{-2} s^{-1} and temperature of 25°C in Allen's culture medium) of *F. muscicola* were different from those conditions which stimulated toxicity (light intensity of 10 μmol m^{-2} s^{-1} and temperature of 10°C and 2.625 g/l of nitrogen and 0.038 g/l of phosphorus in Allen's culture medium) of the cyanobacterium.

- High light intensity (60 μmol m^{-2} s^{-1}) together with high temperature (30°C) promoted the leakage of inhibitory metabolites from the cell into the culture medium.

Chapter 10

Recommendations

Towards the end of this project when the results were beginning to be analyzed it became obvious that the questions raised by the work were likely to exceed the questions which may have been answered. There is still much to be learned about cyanobacterium *F. muscicola* and its toxin(s) as well as their effects in the aquatic ecosystem. The need for future research and development therefore falls into the following categories:

- The identification and structural conformation of compounds causing toxicity. These include fischerellin and other new toxins which this current research indicates are present.

- The determination of the biological nature of the toxic (harmful) effect(s) of these toxins on the organisms of other trophic levels in the food chain.

- The establishment of the role of other environmental factors such as pH, carbon and micronutrients which possibly influence the toxin production. The extension of this study of the concentrations of nitrogen and phosphorus beyond the range used in this present study to observe the affects on toxicity of the cyanobacterium.

- The recognition of those mechanisms responsible for the release of toxins from the cell into the culture medium and aquatic environment.

- The development of sensitive chemical assays to confirm and quantify specific toxins.

- The quantification of the toxic effects of toxins produced by *F. muscicola* on other known biotoxin producing microorganisms.

- The elucidation of those genetic and biochemical mechanisms which are responsible for the regulation of toxin synthesis in the cell.

Bibliography

Aliotta, G. et al. (1990). In vitro algal growth inhibition by phytotoxins of Typha latifolia L. *Journal of Chemical Ecology*, **16**(9), 2637-2646.

Aliotta, G. et al. (1991). Potential allelochemical from Pistia stratiotes L. *J Chem Ecol*, **17**(11), 2223-2234.

Al-Layl, J. K. S. (1989). *Studies on the toxicity of cyanobacteria Anabaena flos-aquae and Microcystis aeruginosa.* Unpublished doctoral dissertation, The University of Dundee, Dundee.

Al-Layl, J. K., Poon, G. K., & Codd, G. A. (1988). Isolation and purification of peptide and alkaloid toxins from Anabaena flos- aquae using high performance thin layer chromatography. *Journal of Microbiological Methods* , **7**, 251-258.

Allen, M. M. (1968). Simple conditions for growth of unicellular blue-green algae on plates. *J Phycol*, **4**, 1-4.

Allen, M. M., & Stanier, R. Y. (1968). Selective isolation of blue-green algae from water and soil. *J Gen Microbiol*, **51**, 203- 209.

Anon. (1990). *Toxic blue-green algae*(First). (Water Quality Series No. 2). London: National Rivers Authority.

Armstrong, J. E. et al. (1991). Cytotoxin production by a marine Lyngbya strain (cyanobacterium) in a large-scale laboratory bioreactor. *J Appl Phycol* , **3**, 277-282.

Baalen, C. V. (1987). Nitrogen Fixation. In: P. Fay, & C. V. Baalen*The Cyanobacteria.* (pp. 187-198). The Netherlands: Elsevier.

Bagchi, S. N., & Marwah, J. B. (1994). Production of an algicide from cyanobacterium Fischerella sp. which inhibits photosynthetic electron transport. *Microbios*, **79**(320), 187.

Baker, P. D., & Humpage, A. R. (1994). Toxicity associated with commonly occurring cyanobacteria in surface waters of the Murray- Darling Basin, Australia. *Aust J Mar Freshwater Res* , **45**, 773- 786.

Bandin, I. et al. (1991). Lack of biological activities in the extracellular products of Renibacterium salmoninarum. *Can J Fish Aquat Sci*, **48**, 421-425.

Barchi, J. J. J., Moore, R. E., & Patterson, G. M. L. (1984). Acutiphycin and 20,21-didehydroacutiphycin, new antineoplastic agents from the cyanophyte Oscillatoria acutissima. *J Am Chem Soc*, **106**, 8193-8197.

Becker, E. W. (1994). **10***Microalgae- biotechnology and microbiology*. (pp. 293). Cambridge: Cambridge University press.

Bloor, S., & England, R. R. (1989). Antibiotic production by the cyanobacterium Nostoc muscorum. *J Appl Phycol* , **1**, 367-372.

Bloor, S., & England, R. R. (1991). Elucidation and optimization of the medium constituents controlling antibiotic production by the cyanobacterium Nostoc muscorum. *Enzyme Microb Technol* , **13**, 76-81.

Bold, H. C., & Wynne, M. J. (1985). *Introduction to the algae: structure and reproduction.* (second edition). : Prentice-Hall, Inc., N.J.

Borowitzka, M. A. (1995). Microalgae as sources of pharmaceuticals and other biologically active compounds. *J Appl phycol*, **7**, 3-15.

Borowitzka, M. A., & Borowitzka, L. J. (1988). *Micro-algal biotechnology*. Cambridge: Cambridge University Press.

Bowling L. (1994). Occurrence and possible causes of a severe cyanobacterial bloom in lake Cargelligo, New South Wales. *Aust J Mar Freshwater Res* , **45**, 737-745.

Bradford, M. M. (1976). A rapid and sensitive method for the quatitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**, 248-254.

Brooks, W. P., & Codd, G. A. (1986). Extraction and purification of toxic peptides from natural blooms and laboratory isolates of the cyanobacterium Microcystis aeruginosa. *Letters in Applied Microbiology*, **2**, 1-3.

Brooks, W. P., & Codd, G. A. (1988). Immunoassay of hepatotoxic cultures and waterblooms of cyanobacteria using Microcystis aeruginosa peptide toxin polyclonal antibodies. *Environmental Technology Letters*, **9**, 1343-1348.

Busby, P. (1993). Regulatory issues - research perspective. *Marine toxins and New Zealand Shellfish.* . (Miscellaneous Series 24, p 45-47.). : The Royal Society of New Zealand, MAF .

Cannell, R. J. P., Owsianka, A. M., & Walker, J. M. (1988). Results of a large-scale screening programme to detect antibacterial activity from freshwater algae. *Br Phycol*, **23**, 41- 44.

Carmeli, S., Moore, R. E., & Patterson, G. M. L. (1990). Tolytoxin and new scytophycins from three species of Scytonema. *Journal of Natural Products* , **53**(6), 1533-1542.

Carmichael, W. W. et al. (1985). Algal toxins and water-based diseases. **15In**: C.P. Strub(pp. 275-313). Chemical Rubber Co. Press, Florida: CRC Critical reviews in Environmental Control .

Carmichael, W. W. (1986). Algal toxins. *Advances in botanical research*, **12**, 47-101.

Carmichael, W. W. (1989). Freshwater cyanobacteria (blue-green algae) toxins. In: C.L. Ownby, & G.V. Odell*Natural Toxins: characterization, pharmacology and therapeutics.* (pp. 211). : Pergamon Press.

Carmichael, W. W. (1992). A Review: Cyanobacteria secondary metabolites-the cyanotoxins. *J Appl Bacteriol*, **72**, 445-459.

Carmichael, W. W. (June 1992). *A status report on planktonic cyanobacteria (Blue-Green Algae) and their toxins.* USA: United States Environmental Protection Agency Report No. EPA/600/R-92/ 079. 141 pp.

Carmichael, W. W. et al. (1988). Toxicity and partial structure of a hepatotoxic peptide produced by the cyanobacterium Nodularia spumegina mertens emend. L575 from New Zealand. *Appl Env Microbiol* , **54**(9), 2257-2263.

Carmichael, W. W. (1994.). *The toxins of cyanobacteria.* : Scientific American 270:64-70.

Carmichael, W. W. (1981.). *The water environment: Algal toxins and health.* : Environmental Science Research, Vol. 20. Plenum Press, New York. p 492.

