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Health Effects Support Document for the Cyanobacterial Toxin Microcystins

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U.S. Environmental Protection Agency
Office of Water (4304T)
Health and Ecological Criteria Division
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FOREWORD

The Safe Drinking Water Act (SDWA), as amended in 1996, requires the Administrator of the U.S. Environmental Protection Agency (EPA) to establish a list of unregulated microbiological and chemical contaminants that are known or anticipated to occur in public water systems and that may need to be controlled with a national primary drinking water regulation. The SDWA also requires that the Agency make regulatory determinations on at least five contaminants on the list every five years. For each contaminant on the Contaminant Candidate List (CCL), the Agency will need to obtain sufficient data to conduct analyses on the extent of occurrence and the risk posed to populations via drinking water. Ultimately, this information will assist the Agency in determining the appropriate course of action (e.g., develop a regulation, develop guidance or make a decision not to regulate the contaminant in drinking water).

This document presents information, including occurrence, toxicology and epidemiology data, for the cyanobacterial toxins microcystins to be considered in the development of a Drinking Water Health Advisory (DWHA). DWHAs serve as the informal technical guidance for unregulated drinking water contaminants to assist federal, state and local officials, and managers of public or community water systems in protecting public health as needed. They are not to be construed as legally enforceable federal standards.

To develop the Health Effects Support Document (HESD) for microcystins, a comprehensive literature search was conducted from January 2013 to May 2014 using Toxicology Literature Online (TOXLINE), PubMed component and Google Scholar to ensure the most recent published information on microcystins was included. The literature search included the following terms: microcystin, microcystin congeners, congeners, human toxicity, animal toxicity, *in vitro* toxicity, *in vivo* toxicity, occurrence, environmental fate, mobility and persistence. EPA assembled available information on: occurrence; environmental fate; mechanisms of toxicity; acute, short term, subchronic and chronic toxicity and cancer in humans and animals; toxicokinetics and exposure.

Additionally, EPA relied on information from the following risk assessments in the development of the HESD for microcystin.

- Health Canada (2012) Toxicity Profile for Cyanobacterial Toxins
- Enzo Funari and Emanuela Testai (2008) Human Health Risk Assessment Related to Cyanotoxins Exposure
- Tai Nguyen Duy, Paul Lam, Glen Shaw and Des Connell (2000) Toxicology and Risk Assessment of Freshwater Cyanobacterial (Blue-Green Algal) Toxins in Water

A Reference Dose (RfD) determination assumes that thresholds exist for certain toxic effects, such as cellular necrosis, significant body or organ weight changes, blood disorders, etc. It is expressed in terms of milligrams per kilogram per day (mg/kg/day) or micrograms per kilogram per day (µg/kg/day). In general, the RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime.

The carcinogenicity assessment for microcystins includes a formal hazard identification and an estimate of tumorigenic potency if applicable. Hazard identification is a weight-of-evidence judgment of the likelihood that the agent is a human carcinogen via the oral route and of the conditions under which the carcinogenic effects may be expressed.

Development of this hazard identification and dose-response assessment for microcystins has followed the general guidelines for risk assessment as set forth by the National Research Council (1983) the EPA's (2014b) *Framework for Human Health Risk Assessment to Inform Decision Making*. EPA guidelines used in the development of this assessment include the following:

- *Guidelines for the Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 1986a)
- *Guidelines for Mutagenicity Risk Assessment* (U.S. EPA, 1986b)
- *Recommendations for and Documentation of Biological Values for Use in Risk Assessment* (U.S. EPA, 1988)
- *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991)
- *Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies* (U.S. EPA, 1994a)
- *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b)
- *Use of the Benchmark Dose Approach in Health Risk Assessment* (U.S. EPA, 1995)
- *Guidelines for Reproductive Toxicity Risk Assessment* (U.S. EPA, 1996)
- *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998)
- *Science Policy Council Handbook: Peer Review (2nd edition)* (U.S. EPA, 2000a)
- *Supplemental Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 2000b)
- *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002)
- *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a)
- *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (U.S. EPA, 2005b)
- *Science Policy Council Handbook: Peer Review* (U.S. EPA, 2006a)
- *A Framework for Assessing Health Risks of Environmental Exposures to Children* (U.S. EPA, 2006b)
- *Highlights of the Exposure Factors Handbook* (U.S. EPA, 2011)
- *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2012)
- *Child-Specific Exposure Scenarios Examples* (U.S. EPA, 2014a)
- *Framework for Human Health Risk Assessment to Inform Decision Making* (U.S. EPA, 2014b)

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

A	Alanine
Adda	3-amino-9-methoxy-2, 6, 8,-trimethyl-10-phenyldeca-4, 6-dienoic acid
ADHD	Attention deficit hyperactivity disorders
AFA	*Aphanizomenon flos-aquae*
ALDH2	Aldehyde dehydrogenase 2
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
AWWARF	American Water Works Association Research Foundation
BGAS	Bluegreen algae supplements
BSO	Buthionine sulfoximine
BUN	Blood urea nitrogen
BW	Body weight
CAS	Chemical Abstracts Service
CEGLHH	Center of Excellence for Great Lakes and Human Health
CCL	Contaminant Candidate List
CHO	Chinese hamster ovary
CI	Confidence Interval
CYP2E1	Cytochrome P450 2E1
CYP450	Cytochrome P450
DMBA	Dimethylbenzanthracene
DNA	Deoxyribonucleic acid
DW	Dry Weight
DWHA	Drinking Water Health Advisories
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
ERK	Extracellular signal-regulated protein kinase
ETC	Electron transport chain
F	Phenylalanine
Fe	Iron
FEL	Frank effect level
FSH	Follicle stimulating hormone
FT3	Free triiodothyronine
FT4	Free thyroxin
g	Gram
GD	Gestation day
GC/MS	Gas chromatograph/mass spectrometry
GFR	Glomerular filtration rate
GGT	y-Glutamyl transpeptidase
GI	Gastrointestinal
GIS	Geographical information system
GSH	Glutathione
GST	Glutathione S-transferase

GST-P	Glutathione *S*-transferase placental form-positive
HA	Health advisory
HAB	Harmful algal bloom
HEK	Human embryonic kidney cells
HPLC	High-performance liquid chromatography
IARC	International Agency for Research on Cancer
i.p.	Intraperitoneal
i.v.	Intravenous
JNK	c-Jun N-terminal protein kinase
kg	Kilogram
L	Leucine
LC/MS	Liquid chromatography/mass spectrometry
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
LOAEL	Lowest-observed-adverse-effect level
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
Mdha	Methyldehydroalanine
MERHAB-LGL	Monitoring and Event Response to Harmful Algal Blooms in the Lower Great Lakes
µg	Microgram
µM	Micromole
mg	Milligram
mL	Milliliter
MMP	Metalloproteinase
Mn	Manganese
MOA	Mode of action
MPT	Mitochondrial permeability transition
mRNA	Messenger RNA
nm	Nanometer
nM	Nanomole
N	Nitrogen
N/A	Not Applicable
NDEA	N-nitrosodiethylamine
NLA	National Lakes Assessment
NMR	Nuclear magnetic resonance
NOAA	National Oceanic and Atmospheric Administration
NOAEL	No-observed-adverse-effect level
NRC	National Research Council
NRPS	Nonribosomal peptide synthetase

OATp	Organic acid transporter polypeptides
OR	Odds ratio
OXPHOS	Oxidative phosphorylation
P	Phosphorus
PCE	Polychromatic erythrocyte
PKS	Polyketide synthase
PMN	Polymorphonuclear leukocyte
PP2A	Protein phosphatase 2A
PP1	Protein phosphatase 1
PP4	Protein phosphatase 4
R	Arginine
RfD	Reference dose
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RR	Relative risk
qPCR	Quantitative polymerase chain reaction
SDWA	Safe Drinking Water Act
SE	Standard error
SOD	Superoxide dismutase
SRR	Standardized rate ratios
TEF	Toxicity equivalency factors
TH	Thyroid hormone
TNF-α	Tumor necrosis factor-alpha
Trα	TH receptor
TOXLINE	Toxicology Literature Online
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling assay
UF	Uncertainty factor
USGS	United States Geological Survey
UV	Ultraviolet
W	Tryptophan
WHO	World Health Organization
Y	Tyrosine

EXECUTIVE SUMMARY

Microcystins are toxins produced by various cyanobacterial species, including members of *Microcystis, Anabaena, Nodularia, Planktothrix, Fischerella, Nostoc, Oscillatoria, and Gloeotrichia*. Structurally, the microcystins are monocyclic heptapeptides that contain seven amino acids joined end-to-end and then head to tail to form cyclic compounds that are comparatively large, (molecular weights ranging from ~ 800 to 1,100 g/mole).

