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OCCURRENCE AND TOXICITY OF MICROCYSTINS IN THE FRESHWATER PULMONATE SNAIL *LYMNAEA STAGNALIS*

by

RONALD WILLIAM ZURAWELL

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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The more that you read,

the more things you will know.

The more that you learn,

the more places you'll go.

– Dr. Seuss

The solution to any problem

–work, love, money, whatever–

is to go fishing,

and the worse the problem,

the longer the trip should be.

– John Gierach

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Occurrence and Toxicity of Microcystins in the Freshwater Pulmonate Snail *Lymnaea stagnalis*** submitted by **Ronald William Zurawell** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

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Date: 17 April 2001

I dedicate this thesis to my family and friends who have stood by me in support;

especially to my father, whose unpretentious sagacity instilled in me a deep appreciation for nature;

and to Diane, my wife, for accompanying me through this often arduous journey.

Abstract

Microcystins (MCYSTs) are potent liver toxins produced by cyanobacteria in fertile waters and have been implicated in poisonings of livestock, pets, wildlife and humans. Information concerning the fate of MCYSTs within food webs is scarce. This thesis explores aspects regarding the occurrence and effects of MCYSTs in pulmonate snails to ascertain whether gastropods figure in the uptake, accumulation and fate of cyanobacterial toxins in aquatic food webs.

A survey of lakes of varying primary productivity determined the concentration of MCYST in the tissue of *Lymnaea stagnalis*, *Helisoma trivolvis* and *Physa gyrina* was correlated with toxin in the phytoplankton ($P \leq 0.03$). Additionally, toxin concentrations in *L. stagnalis* and *P. gyrina* were also correlated with the relative abundance of *Microcystis* spp. ($P < 0.01$). Considering *Microcystis* spp. abundance and phytoplankton toxicity are correlated with indicators of productivity, it is apparent that trophic status influences the seasonal occurrence and concentration of MCYST in snails. Subsequently, I showed that mean toxin concentrations in *L. stagnalis* collected from ten sites within Hastings Lake, Alberta, differs spatially, as 60% of the variation was attributed to sampling site differences and body mass.

I also demonstrated the majority of toxin detected in *L. stagnalis* originates from indigestible cyanobacteria, which is eliminated in $\approx$ 8 h. Nevertheless, lower concentrations (49 ng g^{-1}) detected in snails beyond 24 h, confirms *Microcystis* digestion. In support, I determined that 83% of the total MCYST concentration was contained within the alimentary tract, while 17% was contained within the digestive gland, providing evidence for toxin uptake.

Depuration of MCYST from *L. stagnalis* was bi-phasic. Microcystin declined 80 and 95% (10 and 22°C, respectively) over the first 6 d (fast-phase), followed by reduced rates beyond 6 d (slow-phase). Temperature influenced depuration as fast-phase rates differed.

Orally administered MCYST caused histopathological changes in digestive glands of *L. stagnalis*. Severities were dose dependent and reminiscent of observed pathologies in liver of mammals and fish. Yet, aqueous MCYST did not affect developing *L. stagnalis* embryo, as the mean proportion of living snails in control and treatment groups were equal.

This thesis affords necessary insight on the importance of gastropods in accumulation and transfer of MCYSTs within the food webs of bloom-prone waters.

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List of Nomenclature and Abbreviations

ADDA	=	(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
Artificial pond water	=	Dechlorinated, carbon filtered municipal tap water
Chl-*a*	=	Chlorophyll-*a*
D-MeAsp	=	D-*erythro*-β-methylaspartic acid
HPLC	=	High performance liquid chromatography
IF	=	Intermediate filaments
k_{fast}	=	Fast-phase depuration rate constant
k_{slow}	=	Slow-phase depuration rate constant
Mdha	=	*N*-methyldehydroalanine
MCLR	=	Microcystin-LR
MCYST	=	Microcystin
MF	=	Microfilament
MT	=	Microtubule
NDLN	=	Nodularin
OCDD	=	1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin
PAR	=	Photosynthetically active radiation
PP1c	=	Protein phosphatase type 1c
PP2A	=	Protein phosphatase type 2A
TN	=	Total nitrogen
TP	=	Total phosphorus
Z_{eu}	=	Depth of euphotic zone
Z_m	=	Depth of mixing zone

Chapter 1

General Introduction and Scope of Thesis

1. General Introduction

Cyanobacterial blooms are dense accumulations of cyanobacteria or blue-green algae that typically appear as thick green scums on the surface of productive waters. While blooms are a natural phenomenon in fertile lakes and reservoirs worldwide, it is known that increased inputs of the nutrients phosphorus and nitrogen arising from urban, industrial, and agricultural sources, are resulting in an increase in the occurrence and severity of these blooms (Codd and Bell, 1985; Carmichael, 1994). Not only is the frequency of bloom production increasing but so is the duration and magnitude of these blooms. A potentially hazardous and often lethal consequence associated with cyanobacterial blooms, is the production of potent hepato- (liver) toxins by a number of bloom-forming species of cyanobacteria (Kiviranta *et al.*, 1991; Carmichael, 1992). Comprised of over 70 related variants, the cyclic heptapeptides known as microcystins (MCYSTs) are undoubtedly the most prevalent in the world's freshwaters.

With respect to biological implications and toxicity, MCYSTs are preferentially taken up by hepatocytes (the functional cells of the liver) where they irreversibly bind to specific enzymes known as protein phosphatases type 1 and type 2A (Carmichael, 1994; Bagu *et al.*, 1997). Generally, protein phosphatases function in concert with another group of enzymes known as protein kinases. Through continuous phosphorylation and dephosphorylation of the serine/threonine residues of various proteins, kinases and phosphatases (respectively) regulate many cellular events such as: carbohydrate and lipid metabolism, contractility, membrane transport and secretion, cell division, and gene expression (Cohen, 1989; Isobe *et al.*, 1995). However, once bound with MCYSTs, the enzymatic ability of protein phosphatases are effectively inhibited resulting in the alteration of hepatocyte shape (e. g., hepatocyte plasma membrane bleb formation, loss of microvilli and reorganization of microfilaments) and function (e. g., cell division and glycogen metabolism) (MacIntosh *et al.*, 1990; Carmichael, 1994). At sublethal levels, MCYSTs can cause intestinal and liver dysfunction, as well as the promotion of liver tumors and at higher levels, cause severe liver damage and death via intrahepatic

hemorrhage and hypovolaemic shock (Carmichael, 1992, 1994; Codd, 1995; Bagu *et al.*, 1997). Consequently, MCYSTs have been implicated in a number of poisonings of domestic livestock (cattle, pigs and sheep), pets (dogs), and wildlife (deer, ducks and fish) throughout the world (Schwimmer and Schwimmer, 1968; Codd, 1995). More recently, they have been implicated in the deaths of dialysis patients in a Brazilian hospital that used water from a reservoir experiencing blooms of toxin producing *Microcystis aeruginosa*.

Traditionally, research has focused on the acute toxicity of MCYSTs to laboratory mammals such as mice (Robinson *et al.*, 1991) and rats (Miura *et al.*, 1991). A number of studies have focused on the toxicity of MCYSTs to fish (such as: Carp – Råbergh *et al.*, 1991 and Carbis *et al.*, 1996; Tilapia – Beveridge *et al.*, 1993 and Keshavanath *et al.*, 1994; Rainbow and Brown Trout – Phillips *et al.*, 1985, Bury *et al.*, 1995 and Sahin *et al.*, 1996; and Chinook and Atlantic Salmon – Stephen *et al.*, 1993 and Kent *et al.*, 1996). Few studies have involved other aquatic organisms that typically inhabit bloom prone lakes and reservoirs, yet, may occupy vital roles in the food webs of these water bodies, such as: minute protozoans (e. g., *Heteromita globosa* and *Spumela* sp. – Christoffersen, 1996), large zooplankton (e. g., *Daphnia* spp. – DeMott *et al.*, 1991 and Hietala *et al.*, 1995 and *Artemia salina* – Reinikainen *et al.*, 1995 and Feuillade *et al.*, 1996) and macroinvertebrates (e. g., the mussel, *Anodonta cygnea* – Eriksson *et al.*, 1989 and the clam, *Mytilus galloprovincialis* – Vasconcelos, 1995). Finally, even fewer have focused on the fate of MCYSTs with respect to accumulation patterns within aquatic food webs (Eriksson *et al.*, 1989; Watanabe *et al.*, 1992; Kotak *et al.*, 1996).

Gastropods are found in all types of freshwater habitats, from pristine alpine lakes to polluted prairie ponds (Clarke, 1981; Elder and Collins, 1991). While some species are either carnivores or detritivores, the majorities are herbivorous grazers, feeding on various types of periphytic algae (Elder and Collins, 1991). In turn, gastropods are an important food source for many economically important sport fish (trout, perch and whitefish) and waterfowl (ducks, geese and shorebirds), as well as many less economically important groups (suckers, carp, crayfish and amphibians) (Stein *et al.*, 1984; Lodge, 1986). Consequently, gastropods are used extensively as indicators of polluted conditions in freshwater habitats (Elder and Collins, 1991). Recently, Kotak *et*

al. (1996) reported that microcystin-LR (MCLR), the predominant analogue in Alberta prairie lakes, was detectable in three species of indigenous pulmonate snail (*Lymnaea stagnalis*, *Helisoma trivolvis* and *Physa gyrina*) collected from several hypereutrophic lakes in concentrations up to 120 $\mu g\ g^{-1}$. Furthermore, changes in the MCLR concentration of gastropod tissue roughly followed changes in toxin concentration within the phytoplankton. Pulmonate snails inhabiting eutrophic and hypereutrophic waters, which experience cyanobacterial blooms, may feed directly on toxin-producing cyanobacteria thus, exposing organisms that typically ingest snails, to MCYSTs.

2. Scope of Thesis

This thesis explores several aspects regarding the occurrence and toxicity of MCYSTs in pulmonate snails (particularly *Lymnaea stagnalis*) in efforts to ascertain whether gastropods play a substantive role in the uptake, accumulation and fate of cyanobacterial hepatotoxins in aquatic food webs. Foremost, this work documents the occurrence of MCYSTs in resident pulmonate snails from Alberta lakes and identifies the environmental variables affecting temporal and spatial variation in tissue toxin concentrations (Chapters 3 and 4). As well, the extent and organ specificity of toxin accumulation within the pulmonate snail *L. stagnalis*, is examined (Chapters 5 and 6). Lastly, the toxicological effects (*i. e.*, digestive gland tissue pathology and embryo survival) of MCYST on *L. stagnalis* are evaluated (Chapter 7).

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Chapter 2

Literature Review

1. Cyanobacteria

Cyanobacteria comprise a unique assemblage of undeniably prokaryotic, plant-like organisms. That is, similar to other bacteria, cyanobacteria do not possess membrane-bound nuclei or specialized organelles and are incapable of sexual reproduction (Echlin, 1966). Yet like eukaryotic photoautotrophs (*i. e.*, true algae and higher plants), they utilize chlorophyll-*a* (Chl-*a*) pigment to harness light energy from the sun to convert inorganic carbon from their surroundings into organic compounds, while simultaneously liberating oxygen. Thus cyanobacteria are commonly (albeit incorrectly) referred to as blue-green algae (Stanier and Cohen-Bazire, 1977).

1.1 Importance – Recognized as the first organisms with the capacity for oxygenic photosynthesis beginning between 3.3 and 3.5 billion years ago, cyanobacteria played a paramount role in the oxygenation of Earth's atmosphere (Carmichael, 1994). Coupled with its role as primary producer and thus the basis of the food chain then, cyanobacteria are thought to have paved the way for the evolution of all other higher life forms that exist today (Campbell, 1987).

1.2 Requirements – Requirements fundamental to cyanobacterial, as well as eukaryotic algal growth (reproduction), are related to their need to maintain photosynthesis. Hence, light intensity and quality, the supply of inorganic carbon (CO_2) and water temperature, are of paramount importance. Additionally, nitrogen and phosphorus are critical to intracellular processes, with calcium, magnesium, sodium, potassium, sulphur, chloride and several trace elements (iron, manganese, zinc, copper, cobalt and molybdenum) considered subsidiary (Reynolds, 1984; Paerl, 1988).

1.3 Population dynamics – Ideal levels of the preceding physical and chemical factors may exist for various cyanobacteria and algae, however, as a general rule optimal conditions are unique to a given species and likely not optimal for others (Bucka, 1989). Furthermore, temporal and spatial fluctuations in these factors occur on varying scales, such that freshwater ecosystems are regarded as heterogeneous environments (Richerson *et al.*, 1970). Consequently, specific growth responses to these fluctuations, in

conjunction with various loss processes (*i. e.*, hydraulic washout, sedimentation, zooplankton grazing, bacterial, fungal and viral pathogens), result in temporal and spatial variations in abundance and composition of cyanobacterial and algal populations (George and Heaney, 1978; Reynolds, 1984; Carpenter, 1989).

While, the precise timing and involvement of individual species may fluctuate creating difficulties in establishing patterns of cyanobacterial and algal dominance, Reynolds (1984) suggested that at higher levels of categorization, prediction of dominance could be made with certainty. Accordingly, based on lake morphometry and water chemistry, characteristic patterns of seasonal succession have long been established (Hutchinson, 1967). Not only are these generalized cycles common to similar, adjacent waters, but also repeated in geographically isolated waterbodies of comparable properties (Reynolds, 1984).

2. Eutrophication

Eutrophication refers to the natural or anthropogenic acceleration in the rates of organic productivity (primary or otherwise) within aquatic ecosystems and is attributable to nutrient, primarily phosphorus and nitrogen, enrichment (Vollenweider, 1968; Wetzel, 1983). Natural eutrophication occurs over relatively short periods of geologic time, such that unproductive systems gradually become more productive. This has been ascribed to a flux in relative contributions of allochthonous (catchment basin-derived) and autochthonous (lake basin-generated) materials to the sediment, as influenced by climatic changes on catchment geology (*i. e.*, weathering rate, throughflow and leaching of soils) and lakewater (*i. e.*, retention time and chemistry) properties (Hutchinson, 1973; Reynolds, 1979). In contrast, anthropogenic eutrophication is attributed to nutrient inputs from urbanization (*i. e.*, elevated runoff due to land clearing/pavement and direct sewage disposal), agriculture (*i. e.*, tillage, animal waste and fertilizer use) and industrialization (*i. e.*, forest clearance and waste/by-product disposal) effectively and significantly magnifying natural processes (Prescott, 1948; Vallentyne, 1974).

2.1 Trophic status classification – Founded in the early works of Thienemann (1918) and Naumann (1919), the classification of waterbodies based on their nutrient status and subsequent levels of organic productivity, from oligotrophy (*i. e.*, nutrient

deficient/low rates of production) through various degrees of eutrophy (*i. e.*, nutrient rich/high rates of production), has since received much attention (e. g., Vollenweider, 1968; Rodhe, 1969; Dobson *et al.*, 1974). Characteristics associated with nutrient and productivity levels are indicative of lake trophic status and include water depth and transparency, hypolimnetic oxygen concentrations and sediment type and deposition rates. Hence nutrient deficient oligotrophic lakes, characterized by low rates of primary productivity, maintain greater water depth and transparency, elevated hypolimnetic oxygen concentrations and low deposition rates of primarily inorganic sediments (Table 1). Accordingly, high rates of productivity and thus shallower water depth and transparency, high deposition rates of primarily organic sediments and depressed hypolimnetic oxygen concentrations (Table 1) characterize nutrient enriched eutrophic lakes.

2.2 Trophic status and cyanobacterial dominance – As alluded to earlier (section 1.3), the physical and chemical factors associated with lake trophic status not only influences the overall biomass, but also the composition of cyanobacterial and algal communities. Consequently, the progression from an oligotrophic to eutrophic state is often accompanied by abrupt, long-term compositional shifts to species tolerant of eutrophic conditions (Hutchinson, 1967; Reynolds, 1984) and results in dominance by groups less common in oligotrophic waters (Table 2).

It is well established that cyanobacteria often dominate the phytoplankton communities of warm, nutrient enriched, hardwaters, such as the warm summer periods in eutrophic temperate lakes or perennially in eutrophic subtropical lakes (e. g., Pearsall, 1932; Hutchinson, 1967; Lin, 1972; Sommer *et al.*, 1986; Zohary and Robarts, 1989). In support, strong correlative and experimental evidence suggest several environmental factors critical to cyanobacterial growth and proliferation and include a stable water column, warm water temperature, high epilimnetic nutrient concentrations (phosphorus, nitrogen and organic compounds), low nitrogen:phosphorus ratios (N:P), high pH, low available CO_2 concentrations and reduced grazing by large zooplankton (summarized in Table 3).

3. Cyanobacterial Blooms

It is also apparent that cyanobacteria possess myriad physiological, morphological and ecological adaptations (Table 3) to optimize growth and persistence during physically and chemically favourable conditions (Stanier and Cohen-Bazire, 1977; Paerl, 1988, 1996). As a result, many genera develop population maxima or "blooms" in the epilimnion of eutrophic lakes, reservoirs and estuaries (Table 4). In addition, several species, primarily *Oscillatoria* spp., form metalimnetic blooms in moderately eutrophic waters, while benthic species form maxima in oligotrophic environments (Table 4).

3.1 Buoyancy regulation – The adaptation believed largely responsible for the success of epi- and metalimnetic bloom forming cyanobacteria, is the regulation of buoyancy (Fogg and Walsby, 1971; Reynolds and Walsby, 1975; Humphries and Lyne, 1988). Through the formation of intracellular gas vacuoles composed of numerous proteinaceous vesicles, cyanobacteria reduce cell/colony density and become positively buoyant (reviewed by Walsby, 1977, 1994). Additionally, the accumulation of storage polymers (polysaccharides) and increases in cellular turgor pressure, both products of photosynthesis, impart negative effects on buoyancy by acting as ballast molecules or causing pressure-sensitive gas vesicles to collapse, respectively (Kromkamp, 1987). Together, these mechanisms allow cyanobacteria to migrate vertically through stable water columns, affording access to optimal levels of photosynthetically active radiation (PAR) and inorganic carbon (Paerl *et al.*, 1985; Klemer and Konopka, 1989; Klemer *et al.*, 1996).

Once established, cyanobacteria may further alter epilimnetic conditions (elevated pH via CO_2 depletion) to favour their own growth, while concomitantly reducing growth rates of subsurface species by reducing PAR availability (Paerl, 1988). Consequently, it is by these means that species maintaining large benthic overwintering populations (*i. e.*, *Microcystis*), may come to dominate the euphotic zone of eutrophic lakes during summer periods of water column stability (Sirenko *et al.*, 1969; Reynolds *et al.*, 1981).

3.2 Surface bloom formation – An extraordinary phenomenon that occurs in eutrophic lakes and reservoirs following periodic mixing events, is the formation of dense surface accumulations of cyanobacteria that appear as thick green scums. During such events when the mixed depth is greater than the euphotic depth (*i. e.*, $Z_m > Z_{eu}$), in an

attempt to remain in the illuminated surface waters required for photosynthesis, gas vacuolate cyanobacteria often produce excess gas vesicles. However, if sudden calm conditions ensue, the cyanobacteria, unable to rapidly remove excess vesicles, continue to rise. Furthermore, strong light intensity at the surface (or possibly CO_2 depletion) may inhibit photosynthesis and its concomitant increase in turgor pressure required to down-regulate gas vesicles (and buoyancy), thus stranding cyanobacteria at the surface (mechanism summarized in Figure 1). Once at the surface, light winds transport cyanobacteria laterally towards lee shores. Hence, surface blooms do not result from severe increases in reproduction by cyanobacteria, but from mass recruitment of over-buoyant "existing" cells and/or colonies from the water column to the surface (reviewed by Reynolds and Walsby, 1975).

While surface blooms are a natural phenomenon in fertile lakes and reservoirs worldwide, it is widely accepted that anthropogenic eutrophication is resulting in an increase in the occurrence and severity of these blooms (Reynolds and Walsby, 1975; Codd and Bell, 1985; Carmichael, 1988). Thus, not only is the frequency of surface blooms increasing but also the magnitude and duration of these blooms.

4. Cyanobacterial Toxins

Aside from the adverse effects of blooms with respect to aesthetics and recreation (swimming, boating and fishing), a number of other water-related problems often results including foul odours, unpalatability and severe fish kills due to oxygen depletion and ammonia release by decaying cyanobacteria (Barica, 1975; Paerl, 1988). In addition, a potentially hazardous and often lethal consequence associated with blooms, is the production of potent neuro- and hepato- (liver)-toxins by a number of bloom-forming species of cyanobacteria (Gorham, 1964a; Carmichael, 1992).

4.1 Neurotoxins – The neurotoxins interfere with nervous system function by disrupting the normal propagation of nerve impulses from neurons to muscles, causing paralysis and death via respiratory failure and suffocation in animals (Carmichael, 1994). Saxitoxin and neosaxitoxin are alkaloids produced by freshwater cyanobacteria (Table 5) and coincidentally by marine dinoflagellates *Protogonyaulax tamarensis* and *P. catenella* associated with "Red Tide" paralytic shellfish poisoning (Baden, 1983). The toxins

inhibit nerve impulse propagation along axons by blocking sodium ions from entering nerve cells through the sodium channels, effectively suppressing stimulation of muscles including those of the respiratory system (Adelman *et al.*, 1982).

In contrast, anatoxin-a and anatoxin-a(s) are unique to cyanobacteria (Table 5) and exert toxicity at the neuromuscular junction. Anatoxin-a is a low molecular weight secondary amine that mimics the neurotransmitter acetylcholine and binds to receptors on muscle cells triggering contractions. However, acetylcholinesterase enzyme is unable to break down the toxin, which overstimulates and exhausts effected muscles (Carmichael, 1994). Anatoxin-a(s), a naturally occurring organophosphate also causes muscle fatigue and failure. In this case however, the toxin binds to acetylcholinesterase rendering it incapable of breaking down acetylcholine, resulting in overstimulation of the muscle cells (Carmichael, 1994). Saxitoxin/neosaxitoxin, anatoxin-a(s) and anatoxin-a, are lethal to animals with LD_{50}-values (interperitoneal injection in mice) of 10, 20 and 200 $\mu g\ kg^{-1}$, respectively (reviewed by Carmichael, 1992) and are responsible for the deaths of animals primarily in North America, United Kingdom, Scandinavia and Australia.

4.2 Hepatotoxins – Compared to neurotoxins, the hepatotoxins are a ubiquitous group of peptide toxins produced by several genera and species of bloom-forming cyanobacteria (Table 6) common to freshwater, estuarine and marine environments throughout the world (Table 7). Comprised of microcystins (MCYSTs) and nodularins (NDLNs), cyclic hepta- and pentapeptides, respectively, these toxins are preferentially taken up by hepatocytes (functional cells of the liver) resulting in a range of ill effects. Consequently, hepatotoxins are responsible for numerous poisonings of domestic livestock (cattle, pigs and sheep), pets (dogs) and wildlife (deer, ducks and fish) worldwide (Schwimmer and Schwimmer, 1968; Codd, 1995).

5. Microcystins

Recognition of toxic surface blooms involving *Nodularia spumigena*, the cyanobacteria responsible for the production of NDLNs, date back to observations made by Francis (1878), but as Jones *et al.* (1994) suggests, occur primarily in brackish waters (*i. e.*, estuaries and coastal lagoons). In contrast, blooms of *Microcystis aeruginosa*, the cyanobacteria primarily responsible for the production of MCYSTs, often dominate

productive freshwaters and have been directly linked to numerous livestock and wildlife deaths since early reports by Steyn (1945). Reynolds *et al.* (1981) attributes the success of *M. aeruginosa* to its ability to survive and perennate in these eutrophic conditions and Codd *et al.* (1995) concluded that 50 to 75% of blooms might contain toxins. Considering the cosmopolitan distribution of *M. aeruginosa* and associated toxins, significant effort to better understand the resulting economic and health implications with respect to the world's freshwater resources, is required. Consequently, the focus of this thesis primarily involves MCYSTs and only brief mention of NDLNs will follow.

5.1 Structure and variation – Microcystins are small monocyclic peptides composed of seven amino acids (Figure 2) and possess the generalized structure, cyclo (-D-Ala-**X**-D-MeAsp-**Y**-Adda-D-Glu-Mdha-), where D-Ala and D-Glu are alanine and glutamic acid (in the D configuration) respectively, D-MeAsp is D-*erythro*-β-methylaspartic acid and Mdha is *N*-methyldehydroalanine (Botes *et al.*, 1982, 1984). A novel C-20, β-amino acid residue, abbreviated Adda, is unique to cyanobacteria and has the structural formula (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Botes *et al.*, 1984; Rinehart *et al.*, 1988). In addition, **X** and **Y** represent two variable L-amino acids (Botes *et al.*, 1985), substitutions of which, give rise to at least 20 known primary MCYST analogues (summarized in Table 8). Of these, microcystin-LR, which possesses leucine (L) and arginine (R) at the two variable amino acid positions (Figure 3), is one of the most common forms (Carmichael, 1992; Kotak *et al.*, 1993; Sivonen, 1996).

Various alterations in other constituent amino acids result in numerous additional analogues. For instance, demethylation of D-MeAsp and/or Mdha at positions **3** and **7**, respectively, yield 13 demethylated variants of the primary analogues (Table 9). As well, alterations including methyl esterification of D-Glu (**6**) or substitution (modification) of Mdha (**7**), with/without demethylation of D-MeAsp (**3**), yield another 25 (Table 10). Finally, modifications in Adda (**5**), including geometric isomerization at the C-7 position, demethylation or substitution of the methoxyl group with an acetoxyl group at the C-9 position, with/without variation in amino acids **1**, **3** and/or **7**, gives rise to an additional 12 analogues (Table 11).

Similarly, NDLNs are monocyclic peptides consisting of five amino acids (Figure 4) and possess the structure, cyclo (-D-MeAsp-L-Arg-Adda-D-Glu-Mdhb-). However, NDLNs contain Mdhb (*N*-methyldehydrobutyrine) instead of Mdha (Rinehart *et al.*, 1988). As well, L-arginine, which is present in position **2**, may be substituted by L-valine in a primary variant, nodularin-V, commonly known as motuporin (deSilva *et al.*, 1992). Other analogues result from demethylation of D-MeAsp (**1**) or C-9 of Adda (**3**), methyl esterification of D-MeAsp (**1**) and D-Glu (**4**), substitution of Mdhb (**5**) or geometric isomerization of Adda (**3**) at the C-7 position (Table 12).

Recently, the three-dimensional solution structures of microcystin-LR (MCLR), microcystin-LL (MCLL), NDLN and motuporin (NDLN-V) were determined via ^{1}H nuclear magnetic resonance (NMR) spectroscopy (Rudolph-Böhner *et al.*, 1994; Bagu *et al.*, 1995, 1997; Annila *et al.*, 1996). Calculated NMR structures indicate the heptapeptide rings of MCLR and MCLL maintain well-defined saddle-shaped motifs with the free carboxyl groups of D-MeAsp and D-Glu projecting laterally. In addition, the Adda moiety exists as a highly flexible, hydrophobic tail, extending posteriorly from the rigid cyclic backbone (Bagu *et al.*, 1995, 1997). Similarly, the pentapeptide rings of NDLNs form smaller, rigid backbones, again with the carboxyl groups of D-MeAsp and D-Glu projecting laterally and the hydrophobic Adda side chain extending posteriorly. However, the absence of D-Ala and the first variable L-amino acid (*i. e.*, L-Leu in the case of MCLR and MCLL) shifts the position of the Mdhb moiety within NDLNs relative to Mdha in MCYSTs. As a result, the saddle-shaped motifs of NDLNs display reduced upward curvature towards the anterior (Bagu *et al.*, 1995; Annila *et al.*, 1996).

5.2 Properties and toxicity – The invariable and variable amino acid constituents of MCYSTs and NDLNs include both polar (hydrophilic) and nonpolar (hydrophobic) residues (Table 13). Overall however, regardless of the variable amino acids present, MCYSTs and NDLNs are soluble in water – albeit to varying degrees. Considering the presence of two free ionizable carboxyl groups on the D-MeAsp and D-Glu residues as well as the free ionizable amino group of arginine containing variants, de Maagd *et al.* (1999) suggested that speciation and partitioning between octanol and water may be pH dependant. Indeed, they determined that the dominant ion species (of the free ionizable carboxyl and amino groups), the resulting net charge and consequently, the octanol/water

distribution ratio of MCLR varied with pH (Table 14). Subsequently, it was concluded that "in the pH range 6 to 9 at which cyanobacteria may flourish", only limited bioconcentration of MCLR direct from water into biota via passive diffusion, can be expected (de Maagd *et al.*, 1999).

It is not surprising then that the toxicity, based on interperitoneal injection LD_{50}-values in mice, of the seventy described MCYSTs and nine NDLNs varies widely from approximately 50 to 1000 μg kg^{-1} (summarized in Table 8 to 12). According to Stoner *et al.* (1989) substitutions in the variable amino acid positions of the primary toxin analogues "markedly affects the dosimetric potency, but not the pathophysiology". Furthermore, Stotts *et al.* (1993) concluded that replacement of hydrophobic L-Leu in the first variable position with another hydrophobic L-amino acid (*i. e.*, alanine, phenylalanine or tryptophan), maintained toxicity. However, substitution with hydrophilic amino acids, such as arginine, resulted in large decreases in toxicity. Consequently, MCYSTs that contain polar (hydrophilic) substitutions in both of the variable amino acid positions, such as microcystin-RR (*i. e.*, arginine and arginine) and microcystin-M(O)R (*i. e.*, methionine sulfoxide and arginine), are the least toxic (Table 8).

Alterations to invariable residues may or may not impart reduced toxicity. For instance, demethylation of D-MeAsp or Mdha (Table 9) has little effect on toxicity, however esterification at the free carboxyl group of D-Glu (Table 10) leads to a loss of toxicity (Meriluoto *et al.*, 1989; Harada *et al.*, 1991a; Namikoshi *et al.*, 1992a; Stotts *et al.*, 1993). In addition, replacement of Mdha with *N*-methylalanine (L- and D-), *N*-methylserine, Mdhb or geometrical isomers of dehydrobutyrine (*i. e.*, (*E*)-Dhb and (*Z*)-Dhb) maintains toxicity. Yet replacement with *N*-methyllanthionine reduces toxicity somewhat (≈ 1000 μg kg^{-1}, Table 10). More importantly, certain modifications to the Adda residue can either alter, or have no appreciable effect on toxicity. For example, Namikoshi *et al.* (1990, 1992a) concluded that substitution of the methoxy group with an acetoxy group or demethylation at the C-9 position (*i. e.*, *O*-acetyl-*O*-demethylAdda and *O*-demethylAdda, respectively), has little effect on toxicity. However, isomerization of Adda at the C-7 position (*i. e.*, 6(*Z*)-Adda) yields non-toxic MCYST (> 1200 μg kg^{-1}) or NDLN (2000 μg kg^{-1}) analogues (Harada *et al.*, 1990; Namikoshi *et al.*, 1994).

5.3 Stability and degradation – Microcystins and NDLNs are endotoxins and thus remain within viable cyanobacterial cells throughout growth. However, as natural populations proceed through a subsequent stationary phase (senescence) leading to death, autolysis causes membranes to become susceptible to microbial attack (Berg *et al.*, 1987). As a result, cells become permeable allowing soluble intracellular compounds, including MCYSTs and NDLNs, to diffuse out into the surrounding environment. Previously, Berg *et al.* (1987) demonstrated that after an initial period of "hesitating decomposition" (≈ 14 d), at which time only minor changes to water quality occurred, "accelerated decomposition" lead to a significant release of toxin from bloom material into surrounding water. Under some circumstances, the cell wall and membrane of viable cells can be compromised as well. For example, Sugiura *et al.* (1992, 1993) confirmed the lytic capabilities of the bacterium *Pseudomonas* sp. and the microflagellate *Monas guttula* on viable *M. aeruginosa* cells. Alternatively, the use of chemicals to control cyanobacterial blooms (which act by inhibiting new cell wall synthesis, enzymatic reactions or photosynthesis), or used as flocculents in water treatment processes, may cause cell lysis and result in a significant increase in extracellular toxin concentration (Table 15). Once released into the aqueous environment, aside from dilution, several pathways contribute to natural detoxification of MCYSTs: (1) biological degradation, (2) photolysis, (3) thermal decomposition and (4) adsorption (Harada and Tsuji, 1998).

Pure microcystins are chemically stable in the absence of bacteria and photosynthetic pigments, experiencing limited hydrolysis with time. For instance, Wannemacher (1989) reported that MCLR degraded 0.37% d^{-1} at 45°C, while degradation at 4°C was negligible. These findings were confirmed by Lam *et al.* (1995a), who reported insignificant reductions in MCLR concentration when incubated with sterilized effluent from a wastewater treatment plant. However, in the presence of natural microbial populations, biodegradation may rapidly reduce the concentration of extracellular toxin. Berg *et al.* (1987) concluded that 90% of the toxin released from decomposing bloom material degraded over the following 26-d period. In addition, Kenefick *et al.* (1993) determined the half-life of MCLR from laboratory incubations of copper sulfate-treated bloom samples to be 3 d from the time of maximum toxin release (approximately 2 to 3 d post-treatment) and suggested that a 99% reduction would take

approximately three weeks. Similarly, Jones and Orr (1994) determined the *in situ* degradation of MCLR following algicide treatment of a *M. aeruginosa* bloom on Lake Centenary, Australia. Degradation was bi-phasic, consisting of an initial rapid phase (3 d) during which 90 to 95% of the extracellular toxin decomposed, followed by a second slower phase. In contrast, Kiviranta *et al.* (1991) concluded that hepatotoxin biodegradation in cultures enriched with water from the Vantaanjoki River, Finland was low, with toxin persisting for 2 to 3 months. However, Lam *et al.* (1995a) who incubated purified MCLR with heterotrophic microbes from wastewater treatment plant effluent, observed significant reductions in toxin concentration and toxicity, estimating the half-life of purified MCLR to range between 0.2 to 3.6 d. As well, they suggested that loss of toxicity results from biotransformation of the toxin, citing modifications to the heptapeptide ring and Adda, responsible (Lam *et al.*, 1995a).

More recently, Bourne *et al.* (1996) described the biotransformation dynamics of MCLR by bacterium *Sphingomonas* sp. and proposed at least three enzymes be involved. In the first step, enzyme 1, a metalloprotease (metal-activated protease), catalyzes the hydrolytic cleavage of the Adda-Arg peptide bond. This causes opening of the cyclic ring to yield acyclo-MCLR (NH_2-Adda-D-Glu-Mdha-D-Ala-L-Leu-D-MeAsp-L-Arg-OH), which produces a concomitant 160-fold reduction in toxicity from the parent compound. Next, enzyme 2, a serine protease, catalyzes the cleavage of the Ala-Leu peptide bond to yield a non-toxic tetrapeptide (NH_2-Adda-D-Glu-Mdha-D-Ala-OH). In the last step, enzyme 3, another metalloprotease, catalyzes the cleavage of other bonds yet to be described, yielding undetected peptide fragments and amino acids (Bourne *et al.*, 1996). Thus, biodegradation (biotransformation) is regarded as an important detoxification route for MCYSTs following collapse of severe cyanobacterial blooms, however, variation with respect to microbial abundance and speciation must be considered.

The photolytic effects of light on MCYSTs have been the focus of recent investigations. Foremost, Tsuji *et al.* (1994) reported that MCLR and its non-toxic geometric isomer, [6(*Z*)-Adda^5] MCLR, were stable to fluorescent light and experienced only slight isomerization (*i. e.*, to [6(*Z*)-Adda^5] MCLR and MCLR, respectively) under natural sunlight throughout a 26-d period. However, the presence of photosynthetic pigments (particularly water-soluble phycocyanins) significantly accelerated

isomerization and decomposition of MCLR and [6(*Z*)-Adda^5] MCLR under natural sunlight, but not fluorescent light. Hence, they concluded that while isomerization and decomposition rates of MCYSTs are dependent on both pigment type and concentration, decomposition might predominate at higher levels (Tsuji *et al.*, 1994). Considering that the lowest pigment concentrations used in these studies far exceed natural levels typical of hypereutrophic lakes in Alberta, Canada, Kotak (1995) suggested that photolytic degradation of MCYSTs would take several months – an unacceptably lengthy period for drinking water supplies.

Welker and Steinberg (1999) suggested that humic substances (HS) might also act as photosensitizers, which in the presence of sunlight, mediate the photolytic degradation of MCYSTs. Through the absorption of UV-visible light, HS form highly reactive molecules (*i. e.*, hydroxy-radicals or hydrogen peroxide) that can indirectly lead to transformations of otherwise photochemically inert compounds (Zepp *et al.*, 1985; Cooper *et al.*, 1989). Indeed, Welker and Steinberg (1999) demonstrated indirect photolytic degradation of several MCYSTs (45, 47 and 56% degradation of MCLR, MCYR and MCRR, respectively) in the presence of HS and natural sunlight following an 8-h period and experimentally estimated the half-life of MCRR to be about 10.5 h. Since HS are present in most natural waters in concentrations of several mg L^{-1}, the authors concluded such a process could contribute to the elimination of MCYSTs from natural environments.

In addition, Tsuji *et al.* (1995), who examined the effects of UV light on MCYST stability, determined the half-life of MCLR at 147 $\mu W/cm^2$ to be 10 min with complete decomposition at 2550 $\mu W/cm^2$ occurring after 10 min. They reported significant decomposition, which occurred at wavelengths near the absorption maxima of the toxins, dependent on UV irradiation levels and that lower intensities be accompanied by random isomerization of the Adda moiety (*i. e.*, formation of [6(*Z*)-Adda^5] MCLR, [4(*Z*)-Adda^5] MCLR and an unknown compound). Kaya and Sano (1998) subsequently characterized the unknown to be tricyclo-Adda [(2*S*,3*S*,1'*R*,3'*S*,4'*S*,5'*R*,6'*R*,7'*R*)-3-amino-5-(4',6'-dimethyl-3'-methoxytricyclo[5.4.0.0$^{1',5'}$]undeca-8',10'-dien-6'-yl)-2-methyl-4(*E*)-pentenoic acid]-containing MCLR, which is formed by [2 + 2] addition between the benzene ring and the C-6, C-7 double bond of the Adda moiety. Furthermore, the formation of

[tricyclo-Adda^5] MCLR from MCLR was reversible and decomposed under UV light, suggesting that the photolytic degradation of MCLR by UV irradiation proceeds via [tricyclo-Adda^5] MCLR (Kaya and Sano, 1998). Since non-toxic products are formed, it has been concluded that water treatment involving UV irradiation might be possible for removing MCYSTs from raw water (Tsuji *et al.*, 1995). Consequently, several groups have developed methods involving UV irradiation in the presence of titanium dioxide (TiO_2) catalyst to degrade and remove MCYSTs from drinking water supplies (see Robertson *et al.*, 1998; Shephard *et al.*, 1998).

As previously mentioned, MCYSTs are heat stable and as Wannemacher (1989) noted, autoclaving pure MCLR in distilled water destroyed less than 50% of the toxin. Microcystins are also stable in acidic and basic environments. For example, Berg and Soli (1985) reported no reduction in toxicity of MCLR at pH 2 or 12 and Harada *et al.* (1996) found at water temperatures typical of natural environments (*i. e.*, 5 and 20°C), limited decomposition occurred at pH 1 or 9. However, increased temperatures (*i. e.*, 40°C) in conjunction with acidic (pH 1) or basic (pH 9) conditions lead to toxin degradation and yielded half-life estimates of 3 and 10 weeks, respectively (Harada *et al.*, 1996). Since water of bloom-prone lakes usually range between 20 to 30°C and pH 8 to 10, thermal degradation aided by pH may be considered subsidiary to other detoxification pathways.

Lastly, early reports by Rapala *et al.* (1994) suggest that MCYSTs be adsorbed by natural sediments (13 to 24 μg toxin mL^{-1} sediment). Preliminary research by Harada and Tsuji (1998) ascertained that MCYSTs, particularly hydrophilic variants (e. g., MCRR), are strongly adsorbed on sediment. Recently, Morris *et al.* (2000) suggested the novel Adda moiety make MCYSTs susceptible to scavenging by fine-grained particles such as suspended clay minerals of the smectite group. Indeed, their results showed that more than 81% of MCLR is removed from solution by clay mineral. Considering clay minerals can remain in suspension for long periods and may protect otherwise labile adsorbed organic compounds, serious implications exist with respect to the bio-/geochemical fate (*i. e.*, toxin transport, degradation and food-web availability) of MCYSTs in natural environments (Morris *et al.*, 2000).

6. Microcystin Production and Variability

As mentioned above (section 4.2), several genera of cyanobacteria produce MCYSTs, however it is well established that the production of specific analogues is not restricted to particular species or strains. Furthermore, some produce various analogues simultaneously, while others are incapable of producing toxins, which raises the question, how and why do some (but not all) species and strains of cyanobacteria produce toxin?

6.1 Genetic basis of toxin production – Microcystins are regarded as secondary metabolites, that is, those compounds produced by organisms that are not to be used for its primary metabolism, growth or cell division, yet may assume particular and definite endo- or exogenous functions (Kleinkauf and von Döhren, 1990; Carmichael, 1992). It is widely known that bacteria and fungi produce numerous peptides and lipopeptides that act as antibiotics, enzyme inhibitors, immunosuppressants, plant and animal toxins (Marahiel *et al.*, 1993; Stachelhaus *et al.*, 1995). Many of these are synthesized ribosomally via transcription/translation with subsequent post-translational modification. However, linear, branched and cyclic peptides often containing D-, hydroxy- and/or otherwise extensively modified (acylated, glycosylated, *N*-methylated etc.) amino acids, are produced by a non-ribosomal, thio-template mechanism. This proposed mechanism (summarized in Figure 5), which functions in the absence of RNA message or protein synthesis, utilizes large, modular, multifunctional enzyme complexes known as peptide synthetases to activate, modify and condense amino acids into peptides (reviewed by Laland and Zimmer, 1973; Kleinkauf and von Döhren, 1990; Arment and Carmichael, 1996). Peptide synthetases possess various multiple domains composed of distinct modules responsible for catalyzing the activation of specific amino acids (as acyladenylates) and subsequent thio-ester bond formation of the aminoacyl groups (Stachelhaus *et al.*, 1995). Additional modules may then induce extensive modifications to the resulting carboxy thio-esters, such as epimerization (racemization) into D-amino acids or *N*-methylation, prior to being transferred to a thiol group on a 4'-phosphopantetheine cofactor (Marahiel, 1992). Peptide elongation is accomplished between adjacent amino acids, via repeated trans-peptidation and trans-thiolation reactions of the enzyme bound cofactor (Marahiel, 1992). Hence, the specific organization of functional domains and constituent modules within peptide synthetases,

which act as independent enzymes, form the peptide-template that determines the structure and hence the activity of the peptide product (Stachelhaus and Marahiel, 1995; Kleinkauf and von Döhren, 1996).

Recently, Arment and Carmichael (1996) proposed that several commonalties between MCYSTs and known thio-template products of eubacteria and fungi suggests that MCYSTs may also be produced by a non-ribosomal, thio-template mechanism. These characteristics include their cyclic nature, small size (up to $\approx$ 50 amino acids), presence of D- and highly substituted or unusual amino acids (*i. e.*, Adda) and production of multiple analogues within one culture. Considering the non-ribosomal nature of this mechanism, they hypothesized that thio-template systems, if present in cyanobacteria, will be unaffected by protein synthesis or translation inhibitors (*i. e.*, chloramphenicol and actinomycin D, respectively) that completely arrest transcription/translation. Indeed, they not only detected MCYST, but observed a threefold increase in toxin concentration per cell in exponentially growing cultures of *Microcystis* strain PCC7820 treated with chloramphenicol compared with untreated control cultures (0.32 pg $\pm$ 0.033 and 0.11 pg $\pm$ 0.012 MCYST cell^{-1}, respectively).