Carmichael, W. W., Biggs, D. F., & Gorham, P. R. (1974). Toxicology and pharmacological action of Anabaena flos-aquae toxin. *Science* , **187**, 542-544.

Carmichael, W. W., & Gorham, P. R. (1978). Anatoxins from clones of Anabaena flos-aquae isolated from lakes of westerns Canada. *Mitt Internat Verein Limnol* , **21**, 285-295.

Carmichael, W. W., & Gorham, P. R. (1981). The mosaic nature of toxic blooms of cyanobacteria. In: W. W. Carmichael*The Water Environment: Algal toxins and Health.* (pp. 161-172). New York: Plenum Press.

Carmichael, W. W., Mahmood, N. A., & Hyde, E. G. (1990). Natural toxins from cyanobacteria (Blue-green algae). In: S. Hall and G. Strichartz*Marine Toxins-Origin, Structure, and Molecular Pharmacology.* Washington,DC: American Chemical Society.

Carr, N. G., & Whitton, B. A. (1982). *The biology of cyanobacteria.* : Blackwell Scientific Publications.

Carter, D. C., Moore, R. E., & Mynderse, J. S. (1984). Structure of majusculamide C, a cyclic depsipeptide from Lyngbya majuscula. *J Org Chem*, **49**, 236-241.

Cassie, V. (1984). (Revised checklist of the freshwater algae of New Zealand (excluding diatoms and charophytes) Part I- Cyanophyta, Rhodophyta and Chlorophyta)). *Water & Soil Technical Publication No. 25.* Wellington: Water and Soil Directorate, Ministry of Works and Development, New Zealand.

Castenholz, R. W. (1989). Subsection V. Order Stigonematales. **3***Bergey's Manual of Systematic Bacteriology.* (pp. 1794-1798). : Williams & Wilkins, Baltimore, Maryland.

Chauhan, V. S., Marwah, J. B., & Bagchi, S. N. (1992). Effect of an antibiotic from Oscillatoria sp.on phytoplankters, higher plants and mice. *New Phytol*, **120**, 251-258.

Chetsumon, A. et al. (1994). Antibiotic production by the immobilized cyanobacterium, Scytonema sp. TISTR 8208 in a seaweed-type bioreactor. *J Appl Phycol*, **6**(5-6), 539-544.

Christoffersen, K. et al. (1990). Qualitative importance of the microbial loop and plankton community structure in a eutrophic lake during a bloom of cyanobacteria. *Microb Ecol* , **20**, 253-272.

Chu, F. S. et al. (1989.). Production and characterization of antibodies against microcystins. *Appl Env Microbiol* , **55**(8), 1928-1933.

Chu, F. S., Huang, X., & Wei, R. D. (1990). Enzyme-linked immunosorbent assay for microcystin in blue-green algal blooms. *J Assoc Off Anal Chem* , **73**, 451-456.

Codd, G. A. et al. (1989). Cyanobacterial toxins in european waters: occurrence, properties, problems and requirements. M. L. Richardson, & J. B. D. Wheeler, *Watershed 89: The future for water quality in europe. II.* Univ Surry, Guildford, U.K. *Proceedings of the IAWPRC Conference.* (pp. 211-220). Oxford: Pergamon Press.

Codd, G. A. et al. (1989). Production, detection and quantification of cyanobacterial toxins. *Tox Assess*, **4**, 499-511.

Codd, G. A. et al. (1994). Toxic blooms of cyanobacteria in lake Alexandrina, South Australia-learning from history. *Aust J Mar Freshwater Res* , **45**, 731-736.

Codd, G. A. (1984). Toxins of freshwater cyanobacteria. *Microbiol Sci*, **1**(2), 48-52.

Codd, G. A., Bell, S. G., & Brooks, W. P. (1989). Cyanobacterial toxins in water. *Wat Sci Tech* , **21**(3), 1-13.

Codd, G. A., & Bell, S. G. (1985). Eutrophication and toxic cyanobacteria in freshwaters. *J Water Pollut Control* , **84**, 225- 232.

Codd, G. A., & Poon, G. K. (1988). Cyanobacterial toxins. L.J. Rogers, & J.R. Gallon, *Proceedings of the Phytochemical Society of Europe. Biochemistry of the algae and cyanobacteria* . : Oxford Science Publications.

Cohen, Y., & Gurevitz. (1991). The Cyanobacteria- Ecology, Physiology and Molecular Genetics. (second). In: A. Balow et al.*The Prokaryotes- A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications.* II. (pp. 2079-2104). Springer-Verlag.

Cohen, Y., & Rosenberg, E. (1989). *Microbial mats - physiological ecology of benthic microbial communities.* : American Society for Microbiology, Washington D.C., p 494.

Collins, M. (1978). Algal toxins. *Microbiol Rev*, **42**(4), 725-746.

Davies, O. L. (1978). *The design and analysis of industrial experiments.* New York: Longman Group Limited.

de Cano. M.M.S. et al. (1990). Inhibition of Candida albicans and Staphylococcus aureaus by phenolic compounds from the terrestrial cyanobacterium Nostoc muscorum. *J Appl Phycol*, **2**, 79-81.

DeMott, W. R., & Moxter, F. (1991). Foraging on cyanobacteria by copepods: responses to chemical defenses and resource abundance. *Ecology* , **72**(5), 1820-1834.

DeMott, W. R., Zhang, Q. X., & Carmichael, W. W. (1991). Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of Daphnia. *Limnol Oceanogr* , **36**(7), 1346-1357.

Dierstein, R. et al. (1990). Two closely related peptide toxins in axenically grown Microcystis aeruginosa PCC 7806. *System Appl Microbiol*, **13**, 86-91.

Dierstein, R., Kaiser, I., & Weckesser, J. (1988). Rapid determination of Microcystis sp. toxins by reversed-phase liquid chromatography. *FEMS Microbiol Lett*, **49**(2), 143-147.

Einhellig, F. A. (1986). Mechanisms and modes of action of allelochemcials. In: A. R. Putnam, & C. S. Tang*The Science of Allelopathy.* (pp. 171-188). : John Wiley & Sons.

Eloff, J. N., Siegelman, H. W., & Kycia, H. (1982). Extraction, isolation and stability of toxins from Microcystis aeruginosa. *S Afr J Sci*, **78**, 377-378.

Eriksson, J. E. et al. (1988). A comparison of toxins isolated from the cyanobacterium Oscillatoria agardhii and Microcystis aeruginosa. *Comp Biochem Physiol*, **89c**(2), 207-210.

Eriksson, J. E. et al. (1988). Preliminary characterization of a toxin isolated from the cyanobacterium Nodularia spumigena. *Toxicon*, **26**(2), 161-166.

Eriksson, J. E., Hagerstrand, H., & Isomaa, B. (1987). Cell selective cytotoxicity of a peptide toxin from cyanobacterium Microcystis aeruginosa. *Biochimica et Biophysica Acta*, **930**(3), 304-310. (Technical Note)

Eriksson, J. E., Meriluoto, J. A. O., & Lindholm, T. (1989). Accumulation of a peptide toxin from the cyanobacterium Oscillatoria agardhii in the freshwater mussel Anadonta cygnea. *Hydrobiologia*, **183**(3), 211-216.

Falch, B. S. et al. (1993). Ambigol A and B: New biologically active polychlorinated aromatic compounds from the terrestrial blue green alga Fischerella ambigua. *J Org Chem* , **58**(24), 6750- 6575.

Falch, B. S. et al. (1992). Antibacterial and cytotoxic compounds from the blue-green alga Fischerella ambigua. *Planta Medica, Supplement 1*, **58**, 654-.

Falch, B. S. et al. (1995). Biological activities of cyanobacteria: evaluation of extracts and pure compounds. *Planta Med* , **61**(4), 321-328.

Falconer, I. R. et al. (1983). The occurrence and consequences of blooms of the toxic blue-green alga Microcystis aeruginosa in eastern Australia. *Toxicon suppl* , **3**, 119-121.