Microcystin congeners vary based on their amino acid composition and through methylation or demethylation at selected sites within the cyclicpeptide. The variations in composition and methylation account for the large number of toxin congeners (approximately 100). The microcystins are named based on their variable amino acids. For example, microcystin-LR, the most common congener, contains leucine (L) and arginine (R). The preponderance of toxicological data on the effects of microcystins is restricted to the microcystin-LR congener.

Microcystins are the most common cyanotoxins found worldwide and are relatively stable in the environment as they are resistant to hydrolysis at near neutral pH. In the presence of full sunlight, photochemical breakdown can occur in as little as two weeks or longer than six weeks, depending on the microcystin congener. They are susceptible to degradation by aquatic bacteria found naturally in rivers and reservoirs. In aquatic environments the toxin tends to remain contained within the cyanobacterial cell and is released in substantial amounts only upon cell lysis. Microcystins have been reported to remain potent even after boiling. Microcystins may adsorb onto naturally suspended solids and dried crusts of cyanobacteria and can precipitate out of the water column and reside in sediments for months. Concentrations associated with blooms in surface waters in the U.S. and Europe typically range from very low levels (detection limit) and have been measured as high as 150,000 μg/L.

Drinking water is an important source of potential exposure to cyanotoxins. Exposure to cyanobacteria and their toxins may also occur by ingestion of toxin-contaminated food, by inhalation and dermal contact during bathing or showering, and during recreational activities in waterbodies with the toxins. However, these types of exposures are considered minimal due to various factors including lack of biomagnification and biodilution via food. Due to the seasonality of cyanobacterial blooms, exposures are usually not chronic. Symptoms reported after acute recreational exposure to cyanobacterial blooms (including microcystin-producing genera) included skin irritations, allergic reactions or gastrointestinal illnesses.

Limited data in humans and animals demonstrate that the absorption of microcystins from the intestinal tract into liver, brain, and other tissues, and the export from the body, requires facilitated transport using receptors belonging to the organic acid transporter polypeptide (OATp) family. Data in humans and animals suggests that the liver is a primary site for binding these proteins (i.e., increased liver weight in laboratory animals and increased levels of serum enzymes in laboratory animals and humans). Once inside the cell, these toxins covalently bind to cytosolic proteins (PP1 and PP2) resulting in their retention in the liver. Limited data are available on the metabolism of microcystins, but most of the studies show that conjugation with glutathione and cysteine increases solubility and facilitates excretion.

Human data on the oral toxicity of MC-LR are limited by lack of quantitative information and by potential co-exposure to other cyanobacterial toxins and microorganisms. Acute, short-term and subchronic experimental studies all provide evidence of hepatotoxicity, and chronic studies, that are limited by lack of evaluation of comprehensive endpoints and comprehensive reporting, support these findings. Several studies of microcystin-LR reported findings of lesions in the testes and decreased sperm counts and motility.

EPA estimated a reference dose (RfD) for microcystins of 0.05 μg/kg/day based on increased liver weight, slight to moderate liver lesions with necrosis with hemorrhages, and increased enzyme levels in

rats from the study by Heinze (1999). This study identified a LOAEL of 50 µg/kg/day, based on these effects. The drinking water route of exposure and shorter duration of the study (28 days) closely match potential short-term exposure scenarios that are the focus of a Ten-day health advisory for microcystin. The composite uncertainty factor includes application of a 10 for intraspecies variability, 10 for interspecies variability, 3 ($10^{½}$) for converting a LOAEL to a NOAEL, and 3 ($10^{½}$) for uncertainties in the database.

Applying the Guidelines for Carcinogen Risk Assessment, there is *inadequate evidence to determine the carcinogenicity* of microcystins. The few available epidemiological studies suggest an association between liver or colorectal cancers and microcystin exposures, but are limited by their ecological study design, lack of individual exposure measurements, potential co-exposure to other microbial or chemical contaminants and, in some cases, failure to control for known liver and colorectal risk factors. No long term animal studies designed to evaluate dose-response for tumorigenicity of microcystin following lifetime exposures were available. Other studies evaluating the tumor promotion potential of microcystin following pretreatment with a potent initiator such as NDEA or N-methyl-N-nitroso urea, found an increase in the number and/or size of GST-P positive foci observed (Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994; Falconer and Humpage, 1996; Sekijima et al., 1999; Humpage et al., 2000; Ito et al., 1997b). In two promotion studies, MC-LR alone showed no initiating activity (Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994).

1.0 IDENTITY: CHEMICAL AND PHYSICAL PROPERTIES

1.1 Chemical and Physical Properties

Cyanobacteria, formerly known as blue-green algae (Cyanophyceae), are a group of bacteria containing chlorophyll-a that can carry out the light and dark phases of photosynthesis (Castenholz and Waterbury, 1989). In addition to chlorophyll-a, other pigments such as carotene, xanthophyll, blue *c* phycocyanin and red *c* phycoerythrin are also present in cyanobacteria (Duy et al., 2000). Most cyanobacteria are aerobic photoautotrophs, requiring only water, carbon dioxide, inorganic nutrients and light for survival, but others have heterotrophic properties and can survive long periods in complete darkness (Fay, 1965). Some species also are capable of nitrogen fixation (i.e., diazotrophy) (Duy et al., 2000) producing inorganic nitrogen compounds to synthesize nitrogen-containing biomolecules, such as nucleic acids and proteins. Cyanobacteria can form symbiotic associations with animals and plants, such as fungi, bryophytes, pteriodophytes, gymnosperms and angiosperms, supporting their growth and reproduction (Sarma, 2013; Hudnell, 2008; Hudnell, 2010; Rai, 1990).

Cyanobacteria can be found in unicellular, colony and multicellular filamentous forms. The unicellular form occurs when the daughter cells separate after binary fission reproduction. These cells can aggregate into irregular colonies held together by a slimy matrix secreted during colony growth (WHO, 1999). The filamentous form occurs when repeated cell divisions happen in a single plane at right angles to the main axis (WHO, 1999). Reproduction is asexual.

Cyanobacteria are considered gram-negative, even though the peptidoglycan layer is thicker than most gram-negative bacteria. However, studies using electron microscopy show that cyanobacteria possess properties of both gram-negative and gram-positive bacteria (Stewart et al., 2006a). Compared to heterotrophic bacteria, the cyanobacterial lipopolysaccharides (LPS) have little or no 2-keto-3-deoxy-D-manno-octonic acid, and they lack phosphate groups, glucosamine and L-glycero-D-mannoheptose. Cyanobacteria also have long-chain saturated and unsaturated fatty acids.

Under the optimal pH, nutrient availability, light and temperature conditions, cyanobacteria can reproduce quickly forming a bloom. Studies of the impact of environmental factors on cyanotoxin production are ongoing, including such factors as nutrient (nitrogen, phosphorus and trace metals) concentrations, light, temperature, oxidative stressors and interactions with other biota (viruses, bacteria and animal grazers), as well as the combined effects of these factors (Paerl and Otten 2013a; 2013b). Fulvic and humic acids also have been reported to encourage cyanobacteria growth (Kosakowska et al., 2007).

Cyanobacteria can produce a wide range of bioactive compounds, some of which have beneficial or therapeutic effects. These bioactive compounds have been used in pharmacology, as dietary supplements and as mood enhancers (Jensen et al., 2001). Other cyanobacteria can produce bioactive compounds that may be harmful, called cyanotoxins. The most commonly recognized bioactive compounds produced by cyanobacteria fall into four broad groupings: cyclic peptides, alkaloids, amino acids and LPS.