Additional evidence, in support of the thio-template mechanism first characterized in the eubacteria *Bacillus*, comes from Dittmann *et al.* (1996) and Meißner *et al.* (1996), who recently identified homologous peptide synthetase genes from several strains of *M. aeruginosa*. Not only did these genes maintain the characteristic modular structure, but also highly conserved sequences encoding peptide synthetase units prevalent in eubacteria and fungi. While toxic and non-toxic strains both possessed the conserved motifs A and B (both part of the adenylate-forming domain), Meißner *et al.* (1996) concluded that they differ "in their content of genes for specific peptide synthetases". For example, regions of the *mapep1* nucleotide sequence encoding the Mapep1 peptide synthetase exclusive to toxin-producing strains showed homology to motif Q, part of the domain responsible for epimerizing L- into D-amino acids (Dittmann *et al.*, 1996). Further, Dittmann *et al.* (1997) partially characterized two genes, *mcyA* and *mcyB*, that form a cluster incorporating the *mapep1* sequence and demonstrated that insertional inactivation of the *mcyB* gene from MCYST-producing *M. aeruginosa* strain PCC7806 results in transformation to a non-toxic state. Subsequently, Neilan *et al.* (1999) has

shown MCYST synthetase gene orthologues present in all MCYST-producing strains of *Microcystis*, *Anabaena*, *Oscillatoria*, and *Nostoc*. In addition, the NDLN-producing cyanobacteria, *Nodularia*, also possesses the MCYST synthetase gene orthologue responsible for toxin production. Since the strains used in their study originated from distinct geographic regions, Neilan *et al.* (1999) suggests this may imply a high degree of gene conservation or a cosmopolitan distribution of hepatotoxic strains.

The non-ribosomal, thio-template system provides a mechanism for the production of myriad secondary metabolites, including hepatotoxic MCYSTs and NDLNs by genetically diverse cyanobacteria. While several hypotheses have been suggested, including both cellular (*i. e.*, cell signaling, light regulation or iron transport) and ecological (*i. e.*, anti-herbivore or allelopathic compounds) functions, the precise role of MCYSTs in organisms producing them remains speculative (Demott *et al.*, 1991; Lukač and Aegerter, 1993; Sheen, 1993; Arment and Carmichael, 1996).

6.2 Factors regulating toxin production – Numerous laboratory studies attempted to elucidate factors related to MCYST production. Foremost, growth phase (culture age) of cyanobacteria populations or cultures has been linked to toxicity as toxin concentrations, per cell, is reported to peak during late exponential phase (van der Westhuizen and Eloff, 1985). Furthermore, van der Westhuizen *et al.* (1988) suggested that changes in toxicity are due to fluctuations in toxin concentrations and hence the relative composition of MCYST analogues, but not to structural alterations of the constituent peptide toxins. More recently, investigations by Arment and Carmichael (1996) concluded that MCYST is produced continuously in *Microcystis* strain PCC7820 and that concentrations peak during late exponential to stationary growth phase (0.53 pg $\pm$ 0.055 MCYST cell^{-1}) due to toxin accumulation and not increased synthesis.

Water temperature and light intensity are also well studied and although results appear to be species/strain specific, they have been linked to toxicity. For instance, van der Westhuizen and Eloff (1985) concluded that cultures of *M. aeruginosa* strain UV-006 grown at pH 9.5, yielded maximum toxin content at water temperatures of 20 to 24°C and a fluence rate of 145 µmol photons m^{-2} s^{-1}. Similarly, Gorham (1964b) found toxicity optima for *M. aeruginosa* strain NRC-1 at 25°C, however toxin content did not change with varying light intensity. Sivonen (1990) also reported differing temperature optima

for two strains of *Oscillatoria agardhii* (*i. e.*, 25°C for strain 97 and 15 to 25°C for CYA 128), yet both strains possessed similar low light intensity optima (*i. e.*, $\approx$ 12 $\mu E\ m^{-2}\ s^{-1}$). Sivonen (1990) concluded that conflicting results might be due to differences in light sources (spectrum), species/strain behavior (recall from Table 4, *O. agardhii* is a metalimnetic species and thus prefers lower light intensities), culture media and/or toxin detection methods utilized in the respective studies.

Recent investigations by Utkilen and Gjølme (1992), however, demonstrated only minor effects of light quality on toxicity, thus discounting the role of light spectrum as previously suggested. Nevertheless, with respect to light intensity they revealed a 2.5-fold increase in toxicity (expressed as the ratio of MCYST to biomass) when raised from 20 to 40 $\mu E\ m^{-2}\ s^{-1}$, while further elevations caused decreases in toxicity. As well, the ratio of peptide toxin to total cellular protein also increased with intensity (up to 40 $\mu E\ m^{-2}\ s^{-1}$), but in contrast to toxicity as expressed above, was unaffected by further elevations. Since the ratio of peptide toxin to total protein remains constant beyond 40 $\mu E\ m^{-2}\ s^{-1}$, they suggested that *M. aeruginosa* should reach maximal toxin content at the surface and should decrease with depth as light is attenuated (Utkilen and Gjølme, 1992). More importantly, they noted that decreased toxicity at high intensities ($>$ 40 $\mu E\ m^{-2}\ s^{-1}$), when expressed as the ratio of MCYST to cyanobacterial biomass, results from accumulation of photosynthesis-derived polysaccharides effectively increasing biomass, a fact often ignored and thought responsible for early incongruous studies. Subsequently, Utkilen and Gjølme (1995) suggested that toxin content to protein content rather than dry weight or LD_{50}, be used when expressing changes in toxin production, as use of either the latter two, be misleading.

Several studies considered the influence of nutrients, primarily nitrogen and phosphorus, on the toxin content of cyanobacteria. Codd and Poon (1989) found that omitting the nitrogen source from growth media reduced the toxin content of *M. aeruginosa* in culture by a factor of 10 compared to reference cultures. As well, Sivonen (1990) reported a direct positive relationship between nitrate concentration and production of [D-Asp^3] MCRR in *O. agardhii* strain 97 and CYA 128. Subsequently, they suggested this effect on toxin production by non-heterocystous (non-nitrogen-fixing) species, such as *M. aeruginosa* and *O. agardhii*, "be explained logically by the peptide

nature of the toxins" (Sivonen, 1990). In contrast, Rapala *et al.* (1997) found that two heterocystous strains of *Anabaena* (90 and 202A1) grown in nitrogen-free media, contained greater MCYST concentrations than if cultured in nitrogen-rich conditions. Similarly, Rapala *et al.* (1993) detected greater production of the neurotoxin, anatoxin-a, by nitrogen-fixing strains of *Anabaena* and *Aphanizomenon* grown in the absence of nitrogen as opposed to cells in the presence of nitrogen. Hence it appears that heterocystous and non-heterocystous cyanobacteria differ in their responses to fluctuations in ambient nitrogen (Rapala *et al.*, 1997).

Unlike nitrogen, studies have linked phosphorus concentrations with toxicity of both heterocystous and non-heterocystous species. For example, Sivonen (1990) determined that orthophosphate additions (up to 0.4 mg L^{-1}) increased the production of [D-Asp^3] MCRR in non-heterocystous *O. agardhii* strain 97 and CYA 128, while Rapala *et al.* (1997) reported similar findings with heterocystous *Anabaena* strains. Furthermore, nitrogen-fixing *Nodularia spumigena* isolated from the Baltic Sea, produced more NDLN when cultured with increasing orthophosphate ($\geq$ 0.2 mg L^{-1}) concentrations (Lehtimäki *et al.*, 1997). In contrast, Utkilen and Gjølme (1995) found that intracellular carbohydrate content increased under nitrate- and phosphate-limited conditions and by subtracting carbohydrate content from the dry weight revealed that the ratio of toxin to corrected dry weight remained unchanged. Thus, similar to the conflicting results with respect to light intensity (above), they suggested that increasing cyanobacterial biomass resulting from the accumulation of photosynthesis-derived storage polymers caused decreases in toxicity. As such, they concluded nitrate and phosphate have no influence on toxin content of *M. aeruginosa* and reiterate toxin to protein content rather than dry weight be used when expressing these changes (Utkilen and Gjølme, 1995).

Lastly, since non-ribosomal, cyclic peptide antibiotics, produced by several bacteria are regulated by heavy metals, Lukač and Aegerter (1993) suggested that these metals also influence MCYST production. Of the metals tested, only zinc and iron were shown to significantly affect toxin yield whilst aluminum, cadmium, chromium, copper, manganese, nickel and tin did not. Indeed, both zinc and iron were found to be essential for growth of batch cultures of *M. aeruginosa* strain PCC 7806 and while zinc was positively related to toxin production, iron was negatively related, producing 20 to 40%

more toxin (dry weight basis) at low iron concentrations ($\leq$ 2.5 μM). However, Utkilen and Gjølme (1995) found a decrease in toxicity of *M. aeruginosa* strain CYA 228/1 in continuous culture, when iron was reduced from 10 to 0.3 μM. Lyck *et al.* (1996), who also found increases in toxicity (measured as the ratios of toxin to Chl-*a* and toxin to protein) with iron depletion, attributed these conflicting results to differing methods (*i. e.*, batch versus continuous culture) of the aforementioned investigators.

Other findings of significance include a 5-fold decrease in Chl-*a* and a 3-fold decrease in protein (other than MCYST) content of both toxic and non-toxic strains of *M. aeruginosa* under iron-limiting conditions (Lyck *et al.*, 1996). It has been previously shown that decreases in cellular pigmentation known as chlorosis or bleaching, occurs during iron-limitation (Grossman *et al.*, 1994). As well, the observation by Lyck *et al.* (1996) that the toxic strain maintained a greater tolerance to iron-depletion corresponds with the findings of Utkilen and Gjølme (1995) that MCYST-producing *M. aeruginosa* possess more efficient iron uptake systems than non-toxic strains. Whereas the importance of iron to cyanobacterial growth (*i. e.*, photosynthesis) seems obvious, the relationship between iron and MCYST content remains only speculative. For instance, Utkilen and Gjølme (1995) suggested MCYST be an intracellular chelator, "which inactivates free cellular Fe^{2+} and that MCYST is produced by an enzyme (synthetase) whose activity is controlled by the amount of free Fe^{2+} present".

6.3 Toxin production within natural environments – Given conflicting results and considering that the saturating conditions for most variables used in prior laboratory studies rarely occur in natural settings, Kotak (1995) recognized that extrapolation from these experiments to natural environments would be both difficult and invalid. Alternatively, he proposed field studies correlating environmental variables with toxin concentration in samples of mixed phytoplankton assemblages are necessary.

Earlier, Wicks and Thiel (1990) examined the concentration of several MCYST analogues in phytoplankton collected from Hartbeespoort Dam, South Africa over a 2.5-yr period. Solar radiation, biomass-normalized primary production, oxygen saturation, temperature and pH of lake water, were positively correlated with MCYST concentrations, while Chl-*a* and orthophosphate concentrations were weakly negatively correlated. It is well established that MCYST concentrations in natural phytoplankton

samples are principally influenced by phytoplankton species composition (discussed below, section 6.4). Thus, it is difficult to distinguish between factors that govern actual MCYST production (or loss) within toxin-producing species from those that affect toxin production through the regulation of species abundance or biomass (*i. e.*, autocorrelation). Consequently, in order to compare correlations between MCYST concentration and environmental variables with those of toxin concentrations and species composition (abundance or biomass), Kotak (1995) recommended expressing toxin concentration per unit biomass of toxin-producing species (e. g., μg toxin g^{-1} *M. aeruginosa*). Since the parameters measured by Wicks and Thiel (1990) involved small sample size (n = 16 to 20) and expressed toxin concentration per unit phytoplankton biomass (e. g., μg toxin g^{-1} phytoplankton) and not per unit biomass of toxin-producing species, their results must be interpreted with caution. Moreover, because Hartbeespoort Dam does become ice-covered during winter months (water temperatures did not fall below 15 °C) and nutrient concentrations were in excess of phytoplankton requirements, the applicability of their results to temperate lakes remains questionable (Kotak, 1995).

Recent studies by Kotak *et al.* (1995, 2000) investigated the influence of environmental factors on both the biomass of *M. aeruginosa* (the principal producer of MCLR) and the concentration of toxin within cells from several eutrophic and hypereutrophic temperate lakes in central Alberta, Canada. Of the factors examined, total phosphorus (TP) was the strongest correlate ($P < 0.005$) with both *M. aeruginosa* biomass and cellular MCLR (expressed as μg MCLR g^{-1} *M. aeruginosa*). Likewise, Chl-*a*, Secchi depth and pH were also significantly correlated with both, indicating that these variables may affect *M. aeruginosa* biomass as well as toxin production per unit of *M. aeruginosa*. In contrast, turbidity was only correlated with *M. aeruginosa* biomass, while color and conductivity were exclusively associated with toxin concentration. Thus, it appears certain factors likely act directly on *M. aeruginosa* growth (and hence the biomass produced), while others on toxin production.

In addition, non-linear, negative relationships existed between forms of inorganic nitrogen (NO_3^-+NO_2^- and NH_4^+) and either *M. aeruginosa* biomass or MCLR concentration. This was evident as maximum toxin concentrations occurred during periods when sources of inorganic nitrogen were at their lowest seasonal values (Kotak *et*

al., 2000). Also, dramatic reductions in MCLR concentration within phytoplankton were noted when ratios of total nitrogen to phosphorus (TN:TP) were greater than 5:1. Correspondingly, a univariate regression model of TN:TP explained the most variation ($r^2 = 0.70$, $P < 0.02$, df = 1, 36) in MCLR concentration (ng cellular toxin L^{-1}) in mixed phytoplankton communities (Kotak *et al.*, 2000). Though *M. aeruginosa* biomass and MCLR concentration within phytoplankton were strongly, negatively related to TN:TP, toxin concentration per unit *M. aeruginosa* was not. Hence, they ascertained that the impact of TN:TP ratio on toxin concentration within phytoplankton is manifested primarily through the affect of TN:TP on *M. aeruginosa* biomass (and not on MCLR within *M. aeruginosa*). Indeed, these findings have recently been corroborated by a laboratory study utilizing *M. aeruginosa* strain PCC7820, which indicated that decreasing the N:P of growth media not only increased growth, but also the volumetric MCLR concentration (*i. e.*, ng toxin L^{-1}) as a consequence (Kotak, van Tegham and Prepas, unpublished data). Yet, MCLR concentration per unit biomass of *M. aeruginosa*, decreased with decreasing N:P ratio. Thus, Kotak *et al.* (2000) concluded that toxin dynamics in natural phytoplankton assemblages (of their study lakes) is primarily related to changes in the concentration and ratio of phosphorus and nitrogen.

6.4 Natural toxin variability – Microcystin concentrations within natural phytoplankton communities may be regarded as a balance between biomass of the (toxin) producer and cellular production of the toxin and "that conditions which may be optimal for growth may not be optimal for toxin production" (Kotak *et al.*, 2000). Coincidentally, several researchers have noted that within individual waterbodies, substantial temporal and spatial variability in MCYST concentration might occur. Early on, Carmichael and Gorham (1981) reported that toxin concentration based on mouse toxicity tests, varied daily throughout the growing season (July – September) as well as from one year to the next within Hastings Lake, Alberta. Others have subsequently demonstrated, based on HPLC analysis, similar variability in MCYST concentrations on both seasonal and annual scales (e. g., Wicks and Thiel, 1990; Henriksen and Moestrup, 1997; Park *et al.*, 1998).

Carmichael and Gorham (1981) also reported spatial variation in bloom toxicity, as toxin concentrations differed between sampling stations, some no more than 10 m apart, on a given day. They attributed this "mosaic structure or pattern" of toxic blooms

to temporal and spatial differences not only in species composition, but also in relative proportions of toxic and non-toxic cyanobacterial strains within the same species. Lindholm and Meriluoto (1991) reported seasonal changes in the vertical profile of a demethylated-MCRR analogue to correspond with the vertical positioning of *O. agardhii* in Lake Östra Kyrksundet, southwest Finland. Toxin (20 to 40 μg cellular toxin L^{-1}) was detected at depths of 5 to 9 m, the recurrent depth maxima for this metalimnetic bloom-forming species (Table 4).

Kotak *et al.* (1995) studied the temporal and spatial patterns of MCLR occurrence in three hypereutrophic central Alberta lakes – Coal, Driedmeat and Little Beaver. Seasonal (within-year) and annual (between-year) MCLR concentrations (expressed as either: μg (g total phytoplankton biomass)$^{-1}$, μg (g *M. aeruginosa* biomass)$^{-1}$ or ng L^{-1} lake water) were highly variable for each lake and correlated with the abundance (cells mL^{-1}) and biomass (μm^3 mL^{-1}) of *M. aeruginosa*. Microcystin-LR concentrations also varied over a 24-hr period in Driedmeat Lake. Diel concentrations decreased from an average of 2376 ± 72 μg g^{-1} *M. aeruginosa* biomass, between 1200 to 1800 hrs on 9 September, to a low of 387 ± 101 at 0300 hrs on 10 September, then increased to 1544 ± 5 by 0900 hrs (Kotak *et al.*, 1995). While the underlying mechanism of diel variation remains unclear, Kotak *et al.* (1995) proposed MCLR concentration may be coupled to photoperiod and is perhaps, utilized within or lost from the cell over night. Lastly, substantial spatial variation was found along the lengths of Coal (45 sites) and Little Beaver (29 sites) lakes. In both cases, the concentration of MCLR (ng L^{-1} lake water) was highly correlated with the density and biovolume of *M. aeruginosa* expressed as a percentage of total cyanobacteria density and biovolume, respectively (Kotak *et al.*, 1995).

These studies demonstrate the dynamic properties of MCYSTs within natural aquatic environments. While biomass of toxin-producing species partially explicate changes in MCYST concentrations within phytoplankton assemblages, it is clear that environmental factors that influence toxin production, as well as the occurrence of non-toxic strains, account for significant residual variation (Kotak *et al.*, 2000). Accordingly, toxin variability may be ascribed to four major factors: 1) phytoplankton dynamics (*i. e.*, the relative abundance or biomass of toxin-producing species), 2) variable presence of

distinct toxin- and non-toxin-producing cyanobacterial strains, 3) physiological condition of toxin-producing cells/colonies and 4) effects of environmental variables on toxin production (Carmichael and Gorham, 1981; Kotak, 1995).

7. Toxicology and Biological Effects of Hepatotoxins

In 1878, George Francis published his observations of sickness and mortality in animals (sheep, horses, pigs, cattle and dogs) that consumed water from the Murray River estuary, Lake Alexandria, Australia, laden with the cyanobacteria *Nodularia spumigena*, the principal producer of NDLNs. More than fifty years passed before the earliest toxicological studies linking cyanobacteria to animal (and human) intoxications were published. One such study by Ashworth and Mason in 1946, described the pathological changes in rats administered lethal and sublethal (≈ 0.01 mL g^{-1}, intraperitoneal injection) doses of unknown substance extracted from *M. aeruginosa* (summarized in Table 16). However, another forty years passed before the isolation and chemical characterization of the hepatotoxins were achieved. Consequently, numerous contemporary studies have elucidated the mechanisms by which hepatotoxins exert toxicity to animals.

7.1 Toxin uptake and hepatotropism – The ability of MCYSTs to produce severe deformations such as plasma membrane bleb formation and loss of microvilli, was first demonstrated *in vitro* with isolated rat hepatocytes by Runnegar *et al.* (1981). Moreover, they noted concordance between MCYSTs and phalloidin, a bicyclic peptide hepatotoxin produced by the fungus *Amanita phalloides* that is known to be transported into hepatocytes via the bile acid transport system. Shortly after, *in vivo* studies involving [^{125}I]-labelled MCYST, confirmed the organ specificity (organotropism) of the toxin, as radioactivity (up to 62%) concentrated primarily in the liver (and to a lesser extent in the kidneys) of both mice (Runnegar *et al.*, 1986) and rats (Falconer *et al.*, 1986). Cell selective cytotoxicity was shown by Eriksson *et al.* (1987). Microcystin induced morphological alterations to isolated hepatocytes but not to human erythrocytes or mouse fibroblasts suggesting that some mechanism other than a non-specific plasma membrane interaction be responsible for MCYST uptake. Accordingly, several lines of evidence implicate the multispecific bile acid transport system with the uptake of toxins. For instance, Dahlem *et al.* (1989) ascertained that rats experienced greater hepatotoxicity

(measured as changes in liver mass) when purified MCLR was infused into the isolated ileal region of the intestine as opposed to the isolated jejunal region. These findings are not surprising since specific carriers for bile acids are present in the former, while bile acids are only passively absorbed in the latter (Dahlem *et al.*, 1989). More importantly, Eriksson *et al.* (1990) found the uptake of radiolabelled MCLR derivative, [^{3}H]-dihydromicrocystin-LR, specific to hepatocytes, which are known to possess active bile acid carriers, yet was negligible in several cell lines devoid of such transport systems (*i. e.*, human hepatocarcinoma and neuroblastoma and mouse fibroblast cell lines). Furthermore, they demonstrated that hepatocellular uptake of MCLR could be inhibited in the presence of the bile acid transport inhibitors, antamanide, sulfobromophthalein and rifampicin or following incubation with the bile salts cholate and taurocholate. Lastly, while they recognized that some amphiphatic cyclic peptides are capable of penetrating cell membranes (e. g., cyclosporin, valinomycin and gramicidin) it was shown that the membrane penetrating capacity (surface activity) of MCLR was low. This indicated that MCYST requires an active uptake mechanism, namely the bile acid transport system (Eriksson *et al.*, 1990).

Subsequent investigations involving isolated purfused rat livers have not only illustrated the hepatotropism of MCYSTs, but the subcellular localization of the toxin as well. Hooser *et al.* (1991a) found that the liver took up 13.1% of the total radioactivity, following a 45-min purfusion with tritiated dihydromicrocystin-LR ([^{3}H]-2HMCLR). Of this, the majority (77.8%) was associated with the cytosolic fraction, while less was measured in the nuclear/plasma membrane or microsomal fractions (0.7 and 15.8%, respectively). When the cytosolic fraction was treated with trichloroacetic acid, majority of the radiolabel remained in the insoluble precipitate (e. g., insoluble actin and related elements) suggesting that MCYST binds to cytosolic proteins. Similarly, Robinson *et al.* (1991) who dosed mice with [^{3}H]-MCLR via sublethal (i.v.) injection, found $\approx$ 67% of the radiolabel in the liver by 60 min., which remained throughout the 6-d study. Again, analysis of hepatic cytosol revealed that $\approx$ 83% was covalently bound to cytosolic components within 1 hr post injection and decreased to $\approx$ 42% by day 6. In addition to liver-associated radiolabel, $\approx$ 24% of total administered radiolabel was excreted within the 6 d ($\approx$ 9% and 15% in the urine and feces respectively). However, unlike the hepatic-

cytosolic radiolabel, these fractions did not appear to be protein bound as 60% remained as parent (untransformed) compound (Robinson *et al.*, 1991).

7.2 Mode of toxicity – A study by Elleman *et al.* (1978), one of the first to utilize purified MCYSTs, not only corroborated the earlier pathological findings of Ashworth and Mason (1946), but also those of Gorham (1960) involving an unidentified cyclic peptide toxin described then, as the "fast-death factor". Concurrent with research regarding MCYST uptake and hepatotropism comes an observation by Runnegar *et al.* (1987) that phosphorylase-*a* is activated in hepatocytes following treatment with MCYST. This was the first indication that these hepatotoxins exterted toxicity via the inhibition of protein phosphatases. Consequently, MCYSTs and NDLNs belong to a family of serine/threonine specific protein phosphatase inhibitors that include among others, the marine dinoflagellate toxins okadaic acid and dinophysistoxin-1, the causative agents of diarrhetic shellfish poisoning (Cohen *et al.*, 1990; MacKintosh *et al.*, 1990; Honkanen *et al.*, 1994).

Within normal eukaryotic cells, the post-translational modification of intracellular proteins, attributed to conformational changes resulting from reversible phosphorylation of serine, threonine or tyrosine residues, is recognized as a primary mechanism in controlling numerous cellular events (Cohen, 1989). Protein kinases are the enzymes responsible for catalyzing the addition of γ-phosphoryle from adenosine triphosphate to the hydroxyl group (*i. e.*, phosphorylation) of serine, threonine or tyrosine containing proteins (Toivola, 1998). In contrast, protein phosphatases function in opposition to kinases and effectively catalyze the removal (hydrolysis) of phosphate (*i. e.*, dephosphorylation) from either phospho-serine/threonine or tyrosine residues (reviewed by Cohen, 1989; Jia, 1997). A critical balance between the phosphorylation/dephosphorylation activities of protein kinases and phosphatases (respectively) on regulatory proteins, thus, provides the basis for intracellular signal transduction pathways (Lambert *et al.*, 1994). In this concerted manner, diverse biological processes such as those triggered by extracellular effectors (*i. e.*, hormones, mitogens, cytokines and neurotransmitters), are regulated and include carbohydrate and lipid metabolism, gene expression, protein synthesis, cell division, proliferation and differentiation, membrane transport and secretion, neuro-transmission, contractility and

locomotion, (Cohen, 1989; Shenolikar, 1994; Jia, 1997). Consequently, reversible protein phosphorylation is regarded as "probably the most crucial chemical reaction taking place in living organisms" (Jia, 1997). By inhibiting protein phosphatases then, MCYSTs and related compounds disrupt the balance with protein kinases, producing a hyperphosphorylated state of the cytosolic and cytoskeletal proteins (Toivola *et al.*, 1994).

7.3 Mechanisms of protein phosphatase inhibition by hepatotoxins – Elucidation of the NMR solution structures of MCYSTs and NDLNs (Bagu *et al.*, 1995, 1997; Annila *et al.*, 1996), yield insights as to the molecular basis of hepatotoxin/protein phosphatase interactions. As discussed above (section 5.1), these peptide hepatotoxins possess rigid cyclic backbones with similar positioning of the D-MeAsp/D-Glu carboxyl groups and the novel hydrophobic Adda moiety. The essential nature of the D-Glu and Adda residues is well established in the literature, as specific modifications to either results in loss of toxicity (Tables 10 to 12). Hence, many have proposed these two residues responsible for recognizing and inhibiting the catalytic subunits of PP-1c/PP-2Ac (Bagu *et al.*, 1995; Taylor and Quinn, 1996). Indeed, x-ray crystallographic studies of the MCLR/PP-1c complex indicate that the highly flexible hydrophobic Adda moiety initiates binding and inhibition by adjusting to and interacting with hydrophobic residues, which exist as a cleft on the surface of PP-1c (Goldberg *et al.*, 1995). Additional interactions (*i. e.*, salt-bridges or hydrogen bonds) required for inhibition, occur between the D-Glu carboxyl group of MCLR and Arg-96 and Arg-221 of PP-1c, as well as the D-MeAsp carboxyl group and Arg-96 and Tyr-134 (Goldberg *et al.*, 1995).

Following rapid (within minutes) binding/inactivation of the phosphatase catalytic subunit, formation of a time-dependent (within hours), non-dissociable covalent interaction between the Mdha residue of MCLR and the cysteine-273 residue of PP-1c (alternatively the Cys-266 residue of PP-2Ac) occurs (MacKintosh *et al.*, 1995; Craig *et al.*, 1996). Specifically, this bond results from a Michael addition reaction between the electrophilic α,β-unsaturated carbonyl of Mdha (Michael acceptor) and the nucleophilic thiol groups (Michael donor) of the aforementioned Cys residues (Runnegar *et al.*, 1995). Consequently, site directed mutagenesis of Cys-273 of PP-1c to Ala leads to a loss in the ability to form a covalent MCYST/PP-1c adduct (Runnegar *et al.*, 1995).

Nodularin and motuporin (NDLN-V) also bind/inhibit PP-1c and PP-2Ac. However, the displacement (7.13 Å) of the Mdhb residue of NDLNs due to the reduced upward curvature of the saddle-shaped backbone, increases the distance between its β-carbon and the thiol group of Cys-273 of PP-1c (or Cys-266 of PP-2Ac) by more than 10 Å (see Bagu *et al.*, 1997). As a result, NDLNs do not form covalent linkages with these enzymes (Bagu *et al.*, 1997; Annila *et al.*, 1996). These findings indicate that the formation of a covalent bond is not essential for PP-1c/PP-2Ac inhibition and further explains why NDLNs are carcinogenic while MCYSTs are not (Bagu *et al.*, 1997).

7.4 Toxic effects – At the cellular level, the predominant effect of MCYSTs is the alteration of cytoskeletal components – namely the microfilaments (MFs), intermediate filaments (IFs) and microtubules (MTs). Normally, these filamentous protein components and their associated proteins function in maintaining cell shape, support and movement, cell-to-cell adhesion and cell division (reviewed by Malhotra and Shnitka, 1996). Microfilamentous actin is the predominant protein of the cytoskeleton, forming a mesh-like cortex near the surface of the plasma membrane as well as comprising the structure of cellular microvilli (Alberts *et al.*, 1989). Following treatment with MCYST, hepatocytes experience dose- and time-dependent reorganizations of their actin MFs, resulting in the concentration (aggregation) of MFs away from the plasma membrane (Eriksson *et al.*, 1989a; Hooser *et al.*, 1991b). Consequently, plasma membrane bleb formation, invagination and loss of microvilli occurs (Eriksson *et al.*, 1989a; Hooser *et al.*, 1990, 1991b). It is noteworthy that these changes are distinct (*i. e.*, no change in actin polymerization state) from those of MF-modifying drugs cytochalasin B or phalloidin, which act to prevent either polymerization or depolymerization of actin filaments, respectively (Eriksson *et al.*, 1989a; Hooser *et al.*, 1991b).

Intermediate filaments, which function primarily in structural roles and link cells together, may also be "cytoskeleton-associated targets for microcystin-LR-induced phosphorylation" (Toivola *et al.*, 1997). For instance, the hyperphosphorylation of cytokeratins 8 and 18 leads to the redistribution of cytokeratin IFs in MCYST treated cells (Falconer and Yeung, 1992; Ohta *et al.*, 1992). In addition, hepatocytes have been observed to lose cell-to-cell adhesion following treatment with MCLR (Miura *et al.*, 1989; Hooser *et al.*, 1991b). Subsequently, this may be attributed to the reorganization of

IFs (tonofilaments) comprising desmosome junctions between cells and ultimately results from hyperphosphorylation of desmoplakin I/II, the protein comprising tonofilaments (Miura *et al.*, 1989; Toivola *et al.*, 1997). It must be stressed that the effects of MCYSTs on the cytoskeleton are not exclusive to individual components, as MFs, IFs and MTs remain closely associated (Toivola *et al.*, 1997). To this end, Khan *et al.* (1996) recently described the condensation and collapse of IFs and MTs around the nucleus, followed immediately by the MFs aggregating toward the center as well as the periphery of the cell. This response was observed in both hepatocytes and non-hepatocytes, though sequential differences among cell types were noted (Khan *et al.*, 1996). Lastly, additional ultrastructural changes included dose-dependent vesiculation of the rough endoplasmic reticulum with total or partial ribosomal loss, hydropic mitochondria and intracytoplasmic vacuolization (Dabholkar and Carmichael, 1987; Berg *et al.*, 1988; Miura *et al.*, 1989).

Considering the preferential uptake of MCYSTs by hepatocytes (section 7.1 above) and the subsequent cellular alterations, it is not surprising that the main systemic effects predominantly involve the liver. Following MCYST induced redistribution of cytoskeletal components and concomitant separation of hepatocytes, breakdown of the sinusoidal endothelium and development of hepatic lesions, ensue. Consequently, disruption of the hepatic architecture (*i. e.*, loss of central veins) and widespread hepatocellular necrosis allows blood (up to 50% original systemic blood volume) to leak into the widened interstitial spaces causing severe intrahepatic haemorrhage within the centrilobular region (Theiss *et al.*, 1988; Hooser *et al.*, 1990). This results in significant liver enlargement (*i. e.*, increase in liver mass) and decreased systemic arterial blood pressure, which eventually leads to death via hypovolaemic shock (Dabholkar and Carmichael, 1987; Theiss *et al.*, 1988). In addition, *in vivo* and *in vitro* studies involving MCYSTs and NDLNs have observed other related changes consistent with hepatic degeneration and insufficiency. For example, plasma levels of liver enzymes, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT) and sorbitol dehydrogenase (SDH), bilirubin and lactic acid increased (Runnegar *et al.*, 1988; Theiss *et al.*, 1988; Miura *et al.*, 1989, 1991). Reductions in hepatic glycogen and glutatione, as well as blood glucose levels,

have also been observed (Runnegar *et al.*, 1987; Miura *et al.*, 1991). While the primary effects of MCYST intoxication involve liver damage and insufficiency, experiments have shown similar, but less severe effects within the gastrointestinal tract and kidneys. Gastrointestinal effects typically include enterocyte deformation, gastroenteritis and haemorrhage and may be a direct consequence of toxin uptake via intestinal epithelia (Galey *et al.*, 1987; Dahlem *et al.*, 1989; Falconer *et al.*, 1992a). Kidney effects consist of lesions and cell damage in both the glomeruli and collection tubules with attendant increases in blood urea nitrogen and creatinine levels (Meriluoto *et al.*, 1989; Hooser *et al.*, 1990). However, it remains unclear whether these responses are due directly to MCYST intoxication or are secondary effects due to the collection of hepatocyte debris within renal capillaries and tubules (Meriluoto *et al.*, 1989; Khan *et al.*, 1996).

Research regarding the involvement of cytoskeletal components in maintaining cell shape and function (e. g., cell adhesion, signal transduction across the plasma membrane and cell division), has led to the observation that cytoskeletal disorganization accompanies the transformation of a normal cell into a tumoral cell (Decloitre *et al.*, 1990). Subsequently, numerous chemical agents capable of altering cytoskeletal components, thus causing the promotion of tumors, have been identified (Schliwa *et al.*, 1984; Decloitre *et al.*, 1990). "Tumor promoters are molecules that are not carcinogenic themselves but that are able to increase the incidence of tumors in tissues pretreated with a carcinogenic agent" (Schliwa *et al.*, 1984). For example, the macrocyclic diterpene ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces cytoskeletal alterations in cultured cells, including the redistribution of actin and the cell adhesion plaque protein, vinculin (Schliwa *et al.*, 1984). Phenobarbital (PB) and biliverdin (BV) are two additional tumor-promoting chemicals known to act specifically on the MFs and MTs within hepatic epithelial cells (Decloitre *et al.*, 1990). Considering MCYSTs and NDLNs also cause cytoskeletal alterations within hepatocytes, many have suggested similar tumor-promoting characteristics (Falconer and Buckley, 1989; Matsushima *et al.*, 1990; Yoshizawa *et al.*, 1990). Indeed, tumor-promoting compounds produced by the marine cyanobacterium, *Lyngbya majuscula*, have been identified previously (Fujiki *et al.*, 1985). Consequently, MCYSTs have been shown to be powerful tumor-promoters. For instance, MCYSTs have been demonstrated to promote skin tumor development

following initiation with topical dimethylbenzanthracene (Falconer and Buckley, 1989) as well as, liver tumor development (measured as number and % area of glutathione *S*-transferase placental form-positive foci) following initiation with diethylnitrosamine (DEN) (Nishiwaki-Matsushima *et al.*, 1992). Similar tumor-promoting capabilities have been demonstrated for NDLNs (Ohta *et al.*, 1994). Unlike MCYST however, which only stimulated glutathione *S*-transferase placental form-positive foci in rat liver following initiation with DEN, NDLN alone induced glutathione *S*-transferase placental form-positive foci (Ohta *et al.*, 1994). Hence, in addition to its tumor-promoting properties, NDLN may be regarded a carcinogen as well.

7.5 Routes of hepatotoxin exposure – The primary route of hepatotoxin exposure to animals is through the ingestion of toxin-producing cyanobacteria as a consequence of consuming water from lakes and reservoirs experiencing cyanobacterial blooms. Circumstances in such environments often deteriorate further as light winds cause buoyant cyanobacteria to accumulate leeward along shorelines. While most animals attempt to avoid cyanobacteria-infested waters due to the foul odors emitted from such blooms, those with no alternative sources have few options but to consume contaminated water. Consequently, numerous accounts regarding the often fatal hepatic intoxications of domestic livestock, pets and wildlife during periods of intense cyanobacterial surface accumulations have been documented (reviewed by Schwimmer and Schwimmer, 1968; Beasley *et al.*, 1989; Kotak, 1995). As well, following the maturation and collapse of severe blooms, significant concentrations of hepatotoxin may be released into surrounding water (discussed above, section 5.3). This dissolved, aqueous fraction is regarded, as a direct source of pure toxin that could potentially be ingested by organisms that otherwise would not consume cyanobacteria. Although a large percentage of this fraction degrades within the first two weeks, lower levels may persist over a month or more following the cessation of bloom events (discussed above). Thus, a disappearance of toxin-producing cyanobacteria is not necessarily indicative of non-toxic conditions.

Humans too, may become susceptible to hepatotoxins, both through daily drinking water requirement and accidental recreational intake. Considering humans undoubtedly avoid consuming bloom material, the accidental intake of water during recreational aquatic activities (e. g., swimming, canoeing and water-skiing) is principally

recognized as the avenue for the direct ingestion of toxin containing cyanobacteria cells. Albeit rare, reports of such incidents exist, for example Dillenberg and Dehnel (1960) published the detailed account of a physician who accidentally swallowed cyanobacterial scum while swimming. Symptoms initially included stomach pains and nausea ≈3 h following the incident, progressing to fever, muscle and joint pain within the limbs and painful diarrhoea. Furthermore, *Microcystis* and *Anabaena* were present in the faeces. Since then similar incidents have been documented and are reviewed by Codd *et al.* (1999a).

Far more likely, is the ingestion of drinking water contaminated with toxic cells or dissolved MCYST as a result of insufficient or ineffective drinking water treatment practices. This is especially true for smaller municipalities or rural communities that, at best, utilize traditional treatment processes involving flocculation (with ferric chloride or aluminum sulphate), sedimentation, sand filtration and chlorination. Reportedly, such methods can remove up to 30% of the initial MCYST concentration (Hoffman, 1976; Himberg *et al.*, 1989). However, the use of some chemicals, such as ferric chloride (Himberg *et al.*, 1989) and potassium permanganate (Lam *et al.*, 1995b), may actually increase MCYST concentrations in drinking water by causing intact cyanobacterial cells to lyse (Table 15).

Laboratory and pilot studies demonstrated that the inclusion of powdered or granular activated carbon filtration (Falconer *et al.*, 1983a) along with traditional treatment practices, could remove MCYSTs from drinking water. Unfortunately, subsequent research by Jones *et al.* (1993) found that high doses of powdered activated carbon (up to 100 mg L^{-1}) and lengthy contact times (up to 120 min) were required to reduce MCYST concentrations below target levels (< 1 μg L^{-1}). Hence, they deemed this practice cost prohibitive and unreasonable for typical large-scale operations. Furthermore, Donati *et al.* (1994) found that competition by natural organic compounds in raw water could reduce the adsorption efficiency of powdered activated carbon by 52%, while Craig and Bailey (1995) demonstrated only limited effectiveness of granular activated carbon as constant use exhausted carbon in a relatively short period of time.

Chlorination has also been shown to be generally ineffective in removing hepatotoxins from drinking water (Keijola *et al.*, 1988; Himberg *et al.*, 1989). However,

Nicholson *et al.* (1994) determined that the form of chlorine compound and pH of water greatly influence its ability to destroy MCYSTs. In their study, aqueous chlorine (1 to 2 mg L^{-1}) and calcium hypochlorite (2.5 to 16 mg L^{-1}) removed 90% of MCLR in 30 min, while sodium hypochlorite (15 to 30 mg L^{-1}) removed 50% in 30 min. Monochloramine (20 mg L^{-1}) was ineffective at removing toxin even after 5 d (Nicholson *et al.* 1994). Considering: (1) that the high pH common to waters contaminated with cyanobacteria would cause difficulty maintaining chlorine residual and (2) that the high concentrations of organic compounds would compete with MCYST to form chloramine (a weak oxidant), Kotak (1995) suggested that little MCYST would be destroyed.

In contrast, researchers have shown those treatment processes incorporating ozonation (Keijola *et al.*, 1988) and particularly ozonation combined with activated carbon filtration (Himberg *et al.*, 1989) most effective in removing cyanobacterial toxins from water. Early findings are supported by the work of Rositano *et al.* (1998) that recently demonstrated the rapid destruction of pure hepatotoxins by ozone (166 mg L^{-1} MCLR oxidized below detection limit with less than 0.2 mg L^{-1} of ozone in 4 min). However, in the presence of organic material (*i. e.*, cyanobacterial cell contents) they noted that increased dose rates of ozone were required to oxidize any toxin present. This is likely due to competing reactions between the toxins and organic material for reaction with ozone. Nevertheless, 1.0 mg L^{-1} ozone almost completely removed 220 mg L^{-1} MCYST from a cyanobacterial extract in 5 min (Rositano *et al.*, 1998). Also, the effectiveness of ozone was pH dependent as MCYST oxidation was reduced at higher pH values (> 7.5) reflecting the lower oxidation potential of ozone under alkaline (1.24V) versus acidic conditions (2.07V). Consequently, they concluded that ozone or ozone /hydrogen peroxide, are the only oxidants capable of destroying a range of cyanobacterial toxins (e. g., hepatotoxins and neurotoxins) and are thus preferred methods for disinfecting drinking water (Rositano *et al.*, 1998).

Regardless of the advances in water treatment processes, reports of human illness associated with exposure to cyanobacteria cells/toxins via drinking water have been documented in many countries worldwide and are most often attributable to ignorance or accident. Given that most cyanobacterial toxins were only identified over the past twenty years, many of these earlier cases can only be regarded as suspected cyanobacterial-

related incidents as they rely predominantly on anecdotal and epidemiological evidence (Codd *et al.*, 1999a). One such case of suspected cyanobacterial poisoning occurred at Palm Island, Queensland, Australia in November 1979. Over 130 children and 10 adults suffered from hepatoenteritis over a 21-d period. Symptoms included lethargy, vomiting, diarrhoea, severe abdominal pain, and injury to the liver, kidneys, lungs, adrenals and intestine (Byth, 1980). Upon further investigation, it was learned that one month prior to the outbreak a dense bloom of cyanobacteria occurred in the Solomon Dam, which served as the drinking water supply reservoir. Due to complaints of taste and odour in the treated drinking water, the bloom was subsequently treated with copper sulphate (Bourke *et al.*, 1983). Shortly thereafter, the first reports of illness appeared. Copper sulphate has been suspected as a possible cause of the illnesses (Prociv, 1987). Nevertheless, the confirmed toxicity of *Cylindrospermopsis raciborskii* isolates from Soloman Dam (Hawkins *et al.*, 1985) and the identification of cylindrospermopsin toxin from *C. raciborskii* (Ohtani *et al.*, 1992), implicates this cyanobacteria (and its toxin) in the incident (Codd *et al.*, 1999a).

Human illness attributable to *M. aeruginosa* exposure via drinking water was first documented in Salisbury, Rhodesia from 1960-1965 (Zilberg, 1966). During this period, gastroenteritis was reported in a population whose drinking water came from a lake experiencing blooms of *M. aeruginosa*. Since then, epidemiological studies have linked *M. aeruginosa* and its toxins to several incidents of gastroenteritis and liver damage. For example, Falconer *et al.* (1983b) examined the results of routine assays for hepatic enzymes indicative of toxic liver damage from residents of Armidale, New South Wales, Australia hospitalized prior, during and following *M. aeruginosa* blooms in the Malpas Dam reservoir. They discovered a statistically significant correlation between elevated hepatic γ-glutamyltransferase (GGT) activity from plasma of patients who consumed water from the Malpas Dam reservoir containing blooms of *M. aeruginosa*. In contrast, those who did not consume water from this source during bloom events and others from an adjacent population with alternate drinking water sources did not experience elevated GGT activities. Hence, they concluded that cyanobacteria caused the observed elevations in liver enzymes as a result of liver damage (Falconer *et al.*, 1983b).