Falconer, I. R. (1991). Tumor promotion and liver injury caused by oral consumption of cyanobacteria. *Environ Toxicol Water Quality*, **6**, 177-184.

Falconer, I. R. et al. (1989). Using activated carbon to remove toxicity from drinking water containing cyanobacterial blooms. *J Am Water Works Assoc*, **81**(2), 102-105.

Falconer, I. R., Buckley, T., & Runnegar, M. T. C. (1986). Biological half life, organ distribution and excretion of 125I- labelled toxic peptide from the blue-gree alga Microcystis aeruginosa. *Aust J Biol Sci* , **39**, 17-21.

Federal Water Pollution Control Administration, C. O., & Pacific Northwest Water Lab and Washington Uni., S. (1966). *Environmental requirements of blue-green algae*. **FWPCA Program 16010---10/67**(pp. 111). Washington.

Fedorak, P. M., & Huck, P. M. (1988). Microbial metabolism of cyanobacterial products: batch culture studies with applications to drinking water treatment. *Wat Res*, **22**(10), 1267-1277.

Ferris, M. J., & Hirsch, C. F. (1991). Method for isolation and purification of cyanobacteria. *Appl Env Microbiol*, **57**(5), 1448- 1452.

Feuillade, M., Feuillade, J., & Fiala, V. (1990). The effect of light on the release of organic compounds by the cyanobacterium Oscillatoria rubescens. *Aquatic Sciences* , **52**(4), 345-359.

Flint, E. A. (1966). Toxic algae in some New Zealand freshwater ponds. *New Zealand Veterinary Journal* , **14**(11), 181-185.

Flores, E., & Wolk, C. P. (1986). Production, by filamentous, nitrogen-fixing cyanobacteria, of a bacteriocin and of other antibiotics that kill related strains. *Arch Microbiol* , **145**, 215- 219.

Fogg, G. E. et al. (1973). *The blue-green algae.* London: Academic Press.

Forsberg, C. et al. (1990). Absence of allelopathic effects of Chara on phytoplankton in situ. *Aquatic Botany*, **38**, 289-294.

Forsyth, D. J., Haney, J. F., & James, M. R. (1992). Direct observation of toxic effects of cyanobacterial extracellular products on Daphnia. *Hydrobiologia* , **228**, 151-155.

Foxall, T. L., & Sanser, J. J. J. (1981). Effects of hepatic toxin from the cyanophyte Microcystis aeruginosa. In: W. W. Carmichael*The Water Environment: Algal Toxins and Health.* (pp. 365-387). New York: Plenum Press.

Francis, G. (1878). Poisonous Australian lake. *Nature*, **18**, 11-12.

Fujiki, H., & Sugimura, T. (1987). New classes of tumor promoters: teleocidine, aplysiatoxin and palytoxin. *Advances Cancer Res*, **49**, 223-264.

Fujiki, H., & Sugimura, T. (1983). New potent tumor promoters: teleocidin, lyngbyatoxin A and aplysiatoxin. *Cancer Surveys* , **2**(4), 539-556.

Fulton, R. S. I., & Paerl, H. (1987). Effects of blue-green alga Microcystis aeruginosa on herbivorous zooplankton. *J Plankton Res*, **9**(5), 837-855.

Garcia-pichel, F., & Castenholz, R. W. (1991). Characterization and biological implications of scytonemin, a cyanobacterial sheath pigment. *J Phycol*, **27**, 395-409.

Gathercole, P. S., & Theil, P. G. (1987). Liquid chromatographic determination of the cyanoginosins, toxins produced by the cyanobacterium Microcystis aeruginosa. *J Chromat*, **408**, 435-440.

Gehrke, P. C., & Harris, J. H. (1994). The role of fish in the cyanobacterial blooms in Australia. *Aust J Mar Freshwater Res* , **45**, 905-915.

Gentile, J. H., & Maloney, T. E. (1969). Toxicity and environmental requirements of a strain of Aphanizomenon flos- aquae (L.) Ralfs. *Can J Microbiol*, **15**(2), 165-173.

Gibson, M. T. (1990). Developing biocidal compounds as freshwater algicides. *International Biodeterioration*, **26**(2-4), 149-158.

Gilbert, J. J. (1990). Differential effects of Anabaena affinis on cladocerans and rotifers: mechanisms and implications for zooplankton community structure. *Ecology*, **71**, 1727-1740.

Gilbert, J. J., & Durand, M. W. (1990). Effect of Anabaena flos- aquae on the abilities of Daphnia and Keratella to feed and reproduce on unicellular algae. *Freshwater Biol*, **24**, 577-596.

Gleason, F. K. (1990). The natural herbicide, cyanobacteria, specifically disrupts thylakoid membrane structure in Euglena gracilis strain z. *FEMS Microbiol Lett*, **68**, 77-82.

Gleason, F. K., & Baxa, C. A. (1986). Activity of the natural algicide, cyanobacterin, on eukaryotic microorganisms (algae; fungi; photosynthesis; resistance). *FEMS Microbiol Lett*, **33**, 85- 88.

Gleason, F. K., & Paulson, J. L. (1984). Site of action of the natural algicide, cyanobacterin, in the blue-green alga, Synechococcus sp. *Arch Microbiol* , **138**, 273-277.

Gleason, F. K., & Porwoll, J. (1986). X-ray structure determination of the naturally occurring isomer of cyanobacterin. *J Org Chem*, **51**, 1615-1616.

Gleason, F. K., & Wood, J. M. (1987). Secondary metabolism in the cyanobacteria. In: P. Fay, & C.V. Baalen*The Cyanobacteria*. (pp. 437-452). : Elsevier.

Glombitza, K. W., & Koch, M. (1989). Secondary metabolites of pharmaceutical potential. In: R.C. Cresswell, T.A.V. Rees, & M. Shah*Algal and cyanobacterial biotechnology* . (pp. 161-238). Harlow : Longman Scientific & Technical.

Gorham, P. R. et al. (1964). Isolation and culture of toxic strains of Anabaena flos-aquae (Lyngb.) de Breb. *Verh Internat Verein Limnol*, **XV**, 796-804.

Gorham, P. R. (1962). Laboratory studies on the toxins produced by waterblooms of blue-green algae. *Am J Publ Health*, **52**(12), 2100-2105.

Gorham, P. R. (1964). Toxic algae. In: D.F. Jackson*Algae and Man* . (pp. 307-336). New York: Plenum Press.

Gorham, P. R. (1964). Toxic algae as public health hazard. *J Am Water Works Assoc*, **56**, 1481-1488.

Gorham, P. R. (1960). Toxic waterblooms of blue-green algae. *Can Vet J*, **1**(6), 235-245.

Gorham, P. R., & Carmichael, W. W. (1988). Hazards of freshwater blue-greens (cyanobacteria). In: C. A. Lembi, & J. R. Waaland*Algae and Human Affairs*. (pp. 403-431). Cambridge: Cambridge University Press.

Gorham, P. R., & Carmichael, W. W. (1979). Phycotoxins from blue- green algae. *Pure and Appl Chem* , **52**, 165-174.

Gorham, P. R., & Carmichael, W. W. (1980). Toxic substances from freshwater algae. *Progr Water Technol* , **12**, 189-198.

Grabow, W. O. K. et al. (1982). Microcystis aeruginosa toxin: cell culture toxicity, hemolysis and mutagenicity assays. *Appl Environ Microbiol*, **43**(6), 1425-1433.

Grant, G. A., & Hughes, E. O. (1953). Development of toxicity in blue-green algae. *Can J Publ Health*, **44**, 334-339.

Greber, B. L., & Wickstrom, C. E. (1990). Response of Anabaena flos-aquae (cyanophyta) nitrogenase activity to sudden phosphate deprivation. *J Phycol* , **26**, 650-655.

Gromov, B. V. et al. (1991). Production of the antibiotic cyanobacterin LU-1 by Nostoc linckia CALU 892 (cyanobacterium). *J Appl Phycol* , **3**, 55-59.