Microcystins are produced by several cyanobacterial species, including species of *Anabaena, Nodularia, Nostoc Oscillatoria, members of Microcystis, Fischerella, Planktothrix, and Gloeotrichia echinulata* (Duy et al., 2000; Codd et al., 2005; Stewart et al., 2006a; Carey et al., 2012).

1.2 Microcystin Congeners

The cyclic peptides include six congeners of nodularins and around 100 congeners of microcystins. Figure 2-1 provides the structure of microcystin, a monocyclic heptapeptide, where X and Y represent

variable amino acids as presented in Table 1-1. Although substitutions mostly occur in positions X and Y, other modifications have been reported for all of the amino acids (Puddick et al., 2015). The amino acids are joined end-to-end and then head to tail to form cyclic compounds that are comparatively large, (molecular weights ranging from ~800 to 1,100 g/mole). Table 2-1 lists only the most common microcystin congeners, of which currently around 100 different congeners have been identified.

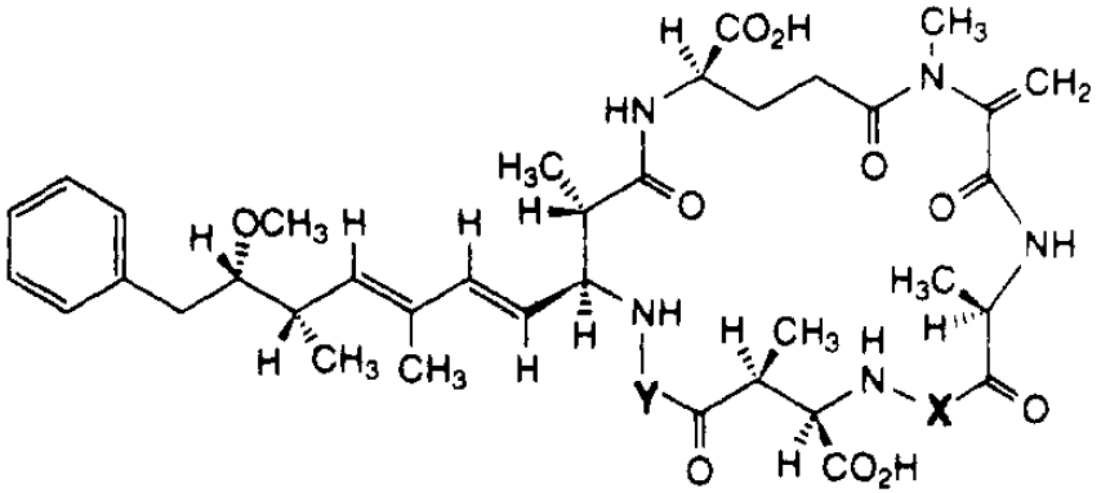


Figure 1-1. Structure of Microcystin (Kondo et al., 1992).

Nodularin has a similar structure to microcystin and a similar mode of toxicity (McElhiney et al., 2005). Nodularins show hepatotoxic effects through the inhibition of protein phosphatases just like microcystins and some have suggested carcinogenic potential of nodularins (Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994). However, there are no published animals studies evaluating the health effects associated with exposure to nodularin.

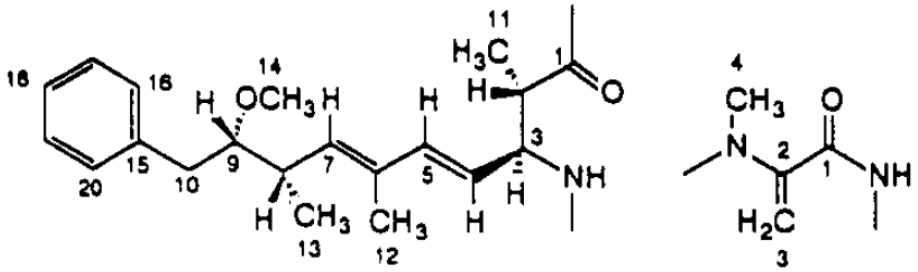
Microcystin congeners vary based on their amino acid composition and through methylation or demethylation at selected sites within the cyclicpeptide (Table 1-1; Duy et al., 2000). The variations in composition and methylation account for the large number of toxin congeners. The microcystins are named based on their variable amino acids, although they have had many other names (Carmichael et al., 1988). For example, microcystin-LR, the most common congener, contains leucine (L) and arginine (R). The letters used to identify the variable amino acids are the standard single letter abbreviations for the amino acids found in proteins. The variable amino acids are usually the L-amino acids as found in proteins. There has been at least one microcystin where the leucine was D-leucine (Carmichael, 1992).

Table 1-1. Amino Acid Composition of Various Microcystin Congeners (Yuan et al., 1999)

Microcystins Congener	Amino Acid in X	Amino Acid in Y
microcystin-LR	Leucine	Arginine
microcystin-RR	Arginine	Arginine
microcystin-YR	Tyrosine	Arginine
microcystin-LA	Leucine	Alanine
microcystin-LY	Leucine	Tyrosine
microcystin-LF	Leucine	Phenylalanine
microcystin-LW	Leucine	Tryptophan

Most research has concentrated on microcystin-LR with lesser amounts of data available for the other amino acid combinations. Structurally, the microcystins are monocyclic heptapeptides that contain seven amino acids: two variable L-amino acids, three common D-amino acids or their derivatives, and two novel D-amino acids (Adda and Mdha). Adda (3S-amino-9S-methoxy-2,6,8S,-trimethyl-10-phenyldeca-4,6-dienoic acid) is characteristic of all toxic microcystin structural congeners and is essential for their biological activity (Rao et al., 2002; Funari and Testai, 2008). Mdha (methyldehydroalanine) is the second unique component of the microcystins. It plays an important role in the ability of the microcystins to inhibit protein phosphatases. Figure 1-2 illustrates the structures of the two unique amino acid microcystin components.

Microcystins are water soluble. In aquatic environments, the cyclic peptides tend to remain contained within the cyanobacterial cell and are released in substantial amounts only upon cell lysis. Microcystins are most frequently found in cyanobacterial blooms in fresh and brackish waters (WHO, 1999). Table 1-2 provides chemical and physical properties of microcystin-LR.



Adda Mdha

Figure 1-2. Structure of the Amino Acids Adda and Mdha (Harada et al., 1991).

Table 1-2. Chemical and Physical Properties of Microcystin-LR

Property	Microcystin-LR
Chemical Abstracts Registry (CAS) #	101043-37-2
Chemical Formula	$C_{49}H_{74}N_{10}O_{12}$
Molecular Weight	995.17 g/mole
Color/Physical State	Solid
Boiling Point	N/A
Melting Point	N/A
Density	1.29 g/cm^3
Vapor Pressure at 25°C	N/A
Henry's Law Constant	N/A
K_{ow}	N/A
K_{oc}	N/A
Solubility in Water	Highly
Other Solvents	Ethanol and methanol

Sources: Chemical Book, 2012; TOXLINE, 2012

2.0 TOXIN SYNTHESIS AND ENVIRONMENTAL FATE

2.1 Cyanotoxin Synthesis

Toxin production varies between blooms and within an individual bloom over time (Duy et al., 2000). Cyanotoxins can be produced by more than one species of cyanobacteria and some species may produce more than one toxin at a time, resulting in blooms with different cyanotoxins (Funari and Testai, 2008). The toxicity of a particular bloom is complex, determined by the mixture of species and the variation of strains with toxic and nontoxic genotypes involved (WHO, 1999). Generally, toxins in cyanobacteria are retained within the cell unless conditions favor cell wall lysis (ILS, 2000).

The synthesis of cyanotoxins is the focus of much research with evidence suggesting that the production and accumulation of toxin(s) correlates with cyanobacterial growth rate, with the highest amount being produced during the late logarithmic phase (Funari and Testai, 2008). For example, Long et al. (2001) described a positive linear relationship between the content of microcystins in cells and their specific growth rate.