Similarly, MCYSTs have been epidemiologically linked to an increased frequency of primary liver cancer (PLC) in several regions in China (Yu, 1989; Carmichael, 1994; Ueno *et al.*, 1996). Indeed, high levels of MCYSTs have been detected in the ponds/ditches and rivers, but not deep wells, which serve as drinking water sources in regions with high incidences of PLC (Ueno *et al.*, 1996). While it is generally acknowledged that MCYSTs lack carcinogenic (tumor-initiating) properties, they have been shown to promote liver tumor development (section 7.4 above). Consequently, researchers hypothesized the widespread dietary intake of aflatoxin B_1, a hepatocarcinogenic mycotoxin often contaminating foodstuffs, combined with the intake of MCYSTs in drinking water, could account for significant correlations between PLC rates and the presence of *Microcystis* in the drinking water (Ueno *et al.*, 1996; Codd *et al.*, 1999a).

The consumption of shellfish (e. g., clams and mussels) and other aquatic organisms (e. g., fish) that may accumulate hepatotoxin by feeding directly on toxin-producing cyanobacteria represents a dietary route of toxin exposure. This is often the case in marine environments where shellfish (primarily bivalves) have been implicated in reports of human and animal intoxication involving paralytic (saxitoxins and gonyautoxins), neurotoxic (brevetoxins), amnesic (domoic acid) and diarrhetic (okadaic acid and dinophysistoxins) shellfish toxins (Baden, 1983; Yasumoto *et al.*, 1985; Bates *et al.*, 1989). Freshwater clams and mussels can indeed accumulate cyanobacterial toxins as demonstrated by Eriksson *et al.* (1989b) who reared freshwater mussels (*Anodonta cygnea*) in laboratory cultures of *O. agardhii*. However, considering that freshwater bivalves are rarely harvested for human consumption, this route of exposure would likely be of significance to wildlife only. Nonetheless, estuarine bivalves (e. g., blue mussels, *Mytilus edulis*) are harvested for human consumption and have been shown to accumulate NDLN during blooms of *Nodularia spumigena* (Falconer *et al.*, 1992b). By coincidence, illness and death following the consumption of eel, pike and burbot (especially burbot liver) by residents and animals (fish, seabirds, foxes and cats) at the Koenigsberg Haff in East Prussia between 1920 and 1940, implicates hepatotoxins produced by blooms of *Oscillatoria* spp. (Berlin, 1948). In residents, the disease occurred only after eating fish

and symptoms included severe muscle and abdominal pain, vomiting and discolored urine.

Recent studies indicate that humans and animals could potentially be exposed to cyanobacterial toxins via the consumption of crop plants irrigated with water containing toxin-producing cyanobacteria. Abe *et al.* (1996) demonstrated that MCLR applied topically to bean (*Phaseolus vulgaris*) leaf surfaces may enter the plants, causing the inhibition of whole leaf photosynthesis. Moreover, Codd *et al.* (1999b) demonstrated that lettuce (*Lactuca sativa*) spray-irrigated with water containing toxic *M. aeruginosa* not only harbored viable cyanobacteria on leaf surfaces, but contained MCYST at varying concentrations throughout the plant. Animals associated with aquatic environments may also risk exposure to hepatotoxins through the consumption of aquatic macrophytes. Investigations by Pflugmacher *et al.* (1998) demonstrated that two submergent macrophytes, *Ceratophyllum demersum* and *Elodea canadensis*, and the bryophyte, *Vesicularia dubyana*, uptake pure MCLR from the surrounding environment. *Vesicularia dubyana* attained the greatest uptake of toxin followed by *C. demersum*. These apparent differences between uptake rates were subsequently, attributed to the surface area of the leaves. As well, the leaves and shoots were more effective at toxin uptake compared to roots and stems (Pflugmacher *et al.*, 1998).

It has also been suggested that humans and animals could be exposed to hepatotoxins through the consumption of dairy products. Indeed antibiotics used in dairy cattle healthcare can result in the presence of these compounds and/or their residues in milk (Schenk and Callery, 1998). Given the similar size and cyclic nature of both antibiotics and hepatotoxins, there exists the possibility that MCYSTs and NDLNs could be secreted in milk of cattle that previously consumed water from a toxin-containing reservoir (Codd *et al.*, 1999a). However, to my knowledge no such cases exist in the literature.

The increasingly popular consumption of cyanobacteria as a dietary (health food) supplement remains a major concern, as few government regulations regarding the mass-culture (in open systems) or harvest of cyanobacteria from natural environments are in place. Thus, failure to test harvested or mass-cultured species or strains for toxin production and toxicity by both short- and long-term bioassays may potentially expose

consumers to various levels of cyanobacterial toxins. Considering the recommended daily dose (1 to 2 g d^{-1}) of such supplements, Kotak (1995) suggested that the amount of MCYST ingested could conceivably exceed the Tolerable Daily Intake (TDI) of 13.3 ng kg bodyweight^{-1} d^{-1} proposed by Kuiper-Goodman *et al.* (1994). Consequently, the continued use of dietary supplements constitutes a potential avenue for the long-term, chronic exposure of humans to hepatotoxins.

Other sources of concern particularly to humans include skin contact (dermal exposure) and inhalation during recreational water activities. Numerous cases, primarily concerning fishermen and swimmers, of irritation and allergic reactions following dermal contact with cyanobacteria, have been reported over the past 30 years (Codd *et al.*, 1999a). However, most instances occurred in marine coastal waters and subsequently have been attributed to highly inflammatory agents, lyngbyatoxin and aplysiatoxin, produced by blooms of *Lyngbya majuscula* (Cardellina *et al.*, 1979; Moore, 1996). Nevertheless, isolated episodes have been documented in freshwater environments during blooms and surface accumulations of *Oscillatoria*, *Microcystis*, *Nodularia*, *Aphanizomenon*, *Anabaena* and *Gloeotrichia*. In one instance, 10 young soldiers suffered blistering around the mouth following aquatic training exercises in Rudyard Lake, England, during a bloom of MCYST-containing *M. aeruginosa* (Turner *et al.*, 1990). In the other case, 11 sea cadets reported facial rashes, asthmatic symptoms and dry sporadic cough with vomiting, following similar aquatic training exercises at Hollingworth Lake, England, which contained hepatotoxic *O. agardhii* (Codd *et al.*, 1999a). As well, mild irritation was also experimentally induced on the skin of guinea pigs treated topically with 500 μg kg^{-1} MCLR dissolved in dimethyl sulfoxide (90% DMSO), however, considering MCYSTs are slowly absorbed through skin, mortality seems unlikely (Wannemacher *et al.*, 1988).

In contrast, reports of respiratory problems as a result of inhalation are relatively rare, although Codd *et al.* (1999a) noted that in both cases of human intoxication above, it is likely that accidental ingestion and inhalation were involved to some extent. Only two laboratory studies appear to have attempted to quantify the intranasal exposure of animals to MCYSTs. First, Creasia (1990) concluded that nasal uptake of MCLR by mice was indeed possible and determined the LD_{50} by this route of exposure to be 18 μg L^{-1} of air.

More recently, Fitzgeorge *et al.* (1994) ascertained the sensitivity of mice to MCLR (LD_{50} values) to be ≈ 10 times greater than oral dose exposures and noted that in addition to liver lesions, extensive necrosis of olfactory and respiratory epithelium was evident. This implies that if the natural formation of aerosols containing dissolved microcystins were to occur (for instance, on lakeshores during windy periods), "the concentrations are not likely to cause adverse health affects" (Kotak, 1995).

Finally, haemodialysis treatment for kidney patients has recently been identified as a special concern. This follows a fatal incident that occurred at a haemodialysis clinic in Caruaru, Brazil in 1996, in which inadequately treated water from the Tabocas Reservoir was used to treat dialysis patients (Dunn, 1996). Out of 124 patients treated at the center, 101 developed some signs of acute hepatotoxicity (Jochimsen *et al.*, 1998). As a result, sixty deaths, either directly related to hepatoxicity or indirectly from gastrointestinal complications or cardiovascular problems, were reported (Pouria *et al.*, 1998). Histological examination of liver tissue obtained at autopsy from 16 patients, revealed cell deformity, extensive necrosis, apoptosis, severe cholestasis, cytoplasmic vacuolization, mixed-leukocyte infiltration, occasional multinucleated hepatocytes, intracellular edema, mitochondrial changes and injuries to the rough and smooth endoplasmic reticula, lipid vacuoles, and residual bodies (Jochimsen *et al.*, 1998). Subsequently, MCLR was identified in clinic water filter material and in the serum available from one victim, while at least three MCYST variants have been detected in the liver tissue of several others (Carmichael, 1998; Pouria *et al.*, 1998).

8. Aquatic Food Web Implications of Hepatotoxins

It is evident that the majority of research has traditionally, focused on the acute toxicity of microcystins to laboratory mammals such as mice and rats (discussed in sections 7.4 and 7.5 above). Reports concerning the toxicity of cyanobacteria and related hepatotoxins to livestock, pets and wildlife are numerous (summarized in Table 17). Consequently, several have studied the toxic effects of MCYSTs and NDLNs to livestock such as chicken (Konst *et al.*, 1965, Jackson *et al.*, 1986 and Falconer *et al.*, 1992a), rabbits (Konst *et al.*, 1965, Stewart *et al.*, 1950 and Rojas *et al.*, 1990), sheep (Konst *et al.*, 1965 and Jackson *et al.*, 1984), pigs (Falconer *et al.*, 1994) and cattle (Konst *et al.*,

1965). With the exception of zooplankton, acute and chronic toxicity studies involving fish and other aquatic organisms that typically inhabit bloom-prone lakes and reservoirs, remain recent endeavors. Finally, few have focused on the fate of MCYSTs and NDLNs with respect to accumulation patterns within aquatic food webs.

8.1 Phytoplankton and Macrophytes – Little is known regarding the allelopathic effects of MCYSTs and NDLNs on phytoplanktonic species that often coexist with toxin-producing cyanobacteria. *Oscillatoria* isolates have been shown to reduce growth of other cyanophytes (e. g., *Anacystis*, *Phormidium* and *Plectonema*), as well as green algae (e. g., *Chlorella*), in laboratory cultures (Bagchi *et al.*, 1990). However, the compound(s) responsible for the observed effects were not determined. Ikawa *et al.* (1994, 1996) observed inhibition of *Chlorella pyrenoidosa* growth in the presence of fatty acid-containing fractions isolated from *Aph. flos-aquae* and *M. aeruginosa*. In both cases, linoleic and linolenic acids were sited, at least, partly responsible for any allelopathic effects of cyanophytes on true algae. Nevertheless, Christoffersen (1996a) indicated that MCLR inhibited the reproduction of the prasinophyte, *Nephroselmis olivacea* (Stein). While short-term (first 4 days) reductions in growth were evident, differences between treatment and control cultures disappeared by day 10 and were subsequently attributed to toxin degradation. Kirpenko (1986) demonstrated an allelopathic effect of cyanobacterial toxins towards macrophytes. Toxin concentrations of 0.04 to 200 μg L^{-1} inhibited growth of *Elodea* and *Lemna*. After 7 days, chlorophyll in the leaves disappeared and the roots fell off. Beyond 15 days, all plants in the high concentration treatment group died.

8.2 Protozoa – Toxicity of cyanobacteria to protozoans was demonstrated early on by Ransom *et al.* (1978). In their study, lyophilized cells of the cyanobacteria, *Fischerella epiphytica* (Ghose) and *Gloeotrichia echinulata* (Richter) and sonified cells of *Nostoc linckia* (Bornet and Thuret) caused death or reduced activity in the ciliate, *Paramecium caudatum* (Ehrenberg). Reductions in numbers and *in situ* growth rates of natural populations of heterotrophic nanoflagellates have been observed following blooms of toxic *Microcystis* in a eutrophic Danish lake (Christoffersen, 1996b). Subsequent laboratory experiments with isolated mixed flagellate populations demonstrated a 24 to 28% and a 36 to 41% reduction in growth rates at 1 and 10 μg MCYST L^{-1}, respectively. In contrast, similar experiments with cultures of *Heteromita*

globosa (Dujardin) and *Spumela* sp. showed little reduction in growth rate, suggesting that species-specific differences may exist (Christoffersen, 1996b). Ward and Codd (1999) tested the toxicity of four MCYST variants to the protozoan, *Tetrahymena pyriformis*. All four variants inhibited population growth rate and maximum culture density. As well, respiration rates of *T. pyriformis* were also inhibited in both a time- and dose-dependent manner. However, the magnitude of these reductions were directly correlated to the estimated octanol/water partition coefficients (2.16, 2.92, 3.46 and 3.56, MCLR, MCLY, MCLW and MCLF respectively) of the toxin variants studied. Consequently, greater toxicity was observed with increasing hydrophobicity (*i. e.*, higher octanol/water partition coefficient), such that the 24-h LC_{50} values followed the order MCLR > MCLY > MCLW ≈ MCLF (Ward and Codd, 1999).

8.3 Zooplankton – The history of research regarding the effects of cyanobacteria and their related toxins to zooplankton, especially large-bodied cladocerans (e. g., *Daphnia* spp.), is extensive and somewhat contradictory. Some studies have observed declines in biomass and changes in species composition of zooplankton communities during cyanobacterial bloom events. This has been attributed to several factors including, manageability of large colonial forms (Burns, 1968; Gliwicz, 1977; Gliwicz and Siedlar, 1980; Fulton, 1988a), indigestibility (Porter, 1973), poor nutritional quality (Porter and Orcutt, 1980) and/or toxicity (Arnold, 1971; Lampert, 1981, 1982; Nizan *et al.*, 1986). In contrast, others have reported successful population growth on diets of cyanobacteria. For instance, De Bernardi *et al.* (1981) who fed small colonies of *M. aeruginosa* to three species of *Daphnia* (*D. obtusa*, *D. hyalina* and *D. cucullata*) concluded this cyanobacteria could be efficiently utilized as a food source. However, the presence of toxic strains and colony size was recognized paramount in determining the extent utilization of *M. aeruginosa* by cladocerans. Similarly, Benndorf and Henning (1989) reported the greatest biomass of *Daphnia galeata* in Bautzen Reservoir (Germany) occurred during periods of maximum *Microcystis* development.

Stangenberg (1968) was first to report on the possible toxic effects of cyanobacteria to zooplankton. In laboratory feeding studies, *Daphnia longispina* (Müller) exposed to fresh *M. aeruginosa* displayed no toxic effects. However, prior freezing and thawing of the *M. aeruginosa* cells caused mortality to both *D. longispina*

and *Eucypris virens* (Jurine). Since MCYSTs are endotoxins, ingestion and some degree of digestion and assimilation must occur in order for toxin-producing cyanobacteria to elicit a toxic response. While Stangenberg (1968) suggested that fresh cyanobacteria be non-toxic to zooplankton, a more plausible explanation (assuming ingestion occurred) might be that *D. longispina* was incapable of digesting *M. aeruginosa.* In addition, freezing and thawing *M. aeruginosa* compromises cell membrane integrity, resulting in the release of dissolved intracellular compounds to the surrounding environment. Thus, exposure to previously frozen *M. aeruginosa* would increase the potential for ingestion of pure toxin from both the aqueous environment and that contained in thawed cyanobacterial cells. This might explain the mortality observed in *D. longispina* and *E. virens* exposed to *M. aeruginosa* that was previously frozen.

In general, large-bodied cladocerans seem more sensitive to hepatotoxins than smaller species. Out of 13 species examined, Lampert (1982) demonstrated greater reductions in the filter-feeding rate of large *Daphnia* spp. compared to smaller *Ceriodaphnia reticulata* and *Bosmina longirostris.* Likewise, Fulton (1988b) found *B. longirostris* able to consume a toxic strain of *M. aeruginosa* and concluded that, although cyanobacteria offer little nutrition, this cladoceran remains resistant to cyanobacterial toxins. Furthermore, as *B. longirostris* showed no evidence of avoiding the consumption of *M. aeruginosa* by selective feeding or depressing filter-feeding rates, the mechanism of toxin resistance must function post-ingestion via reduced toxin assimilation or some manner of detoxification (Fulton, 1988b). Unlike daphnids, copepods appear to feed size-selectively on filamentous and colonial cyanobacteria and able to discriminate against toxic strains (DeMott and Moxter, 1991). However, acute toxicity experiments with purified MCLR (DeMott *et al.*, 1991), indicate the copepod, *Diaptomus birgei*, to be more sensitive (48-h LC_{50}, 0.45 to 1.00 μg mL^{-1}) than either *Daphnia pulicaria, D. hyalina* or *D. pulex* (48-h LC_{50}, 21.4, 11.6 and 9.6 μg mL^{-1}, respectively). Subsequent studies show *Diaptomus birgei, D. pulicaria* and *D. pulex* not only differ in their levels of PP1 and PP2A activities, but also in the respective sensitivities of these enzymes to inhibition by MCLR (DeMott and Dhawale, 1995). Furthermore, DeMott and Dhawale (1995) suggested that the ability to synthesize additional protein phosphatase enzymes in

the presence of hepatotoxins, likely influence the potencies of these toxins to zooplankton.

Conflicting results have been reported with microzooplankton as well. For example, the rotifer *Brachionus calyciflorus*, which is known to consume individual *M. aeruginosa* cells, was shown to be susceptible to MCYSTs directly through ingestion of toxin containing cells and indirectly via passive uptake of dissolved toxin from the surrounding environment (Starkweather and Kellar, 1987). Similarly, a toxic strain of *M. aeruginosa* reduced ingestion rates in *Brachionus rubens* and increased mortality compared to non-fed controls (Rothhaupt, 1991). However, Fulton and Paerl (1987) showed that *Br. calyciflorus* was not only unaffected by these toxins, but displayed the ability to grow and reproduce on a diet of *M. aeruginosa*.

Regarding the toxicity of cyanobacteria to zooplankton, it is suggested that numerous factors have contributed to the obvious disparity among the literature. Paramount to this discussion are findings of several authors that a general lack of correlation exists between the toxin content of natural populations or isolated strains of *Microcystis* and their respective toxicity to various zooplankters (Nizan *et al.*, 1986; Henning *et al.*, 1991; Jungmann, 1992; Jungmann and Benndorf, 1994). To this end, several have suggested other metabolites rather than MCYSTs be responsible for toxicity of cyanobacteria to zooplankton (Jungmann, 1992; Jungmann and Benndorf, 1994). Jungmann (1992) found an aqueous MCYST-free fraction partially purified from *M. aeruginosa* strain PCC7806 to contain the compound responsible for *Daphnia* toxicity. Likewise, Reinikainen *et al.* (1995) isolated a fraction from *Oscillatoria* (*Planktothrix*) *agardhii* that was non-toxic to mice, yet toxic to *Artemia salina* and *D. pulex*. However, DeMott and Dhawale (1995) cautioned against this use of purified toxins and cells extracts in quantifying relative toxicities of ingested compounds to zooplankton, as these must be absorbed through the carapace, whereas naturally ingested toxin, via intact *Microcystis* cells, is absorbed through the gut.

Incongruous conclusions may also emanate from strain-specific differences between the cyanobacteria used in previous studies. Typically, experiments have compared the toxicity of *Microcystis* strains that differ in MCYST content. However, other strain-specific properties may impart variation in the results obtained in comparable

investigations. For instance, strain-specific differences in *Microcystis* ingestibility by zooplankton could influence the overall ingestion rate of cells/colonies and thus, the resulting dose of MCYST (Rohrlack *et al.*, 1999a). Indeed, some strains are able to directly alter the ingestion rate of zooplankters, whereas others cannot (Lampert, 1981, 1982; Fulton and Paerl, 1987; Henning *et al.*, 1991). As well, *Microcystis* strains differ in their content of toxic oligopeptides (Weckesser *et al.*, 1996), which could exacerbate or alleviate any toxic effects attributable to MCYSTs (Rohrlack *et al.*, 1999a). Recent studies have addressed several of these problems. By comparing the effects of MCYST-producing (wild-type) *Microcystis aeruginosa* strain (mcy^+ PCC7806) and its non-MCYST-producing (mutant) variant (mcy^- PCC7806) to *D. galeata*, Rohrlack *et al.* (1999a) demonstrated that MCYSTs are the most likely cause of the daphnid poisoning observed when wild-type mcy^+ PCC7806 is fed to the animals. Additionally, since both mcy^+ PCC7806 and mcy^- PCC7806 were able to reduce ingestion rate in *D. galeata*, they concluded MCYSTs "are not responsible for inhibition of the ingestion process" (Rohrlack *et al.*, 1999a). In subsequent investigations early suppositions were extended. Consequently, it was concluded that MCYSTs assume a decisive role in daphnid toxicity by *M. aeruginosa*, while some unidentified factor, which affects the frequency and food transport efficiency of the maxillules, be responsible for the inhibitory effect of *Microcystis* on daphnid ingestion rate (Rohrlack *et al.*, 1999b, 1999c).

Clonal differences in the sensitivity of various zooplankters to toxic cyanobacteria have also been demonstrated and may influence the clonal composition of *Daphnia* populations. This was recently illustrated in a chronic lifetable experiment, in which three clones of *D. pulex* and two of *D. longispina* were exposed to toxic *M. aeruginosa* for 21 days (Hietala *et al.*, 1995). As a result of nutritional deficiency and toxic effects, they found exposure to *Microcystis* increased mortality, delayed maturation and decreased both growth and offspring production. Furthermore, variations in life history responses between species and among clones were observed (Hietala *et al.*, 1995). Similar findings were subsequently found when comparing survivorship of ten *D. pulex* clones during acute exposure to toxic *M. aeruginosa* (Hietala *et al.*, 1997).

Environmental factors can also influence the toxicity of *M. aeruginosa* and related toxins to zooplankton. Increasing water temperature has been shown to cause dramatic

increases in the acute toxicity of *Microcystis* to *Daphnia* (Hietala *et al.*, 1997). Similarly, temperature increased the sensitivity of *D. pulex* to neurotoxic *Anabaena affinis*, as well as purified anatoxin-a (Claska and Gilbert, 1998). Since the effect of temperature does not depend on encounters with cyanobacteria (*i. e.*, increase the extent of mechanical interference with ingestion), it was concluded that increasing temperature likely modifies the response of *Daphnia* to ingested or assimilated toxin. This is not surprising considering "temperature has a major impact on zooplankton physiology and ecology by changing metabolic rate and activity level" (Claska and Gilbert, 1998).

It is apparent that MCYSTs impact living zooplankton and that individual response likely depends on complex interactions between numerous behavioral, physiological and environmental factors. Consequently, toxic cyanobacteria are being regarded as the modifying agent of zooplankton communities at both the species and clonal levels (Hietala *et al.*, 1995). Nevertheless, a reciprocal and equally abstruse relationship in which zooplankton populations influence and otherwise alter cyanobacterial (and algal) community species composition must not be ignored. On the one hand, indiscriminate grazers capable of ingesting cyanobacteria and/or those resistant to toxic effects of MCYSTs may prevent the formation of cyanobacterial blooms (Matveev *et al.*, 1994). Indeed, some have hypothesized (Ganf, 1983; Gliwicz, 1990a) and demonstrated (Christoffersen *et al.*, 1993) large-bodied cladocerans capable of preventing cyanobacteria from forming large ungrazable colonies by feeding on smaller antecedent colonies. Yet others have noted various zooplankters able to graze on relatively large (up to 1 mm in length) colonies and filaments (Ganf, 1983; DeMott and Moxter, 1991; Kobayashi, 1993). On the other hand however, the ability of zooplankton to control cyanobacteria density has limits possibly defined by a critical colony concentration beyond which, zooplankton growth rate and reproduction becomes impaired (Gliwicz, 1990b). Benndorf and Henning (1989) suggested that patchy distribution of *Microcystis* strains in conjunction with selective grazing by zooplankton might reduce the biomass of non-toxic strains, thus increasing the proportion and biomass of toxic strains. Thus, zooplankton can assume primary roles in both the suppression and progression of often toxic cyanobacteria blooms. Furthermore, it has recently been shown that zooplankton such as *Daphnia* spp. accumulate MCYSTs (Kotak *et al.*, 1996a;

Thostrup and Christoffersen, 1999) and in this regard act as potential vectors for the transfer of cyanobacterial toxins to higher trophic levels in aquatic food webs.

8.4 Macroinvertebrates – Relatively few studies have focussed on the accumulation and toxicity of hepatotoxins in macroinvertebrates (benthic or otherwise). Nevertheless, reports of mass mortality of macroinvertebrates following severe blooms of toxic cyanobacteria exist. For instance, significant mortality of chironomid, oligochaete and ceratopogonid larvae, crayfish (*Orconectes limosus* Rafinesque) and bivalves (*Anodonta piscinalis* and *Unio tumidus*) were observed on the Goczałkowice Reservoir, Southern Poland, during intense cyanobacterial blooms of the summer 1992 (Krzyżanek *et al.*, 1993). In this case, toxins produced by *Aphanizomenon flos-aquae* were cited responsible, as sufficient oxygen was present in overlying waters. Laboratory studies have confirmed the toxicity of hepatotoxins to aquatic larvae of the phantom midge *Chaoborus* sp. (Laurén-Määttä *et al.*, 1995) and mosquito *Aedes aegypti* (Linnaeus) (Kiviranta *et al.*, 1993), as well as to adult mosquito *Culex pipiens* (Linn.) (Turell and Middlebrook, 1988). Microcystin-LR was demonstrated to be toxic to larvae (e. g., diamond-backed moth *Plutella xylostella*, cotton leafworm *Spodoptera littoralis* and cabbage white butterfly *Pieris brassicae*) and adult (e. g., house fly *Musca domestica*) terrestrial insects at doses comparable to the insecticides, rotenone, malathion and carbofuran (Delaney and Wilkins, 1995).

Within aquatic environments, the most obvious group of organisms threatened by the presence of toxic cyanobacteria may be the sessile, filter-feeding bivalves. As previously mentioned (section 7.5 above), a laboratory study by Eriksson *et al.* (1989b) demonstrated the freshwater mussel *Anodonta cygnea* accumulates peptide toxin when reared in cultures of *Oscillatoria agardhii* and suggested that organisms feeding on toxin-producing cyanobacteria in eutrophic environments could potentially accumulate toxin. In this regard, several have observed the occurrence and accumulation of hepatotoxins in tissues of freshwater mussels and clams sampled from bloom-prone lakes and reservoirs. For instance, Watanabe *et al.* (1997) reported the accumulation of MCYST by the bivalves, *Anodonta woodiana* and *Unio douglasiae*, collected from Lake Suwa, Japan. Similarly, Prepas *et al.* (1997) illustrated the accumulation of MCYST in tissue of *Anodonta grandis simpsoniana* from several eutrophic Alberta lakes and the persistence

of toxin more than 21 days after clams were removed from the toxin source. However, direct toxic effects of MCYSTs on bivalve survival have not been observed and hepatotoxins appear not to be metabolized in these organisms (Eriksson *et al.*, 1989b; Vasconcelos, 1995).

Kotak *et al.* (1996a) suggested that MCLR might occur in a variety of aquatic organisms that inhabit hypereutrophic Alberta lakes. Out of the macroinvertebrates examined, MCLR was detected only in herbivorous grazers, including resident zooplankton and gastropods. Nevertheless, recent investigations indicate omnivorous decapods (*i. e.*, crayfish) may also accumulate peptide hepatotoxins from toxin-producing benthic cyanobacteria. Benthic *Oscillatoria sancta* (Gomont) were found in the gut contents of adult signal crayfish, *Pacifastacus leniusculus* (Dana) sampled from a Swedish pond (Lirås *et al.*, 1998). Subsequent laboratory studies confirmed the ability of crayfish (up to 50%) feeding on toxic *Oscillatoria* (*Planktothrix*) *agardhii* (Gomont), to accumulate MCYSTs within the hepatopancreas (digestive gland), though toxic effects were not observed (Lirås *et al.*, 1998). Considering this apparent resistance to cyanobacterial toxins, it has recently been suggested that crayfish use cyanobacteria as a food source to increase their biomass in hypereutrophic waters (Vasconcelos, 1999).

8.5 Fish – The occurrence of massive fish kills occasionally accompany reports of severe cyanobacterial blooms (Steyn, 1945; Schwimmer and Schwimmer, 1968). However, many of these incidents have been attributed to indirect effects, such as oxygen depletion (Peñaloza *et al.*, 1990) or increased ambient ammonia concentrations (Paley *et al.*, 1993; Bury *et al.*, 1995) resulting from decaying cyanobacteria (Pillay, 1992). Nevertheless, accounts implicating cyanobacterial hepatotoxins in the mortality of fish from bloom-prone lakes and reservoirs exist. In one instance, about 1000 dead or dying brown trout (*Salmo trutta* L.) were found over a 2-day period in Loch Leven, Scotland June 1992 (Rodger *et al.*, 1994). During this period, a substantial bloom of *Anabaena flos-aquae*, containing three MCYST variants totaling 539 μg MCLR equivalents g^{-1} dry weight phytoplankton, was evident. Furthermore, dissolved concentrations of the three variants in water samples ranged between 16 and 19 μg MCLR equivalents L^{-1}. Histological observations of tissues from moribund and apparently healthy fish, revealed pathology consistent with experimentally induced hepatic changes in rainbow trout

(*Oncorhynchus mykiss* Walbaum) injected intraperitoneally (i.p.) with *M. aeruginosa* (Phillips *et al.*, 1985) and common carp (*Cyprinus carpio* Linneo) injected with purified MCLR (Råbergh *et al.*, 1991). Microcystins have also been linked to the occurrence of "netpen liver disease" (NLD), a severe condition afflicting netpen-reared Atlantic (*Salmo salar*) and Chinook (*Oncorhynchus tshawtyscha*) salmon and steelhead (*Oncorhynchus mykiss*) trout (Kent *et al.*, 1988; Kent, 1990). In this case, histopathologic changes observed in Atlantic salmon injected with MCLR appeared similar to changes in fish suffering from NLD, including diffuse necrosis and hepatic megalocytosis (Andersen *et al.*, 1993). In addition, extracts from livers of fish with NLD contained a protein phosphatase inhibitor chromatographically indistinguishable from MCLR providing evidence for the presence of MCYST. Subsequently, MCLR was detected in Dungeness crab larvae (*Cancer magister*) in the vicinity of netpen farms, supporting anecdotal evidence that NLD is associated with salmon feeding on an abundance of crab larvae (Williams *et al.*, 1997a).

In general, gross, histopathological and ultrastructural changes reported in various fish species following acute exposure to either *M. aeruginosa* and/or purified MCYST analogs (summarized in Table 18), are similar in many respects to those within mammals (Theiss *et al.*, 1988; Miura *et al.*, 1989; Hooser *et al.*, 1990). Nevertheless, several dissimilarities do exist. For example, while the liver to body mass ratio increases in fish and mice alike, the increase in fish liver mass is attributable to water retention as opposed to the pooling of blood (haemorrhage) in mice liver tissue (Kotak *et al.*, 1996b). In addition to severe damage and dysfunction of the liver, MCYSTs also induce pathological changes within the kidneys (Table 18) and gills of fish (Råbergh *et al.*, 1991; Kotak *et al.*, 1996b; Carbis *et al.*, 1997). Elevations of the liver-associated enzymes, LDH, ALT and AST, have also been observed in blood following exposure to MCYSTs (Råbergh *et al.*, 1991; Carbis *et al.*, 1996; Navrátil *et al.*, 1998). However, species-specific differences in MCYST tolerance, as indicated by degree liver damage and liver enzyme activities in blood of exposed fish, are evident (Bury *et al.*, 1997).

Recent investigations suggest that MCYSTs disrupt internal ion homeostasis by blocking normal gill function. Foremost, Gaete *et al.* (1994) demonstrated the ability *M. aeruginosa* extracts to upset homeostatic processes by inhibiting the ion pumps of gill

chloride cells. Treatment of a microsomal fraction isolated from gill of common carp with nM concentrations of *M. aeruginosa* extract, caused the dose-dependent inhibition of several ion pump enzymes including Mg^{2+}-dependent (Na^+/K^+), Na^+, HCO^-_3 and Ca^{2+}-stimulated ATPases. Subsequently, MCLR was shown to inactivate K^+-sensitive hydrolysis of Na^+-dependent phosphoenzyme formation and oubain binding indicating MCYSTs influence Na^+ ion transport through the gills by inhibiting the dephosphorylation of the sodium pump enzymes (Zambrano and Canelo, 1996). As a result, plasma Na^+ and Cl^- concentrations decreased in fish exposed to MCYSTs via i.p. injection, oral gavage and immersion (Bury *et al.*, 1996; Carbis *et al.*, 1996). In contrast, pure MCLR failed to inhibit whole-body Ca^{2+} influx in freshwater tilapia (*Oreochromis mossambicus* Peters), while hydrophobic fractions of *M. aeruginosa* strains PCC 7820 (high toxin content) and CYA 43 (low toxin content) were able (Bury *et al.*, 1996). Hence, in addition to MCYSTs, unidentified compounds from cyanobacteria have the potential to interfere with ionic transport processes in freshwater fish resulting in homeostatic disturbances.

Exposure to MCYSTs may induce other behavioral and/or developmental alterations in various fish species. For instance, Baganz *et al.* (1998) demonstrated that MCLR causes dose-dependent changes in spontaneous locomotor activity of zebrafish (*Danio rerio*). At low concentrations (*i. e.*, 0.5 and 5 $\mu g\ L^{-1}$) daytime motility increased and was consequently, interpreted as an escape reaction or an increased spatial orientation behavior to cope with changing ecological conditions (Baganz *et al.*, 1998). Yet high concentrations (*i. e.*, 50 $\mu g\ L^{-1}$) caused a significant decrease in movement, which was thought to be an energy saving behavior. Interestingly, the effects of MCLR on night-time motility were to the contrary (*i. e.*, low concentrations decreased motility, while high concentrations increased motility). This "phase shifting of the circadian activity patterns and the elevated activities during night-times may be interpreted as a sheltering behavior" (Baganz *et al.*, 1998). Furthermore, concomitant reductions in activity during spawning time periods and during feeding pose serious ecological implications and characterize MCYSTs as severe stressors for zebrafish. Toxicity studies with pure toxin on the developing zebrafish indicate that embryonic exposure to MCYST (5 and 50 μg MCLR L^{-1}) reduces larval survival rate and the growth:weight ratio by 40

and 25%, respectively (Oberemm *et al.*, 1997). However, exposure to crude cyanobacterial extracts elicited far more pronounced effects, including gross malformations and high mortalities during embryonic development such that progression to larval stage ceased (Oberemm *et al.*, 1997, 1999). Subsequent research concluded that chronic exposure to low MCYST levels (0.5 μg MCLR L^{-1}) may reduce growth by increasing energy demands required by detoxication processes, suggesting MCYSTs are metabolized *in vivo* (Wiegand *et al.*, 1999).

It is well recognized that cyanobacteria are an important dietary component of many tropical cichlids (e. g., tilapia, *Oreochromis niloticus* L.) and cyprinids (e. g., silver carp, *Hypophthalmichthys molitrix* Valenciennes) (Bowen, 1982; Spataru and Gophen, 1985). Laboratory research indicates both tilapia and silver carp display marked differences in grazing response to non-toxic versus toxic *M. aeruginosa* strains. Beveridge *et al.* (1993) noted fish exposed to a non-toxic strain sustained greater opercular beat rates, which effectively maintains the flow of water and suspended food particles over the gills, compared to those exposed to a toxic strain. Furthermore, gut content analysis confirmed few particles ($< 10^3$) of toxic *M. aeruginosa* had been consumed by fish in contrast to those of the non-toxic strain ($> 10^7$). Subsequently, in mixed populations of toxic and non-toxic *M. aeruginosa*, ingestion by tilapia was dependent on the proportion of toxic cells, irrespective of particle density (Keshavanath *et al.*, 1994). Grazing response decreased "in an approximately linearly fashion as the proportion of toxic cells in the population" increased beyond 25% (Keshavanath *et al.*, 1994). Consequently, these results suggest that ingestion and hence growth would be suppressed (Beveridge *et al.*, 1993). Yet in the natural environment of eutrophic lakes and reservoirs, there are periods when the phytoplankton community may become completely dominated by toxin-producing cyanobacteria. Observations of the gut contents of common carp collected from Lake Mokoan, Australia, indicate *M. aeruginosa* is the dominant food constituent from February through April (Carbis *et al.*, 1997). Though fish appeared healthy and no mortalities were reported during this period, 66% of those collected possessed signs of hepatocyte atrophy of which 37% also had gills displaying folded lamellar tips with epithelial ballooning and localized necrosis. In addition, serum AST activities and bilirubin concentrations were elevated from January

through April, while serum sodium and chloride concentrations were depressed from December through April. In contrast, fish collected during the same period from Lake Wellington, which did not contain significant growths of *M. aeruginosa*, experienced none of these histopathological symptoms or effects on serum biochemistry.

In light of these laboratory studies and field observations, it is apparent that fish are susceptible to the toxic effects of hepatotoxins at various life stages. However, evidence also suggests that oral and immersion exposure of these organisms to MCYSTs is slow to cause adverse effects and mortality, such that acute toxic episodes are generally quite rare (Carbis *et al.*, 1996). This raises the question as to why fish are significantly less sensitive to MCYSTs than mammals? Gill and skin epithelia of freshwater fish are considered barriers to MCYST transport (Bury *et al.*, 1995, 1998). Nevertheless, toxin uptake studies utilizing oral routes of exposure and i.p. injections of radio-labeled MCLR, not only confirm the hepatotropism of MCYSTs within fish, but also yield insights regarding the ostensible resistance of fish to the acute hepatotoxic effects of MCYSTs compared to mammals. Tencalla and Dietrich (1997) demonstrated the rapid, albeit limited (*i. e.*, less than 5% of the total applied MCYST dose), absorption of MCYST from the gastrointestinal tract into the blood of rainbow trout following gavage with toxic *M. aeruginosa*. Once in the blood, toxin (estimated to be 1.7% of applied dose) rapidly accumulated in the liver tissue, where $\approx$ 63% became covalently bound to protein phosphatases (Tencalla and Dietrich, 1997). These findings compare favorably with Williams *et al.* (1997b), who concluded that $\approx$ 60% of MCYST in liver of Atlantic salmon i.p. injected with MCYST, remains covalently bound to protein phosphatases. Furthermore, beyond 24 h, metabolism occurred forming products more polar than MCLR, half of which lacked protein phosphatase-inhibiting activity (Williams *et al.*, 1997b). Biochemically active (unbound) MCYSTs have been detected in the biliary tract of rainbow trout, following oral administration of toxic *M. aeruginosa*, suggesting bile plays an important role in elimination of MCYSTs from liver of fish (Sahin *et al.*, 1996). The fact that MCYST clearance from tissue of fish proceeds expeditiously compared to mammals (*i. e.*, mice), might explain in part the greater resistance of fish to cyanobacterial hepatotoxins (Williams *et al.*, 1995). As a result, reported acute LD_{50} values are typically > 500 $\mu g\ Kg^{-1}$ for fish, while values for mammals, such as mice, are

considerably lower (≈ 50 μg Kg^{-1}) (Kotak, 1995). Finally, the relatively insignificant accumulation of MCYSTs in the flesh of fish somewhat precludes them in the transfer of hepatotoxins to other organisms associated with the food webs of bloom-prone waters, though further studies addressing this issue are warranted.

9. Conclusion

Cyanobacteria comprise a group of true bacteria that possess the photosynthetic processes common to true algae and higher plants. Additionally, they have evolved through a myriad adaptations allowing them to successfully compete against true algae and as a consequence, often dominate the phytoplankton communities of eutrophic lakes and reservoirs. During calm weather periods following lengthy turbulent conditions, the ability of cyanobacteria to regulate buoyancy may inadvertently cause them to become stranded at the water's surface, resulting in the formation of dense accumulations that appear as thick green scums. Historically, these blooms have been associated with numerous reports of toxicity to aquatic and terrestrial organisms that accidentally or reluctantly ingest cyanobacteria. Subsequently, secondary metabolites produced by cyanobacteria that act as potent neuro and liver toxins were discovered in several bloom-forming species. Of these toxins, the cyclic heptapeptide MCYSTs are undoubtedly the most common to the world's eutrophic freshwaters. Comprised of over 70 related variants, these toxins are preferentially taken up by hepatocytes where they effectively inhibit the activity of specific protein phosphatases. In conjunction with protein kinases, these enzymes normally mediate the reversible phosphorylation of the serine/threonine residues of regulatory proteins. Microcystins then, disrupt the critical balance between the phosphorylation/dephosphorylation activities of protein kinases and phosphatases (respectively), producing a hyperphosphorylated state of the cytosolic and cytoskeletal proteins within hepatocytes. The predominant effect is the reorganization of cytoskeletal components. As a result, plasma membrane bleb formation, loss of microvilli and cell-cell adhesion, as well as numerous ultrastructural changes occur. In animals, predominantly mammals and fish, these hepatocellular alterations ultimately cause the disruption of the hepatic architecture and widespread necrosis potentially leading to death via severe hepatic damage (mammals) or dysfunction (fish). The majority of research has

focussed on the acute toxicity of MCYSTs to mammals, while few have studied the long-term chronic effects of MCYSTs to susceptible organisms. Still, fewer have investigated the potential accumulation and transfer of these toxins in aquatic and terrestrial organisms associated with the food webs of bloom-prone lakes and reservoirs. Consequently, a better understanding of the mechanisms, risks and impacts of toxic blooms are necessary in order to develop mitigative strategies regarding the malady and mortality of livestock, pets, wildlife and humans. Thus, future research is imperative.

Table 1. Physical, chemical and biological characteristics of lakes and reservoirs based on trophic status

Characteristic	Oligotrophic	Eutrophic
Nutrient status	Deficient in phosphorus and possibly nitrogen as a result of low inputs of primarily catchment-derived inorganic nutrients. Oxic hypolimnion limits nutrient cycling from sediments.	Natural (catchment-derived) or anthropogenic enrichment of phosphorus and nitrogen increases lake basin-derived, organic nutrients. Anoxic hypolimnion permits nutrient cycling from sediments.
Organic productivity	Low rates of primary and subsequent levels of production.	High rates of primary and subsequent levels of production.
Water transparency	High, due to low light attenuation by phytoplankton.	Low, due to high light attenuation by phytoplankton.
Euphotic zone depth	Greater euphotic zone depth due to high water transparency.	Less euphotic zone depth due to low water transparency.
Sediment deposition rate	Low rates of deposition to sediments due to reduced levels of organic productivity.	High rates of deposition to sediments due to elevated levels of organic productivity.
Sediment type	Sediments remain primarily inorganic from catchment-derived material.	Sediments becoming primarily organic due to greater contributions from lake basin-derived material.
Water depth	Lower productivity and sedimentation rates results in lake basin infilling at slow rate, thus depth is maintained.	Greater productivity and sedimentation rates results in lake basin infilling at rapid rate, thus becoming shallower.
Hypolimnetic oxygen	Elevated concentrations of dissolved oxygen due to the depressed rates of decomposition resulting from small proportion of organic sediments.	Depressed concentrations of dissolved oxygen due to the elevated rates of decomposition resulting from large proportion of organic sediments.
Benthic community	Consist of high diversity of low oxygen intolerant species.	Consist of low diversity of low oxygen tolerant (hemoglobin possessing) species.
Fish community	Contain deep-water species (char and whitefish).	Lack of deep-water species (char and whitefish).

Adapted from Hutchinson (1973) and Wetzel (1983).