Gross, E. M., Elert, E. V., & Juttner, F. (1994). Production of allelochemicals in Fischerella muscicola under different environmental conditions. *Verh Internat Limnol*, **25**, 2231-2233.

Gross, E. M., Wolk, C. P., & Juttner, F. (1991). Fischerellin, a new allelochemical from the fresh water cyanobacterium, Fischerella muscicola. *J Phycol*, **27**, 686-692.

Hadar, D. P. (1987). Photomovement. In: P. Fay, & C. V. Baalen*The Cyanobacteria.* (pp. 325-345). : Elsevier.

Haney, J. F. et al. (1995). Effects of products released by Aphanizomenon flos-aquae and purified saxitoxin on the movements of Daphnia carinata feeding appendages. *Limnol Oceanogr*, **40**(2), 263-272.

Hannah, D. J. et al. (1993). Toxins and assays techniques. J. A. Jasperse, *Marine toxins and New Zealand shellfish. Miscellaneous series.* 24. (pp. 28-30). Wellington: The Royal Society of New Zealand.

Harada, K. I. et al. (1988). Analysis and purification of toxic peptides from cyanobacteria by reversed-phase high-performance liquid chromatography. *Journal of Chromatography* , **448**, 275-283.

Harada, K. I. et al. (1991). Microcystins from Anabaena flos- aquae NRC-525-17. *Chem Res Toxicol*, **4**, 535-540.

Harada, K. I. et al. (1989). A new procedure for the analysis and purification of naturally occurring anatoxin-a from the blue- green algae Anabaena flos-aquae. *Toxicon*, **27**(12), 1289-1296.

Harris, G. P. (1986). *Phytoplankton ecology: structure, function and fluctuation.* : Chapman and Hall, London, New York.

Hauman, J. H. (1981). Is a plasmid involved in the toxicity of Microcystis aeruginosa? In: W. W. Carmichael*The Water Environment: Algal Toxins and Health.* (pp. 97-102). New York: Plenum Press.

Hawser, S. D. et al. (1992). Toxicity of bloom of the cyanobacterium Trichodesmium to zooplankton. *J Appl Phycol*, **4**, 79-.

He, Z. R. et al. (1989). Study on the toxicity of blue-green algal blooms in Donghu Lake. II. Seasonal variation of toxicity and characteristics of Microcystis toxin. *Acta Hydrobiologica Sinica*, **13**(3), 201-209.

Hellebust, J. A., & Craigie, J. S. (1978). *Handbook of phycological methods: physiological and biochemical methods.* Cambridge: Cambridge University Press.

Herman, D. C. et al. (1990). Impact of volatile aromatic hydrocarbons, alone and in combination, on growth of the freshwater alga Selenastrum capricornutum. *Aquatic Toxicology*, **18**, 87-100.

Hewitt, W. (1977). *Microbiological assay- An Introduction to quantitative principles and evaluation.* London: Academic Press.

Himberg, K. et al. (1989). The effect of water treatment processes on the removal of hepatotoxins from Microcystis and Oscillatoria cyanobacteria: a laboratory study. *Water REs*, **23**(8), 979-984.

Honkanen, R. E. et al. (1990). Characterization of microcystin- LR, a potent inhibitor of type 1 and type 2A protein phosphatases. *J Biol Chem*, **265**(32), 19401-19404.

Honkanen, R. E. et al. (1995). Protein phosphatase inhibitory activity in extracts of cultured blue-green algae (cyanophyta). *J Phycol*, **31**(3), 478-.

Hornsey, I. S. (1985). The production of antimicrobial compounds by British marine algae IV. Variation of antimicrobial activity with algal generation. *Br Phycol J*, **20**, 21-25.

Hughes, E. O., Gorham, P. R., & Zehnder, A. (1958). Toxicity of a unialgal culture of Microcystis aeruginosa. *Can J Microbiol* , **4**, 225-236.

Hyde, E. G., & Carmichael, W. W. (1991). Anatoxin-a(s): a naturally occurring organophosphate is an irreversible active site-directed inhibitor of acetylcholinesterase. *J Biochem Toxicol*, **6**(3), 195-201.

Ikawa, M., & Sanser, J. J. (1990). The chemistry and physiology of algal toxins. In: I. Akatsuka*Introduction to Applied Phycology*. (pp. 27-65). The Hague, The Netherlands : SPB Academic Publishing bv.

Ishibashi, M. et al. (1986). Scytophycins, cytotoxic and antimycotic agents from the cyanophyte Scytonema pseudohofmanni. *J Org Chem* , **51**, 5300-5306.

Jones, A. K. (1988). Algal extracellular products - antimicrobial substances. L. J. Rogers, & J. R. Gallon, *Biochemistry of the algae and cyanobacteria*. **28**Oxford, U.K. *Proceedings of the Phytochemical Society of Europe*. (pp. 257-281). : Oxford Science Publications.

Jones, G. J., Balckburn, S. I., & Parker, N. S. (1994). A toxic bloom of Nodularia spumegina mertens in Orielton lagoon, Tasmania. *Aust J Mar Freshwater Res* , **45**, 787-800.

Jones, G. J., Falconer, I. R., & Wilkins, R. M. (1995). Persistence of cyclic peptide toxins in dried Microcystis aeruginosa crusts from lake Mokoan, Australia. *Env Toxicity and Water Quality*, **10**(1), 19-24.

Jorgensen, B. B. (1989). Light penetration, absorption and action spectra in cyanobacterial mats. In: Y. Cohen, & E. Rosenberg*Microbial Mats- physiological ecology of benthic microbial communities.* (pp. 123-137). Washington, DC: American Society for Microbiology.

Juttner, F., Leonhardt, J., & Mohren, S. (1983). Environmental factors affecting the formation of mesityloxide, dimethylallylic alcohol and other volatile compounds excreted by Anabaena cylindrica. *J Gen Microbiol* , **129**, 407-412.

Keating, K. I. (1976). *Algal metabolite influence on bloom sequence in eutrophied freshwater ponds.* (EPA-600/3-76-081). : US Environmental Protection Agency.

Keleti, G. et al. (1981). Isolation and characterization of endotoxin from cyanobacteria (Blue-green algae). In: W.W. Carmichael*The water environment - Algal toxins and health* . New York: Plenum Press.

Keleti, G., & Sykora, J. L. (1982). Production and properties of cyanobacterial endotoxins. *Appl Env Microbiol* , **43**(1), 104-109.

Kellam, S. J. et al. (1988). Results of a large-scale screening programme to detect antifungal activity from marine and freshwater microalgae in laboratory culture. *Br Phycol J*, **23**, 45- 47.

Kenefick, S. L. et al. (1992). Odorous substances and cyanoabcterial toxins in Prairie drinking water sources. *Wat Sci Tech*, **25**(2), 147-154.

Kiviranta, J. et al. (1991). Detection of toxicity of cyanobacteria by Artemia salina bioassay. *Environ Toxicol Water Quality*, **6**,

Kiviranta, J. (1991). Production and biodegradation of cyanobacterial toxins - a laboratory study. *Arch Hydrobiol*, **121**(3), 281-294.

Kotak, B. G. et al. (1995). Variability of hepatotoxin microcystin-lr in hyper eutrophic drinking water lakes. *J Phycol* , **31**, 248-263.

Kratz, W. A., & Myers, J. (1955). Nutrition and growth of several blue-green algae. *Am J Bot* , **42**, 282-287.

Kruger, G. H. J., Eloff, J. N., & Pretorius, J. A. (1981). Morphological changes in toxic and non-toxic Microcystis isolates at different irradiance levels. *J Phycol*, **17**, 52-56.

Lahti, K. et al. (1995). Assessment of rapid bioassays for detecting cyanobacterial toxicity. *Letters in Applied Microbiology* , **21**, 109-114.

Lambert, T. W. et al. (1994). Quantitation of the microcystin hepatotoxins in water at environmentally relevant concentrations with protein phosphatase bioassay. *Environ Sci Technol*, **28**, 753- 755.