Evidence suggests that the environmental conditions in which a bloom occurs may alter the levels of toxin produced. Several culture experiments have suggested that the biosynthesis of microcystin is regulated by environmental and nutritional factors including light intensity, temperature, and nutrients such as nitrogen, phosphorus, and iron (Neilan et al., 2007). However, the physiological function of iron is still unclear. Studies on the effect of different light intensities on microcystin production have yielded contradictory conclusions (Neilan et al., 2007). The effects of environmental conditions on bloom growth and toxin production are discussed in more detail in section 2.2.

Although there is little information on the genetic regulation of microcystin production, Dittman et al. (1997) showed that peptide synthetase genes are responsible for microcystin production. Studies conducted by Kaebernick et al. (2000) on *Microcystis aeruginosa* suggest that microcystin is produced nonribosomally through large multifunctional enzyme complexes consisting of both nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules coded by the *mcy*S (microcystin) gene cluster. According to Gewolb (2002), most NRPSs are made up of a series of four to 10 modules, each of which is responsible for specific steps of activation, modification, and condensation during the addition of one specific amino acid or other compound to the growing linear peptide chain that is then cyclized to produce microcystin. The sequence of modules in an enzyme determines the type of microcystin produced (Gewolb, 2002).

The difference in toxicity of microcystin congeners depends on the amino acid composition (Falconer, 2005). Stoner et al (1989) administered by intraperitoneal (i.p.) purified microcystin congeners (-LR, -LA, -LY and –RR congeners) into ten or more adult male and female Swiss albino mice. Necropsies were performed to confirm the presence of the pathognomonic hemorrhagic livers. The authors reported LD50 doses of 36 ng/g-bw for microcystin-LR, 39 ng/g-bw for microcystin -LA, 91 ng/g-bw for microcystin -LY and 111 ng/g-bw for microcystin –RR. Similarly, Gupta et al., (2003) determined LD50s for the MC congeners LR, RR and YR in female mice using DNA fragmentation assay and histopathology examinations of the liver and lung. The acute LD50 determinations showed that the most toxic variant was microcystin -LR (43.0 µg/kg), followed by microcystin-YR (110.6 µg/kg) and microcystin-RR (235.4 µg/kg). The most toxic microcystins are those with the more hydrophobic L-amino with one or two hydrophobic amino acids (-LA, -LR, -and -YM) and the least toxic are those with hydrophilic amino acids, such as microcystin-RR. The Adda group is also important since its removal or saturation of its double bonds greatly reduces toxicity.

2.2 Environmental Factors that Affect the Fate of Cyanotoxins

Cyanotoxin production is strongly influenced by the environmental conditions that promote growth of particular cyanobacterial species and strains. Nutrient concentrations, light intensity, temperature, and other environmental factors affect growth and the population dynamics of cyanobacteria production, as described below. Although environmental conditions affect the formation of blooms, the number of cyanobacteria and the concentration of toxins produced are not always closely related. Cyanotoxin concentrations depend on the dominance and diversity of strains within the bloom along with environmental and ecosystem influences on bloom dynamics as shown in Figure 2-1 below (Hitzfeld et al., 2000; WHO, 1999).

2.2.1 Nutrients

Nutrients are key environmental drivers that influence the proportion of cyanobacteria in the phytoplankton community, the cyanobacterial biovolume, toxin production, and the impact that cyanobacteria may have on ecosystem function and water quality (Paerl et al., 2011). Cyanobacteria production and toxin concentrations are dependent on nutrient levels (Wang et al., 2002); however, different cyanobacteria species use organic and inorganic nutrient forms differently. Loading of nitrogen (N) and/or phosphorus (P) to water bodies from agricultural, industrial and urban sources influence the development of cyanobacterial blooms and may be related to cyanotoxin production (Paerl et al., 2011). Nitrogen loading can enhance the growth and toxin levels of *Microcystis sp.* blooms and microcystin synthetase gene expression (Gobler et al., 2007; O'Neil et al., 2012). Gobler et al. (2007) suggest that dominance of *Microcystis sp*. blooms during summer is linked to N loading, which stimulates growth and toxin synthesis. This may cause the inhibition of grazing by mesozooplankton and further accumulation of cyanobacterial cells.

Optimal concentrations of total and dissolved phosphorous (Wang et al., 2002) and soluble phosphates and nitrates (ILS, 2000; Paerl and Scott, 2010; Wang et al., 2010; O'Neil et al., 2012) may also result in the increased production of microcystins. Some studies have observed a decrease in toxicity of *Microcystis sp.* after removal of N or inorganic carbon, but no changes was observed when P was removed from a cyanobacteria culture media (Codd and Poon, 1988). Similarly, Sivonen (1990) found a relationship between high toxicity and high N concentration, but no effect at higher concentrations of phosphorus.

Smith (1983) first described a strong relationship between the relative amounts of N and P in surface waters and cyanobacterial blooms. Smith proposed that cyanobacteria should be superior competitors under conditions of N-limitation because of their unique capacity for N-fixation. While the dominance of N-fixing cyanobacteria at low N:P ratios has been demonstrated in mesocosm- and ecosystem-scale experiments in prairie and boreal lakes (Schindler et al., 2008) the hypothesis that low N:P ratios favor cyanobacteria formation has been debated and challenged for its inability to reliably predict cyanobacterial dominance (Downing et al., 2001). Eutrophic systems already subject to bloom events are prone to further expansion of these blooms due to additional N inputs, especially if these nutrients are available from internal sources. As the trophic state increases, aquatic systems absorb higher concentrations of N (Paerl and Huisman, 2008; Paerl and Otten, 2013b). Recent surveys of cyanobacterial and algal productivity in response to nutrient pollution across geographically diverse eutrophic lakes, reservoirs, estuarine and coastal waters, and in different experimental enclosures of varying sizes demonstrate that greater stimulation is routinely observed in response to both N and P additions. Further, this evidence suggests that nutrient colimitation is widespread (Elser et al., 2007; Lewis et al., 2011; Paerl et al., 2011). These results strongly suggest that reductions in both N and P inputs are needed to stem eutrophication and cyanobacterial bloom expansion.

Analysis of observational data collected at larger spatial scales support the idea that controlling Total Phosphorus (TP) and Total Nitrogen (TN) could reduce the frequency of high MC events by reducing the biomass of cyanobacteria in the system (Yuan et al., 2014, Orihel et al., 2012; Scott et al., 2013). Some of these analyses have also found that TN concentrations are the strongest predictors of high MC across large spatial scales, but the causal mechanisms for this correlation are still not clear (Scott et al., 2013; Yuan et al., 2014). Subsequent experiments should manipulate N:P ratios at scales relevant to ecosystem management to further develop/evaluate the need for a dual nutrient strategy as discussed in Paerl et al. (2011) and Paerl and Otten (2013b).

2.2.2 Light Intensity

Sunlight availability and turbidity have a strong influence on the cyanobacteria species that predominate, as well as the depth at which they occur (Falconer et al., 2005; Carey et al., 2012). For example, *Microcystis aeruginosa* occurs mostly at the surface with higher light intensities and in shallow lakes. The relationship of light intensity to toxin production in blooms is somewhat unclear and continues to be investigated (Duy et al., 2000). Some scientists have found evidence that toxin production increases with high light intensity (Watanabe and Oishi, 1985) while others have found little variation in toxicity at different levels of light intensity (Codd and Poon, 1988; Codd, 1995).

Kosten et al. (2011) surveyed 143 shallow lakes along a latitudinal gradient (between 5-55°S and 38-68°N) from subarctic Europe to southern South America). Their analyses found a greater proportion of the total phytoplankton biovolume attributable to cyanobacteria in lakes with high rates of light absorption. Kosten et al. (2011) could not establish cause and effect from these field data, but other controlled experiments and field data have demonstrated that light availability can affect the competitive balance among a large group of shade-tolerant species of cyanobacteria, mainly *Oscillatoriales* and other phytoplankton species (Smith, 1986; Scheffer et al., 1997). Overall, results from Kosten et al. (2011) suggest that higher temperatures interact with nutrient loading and underwater light conditions in determining the proportion of cyanobacteria in the phytoplankton community in shallow lakes.