Table 2. Common major phytoplankton associations based on lake trophic status and other water characteristics

Trophic status	Water characteristics	Dominant groups/species	Common groups/species
Oligotrophic	Slightly acidic; very low salinity	Desmids *Staurodesmus*, *Staurastrum*	*Sphaerocystis*, *Gloeocystis*, *Rhizosolenia*, *Tabellaria*
Oligotrophic	Neutral to slightly alkaline; nutrient-poor lakes	Diatoms, especially *Cyclotella* and *Tabellaria*	Some *Asterionella* spp., *Melosira* spp. and *Dinobryon*
Oligotrophic	Neutral to slightly alkaline; nutrient-poor lakes or more productive lakes at seasons of nutrient reduction	Chrysophycean algae, especially *Dinobryon*, some *Mallomonas*	Other chrysophyceans, e. g., *Synura*, *Uroglena*; diatom *Tabellaria*
Oligotrophic	Neutral to slightly alkaline; nutrient-poor lakes	Chlorococcal *Oocystis* or chrysophycean *Botryococcus*	Oligotrophic diatoms
Oligotrophic	Neutral to slightly alkaline; generally nutrient poor; Common in shallow Arctic lakes	Dinoflagellates, especially some *Peridinium* and *Ceratium* spp.	Small chrysophytes, cryptophytes and diatoms
Meso- or Eutrophic	Neutral to slightly alkaline; annual dominants or in eutrophic lakes at certain seasons	Dinoflagellates, some *Peridinium* and *Ceratium* spp.	*Glenodinium* and many other algae
Eutrophic	Usually alkaline lakes with nutrient enrichment	Diatoms much of year, especially *Asterionella* spp., *Fragilaria crotonensis*, *Synedra*, *Stephanodiscus* and *Melosira granulata*	Many other algae, especially cyanobacteria and green algae during warmer periods of year; desmids if dissolved organic matter is fairly high
Eutrophic	Usually alkaline; nutrient enriched; common in warmer periods of temperate lakes or perennially in enriched tropical lakes	Cyanobacteria, especially *Microcystis* spp., *Aphanizomenon* spp. and *Anabaena* spp.	Other cyanobacteria; euglenophytes if organically enriched or polluted

Adapted from Wetzel (1983) after Hutchinson (1967).

Table 3. Physical, chemical and biological factors and physiological and morphological adaptations associated with cyanobacterial growth and proliferation.

Factor/Adaptation	Effects/Mechanisms	References
Catchment size, geology and land use	Determines catchment:lake area ratio, lake morphometry, surface and ground water contributions, natural and anthropogenic nutrient loading rates and water flushing rates and chemistry.	Schindler, 1971; Dillon and Kirchner, 1975; Stumm and Morgan, 1981; Prairie and Kalff, 1986; Duarte and Kalff, 1989
Lake morphometry	Influences mean depth and volume, epilimnion:hypolimnion ratio, water column stability, hypolimnetic O_2 levels (thus, phosphorus exchange with sediments).	Rawson, 1955; Straskraba, 1980; Duarte and Kalff, 1989
Water residence time	Influences water chemistry (*i. e.*, nutrient dilution). Loss mechanism due to washout of algal inocula and increased loss of slow growing species.	Pearl, 1996
Water column stability	Low turbulence or low mixing:euphotic depth ratio (Z_m:Z_{eu} < 1) favors gas vacuolate cyanobacteria.	Reynolds and Walsby, 1975; Steinberg and Hartmann, 1988
Water temperature	Increased nutrient uptake, photosynthetic and growth rates with increasing temperatures to (15-30°C), beyond which, inhibition may occur.	McQueen and Lean, 1987; Zhang and Prepas, 1996
Light intensity (attenuation)	Diurnal migrations/chromatic adaptations accommodate changing light intensity, greater tolerance for high light intensity (high UV).	Reynolds and Walsby, 1975; Klemer, 1976; Paerl *et al.*, 1985
CO_2 concentrations	Low available CO_2 favors cyanobacteria due to superior uptake kinetics for inorganic carbon. Low CO_2 increases pH.	King, 1970; Shapiro, 1973, 1984; Caraco and Miller, 1998
pH	High pH (pH > 8) may reduce loss rates by decreasing cyanophages or disease bacteria. High pH optima for nutrient uptake enzymes. Influences form of nutrients and metals and thus availability and uptake.	Brock, 1973; Moss, 1973; Shapiro, 1984; Caraco and Miller, 1998
Phosphorus concentrations	Phosphorus is often the limiting nutrient in freshwater systems.	Vollenweider, 1976; Trimbee and Prepas, 1987
Nitrogen:Phosphorus ratio	Low N:P ratios favor nitrogen-fixing (heterocystous) species over non nitrogen-fixing eukaryotic algae.	Schindler, 1977; Flett *et al.*, 1980; Smith, 1983; Pick and Lean, 1987

Table 3. (continued)

Factor/Adaptation	Effects/Mechanisms	References
Organic matter	Act as metal chelators and nutrient sources (heterotrophy).	Pearsall, 1932; Elder and Horne, 1977; Paerl, 1988
Production of resting stages (seed beds)	Many species produce resting stages (specialized akinetes in N_2-fixing species) or overwintering vegetative cells (*Microcystis* sp.) that remain on the sediments until suitable conditions arise	Preston *et al.*, 1980; Nichols and Adams, 1982
Production of allelopathic compounds	Linoleic and linolenic fatty acids produced by several cyanobacteria inhibit growth of competing algae.	Ikawa *et al.*, 1996
Buoyancy regulation	Production of gas vacuoles regulates cells/colonies vertically in the water column, affording access to optimal levels of light and nutrients	Reynolds and Walsby, 1975
Colony/Filament morphology	Large colonies (multiple filaments) move more rapidly than smaller of same density, thus regulate vertical position faster.	Reynolds and Walsby, 1975
Heterotrophic bacterial synergism	Nutritional and biotic associations enhance growth of cyanobacteria (*i. e.*, exchange of organic nutrients and increased N_2 fixation in anoxic microsphere provided by bacteria)	Paerl, 1988
Micrograzer (protozoans and rotifers) synergism	Micrograzers may graze on heterotrophic bacteria thus enhancing nutrient cycling for cyanobacterial growth.	Paerl, 1988
Macrograzers (large zooplankton, crustaceans and fish)	Loss mechanism for edible or preferred species. Filamentous and large colonial cyanobacteria inedible, indigestible or poorly assimilated by most macrograzers.	Porter, 1973; Kalff and Knoechel, 1978; Porter and Orcutt, 1980

Table 4. Common genera and species of bloom forming freshwater/estuarine cyanobacteria

Lake strata	Genera/Species	References
Epilimnetic	*Anabaena*, *Aphanizomenon*, *Coelosphaerium*, *Gloeotrichia*, *Gomphosphaeria*, *Lyngbya*, *Microcystis*, *Spirulina* and *Nodularia spumigena*†	Francis, 1878; Prescott, 1948; Reynolds, 1971; Reynolds and Walsby, 1975; Reynolds, 1984; Paerl, 1988;
Metalimnetic	*Ana. planktonica*, *Oscillatoria agardhii*, *O. agardhii* var. *isothrix*‡, *O. lauterbornii*, *O. limosa*, *O. prolifica*, *O. rubescens* and *Spirulina laxa*	Reynolds and Walsby, 1975; Klemer, 1976; Lindholm and Meriluoto, 1991
Benthic	*O. granulata*, *O. limosa*, *O sensulato* and *O. tenuis*	Codd *et al.*, 1992; Mez *et al.*, 1998

† *Nodularia spumigena* blooms occur primarily in brackish waters – estuaries and coastal lagoons (Jones *et al.*, 1994).
‡ *O. agardhii* var. *isothrix* also stratifies under ice cover (Reynolds and Walsby, 1975).

Table 5. Common genera and species of freshwater/estuarine cyanobacteria known to produce neurotoxins

Toxin	Genera/Species	References
Saxitoxin/Neosaxitoxin	*Aphanizomenon flos-aquae* and *Anabaena circinalis*	Ikawa *et al.*, 1982; Humpage *et al.*, 1994
Anatoxin-a	*Ana. flos-aquae*, *Ana. circinalis*, *Ana. lemmermannii*, *Ana. macrospora*, *Ana. planctonica*, *Ana. spiroides*, *Aph. flos-aquae*, *Cylindrospermum* sp., *Microcystis aeruginosa* and *Oscillatoria agardhii*[†]	Devlin *et al.*, 1977; Sivonen *et al.*, 1989; Edwards *et al.*, 1992[†]; Park *et al.*, 1993
Anatoxin-a(s)	*Ana. flos-aquae* and *O. agardhii*	Mahmood and Carmichael, 1986; Hawser *et al.*, 1990

[†] Benthic *Oscillatoria* spp. also known to produce anatoxin-a.

Table 6. Common genera and species of freshwater/estuarine cyanobacteria known to produce hepatotoxins

Toxin	Genera/Species	References
Microcystins	*Microcystis aeruginosa*, *M. flos-aquae*, *M. viridis*, *M. wesenbergii*, *Anabaena flos-aquae*, *Ana. circinalis*, *Ana. lemmermannii*, *Ana. viguieri*, *Anabaenopsis milleri*, *Oscillatoria agardhii* and *Nostoc* sp.	Botes *et al.*, 1985; Kusumi *et al.*, 1987; Krishnamurthy *et al.*, 1986; Meriluoto *et al.*, 1989; Lanaras *et al.*, 1989; Namikoshi *et al.*, 1990; Sivonen *et al.*, 1990b; Namikoshi *et al.*, 1992a; Jungmann, 1992
Nodularins	*Nodularia spumigena*[†]	Eriksson *et al.*,1988; Jones *et al.*, 1994

[†] Toxic blooms of *Nodularia spumigena* occur primarily in brackish waters – estuaries and coastal lagoons (Jones *et al.*, 1994).

Table 7. Global occurrence of toxic cyanobacterial blooms in freshwater, estuarine and marine environments

Region	Country
Europe	Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Netherlands, Norway, Poland, Portugal, Russia, Slovakia, Sweden, Switzerland, Ukraine, United Kingdom
Americas	Argentina, Bermuda, Brazil, Canada, Chile, USA, Venezuela
Middle East and Asia	Bangladesh, India, Israel, Japan, Malaysia, Peoples' Republic of China, Saudi Arabia, South Korea, Thailand
Africa	Egypt, Ethiopia, Morocco, South Africa, Zimbabwe
Australasia	Australia, New Caledonia, New Zealand
Marine	Atlantic Ocean, Baltic Sea, Caribbean Sea, Indian Ocean

Adapted from Codd (1995).

Table 8. Primary microcystin analogues with variations in amino acids **2 (X)** and **4 (Y)**

Analogue	Structure	Molecular weight	Toxicity*
Microcystin-AR	cyclo (-D-Ala-L-**Ala**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	952[13]	250[13]
Microcystin-FR	cyclo (-D-Ala-L-**Phe**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	1028[8,13]	250[13]
Microcystin-HilR	cyclo (-D-Ala-L-**Hil**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	1008[14]	100[14]
Microcystin-HtyR	cyclo (-D-Ala-L-**Hty**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	1058[9]	80-100[9]
Microcystin-LA	cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Ala**-Adda-D-Glu-Mdha-)	909[2,3]	50[2]
Microcystin-LAba	cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Aba**-Adda-D-Glu-Mdha-)	923[8]	na
Microcystin-LF	cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Phe**-Adda-D-Glu-Mdha-)	985[1]	na
Microcystin-LL	cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Leu**-Adda-D-Glu-Mdha-)	951[5]	na
Microcystin-LM	cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Met**-Adda-D-Glu-Mdha-)	na[5]	na
Microcystin-LR	cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	994[4]	50[10]
Microcystin-LV	cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Val**-Adda-D-Glu-Mdha-)	na[5]	na
Microcystin-LY	cyclo (-D-Ala-L-**Leu**-D-MeAsp-L-**Tyr**-Adda-D-Glu-Mdha-)	1001[15]	90[15]
Microcystin-M(O)R	cyclo (-D-Ala-L-**Met(O)**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	1028[13]	700-800[13]
Microcystin-RA	cyclo (-D-Ala-L-**Arg**-D-MeAsp-L-**Ala**-Adda-D-Glu-Mdha-)	953[12]	na
Microcystin-RR	cyclo (-D-Ala-L-**Arg**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	1037[11]	500-800[13]
Microcystin-WR	cyclo (-D-Ala-L-**Try**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	1067[13]	150-200[13]
Microcystin-YA	cyclo (-D-Ala-L-**Tyr**-D-MeAsp-L-**Ala**-Adda-D-Glu-Mdha-)	959[4]	60-70
Microcystin-YM(O)	cyclo (-D-Ala-L-**Tyr**-D-MeAsp-L-**Met(O)**-Adda-D-Glu-Mdha-)	1035[4]	56-110[6,7]
Microcystin-YR	cyclo (-D-Ala-L-**Tyr**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	1044[4]	150-200[13]
Microcystin-(H4)YR	cyclo (-D-Ala-L-**(H4)Tyr**-D-MeAsp-L-**Arg**-Adda-D-Glu-Mdha-)	1048[14]	na

* Interperitoneal LD_{50} in mice ($\mu g\ kg^{-1}$).

Aba, L-aminoisobutyric acid. Hil, L-homoisoleucine. Hty, L-homotyrosine. M(O), methionine *S*-oxide. (H4)Y, 1,2,3,4-tetrahydrotyrosine. na, not available.

[1]Azevedo *et al.*, 1994. [2]Botes *et al.*, 1982. [3]Botes *et al.*, 1984. [4]Botes *et al.*, 1985. [5]Craig *et al.*, 1993. [6]Elleman *et al.*, 1978. [7]Falconer and Runnegar, 1987. [8]Gathercole and Thiel, 1987. [9]Harada *et al.*, 1991b. [10]Krishnamurthy *et al.*, 1986. [11]Kusumi *et al.*, 1987. [12]Lee *et al.*, 1998. [13]Namikoshi *et al.*, 1992a. [14]Namikoshi *et al.*, 1995. [15]Stoner *et al.*, 1989.

Table 9. Microcystin analogues with demethylation of amino acids **3** and/or **7**

Analogue	Structure	Molecular weight	Toxicity*
[D-Asp^3]microcystin-HtyR	cyclo (-D-Ala-L-Hty-**D-Asp**-L-Arg-Adda-D-Glu-Mdha-)	1044[2]	160-300[2]
[D-Asp^3]microcystin-LR	cyclo (-D-Ala-L-Leu-**D-Asp**-L-Arg-Adda-D-Glu-Mdha-)	980[5]	50[4]
[D-Asp^3]microcystin-RR	cyclo (-D-Ala-L-Arg-**D-Asp**-L-Arg-Adda-D-Glu-Mdha-)	1023[7]	250[7]
[D-Asp^3]microcystin-YR	cyclo (-D-Ala-L-Tyr-**D-Asp**-L-Arg-Adda-D-Glu-Mdha-)	1030[10]	na
[Dha^7]microcystin-FR	cyclo (-D-Ala-L-Phe-D-MeAsp-L-Arg-Adda-D-Glu-**Dha**-)	1014[6]	na
[Dha^7]microcystin-HphR	cyclo (-D-Ala-L-Hph-D-MeAsp-L-Arg-Adda-D-Glu-**Dha**-)	1028[9]	na
[Dha^7]microcystin-HtyR	cyclo (-D-Ala-L-Hty-D-MeAsp-L-Arg-Adda-D-Glu-**Dha**-)	1044[9]	na
[Dha^7]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-**Dha**-)	980[1]	250[8]
[Dha^7]microcystin-RR	cyclo (-D-Ala-L-Arg-D-MeAsp-L-Arg-Adda-D-Glu-**Dha**-)	1023[3]	180[3]
[Dha^7]microcystin-YR	cyclo (-D-Ala-L-Tyr-D-MeAsp-L-Arg-Adda-D-Glu-**Dha**-)	1030[11]	na
[D-Asp^3, Dha^7]microcystin-HtyR	cyclo (-D-Ala-L-Hty-**D-Asp**-L-Arg-Adda-D-Glu-**Dha**-)	1030[9]	na
[D-Asp^3, Dha^7]microcystin-LR	cyclo (-D-Ala-L-Leu-**D-Asp**-L-Arg-Adda-D-Glu-**Dha**-)	966[1]	200-250
[D-Asp^3, Dha^7]microcystin-RR	cyclo (-D-Ala-L-Arg-**D-Asp**-L-Arg-Adda-D-Glu-**Dha**-)	1009[5]	na

* Interperitoneal LD_{50} in mice ($\mu g\ kg^{-1}$).

Dha, dehydroalanine. Hph, L-homophenylalanine. na, not available. For other abbreviations, see Table 8.

[1]Harada *et al.*, 1991a. [2]Harada *et al.*, 1991b. [3]Kiviranta *et al.*, 1992. [4]Krishnamurthy *et al.*, 1986. [5]Krishnamurthy *et al.*, 1989. [6]Luukkainen *et al.*, 1994. [7]Meriluoto *et al.*, 1989. [8]Namikoshi *et al.*, 1992a. [9]Namikoshi *et al.*, 1992b. [10]Namikoshi *et al.*, 1992d. [11]Sivonen *et al.*, 1992b.

Table 10. Microcystin analogues with variation in amino acids **6** or **7**, with/without demethylation of amino acid **3**

Analogue	Structure	Molecular weight	Toxicity*
[D-Glu(OCH_3)^6]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-**D-Glu-(OCH_3)**-Mdha-)	1008[12]	> 1000[12]
[D-Glu-$OC_2H_3(CH_3)OH^6$]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-**D-Glu-$OC_2H_3(CH_3)OH$**-Mdha-)	1052[2]	> 1000[2]
[Mdhb^7]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-D-Glu-**Mdhb**-)	1008[11]	na
[L-MeAla^7]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-**L-MeAla**-)	996[5]	85[5]
[D-MeAla^7]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-**D-MeAla**-)	996[5]	100[5]
[L-MeLan^7]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-**L-MeLan**-)	1115[6]	1000[6]
[L-MeSer^7]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-**L-MeSer**-)	1012[2]	150[6]
[L-Ser^7]microcystin-HtyR	cyclo (-D-Ala-L-Hty-D-MeAsp-L-Arg-Adda-D-Glu-**L-Ser**-)	1062[3]	na
[L-Ser^7]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-**L-Ser**-)	998[4]	na
[L-Ser^7]microcystin-RR	cyclo (-D-Ala-L-Arg-D-MeAsp-L-Arg-Adda-D-Glu-**L-Ser**-)	1041[4]	na
[Dha^7]microcystin-EE(OMe)	cyclo (-D-Ala-**L-Glu**-D-MeAsp-**L-Glu(OMe)**-Adda-D-Glu-**Dha**-)	na[7]	na
[Dha^7]microcystin-E(OMe)E(OMe)	cyclo (-D-Ala-**L-Glu(OMe)**-D-MeAsp-**L-Glu(OMe)**-Adda-D-Glu-**Dha**-)	997[7]	na
[L-Ser^7]microcystin-EE(OMe)	cyclo (-D-Ala-**L-Glu**-D-MeAsp-**L-Glu(OMe)**-Adda-D-Glu-**L-Ser**-)	na[7]	na
[L-Ser^7]microcystin-E(OMe)E(OMe)	cyclo (-D-Ala-**L-Glu(OMe)**-D-MeAsp-**L-Glu(OMe)**-Adda-D-Glu-**L-Ser**-)	1015[7]	na
[D-Asp^3, D-Glu(OCH_3)^6]microcystin-LR	cyclo (-D-Ser-L-Leu-**D-Asp**-L-Arg-Adda-**D-Glu(OCH_3)**-Mdha-)	994[12]	na
[D-Asp^3, MeSer^7]microcystin-RR	cyclo (-D-Ala-L-Arg-**D-Asp**-L-Arg-Adda-D-Glu-**MeSer**-)	1041[1]	na
[D-Asp^3, L-Ser^7]microcystin-XR	cyclo (-D-Ala-UNK-**D-Asp**-L-Arg-Adda-D-Glu-**L-Ser**-)	998[4]	na
[D-Asp^3, (*E*)-Dhb^7]microcystin-RR	cyclo (-D-Ala-L-Arg-**D-Asp**-L-Arg-Adda-D-Glu-**(*E*)-Dhb**-)	1023[8]	250[9]
[D-Asp^3, (*E*)-Dhb^7]microcystin-LR	cyclo (-D-Ala-L-Leu-**D-Asp**-L-Arg-Adda-D-Glu-**(*E*)-Dhb**-)	980[9]	70[9]
[D-Asp^3, (*E*)-Dhb^7]microcystin-HtyR	cyclo (-D-Ala-L-Hty-**D-Asp**-L-Arg-Adda-D-Glu-**(*E*)-Dhb**-)	1044[9]	70[9]
[D-Asp^3, (*Z*)-Dhb^7]microcystin-LR	cyclo (-D-Ala-L-Leu-**D-Asp**-L-Arg-Adda-D-Glu-**(*Z*)-Dhb**-)	980[10]	†[10]
[D-Asp^3, (*Z*)-Dhb^7]microcystin-HtyR	cyclo (-D-Ala-L-Hty-**D-Asp**-L-Arg-Adda-D-Glu-**(*Z*)-Dhb**-)	1044[10]	†[10]

Table 10. (continued)

Analogue	Structure	Molecular weight	Toxicity*
[D-Asp^3, Dha^7]microcystin-E(OMe)E(OMe)	cyclo (-D-Ala-L-**Glu(OMe)**-D-**Asp**-L-**Glu(OMe)**-Adda-D-Glu-**Dha**-)	983[7]	na
[D-Asp^3, L-Ser^7]microcystin-E(OMe)E(OMe)	cyclo (-D-Ala-L-**Glu(OMe)**-D-**Asp**-L-**Glu(OMe)**-Adda-D-Glu-L-**Ser**-)	1001[7]	na
[D-Asp^3, Dha^7]microcystin-EE(OMe)	cyclo (-D-Ala-L-**Glu**-D-**Asp**-L-**Glu(OMe)**-Adda-D-Glu-**Dha**-)	na[7]	na

* Interperitoneal LD_{50} in mice ($\mu g\ kg^{-1}$).

† Isolated from toxic bloom samples, but LD_{50} in mice not reported.

Dhb, dehydrobutyrine. E, L-glutamic acid. E(OMe), L-glutamic acid δ-methyl ester. MeAla, *N*-methylalanine. MeLan, *N*-methyllanthionine. MeSer, *N*-methylserine. na, not available. For other abbreviations, see Table 8 and 9.

[1]Luukkainen *et al.*, 1993. [2]Namikoshi *et al.*, 1992a. [3]Namikoshi *et al.*, 1992b. [4]Namikoshi *et al.*, 1992c. [5]Namikoshi *et al.*, 1993. [6]Namikoshi *et al.*, 1995. [7]Namikoshi *et al.*, 1998. [8]Sano and Kaya, 1995. [9]Sano and Kaya, 1998a. [10]Sano and Kaya, 1998b. [11]Sivonen *et al.*, 1990a. [12]Sivonen *et al.*, 1992c.

Table 11. Microcystin analogues with modification of amino acid **5** (Adda), with/without variation(s) in amino acids **1**, **3** or **7**

Analogue	Structure	Molecular weight	Toxicity*
[6(*Z*)-Adda^5]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-**6(*Z*)Adda**-D-Glu-Mdha-)	994[2]	> 1200[2]
[6(*Z*)-Adda^5]microcystin-RR	cyclo (-D-Ala-L-Arg-D-MeAsp-L-Arg-**6(*Z*)Adda**-D-Glu-Mdha-)	1037[2]	> 1200[2]
[ADMAdda^5]microcystin-LHar	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Har-**ADMAdda**-D-Glu-Mdha-)	1036[3]	60[5]
[ADMAdda^5]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-**ADMAdda**-D-Glu-Mdha-)	1022[3]	60[5]
[DMAdda^5]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-**DMAdda**-D-Glu-Mdha-)	980[4]	90-100[4]
[D-Ser^1, ADMAdda^5]microcystin-LR	cyclo (-**D-Ser**-L-Leu-D-MeAsp-L-Arg-**ADMAdda**-D-Glu-Mdha-)	1038[6]	na
[D-Asp^3, ADMAdda^5]microcystin-LR	cyclo (-D-Ala-L-Leu-**D-Asp**-L-Arg-**ADMAdda**-D-Glu-Mdha-)	1008[3]	160[5]
[D-Asp^3, ADMAdda^5]microcystin-LHar	cyclo (-D-Ala-L-Leu-**D-Asp**-L-Har-**ADMAdda**-D-Glu-Mdha-)	1022[6]	na
[ADMAdda^5, MeSer^7]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-**ADMAdda**-D-Glu-**MeSer**-)	1040[6]	na
[D-Asp^3, ADMAdda^5, Dhb^7]microcystin-LR	cyclo (-D-Ala-L-Leu-**D-Asp**-L-Arg-**ADMAdda**-D-Glu-**Dhb**-)	1008[1]	na
[D-Asp^3, ADMAdda^5, Dhb^7]microcystin-RR	cyclo (-D-Ala-L-Arg-**D-Asp**-L-Arg-**ADMAdda**-D-Glu-**Dhb**-)	1051[1]	200[1]
[D-Asp^3, ADMAdda^5, Dhb^7]microcystin-HtyR	cyclo (-D-Ala-L-Hty-**D-Asp**-L-Arg-**ADMAdda**-D-Glu-**Dhb**-)	1072[1]	100[1]

* Interperitoneal LD_{50} in mice ($\mu g\ kg^{-1}$).

ADMAdda, *O*-Acetyl-*O*-demethylAdda. DMAdda, *O*-DemethylAdda. Har, L-Homoarginine. 6(*Z*)-Adda, stereoisomer of Adda at the Δ^6 double bond. na, not available. For other abbreviations, see Table 8, 9 and 10.

[1]Beattie *et al.*, 1998. [2]Harada *et al.*, 1990. [3]Namikoshi *et al.*, 1990. [4]Namikoshi *et al.*, 1992a. [5]Sivonen *et al.*, 1990a. [6]Sivonen *et al.*, 1992a.

Table 12. Nodularin analogues with/without variation(s) in amino acids **1**, **2**, **3** (Adda), **4** or **5**

Analogue	Structure	Molecular weight	Toxicity*
Nodularin	cyclo (-D-MeAsp-L-Arg-Adda-D-Glu-Mdhb-)	824[5]	50[2]
[D-Asp[1]]nodularin	cyclo (-**D-Asp**-L-Arg-Adda-D-Glu-Mdhb-)	810[4]	75[4]
[L-Val[2]]nodularin (Motuporin)	cyclo (-D-MeAsp-**L-Val**-Adda-D-Glu-Mdhb-)	na[1]	na
[DMAdda[3]]nodularin	cyclo (-D-MeAsp-L-Arg-**DMAdda**-D-Glu-Mdhb-)	810[4]	150 [4]
[6(*Z*)Adda[3]]nodularin	cyclo (-D-MeAsp-L-Arg-**6(*Z*)Adda**-D-Glu-Mdhb-)	824[4]	> 2000[4]
[D-Glu(OCH_3)[4]]nodularin	cyclo (-D-MeAsp-L-Arg-Adda-**D-Glu(OCH_3)**-Mdhb-)	na	> 1200
[D-MeAsp(OCH_3)[1], D-Glu(OCH_3)[4]]nodularin	cyclo (-**D-MeAsp(OCH_3)**-L-Arg-DMAdda-**D-Glu(OCH_3)**-Mdhb-)	na	> 1000
[L-MeAbu[5]]nodularin	cyclo (-D-MeAsp-L-Arg-Adda-D-Glu-**L-MeAbu**-)	826[3]	150[3]
[D-MeAbu[5]]nodularin	cyclo (-D-MeAsp-L-Arg-Adda-D-Glu-**D-MeAbu**-)	826[3]	150[3]

* Interperitoneal LD_{50} in mice ($\mu g\ kg^{-1}$).
MeAbu, *N*-methylamino butyrine. na, not available. For other abbreviations, see Table 8, 9, 10 and 11.
[1]deSilva *et al.*, 1992. [2]Eriksson *et al.*, 1988. [3]Namikoshi *et al.*, 1993. [4]Namikoshi *et al.*, 1994. [5]Rinehart *et al.*, 1988.

Table 13. Hydrophobic/hydrophilic properties of invariable and variable amino acid constituents of microcystins (MCYSTs) and nodularins (NDLNs)

Property	Invariable amino acids	Variable L-amino acids
Hydrophobic	D-Alanine, *N*-methyldehydroalanine (MCYSTs), *N*-methyldehydrobutyrine (NDLNs) and Adda	Alanine, Isoleucine, Leucine, Methionine, Phenylalanine, Tryptophan, Tyrosine[†] and Valine
Hydrophilic	D-*erythro*-β-methylaspartic acid and D-Glutamic acid	Arginine

[†] Tyrosine is strongly hydrophobic due to benzene ring, but its hydroxyl group permits interaction with water (Darnell *et al.*, 1990).

Table 14. pH-dependent dominant ion speciation (of the two free ionizable carboxyl groups and single free amino group), net charge and log transformed octanol/water distribution ratio of microcystin-LR

pH	Dominant ion species	Net charge	log D_{ow}
< 2.09	$(COOH)_2$ (NH_2^+)	+	*
2.09 – 2.19	(COO^-) (COOH) (NH_2^+)	o	*
2.19 – 12.48	$(COO^-)_2$ (NH_2^+)	–	*
> 12.48	$(COO^-)_2$ (NH)	– –	*

* Determined log D_{ow} values range from 2.18 at pH = 1 to –1.76 at pH = 10.
Adapted from de Maagd *et al.* (1999).

Table 15. Lytic characteristics of common chemicals used in drinking water treatment and/or the control of cyanobacterial blooms

Common chemical name	Chemical formula	Cell lysis/ toxin release
Copper sulphate	$CuSO_4$	yes[2,3]
Reglone A (diquat)	1,1-ethylene-2,2-dipyridilium dibromide	yes[4,5]
Simazine	2-chloro-4,6-bis(ethylamino)-*s*-triazine	yes[4]
Aluminum sulphate (alum)	$Al_2(SO_4)_3 \cdot 14H_2O$	na[4,5]
Calcium hydroxide (lime)	$Ca(OH)_2$	na[3,4,5]
Ferric chloride	$FeCl_3$	na[1]
Potassium permanganate†	$KMnO_4$	yes[4]
Sodium hypochlorite (chlorine)‡	NaOCl	yes[4]

† Potassium permanganate is used in drinking water treatment to reduce phytoplankton-related odour problems (Cherry, 1962).
‡ Chlorine is used in drinking water treatment to disinfect and reduce biological activity (Montgomery, 1985).
na, no appreciable toxin release.
[1]Chow *et al.*, 1998. [2]Jones and Orr, 1994. [3]Kenefick *et al.*, 1993. [4]Lam *et al.*, 1995b. [5]Lam and Prepas, 1997.

Table 16. Summary of gross pathological and histological changes to liver of laboratory rats injected intraperitoneally with *M. aeruginosa* extract

Post injection time	Gross pathological changes	Histological changes
15-30 min	Enlargement of the liver due to increased centrilobular blood content.	Hepatocytes appeared swollen and vacuolated. Cytoplasm was granular. Pyknotic nuclei. Central sinusoids slightly distended. Some areas of coagulative necrosis.
3-6 hrs	25% increase in liver mass. Liver appeared softened and friable with partially liquefied parenchyma. Severe intrahepatic haemorrhaging in acutely poisoned animals resulting in terminal circulatory deficiency (hypovolumic shock and heart failure) and death.	Marked necrosis of the centrilobular region with complete cell lysis. Peripheral cells appeared swollen and vacuolated. Distended sinusoids filled with red blood cells. Destruction of the sinusoidal endothelium causing pooling of blood.
2-3 days	Liver two thirds of normal size. Soft with mottled appearance. Slightly enlarged central veins.	Number of dead hepatocytes diminished. Lobular structure becoming restored. Reduced blood content of sinusoids. Some parenchymal cells still swollen and granular.
5 days	Liver almost normal with slight mottled appearance.	Hepatic lobules normal in size and arrangement. Normal blood content within sinusoids. Few hepatocytes swollen and granular. Mitotic figures seen in parenchymal cells indicating regeneration.

Summarized from Ashworth and Mason (1946).

Table 17. Summary of observed cyanobacterial hepatotoxin related intoxications involving livestock, pets and wildlife

Animal	References
Cattle	Steyn, 1945; Stewart *et al.*, 1950; O'Donoghue and Wilton, 1951; McLeod and Bondar, 1952; MacDonald, 1960; Senior, 1960; Galey *et al.*, 1987; Kerr *et al.*, 1987; McKenzie *et al.*, 1988; Fitzgerald and Poppenga, 1993; Van Halderen *et al.*, 1995; Mez *et al.*, 1997; Frazier *et al.*, 1998; Puschner *et al.*, 1998
Sheep	Steyn, 1945; Done and Bain, 1993; Van Halderen *et al.*, 1995
Horses	Steyn, 1945; O'Donoghue and Wilton, 1951; McLeod and Bondar, 1952
Pigs	O'Donoghue and Wilton, 1951; McLeod and Bondar, 1952; Chengappa *et al.*, 1989
Dogs	Steyn, 1945; O'Donoghue and Wilton, 1951; McLeod and Bondar, 1952; Senior, 1960; Edler *et al.*, 1985; Kelly and Pontefract, 1990; DeVries *et al.*, 1993; Harding *et al.*, 1995
Cats	McLeod and Bondar, 1952
Poultry	Steyn, 1945; O'Donoghue and Wilton, 1951; Dillenberg and Dehnel, 1960
Waterfowl	Steyn, 1945; O'Donoghue and Wilton, 1951; Bossenmaier *et al.*, 1954; Dillenberg and Dehnel, 1960

Numerous additional observations summarized by Schwimmer and Schwimmer (1968).

Table 18. Summary of gross pathological, histological and ultrastructural changes to liver and kidneys of fish exposed to *M. aeruginosa* and/or purified MCYST

Pathology	Liver	Kidney
Gross changes	General vasodilation and severe liver congestion and damage.	
Histological changes	Loss of cell-cell junctions resulting in hepatocyte dissociation, massive liquifactive necrosis and loss of the hepatic parenchymal architecture. Disruption of bile canaliculi.	Dilation of Bowman's capsules of kidney glomeruli and progressive degeneration of the tubuli leading to coagulative necrosis of the tubular epithelium.
Ultrastructural changes	Condensed mitochondria, fragmentation and vesiculation of rough endoplasmic reticulum with ribosomal loss. Peripheral accumulation of cytoskeletal microfilaments. Cellular vacuolization, nuclear pyknosis and eventual collapse of the nuclear membrane.	Lysis of the tubular epithelial lining.

Phillips *et al.*, 1985; Råbergh *et al.*, 1991; Bingsheng *et al.*, 1993; Kotak *et al.*, 1996b; Bury *et al.*, 1997.

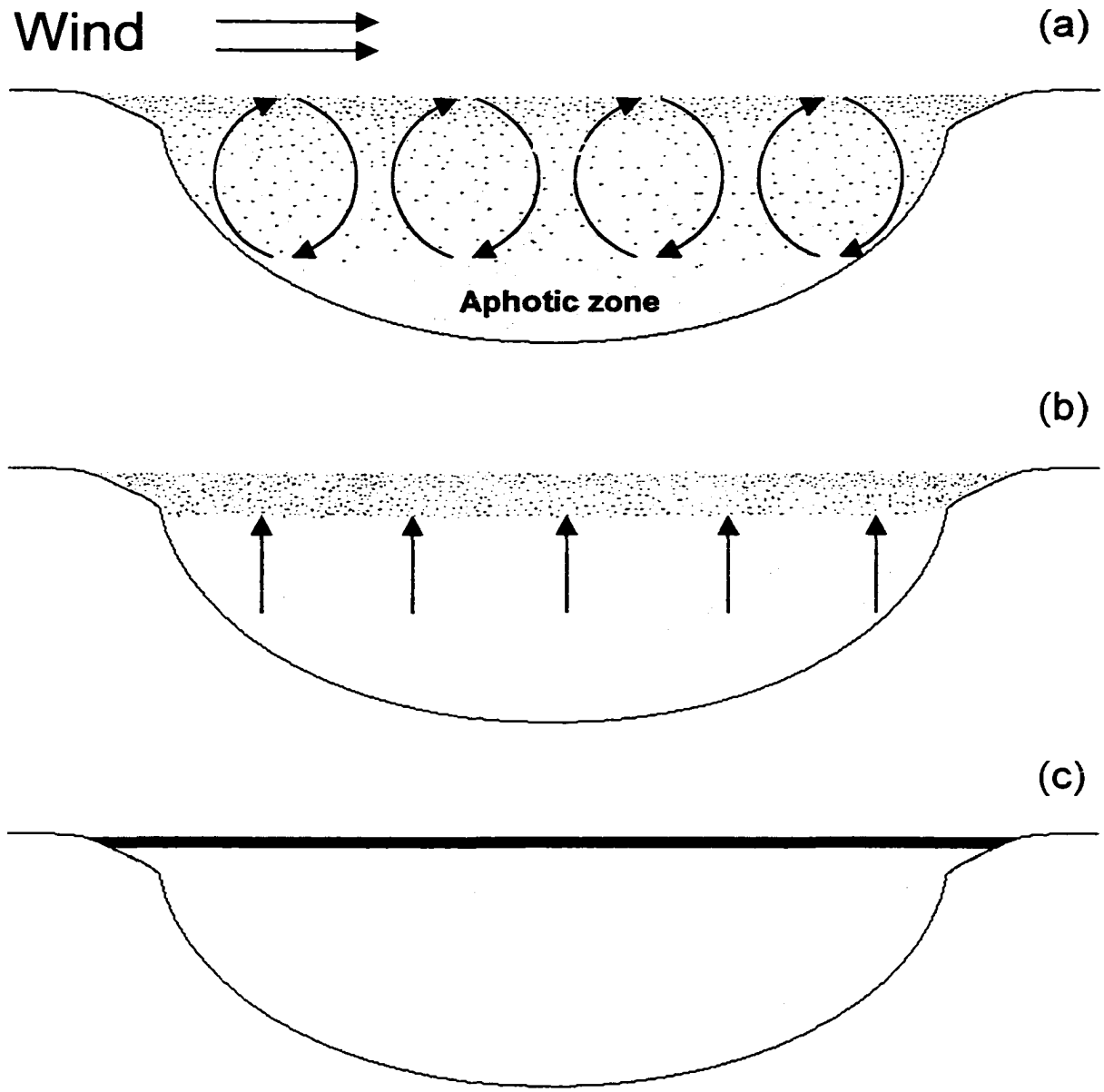

Figure 1. Mechanisms of surface bloom formation: (a) abundance of existing, gas vacuolate cyanobacterial cells/colonies entrained throughout water column by wind induced mixing beyond the euphotic zone (depth of illumination) into the aphotic zone (no illumination); (b) cessation of turbulence results in mass migration of over-buoyant cyanobacteria towards surface; (c) unable to rapidly down-regulate buoyancy, cells/colonies become stranded at surface resulting in severe surface accumulation or bloom.

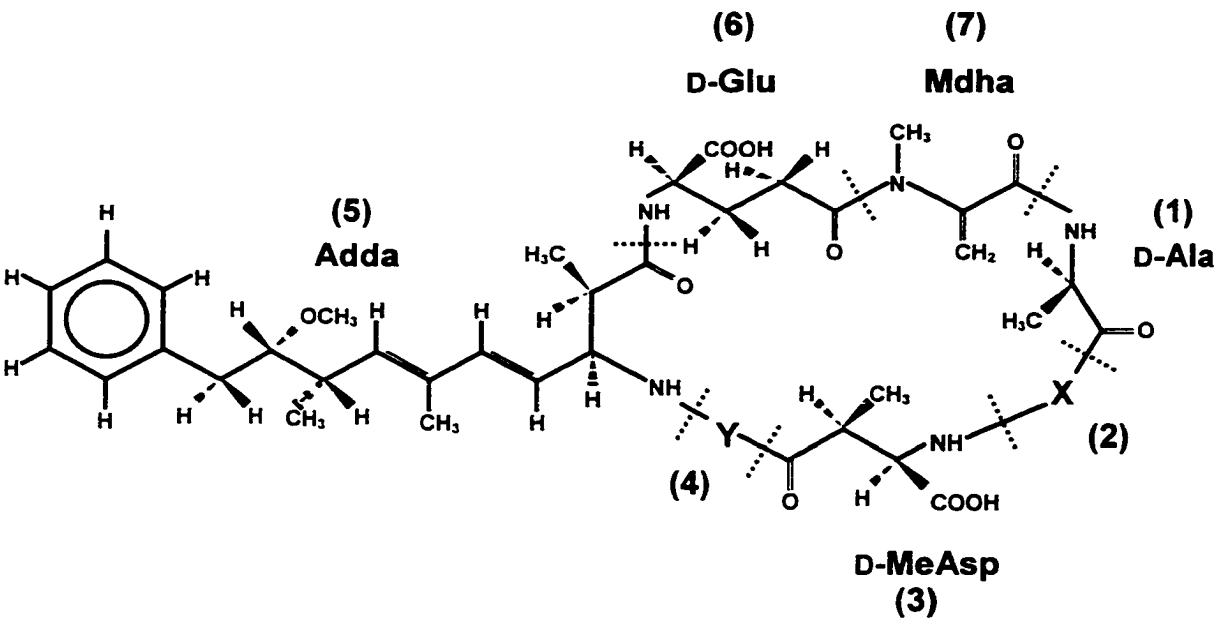

Figure 2. Generalized chemical structure of microcystin: where position (1) is D-Alanine; (2) **X** is a variable L-amino acid; (3) is D-*erythro*-β-methylaspartic acid; (4) **Y** is another variable L-amino acid; (5) is Adda, (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; (6) is D-Glutamic acid and (7) is *N*-methyldehydroalanine.

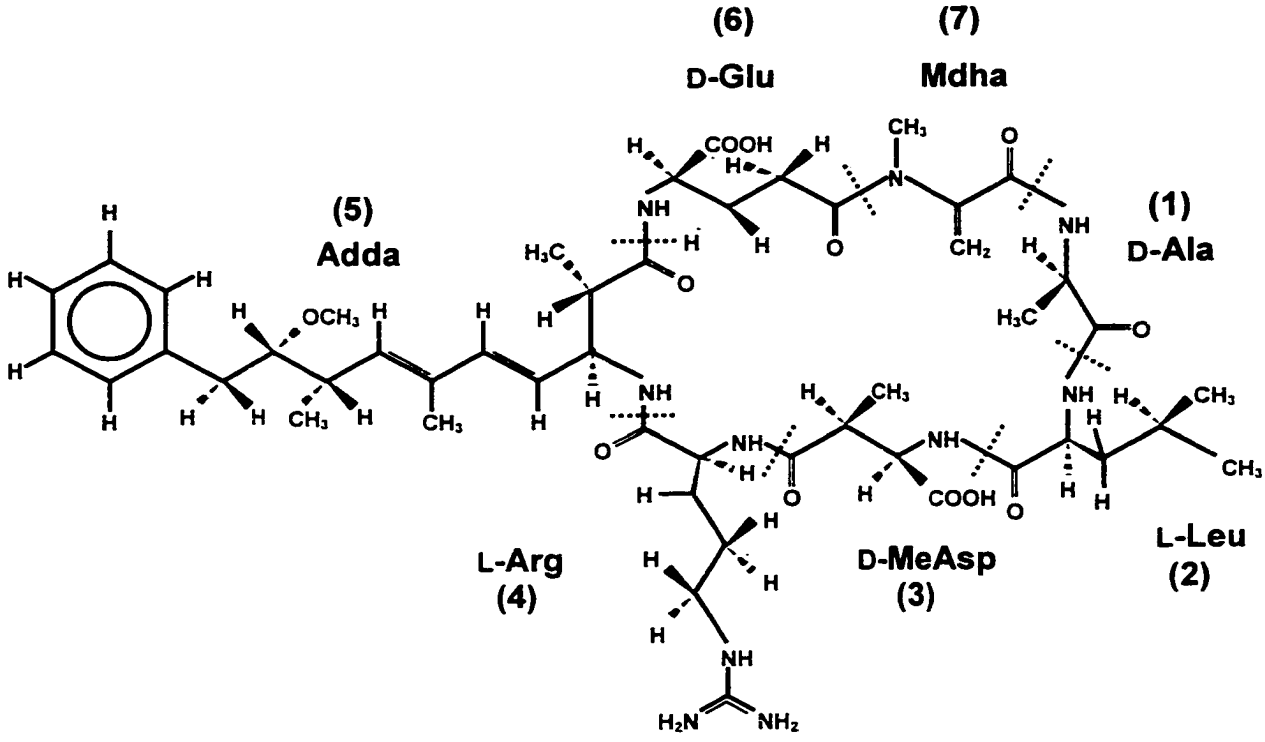

Figure 3. Chemical structure of microcystin-**LR**: where position (1) is D-Alanine; (2) is L-**L**eucine; (3) is D-*erythro*-β-methylaspartic acid; (4) is L-**A**rginine; (5) is Adda; (6) is D-Glutamic acid and (7) is *N*-methyldehydroalanine.