Lampert, W. (1987). Laboratory studies on zooplankton- cyanobacteria interactions. *NZ Mar Freshwater Res*, **21**(3), 483- 490.

Lampert, W. (1981). Toxicity of blue-green Microcystis aeruginosa: effective defense mechanism against grazing pressure by Daphnia. *Verh Int Verein Limnol*, **21**(3), 1436-1440.

Lauren-Maatta, C. et al. (1995). Do Microcystis aeruginosa toxins accumulate in the food web: a laboratory study. *Hydrobiologia* , **304**, 23-27.

Lawton, L. A. et al. (1990). Use of rapid bioluminescence assay for detecting cyanobacterial microcystin toxicity. *Lett Appl Microbiol*, **11**(4), 205-207.

Lawton, L. A., & Codd, G. A. (1991). Cyanobacterial (blue-green algal) toxins and their significance in UK and european waters. *J IWEM* , **5**, 460-465.

Lawton, L., & Codd, G. A. (1988). Effects of cyanobacterial peptide toxins on the ciliate Paramecium. *Br Phycol J* , **23**(3), 292.

Lindholm, T., Eriksson, J. E., & Meriluoto, J. A. O. (1989). Toxic cyanobacteria and water quality problems-examples from a eutrophic lake on Aland, southwest Finland. *Water Res* , **23**, 481- 486.

Luukkainen, R. et al. (1993). Isolation and identification of eight microcystins from thirteen Oscillatoria agardhii strains and structure of a new microcystin. *Appl Env Microbiol* , **59**(7), 2204-2209.

Maestrini, S. Y., & Bonin, D. J. (1981). Allelopathic relationships between phytoplankton species. *Canadian Bulletin of Fisheries And Aquatic Sciences* , **210**, 323-338.

Martin, T. C., & Waytt, J. T. (1974). Extracellular investments in blue-green algae with particular emphasis on the genus Nostoc. *J Phycol* , **10**, 204-210.

Marvan, P., Pribil, S., & Lhotsky, O. (1979). *Algal assays and monitoring eutrophication.* Stuttgart: E. Schweizerbart'sche Verlagsbuchhandlung (Nagele u. Obermiller).

Mason, C. P. et al. (1982). Isolation of chlorine-containing antibiotic from the freshwater cyanobacterium Scytonema hofmanni. *Science* , **215**, 400-402.

McCormick, P. V., & Stevenson, R. J. (1991). Mechanisms of benthic algal succession in lotic environments. *Ecology*, **72**(5), 1835-1848.

McInnes, A. et al. (1983). The clinical pathology of sheep experimentally poisoned with the blue-green alga Microcystis aeruginosa. *Toxicon, Suppl*, **3**, 281-284.

Mead, R., Curnow, R. N., & Hasted, A. M. (1993). Statistical methods in agriculture and experimental biology. (Second). (pp. 415). London: Chapman & Hall.

Meriluoto, J. A., & Eriksson, J. E. (1988). Rapid analysis of peptide toxins in cyanobacteria. *Journal of Chromatography* , **438**, 93-99.

Metting, B., & Pyne, J. W. (1986). Biologically active compounds from microalgae. *Enzyme Microbiol Technol*, **8**(7), 386-394.

Millington, L. A., Goulding, K. H., & Adams, N. (1988). The influence of growth medium composition on the toxicity of chemicals to algae. *Wat Res*, **22**(12), 1593-1597.

Mingazzini, M. (1993). Comparison of different methods for quantitative toxicity measurements on natural waters using S. capricornutum. *Wat Res*, **27**(6), 1055-1062.

Moore, R. E. et al. (1984). Absolute stereochemistries of the aplysiatoxins and oscillatoxin A. *J Org Chem* , **49**, 2484-2489.

Moore, R. E. et al. (1984). Hapalindoles: New alkaloids from the blue-green alga Hapalosiphon fontinalis. *J Am Chem Soc* , **106**, 6456-6457.

Moore, R. E. et al. (1987). Hapalindoles, antibacterial and antimycotic alkaloids from the cyanophyte Hapalosiphon fontinalis. *J Org Chem* , **52**, 1036-1043.

Moore, R. E. (1981). Toxins from marine blue-green algae. In: W.W. Carmichael*The water environment - Algal toxins and health.* New York: Plenum Press.

Moore, R. E., Yang, X. G., & Patterson, G. M. L. (1987). Fontonamide and anhydrohapaloxindole A, two new alkaloids from the blue-green alga Hapalosiphon fontinalis. *J Org Chem* , **52**, 3773-3777.

Nisbet, L. J. (1982). Current strategies in the search for bioactive microbial metabolites. *J Chem Tech Biotechnol*, **32**, 251- 270.

Ohtake, A. et al. (1989). Toxicity of Microcystis species isolated from natural blooms and purification of the toxin. *Appl Env Microbiol*, **55**(12), 3202-3207.

Ojanpera, I. et al. (1991). Facile detection of anatoxin-a in algal material by thin layer chromatography with fast black K salt. *Analyst* , **116**, 265-267.

Ott, R. L., & Mendenhall, W. (1995). Understanding Statistics. (sixth). (pp. 598). California: Duxbury Press.

Paerl, H. W. (1988). Growth and reproductive strategies of fresh water blue-green algae (cyanobacteria). In: C. D. Sandgren*Growth and reproductive strategies of freshwater phytoplankton.* (pp. 261-315). Cambridge: Cambridge University Press.

Park, A., Moore, R. E., & Patterson, G. M. L. (1992). Fischerindole L, a new isonitrile from the terrestrial blue-green alga Fischerella muscicola. *Tetrahedron Letters*, **33**(23), 3257- 3260.

Patterson, G. M. L. et al. (1991). Antineoplastic activity of cultured blue-green algae (Cyanophyta). *J Phycol* , **27**, 530-536.

Patterson, G. M. L., & Bolis, C. M. (1993). Regulation of scytophycin accumulation in cultures of Scytonema ocellatum.I.Physical factors. *Appl Microbiol Biotechnol* , **40**, 375-381.

Patterson, G. M. L., & Carmeli, S. (1992). Biological effects of tolytoxin (6-hydroxy-7-0-methyl-scytophycin b), a potent bioactive metabolite from cyanobacteria. *Arch Microbiol* , **157**, 406-410.

Patterson, G. M. L., & Harris, D. O. (1983). The effect of Pandorina morum (Chlorophyta) toxin on the growth of selected algae, bacteria and higher plants. *Br Phycol J*, **18**, 259-266.

Penaloza, R. et al. (1990). Toxicity of a soluble peptide from Microcystis sp. to zooplankton and fish. *Freshwater Biology* , **24**, 233-240.

Philippis, R. D. et al. (1991). Effects of growth conditions on exopolysaccharide prodcution by Cyanospira capsulata. *Bioresources Technology*, **38**(2-3), 101-104.

Phillips, M. J. (1985). The toxicity of the cyanobacterium Microcystis aeruginosa to rainbow trout, Salmo gairdneri Richardson. *J Fish Dis* , **8**, 339-344.

Pick, F. R., & Lean, D. R. S. (1987). The role of macronutrients (C,N,P) in controlling cyanobacterial dominance in temperate lakes. *NZ Mar Freshwater Res*, **21**(3), 425-434.

Pignatello, J. J. et al. (1983). Structure of the antibiotic cyanobacterin, a chlorine containing gamma-Lactone from the freshwater cyanobacterium Scytonema hofmanni. *J Org Chem* , **48**, 4035-4038.

Pirt, S. J. (1985). *Principles of Microbe and Cell Cultivation.* (pp. 273). : Blackwell Scientific Publications.

Poon, G. K. et al. (1987). Purification procedure for peptide toxins from the cyanobacterium Microcystis aeruginosa involving high-performance thin-layer chromatography. *Journal of Chromatography* , **387**, 551-555.

Priestley, I. M. (1989). *Properties and in vitro behaviour of toxins from the cyanobacterium Microcystis aeruginosa.* Unpublished doctoral dissertation, The University of Dundee, Dundee.