2.2.3 Temperature

The increasing body of laboratory and field data (Weyhenmeyer, 2001; Huisman et al., 2005; Reynolds, 2006; De Senerpont Domis et al., 2007; Jeppesen et al., 2009; Wagner and Adrian, 2009; Kosten et al., 2011; Carey et al., 2012) suggest that an increase in temperature may influence cyanobacterial dominance in the phytoplankton community. Cyanobacteria may benefit more from warming than other phytoplankton groups due to their higher optimum growth temperatures. The optimum temperatures for microcystin production range from 20 to 25°C (WHO, 2003). The increase in water column stability associated with higher temperatures also may favor cyanobacteria (Wagner and Adrian, 2009; Carey et al., 2012). Kosten et al. (2011) demonstrated that during the summer, the percentage of the total phytoplankton biovolume attributable to cyanobacteria increased steeply with temperature in shallow lakes sampled along a latitudinal transect ranging from subarctic Europe to southern South America. Furthermore, warmer temperatures appear to favor the growth of toxigenic strains of *Microcystis* over nontoxic ecotypes (Dziallas and Grossart, 2011; Paerl and Otten, 2013b).

Indirectly, warming also may increase nutrient concentrations by enhancing mineralization (Gudasz et al., 2010; Kosten et al., 2009 and 2010) by temperature- or anoxia-mediated sediment phosphorus release (Jensen and Andersen, 1992; Søndergaard et al., 2003). Thus, temperature may indirectly increase cyanobacteria biomass through its effect on nutrient concentrations. Others have suggested that warmer conditions may raise total phytoplankton biomass through an alteration of top-down regulation by selective grazing that favors larger size phytoplankton species and cyanobacteria blooms (Jeppesen et al., 2009, 2010; Teixeira-de Mello et al., 2009). The relationship between temperature and cyanobacterial

dominance may be explained not only by temperature effect on the competitive advantage of cyanobacteria, but also factors such as the percent area covered and the volume of the lake taken up by submerged macrophytes (Kosten et al., 2011; Carey et al., 2012).

Rising global temperatures and changing precipitation patterns may stimulate cyanobacteria blooms. Warmer temperatures favor surface bloom-forming cyanobacterial genera because they are heat-adapted and their maximal growth rates occur at relatively high temperatures, often in excess of 25°C (Robarts and Zohary 1987; Reynolds, 2006). At these elevated temperatures, cyanobacteria routinely out-compete eukaryotic algae (Elliott, 2010; Paerl et al., 2011). Specifically, as the growth rates of the eukaryotic taxa decline in response to warming, cyanobacterial growth rates reach their optima. Warmer surface waters, especially in areas of reduced precipitation, are prone to intense vertical stratification. The strength of vertical stratification depends on the density difference between the warm surface layer and the underlying cold water which is influenced by amount of precipitation. As temperatures rise due to climate change, stratification is expected to occur earlier in the spring and persist longer into the fall (Paerl and Otten, 2013b). The increase in water column stability associated with higher temperatures and climate change may therefore favor cyanobacteria production and possibly the prevalence of cyanotoxins such as microcystins (Wagner and Adrian, 2009; Carey et al., 2012).

2.2.4 Other Environmental Factors

Cyanobacteria blooms have been shown to intensify and persist at pH levels between six and nine (WHO, 2003). When these blooms are massive or persist for a prolonged period, they can become harmful. Kosten et al. (2011) noted the impact of pH on cyanobacteria abundance in lakes along a latitudinal transect from Europe to southern South America. The percentage of cyanobacteria in the 143 shallow lakes sampled was highly correlated with pH, with an increased proportion of cyanobacteria at higher pH.

Cyanobacteria have a competitive advantage over other phytoplankton species because they are efficient users of carbon dioxide in water (Shapiro, 1984; Caraco and Miller, 1998). This characteristic is especially advantageous for cyanobacteria under conditions of higher pH when the concentration of carbon dioxide in the water column is diminished due to photosynthetic activity. Although this could explain the positive correlation observed between pH and the proportion of cyanobacteria, the high proportion of cyanobacteria at high pH could be the result of an indirect nutrient effect as described previously (see discussion in Temperature Section). As photosynthesis intensifies, pH increases due to carbon dioxide uptake by algae, resulting in a shift in the carbonic buffer equilibrium and a higher concentration of basic forms of carbonate. Thus, higher water column pH may be correlated with a higher proportion of cyanobacteria because of higher photosynthetic rates, which can be linked with high nutrient concentrations (Duy et al., 2000) that stimulate phytoplankton growth and bloom formation. High iron concentrations (more than 100 μM) have also been shown to increase cell density and chlorophyll content in *Microcystis aeruginosa* (Kosakowska et al., 2007).

Most phytoplankton-cyanobacteria blooms occur in late summer and early fall when deeper lakes or reservoirs are vertically stratified and phytoplankton species may be stratified as well. Vertical phytoplankton biomass structure and cyanotoxin production can be influenced by seasonal changes as well as severe weather conditions (e.g., strong wind or rainfall), and also by runoff. At times, the hypolimnion (bottom layer of the water column) can have a higher phytoplankton-cyanobacteria biomass and display different population dynamics than the epilimnion (upper layer of the water column). Conversely, seasonal effects of increasing temperatures and changes in wind patterns may favorably influence the upper water column cyanobacterial community. This vertical variability is common and attributed to four causes, each of which may occur at different times, including: (a) sinking of dead/dying cells; (b) density stratification of the water column, especially nutrient concentrations and light, which affects all aspects of cyanobacteria growth; (c) increased nutrient supply from organic-rich bottom

sediment (even when the water body is not density-stratified), encouraging cyanobacteria growth at or near the bottom sediment; and (d) species-specific factors such as the tendency to form surface scums in the case of *M. aeruginosa* or the presence of resting spores in the sediment in the case of *N. spumigena* (Drake et al., 2010). In addition, there are microbial interactions that may occur within blooms, such as competition and adaptation between toxic and nontoxic cyanobacterial strains, as well as impacts from viruses. Each of these factors can cause fluctuations in bloom development and composition. When the composition of the cyanobacteria bloom changes, so do the toxins present and their concentrations (Honjo et al., 2006; Paerl and

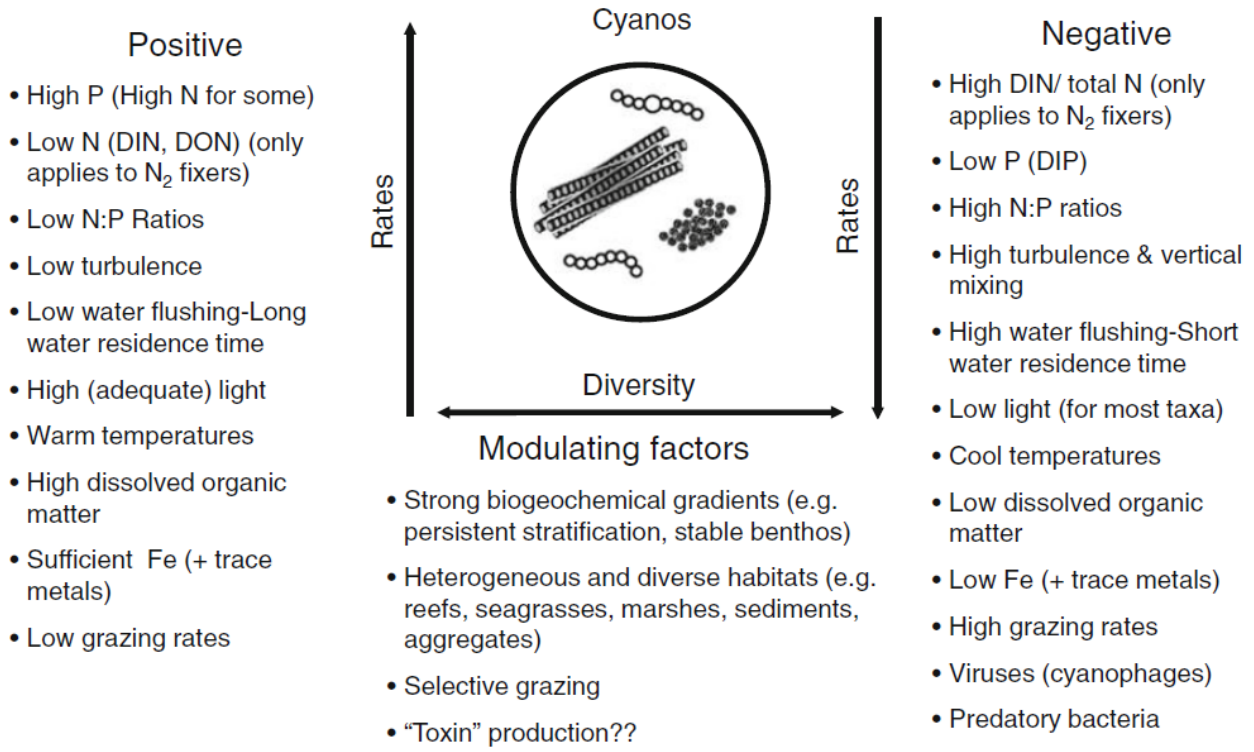


Figure 2-1. Environmental Factors Influencing Cyanobacterial Blooms.