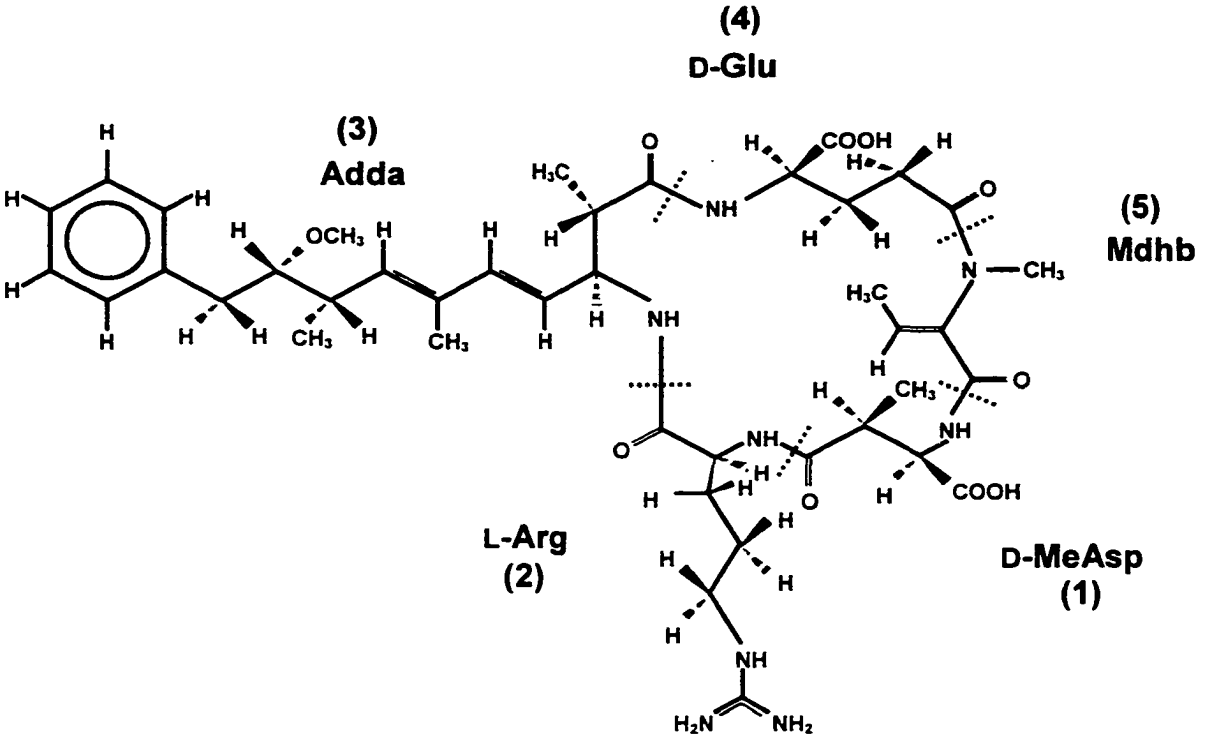

Figure 4. Chemical structure of nodularin: where position (1) is D-*erythro*-β-methylaspartic acid; (2) is L-Arginine; (3) is Adda; (4) is D-Glutamic acid and (5) is *N*-methyldehydrobutyrine.

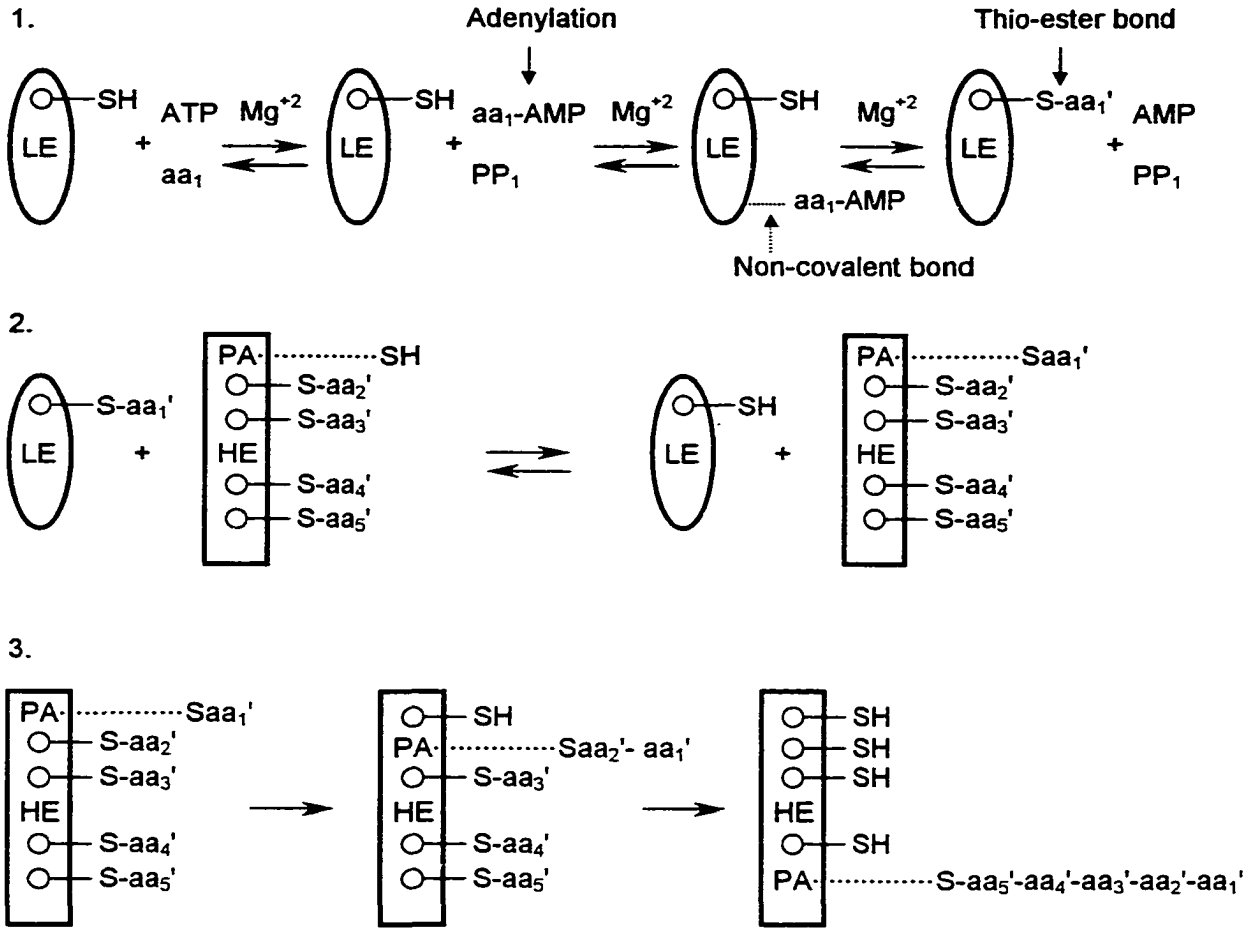

Figure 5. Proposed generalized scheme of multienzymatic thio-template mechanism. Step 1. In the presence of adenosine triphosphate (ATP) and magnesium (Mg^{+2}) substrate amino acid (aa_1) is activated through an adenylation reaction, which hydrolyzes ATP to adenosine monophosphate (AMP) and inorganic pyrophosphate (PP_1). The resulting aminoacyl adenylate (aa_1-AMP) first, binds non-covalently to light enzyme synthetase (LE), then following the transfer of the aminoacyl group (aa_1') from the adenylate to a thiol site (SH) on the enzyme, a covalent thio-ester bond is formed. Step 2. Similar adenylation reactions involving other substrate amino acids occur. These are transferred to heavy enzyme synthetase (HE), where specific aminoacyl groups (aa_2'-aa_5') form thio-ester bonds. Then the light enzyme component passes its aminoacyl group (aa_1') to the thiol site of a 4'-phosphopantetheine arm (PA). Step 3. Peptide elongation is initiated with the movement of PA and attached aminoacyl group (aa_1') to the adjacent module and results in the formation of a peptide bond between the two aminoacyl groups (e.g. aa_1'-aa_2'). Consequently, elongation continues as the arm moves through the various modules on the heavy enzyme. Adapted from Kleinkauf and von Döhren (1990) and Arment and Carmichael (1996).

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Chapter 3

Influence of lake trophic status on the occurrence of microcystin-LR in the tissue of pulmonate snails [1]

1. Introduction

The lakes and reservoirs of Alberta, Canada, often experience severe blooms of cyanobacteria (Kotak *et al.*, 1993). It is well recognized that bloom-forming species and strains of cyanobacteria, commonly of the genera *Microcystis* (Botes *et al.*, 1985), *Anabaena* (Krishnamurthy *et al.*, 1986), *Oscillatoria* (Meriluoto *et al.*, 1989) and *Nostoc* (Namikoshi *et al.*, 1990), can produce potent hepato- (liver) toxins termed microcystins. Of approximately 50 known cyclic heptapeptide variants, microcystin-LR (MCLR), which contains leucine and arginine at two variable amino acid positions, is the most common form in lakes and reservoirs of Alberta (Kotak, 1995). At sublethal levels, MCLR can cause intestinal and liver dysfunction in animals as well as promote liver tumours often leading to severe liver damage and death via intrahepatic haemorrhage and hypovolaemic shock (Carmichael, 1992, 1994; Codd, 1995; Bagu *et al.*, 1997). Consequently, MCLR has been implicated in a number of poisonings of domestic livestock (e. g., cattle, pigs and sheep), pets (e. g., dogs) and wildlife (e. g., deer, ducks and fish) world-wide (Schwimmer and Schwimmer, 1968; Codd, 1995) and more recently, in the deaths of human dialysis patients occupying a Brazilian hospital (Dunn, 1996).

Traditionally, research has focused on the acute toxicity of MCLR to laboratory mammals; for example, mice (Dabholkar and Carmichael, 1987; Robinson *et al.*, 1991) and rats (Eriksson *et al.*, 1989; Miura *et al.*, 1991). Comparatively few studies have examined the occurrence or toxicity of MCLR to other organisms that typically inhabit bloom-prone lakes and reservoirs, yet may occupy vital ecological roles in the food webs of these water bodies.

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Recently, Kotak *et al.* (1996) suggested that MCLR might occur in a variety of aquatic organisms that inhabit hypereutrophic (highly productive), but not oligo-mesotrophic (unproductive), Alberta lakes. Out of the macroinvertebrates examined, MCLR was detected only in herbivorous grazers, including the pulmonate snail, *Lymnaea stagnalis*, *Helisoma trivolvis* and *Physa gyrina*. It was further noted that stomach contents of northern pike, *Esox lucius*, and white sucker, *Catostomus commersoni*, contained appreciable numbers of these gastropods. Since snails are not only an important food source for fish, but also waterfowl (e. g., ducks, geese and shorebirds), crayfish and amphibians (Stein *et al.*, 1984; Lodge, 1986), serious implications exist for the transfer of MCLR to other organisms that typically inhabit bloom-prone waters.

In light of recent findings and considering the general lack of research involving aquatic organisms that may experience toxin-producing cyanobacterial blooms, I conducted a study on the occurrence of MCLR in tissues of resident pulmonate snails from Alberta lakes. My objectives were to determine the extent to which MCLR occurs in tissue of resident pulmonate snails from lakes of varying trophic status and to determine the seasonal variation in toxin concentrations within snail tissue from these lakes. I hypothesized that the concentration of MCLR in the tissue of pulmonate snails is positively related to lake trophic status and varies seasonally in response to changes in the abundance and toxicity of *Microcystis* spp.

2. Materials and Methods

2.1 Study sites

Seven lakes, four located in north-central (*i. e.*, the Baptiste, Narrow, Skeleton and Steele lakes) and three located in central Alberta (*i. e.*, the Coal, Little Beaver and Driedmeat lakes) were sampled every 2 weeks from mid-May through mid-September 1995 (Figure 1). While all were considered hardwater lakes based on the total alkalinity (measured as mg L^{-1} $CaCO_3$), these bodies of water varied dramatically in basin morphometry and the ratio of catchment area to lake surface area (Mitchell and Prepas, 1990). Based on summer total nitrogen (TN), total phosphorus (TP) and chlorophyll-*a* (Chl-*a*) concentrations in the euphotic zone, the lakes represent a cross-section in trophic

status (Table 1) from the oligo-mesotrophic Narrow Lake (lowest potential for toxin production), through the eutrophic Skeleton, Baptiste and Coal lakes, to the hypereutrophic Steele, Little Beaver and Driedmeat lakes (greatest potential for toxin production). Except for Narrow Lake, all of the study lakes have a documented history of cyanobacterial blooms of varying magnitudes (Kotak *et al.*, 1993, 1995, 1996).

2.2 Environmental variables

A variety of physical (water temperature and transparency), chemical (total phosphorus, total nitrogen and total aqueous microcystin concentration) and biological (phytoplankton biomass and species composition) variables were measured for each sample date. Physical parameters were measured *in situ* at one site on each lake. Temperature was measured with a Flett model MK II electronic thermistor (accurate to 0.1°C) at the surface and every 1-m depth interval until the sediment was reached. Water transparency was estimated with a Secchi disk. For chemical and biological parameters, an integrated water sample was collected from the surface to a depth of 2 m (except Little Beaver Lake, which was sampled from the surface to a depth of 1 m) with Tygon™ tubing (Fisher Scientific Ltd. Nepean, ON, Canada) fitted with a one-way valve on the subsurface end. Four samples, taken along a transect of the lake length, were then combined to yield a composite integrated water sample used for analysis. Total phosphorus concentrations were determined colorimetrically following potassium persulphate digestion (Menzel and Corwin, 1965) as modified by Prepas and Rigler (1982), except that particles larger than 250 μm were not filtered out. Total dissolved- and total particulate nitrogen were measured and total nitrogen calculated as described by Stainton *et al.* (1977). A subsample (≈ 7 mL) of water was filtered through a pre-rinsed, 0.45 μm Millipore HA filter (Millipore Corp., Bedford, MA, U.S.A.) and frozen for subsequent analysis of total aqueous microcystin concentration by the protein phosphatase (PP1c) assay (Luu *et al.*, 1993). Phytoplankton biomass was estimated by determining the Chl-*a* concentration of the composite, integrated water samples (in triplicate). Subsamples were filtered onto 43 mm Whatman GF/C filters (Whatman Inc., Fairfield, NJ, U.S.A.), extracted in 95% ethanol and measured spectrophotometrically as

described in Bergmann and Peters (1980). Phytoplankton community composition was determined via enumeration with an inverted light microscope (Prescott, 1978).

2.3 Phytoplankton

Phytoplankton for the determination of cellular MCLR concentration was obtained at the same four locations as the water samples. At each site, phytoplankton was collected from the surface to a depth of 2 m (surface to 1 m for Little Beaver Lake) and concentrated with repeated vertical hauls of a 64 μm mesh (Nitex™) plankton net. For each lake, material from the four sites was combined and excess water removed by placing the sample in a acrylic cylinder (100 mm in diameter, 120 mm in height) covered by 64 μm mesh (Nitex™) at one end. The resulting sample was immediately frozen upon arrival at the laboratory.

2.4 Gastropods

Resident pulmonate snails were collected with a mesh dip net from the littoral zone, usually in association with dense growths of pondweed [*Potamogeton pectinatus* L. and *P. richardsonii* (Benn.) Rydb.], coontail (*Ceratophyllum demersum* L.), bullrush (*Scirpus* sp.) and occasionally, yellow water lily (*Nuphar variegatum* Engelm.). Once collected, snails were placed on ice to reduce metabolic activity (digestion). Upon arrival at the laboratory, snails were frozen. Snails were identified to the subspecies based on descriptions of shell morphology by Clarke (1981) and Clifford (1993). Although three gastropod species were common in the seven study lakes, often not enough tissue was collected for MCLR analysis. Consequently, not all of the species are represented for each sampling date (Figure 5).

2.5 Microcystin-LR analysis

Frozen phytoplankton and gastropods (excluding shell) were lyophilized and the tissues homogenized with mortar and pestle. Approximately 100 mg (dry weight) of tissue homogenate was placed in a 20 mL glass vial to which 10 mL of 100% methanol was added. The sample was sonicated for 20 s to disrupt the cells, then left to settle overnight. The liquid portion was carefully decanted and filtered through a Whatman

GF/A filter, then placed in a glass vial. An additional 5 mL of 100% methanol was added to the sample solids and resuspended by mixing. After settling overnight, the sample was again mixed and filtered through a Whatman GF/A filter. The resulting two extracts were then combined and evaporated under a constant stream of nitrogen gas with a Reactivap™ heater. The semipurified extract was resuspended in 2 mL of 100% methanol and analyzed for MCLR concentration by reverse-phase, high-performance liquid chromatography (HPLC). The separation was performed with a Waters LC Module 1 HPLC equipped with a 150 mm NovaPak C-18 reverse-phase column (Waters Ltd., Mississaga, ON, Canada). Microcystin-LR was eluted under linear gradient conditions of 20 to 30% acetonitrile in 10 mM ammonium acetate over 10 min at a flow rate of 1 mL min^{-1}. Identification and quantification of MCLR was based on UV absorbance at 238 nm with subsequent comparison of retention times and peak areas, respectively, with a MCLR standard (Calbiochem-Novabiochem Corp, La Jolla, CA, U.S.A.). Given these conditions, the detection limit of the HPLC system was ≈ 1 ng per 20 μL injection.

2.6 Statistical analysis

Correlation analyses were performed with the SPSS for Windows (Version 7.5) statistical software (SPSS Inc., Chicago, IL, U.S.A.). All variables were log-transformed (except relative *Microcystis* spp. abundance data which were arcsine-square root transformed) to normalize the data.

3. Results

3.1 Phytoplankton community composition, biovolume and abundance

The relative biovolume of phytoplankton phyla (*i. e.*, biovolume for each phyla expressed as a percentage of the total μm^3 mL^{-1} for all phyla), varied within and among lakes throughout the sampling period (Figure 2). In general, the phytoplankton community in the least productive system (*i. e.*, the oligo-mesotrophic Narrow Lake) was dominated by diatoms, chrysophytes and cryptophytes through the months of May and June, yielding somewhat to dinoflagellates and chlorophytes through July and August.

Although cyanophytes were present throughout the sampling period, these comprised a relatively small proportion of the total phytoplankton biovolume (Figure 2a). However, with increasing productivity levels, cyanophytes constituted a greater proportion of the phytoplankton community, often dominating throughout much of the sampling period (Figure 2b-g). Similarly, the relative abundance of *Microcystis* spp. (*i. e.*, the number of *Microcystis* cells expressed as a percentage of the total number of cells mL^{-1} for all species) increased with increasing trophic status. *Microcystis* spp. often comprised a large proportion of the phytoplankton community within the highly productive lakes (*i. e.*, Steele, Little Beaver and Driedmeat lakes; Figure 3e-g), but less important in the moderately productive systems (*i. e.*, Skeleton, Baptiste and Coal lakes; Figure 3b-d) and essentially non-existent in Narrow Lake (Figure 3a). Considering all seven lakes and all nine sampling dates together, total phytoplankton, total cyanophyte and *Microcystis* spp. abundance (expressed as cells mL^{-1}) were associated with measures of lake trophic status (Table 2).

3.2 Microcystin-LR in phytoplankton

The cellular concentration of MCLR in the phytoplankton varied between and within lakes throughout the summer months. Narrow Lake, which only contained a relatively small amount of *Microcystis* spp. ($\approx$ 3%) on one sampling date (26 June 1995), had no detectable concentrations of MCLR throughout the study (Figure 3a). In contrast, phytoplankton from the eutrophic Skeleton, Baptiste and Coal lakes (Figure 3b-d) contained moderate toxin concentrations (213, 212 and 285 $\mu g\ g^{-1}$, respectively), while the hypereutrophic Steele, Little Beaver and Driedmeat lakes (Figure 3e-g) experienced the highest levels (974, 1278 and 1526 $\mu g\ g^{-1}$, respectively). Furthermore, the concentration of MCLR in phytoplankton (expressed as μg MCLR g^{-1} phytoplankton) was positively correlated with both absolute and relative abundance of *Microcystis* spp., and was expectedly associated with measures of trophic status (Table 3).

3.3 Microcystin-LR in water

The total aqueous microcystin concentration, expressed as μg MCLR equivalents L^{-1} lake water, also varied between and within lakes through the sampling period. In

general, the frequency of detectable concentrations of total aqueous microcystin increased with increasing trophic status since it was positively correlated with *Microcystis* spp. abundance and the concentration of MCLR in the phytoplankton (Table 3). For example, microcystin was never detected in Narrow Lake (Figure 4a), while Skeleton, Baptiste and Coal lakes (Figure 4b-d), occasionally had low toxin concentrations (0.1 to 0.4 $\mu g\ L^{-1}$) of toxin with the exception of Coal Lake reaching 1.3 $\mu g\ L^{-1}$ on 19 September 1995. In contrast, Steele, Little Beaver and Driedmeat lakes (Figure 4e-g) regularly experienced higher concentrations (up to 1.2 $\mu g\ L^{-1}$) of aqueous microcystin.

3.4 Microcystin-LR in gastropods

The three species of gastropod collected from the study lakes were the common pond snail, *Lymnaea stagnalis jugularis* (Say), the ramshorn snail, *Helisoma trivolvis subcrenatum* (Carpenter), and the tadpole snail, *Physa gyrina gyrina* (Say). In general, MCLR concentrations in the tissue of these species followed changes in the toxin content of the phytoplankton since the variables were positively correlated (Table 4). Additionally, the concentration of toxin within the tissue of *L. stagnalis* and *P. gyrina* was also positively associated with the relative abundance of *Microcystis* spp. As a consequence, MCLR was detected more often and at greater concentrations in gastropods from highly productive lakes than those from lower productivity (Figure 5). For instance, gastropods from Narrow Lake contained no detectable concentrations of MCLR during the study (Figure 5a). Similarly, Baptiste Lake, which contained relatively few *Microcystis* spp. and low concentrations of MCLR in the phytoplankton (Figure 3c), experienced detectable toxin levels in tissue of gastropods on only one occasion (Figure 5c). However, snails collected from the eutrophic Skeleton and Coal lakes (Figure 5b, d, respectively), experienced low to moderate toxin concentrations (up to 77 $\mu g\ g^{-1}$) during periods of increased *Microcystis* spp. abundance and toxin within the phytoplankton (Figure 3b, d, respectively). Except for Little Beaver Lake (Figure 5f) where few samples were sufficient for MCLR analysis (about 50 mg freeze-dried weight excluding shell), snail tissue from the hypereutrophic lakes contained the greatest levels of MCLR (up to 140 and 129 $\mu g\ g^{-1}$ in Steele and Driedmeat lakes; Figure 5e, g, respectively).

This typically occurred during or immediately subsequent to periods of increased *Microcystis* spp. abundance and toxin within the phytoplankton. Finally, for all three gastropod species, the concentration of MCLR in tissue was not associated with aqueous total microcystin concentration of the lake water (Table 4).

4. Discussion

These results indicate that phytoplankton community composition is influenced by lake trophic status, since total phytoplankton, total cyanophyte and *Microcystis* spp. abundance were associated with measures of lake trophic status (Table 2). Although other genera of cyanobacteria (*i. e.*, *Anabaena*, *Oscillatoria* and *Nostoc*) produce MCLR in waterbodies world-wide, Kotak (1995) concluded that *Microcystis* spp. are responsible for toxins occurring within lakes and reservoirs of Alberta. Consequently, the concentration of MCLR in the phytoplankton varied within each lake throughout the study (Figure 3), probably in response to fluctuations in the relative and the absolute abundance of *Microcystis* spp. As a result, not only did phytoplankton from the hypereutrophic lakes (*i. e.*, Steele, Little Beaver and Driedmeat lakes) contain detectable levels of toxin more often than less eutrophic lakes (*i. e.*, Coal, Skeleton, Baptiste and Narrow lakes), but concentrations in the former were an order of magnitude greater than the latter. Thus, the potential for an oligo- or mesotrophic lake to experience toxin-producing blooms of *Microcystis* spp. during the summer months is relatively low compared to eutrophic and hypereutrophic lakes. These findings are consistent with those of Kotak *et al.* (1993, 1995), who demonstrated that trophic status influenced the concentration of MCLR in phytoplankton by regulating the occurrence of *Microcystis* spp. in several central and north-central Alberta lakes.

Aqueous microcystin, the fraction of which is typically released by *Microcystis* cells during natural senescence, was detected more often in the hypereutrophic lakes than the others probably because of its association with the absolute abundance of *Microcystis* spp. and the concentration of MCLR in the phytoplankton. Unlike toxin in the phytoplankton, these concentrations were highly variable throughout the sampling period within each lake. This probably results from factors that alter toxin availability and/or

stability and include algal growth phase, toxin degradation (microbial and photolytic) and dilution (Tsuji *et al.*, 1994; Lam *et al.*, 1995).

The concentration of toxin in the tissue of resident pulmonate snails was highly variable, both within (seasonally) and among lakes (Figure 5). Although fluctuations generally followed changes in the toxin content of the phytoplankton for all three gastropod species, differences in the strength of this association were evident (Table 4). Additionally, the concentrations of toxin in the tissue of *L. stagnalis* and *P. gyrina* were also associated with the relative abundance of *Microcystis* spp.; however, this was not the case for *H. trivolvis*. Albeit speculative, several possibilities may explain this lack of correlation. For instance, pulmonate snails are considered indiscriminate grazers that feed on available periphytic algae (Elder and Collins, 1991). While *Microcystis* spp. are primarily planktonic, they do become entrapped in dense macrophytes and settle on rocks and sediments during bloom events. If the snail species sampled are indeed indiscriminate, then their diets should reflect the relative availability (abundance) of various algal species present. However, this would not be the case for snails able to avoid toxin-producing algal cells, either by discriminating between and selectively ingesting other algal species (or alternative food sources such as macrophytes or detritus), egesting *Microcystis* spp., or altering ingestion rates. Alternatively, the extracellular fraction represents a second possible route for the exposure of gastropods to MCLR. Gastropods are known to take up various dissolved compounds from the aquatic environment by drinking (Thomas *et al.*, 1990) or during normal feeding since the intake of water is a consequence of the bite-swallow cycle (DeWith, 1996). Also, dissolved and particulate matter may be taken up through the epidermis through respiration, osmoregulation and digestion (Zylstra, 1972). Certainly, gastropods inhabiting hypereutrophic lakes such as Steele, Little Beaver and Driedmeat would, at times, be subject to dissolved MCLR (up to 1.3 $\mu g\ L^{-1}$ in the present study; Figure 4). Whether snails can avoid or restrict the uptake of dissolved toxin from the aquatic environment is not currently known. Nevertheless, the great seasonal variability and lack of association with tissue concentration of the three gastropod species precludes the dissolved fraction as the primary avenue for exposure to MCLR. Likewise, studies with the freshwater clam, *Anodonta grandis simpsoniana* (Prepas *et al.*, 1997) and the blue mussel, *Mytilus*

edulis (Andrasi, 1985; Novaczek *et al.*, 1991), reported far greater uptake of algal toxins through diet rather than through aqueous routes.

Other factors probably also contributed to the inter- and intraspecific variation in MCLR concentration of gastropods. Foremost is the distribution and previous exposure of gastropods to toxic phytoplankton. Phytoplankton community composition and abundance (and thus toxin concentration) were spatially (vertically and horizontally) and temporally variable within a lake (Kotak *et al.*, 1995). Indeed, Carmichael and Gorham (1981) attributed spatial variation in bloom toxicity in Hastings Lake, Alberta, to spatial differences not only in species composition, but also in relative proportions of toxic and nontoxic cyanobacterial strains within the same species. Secondly, gastropods, which are known to feed almost continuously day and night (Veldhuijzen, 1974), may move both vertically (self-regulation of buoyancy) and horizontally (wave action) about the water column. Together, these factors may contribute to variation in toxin concentration between and within sites. Finally, the fact that phytoplankton were collected and combined at four points along transects of a lake's length, while gastropods nearer the shoreline, may also contribute to the variation found in the present study.

Periodic observations of gastropod faecal strings from *L. stagnalis* collected from Driedmeat Lake often revealed whole and partially intact colonies of *Microcystis* spp. comprising the gizzard fraction. This portion (in the case of *L. stagnalis* at least) of the faeces composed of indigestible matter, forms when food is passed from the gizzard into the pro-intestine without further digestion in the caecum or digestive gland (Carriker, 1946). Hence, the presence of intact *Microcystis* spp. colonies suggests the crop and gizzard fail to mechanically disrupt these cells. Since MCLR is an endotoxin typically released during cell lysis, insufficient comminution of *Microcystis* colonies would limit the uptake of toxin by the digestive system. Additionally, Carriker (1946) noted that the pulmonate snail *Lymnaea stagnalis* is known to ingest excreted faecal strings, even in the presence of fresh food, since some nourishment is derived from them. It is unclear whether small fragments (individual cells), and/or re-ingested cells and colonies are mechanically processed by these organs, and then further digested in the caecum or digestive gland by enzymatic means. Since snails were immediately cooled then frozen

upon collection, gut contents were not eliminated. Consequently, any toxin detected was probably derived from *Microcystis* cells/colonies contained in the alimentary system.

5. Conclusion

The present study identifies the concentration of the toxin within the phytoplankton and composition of the phytoplankton community (*i. e.*, abundance of *Microcystis* spp.), as influenced by lake trophic status, paramount in determining seasonal variation of toxin within pulmonate snail tissue. Except for the most severe and abrupt cyanobacterial die-offs following extreme bloom events, the extracellular toxin fraction probably contributes minimally to MCLR levels within the tissue of gastropods. Species-specific differences may also exist in the response of pulmonate snails to MCLR. Finally, the presence of intact colonies within the alimentary tract of snails is of interest when considering food web interactions, and since predators typically ingest whole organisms, snails may be regarded as a direct source of toxin-containing *Microcystis* cells to higher organisms that would otherwise not ingest cyanobacteria. In view of my findings and given their importance as food sources for many aquatic and terrestrial organisms, it seems that pulmonate snails play an important role in the occurrence and fate of MCLR in the aquatic environment.

Table 1. Water quality and morphometric characteristics of the seven study lakes. Results are presented as means; ± *SE*

Variable	Lake						
	Narrow	Skeleton*	Baptiste	Coal	Steele	Little Beaver	Driedmeat
Maximum depth (m) †	38.0	11.0	27.5	5.5	6.1	2.0	3.7
Catchment:lake surface area ratio †	6	4	29	114	38	‡	437
Total alkalinity (mg L^{-1} $CaCO_3$) †	155 ± 1.0	178 ± 0.6	164 ± 3.0	201 ± 2.2	123 ± 20.7	332 ± 11.0	219 ± 3.9
Total nitrogen (μg L^{-1})	537 ± 18	1274 ± 84	1380 ± 131	1448 ± 108	1591 ± 127	3769 ± 176	2213 ± 223
Total phosphorus (μg L^{-1})	11 ± 1	42 ± 3	57 ± 7	71 ± 8	109 ± 12	207 ± 11	401 ± 64
Total nitrogen:total phosphorus ratio	51 ± 4	31 ± 1	25 ± 2	22 ± 1	15 ± 1	18 ± 1	7 ± 1
Chlorophyll-*a* (μg L^{-1})	1 ± 0	17 ± 4	30 ± 7	23 ± 5	28 ± 9	55 ± 8	61 ± 10
Secchi depth (m)	7.06 ± 0.25	1.84 ± 0.22	1.27 ± 0.19	1.53 ± 0.15	2.01 ± 0.36	0.47 ± 0.09	0.64 ± 0.11

* Values for Skeleton Lake south basin only.

† Morphometric characteristics (maximum depth and catchment:lake surface area ratio) and total alkalinity were obtained from Mitchell and Prepas (1990) and Kotak *et al.* (1993).

‡ Unknown value.

Table 2. Summary of correlation analyses for total phytoplankton, total cyanophyte and *Microcystis* spp. abundance (cells mL^{-1}) versus measures of lake trophic status. Correlation analyses were performed on log-log transformed data from the seven study lakes on nine sampling dates (degrees of freedom = 60)

	Total phytoplankton		Total cyanophyte		*Microcystis* spp.	
Measures of lake trophic status	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value
Total nitrogen ($\mu g\ L^{-1}$)	0.88	*	0.86	*	0.85	*
Total phosphorus ($\mu g\ L^{-1}$)	0.79	*	0.78	*	0.81	*
Total nitrogen:total phosphorus ratio	-0.62	*	-0.55	*	-0.61	*
Chlorophyll-*a* ($\mu g\ L^{-1}$)	0.91	*	0.90	*	0.85	*
Secchi depth (m)	-0.89	*	-0.87	*	-0.81	*

* $P < 0.00001$.

Table 3. Summary of correlation analyses for microcystin-LR (MCLR) in phytoplankton (μg g^{-1}) and water (μg MCLR eq. L^{-1}) versus absolute and relative *Microcystis* spp. abundance (cells mL^{-1} and percentage, respectively), and various measures of lake trophic status. Correlation analyses were performed on log-log transformed data (except relative *Microcystis* spp. abundance, which were arcsine-square root transformed) from the seven study lakes on nine sampling dates

	MCLR in phytoplankton (μg g^{-1})			MCLR in water (μg MCLR eq. L^{-1})		
Measures of lake trophic status	*r*	*P*-value	df	*r*	*P*-value	df
MCLR in phytoplankton (μg g^{-1})	–	–	–	0.47	0.0001	61
Absolute *Microcystis* spp. abundance (cells mL^{-1})	0.91	*	60	0.41	0.001	60
Relative *Microcystis* spp. abundance (%)	0.71	*	60	–	–	–
Total nitrogen (μg L^{-1})	0.85	*	61	–	–	–
Total phosphorus (μg L^{-1})	0.85	*	61	–	–	–
Total nitrogen:total phosphorus ratio	-0.68	*	61	–	–	–
Chlorophyll-*a* (μg L^{-1})	0.82	*	61	–	–	–
Secchi depth (m)	-0.79	*	61	–	–	–

* $P < 0.00001$.

Table 4. Summary of correlation analyses for the tissue concentration of microcystin-LR (MCLR) in three species of pulmonate snail versus MCLR in phytoplankton, MCLR in water and relative *Microcystis* spp. abundance. Correlation analyses were performed on log-log transformed data (except relative *Microcystis* spp. abundance, which was arcsine-square root transformed) from the seven study lakes on nine sampling dates.

	MCLR in *Lymnaea stagnalis* ($\mu g\ g^{-1}$)			MCLR in *Physa gyrina* ($\mu g\ g^{-1}$)			MCLR in *Helisoma trivolvis* ($\mu g\ g^{-1}$)		
Measures of lake trophic status	*r*	*P*-value	df	*r*	*P*-value	df	*r*	*P*-value	df
MCLR in phytoplankton ($\mu g\ g^{-1}$)	0.50	0.0004	44	0.37	0.03	29	0.41	0.01	31
MCLR in water (μg MCLR eq. L^{-1})	0.16	> 0.29	44	0.14	0.45	29	0.19	> 0.28	31
Relative *Microcystis* spp. abundance (%)	0.49	0.0007	43	0.47	0.008	29	0.22	> 0.22	31

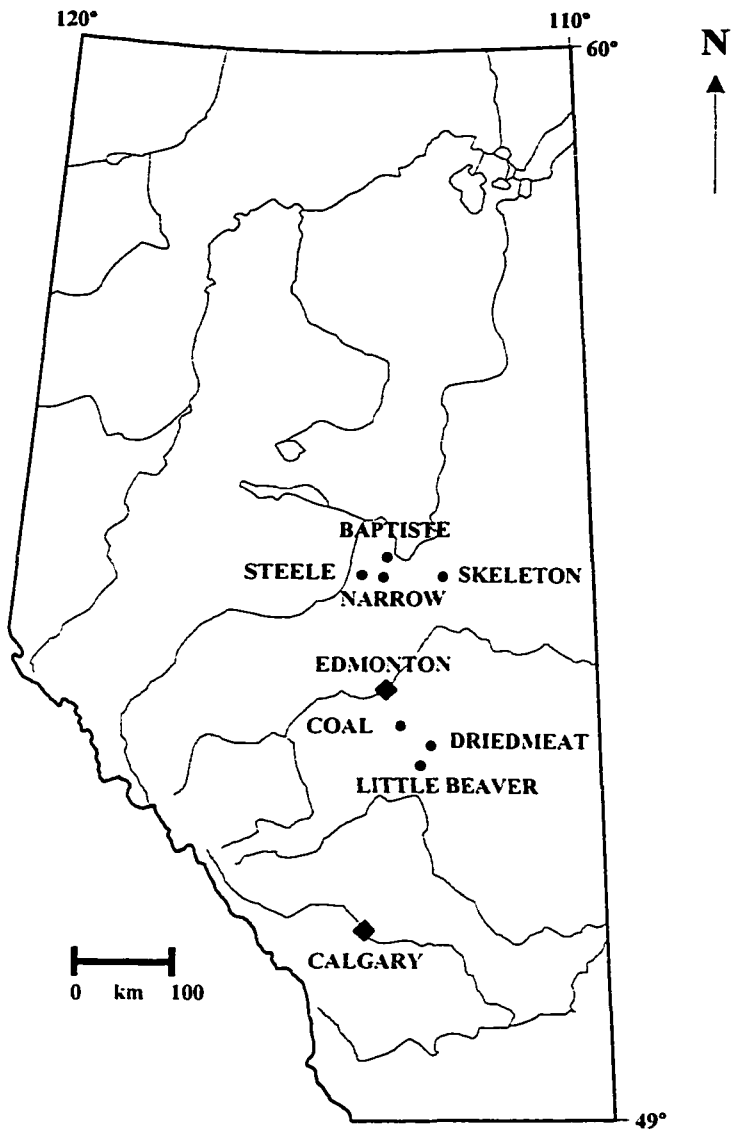

Figure 1. Map of Alberta, Canada showing geographic locations of the seven study lakes.

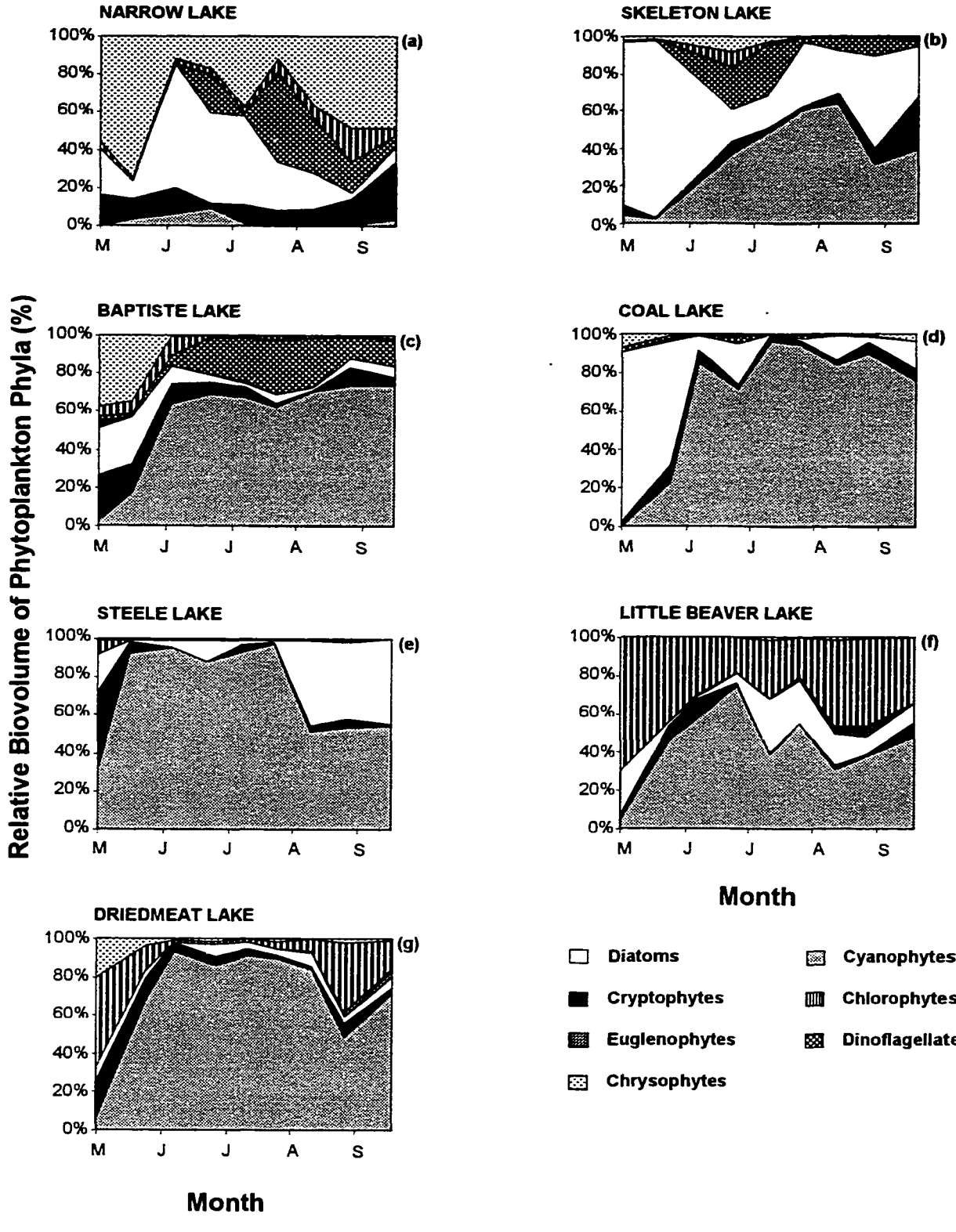

Figure 2. Seasonal changes (mid-May through mid-September 1995) of relative (%) biovolume of phytoplankton phyla in the seven study lakes: (a) Narrow Lake; (b) Skeleton Lake; (c) Baptiste Lake; (d) Coal Lake; (e) Steele Lake; (f) Little Beaver Lake; and (g) Driedmeat Lake.