Prinsep, M. R. et al. (1992). Microcystin-LA from a blue-green alga belonging to the stigonematales. *Phytochemistry*, **31**(4), 1247-1248.

Radway, J. C. et al. (1994). Nutrient reomval by thermophilic Fischerella (Mastigocladus laminosus) in a simulated algaculture process. *Bioresources Technology* , **50**, 227-233.

Ransom, R. E., Nerad, T. A., & Meier, P. G. (1978). Acute toxicity of some blue-green algae to the protozoan Paramecium caudatum. *J Phycol*, **14**, 114-116.

Repavich, W. M. et al. (1990). Cyanobacteria (blue-green algae) in Wisconsin waters: acute and chronic toxicity. *Wat Res*, **24**(2), 225-231.

Reynolds, C. S. (1987). Cyanobacterial water blooms. *Advances in Botanical Research* , **13**, 67-143.

Reynolds, C. S. (1972). Growth, gas vacuolation and buoyancy in natural population of a planktonic blue-green alga. *Freshwat Biol*, **2**, 87-106.

Reynolds, C. S. (1984). Phytoplankton periodicity: the interaction of form, function and environmental variability. *Freshwater Biology* , **14**, 111-142.

Reynolds, C. S., & Walsby, A. E. (1975). Water-Blooms. *Biol Rev*, **50**, 437-481.

Rice, E. L. (1984). Allelopathy. (2nd edition). Orlando, Florida, USA: Academic Press, Inc.

Rijstenbil, J. W. (1989). Competitive interaction between Ditylum brightwellii and Skeletonema costatum by toxic metabolites. *Netherlands Journal of Sea Research* , **23**(1), 23-27.

Rinehart, K. L., Namikoshi, M., & Choi, B. W. (1994). Structure and biosynthesis of toxins from blue-green algae (cyanobacteria). *J Appl Phycol* , **6**, 159-176.

Robarts, R. D., & Zohary, T. (1987). Temperature effects on photosynthetic capacity, respiration and growth rates of bloom- forming cyanoabcteria. *NZ Mar Freshwater Res*, **21**(3), 391-399.

Rosen, B. H., MacLeod, B. W., & Simpson, M. R. (1992). Accumulation and release of geosmin during the growth phases of Anabaena circinalis (Kutz.) rabenhorst. *Wat Sci Tech*, **25**(2), 185- 190.

Roszell, L. E., Schulman, L. S., & Baden, D. G. (1990). Toxin profiles are dependent on growth stages in cultured Ptychodiscus brevis. In: E. Graneli et al.*Toxic marine phytoplankton.* (pp. 403-406). : Elsevier Science Publishing Co., Inc.

Runnegar, M. T. C. et al. (1983). Toxin production by Microcystis aeruginosa cultures. *Toxicon, Suppl*, **3**, 377-380.

Sand-Jensen, K., & Borum, J. (1991). Interactions among phytoplankton, periphyton and macrophytes in temperate freshwaters and estuaries. *Aquatic Botany*, **41**, 137-175.

Sayed, O. H., & Fadl-allah, E. M. (1992). Influence of aflatoxin B1 on growth, photosynthetic oxygen evolution and regreening of Chlorella fusca (Chorococcales, Chlorophyta). *Cryptogamie, Algol*, **13**(1), 45-48.

Schwartz, R. E. et al. (1990). Parmaceuticals from cultured algae. *J Ind Microbiol*, **5**, 113-124.

Schwimmer, M. (1964). Algae and medicine. In: D. F. Jackson*Algae and Man*. (pp. 368-412). New York: Plenum Press.

Scott, W. E. (1991). Occurrence and significance of toxic cyanobacteria in southern Africa. *Wat Sci Tech* , **23**, 175-180.

Scott, W. E., Barlow, D. J., & Hauman, J. H. (1981). Studies on the ecology, growth and physiology of toxic Microcystis aeruginosa in South Africa. In: W. W. Carmichael*The Water Environment: Algal Toxins and Health*. (pp. 49-69). New York: Plenum Press.

Shapiro, J. (1973). Blue-green algae: why they become dominant. *Science* , **179**, 382-384.

Shi, L., Carmichael, W. W., & Miller, I. (1995). Immuno-gold localization of hepatotoxins in cyanobacterial cells. *Arch Microbiol* , **163**, 7-15.

Shilo, M. (1989). The unique characteristics of benthic cyanobacteria. In: Y. Cohen, & E. Rosenberg*Microbial Mats- Physiological ecology of benthic microbial communities*. (pp. 207- 213). Washington, DC: American Society for Microbilogy.

Shirai, M. et al. (1990). Toxicity and toxins of natural blooms and isolated strains of Microcystis spp (cyanobacteria) and improved procedure for purification of cultures. *Appl Env Microbiol* , **57**, 1241-1245.

Shirai, M. et al. (1991). Toxicity and toxins of natural blooms and isolated strains of Microcystis spp. (cyanobacteria) and improved procedure for purification of cultures. *Appl Env Microbiol* , **57**(4), 1241-1245.

Silvey, J. K., Henley, D. E., & Wyatt, J. T. (1972). Planktonic blue-green algae: growth and odor-production studies. *Journal AWWA*, 35-39.

Sivonen, K. (1990). Effects of light, temperature, nitrate, orthophosphate and bacteria on growth of and hepatotoxin production by Oscillatoria agardhii strains. *Appl Env Microbiol* , **56**(9), 2658-2666.

Sivonen, K. et al. (1990). Isolation and characterization of hepatotoxic microcystin homologs from the filamentous freshwater cyanobacterium Nostoc sp strain 152. *Appl Env Microbiol* , **56**(9), 2650-2657.

Sivonen, K. et al. (1992). Isolation and characterization of a variety of microsystins from seven strains of the cyanobacterial genus Anabaena. *Appl Env Microbiol* , **58**(8), 2495-2500.

Sivonen, K. et al. (1990). Toxic cyanobacteria (Blue-green algae) in Finnish fresh and coastal waters. *Hydrobiologia* , **190**, 267- 275.

Sivonen, K. et al. (1989). Toxicity and isolation of the cyanobacterium Nodularia spumigena from the southern Baltic Sea in 1986. *Hydrobiologia*, **185**, 3-8.

Skulberg, O. M. et al. (1992). Investigations of a neurotoxic Oscillatorian strain (Cyanophyceae) and its toxin. Isolation and characterization of Homoanatoxin-a. *Environemntal Toxicology and Chemistry* , **11**, 321-329.

Skulberg, O. M., Codd, G. A., & Carmichael, W. W. (1984). Toxic blue-green algal blooms in europe: a growing problem. *Ambio*, **13**, 244-247.

Skulberg, O. M., Codd, G. A., & Carmichael, W. W. (1984). Toxic blue-green algal blooms in Europe: a growing problem. *Ambio*, **13**(4), 244-247.

Srivastava, V. C., Manderson, G. J., & Bhamidimarri, R. (1994). Growth and toxicity of a cyanobacterium Fischerella muscicola. *Proceedings of the inaugural New Zealand postgraduate conference for engineering and techonlogy students.* Palmerston North. (pp. 426-432). : Department of Production Technology, Massey University, Palmerston North.

Stal, L. J. (1991). The metabolic versatility of the mat-building cyanobacteria Microcoleus chthonoplastes and Oscillatoria limosa and its ecological significance. *Algological Studies* , **64**, 453- 467.

Stanier, R. Y., & Cohen-Bazire, G. (1977). Phototrophic Prokaryotes: The cyanobacteria. *Ann Rev Microbiol*, **31**, 225-274.

Starr, R. C., & Zeikus, J. A. (Supplement to September 1987). UTEX - The culture collection of algae at the University of Texas at Austin. *J Phycol*, **23**,

Stein, J. R. (1979). *Handbook of phycological methods- culture methods & growth measurements.* : Cambridge University Press.