Otten, 2013b). The concentration of cyanotoxins observed in a water body when a bloom collapses, such as from cell aging or algaecide treatment, depends on dilution of the toxin due to water column mixing, the degree of adsorption to sediment or particulates and the rate of toxin biodegradation (Funari and Testai, 2008).

In summary, there is a complex interplay of environmental factors that dictates the spatial and temporal changes in the concentration of cyanobacteria cells and their toxins with respect to the dominant species as illustrated in Figure 2-1 (Paerl and Otten, 2013b). Factors such as the N:P ratio, organic matter availability, temperature, and light attenuation, as well as other physico-chemical processes, can play a role in determining harmful algal bloom (HAB) composition and toxin production (Paerl and Huisman, 2008; Paerl and Otten, 2013b). Dynamics of microflora competition as blooms develop and collapse can also impact cyanotoxin concentrations in surface waters. In addition, impacts of climate change, including potential warming of surface waters and changes in precipitation, could result in changes in ecosystem dynamics that lead to more frequent formation of cyanobacteria blooms and their associated toxins (Paerl and Huisman, 2008; Paerl et al., 2011; Paerl and Otten, 2013b).

2.3 Environmental Fate of Microcystins

2.3.1 Hydrolysis

Microcystins are relatively stable and resistant to chemical hydrolysis or oxidation at near neutral pH. Elevated or low pH or temperatures above 30°C may cause slow hydrolysis. They have been reported to remain potent toxins even after boiling (Rao et al., 2002). In natural waters, microcystins may persist for between 21 days and 2-3 months in solution and up to 6 months in dry scum when kept in the dark (Rapala et al., 2006; Funari and Testai, 2008).

2.3.2 Photolysis

In the presence of full sunlight, microcystins undergo slow photochemical breakdown, but this varies by microcystin congener (WHO, 1999; Chorus et al., 2000). The presence of water-soluble cell pigments, in particular phycobiliproteins, enhances this breakdown. Breakdown can occur rapidly in as few as two weeks or longer than six weeks, depending on the concentration of pigment and the intensity of the light (Tsuji et al., 1993; 1995). Microcystin-LR was photodegraded with a half-life (time it takes half of the toxin to degrade) of about 5 days in the presence of 5 mg/L of extractable cyanobacterial pigment. Humic substances can also act as photosensitizers and increase the rate of microcystin breakdown with sunlight. In deeper or turbid water, the breakdown rate is slower.

2.3.3 Metabolism

Microcystins are susceptible to degradation by aquatic bacteria found naturally in rivers and reservoirs (Jones et al., 1994). Bacteria isolates of *Arthrobacter, Brevibacterium, Rhodococcus, Paucibacter*, and various strains of the genus *Sphingomonas (Pseudomonas)* have been reported to be capable of degrading MC-LR (de la Cruz et al., 2011; Han et al., 2012). These degradative bacteria have also been found in sewage effluent (Lam et al., 1995), lake water (Jones et al., 1994; Cousins et al., 1996; Lahti et al., 1997a), and lake sediment (Rapala et al., 1994; Lahti et al., 1997b). Lam et al (1995) reported that the biotransformation of microcystin-LR followed a first-order decay with a half-life of microcystin biotransformation of 0.2 to 3.6 days (Lam et al., 1995). Jones et al. (1994) evaluated degradation of microcystin-LR in different natural surface waters. Microcystin-LR persisted for 3 days to 3 weeks before being degraded. However, degradation was fairly rapid with more than 95% loss happening within 3 to 4 days (Jones et al., 1994). A study by Christoffersen et al. (2002), reported half-lives of microcystin-LR in the laboratory and in the field of approximately 1 day, which were driven largely by bacterial aerobic metabolism. These researchers found that approximately 90% of the initial amount of microcystin disappeared from the water phase within 5 days, irrespective of the starting concentration. Other researchers (Edwards et al., 2008) have reported longer half-lives of 4-14 days, with longer half-lives associated with streams and shorter half-lives associated with lakes.

2.3.4 Transport

Microcystins may adsorb onto naturally suspended solids and dried crusts of cyanobacteria. They can precipitate out of the water column and reside in sediments for months (Han et al., 2012: Falconer, 1998). A study by United States Geological Survey (USGS) and the University of Central Florida determined that microcystins did not sorb in sandy aquifers and were transported along with groundwater (O'Reilly et al., 2011). The authors suggested that the removal of microcystins was due to biodegradation.

2.4 Summary

Microcystins are produced by a variety of cyanobacteria. Currently around 100 different congeners of microcystins have been identified, with Microcystin-LR the most common and best known congener worldwide. Environmental conditions such as nutrients, pH, light intensity and temperature influence the growth of these cyanobacteria and could encourage toxin production. Microcystins are water soluble and tend to remain contained within the cyanobacterial cell until the cell lyses (dies) and they are released in substantial amounts into the water. They are stable and resistant to chemical hydrolysis or oxidation at near neutral pH. Slow hydrolysis may occur at elevated or low pH, or temperatures above 30°C. Microcystins remain potent even after boiling for 15 minutes. In the dark, microcystins may persist from 21 days to 3 months in solution and up to 6 months in dry scum. In the presence of full sunlight, microcystins undergo slow photochemical degradation which varies by microcystin congener and could take about one to a few weeks, or longer than six weeks to degrade. The presence of water-soluble cell pigments, in particular phycobiliproteins, enhances this breakdown. Half-lives vary from 4 to 14 days. Microcystins are susceptible to degradation by aquatic bacteria found naturally in rivers and reservoirs (Jones et al., 1994). Half-lives vary from 0.2 to 14 days.

3.0 CYANOTOXIN OCCURRENCE AND EXPOSURE IN WATER

The presence of detectable concentrations of cyanotoxins in the environment is closely associated with blooms of cyanobacteria. Cyanobacteria flourish in various natural environments including salty, brackish or fresh water, cold and hot springs, and in environments where no other microalgae can exist, including desert sand, volcanic ash and rocks (Jaag, 1945; Dor and Danin, 1996). Cyanobacteria also form symbiotic associations with aquatic animals and plants, and cyanotoxins are known to bioaccumulate in common aquatic vertebrates and invertebrates (Ettoumi et al. 2011).

Currently, there is no national database recording freshwater harmful algal bloom (HAB) events. Instead, state and local governments document HAB occurrences in various ways depending on the monitoring methods used and the availability of laboratories capable of conducting algal toxin analyses.