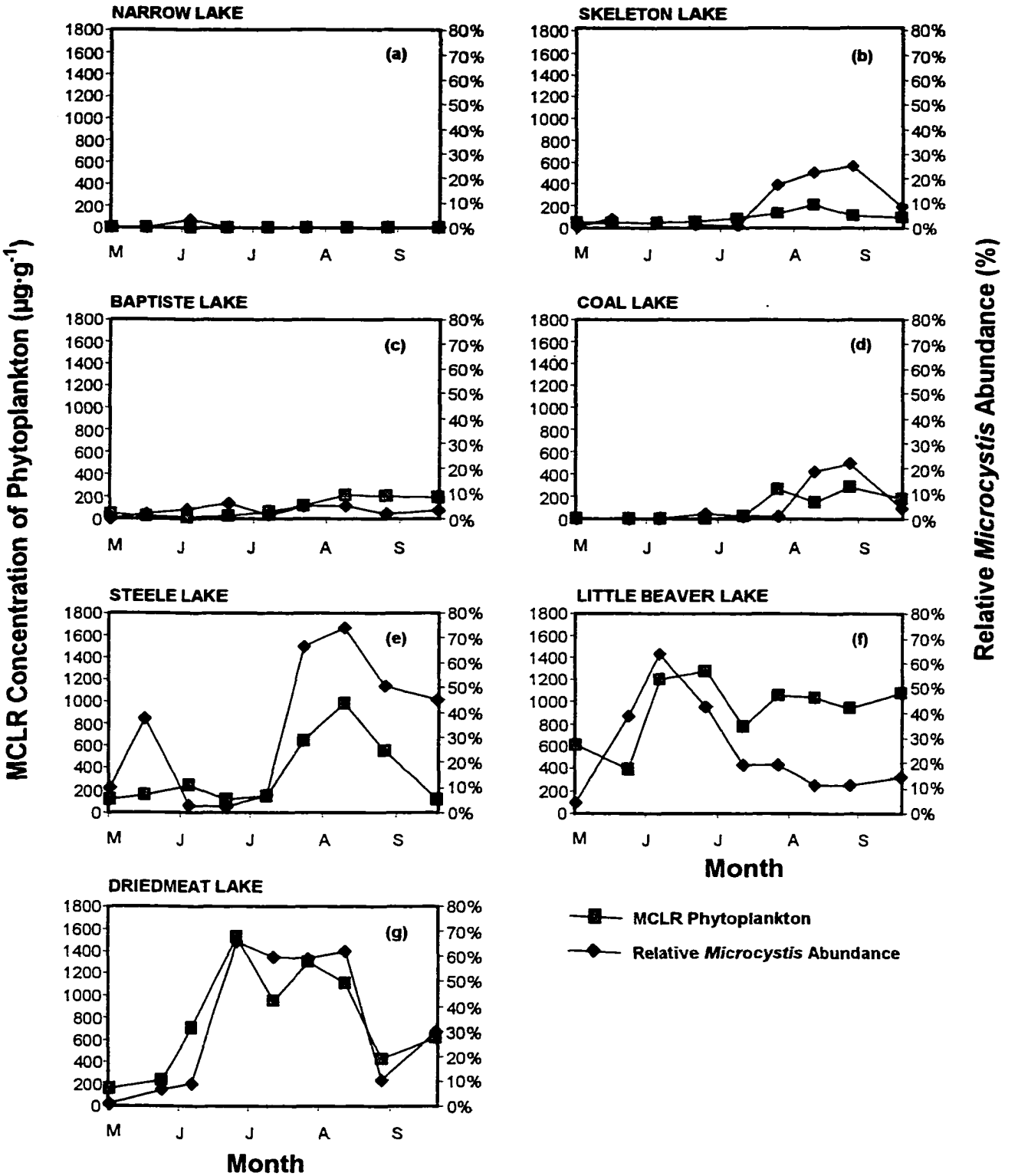

Figure 3. Seasonal changes (mid-May through mid-September 1995) of microcystin-LR (MCLR) concentration in phytoplankton (µg g^{-1}) and relative *Microcystis* spp. abundance (proportion of *Microcystis* spp. cells from total phytoplankton cells mL^{-1}, expressed as a percentage) in the seven study lakes: (a) Narrow Lake; (b) Skeleton Lake; (c) Baptiste Lake; (d) Coal Lake; (e) Steele Lake; (f) Little Beaver Lake; and (g) Driedmeat Lake.

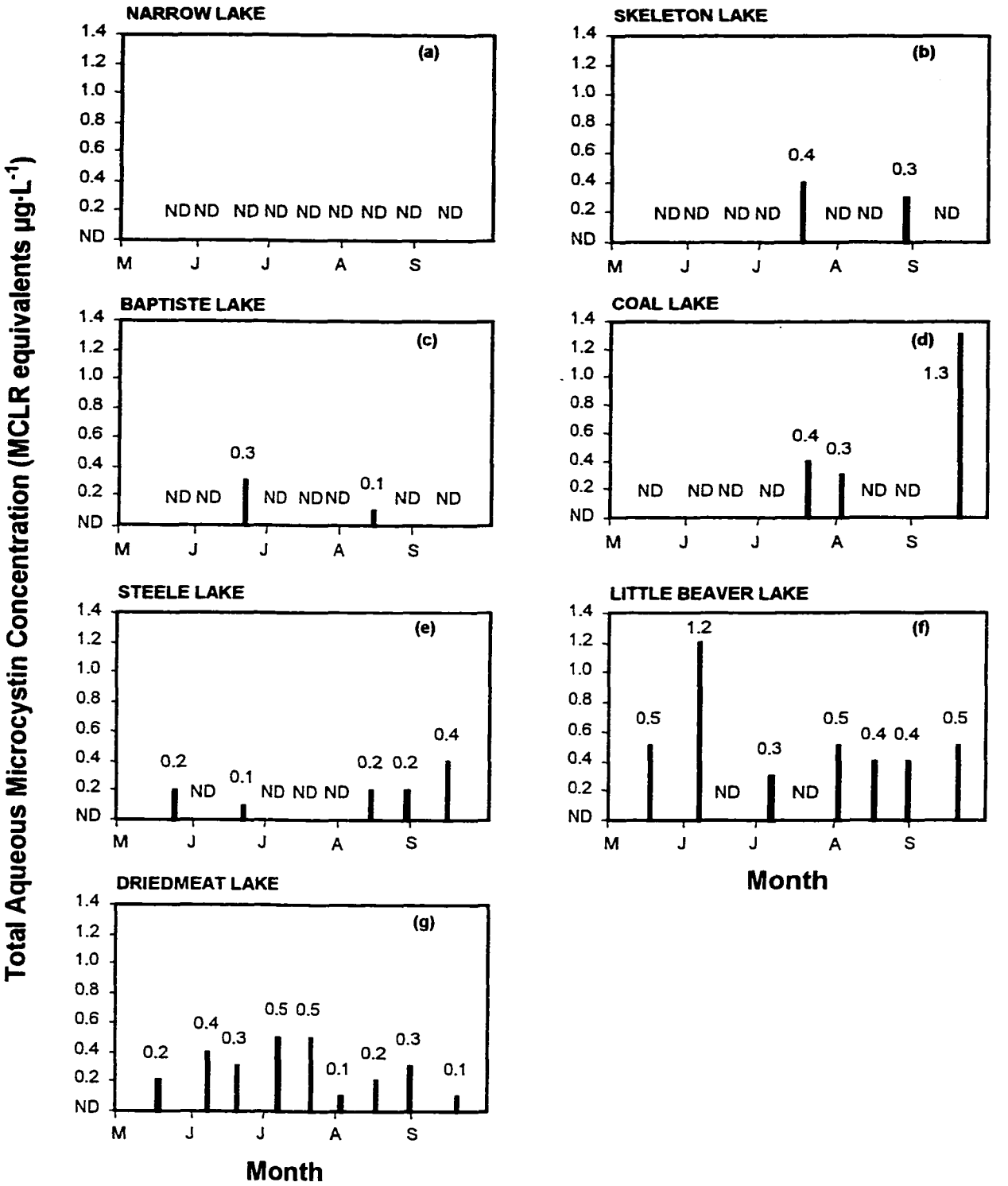

Figure 4. Seasonal changes (mid-May through mid-September 1995) in total aqueous microcystin concentration (expressed as MCLR equivalents µg L^{-1}) for the seven study lakes: (a) Narrow Lake; (b) Skeleton Lake; (c) Baptiste Lake; (d) Coal Lake; (e) Steele Lake; (f) Little Beaver Lake; and (g) Driedmeat Lake. Key: (ND) non-detectable microcystin concentrations.

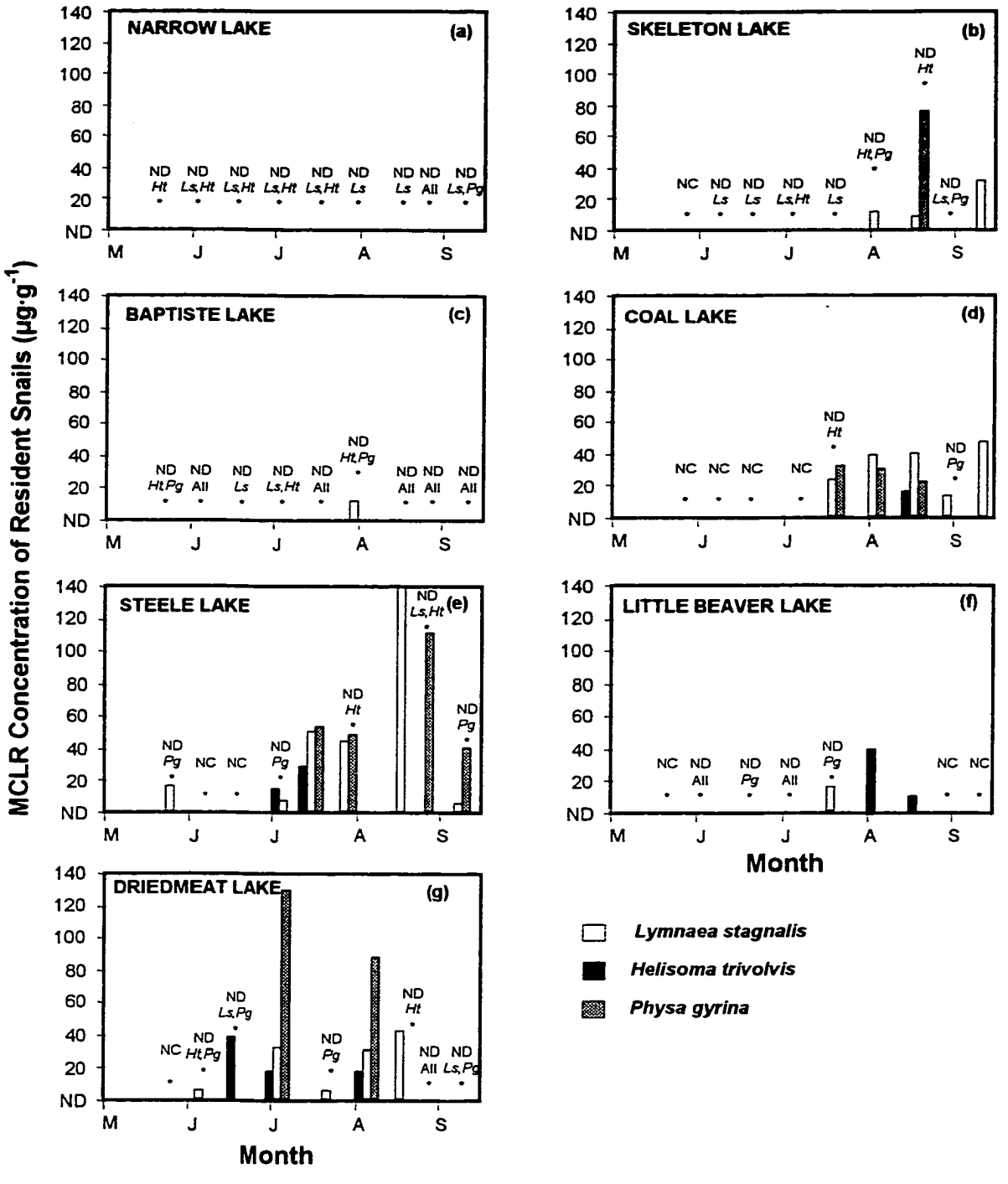

Figure 5. Seasonal changes (mid-May through mid-September 1995) of microcystin-LR (MCLR) concentration (µg g^{-1}) in tissue from resident snail species in the seven study lakes: (a) Narrow Lake; (b) Skeleton Lake; (c) Baptiste Lake; (d) Coal Lake; (e) Steele Lake; (f) Little Beaver Lake; and (g) Driedmeat Lake. Key: (ND) non-detectable MCLR concentrations; (*Ls*) *Lymnaea stagnalis* tissue; (*Ht*) *Helisoma trivolvis* tissue; (*Pg*) *Physa gyrina* tissue; and (NC) either no gastropods collected or insufficient amount of tissue collected for MCLR analysis.

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Chapter 4

Spatial differences in the microcystin in *Lymnaea stagnalis* collected from Hastings Lake, Alberta [2]

1. Introduction

The production of potent toxins by bloom-forming cyanobacteria in eutrophic lakes and reservoirs has been widely reported throughout the world (Carmichael, 1994; Kotak, 1995). In many instances, hepatotoxic microcystins (MCYSTs) have been implicated in sublethal and acute intoxications of domestic livestock (e. g., cattle and sheep), pets (e. g., dogs), wildlife (e. g., waterfowl and fish) and even humans, (Schwimmer and Schwimmer, 1968; Codd, 1995; Dunn, 1996). Historically, research has focused on the acute toxicity of MCYSTs to laboratory mammals (e. g., mice - Dabholkar and Carmichael, 1987; and rats - Miura *et al.*, 1991) and fish (e. g., rainbow trout - Kotak *et al.*, 1996; and carp - Råbergh *et al.*, 1991). Consequently, with the exception of zooplankton (primarily cladocerans, copepods and rotifers), information on the occurrence and toxicity of MCYSTs in most other aquatic organisms that typically inhabit bloom-prone waters is limited (Christoffersen, 1996). Considering the paucity of knowledge, subsequent research evaluating the accumulation and chronic toxicity of MCYSTs in aquatic organisms and the resulting food web implications are warranted.

Several contemporary studies have illustrated the occurrence of MCYSTs in macroinvertebrates collected from eutrophic freshwater environments and include the gastropods *Lymnaea stagnalis*, *Helisoma trivolvis* and *Physa gyrina* (Kotak *et al.*, 1996b) and the bivalves *Anodonta grandis* (Prepas *et al.*, 1997), *Anodonta woodiana* and *Unio douglasiae* (Watanabe *et al.*, 1997). Conjointly with herbivorous zooplankton, such as *Daphnia magna*, macroinvertebrates have been implicated in the transfer of MCYSTs to various compartments within aquatic food webs (Thostrup and Christoffersen, 1999).

[2] A version of this chapter will be submitted as Zurawell R.W., Earle, J.C., Holmes C.F.B. and Prepas E.E. (submitted a) Spatial differences in the microcystin in *Lymnaea stagnalis* collected from Hastings Lake, Alberta.

Recently, Zurawell *et al.* (1999) confirmed earlier suppositions made by Kotak *et al.* (1996b) identifying the toxin concentration of the phytoplankton community, as influenced by the relative abundance and biomass of *Microcystis* spp., "paramount in determining seasonal variation of toxin within pulmonate snail tissue". While MCYST concentrations within the tissue of *L. stagnalis* generally reflected changes in the toxin content of the phytoplankton ($P < 0.001$) and the relative abundance ($P < 0.001$) and biomass ($P = 0.015$) of *Microcystis* spp., considerable disparity among individuals was evident (Zurawell *et al.*, 1999). Consequently, several factors contributing to the heterogeneity in tissue toxin concentration of resident pulmonate snails were proposed. Foremost, spatial differences in phytoplankton community composition (*i. e.*, relative abundance and biomass of *Microcystis* spp.) and thus toxin concentration within phytoplankton, may account for much of this variation, since snails were collected from more than one location within each study lake on any given sampling date (Zurawell *et al.*, 1999). Indeed, Carmichael and Gorham (1981) attributed spatial variation in bloom toxicity in Hastings Lake, Alberta, to spatial differences not only in species composition, but also in relative proportions of toxic and nontoxic cyanobacterial strains within the same species. Presuming pulmonate snails are indiscriminate grazers (Elder and Collins, 1991) and their diets, thus reflect the relative availability (biomass) of various algal species present, the concentration of toxin within tissue of these organisms may vary spatially as well.

Based on these findings, I investigated the spatial heterogeneity in MCYST concentration of the common pond snail *Lymnaea stagnalis jugularis* (Say) collected from Hastings Lake, Alberta, Canada. Objectives of the study were to determine the spatial differences in phytoplankton community composition (*i. e.*, relative biomass of MCYST-producing cyanobacteria) and toxicity and hence, MCYST levels of *L. stagnalis* collected from ten littoral sites within a eutrophic lake experiencing toxic cyanobacterial blooms. It is hypothesized that MCYST concentrations in *L. stagnalis* differ between littoral sites as a consequence of spatial variation in *Microcystis* spp. biomass, as well as the concentration of toxin within phytoplankton.

2. *Materials and Methods*

2.1 Study sites

This study was conducted on September 17, 1999 and involved ten sites (each approximately 5 m^2 in area) chosen at regular intervals along the littoral zone of Hastings Lake (Figure 1). Located in the Boreal Mixedwood Ecoregion of central Alberta, Canada, Hastings Lake is a shallow, medium-sized, hardwater lake (Table 1) that remains well mixed throughout open-water season becoming thermally stratified only intermittently during calm periods (Mitchell and Prepas, 1990). The surrounding drainage basin and consequently, the catchment area to lake surface area ratio are large (31; Table 1) and characterized by a history of both natural and anthropogenic disturbance (e. g., forest fire, timber harvesting, agriculture and residential development). Accordingly, based on summer total nitrogen (TN), total phosphorus (TP), TN:TP ratio and chlorophyll-*a* (Chl-*a*) concentrations in the euphotic zone (Table 1), the lake is considered hypereutrophic and consequently, subject to severe surface accumulations of cyanobacteria often dominated by toxin-producing *Microcystis aeruginosa* (Carmichael and Gorham, 1981).

2.2 Environmental variables

For chemical (chlorophyll-*a* and aqueous microcystin concentrations) and biological (phytoplankton species composition and biomass) parameters, an integrated water sample was collected from the surface to a depth of 1 m with Tygon™ tubing (Fisher Scientific Ltd., Nepean, ON, Canada) fitted with a one-way valve on the subsurface end. A subsample (≈ 7 mL) of water was filtered through a pre-rinsed, 0.45 μm Millipore HA filter (Millipore Corp., Bedford, MA, U.S.A.) and frozen for subsequent analysis of total aqueous MCYST concentration by the protein phosphatase (PP) inhibition assay (below). Phytoplankton biomass was estimated by determining the Chl-*a* concentration of the integrated water samples (in triplicate). Subsamples were filtered onto 43 mm diameter Whatman GF/C filters (Whatman Inc., Fairfield, NJ, U.S.A.), extracted in 95% ethanol and measured spectrophotometrically as described in Bergmann and Peters (1980).

2.3 Phytoplankton

Cyanobacteria community composition (*i. e.*, species identification and biomass) was determined on samples collected in 100-mL amber bottles. A 2.5 mL subsample of each was settled onto glass slides and the number of cells directly enumerated with use of an inverted light microscope. Species biomass was estimated by determining the biovolume and mean number of cells from 15 randomly selected fields of view as described by Prescott (1978). Phytoplankton for the determination of cellular MCYST concentration was collected and concentrated at each site with repeated vertical hauls of a 64 μm mesh (Nitex™) plankton net from the surface to a depth of 1 m. Water was further removed by placing the sample in a acrylic cylinder (100 mm in diameter, 120 mm in height) covered by 64 μm mesh (Nitex™) at one end. The resulting phytoplankton paste was placed in 150-mL specimen cups and immediately frozen upon arrival at the laboratory.

2.4 Gastropods

Ten adult *Lymnaea stagnalis jugularis* (Say) were randomly collected from a 1 m^2 area with a mesh dip net from each site, usually in association with dense growths of pondweed [*Potamogeton pectinatus* L. and *P. richardsonii* (Benn.) Rydb.], coontail (*Ceratophyllum demersum* L.) and bullrush (*Scirpus* sp.). Snail species identification was based on the shell morphology descriptions of Clarke (1981) and Clifford (1993). Once collected, snails were placed on dry ice to reduce metabolic activity (digestion).

2.5 Microcystin analysis

Frozen phytoplankton were lyophilized then homogenized with mortar and pestle. Approximately 100 mg (dry weight) phytoplankton was placed in a 20 mL glass vial to which 10 mL of 100% methanol was added. Each sample was sonicated for 20 s to disrupt the cells, then left to settle overnight. Next day, an additional 5 mL of 100% methanol was added and samples resuspended by mixing. After settling overnight, the liquid portion of each was carefully decanted and filtered through a Whatman GF/A filter, then placed in a glass vial. The resulting extracts were evaporated to dryness under a constant stream of nitrogen gas with a Reactivap™ heater and then resuspended in 4

mL of ultra pure water. The semi-purified extracts were analyzed for MCYST concentration via colorimetric PP inhibition assay as specified by An and Carmichael (1994), utilizing recombinant PP1c expressed in *E. coli* (provided by C.F.B. Holmes). Gastropods (excluding shell) were processed similar to phytoplankton. However, a single 10 mL addition of 100% methanol was combined with the entire mass of soft tissue and sonicated for 20 s with no additional methanol added the following day.

Microcystin concentrations of both gastropod and phytoplankton extracts were extrapolated from curves plotting PP1c inhibition by microcystin-LR (MCLR) standards (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and are thus expressed as mass MCLR equivalents g^{-1} tissue (dry weight). The detection limit of this assay was approximately 3 ng MCLR eq. g^{-1} tissue. While this assay quantifies all MCYSTs capable of inhibiting PP1c, toxin fractions irreversibly bound to endogenous PP1c and PP2A within gastropod tissues would be unavailable for further PP1c inhibition and consequently remain undetected. Hence, the MCYST values reported underestimate the actual total toxin concentrations within tissues.

2.6 Statistical analysis

Species composition (*Microcystis* spp. biomass), Chl-*a* and toxin (aqueous MCYST, toxin concentrations in phytoplankton and mean MCYST concentrations in *L. stagnalis*) data were transformed with the Box-Cox procedure to eliminate heteroscedasticity and improve normality (Sokal and Rohlf, 1995). Mean toxin concentrations in *L. stagnalis* collected from the ten sites were initially regressed on body tissue dry weight to identify statistical confounding due to physiological variation within the snail population of Hastings Lake. Subsequent to the identification of weight as a significant covariate, mean toxin concentrations in *L. stagnalis* were compared with analysis of covariance (ANCOVA) and *a posteriori* comparisons were computed with Tukey's honestly significant difference (HSD) test. Analyses were performed with SYSTAT for Windows (Version 10.0) statistical software (SPSS Inc., Chicago, IL, U.S.A.). Results were considered significant at $P < 0.05$.

3. Results

3.1 Chlorophyll-a concentration and phytoplankton community composition

During the study, an appreciable accumulation of cyanobacteria was evident on the surface of Hastings Lake. Accordingly, Chl-*a* concentrations from the ten sites ranged from 120 to 4080 μg L^{-1} indicating large spatial variations in phytoplankton biomass (Table 2). In general, *Aphanizomenon flos-aquae* dominated the phytoplankton communities within each of the ten sites, though varying amounts of *M. aeruginosa* were present. Since *A. flos-aquae* and *M. aeruginosa* dominated the phytoplankton community, almost to the exclusion of all other cyanobacteria and eukaryotic algae, only these two species were enumerated. Furthermore, the biomass (μm L^{-1}) of these two species varied among sites and not surprisingly, total biomass (*i. e.*, *A. flos-aquae* and *M. aeruginosa* considered together) was strongly associated with Chl-*a* concentrations (r = 0.9; $P < 0.001$). Similarly, the biomass of *M. aeruginosa* relative to total cyanobacterial biomass also varied among sampling sites ranging from 0.5 to 4.3% (Figure 1).

3.2 Microcystin in phytoplankton and water

The cellular concentration of MCYST in phytoplankton (expressed as μg MCLR equivalents·g^{-1} dry wt.) collected from the ten sites was highly variable and ranging from 21 to 65 μg g^{-1} (Table 2). Cellular toxin levels in phytoplankton were associated with the relative biomass of *M. aeruginosa* (r = 0.8; P = 0.008). Similarly, total aqueous MCYST concentration (expressed as μg MCLR equivalents L^{-1} lake water) was variable and ranged from 0.18 to 4.9 μg L^{-1} (Table 2). However, unlike the cellular toxin concentration of the phytoplankton, aqueous MCYST concentrations were evidently out of phase with the relative biomass of *M. aeruginosa* (P = 0.18).

3.3 Microcystin in Lymnaea stagnalis

All individual *L. stagnalis* collected from each of the ten study sites contained detectable amounts of MCYST (expressed as ng MCLR equivalents g^{-1} dry wt.). The tissue toxin concentrations within each site were variable; the coefficients of variation (*CV*) ranged from 42 to 103%. Results of a regression analysis suggests body mass and

toxin concentrations in tissue of *L. stagnalis* be inversely related (r = -0.3; $P < 0.003$). Consequently, mean tissue toxin concentrations between sites were compared considering body mass as a covariate. Indeed, both sampling site ($P = 0.002$) and body mass ($P = 0.0001$) contribute to the disparities in toxin concentrations within *L. stagnalis* and overall, account for 60% of the variation in tissue toxin concentration. Furthermore, subsequent *a posteriori* comparisons suggest that spatial trends in mean tissue toxin concentrations exist, as several sampling sites located on the south shore of Hastings Lake differed from sites located on the north shore (*i. e.*, sites 4, 5 and 6 differed from site 8 and site 5 also differed from site 7). Mean toxin concentrations within *L. stagnalis* were not detectably associated with MCYST concentration in the *A. flos-aquae*-dominated phytoplankton biomass ($P = 0.55$) or dissolved toxin within the water ($P = 0.81$). In addition, the tissue toxin concentration was not correlated with the relative biomass of *M. aeruginosa* ($P = 0.98$), though accounting for the influence of snail body mass strengthened this association ($P = 0.17$).

4. Discussion

Previously, Carmichael and Gorham (1981) and Kotak *et al.* (1995) demonstrated spatial variation in bloom-related toxicity within eutrophic Alberta lakes. These differences were both attributed to variability in phytoplankton species composition and to the relative proportion of toxic versus nontoxic cyanobacterial strains within the same species (Carmichael and Gorham, 1981) and possibly to incongruities "in the mix of cell cycle phases between sites and/or specific toxicities of *M. aeruginosa* strains" (Kotak *et al.*, 1995). The results of the present study are consistent with previous research, which indicated that phytoplankton biomass can vary spatially in the littoral zone of Hastings Lake. Furthermore, the MCYST concentration of phytoplankton also differed spatially, as it was associated with the relative biomass of *M. aeruginosa* and is consistent with earlier findings (Kotak *et al.*, 1995).

Pulmonate snails are indiscriminate grazers (Elder and Collins, 1991) and their diets reflect the relative availability (*i. e.*, biomass) of various algal and cyanobacterial species. Hence, the concentration of toxin within tissue of these organisms was expected to vary spatially. The mean tissue toxin concentrations of *L. stagnalis* collected from ten

littoral sites of Hastings Lake differed and *post hoc* comparisons suggested that spatial trends did exist as several sampling sites located on the south shore differed from others on the north shore. Comparable to the seasonal variation in MCYST concentration of resident pulmonate snails from eutrophic Alberta lakes (Zurawell *et al.*, 1999), there is some evidence that toxin concentrations in *L. stagnalis* are associated with the relative biomass of *M. aeruginosa*. Stronger evidence of a causal link between *L. stagnalis* toxin levels and toxin-producing cyanobacteria should not be expected given the relatively small proportion *M. aeruginosa* comprises of the *L. stagnalis* diet; *M. aeruginosa* representing less than 0.5 to 4.3% of the dietary phytoplankton biomass available to snails within Hastings Lake.

Recently, Zurawell *et al.* (submitted a) concluded the majority of detected MCYST in *L. stagnalis* exposed to toxic *M. aeruginosa*, originates from indigestible cyanobacterial residues contained within the alimentary tract. To this end, factors controlling differences in gut content elimination affect the resulting MCYST concentration. In this study, individual snail body mass most notably influenced toxin concentrations in tissue of *L. stagnalis* ($P < 0.003$). Body size and other parameters of physiological state are known to influence ingestion, assimilation and egestion rates of MCYST in various aquatic invertebrates (see discussion, Zurawell *et al.*, submitted b). While all attempts were made to collect snails of similar size from each of the ten sites, individuals varied from 30 to 50 mm in shell height. Furthermore, distinct discrepancies in the body size of *L. stagnalis* were observed for some sites, possibly resulting from age (cohort) differences. Consequently, 60% of the variation in mean MCYST concentration in *L. stagnalis* was attributed to spatial variation (*i. e.*, sampling site) and body mass. Similarly, accounting for the influence of body mass strengthened the association between the toxin concentration in snails and the relative biomass of *M. aeruginosa* ($P = 0.17$). Failure to produce stronger evidence of this potential causal relationship is likely due in part to the small sample size ($n = 10$) and relative scarcity of *M. aeruginosa* as a food source to *L. stagnalis*.

Other factors may also explain why the toxin concentration in tissue of *L. stagnalis* weekly reflects the instantaneous relative biomass of *M. aeruginosa* or toxicity of the phytoplankton. Foremost, growth responses by phytoplankton to fluctuating

environmental conditions in conjunction with various loss processes (*i. e.*, hydraulic washout, sedimentation, zooplankton grazing, bacterial, fungal and viral pathogens) alter biomass and composition of populations on several temporal and spatial scales (George and Heaney, 1978; Reynolds, 1984; Carpenter, 1989). Moreover, Zurawell *et al.* (submitted a, b) recently demonstrated that indigestible toxin-containing cyanobacterial residues can require up to 8 h before elimination from the alimentary tract of *L. stagnalis*, while detectable concentrations of MCYST within the digestive gland tissue may remain up to 30 d post-ingestion. In contrast, phytoplankton rapidly respond to changing environmental conditions (including the formation and disappearance of cyanobacterial blooms). As a result, a lag period between fluctuations in *M. aeruginosa* biomass and toxicity and the MCYST concentration within the snails surely exists. Indeed, Zurawell *et al.* (1999) observed a delay of 4 to 6 d between peak biomass and toxicity of *M. aeruginosa* and the MCYST content of resident pulmonate snails collected from several eutrophic lakes, which could account for some observed disparities when comparing temporal and spatial studies.

Likewise, spatial differences in water temperature (not recorded in this study) may contribute to these findings, as the influence of water temperature on ingestion and metabolism in gastropods is well established (Calow, 1975). To this end, Zurawell *et al.* (submitted b) demonstrated that cooler water temperature (10°C) mitigates the long-term (30 d) elimination of MCYST from *L. stagnalis* previously exposed to toxic *M. aeruginosa*, compared to those snails maintained in warmer (22°C) water. It is plausible that sites with low water temperature could moderate the ingestion, accumulation and elimination in such a way, that only low concentrations of MCYST exist even when *M. aeruginosa* is relatively abundant. Alternatively, the opposite situation may occur as well (*i. e.*, higher water temperature leading to elevated MCYST concentrations even during periods of reduced *M. aeruginosa* availability).

The concentration of (extracellular) toxin in water, which represents a second possible route for the exposure of snails to MCYST, may also affect the present results. In this study, aqueous MCYST varied by an order of magnitude between sites (Table 2). However, the lack of association between the toxin concentration in water and the concentration in tissue of *L. stagnalis*, which was reported in this study, compares

favorably with the previous findings of Zurawell *et al.* (1999). While gastropods can ingest dissolved compounds from the aquatic environment through drinking (Thomas *et al.*, 1990) or during feeding (DeWith, 1996), it remains uncertain whether *L. stagnalis* are able to consume MCYSTs by these mechanisms. Thus, with the possible exception of extremely high concentrations following the collapse of severe bloom events, the extracellular MCYST fraction may be regarded as an inconsequential source of toxin exposure to snails.

5. Conclusion

Zurawell *et al.* (1999) suggested that spatial differences in phytoplankton community composition (*i. e.*, relative availability of toxin-producing cyanobacteria) and thus MCYST concentration within phytoplankton contributes to heterogeneity in toxin concentration within tissue of pulmonate snails collected from bloom-prone lakes. In the present study, the mean concentration of MCYST within tissue of *L. stagnalis* varied spatially, as several sites on the south shore were significantly different from others on the north. Subsequently, 60% of the variation in tissue toxin concentration was attributed to sampling site differences and body mass. Similarly, adjusting for body mass strengthened the association between the toxin concentration in snails and the relative biomass of *M. aeruginosa*. This is not surprising, as differences in physiological state manifested as body size could affect tissue toxin concentrations of individual snails by influencing toxin uptake and elimination. Other factors such as a lag period between rapid fluctuations in *M. aeruginosa* or its toxicity and the MCYST concentration within the snails resulting from toxin accumulation, could mitigate differences between sites, as could water temperature, which influences ingestion, accumulation and elimination of MCYST from within snails. While this study identifies the ubiquity to which resident pulmonate snails contain MCYST and affirms the potential role of these organisms in the accumulation and transfer of toxin within the food webs of bloom-prone lakes, future investigations regarding the extent utilization of cyanobacteria by gastropods are required.

Table 1. Morphometric and water quality characteristics of Hastings Lake

Characteristic	
Drainage basin area (km^2) †	269
Lake surface area (km^2) †	8.7
Drainage basin:lake surface area ratio †	31
Maximum depth (m) †	7.3
Mean depth (m) †	2.4
Total alkalinity (mg L^{-1} $CaCO_3$) ‡	280 ± 5
Total nitrogen (μg L^{-1}) ‡	2587 ± 151
Total phosphorus (μg L^{-1}) ‡	91 ± 12
Total nitrogen:total phosphorus ratio ‡	34 ± 4
Chlorophyll-*a* (μg L^{-1}) ‡	40 ± 13

† Morphometric characteristics (drainage basin and lake surface area, catchment:lake surface area ratio, maximum and mean depth) were obtained from Mitchell and Prepas (1990).
‡ Water quality values (total alkalinity, total nitrogen, total phosphorus, total nitrogen:total phosphorus ratio and chlorophyll-*a*) were obtained from Alberta Capital Health Authority (unpublished data, 2000) and represent mid-March to mid-October means; ± *SE*.

Table 2. Chlorophyll-*a* and microcystin (MCYST) concentrations in phytoplankton, water and *Lymnaea stagnalis* collected from ten study sites. Results for MCYST concentrations in *L. stagnalis* are presented as means from 10 individuals per site.

Site	Chlorophyll-*a* ($\mu g\ L^{-1}$)	MCYST in phytoplankton (μg MCLR eq. g^{-1})	MCYST in water (μg MCLR eq. L^{-1})	MCYST in *Lymnaea stagnalis* (ng MCLR eq. g^{-1}) ± *SE*
1	202	21.6	0.20	318 ± 76
2	139	55.7	0.15	381 ± 69
3	4080	27.5	3.37	361 ± 76
4	2388	54.3	1.63	169 ± 34
5	2036	42.7	0.74	379 ± 124
6	3358	25.1	0.48	187 ± 35
7	857	20.7	1.47	415 ± 61
8	661	50.0	0.71	343 ± 51
9	2623	57.2	4.85	283 ± 38
10	120	64.5	0.18	344 ± 50

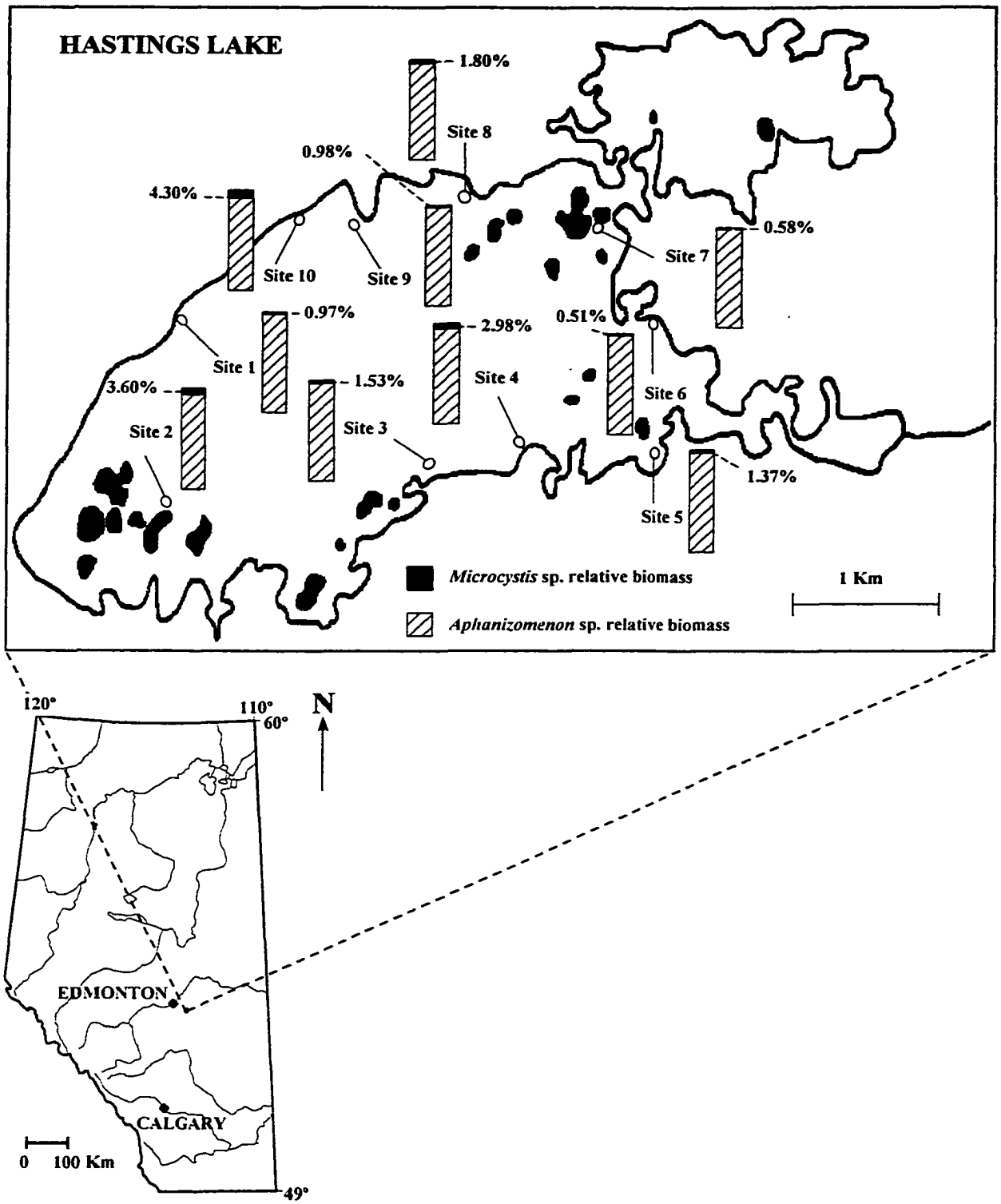

Figure 1. Map of Alberta, Canada, showing geographic location of Hastings Lake and the relative availability (*i. e.*, biomass) of *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* within the ten study sites.

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Zurawell R.W., Holmes C.F.B. and Prepas E.E. (submitted b) Depuration of microcystin from tissue of the freshwater pulmonate snail *Lymnaea stagnalis jugularis* (Say).

Chapter 5

24 hour gut clearance and tissue distribution of microcystin in the freshwater pulmonate snail *Lymnaea stagnalis jugularis* (Say) [3]

1. Introduction

The accumulation of phytoplankton toxins by benthic macroinvertebrates inhabiting marine environments is well-documented (Shumway, 1990). Okadaic acid (OA) and the dinophysistoxins (DTXs), comprise one class of toxins which are responsible for diarrhetic shellfish poisoning (DSP). DSP causes a potentially severe gastrointestinal illness after consuming contaminated shellfish (Yasumoto *et al.*, 1985). Produced by the marine dinoflagellates *Dinophysis* spp. and *Prorocentrum* spp., these compounds are known to specifically inhibit protein phosphatases type 1 (PP1c) and 2A (PP2A) within animal cells and consequently have been identified as potent tumour promoters (Bialojan and Takai, 1988; Fujiki *et al.*, 1988; Suganuma *et al.*, 1988).

In freshwater environments, cyclic heptapeptides produced by the cyanobacteria *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc* (Botes *et al.*, 1985; Krishnamuthy *et al.*, 1986; Meriluoto *et al.*, 1989; Namikoshi *et al.*, 1990), possess similar toxicological properties to OA and DTXs (Boland *et al.*, 1993). Collectively referred to as microcystins (MCYSTs), these toxins also inhibit PP1c and PP2A (MacKintosh *et al.*, 1990) and are recognized as potent tumour promoters (Nishiwaki-Matsushima *et al.*, 1991). Consequently, accidental ingestion of MCYSTs has been implicated in serious illness and death of domestic and wild animals worldwide (Schwimmer and Schwimmer, 1968; Carmichael, 1994).

In contrast to OA and its related congeners, little is known of the potential accumulation of MCYSTs by benthic macroinvertebrates inhabiting freshwater lakes and reservoirs that experience periodic blooms of toxin-producing cyanobacteria. Eriksson *et al.* (1989) previously demonstrated the freshwater mussel *Anodonta cygnea* accumulates

[3] A version of this chapter has been submitted as Zurawell R.W., Holmes C.F.B. and Prepas E.E. (submitted b) 24 hour gut clearance and tissue distribution of microcystin in the freshwater pulmonate snail *Lymnaea stagnalis jugularis*.

peptide toxin when reared in laboratory cultures of *Oscillatoria agardhii* and suggested that organisms feeding on toxin-producing cyanobacteria in eutrophic environments could potentially accumulate toxin. Subsequently, several groups have illustrated the *in situ* accumulation of MCYSTs by other bivalves collected from eutrophic freshwater environments including the mussels *Anodonta grandis* (Prepas *et al.*, 1997), *Anodonta woodiana* and *Unio douglasiae* (Watanabe *et al.*, 1997).

In addition to filter-feeding bivalves, Kotak *et al.* (1996) and Zurawell *et al.* (1999) previously documented the occurrence of microcystin-LR, a predominant MCYST variant, in herbivorous gastropods *Lymnaea stagnalis*, *Helisoma trivolvis* and *Physa gyrina* collected from eutrophic Alberta lakes. Furthermore, Zurawell *et al.* (1999) noted the presence of whole and partially intact colonies of *Microcystis* spp. comprising the gizzard fraction of faecal strings from *L. stagnalis* collected from Driedmeat Lake, Alberta. This portion of the faeces composed of indigestible matter, forms when food is passed from the gizzard into the pro-intestine without further digestion in the caecum or digestive gland (Carriker, 1946). Since, MCYSTs are endotoxins typically released during cell lysis, failure of the gizzard to mechanically disrupt *Microcystis* cells/colonies may limit the uptake of toxin by the digestive system. Considering snails were immediately frozen upon collection, gut contents would not have been eliminated. Consequently, Zurawell *et al.* (1999) suggested that toxin detected in gastropod tissue probably originated from *Microcystis* cells/colonies contained in the alimentary system and not from toxin accumulated in somatic tissues.

The primary goal of this study was to examine whether MCYST detected in *L. stagnalis* originates from non-digested *Microcystis* cells/colonies contained within the alimentary system or from toxin accumulated in tissues (or both). Secondly, if accumulated toxin exists, determine which tissues contain detectable levels of MCYST. It was hypothesized that the concentration of MCYST in *L. stagnalis* previously exposed to toxin-producing cyanobacteria originates from intact cells/colonies within the alimentary tract and decreases significantly as gut contents are eliminated.

2. *Materials and Methods*

2.1 Gastropod and phytoplankton collection

Adult (35 to 50 mm in height) *Lymnaea stagnalis jugularis* (Say) were harvested from Hastings Lake Alberta, Canada, on 17 September 1999 (tissue distribution study) and 8 October 1999 (24 hour gut clearance study). Snails were collected with a mesh dip net from a littoral area approximately 50 m in length and immediately placed in aquaria containing lake water for transport to the laboratory. Species identification was based on shell morphology descriptions of Clarke (1981) and Clifford (1993).