Steinberg, C. E. W., & Hartmann, H. M. (1988). Planktonic bloom forming cyanobacteria and the eutrophication of lakes and rivers. *Freswater Biology* , **20**, 279-287.

Stevens, D. K., & Krieger, R. I. (1988). Analysis of anatoxin-a by GC/ECD. *Journal of Analytical Toxicology* , **12**, 126-131.

Stevens, D. K., & Krieger, R. I. (1991). Stablity studies on the cyanobacterial nicotinic alkaloid anatoxin-a. *Toxicon* , **29**(2), 167-179.

Sykora, J. L., & Keleti, G. (1981). Cyanobacteria and endotoxins in drinking water supplies. In: W.W. Carmichael*The water environment - Algal toxins and health* . New York: Plenum Press.

Tilzer, M. M. (1987). Light dependence of photosynthesis and growth in cyanobacteria: implications for their dominance in eutrophic lakes. *NZ Mar Freshwater Res*, **21**(3), 401-412.

Tsuchiya, Y., Watanabe, M. F., & Watanabe, M. (1992). Volatile organic sulfur compounds associated with blue-green algae from inland waters of Japan. *Wat Sci Tech*, **25**(2), 123-130.

Turner, P. C. et al. (1990). Pneumonia associated with contact with cyanobacteria. *Br Med J*, **300**, 1440-1441.

Utkilen, H., & Gjolme, N. (1992). Toxin production by Microcystis aeruginosa as a function of light in continuous cultures and its ecological significance. *Appl Env Microbiol*, **58**(4), 1321-1325.

van der Westhuizen, A. J., & Eloff, J. N. (1985). Effect of temperature and light on the toxicity and growth of the blue- green alga Microcystis aeruginosa (UV-006). *Planta* , **163**, 55-59.

van der Westhuizen, A. J., Eloff, J. N., & Kruger, G. H. J. (1986). Effect of temperature and light (fluence rate) on the composition of the toxin of the cyanobacterium Microcystis aeruginosa (UV-006). *Arch Hydrobiol*, **108**(2), 145-154.

van der Westhuizen, A. J., Eloff, J. N., & Kruger, G. H. J. (1988). Effect of culture age and pH of the culture medium on the composition of the toxin of the cyanobacterium Microcystis aeruginosa. *S Afr J Bot*, **54**(4), 372-374.

Van Hoof, F. et al. (1994). *Toxicity studies with blue-green algae from Flemish reservoirs. Special Publication*. 149. Great Britain: Royal Society of Chemistry.

Van Vierssen, W., & Prins, T. C. (1985). On the relationship between the growth of algae and aquatic macrophytes in brackish water. *Aquatic Botany*, **21**, 165-179.

Vasconcelos, V. M. (1995). Uptake and depuration of the heptapeptide toxin microcystin-LR in Mytilus galloprovincialis. *Aquatic Toxicology*, **32**(2-3), 227-238.

Vincent, W. F. (1989). Cyanobacterial growth and dominance in two eutrophic lakes: review and synthesis. *Arch Hydrobiol Beih Ergebn Limnol* , **32**, 239-254.

Vincent, W. F., & Dryden, S. J. (1989). Phytoplankton succession and cyanobacterial dominance in a eutrophic lake of the mid- temperate zone (Lake Okaro, New Zealand). *Arch Hydrobiol Beih Ergebn Limnol* , **32**, 137-163.

Vincent, W. F., & Forsyth, D. J. (1987). Geothermally influenced waters. In: A.B. Viner*Inland waters of New Zealand*. (pp. 349- 378). Wellington: DSIR bulletin 241.

Vincent, W. F., & Silvester, W. B. (1979). Growth of blue-green algae in the manukau (New Zealand) oxidation ponds-II. Experimental studeis on algal interaction. *Water Research* , **13**, 717-723.

Vincent, W. F., & Sivester, W. B. (1979). Growth of blue-green algae in the manukau (New Zealand) oxidation ponds-I. Growth potential of oxidation pond water and comparative optima for blue-green and green algal growth. *Water Research*, **13**, 711-716.

Viner, A. B. (1987). Cyanobacteria in New Zealand inland waters. *NZ Mar Freshwater Res*, **21**(3), 503-508.

Vining, L. C. Secondary metabolism. **4**In: H. J. Rehm, & G. Reed*Biotechnology: A comprehensive treatise in 8 volumes*. Canada.

Watanabe, M. F. et al. (1991). Composition of cyclic peptide toxins among strains of Microcystis aeruginosa (Blue-green algae, Cyanobacteria). *Bot Mag Tokyo* , **104**, 49-57.

Watanabe, M. F. et al. (1989). Heptapeptide toxin production during the batch culture of two Microcystis species (Cyanobacteria). *J Appl Phycol* , **1**, 161-165.

Watanabe, M. W., Kaya, K., & Takamura, N. (1992). Fate of the toxic cyclic heptapeptides, the microcystins, from blooms of Microcystis (cyanobacteria) in a hypertrophic lake. *J Phycol* , **28**, 761-767.

Watanabe, M. F., & Oishi, S. (1985). Effects of environmental factors on toxicity of a cyanobacterium (Microcystis aeruginosa) under culture conditions. *Appl Env Microbiol* , **49**(5), 1342-1344.

Watanabe, M. F., & Oishi, S. (1983). A highly toxic strain of blue-gree alga Microcystis aeruginosa isolated from Lake Suwa. *Bull Jap Soc Sci Fish* , **49**(11), 1759.

Watanabe, M. F., & Oishi, S. (1982). Toxic substances from a natural bloom of Microcystis aeruginosa. *Appl Env Microbiol*, **43**(4), 819-822.

Waterbury, J. B. (1991). The Cyanobacteria- Isolation, Purification and Identification. (second). In: A. Balow et al.*The Prokaryotes- A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications.* II. (pp. 2058-2078). : Springer-Verlag.

Wicks, R. J., & Thiel, P. G. (1990). Environmental factors affecting the production of peptide toxins in floating scums of the cyanobacterium Microcystis aeruginosa in hypertrophic african reservoir. *Environ Sci Technol*, **24**, 1414-1418.

Williamson. G. B., & Richardson, D. (1988). Bioassay for allelopathy: measuring treatment responses with independent control. *Journal of Chemical Ecology*, **14**(1), 181-187.

Wium-Andersen, S. (1987). Allelopathy among aquatic plants. *Arch Hydrobiol Beih*, **27**, 167-172.

Wong, S. H., & Hindin, E. (1982). Detecting an algal toxin by high-pressure liquid chromatography. *Am Water Works Assoc J*, **74**(10), 528-529.

Wyman, M., & Fay, P. (1987). Acclimation to the natural light climate. In: P. Fay, & C. V. Baalen*The Cyanobacteria.* (pp. 347- 376). : Elsevier.

Yamane, A., Nishimura, H., & Mizutani, J. (1992). Allelopathy of yellow Fieldcress (Rorippa sylvestris)- Identification and characterization of phytotoxic constituents. *J Chem Ecol*, **18**(5), 683-691.

Blackburn, S.I. *et al.*(1996). Effect of salinity on growth and toxin production in cultures of the bloom-forming cyanobacterium *Nodularia spumigena* from Australian waters. Phycologia. 35(6): in press.

Brock, T.D., Madigan, M.T., Martinko, J.M. and Parker, J. (1994). Biology of Micro-organisms. Seventh edition, Prentice-Hall International, Inc. USA.

Hawker, L.E. and Linton, A.H. (1979). Micro-organisms. Function, Form and Environment. Second edition, Edward Arnold.

Mandelstam, J. and McQuillen, K. (1973). Biochemistry of Bacterial Growth. Blackwell Scientific Publications.

Pelczar, M.J., Chan, E.C.S. and Krieg, N.R. (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc.

Pirt, S.J. (1975). Principles of Microbe and Cell Cultivation. Blackwell Scientific Publications. pp 274.