Human exposure to cyanotoxins, including microcystin, may occur by direct ingestion of toxin-contaminated water or food, and by inhalation and dermal contact during bathing, showering or during recreational activities in waterbodies contaminated with the toxins. Microcystins can be dissolved in drinking water either by the breakdown of a cyanobacterial bloom or by cell lysis. Exposure can occur via drinking water as some water treatment technologies are not designed for removal of cyanotoxins. Because children consume more water per unit body weight than do adults, children may potentially receive a higher dose on a per body-weight basis. Exposure through drinking water can occur if there are toxins in the water source and the existing water treatment technologies were not designed for removal of cyanotoxins. Because children consume more water per unit body weight than do adults, children potentially may receive a higher dose than adults. Exposures are usually not chronic; however, they can be repeated in regions where cyanobacterial blooms are more extensive or persistent. Exposure to microcystin from ambient surface waters is more likely to be acute or subacute as is most likely to occur during a bloom. People, particularly children, recreating close to lakes and beach shores also can be at potential risk from exposure to nearshore blooms.

Livestock and pets potentially can be exposed to higher concentrations of cyanobacterial toxins than humans because they are more likely to consume scum and mats while drinking cyanobacteria-contaminated water (Backer et al., 2013). Dogs are particularly at risk as they may lick cyanobacteria from their fur after swimming in a water body with an ongoing bloom.

3.1 General Occurrence of Cyanobacteria in Water

Species of cyanobacteria are predominantly found in eutrophic (nutrient-rich) water bodies in freshwater and marine environments (ILS, 2000), including salt marshes. Most marine cyanobacteria of known public health concern grow along the shore as benthic vegetation between the low- and high-tide water marks. The marine planktonic forms have a global distribution. They also can be found in hot springs (Castenholz, 1973; Mohamed, 2008), mountain streams (Kann, 1988), Arctic and Antarctic lakes (Skulberg, 1996) and in snow and ice (Laamanen, 1996).

A visibly colored scum formed by floating cells may contain more than 10,000 cells/mL (Falconer, 1998). The floating scum, as in the case of *Microcystis spp.*, may be concentrated by prevailing winds in certain surface water areas, especially at the shore.

3.2 Microcystins Occurrence in Surface Water

The microcystins are the most common cyanotoxins found worldwide and have been reported in surface waters in most of the U.S. and Europe (Funari and Testai, 2008). Dry-weight concentrations of microcystins in surface freshwater cyanobacterial blooms or surface freshwater samples reported worldwide between 1985 and 1996 ranged from 1 to 7,300 µg/g. Water concentrations of extracellular

plus intracellular microcystins ranged from 0.04 to 150,000 µg/L. The concentration of extracellular microcystins ranged from 0.02 to a high of 1,800 µg/L, which occurred following treatment of a large bloom with algaecide (WHO, 1999). A concentration of 150,000 µg/L total microcystins was reported by the USGS in a lake in Kansas (Graham et al., 2012).

According to a survey conducted in Florida in 1999 between the months of June and November, the most frequently observed cyanobacteria were *Microcystis* (43.1%), *Cylindrospermopsis* (39.5%), *and Anabaena spp* (28.7%) (Burns, 2008). Of 167 surface water samples taken from 75 waterbodies, 88 samples were positive for cyanotoxins. Microcystin was the most commonly found cyanotoxin in water samples collected, occurring in 87 water samples.

In 2002, the Monitoring and Event Response to Harmful Algal Blooms in the Lower Great Lakes (MERHAB-LGL) project evaluated the occurrence and distribution of cyanobacterial toxins in the lower Great Lakes region (Boyer, 2007). Analysis for total microcystins was done using Protein Phosphatase Inhibition Assay (PPIA). Microcystins were detected in at least 65% of samples, mostly in Lake Erie, Lake Ontario, and Lake Champlain. The National Oceanic and Atmospheric Administration (NOAA) Center of Excellence for Great Lakes and Human Health (CEGLHH) continues to monitor the Great Lakes and regularly samples algal blooms for microcystin in response to bloom events.

A 2004 study of the Great Lakes found high levels of cyanobacteria during the month of August (Makarewicz et al., 2006). Microcystin-LR was analyzed by PPIA (limit of detection of 0.003 µg/L) and was detected at levels of 0.084 µg/L in the nearshore and 0.076 µg/L in the bays and rivers. The study reported higher levels of microcystin-LR (1.6 to 10.7µg/L) in smaller lakes in the Lake Ontario watershed.

In 2006, the USGS conducted a study of 23 lakes in the Midwestern U.S. in which cyanobacterial blooms were sampled to determine the co-occurrence of toxins (Graham et al., 2010). The study reported that microcystins were detected in 91% of the lakes sampled. Mixtures of all the microcystin congeners measured (LA, LF, LR, LW, LY, RR, and YR) were common and all the congeners were present in the blooms. Microcystin-LR and –RR were the dominant congeners detected with mean concentrations of 104 and 910 µg/L, respectively.

EPA's National Aquatic Resource Surveys (NARS) generate national estimates of pollutant occurrence every 5 years. In 2007, the National Lakes Assessment (NLA) conducted the first-ever national probability-based survey of the nation's lakes, ponds and reservoirs (U.S.EPA, 2009). This baseline study of the condition of the nation's lakes provided estimates of the condition of natural and man-made freshwater lakes, ponds, and reservoirs greater than 10 acres and at least one meter deep. A total of 1,028 lakes were sampled for the NLA during summer 2007. The NLA measured microcystins using Enzyme Linked Immunosorbent Assays (ELISA) with a detection limit of 0.1 µg/L, as well as cyanobacterial cell counts and chlorophyll-a concentrations, as indicators of the presence of cyanobacterial toxins. Samples were collected in open water at mid-lake; no samples were taken nearshore or other areas where scums were present.

A total of 48 states were sampled in the NLA and states with lakes reporting microcystin levels above the WHO's moderate risk[1] threshold in recreational water (>10 µg/L) are shown in Table 3-1. Microcystins were present in 30% of the lakes sampled nationally, with sample concentrations that ranged from the limit of detection (0.1 µg/L) to 225 µg/L. Two states (North Dakota and Nebraska) had 9% of the samples

[1] The WHO established guideline values for recreational exposure to cyanobacteria using a three-tier approach: low risk (<20,000 cyanobacterial cells/ml corresponding to <10 µg/L of MC-LR); moderate risk (20,000-100,000 cyanobacterial cells/ml corresponding to 10-20 µg/L of MC-LR); and high risk (>100,000 cyanobacterial cells/ml corresponding to >20 µg/L for MC-LR) (WHO, 1999).

above 10 µg/L. Other states, including Iowa, Texas, South Dakota, and Utah also had samples that exceeded 10 µg/L. Several samples in North Dakota, Nebraska, and Ohio exceeded the WHO high risk threshold value for recreational waters of 20 µg/L (192, 225 µg/L, and 78 µg/L, respectively). EPA completed a second survey in 2012 but data have not yet been published.

Table 3-1. States Surveyed as Part of the 2007 National Lakes Assessment with Water Body Microcystin Concentrations Above the WHO Advisory Guideline Level for Recreational Water of 10 µg/L (U.S. EPA, 2009)

State	Number of Sites Sampled	Percentage of Samples with Detection of Microcystins >10 µg/L	Maximum Detection of Microcystins
North Dakota	38	9.1%	192 µg/L
Nebraska	42	9.1%	225 µg/L
South Dakota	40	4.9%	33 µg/L
Ohio	21	4.5%	78 µg/L*
Iowa	20	4.5%	38 µg/L*
Utah	26	3.6%	15 µg/L*
Texas	51	1.8%	28 µg/L *

* Single Sample

Microcystins have been detected in most of the states of the U.S. and over the past several years and many studies have been conducted to determine their occurrence in surface water. USGS, for example, did a study in the Upper Klamath Lake in Oregon in 2007 and detected total microcystin concentrations between 1 µg/L and 17 µg/L (VanderKooi et al., 2010). USGS also monitored Lake Houston in Texas from 2006 to 2008, and found microcystins in 16% of samples, with concentrations less than or equal to 0.2 µg/L (Beussink and Graham, 2011). In 2011, USGS conducted a study on the upstream reservoirs of the Kansas River, a primary source of drinking water for residents in northeastern Kansas, to characterize the transport of cyanobacteria and associated compounds (Graham et al., 2012). Concentrations of total microcystins were low in the majority of the tributaries with the exception of Milford Lake, which had higher total microcystin concentrations, some of which exceeded the Kansas recreational guidance level of 20 µg/L. Upstream from Milford Lake, a cyanobacterial bloom was observed with total microcystin concentration of 150,000 µg/L. When sampled a week later, total microcystin concentrations were less than 1 µg/L. This may have been due either to dispersion of microcystins through the water column or to other areas or settling of cyanobacteria out of the water column. Samples taken during the same time from outflow waters contained a total microcystin concentration of 6.2 µg/L.