Phytoplankton to be fed to snails in the laboratory was also collected (on respective dates) from this site and concentrated with repeated vertical hauls of a 64 μm mesh (Nitex™) plankton net from the surface to a depth of 1 m. Material was combined in an 8 L glass container and five 150 mL subsamples removed and immediately frozen for subsequent determination of microcystin concentration within the phytoplankton.

In the laboratory, snails were placed in 20 L aquaria supplied with a continuous flow of artificial pond water (dechlorinated, carbon filtered municipal tap water) at 21°C (± 1°C). Snails were acclimated for five days prior to experimentation, at which time, were fed exclusively phytoplankton collected from Hastings Lake.

2.2 24 hour gut clearance experiment

Gastropods used in the 24 hour gut clearance study were equally divided among three, 4 L continuous-flow holding tanks supplied with artificial pond water at 21°C (± 1°C). At this time, 3 individuals from each tank were set aside for toxin analysis to determine MCYST concentration at time zero. Subsequently, *L. stagnalis* were randomly sampled from each tank in triplicate at 4, 8, 12, 16, 20 and 24 h, immediately frozen to halt metabolic activity and later processed for MCYST analysis. Over the course of the experiment (24 h) snails were not fed.

2.3 Tissue distribution experiment

Thirty-five *L. stagnalis* were removed from the aquaria, blotted dry with paper towel and then frozen. Of these, 25 snails were thawed, removed from their shells and

dissected into three fractions: alimentary tract (including esophagus, crop, gizzard and complete intestine), digestive gland (hepatopancreas) and remaining visceral mass (including head, foot, circulatory system and reproductive organs). Fractions from three consecutive individuals were pooled together and processed for MCYST analysis. The remaining ten snails were thawed, removed from their shells and whole tissues processed for MCYST analysis.

2.4 Microcystin analysis

Frozen gastropods (excluding shell) were lyophilized then homogenized with mortar and pestle. Tissue homogenates of individual snails were weighed to determine freeze-dried body weights and placed in 20 mL glass vials to which 10 mL of 100% methanol was added. Each sample was sonicated for 20 s to disrupt the cells, then left to settle overnight. The liquid portion was carefully decanted and filtered through a Whatman GF/A filter (Whatman Inc., Fairfield, NJ, U.S.A.), then placed in a glass vial. The resulting extract was evaporated under a constant stream of nitrogen gas with a Reactivap™ heater. The semi-purified extract was resuspended in 4 mL of ultra pure water and analyzed for MCYST concentration via colorimetric protein phosphatase (PP) inhibition assay as specified by An and Carmichael (1994), utilizing recombinant PP1c expressed in *E. coli* (provided by C.F.B. Holmes). Phytoplankton were processed and analyzed similar to gastropods, however, approximately 100 mg (dry weight) phytoplankton homogenates were used. As well, after each sample was sonicated and allowed settling overnight, an additional 5 mL of 100% methanol was added to the sample vials. These were then resuspended by mixing and again left to settle overnight. Next day, each sample was filtered, evaporated and resuspended in 4 mL of ultra pure water.

Microcystin concentrations of both gastropod and phytoplankton extracts were extrapolated from curves plotting PP1c inhibition by microcystin-LR (MCLR) standards (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and are thus expressed as mass MCLR equivalents g^{-1} tissue (dry weight). The detection limit of this assay was approximately 3 ng MCLR eq. g^{-1} tissue. While this assay quantifies all MCYSTs capable of inhibiting PP1c, toxin fractions irreversibly bound to endogenous PP1c and

PP2A within gastropod tissues would be unavailable for further PP1c inhibition and consequently remain undetected. Hence, the MCYST values reported undoubtedly underestimate the actual total toxin concentrations within tissues.

2.5 Statistical analysis

With respect to the 24 hour gut clearance study, mean concentrations (± *SE*) of MCYST in *L. stagnalis* sampled (in triplicate) from the three holding tanks were computed for each sampling time (0, 4, 8, 12, 16, 20 and 24 h). Mean toxin concentrations among the three tanks and between sampling times were examined on $\log_{10}$-transformed data with a nested analysis of variance (nested ANOVA). Subsequent *a posteriori* comparisons were computed with Tukey's honestly significant difference (HSD) test. These analyses were performed with SYSTAT for Windows (Version 10.0) statistical software (SPSS Inc., Chicago, IL, U.S.A.). Nonlinear curve estimation of the toxin depuration curve was performed on untransformed data with GraphPad Prism (Version 3.02 Trial) software for Windows (GraphPad Software Inc., San Diego, CA, U.S.A.). Toxin concentrations of snails analyzed wholly and those fractionated for use in the tissue distribution study were tested for normality (Kolmogorov-Smirnov test, $P > 0.19$) and homogeneity of variance (Levene's test, $P = 0.29$). Comparison of total MCYST in whole snails with the summed concentrations of toxin within the three tissue fractions, was made with a non-paired *t*-test (df = 16). Comparison of the mean toxin proportions for each of the three tissue fractions was performed with a one-way ANOVA (df = 2, 21) followed by Tukey's HSD test (df = 2, 21). Analyses were performed with SYSTAT for Windows (Version 10.0) and SPSS for Windows (Version 8.0) statistical software (SPSS Inc., Chicago, IL, U.S.A.). Results were considered significant at $P < 0.05$.

3. Results

3.1 24 hour gut clearance experiment

The mean MCYST concentration of phytoplankton collected simultaneously with *Lymnaea stagnalis* from Hastings Lake and subsequently fed to snails during the acclimation period, was 72 μg g^{-1} dry weight. At no time over this 5-day period did individual snails appear to be adversely affected by the presence of toxin-containing cyanobacteria. Food supply was in great excess relative to that required by the gastropods, thus it was assumed that all individuals had equal opportunity to graze. The resulting mean MCYST concentrations within *L. stagnalis* immediately following acclimation (*i. e.*, time 0) was 136 (± 1) ng MCLR eq. g^{-1}. Over the next two sampling periods (4 and 8 h) mean toxin concentrations declined in snails by 37% to 86 (± 10) ng g^{-1} and by 20% to 59 (± 13) ng g^{-1}, respectively (Figure 1). A further 7% decrease in mean toxin concentration to 47 (± 6) ng g^{-1} MCLR eq. was observed between 8 and 12 h. However, beyond this point little change occurred as mean toxin concentrations of 49 (± 5), 49 (± 3), 48 (± 4) ng g^{-1} MCLR eq. were recorded at 16, 20 and 24 h, respectively (Figure 1). While mean toxin concentrations of snails among the three tanks did not differ detectably ($P = 0.32$), mean concentrations within the snails differed ($P = 0.0007$) over the 24 h. Based on *a posteriori* multiple comparisons of the means, MCYST concentration at the commencement of the study (*i. e.*, time 0) differed ($P < 0.0007$) from those concentrations sampled beyond 4 h. Microcystin elimination over the first 12 h fit a single-phase (*i. e.*, one-compartment) exponential decay equation ($\hat{Y} = 103.6e^{(-0.169*\chi)} + 32.85$; $r^2 = 0.99$). The estimated depuration rate constant ($k = -0.169$ h^{-1}), was used to calculate the half-life ($t_{1/2}$) of MCYST in snails ($t_{1/2} = \ln 2/k$), which was 4 h.

3.2 Tissue distribution experiment

The MCYST concentration within the phytoplankton collected from Hastings Lake and subsequently fed to *L. stagnalis* during the acclimation period was 609 μg g^{-1} dry weight. Like *L. stagnalis* used in the previous study, at no time during the acclimation period did individuals appear to be adversely affected by the presence of toxin-containing cyanobacteria. Similarly, food supply was in great excess of that

required by the gastropods and it was likewise assumed that all snails had equal opportunity to graze. The resulting toxin concentrations of the ten *L. stagnalis* analyzed wholly, ranged from 643 to 2731 ng MCLR eq. g^{-1} with a mean of 1598 (± 192) ng g^{-1}. Thus, considerable individual variation in MCYST concentration existed. When total MCYST was summed from separate tissue fractions (*i. e.*, alimentary tract, digestive gland and remaining tissues) and adjusted to a per snail basis, concentrations of the eight composite groups had mean of 1279 (± 138) ng g^{-1}. The mean concentrations in whole snails and the mean of those summed portions were not detectably different (independent *t*-test; $P = 0.2$). Considering the three tissue fractions separately, the largest mean percentage of total MCYST concentration was contained within the alimentary tract portion (82.8 ± 2.26%; Table 1). The digestive gland portion possessed the second largest mean percentage (16.9 ± 2.24%; Table 1), while the remaining tissues comprised the least (0.3 ± 0.04%; Table 1). A comparison of the mean MCYST proportions concluded differences ($P < 0.0001$) between the three tissue fractions.

4. Discussion

The first reports regarding the occurrence of MCYSTs in freshwater pulmonate snails can be attributed to the aquatic food web studies by Kotak *et al.* (1996). Subsequently, Zurawell *et al.* (1999) also documented the occurrence of MCYSTs within resident gastropods collected from eutrophic lakes. Coincidentally, this latter study observed whole and partially intact colonies of *Microcystis* spp. within the faecal strings of *L. stagnalis*. These findings raise questions as to the actual source of MCYST detected in whole snail tissues as indicated by these previous works. The purpose of the 24 hour gut clearance study then, was to determine if *L. stagnalis* that recently grazed on toxin-producing cyanobacteria contain detectable concentrations of MCYST following digestion and elimination of gut contents.

To appreciate the results of the study, the functioning of the alimentary system of *L. stagnalis* must first be considered. Carriker (1946) and Boer and Kits (1990) have made detailed observations regarding gross morphological, ultrastructural and histochemical aspects of the alimentary tract of *Lymnaea*. As well, Veldhuijzen (1974) studied the sorting and retention time of particles within the digestive system. These

findings, applied to the functioning of the alimentary system, are summarized in Table 2. Coarse, indigestible plant matter consumed by *L. stagnalis*, passes through the gizzard directly to the prointestine and exclusively comprises the gizzard string fraction of the faeces. According to Zurawell *et al.* (1999) the presence of intact *Microcystis* colonies within this fraction, suggests *L. stagnalis* is incapable of digesting colonial cyanobacteria, especially those species possessing mucilaginous sheaths. Scheerboom *et al.* (1978) demonstrated that *L. stagnalis* approximately 35 mm in shell height required 2.3 to 2.7 h post-ingestion for large, indigestible fractions to be eliminated from the alimentary tract. Since snails ranged between 30 to 40 mm in height; if *Microcystis* was not digested by *L. stagnalis*, cyanobacteria ingested prior to the commencement of this study (*i. e.*, time 0) would have been eliminated by the subsequent sampling time (*i. e.*, time 4 h). While, MCYST declined 36.7% in the first 4 h of the study, notable concentrations remained (up to 107 ng MCLR eq. g^{-1} in tank 3).

In the pylorus, food particles up to 0.4 μm are directed towards the hepatic vestibulum. At this point, indigestible matter enters the caecum where it is consolidated into the caecal string to be passed on to the prointestine (Table 2). Veldhuijzen (1974) calculated the time required for the elimination of fine inert particles (≤ 0.4 μm) within the caecum of *L. stagnalis* (≈ 35 mm shell height) to be approximately 4 h. Thus, MCYST detected at 4 h may represent toxin within individual *Microcystis* cells, which typically range between 3 to 6 μm in diameter. If this were the case, snails sampled beyond 4 h would be void of any toxin containing *Microcystis*. The decline (20.4%) in toxin concentration between 4 and 8 h may correspond to the elimination of intact cells from the alimentary tract via the caecum. If *Microcystis* were not digested, MCYST would not be detected in snails at or beyond 8 h post-ingestion. Again, variability in the mean MCYST concentrations indicates disparity in the time required for individual snails to eliminate gut contents.

Although the mean toxin concentrations from 8 to 24 h were not different from each other, the 7% decline in toxin concentration between 8 and 12 h is notable. Previous studies have demonstrated that material processed within the digestive gland of *L. stagnalis* is eliminated in the faeces about every 2 h (Veldhuijzen, 1974). Since no attempt was made to quantify MCYST directly from faecal string fractions, it is difficult

to ascertain whether this decrease represents elimination of excess MCYST from the digestive gland or continued elimination from the caecal (or gizzard) string. Regardless, the presence of toxin in snails beyond 8 h (*i. e.*, time 12 to 24 h) corresponds favourably with Veldhuijzen (1974), who demonstrated that fine and soluble digestible matter entering the digestive gland might remain detectable for at least 100 h post-ingestion. Consequently, these findings indicate that although elimination of the gizzard and caecal string fractions account for the majority (at least 57%) of ingested MCYST, lower, detectable concentrations exist in the digestive gland of *L. stagnalis* beyond 24 h. Furthermore, MCYST may remain for more than 100 h within digestive glands cells before being eliminated in the digestive gland fraction of the faeces.

Though MCYST elimination fit a single-phase exponential loss equation, the insufficient amount of data collected in this study (*i. e.*, the number of sampling periods) made it impossible to estimate toxin elimination based on a two-phase exponential loss equation. However, the initial toxin loss over the first 4 h corresponding to toxin elimination with indigestible *Microcystis* via the gizzard, occurred rapidly with an approximate half-life ($t_{1/2}$) of 1.8 h. In contrast, toxin loss over the subsequent 4 h attributable to smaller indigestible residues eliminated from the alimentary tract via the caecum occurred more slowly with a half-life ($t_{1/2}$) of 3.2 h. Hence, the previous findings above suggest that toxin loss may occur in two separate phases. In this regard, I suggest a subsequent study involving the determination of MCYST concentrations in snails sampled proportionately to the estimated change in MCYST concentration, is required to better quantify and understand short-term toxin elimination from pulmonate snails.

In light of these findings, it would be reasonable to expect that within *L. stagnalis* fractionated into various tissues, the majority of MCYST would occur within those associated with the alimentary system. As well, the digestive gland would be expected to contain detectable toxin concentrations. However, since no published information exists regarding the accumulation of MCYSTs in other tissues (*i. e.*, foot, mantle, kidneys, gonads etc.) of *L. stagnalis*, it is difficult to ascertain whether toxin would be detectable in the remaining tissues.

In this experiment, the summed MCYST concentrations of the three tissue fractions, when expressed on a per snail basis, was similar to those snails analyzed

wholly. There is no evidence to suggest then, that dissecting snails into various tissue fractions results in unaccounted loss of toxin. It is important to recognize, however, the high variability in MCYST concentration within each component may be preventing the identification of toxin loss to an additional component. Considered separately, the mean percent MCYST concentration observed in the alimentary tract was about 82.8% (Table 1). Since all snails were immediately dissected upon removal from the test aquaria, gut contents presumably remained within the tract. Thus, intact *Microcystis* within the buccal cavity, esophagus, crop, gizzard, pylorus and intestine all likely contributed to this MCYST portion. According to histochemical and ultrastructural studies of the alimentary tract of *L. stagnalis*, enzymatic digestion may occur in both the crop and gizzard (Table 2). With the exception of the gizzard, the epithelium lining the remaining regions displays signs of pinocytosis, lysosomal breakdown and storage of glycogen, lipids and proteins. Consequently, the alimentary system, particularly the crop and prointestine, assumes a prominent role in the absorption of nutrients and dissolved substances (Boer and Kits, 1990). In addition to cellular MCYST contained within indigestible *Microcystis*, it is conceivable that a portion of the MCYST detected in the alimentary tract fraction may be attributed to toxin previously taken up by epithelial and related tissues.

The mean percent MCYST concentration observed in the digestive gland, as determined by the tissue distribution study, was approximately 16.9%, while only minor quantities (0.3%) were detected in the remaining tissues (Table 1). Many have previously suggested the significant capacity of the digestive gland in the assimilation of dissolved and particulate nutrients and compounds (Carriker, 1946; Boer and Kits, 1990). Since detectable concentrations of MCYST in this tissue would be expected only if digestion of *Microcystis* occurs, the mere presence of toxin within the digestive gland provides definitive evidence for not only digestion of *Microcystis*, but the cellular uptake of toxin as well. While this is the first study documenting the uptake of MCYST from toxin-containing cyanobacteria by any freshwater gastropod molluscs, others have demonstrated similar toxin uptake by bivalve molluscs. For instance, Eriksson *et al.* (1989) found that freshwater swan mussels (*Anodonta cygnea*) accumulated 70 μg hepatotoxin g^{-1} dry weight, during exposure to laboratory cultures of the cyanobacterium

Oscillatoria agardhii. Of the total toxin content, the majority (40%) was contained within the digestive gland (hepatopancreas), minor quantities were detected in the intestinal and gonadal tissue, while the remaining toxin occurred in the kidneys and connective tissues. Similarly, Watanabe *et al.* (1997) reported the accumulation of both MCLR and microcystin-RR (MCRR) primarily in the digestive gland of *Unio douglasiae* as well as MCLR within *Anodonta woodiana*. With respect to my study, further fractionation of the remaining tissues was not performed. Thus it would be impossible to determine those tissues responsible for the uptake of minor MCYST quantities. However, comparisons with bivalve mollusc studies suggest the kidneys as the potential site for minor toxin accumulation.

5. Conclusion

The previous study by Zurawell *et al.* (1999) raised questions regarding the ability of pulmonate snails to digest cyanobacteria and consequently the source of MCYST detected in whole snails collected from eutrophic environments. The results of the 24 hour gut clearance study clearly demonstrate the majority of detected toxin originates from within indigestible cyanobacterial residues. In adult *L. stagnalis* between 30 to 40 mm in shell height, the time required for elimination of these toxin-containing gut contents was about 8 h. However, it is recognized that in natural environments, *L. stagnalis* may feed continuously both day and night (Veldhuijzen, 1974). Since eutrophic waterbodies experience blooms of toxin-producing cyanobacteria through much of the summer season, significant amounts of indigestible cyanobacteria and related endotoxins may continually exist within the alimentary system of resident snails. More importantly, these studies provide conclusive evidence regarding the limited digestion of *Microcystis* and subsequent accumulation of MCYST primarily within the digestive gland of *L. stagnalis*. While toxin (48.8 ng MCLR eq. g^{-1}) was detected in *L. stagnalis* sampled at 24 h post-ingestion, further studies are required to characterize the long term depuration of MCYST from snail tissue. Nevertheless, the uptake and accumulation of MCYST by *L. stagnalis* not only suggests potential toxicity to those snails inhabiting bloom-prone waters, but also implicates them in the transfer of hepatotoxins to other aquatic and terrestrial organisms associated with these food webs.

Table 1. Percent microcystin (MCYST) concentration contributed by the alimentary tract, digestive gland and remaining tissue portions of *Lymnaea stagnalis*. Each sample represents a composite of tissue portions contributed by three individual snails (sample 8, composite of four individuals). For ease of comparison with the MCYST concentration within whole snails, toxin is expressed on per snail basis (*i. e.*, % ng MCLR equivalents g^{-1} tissue $snail^{-1}$).

	% MCLR eq. ng g^{-1} $snail^{-1}$		
Sample	Alimentary tract	Digestive gland	Remaining tissues
1	83.18	16.59	0.24
2	76.06	23.73	0.21
3	78.22	21.52	0.26
4	87.46	12.14	0.41
5	94.32	5.52	0.16
6	85.00	14.69	0.32
7	75.14	24.40	0.47
8	83.29	16.33	0.38
Mean ± *SE*	82.8 ± 2.26%	16.9 ± 2.24%	0.3 ± 0.04%

Table 2. Summary of digestion and elimination of food within the alimentary system of *Lymnaea stagnalis*

Alimentary Region	Functions
Postesophagus	Temporary storage of food when crop is full. Initiatory site of enzymatic digestion by salivary gland excretions.
Crop	Food storage and initiatory site of mechanical digestion (comminution). Chemical digestion.
Gizzard	Primary site of comminution aided by the presence of sand and fine particles. Chemical digestion by salivary and digestive gland enzymes.
Pylorus	Responsible for the ciliary sorting of food particles by size and digestibility. Procurrent (ventral) passage transports large indigestible particles (> 0.4 μm diameter) directly towards the prointestine. Dorsal passage conducts particles ≤ 0.4 μm towards the hepatic vestibulum. Retrocurrent passage directs partially or non-digested matter back into the gizzard for further comminution.
Hepatic vestibulum	Selectively conducts soluble and suspended particulate matter (< 0.4 μm) from the pylorus into the digestive gland ducts and indigestible matter (≤ 0.4 μm) directly to the caecum.
Digestive gland (hepatopancreas)	Primary site of endocytosis, intracellular (lysosomal) digestion and vacuolate excretion of indigestible residues. Secretion of digestive enzymes (carbohydrases and proteases) to the gizzard.
Caecum	Receives indigestible matter from the hepatic vestibulum. Consolidation of caecal string fraction of faeces to be directed to the prointestine.
Prointestine	Absorption of water. Final consolidation of faecal string fractions (gizzard string, caecal string and digestive gland string) through the secretion of cementing compounds. Further faecal string compaction.
Mid, postintestine and rectum	Further mucous production and final elimination of faecal strings.

Summarized from Carriker (1946), Veldhuijzen (1974) and Boer and Kits (1990).

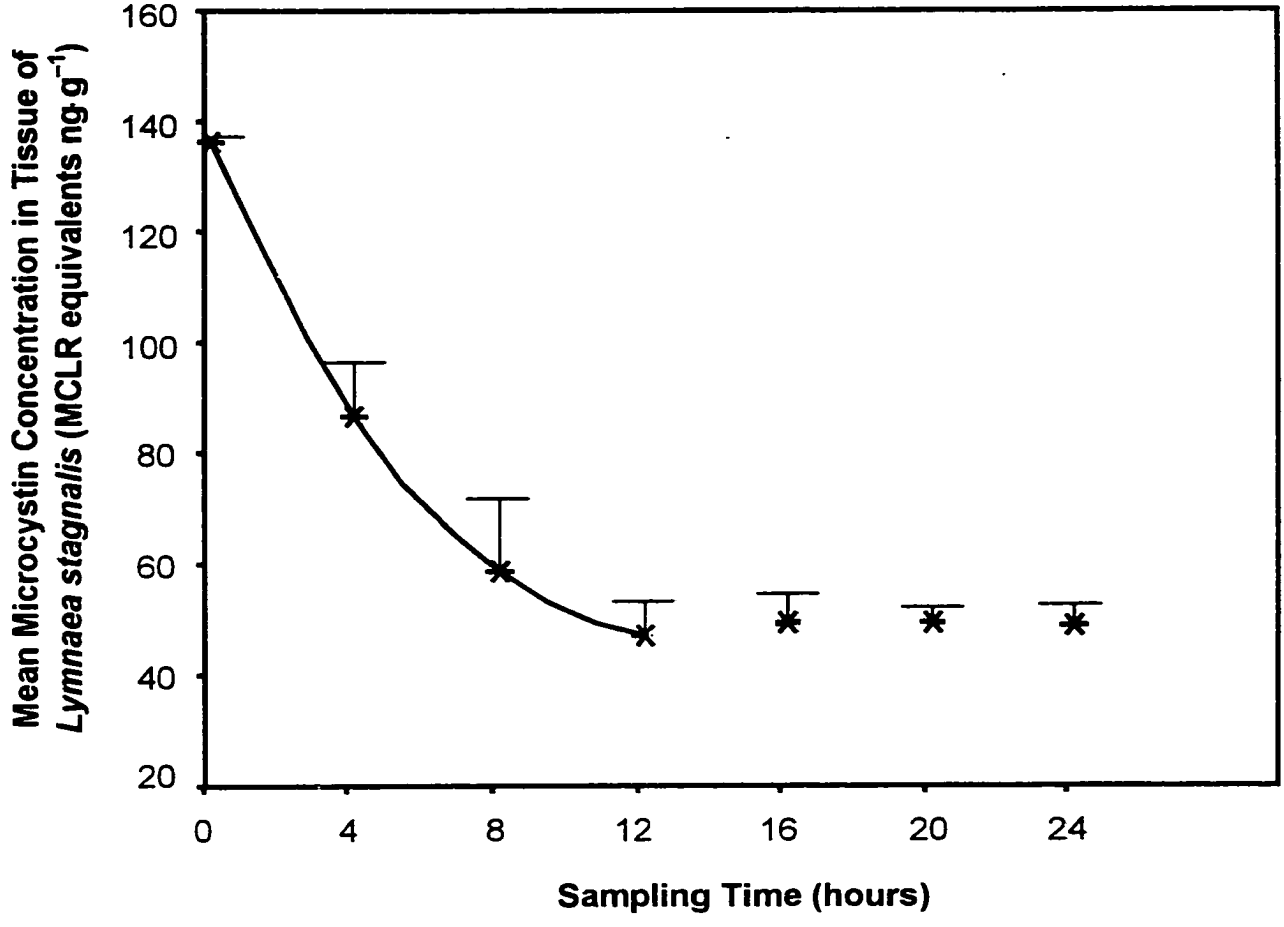

Figure 1. Mean microcystin concentrations (expressed as ng MCLR equivalents g^{-1} dry weight) in tissue of *Lymnaea stagnalis* sampled from three tanks every four hours over a 24-h period. Line represents least squares nonlinear estimation. Error bars = *SE* for triplicate samples.

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Chapter 6

Depuration of microcystin from tissue of the freshwater pulmonate snail *Lymnaea stagnalis jugularis* (Say) [4]

1. Introduction

Numerous studies have affirmed the importance of benthic macroinvertebrates in the accumulation of potent toxins produced by marine dinoflagellates and diatoms (Shumway, 1990). Consequently, clams, mussels, oysters and scallops, have been implicated in myriad reports of human and animal intoxication involving paralytic (saxitoxins and gonyautoxins), neurotoxic (brevetoxins), amnesic (domoic acid) and diarrhetic (okadaic acid and dinophysistoxins) shellfish toxins (Baden, 1983; Yasumoto *et al.*, 1985; Bates *et al.*, 1989). In contrast, information regarding the accumulation of potent cyanobacterial hepatotoxins, termed microcystins (MCYSTs), by freshwater macroinvertebrates remains sporadic (Christoffersen, 1996). Nevertheless, due to the increasing frequency and magnitude of toxic cyanobacterial blooms attributable to the worldwide eutrophication of freshwaters, insights concerning the accumulation of MCYSTs by organisms associated with aquatic food webs are imperative.

Foremost, Eriksson *et al.* (1989) experimentally demonstrated that when reared in laboratory cultures of *Oscillatoria agardhii*, the freshwater mussel *Anodonta cygnea* rapidly accumulates peptide toxin, with the highest levels encountered in hepatopancreatic tissue. Subsequently, Watanabe *et al.* (1997) reported the accumulation of MCYST by the bivalves, *Anodonta woodiana* and *Unio douglasiae*, collected from Lake Suwa, Japan. Similarly, Prepas *et al.* (1997) illustrated the accumulation of MCYST in tissue of *Anodonta grandis* from several eutrophic Alberta lakes and the persistence of toxin more than 21 days after clams were removed from the toxin source.

Recent studies suggest that pulmonate snails (Phylum Mollusca, Class Gastropoda and subclass pulmonata) assume an important role in the occurrence and accumulation of

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MCYSTs in food webs within eutrophic freshwater environments. For example, Kotak *et al.* (1996) concluded that, among invertebrates collected from three eutrophic Alberta lakes, toxin was detected exclusively in herbivorous grazers (*i. e.*, zooplankton and pulmonate snails) and further noted that stomach contents of northern pike, *Esox lucius*, and white sucker, *Catostomus commersoni*, contained appreciable numbers of gastropods. Subsequently, Zurawell *et al.* (1999) concluded that seasonal variation in toxin concentration within tissue of pulmonate snails, specifically *Lymnaea stagnalis*, is influenced primarily by toxin concentration within phytoplankton and *Microcystis* spp. abundance. Moreover, the presence of intact colonies of *Microcystis* spp. in the faecal strings of *L. stagnalis* suggested that toxin detected in gastropod tissue probably originates from *Microcystis* cells/colonies contained in the alimentary system and not from toxin accumulated in tissue (Zurawell *et al.*, 1999). However, Zurawell *et al.* (submitted) recently demonstrated that the concentration of MCYST in *L. stagnalis* previously exposed to toxin-producing cyanobacteria, not only originates from intact *Microcystis* spp. contained within the alimentary tract, but also from toxin accumulated within the digestive gland (hepatopancreas).

Gastropods are important food sources for fish, crayfish, amphibians, and waterfowl (Stein *et al.*, 1984; Lodge, 1986), hence the accumulation of MCYST by these organisms has serious food web implications even after the disappearance of toxin-producing cyanobacteria. Moreover, considering ingestion, digestion and metabolism by snails be temperature dependent (Calow, 1975a, 1975b), the rate of toxin elimination may also vary with ambient temperature, thus influencing the risk of MCYST exposure to predatory organisms. The present study examines the depuration of accumulated MCYST from the tissue of adult *L. stagnalis* maintained in warm (22°C) or cool (10°C) water over a 30-day period. It was hypothesized that tissue toxin concentrations of snails removed from toxin-producing cyanobacteria decreases with time, the rate being dependent on temperature (*i. e.*, slower depuration rate in 10°C than 22°C water).

2. *Materials and Methods*

2.1 Gastropod and phytoplankton collection

Over 300 adult (25 to 30 mm in height) *Lymnaea stagnalis jugularis* (Say) were harvested from Driedmeat Lake Alberta, Canada, during late August 1996. Snails were collected with a mesh dip net from an approximately 20 m^2 littoral area commonly vegetated with dense growths of pondweed [*Potamogeton pectinatus* L. and *P. richardsonii* (Benn.) Rydb.], coontail (*Ceratophyllum demersum* L.), bullrush (*Scirpus* sp.). Individuals were identified on site based on shell morphology descriptions of Clarke (1981) and Clifford (1993) and then immediately placed in aquaria containing lake water for transport to the laboratory. Phytoplankton to be fed to snails in the laboratory was also collected from this site and concentrated with repeated vertical hauls of a 64 μm mesh (Nitex™) plankton net from the surface to a depth of 1 m. This material was combined in an 8 L glass container and five 150 mL subsamples were removed and immediately frozen for subsequent determination of MCYST concentration within the phytoplankton.

2.2 Depuration experiment

In the laboratory, snails were placed in 20 L aquaria supplied with a continuous flow of artificial pond water (dechlorinated, carbon filtered municipal tap water) at 16°C (± 1°C). Snails were acclimated for five days during which time they were fed exclusively phytoplankton collected from Driedmeat Lake. Subsequently, snails were divided equally among six, 4 L continuous-flow holding tanks supplied with artificial pond water, three at 22°C (± 1°C) and three at 10°C (± 1°C). Also at this time, 6 snails were set aside for toxin analysis to determine MCYST concentration at time zero. Over the course of the experiment, snails were fed Unifeed trout ration (United Grain Growers Ltd., Okotoks, AB, Canada) at a rate of three pellets per tank per day. In addition, one 10 cm^2 leaf of lettuce (*Lactuca sativa*) pre-rinsed with distilled water was added to each tank twice a week. Snails were randomly sampled from each tank in triplicate on days 3, 6, 10, 13, 16, 19, 24 and 30.

2.3 Microcystin analysis

Frozen gastropods (excluding shell) and phytoplankton samples were lyophilized and the tissues homogenized with a mortar and pestle. Tissue homogenates of individual snails were weighed to determine freeze-dried body weights and placed in 20 mL glass vials to which 10 mL of 100% methanol was added. Similarly, phytoplankton samples (≈ 100 mg freeze-dried weight) were placed in 20 mL glass vials and 10 mL of 100% methanol added. All samples were sonicated for 20 s to disrupt the cells, then left to settle overnight. For each sample, the liquid portion was carefully decanted and filtered through a Whatman GF/A filter (Whatman Inc., Fairfield, NJ, U.S.A.) and then placed in a glass vial. An additional 5 mL of 100% methanol was added to the remaining solids and resuspended by mixing. After settling overnight, samples were again mixed, then filtered through a Whatman GF/A filter. The resulting two extracts from each sample was combined and evaporated under a constant stream of nitrogen gas with a Reactivap™ heater. The semi-purified extracts were resuspended in 4 mL of ultra pure water and analyzed for MCYST concentration via colorimetric protein phosphatase (PP) inhibition assay as specified by An and Carmichael (1994), utilizing recombinant PP1c expressed in *E. coli* (provided by C.F.B. Holmes).

Microcystin concentrations of both gastropod and phytoplankton extracts were extrapolated from curves plotting PP1c inhibition by microcystin-LR (MCLR) standards (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and are thus expressed as mass MCLR equivalents g^{-1} tissue (dry weight). The detection limit of this assay was approximately 3 ng MCLR eq. g^{-1} tissue. While this assay quantifies all MCYSTs capable of inhibiting PP1c, toxin fractions irreversibly bound to endogenous PP1c and PP2A within gastropod tissues would be unavailable for further PP1c inhibition and consequently remain undetected. Hence, the MCYST values reported undoubtedly underestimate the actual total toxin concentrations within tissues.

2.4 Statistical analysis

Mean MCYST concentrations in *L. stagnalis* at the commencement of the study (*i. e.*, time 0) and those sampled (in triplicate) from the six holding tanks on days 3, 6, 10, 13, 16, 19, 24 and 30 were computed (± *SE*). Nonlinear curve estimation of the

depuration curves (10 and 22°C) was performed on untransformed data with GraphPad Prism (Version 3.02 Trial) software for Windows (GraphPad Software Inc., San Diego, CA, U.S.A.). Fast-phase depuration rate constants (k_{fast}) were compared using Student's *t* test for the slopes of two regression equations as suggested by Zar (1984).

3. Results

The mean MCYST concentration of phytoplankton collected simultaneously with *Lymnaea stagnalis* from Driedmeat Lake and subsequently fed to snails during the acclimation period, was 512 μg g^{-1} dry weight. At no time during the 5 day acclimation period did individual snails appear to be adversely affected by the presence of toxin-containing cyanobacteria. In addition, the phytoplankton added to the aquaria was in great excess of that required by the gastropods. Thus, it was assumed that all individuals had equal opportunity to graze. Immediately following the acclimation period, MCYST concentrations within the six *L. stagnalis* sampled (*i. e.*, time 0) varied from 966 to 1399 ng MCLR eq. g^{-1} with a mean concentration of 1239 (± 65). During the course of the study, MCYST concentrations in snails declined at both temperatures (Figure 1). At either temperature the greatest change occurred over the first 3 d, as mean toxin concentrations in snails sampled from the replicate tanks decreased by 63.7 and 87.7% to 449 (± 13) and 153 (± 30) ng MCLR eq. g^{-1} (10 and 22°C, respectively; Table 1). With respect to snails maintained at 22°C, an additional 7.3% toxin decline by day 6 resulted in an overall (cumulative) toxin loss of 95% (Table 1). This trend continued as mean MCYST concentration decreased another 3.4% by day 10, signifying a 98.4% cumulative loss of toxin from tissue. In contrast, snails at 10°C experienced a 16.1% decrease in mean toxin concentration between days 3 and 6 followed by an additional 9.4% by day 10. Respectively, these represent 79.8 and 89.2% depuration in overall mean toxin concentration (Table 1). Consequently, mean toxin concentration in *L. stagnalis* maintained at 22°C was reduced to 7 (± 1) ng MCLR eq. g^{-1} by day 30, while those at 10°C diminished to 31 (± 6) ng MCLR eq. g^{-1} (Figure 1). These reductions represent a cumulative toxin loss of 99.5 and 97.5% respectively (Table 1).

The findings of the nonlinear curve estimation analyses indicate that regardless of temperature, depuration of MCYST is bi-phasic (*i. e.*, rapid initial rate of toxin

depuration followed by a second slower rate) and fit two-phase exponential loss curves ($r^2 > 0.99$; Figure 1). Estimated depuration rate constants (k_{fast} and k_{slow}; Figure 1) were used to calculate half-life ($t_{1/2}$) of MCYST during both fast and slow toxin loss phases ($t_{1/2} = \ln 2/k$). Consequently, the half-life of MCYST in snails at 22°C was 0.017 d during the initial (fast) phase, but decreased to 2.086 d during the slow phase. On the other hand, the half-life of toxin in snails at 10°C was 0.739 d and 3.713 d during the fast and slow phases, respectively. A comparison of the fast-phase depuration rate constants (k_{fast}) revealed a significant ($P < 0.001$) difference between the rate of toxin loss from tissue of snails maintained at 22°C opposed to those at 10°C.

4. Discussion

Recently, Zurawell *et al.* (submitted) provided conclusive evidence regarding the limited digestion of *Microcystis* by the freshwater pulmonate snail *L. stagnalis*. The elimination of the gizzard and caecal string fractions (*i. e.*, indigestible cyanobacterial cells/colonies), which occurred during the initial 8 h post-ingestion, accounted for the majority (≈ 57%) loss of ingested MCYST. However, lower concentrations (≈ 49 ng MCLR eq. g^{-1}) detected up to 24 h post-ingestion were subsequently attributed to toxin accumulated within the digestive gland tissue (Zurawell *et al.*, submitted). Considering that fine and soluble digestible matter entering the digestive gland might remain detectable beyond 100 h (Veldhuijzen, 1974), this toxin fraction may persist for several days before being eliminated in the digestive gland fraction of the faeces (Zurawell *et al.*, submitted).

Based on previous findings then, complete elimination of indigestible gut contents from the gizzard and caecum (up to 8 h), as well as digestible matter from the digestive gland (up to 100 h), should correspond with the period of maximum toxin loss (Veldhuijzen, 1974; Zurawell *et al.*, submitted). My results indicate that irrespective of temperature, the greatest decline (63.7 and 87.7% at 10 and 22°C respectively) in mean MCYST concentration occurred during the first 3 d post-ingestion. In addition, decreases (16.1 and 7.3% at 10 and 22°C respectively) in toxin concentration between days 3 and 6 are notable and likely coincide with MCYST elimination exclusively from the digestive gland. Hence, the majority (79.8 and 95.0% cumulative decline, 10 and 22°C

respectively) of toxin loss from *L. stagnalis*, which occurred over the first 6 d, may be attributed foremost to digestion/egestion. The depuration of hydrophobic organic compounds from tissues of aquatic invertebrates often assume first-order (log linear) exponential loss kinetics, yet others are known to better fit bi-phasic models. For instance, Pastershank *et al.* (1999) concluded the depuration of 2,3,7,8-tetrachlorodibenzofuran from the filter-feeding caddisfly larvae, *Hydropsyche bidens* (Ross), followed a negative exponential curve, while loss of 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDD) was bi-phasic. Furthermore, they attributed the bi-phasic nature of OCDD depuration to the rapid, concomitant elimination of gut contents (Pastershank *et al.*, 1999). Similarly, Loonen *et al.* (1997) found that the bi-phasic depuration of OCDD from oligochaetes (*Lumbriculus variegatus*) was attributable to an initial rapid loss via gut content elimination, followed by slower depuration from other body compartments.

In the present study, the fact that MCYST remained detectable beyond 6 d provides evidence for the limited accumulation of toxin within *L. stagnalis*. As well, depuration of toxin continued beyond day 6 to the conclusion of the study (*i. e.*, day 30), albeit at lower rates. Consequently, nearly all (97.5 and 99.5%, 10 and 22°C respectively) of the initial MCYST was eliminated from snails by day 30, at either temperature (Table 1). According to Eriksson *et al.* (1989), who demonstrated hepatotoxin accumulation in the freshwater mussel *Anodonta cygnea*, long-term depuration could result from either passive excretion or degradation of toxin with time. Prepas *et al.* (1997) observed a 71% decrease in total MCYST concentration within the freshwater clam *Anodonta grandis*, over the initial 6-d period following removal from toxic cyanobacteria. However, 29% of the toxin remained after 21 d (Prepas *et al.*, 1997). These disparities may result from body size differences between relatively large bivalve molluscs used in other studies and the smaller pulmonate snails used in this experiment. In general, larger organisms ingest food at greater rates and feed less discriminately than smaller organisms (Peters, 1983). Furthermore, small-sized aquatic invertebrates typically possess lower assimilation efficiencies and greater depuration rates for hydrophobic organic compounds, compared to larger organisms (Pastershank *et al.*, 1999). Thus, even within a given species, the implications of body-size suggest a

predisposition towards large aquatic grazers with respect to the accumulation and potential transfer of MCYSTs to aquatic and terrestrial predators associated with the food webs of bloom-prone lakes.

It is apparent that temperature influences the rate of MCYST depuration from tissue of *L. stagnalis*. Consequently, mean toxin concentrations in snails maintained in cooler (10°C) water, decline slower compared to those maintained in warmer (22°C). These findings are not surprising as numerous studies not only indicate a significant relationship between temperature and ingestion rate, but egestion rate as well (Calow, 1975a, 1975b). Since the digestive gland (hepatopancreas) is regarded as the primary site of digestion/absorption (Carriker, 1946; Boer and Kits, 1990), a reduction in egestion rate concomitant with decreasing ambient temperature may afford pulmonate snails greater opportunity to extract nutrients from ingested food particles (Calow, 1975b). Hence, reductions in the rate of MCYST elimination from the digestive gland are likely responsible for mitigating toxin depuration from snails maintained at 10°C as opposed to those at 22°C. Finally, though MCYSTs are stable to the effects of heat, toxin degradation at elevated temperatures might occur in acidic environments (Harada *et al.*, 1996). Thus, it is plausible that greater thermal decomposition of MCYSTs within the alimentary system of *L. stagnalis* would occur at 22°C relative to snails at 10°C.

5. Conclusion

Compared to marine species, freshwater macroinvertebrates (*i. e.*, bivalves and gastropods) seem economically unimportant as they are rarely harvested for human consumption. Nevertheless, these organisms are significant links in the food webs of freshwater environments as they feed directly on primary producers and in turn, become prey for numerous aquatic and terrestrial organisms. The eutrophic and hypereutrophic lakes of Western Canada often experience severe blooms of toxin-producing cyanobacteria during the open water (May to October) season (Kotak *et al.*, 1995). Within many of these lakes, pulmonate snails remain the largest-bodied primary consumer, as bivalve molluscs (*i. e.*, clams and mussels) are typically unable to inhabit shallow, oxygen deficient conditions (Clarke, 1981; Elder and Collins, 1991). Results of the present study demonstrate the ability of *L. stagnalis* to retain detectable MCYST

concentrations up to 30 d, following the cessation of toxic bloom events. The bi-phasic depuration of MCYST reported here, compares favourably with several studies regarding the elimination of heavy metals and hydrophobic organic compounds from tissues of various freshwater molluscs (Elder and Collins, 1991; Pastershank *et al.*, 1999). Consequently, gut content elimination is responsible for the rapid initial clearance of the majority of toxin and is presumably followed by decelerated loss via passive excretion and/or toxin degradation with time. The role of temperature is significant, as cyanobacterial blooms often persist into the autumn when water typically decreases below 10°C. Hence, snails likely contain MCYST well beyond 30 d post-ingestion. In Western Canada, this period coincides with large annual waterfowl migrations and, in light of these findings, *L. stagnalis* and other pulmonate snails can assume paramount roles in the transfer of MCYSTs to these and other organisms associated with the food webs of these bloom-prone lakes. Moreover, since molluscs are used extensively as biomonitoring tools for heavy metals in aquatic environments, I propose pulmonate snails (especially large-bodied Lymnaeids) be useful bioindicators of cyanobacterial hepatotoxins in hypereutrophic freshwaters.

Table 1. Percent decline and cumulative percent decline in mean microcystin (MCYST) concentration in tissue of *Lymnaea stagnalis* at 10 and 22°C (± 1°C) sampled over 30-d period

	% MCYST decline*		Cumulative % MCYST decline	
Time (days)	10°C	22°C	10°C	22°C
3	63.7	87.7	63.7	87.7
6	16.1	7.3	79.8	95.0
10	9.4	3.4	89.2	98.4
13	3.9	0.5	93.1	98.9
16	1.9	0.1	95.0	98.9
19	1.7	0.5	96.7	99.3
24	0.7	0.1	97.4	99.4
30	0.1	0.1	97.5	99.5

* Values represent percent decrease in toxin concentration compared to initial mean concentration at time (day) 0 (*i. e.*, 1239 ng MCLR eq. g^{-1}).