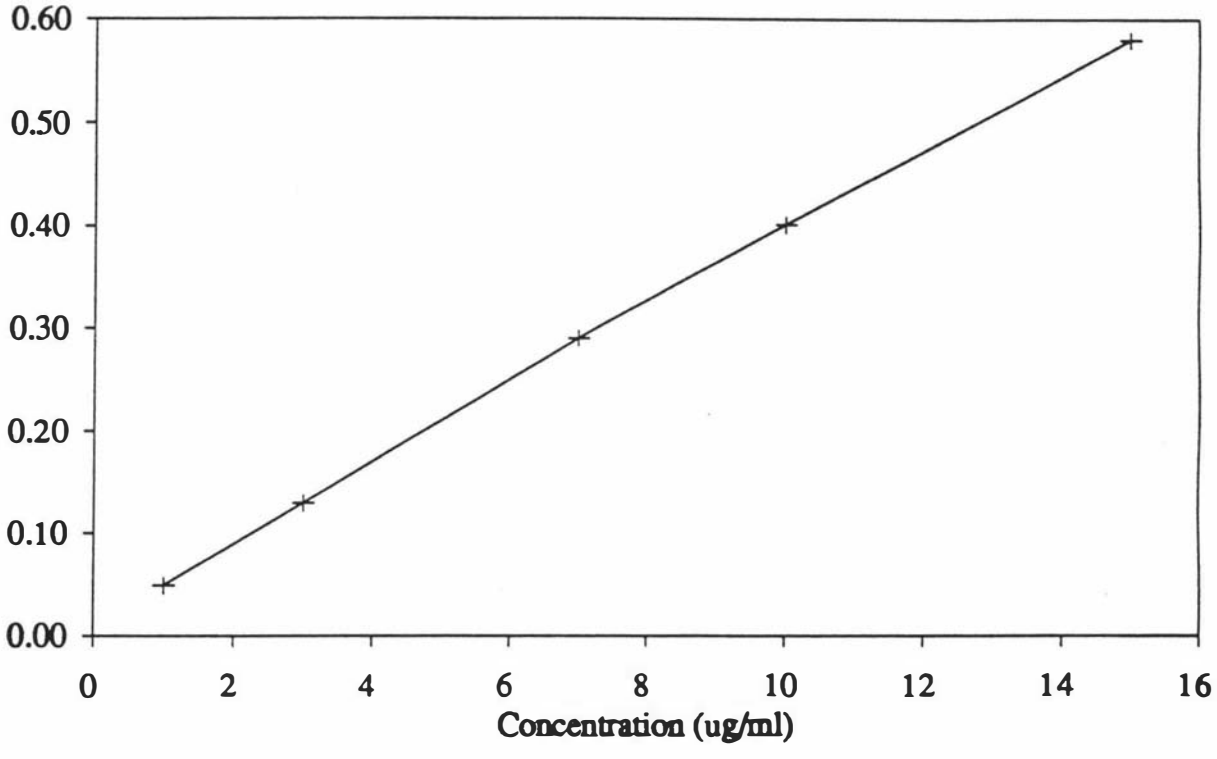
VanLiere, L. and Mur, L.C. (1979). Growth kinetics of Oscillatoria agardhii Gomont in continuous culture, limited in its growth by the light energy supply. J. Gen. Microbiol. 115, 153-160.

Appendices

Appendix A

Standard Curve of Protein

Standard Curve of Protein
Bio-Rad (Bradford's method)



Appendix B

SAS Programming

```
TITLE1 ' ';
options ls=78 ps=60;
/* file h:\fact.dd1 text input */
data dd1;
  infile 'C:\GANESH\CONSULT\fact.dd1';
  do expt = 1 to 8;
     input comb repl $ tox @;
     output;
  end;
run;
data dd1; set dd1;
  if comb='.' then delete;
  if expt=1 then do; lit=1; tmp=1; block=1; end;
  if expt=2 then do; lit=2; tmp=1; block=1; end;
  if expt=3 then do; lit=1; tmp=2; block=1; end;
  if expt=4 then do; lit=2; tmp=2; block=1; end;
  if expt=5 then do; lit=1; tmp=2; block=2; end;
  if expt=6 then do; lit=1; tmp=1; block=2; end;
  if expt=7 then do; lit=2; tmp=2; block=2; end;
  if expt=8 then do; lit=2; tmp=1; block=2; end;
  if comb=1 then do; ng=1; ph=1; end;
  if comb=2 then do; ng=2; ph=1; end;
```

```
  if comb=3 then do; ng=3; ph=1; end;
  if comb=4 then do; ng=1; ph=2; end;
  if comb=5 then do; ng=2; ph=2; end;
  if comb=6 then do; ng=3; ph=2; end;
  if comb=7 then do; ng=1; ph=3; end;
  if comb=8 then do; ng=2; ph=3; end;
  if comb=9 then do; ng=3; ph=3; end;
run;
proc sort;
  by comb repl block expt;
run;
/*
proc print uniform;
run;
*/
PROC GLM DATA=DD1 ORDER=DATA NOPRINT;
CLASS BLOCK LIT TMP NG PH;
MODEL TOX = BLOCK LIT TMP LIT*TMP BLOCK(LIT TMP)
        NG PH NG*PH LIT*NG TMP*NG LIT*TMP*NG LIT*PH TMP*PH
LIT*TMP*PH
        LIT*TMP*NG*PH / SS2;
TEST H=BLOCK LIT TMP LIT*TMP E=BLOCK(LIT TMP) / HTYPE=2 ETYPE=2;
MEANS NG PH / DUNCAN;
```

```
LSMEANS LIT*TMP / E=BLOCK(LIT TMP) PDIFF;
LSMEANS NG*PH LIT*NG TMP*NG LIT*PH TMP*PH LIT*TMP*NG LIT*TMP*PH
      LIT*TMP*NG*PH / PDIFF;
OUTPUT OUT=DD2 R=RESID STUDENT=SRESID P=FITTED;
RUN;
PROC PLOT DATA=DD2;
  PLOT RESID*FITTED;
  PLOT SRESID*FITTED;
RUN;
PROC MEANS NOPRINT;
CLASS LIT TMP NG PH;
VAR TOX;
OUTPUT OUT=NEW MEAN=MTOX;
RUN;
PROC PRINT UNIFORM;
RUN;
```

Appendix C

Pairwise comparison of LIT*TMP*PH

2.

General Linear Models Procedure

Pr > |T| H0: LSMEAN(i)=LSMEAN(j)

i/j	1	2	3	4	5	6	7	8	9
1	.	0.6382	0.6832	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
2	0.6382	.	0.9512	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
3	0.6832	0.9512	.	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
4	0.0001	0.0001	0.0001	.	0.8704	0.2177	0.0002	0.0005	0.0105
5	0.0001	0.0001	0.0001	0.8704	.	0.2843	0.0001	0.0003	0.0066
6	0.0001	0.0001	0.0001	0.2177	0.2843	.	0.0001	0.0001	0.0002
7	0.0001	0.0001	0.0001	0.0002	0.0001	0.0001	.	0.8171	0.2201
8	0.0001	0.0001	0.0001	0.0005	0.0003	0.0001	0.8171	.	0.3292
9	0.0001	0.0001	0.0001	0.0105	0.0066	0.0002	0.2201	0.3292	.
10	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
11	0.0001	0.0001	0.0001	0.0001	0.0001	0.0022	0.0001	0.0001	0.0001
12	0.0001	0.0001	0.0001	0.0001	0.0001	0.0025	0.0001	0.0001	0.0001

Pr > |T| H0: LSMEAN(i)=LSMEAN(j)

i/j	10	11	12
1	0.0001	0.0001	0.0001
2	0.0001	0.0001	0.0001
3	0.0001	0.0001	0.0001
4	0.0001	0.0001	0.0001
5	0.0001	0.0001	0.0001
6	0.0001	0.0022	0.0025
7	0.0001	0.0001	0.0001
8	0.0001	0.0001	0.0001
9	0.0001	0.0001	0.0001
10	.	0.1435	0.1159
11	0.1435	.	0.9328
12	0.1159	0.9328	.

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.