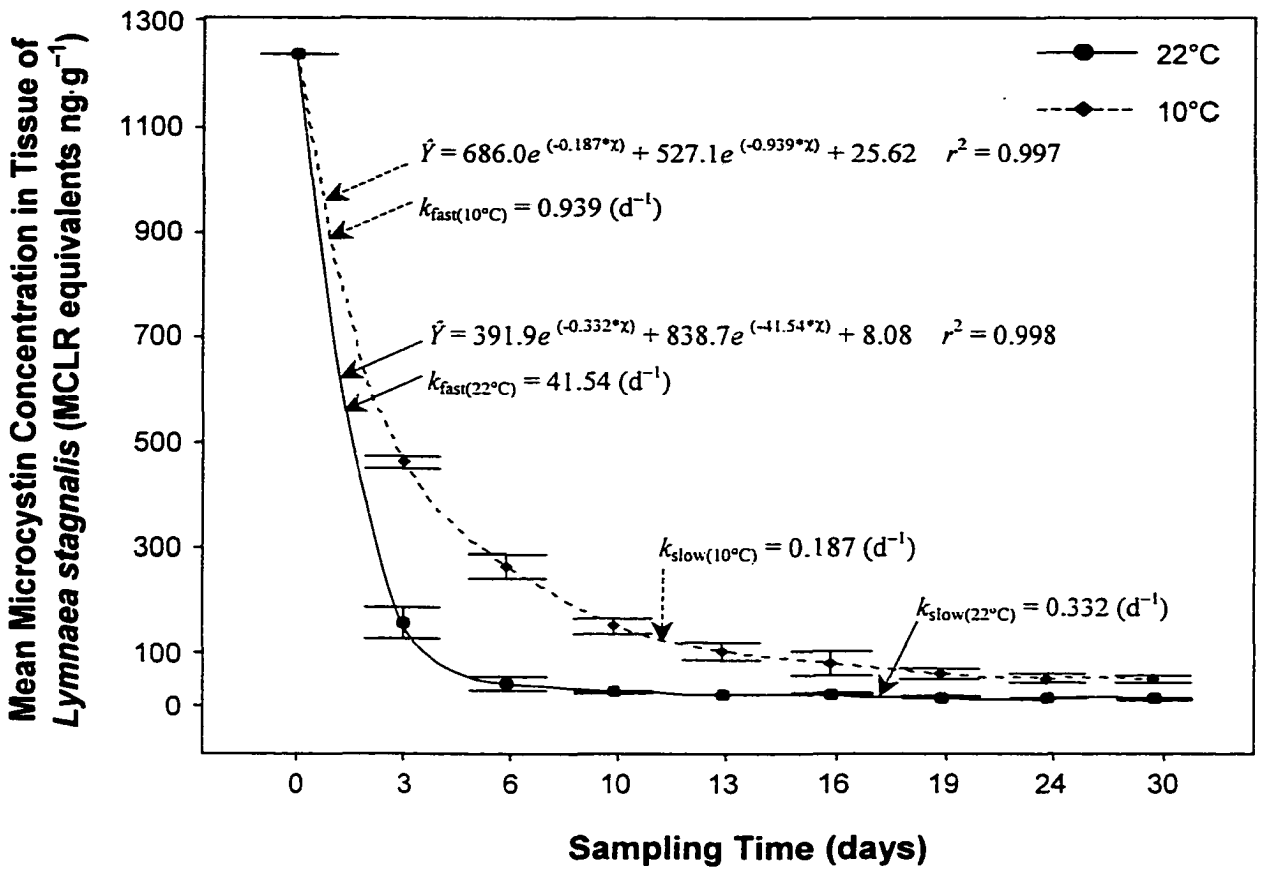

Figure 1. Best-fit nonlinear depuration curves estimated for mean microcystin concentrations (expressed as ng MCLR equivalents g^{-1}) in tissue of *Lymnaea stagnalis* sampled from six tanks (3 replicate tanks each at 10 and 22°C) over a 30-d period. All equation parameters significant ($P < 0.001$). Error bars = *SE* for replicate tanks.

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Chapter 7

Effects of microcystin-LR on the digestive gland tissue and embryo survival of the freshwater pulmonate snail *Lymnaea stagnalis* [5]

1. Introduction

Microcystin-LR (MCLR) is a potent hepato- (liver) toxin produced by common bloom-forming species of cyanobacteria in productive lakes and reservoirs worldwide. In mammals and fish, MCLR is preferentially taken up by hepatocytes (functional cells of the liver) via the multispecific bile acid transport system (Eriksson *et al.*, 1990), where it irreversibly binds to and inhibits specific enzymes known as protein phosphatases 1 and 2A. Normally, these enzymes function in opposition of protein kinases to mediate the reversible phosphorylation of the serine/threonine residues of regulatory proteins (Cohen *et al.*, 1990; MacKintosh *et al.*, 1990). Microcystin-LR then, disrupts the critical balance between the phosphorylation/dephosphorylation activities of protein kinases and phosphatases (respectively), producing a hyperphosphorylated state of various cytosolic and cytoskeletal proteins within hepatocytes. The predominant cellular effect of MCLR is the reorganization of cytoskeletal components – namely the microfilaments (MFs), intermediate filaments (IFs) and microtubules (MTs), resulting in plasma membrane bleb formation, invagination, loss of microvilli and cell-cell adhesion, as well as numerous ultrastructural alterations (Eriksson *et al.*, 1989a; Hooser *et al.*, 1990, 1991). Ultimately, these changes cause disruption of the hepatic architecture and widespread necrosis potentially leading to death via severe hepatic damage (mammals) or dysfunction (fish).

In comparison to mammals and fish, relatively few studies have focussed on the toxicity of hepatotoxins to aquatic macroinvertebrates (benthic or otherwise). The most obvious organisms threatened by the presence of toxic cyanobacteria may be the sessile, filter-feeding mussels and clams (e. g., *Anodonta cygnea* – Eriksson *et al.*, 1989b; *A.*

[5] A version of this chapter will be submitted as Zurawell R.W. and Prepas E.E. (submitted d) Effects of microcystin-LR on the digestive gland tissue and embryo survival of the freshwater pulmonate snail *Lymnaea stagnalis*.

woodiana and *Unio douglasiae* – Watanabe *et al.*, 1997; *A. grandis* – Prepas *et al.*, 1997). Nevertheless, several studies indicate that herbivorous (e. g., gastropods – Kotak *et al.*, 1996a; Zurawell *et al.*, 1999) and omnivorous (e. g., crayfish – Lirås *et al.*, 1998) grazers inhabiting bloom-prone lakes might accumulate hepatotoxins. To this end, Zurawell *et al.* (submitted a) recently demonstrated the microcystin concentration within *Lymnaea stagnalis* (Phylum Mollusca, Class Gastropoda and subclass pulmonata) previously exposed to toxin-producing cyanobacteria, not only originates from intact *Microcystis* spp. contained within the alimentary tract, but also from toxin accumulated within the digestive gland (hepatopancreas). Furthermore, detectable toxin concentrations within the digestive gland tissue may remain up to 30 d post-ingestion (Zurawell *et al.*, submitted b). Since the digestive gland is the primary site of intracellular digestion/detoxication/metabolism (Carriker, 1946; Livingstone, 1985), the potential exists for direct toxic effects by ingested microcystin on this organ.

Considering the paucity of information regarding the toxicity of hepatotoxins to aquatic macroinvertebrates, particularly with respect to the effects on histopathology and reproduction, I examined whether MCLR causes the disruption of digestive gland cells in adult *L. stagnalis*. Moreover, the effects of aqueous MCLR on the survival of *L. stagnalis* embryos were investigated. Specifically, my objectives of the first study were to describe cellular changes within the digestive glands of adult *L. stagnalis* exposed to (orally administered) MCLR and determine if the extent of disruption varies with dose. The goal of my second study was to determine if environmentally relevant concentrations of aqueous MCLR affect the success of embryos developing to 15 d post-oviposition.

2. Materials and Methods

2.1 Oral dosing experiment

Adult *Lymnaea stagnalis* (≈ 30 mm shell length) from an established laboratory colony (provided by J.I. Goldberg) were used in this study. Purified MCLR (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was resuspended in artificial pond water (dechlorinated, carbon filtered municipal tap water) and serially diluted into 4 treatment concentrations (10, 50, 100 or 1000 μg L^{-1}). To anaesthetize *L. stagnalis* prior to

treatment, individuals were removed from their aquarium and placed in a beaker containing Listerine™ and artificial pond water (25% v/v) for 20 min. This process effectively relaxed the snails and prevented them from withdrawing into their shells during handling. Individual snails were placed onto a petri dish containing artificial pond water. Then with aid of a dissecting microscope (5× power), a single 20 μL dose was administered into the esophagus via the buccal cavity with a fine (20 gauge) round-tip syringe. This process was repeated with a total of 6 snails for each of the 4 treatment conditions. Based on a 20 μL dose, snails received 0.0002, 0.001, 0.002 or 0.02 μg of MCLR (equal to 0.0002, 0.001, 0.002 or 0.02 nmol MCLR, respectively). Additionally, six snails were injected with artificial pond water only and served as experimental controls. Following treatment, snails were placed into a 20 L aquarium that was equally subdivided, which separated the 5 groups of snails, yet allowed water to flow through. Snails were maintained in continuous flow of artificial pond water (20 ± 1°C) under a 16:8 h light:dark cycle. After 96 hours, snails were removed from the aquarium and cooled in 4°C (± 1°C) artificial pond water. These were again anaesthetized in a 25% Listerine™ and artificial pond water solution (4°C). Next, the viscera was removed from the shell and the digestive gland immediately excised and placed in a vial containing Bouin's fixative. It was necessary to perform the dissection at a relatively low temperature (4°C) as to minimize cellular degeneration by digestive gland enzymes. The digestive glands were later processed, embedded in paraffin, thin sectioned (5 μm), stained (hematoxylin and eosin) and permanent slides were prepared. Histological observations were made with a light microscope at 100 and 400 times magnification.

2.2 Embryo survival experiment

Adult *Lymnaea stagnalis* (≈ 30 mm shell height) from an established laboratory colony were transferred into a 20 L aquarium containing clean, aerated artificial pond water and a substrate of pre-rinsed, crushed oyster shell. It is recognized that periodic water change stimulates oviposition in *L. stagnalis* (van der Steen, 1967). Within 2 d following placement into clean water, a sufficient number of egg masses (multiple egg cells enveloped by a gelatinous matrix or tunica surrounded by the common tunica capsulae – Bretschneider, 1948) were oviposited for use in this study. Egg masses were

gently dislodged from the glass walls of the aquarium with a scalpel blade and placed into 150 mL glass beakers containing 100 mL of aqueous MCLR (purified MCLR diluted with artificial pond water) at concentrations of either 0.01, 0.1, 1.0 or 10 μg L^{-1}. A total of 5 egg masses were included for each of the 4 treatment conditions. In addition, 5 masses were individually placed into beakers containing 100 mL of artificial pond water only and served as experimental controls. The 25 beakers were randomly placed into a water bath maintained at 20°C (± 1°C) and exposed to a 16:8 h light:dark cycle of illumination. Treatment solutions were replaced every 5 d with freshly diluted MCLR until viable embryos proceeded through the pre-hatch, "hippo" stage of development (up to 15 d). At this point, the number of expired and living individuals within each mass was enumerated with aid of a dissecting microscope.

2.3 Statistical analysis

Embryo survival data were tested for normality (Kolmogorov-Smirnov test, $P > 0.19$) and homogeneity of variance (Levene's test, $P > 0.22$) and the mean proportion of living individuals within the 4 treatments and control groups subsequently compared with a one-way ANOVA ($\alpha = 0.05$; df = 4, 20). Analyses were performed with SPSS for Windows (Version 8.0) statistical software (SPSS Inc., Chicago, IL, U.S.A.).

3. Results

3.1 Oral dosing experiment

Following 96 hr post-injection, casual observations indicated that *L. stagnalis* in the control (water only) and the two lowest treatment groups (*i. e.*, 0.0002, 0.001 nmol MCLR) appeared normal, displaying typical behaviors (*i. e.*, extending from shell, crawling on aquarium walls and periodic surfacing for air). Snails receiving 0.002 nmol MCLR displayed similar behaviors, though to a lesser extent. In contrast, snails receiving the highest dose (*i. e.*, 0.02 nmol MCLR) remained withdrawn in their shells during much of the 96 h suggesting ill effects due to MCLR intoxication. Gross observations of the digestive gland tissue from snails in all five groups, revealed distinct changes in this organ with increasing dose. Tissue from individuals receiving water only,

0.0002 or 0.001 nmol MCLR appeared tan in color, firm in texture and maintained its shaped during removal from the shell. However, tissues from *L. stagnalis* receiving either of the two higher doses appeared pale in color, severely softened and exceedingly friable, making removal somewhat difficult. In contrast to control snails (Figure 1), histological observations of the digestive glands of snails exposed to MCLR, indicated a dose dependent alteration of cells (Figure 2-5). At the lowest treatment dose (0.0002 nmol MCLR; Figure 2), digestive gland cells showed signs of minor bleb formation and alteration of cell shape (*i. e.*, becoming club shaped due to narrowing of cells at base). At higher doses (0.001 and 0.002 nmol MCLR; Figure 3, 4), bleb formation and alteration of cell shape became more pronounced. Nuclei migrated to the base of the cell as vacuolization progressively increased. Furthermore, separation of the basal lamina occurred from the cells, while instances of cell lysis caused cytoplasmic debris to collect within the lumen of the gland. At the highest dose (0.02 nmol MCLR; Figure 5), massive necrosis was evident leading to almost complete disruption of digestive gland tissue integrity.

3.2 Embryo survival experiment

The number of egg cells per oviposited mass used is this study ranged from 61 to 80. *Lymnaea stagnalis* embryos typically progress through several stages of development before hatching from the egg mass. These include trochophore larvae (≈ 3 d), veliger stage (≈ 5 d) and hippo stage (≈ 7 d). Beyond the hippo stage, the heartbeat can be clearly discerned. Thus, the presence of heartbeat in hippo stage *L. stagnalis* was the sole determinant used to indicate whether an individual was living. Alternatively, all individuals lacking a heartbeat, as well as those not progressing through this stage by day 15, were considered expired. In general, the proportion of embryonic *L. stagnalis* successfully progressing through the various stages of development (up to 15 d) varied within each group (summarized in Table 1). However, comparison of the mean proportion of embryos living at 15 d concluded no significant difference (ANOVA, P = 0.35) between snails in the control or either of the 4 treatment groups.

4. Discussion

Several studies have demonstrated the occurrence and accumulation of peptide hepatotoxins in tissues of aquatic macroinvertebrates (primarily bivalves and gastropods) essential in the food webs of bloom-prone lakes (Eriksson *et al.*, 1989b; Kotak *et al.*, 1996a; Watanabe *et al.*, 1997; Prepas *et al.*, 1997; Zurawell *et al.*, 1999, submitted a, b). Until now, however, little research has focussed on the possible adverse affects of hepatotoxins on these organisms. The goal of the first study then, was to identify the effects of orally administered MCLR on the digestive gland histology of *L. stagnalis* and to compare these changes with alterations reported to occur in liver tissue of affected fish and mammals.

Indeed, my results clearly demonstrate the ability of MCLR to elicit cellular changes in the digestive glands of *L. stagnalis*. Even at low treatment doses (0.0002 nmol MCLR; Figure 2) indicative of natural environments, digestive gland cells showed signs of minor bleb formation and alteration of cell shape. This pathology is consistent with that observed in liver tissue of both mammals and fish. The predominant effects in animals, following oral and interperitoneal exposure to peptide hepatotoxins, typically include: plasma membrane bleb formation and invagination, cellular vacuolization and hepatocyte dissociation (Hooser *et al.*, 1990; Råbergh *et al.*, 1991; Kotak *et al.*, 1996b). Cell lysis leading to necrosis and complete disruption of digestive gland integrity evident in snails exposed to the highest dose (0.02 nmol MCLR; Figure 5), also agrees with previous studies showing localized necrosis culminating in the loss of hepatic architecture in mammals and fish (Theiss *et al.*, 1988; Hooser *et al.*, 1990; Kotak *et al.*, 1996b).

Studies involving vertebrates have proved these cellular effects are due to dose- and time-dependent reorganizations of cytoskeletal components (MFs, IFs and MTs), which ultimately result from the inhibition of protein phosphatases 1 and 2A by peptide hepatotoxins (Eriksson *et al.*, 1989a; Hooser *et al.*, 1991; Toivola *et al.*, 1997). The present study precluded observations regarding ultrastructural (*i. e.*, the alteration of cytoskeletal components) or biochemical changes (*i. e.*, the inhibition of protein phosphatases). Thus, my findings do not prove unequivocally, that cellular changes reported for *L. stagnalis*, are in fact due to the inhibition of protein phosphatase 1 and/or

2A. However, it is well recognized that protein phosphatases, as well as the proteins comprising the cytoskeletal components (e. g., actin within MFs or tubulin within MTs), are highly conserved in evolution (Cohen, 1989; Toivola, 1998). As a result, vertebrates and invertebrates alike possess these compounds. Though future investigations are necessary, I suggest the effects of MCLR on the digestive gland of *L. stagnalis* reported here, can most likely be attributed to cytoskeletal reorganization resulting from the inhibition of protein phosphatase 1 and/or 2A.

The general absence of information regarding adverse effects of hepatotoxins on macroinvertebrate reproduction provides the impetus for the embryo survival study. Gastropods are regarded as important food sources for fish, crayfish, amphibians, and waterfowl (Stein *et al.*, 1984; Lodge, 1986). Thus, any negative impacts of hepatotoxins with respect to gastropod reproduction may yield implications for other aquatic and terrestrial organisms associated with the food webs of bloom-prone lakes. The intent of the second study then, was to examine the effects of aqueous MCLR on the survival success of oviposited egg cells of *L. stagnalis*.

The proportion of successfully developed living snails following the 15-d exposure to artificial pond water only or any of the treatments was not different (P = 0.35). Thus, it appears that based on the concentrations used in this experiment (0.01, 0.1, 1.0 or 10 $\mu g\ L^{-1}$), aqueous MCLR has no affect on the survival of *L. stagnalis* embryo. While the concentrations of MCLR used in this study represent a range of environmentally relevant toxin levels known to exist during late summer periods in eutrophic north-temperate lakes and reservoirs of western Canada (Kotak *et al.*, 1993, 1995; Zurawell *et al.*, 1999), it remains uncertain whether higher ($> 10\ \mu g\ L^{-1}$) concentrations that exist in other freshwaters of the world, could reduce the survival success of pulmonate snail embryo. Past research demonstrates the ability of the tunica capsulae to imbibe water over time. In this way, the egg mass softens allowing developed snails to escape into their aquatic environment (Bretschneider, 1948). It is plausible that the tunica capsulae and common matrix surrounding the egg cells or the lamellar membranes enveloping each individual egg cell within the mass provide an effective barrier to aqueous MCLR exposure. In this case, regardless of MCLR

concentration of the external environment, the developing embryo would be protected from any detrimental effects.

Although MCLR failed to elicit a negative response on embryonic survival, other aspects of reproduction may be susceptible to toxin exposure. For instance, adversities with respect to the number of egg cells per oviposited egg mass, size of individual egg cells within each mass and time required between successive ovipositions (latency period), could influence the overall population of snails within affected habitats. Moreover, potential delays in sexual maturation by juvenile *L. stagnalis* exposed to MCLR via aqueous routes (*i. e.*, water intake as a consequence of feeding and/or uptake across the epidermis) or more notably through diet (Zurawell *et al.*, submitted a, b), may also contribute to seasonal reductions in the number of breeding individuals.

5. Conclusion

To my knowledge, the studies presented here are the first to examine the potential capacity of peptide hepatotoxins, such as MCLR, to induce toxic effects in freshwater gastropod molluscs. Histopathological alterations to the digestive glands of *L. stagnalis* exposed to MCLR via oral administration are notable and suggest snails inhabiting bloom-prone lakes and reservoirs, periodically are susceptible to hepatotoxins. Moreover, the similarity existing between the cellular changes in *L. stagnalis* demonstrated here and those reported in mammals and fish elsewhere, indicates a common mode of toxicity in both vertebrates and invertebrates. Microcystin-LR had no adverse affect on the survival of snail embryo progressing through the various stages of development. Reproduction is arguably the single most important function in the lifecycle of an organism and "the inability of an organism to complete any one stage of the reproductive process severely reduces its lifetime reproductive success" (Woin and Brönmark, 1992). Considering the toxic effects of MCLR on adult snails, other aspects of gastropod reproduction may suffer. Hence, to adequately appreciate the impacts of hepatotoxins on aquatic macroinvertebrates and the ensuing food web implications, further investigations addressing these possibilities are necessary.

Table 1. Summary of the embryo survival study. Values represent the proportion of the total number of oviposited *Lymnaea stagnalis* egg cells, considered living* at the conclusion of the 15 d study

	Proportion of living *Lymnaea stagnalis*				
Replicate	Control	0.01 μg L^{-1}	0.1 μg L^{-1}	1.0 μg L^{-1}	10.0 μg L^{-1}
1	0.985	1.000	0.959	0.946	0.934
2	0.952	0.948	0.947	1.000	0.969
3	0.938	0.963	0.985	0.986	0.944
4	0.960	0.985	0.968	0.959	0.949
5	1.000	0.986	0.953	0.955	0.955
Mean (± *SE*)	0.967 (0.011)	0.976 (0.009)	0.963 (0.007)	0.969 (0.010)	0.950 (0.006)

* Living snails were defined as those developed beyond the hippo stage with a discernible heartbeat at 15 d.

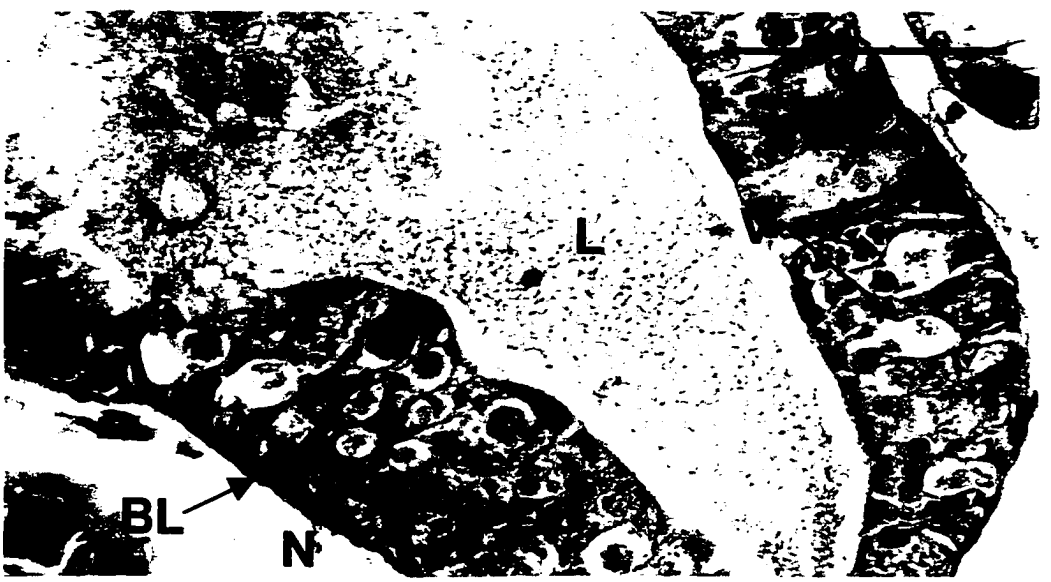

Figure 1. Light micrograph of 5-μm cross section through digestive gland tissue of *Lymnaea stagnalis* injected with artificial pond water only. BL = basal lamina; DC = digestive gland cells; L = lumen; N = nuclei; V = vacuoles. Bar = 50 μm.

Figure 2. Light micrograph of 5-μm cross section through digestive gland tissue of *Lymnaea stagnalis* injected with 0.0002 nmol microcystin-LR. BL = basal lamina; DC = digestive gland cells; L = lumen; N = nuclei; V = vacuoles; bf = bleb formation. Bar = 50 μm.

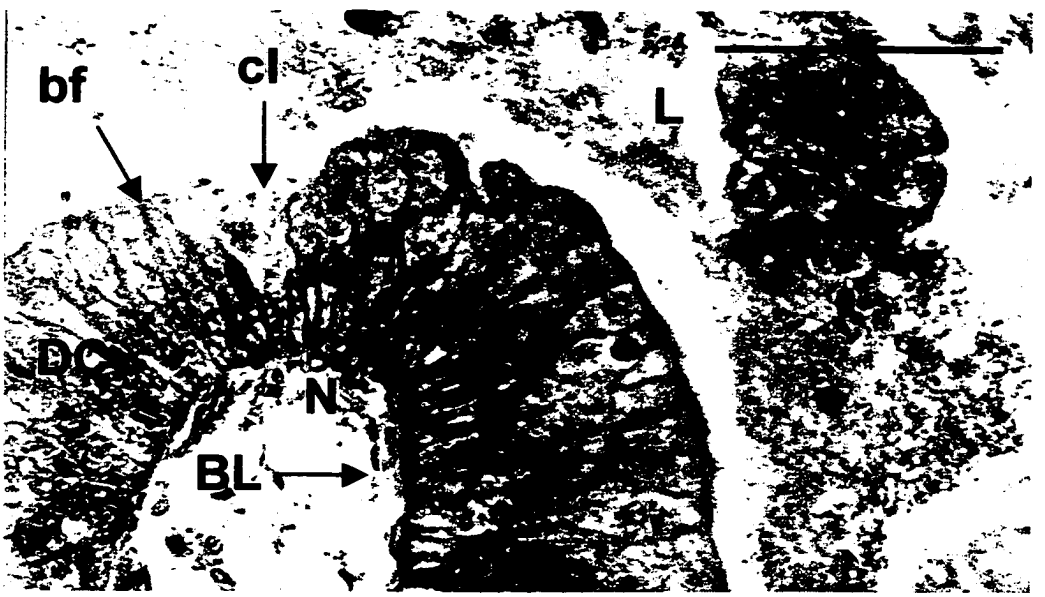

Figure 3. Light micrograph of 5-μm cross section through digestive gland tissue of *Lymnaea stagnalis* injected with 0.001 nmol microcystin-LR. BL = basal lamina; DC = digestive gland cells; L = lumen; N = nuclei; bf = bleb formation; cl = cell lysis. Bar = 50 μm.

Figure 4. Light micrograph of 5-μm cross section through digestive gland tissue of *Lymnaea stagnalis* injected with 0.002 nmol microcystin-LR. DC = digestive gland cells; L = lumen; N = nuclei; V = vacuoles; bf = bleb formation; cd = cytoplasmic debris; cl = cell lysis. Bar = 50 μm.

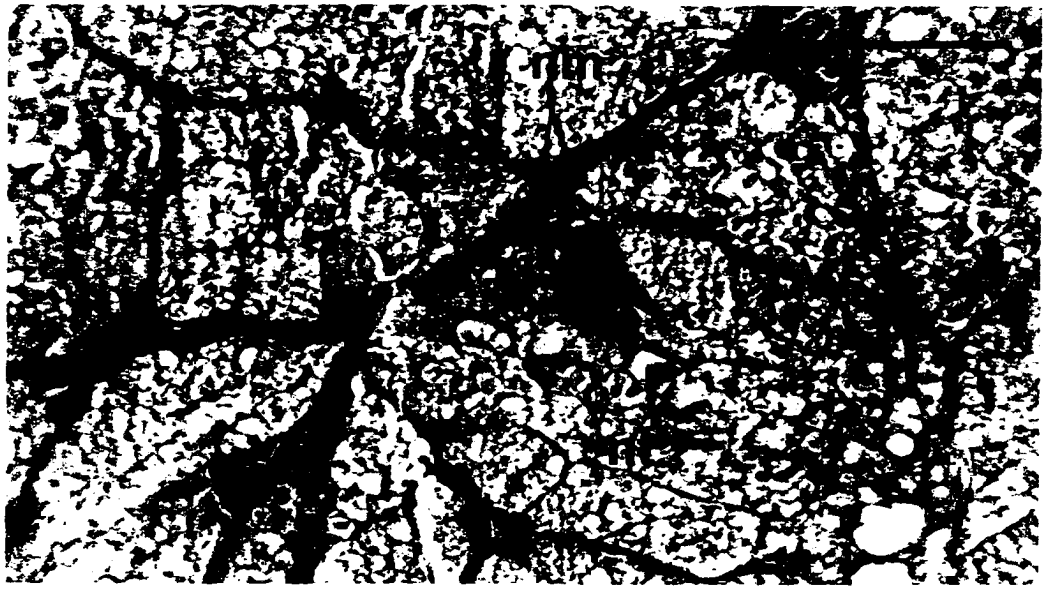

Figure 5. Light micrograph of 5-μm cross section through digestive gland tissue of *Lymnaea stagnalis* injected with 0.02 nmol microcystin-LR. L = lumen; cd = cytoplasmic debris; nm = nuclear material; nt = necrotic tissue. Bar = 50 μm.

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Chapter 8

General Discussion and Conclusions

1. General Discussion

Research involving cyanobacterial hepatotoxins has traditionally focused on the acute toxicity of microcystins (MCYSTs) to laboratory mammals and fish. In addition, several studies have examined the occurrence and toxicity of MCYSTs to zooplankton including minute protozoans (e. g., *Heteromita globosa* and *Spumela* sp. – Christoffersen, 1996) and large-bodied grazers (e. g., *Daphnia* spp. – DeMott *et al.*, 1991 and *Artemia salina* – Reinikainen *et al.*, 1995). Comparatively few have concentrated on the uptake, accumulation and toxicity of hepatotoxins in aquatic macroinvertebrates (benthic or otherwise). The most obvious group threatened by the presence of toxic cyanobacteria may be the sessile, filter-feeding bivalves. In this regard, Eriksson *et al.* (1989) demonstrated the freshwater mussel *Anodonta cygnea* accumulates peptide toxin when reared in cultures of *Oscillatoria agardhii* and suggested that organisms feeding on toxin-producing cyanobacteria in eutrophic environments could potentially accumulate toxin. Subsequently, the natural occurrence of hepatotoxins in tissues of freshwater mussels and clams sampled from bloom-prone lakes and reservoirs has been reported. For instance, Watanabe *et al.* (1997) noted the accumulation of MCYST by the bivalves, *Anodonta woodiana* and *Unio douglasiae*, collected from Lake Suwa, Japan. Moreover, Prepas *et al.* (1997) not only documented the accumulation of MCYST in tissue of *Anodonta grandis simpsoniana* from several eutrophic Alberta lakes, but also showed the persistence of toxin more than 21 days after clams were removed from the toxin source. However, direct toxic effects of MCYSTs on bivalve survival have not been observed and hepatotoxins appear not to be metabolized in these organisms (Eriksson *et al.*, 1989; Vasconcelos, 1995).

A study by Kotak *et al.* (1996a), who suggested microcystin-LR (MCLR) might occur in a variety of aquatic organisms inhabiting bloom-prone waters, detected toxin within indigenous pulmonate snails collected from several hypereutrophic Alberta lakes. Furthermore, the tissue toxin concentrations roughly followed changes in toxin concentrations within phytoplankton. Gastropods are recognized as an important food

source for many economically important sport fish (trout, perch and whitefish) and waterfowl (ducks, geese and shorebirds), as well as many less economically important groups (suckers, carp, crayfish and amphibians) (Stein *et al.*, 1984; Lodge, 1986). As well, pulmonate snails often remain the largest-bodied primary consumer within eutrophic waters, as bivalve molluscs (*i. e.*, mussels and clams) are unable to inhabit shallow, highly productive, oxygen deficient conditions (Clarke, 1981; Elder and Collins, 1991). Thus, as they may comprise the largest proportion of the macroinvertebrate fauna, snails potentially predominate in the transfer of toxins to other aquatic and terrestrial organisms associated with the food webs of bloom-prone lakes and reservoirs. Conjointly, these facts and findings provide the main impetus to ascertain whether gastropods play a substantive role in the uptake, accumulation and fate of cyanobacterial hepatotoxins in aquatic food webs.

In an attempt to further substantiate the findings of Kotak *et al.* (1996a) and assess the influence of lake trophic status on the occurrence of MCLR in the tissue of resident pulmonate snails, seven Alberta lakes of varying primary productivity (measured as chlorophyll-*a* concentration of the phytoplankton) were surveyed. The concentration of toxin in the tissue of three resident gastropod species (*i. e.*, *Lymnaea stagnalis*, *Helisoma trivolvis* and *Physa gyrina*) was correlated with toxin in the phytoplankton ($P \leq 0.03$), but not detectably so with extracellular aqueous microcystin ($P > 0.28$). Moreover, the tissue toxin concentrations of *L. stagnalis* and *P. gyrina* were also correlated with the relative abundance of *Microcystis* spp. ($P < 0.01$). Given that *Microcystis* spp. abundance and MCLR concentration within phytoplankton are correlated with indicators of productivity (*i. e.*, total nitrogen, total phosphorus and chlorophyll-*a* concentrations, total nitrogen:total phosphorus ratio and Secchi depth), it is apparent that trophic status is important in influencing the occurrence and concentration of MCLR in pulmonate snails.

Though concentrations varied within lakes, MCLR was detected more often and at greater concentrations in gastropods from highly productive lakes than in those from lower productivity. Subsequently, several factors contributing to intraspecific variation in MCLR tissue concentrations were proposed. Foremost, spatial differences (vertical and horizontal) in phytoplankton community composition (*i. e.*, relative abundance and biomass of *Microcystis* spp.) and thus toxin concentration within phytoplankton, may

account for much of this variation, since snails were collected from more than one location within each study lake on any given sampling date. Indeed, both Carmichael and Gorham (1981) and Kotak *et al.* (1995) have previously demonstrated spatial variation in bloom toxicity within eutrophic Alberta lakes. In order to investigate the spatial heterogeneity in microcystin (MCYST) concentration in tissue of *L. stagnalis*, a field study was conducted within Hastings Lake, Alberta on September 17, 1999. In this instance, Chl-*a* concentrations from ten study sites differed dramatically, indicating large spatial variations in phytoplankton biomass (predominately *Aphanizomenon flos-aquae* and *M. aeruginosa*). Consequently, the concentration of MCYST in phytoplankton also varied between sites. All snails collected from each of the sites contained detectable amounts of MCYST, though individual variation was high ($CV > 42\%$). Overall, the mean concentration of MCYST within tissue of *L. stagnalis* varied spatially, as several sites on the south shore were significantly different from others on the north. Moreover, 60% of the variation in tissue toxin concentration was attributed to sampling site differences and body mass. Similarly, adjusting for body mass strengthened the association between the toxin concentration in snails and the relative biomass of *M. aeruginosa*. Body size affects tissue toxin concentrations of individual snails by influencing toxin uptake and elimination (Peters, 1983). Nevertheless, other factors such as a lag period between rapid fluctuations in *M. aeruginosa* or its toxicity and the MCYST concentration within the snails resulting from toxin accumulation, could also mitigate spatial variation, as could water temperature, which influences ingestion, accumulation and elimination of MCYST from within snails (Calow, 1975a, 1975b).

Relating to this is the revelation of whole and partially intact colonies of *Microcystis* spp. comprising the gizzard fraction of gastropod faecal strings from *L. stagnalis* collected from Driedmeat Lake during the initial field survey. This portion of the faeces, composed of undigested matter, forms when food is passed from the gizzard into the prointestine without further digestion in the caecum or digestive gland (Carriker, 1946). Hence, the presence of intact *Microcystis* spp. colonies suggests the crop and gizzard fail to mechanically disrupt these cells. Since MCYSTs are endotoxins typically released during cell lysis, insufficient comminution of *Microcystis* colonies would limit the uptake of toxin by the digestive system. As snails for use in the two field studies

were immediately cooled then frozen upon collection, it is plausible that the biomass of *Microcystis* cells/colonies contained in the alimentary system at this instance would determine toxin concentration detected. Thus, snails possessing full intestinal tracts immediately prior to collection would yield higher toxin concentrations compared to those individuals defaecating erstwhile.

To better understand aspects of MCYST uptake and accumulation in pulmonate snails, two laboratory studies examining short-term (24 h) gut clearance and tissue distribution of toxin within the giant pond snail, *L. stagnalis* were conducted. Results of the 24 h gut clearance study clearly demonstrate the majority of toxin in adult *L. stagnalis* previously exposed to toxin-containing cyanobacteria, originates from within indigestible cyanobacterial residues and is eliminated $\approx$ 8 h post-ingestion. In natural environments, *L. stagnalis* may feed continuously both day and night (Veldhuijzen, 1974) and since eutrophic waterbodies experience blooms of toxin-producing cyanobacteria through much of the summer season, significant amounts of toxin may continually exist within the alimentary system of resident snails. Nevertheless, toxin was detected in snails beyond 24 h, suggesting some limited digestion of *Microcystis* does occur. Analyses of *L. stagnalis* fractionated into alimentary tract, digestive gland and remaining visceral tissues support these findings, as the largest mean percentage of the total MCYST concentration was contained within the alimentary tract portion (82.8 $\pm$ 2.3%). Yet, an appreciable percentage (16.9 $\pm$ 2.2%) was also contained within the digestive gland tissue, thus providing conclusive evidence not only for the digestion of *Microcystis* in the gut of *L. stagnalis*, but for the uptake of toxin as well.

Considering the potential for MCYST accumulation within the digestive gland of *L. stagnalis*, a third laboratory study examining the long-term (30 d) depuration of toxin at two relevant ambient temperatures (10 and 22°C) was performed. Toxin loss during the course of the study was bi-phasic in snails from both temperature treatments and compares favourably with several studies regarding the elimination of heavy metals and hydrophobic organic compounds from tissues of various freshwater molluscs (Elder and Collins, 1991; Pastershank *et al.*, 1999). Though the greatest change (fast-phase) occurred over the first 3 days post-ingestion (63.7 and 87.7% MCYST decline at 10 and 22°C, respectively), further decreases (16.1 and 7.3% at 10 and 22°C respectively) in

toxin concentration between days 3 and 6 were notable. Together, this majority (79.8 and 95.0% cumulative MCYST decline, 10 and 22°C respectively) of toxin loss from *L. stagnalis*, which occurred over the first 6 d, can be attributed foremost to digestion/egestion. Toxin depuration continued beyond day 6, albeit at lower rates (slow-phase), providing evidence for the limited accumulation of toxin within *L. stagnalis*. Consequently, these results indicate *L. stagnalis* is able to retain detectable MCYST concentrations up to 30 d following the collapse of toxic bloom events. Lastly, fast-phase depuration rate constants (k_{fast}10 and 22°C) were different at the two ambient temperatures ($P < 0.001$). Thus, faster toxin loss from tissue of pulmonate snails occurs at warmer temperatures. Given cyanobacterial blooms often persist into the autumn when water typically decreases below 10°C, snails likely contain MCYST well beyond the cessation of these bloom events (*i. e.*, > 30 d).

The gastropod digestive gland is the primary site of intracellular digestion/detoxication/metabolism (Carriker, 1946; Livingstone, 1985). Hence, my previous findings with respect to the uptake and accumulation of MCYST by *L. stagnalis* indicate a potential exists for direct toxic effects on this organ. Subsequently, I examined whether MCLR causes: (1) the disruption of digestive gland cells in adult *L. stagnalis* and (2) mortality in developing snail embryo. Results of the former investigation clearly demonstrate the ability of MCLR to elicit changes in digestive glands of adult *L. stagnalis*, even at low treatment doses (0.0002 nmol MCLR). Furthermore, the severities of cellular alterations were dose-dependent and reminiscent of the pathology observed in liver tissue of both mammals and fish following MCYST exposure (Theiss *et al.*, 1988; Hooser *et al.*, 1990; Råbergh *et al.*, 1991; Kotak *et al.*, 1996b). This is notable and indicates the mode of MCYST toxicity is comparable in both vertebrates and invertebrates. In contrast, the latter investigation showed no adverse affects of environmentally relevant concentrations (0.01, 0.1, 1.0 or 10 μg L^{-1}) of aqueous MCLR on the mortality of snail embryo progressing through the various stages of development compared to snails exposed to artificial pond water only (ANOVA, $P = 0.35$).

2. Conclusions

This thesis explores various aspects regarding the occurrence and effects of MCYSTs in freshwater pulmonate snails (particularly *Lymnaea stagnalis*) in efforts to ascertain whether gastropods can play a substantive role in the uptake, accumulation and fate of cyanobacterial hepatotoxins in aquatic food webs. The initial field survey (chapter 3) documents the occurrence of MCYSTs in resident pulmonate snails from Alberta lakes and identifies the environmental variables affecting temporal (seasonal) variation in tissue toxin concentrations. The subsequent field study (chapter 4) not only demonstrates the spatial heterogeneity in tissue toxin concentration of these organisms, but also the omnipresence of toxin-containing snails during severe cyanobacterial blooms. Furthermore, physiological state manifested as body size was shown to affect tissue toxin concentrations of individual snails likely by influencing toxin uptake and elimination.

The 24-h gut clearance, tissue distribution and 30-d depuration studies (chapters 5 and 6) are, to my knowledge, the first to corroborate the uptake, accumulation and elimination of cyanobacterial toxins in freshwater pulmonate snails. While it is now apparent that the majority of toxin exists within indigestible cyanobacteria contained in the alimentary system, detectable concentrations may remain within the digestive gland tissue for up to 30 d during the warm (22°C) water (summer) period. Moreover, the ability of snails to retain toxin well beyond 30 d during the cool (< 10°C) water (autumn) season is significant, as this period coincides with large annual waterfowl migrations in western Canada. Together, these studies not only indicate that resident pulmonate snails inhabiting bloom-prone waters be subject to the potential toxic effects of MCYSTs, but also implicates snails in the transfer of hepatotoxins to other aquatic and terrestrial organisms associated with these food webs even well beyond the seasonal disappearance of toxic cyanobacteria.

The final two investigations regarding the toxicological effects of MCLR on digestive gland histology and embryo survival (chapter 7) are, in essence, preliminary attempts to identify potential adversities with respect to pulmonate snail populations within bloom-prone waters. That is, any significant declines in gastropod populations resulting from increased mortality and/or reductions in snail reproductive success, could circuitously affect organisms that otherwise rely on snails as prey. Microcystin-LR had

no adverse affect on the mortality of snail embryo progressing through various stages of development. However, the direct toxic effects on adult snails described here, may influence other aspects of gastropod reproduction (e. g., reductions in the number of egg cells per oviposited egg mass or increased time required between successive ovipositions). Additionally, potential delays to sexual maturation by juvenile *L. stagnalis* exposed to MCLR via diet or aqueous routes (*i. e.*, water intake as a consequence of feeding) contributing to seasonal reductions in the number of breeding individuals, could reduce the overall population of snails within affected habitats.

To adequately appreciate the impacts of hepatotoxins on gastropods and to evaluate their ensuing food web implications, future investigations are required. For instance, examining the extent to which snails are able to digest *Microcystis* is necessary in order to determine the potential for MCYST uptake via diet. Similarly, alternate routes of toxin uptake (*i. e.*, uptake of dissolved MCYST in water as a consequence of feeding or passive and/or active toxin uptake through the body surface) must be identified, as these contribute to the risks MCYSTs pose to pulmonate snails and other organisms inhabiting productive lakes and reservoirs. Lastly, considering their: (1) tolerance for highly productive conditions, (2) ability to accumulate MCYSTs from natural environments, (3) role in the aquatic food web, (4) ubiquitous distribution and (5) ease of sample collection, pulmonate snails (especially large-bodied Lymnaeids) must be further evaluated as useful bioindicators of cyanobacterial hepatotoxins in eutrophic freshwaters.

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Appendix A

Raw Data and Detailed Methodology

A copy of the raw data and detailed methodology regarding these studies may be obtained from Dr. E.E. Prepas, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9.