In 2005, Washington State Department of Ecology developed the Ecology Freshwater Algae Program to focus on the monitoring and management of cyanobacteria in Washington lakes, ponds, and streams (WSDE, 2012). The data collected have been summarized in a series of reports for the Washington State Legislature (Hamel, 2009, 2012). Microcystin levels ranged from the detection limit (0.05 µg/L) to 4,620 µg/L in 2008, 18,700 µg/L in 2009, 853 µg/L in 2010, and 26,400 µg/L in 2011.

Other surveys and studies have been conducted to determine the occurrence of microcystin in lakes in the United States. A survey conducted during the spring and summer of 1999 and 2000 in more than 50 lakes in New Hampshire found measureable microcystin concentrations in all samples (Haney and Ikawa, 2000). Microcystins were analyzed by ELISA and were found in all of the lakes sampled with a mean concentration of 0.1 µg/L. In 2005 and 2006, a study conducted in New York, including Lake Ontario, found variability in microcystin-LR concentrations within the Lake Ontario ecosystem (Makarewicz et al., 2009). Of the samples taken in Lake Ontario coastal waters, only 0.3% of the samples exceeded the WHO provisional guideline value for drinking water of 1 µg/L. However, 20.4% of the samples taken at upland lakes and ponds within the Lake Ontario watershed, some of them sources of drinking water,

exceeded 1 µg/L. During 2008 and 2009, a study was done in Kabetogama Lake, Minnesota to measure microcystin concentrations associated with algal blooms (Christensen et al., 2011). Microcystins were detected in 78% of bloom samples. Of these, 50% were above 1 µg/L in finished drinking water and two samples were above the high risk WHO recreational level of 20 µg/L.

A study from 2002 evaluated water quality, including chlorophyll-a concentrations, cyanobacterial assemblages, and microcystin concentrations in 11 potable water supply reservoirs within the North Carolina Piedmont during the dry summer growing season (Touchette et al., 2007). Microcystin concentrations were assessed using ELISA. The study found that cyanobacteria were the dominant phytoplankton community, averaging 65-95% of the total cells. Although microcystin concentrations were detected in nearly all source water samples, concentrations were <0.8 µg/L.

Since 2007, Ohio EPA (OHEPA, 2012) has been monitoring inland lakes for cyanotoxins. Of the 19 lakes in Ohio sampled during the NLA, 36% had detectable levels of microcystins. In 2010, OHEPA sampled Grand Lake St. Marys for anatoxin-a, cylindrospermopsin, microcystins, and saxitoxin. Toxin levels ranged from below the detection limit (<0.15 µg/L) to more than 2,000 µg/L for microcystins. Follow-up samples taken in 2011 for microcystins indicated concentrations exceeding 50 µg/L in August. During the same month, sampling in Lake Erie found microcystins levels to exceed 100 µg/L.

In 2008, NOAA began monitoring cyanobacterial blooms in Lake Erie using high temporal resolution satellite imagery. Between 2008 and 2010, *Microcystis* cyanobacterial blooms associated with water temperatures above 18°C were detected (Wynne et al., 2013). Using the Great Lakes Coastal Forecast System (GLCFS) hydrodynamic model, forecasts of bloom transport are created to estimate the trajectory of the bloom and these are distributed as bulletins to local managers, health departments, researchers and other stakeholders. To evaluate bloom toxicity, the Great Lakes Environmental Research Laboratory (GLERL) collected samples at six stations each week for 24 weeks, measuring toxin concentrations as well as chlorophyll biomass and an additional 18 parameters (e.g., nutrients) to improve future forecasts of these blooms. In 2014, particulate toxin concentrations, collected from 1 meter depth, ranged from below detection to 36.7 µg/L. Particulate toxin concentrations peaked in August, 2014 at all sites, with the Maumee Bay site yielding the highest toxin concentration for the entire sampling period. Dissolved toxin concentrations were collected at each site from September until November when the field season ended. During the final months of sampling (October-November) dissolved toxin concentrations were detected with peak concentrations of 0.8 µg/L (mean: 0.28 +/- 0.2 µg/L) whereas particulate toxin concentrations were below detection limits on many dates indicating that a majority of the toxins (mean: 72% +/- 37%) were in the dissolved pool as the bloom declined in intensity.

Concentrations of microcystins were detected during sampling in 2005 and 2006 in lakes and ponds used as a source of drinking water within the Lake Ontario watershed (Makarewicz et al., 2009). A microcystin-LR concentration of 5.07 µg/L was found in Conesus Lake, a source of public water supply that provides drinking water to approximately 15,000 people. Microcystin-LR was also detected at 10.716 µg/L in Silver Lake, a public drinking water supply for four municipalities.

3.3 Microcystins Occurrence in Drinking Water

The occurrence of cyanotoxins in drinking water depends on their levels in the raw source water and the effectiveness of treatment methods for removing cyanobacteria and cyanotoxins during the production of drinking water. Currently, there is no program in place to monitor for the occurrence of cyanotoxins at surface-water treatment plants for drinking water in the U.S. Therefore, data on the presence or absence of cyanotoxins in finished drinking water are limited.

The American Water Works Association Research Foundation (AWWARF) conducted a study on the occurrence of cyanobacterial toxins in source and treated drinking waters from 24 public water systems in the U.S. and Canada in 1996-1998 (AWWARF, 2001). Of 677 samples tested, microcystin was found in 80% (539) of the waters sampled, including treated waters. Only two samples of finished drinking water had microcystin concentrations above 1 µg/L. A survey conducted in 1999 in Florida (Burns, 2008) reported that microcystins were the most commonly found toxin in pre- and post-treated drinking water. Finished water concentrations ranged from below detection levels to 12.5 µg/L.

A study from 2002 conducted during the dry summer growing season, evaluated the water quality and environmental parameters, including phytoplankton chlorophyll a concentrations, cyanobacterial assemblages, and microcystin concentrations in 11 potable water supply reservoirs within the North Carolina Piedmont (Touchette et al., 2007). The study found that cyanobacteria were the dominant phytoplankton community, averaging 65-95% of the total cells. Although microcystin concentrations were detected in nearly all samples, microcystin-LR was detected below 1µg/L.

During the summer of 2003, a survey was conducted to test for microcystins in 33 U.S. drinking water treatment plants in the Northeast and Midwest (Haddix et al., 2007). Microcystins were detected at low levels ranging from undetectable (<0.15 µg/L) to 0.36 µg/L in all 77 finished water samples.

Concentrations of microcystin-LR have been detected during sampling in 2005 and 2006 in lakes and ponds used as a source of drinking water within the Lake Ontario watershed (Makarewicz et al., 2009). A Microcystin-LR concentration of 5.070 µg/L was measured in Conesus Lake, a source of public water supply that provides drinking water to approximately 15,000 people. Microcystin-LR was also detected at 10.716 µg/L in Silver Lake, a public drinking water supply for four municipalities.

In August 2014, the city of Toledo, Ohio issued a "do not drink or boil advisory" to nearly 500,000 customers in response to the presence of total microcystins in the city's finished drinking water at levels up to 2.50 µg/L. The presence of the toxins was due to a cyanobacterial bloom near Toledo's drinking water intake located on Lake Erie. The advisory was lifted two days later, after treatment adjustments led to the reduction of the cyanotoxin concentrations to concentrations below the WHO guideline value of 1 µg/L in all samples from the treatment plant and distribution system.

3.4 Summary

Microcystin-producing cyanobacteria occur in freshwater systems worldwide. No national database recording freshwater microcystins is available. Microcystin monitoring efforts in surface waters and drinking water is being conducted by states and others, including USGS, EPA, and NOAA. A survey done by USGS in 2006 of 23 lakes in the Midwestern U.S., found that microcystin was detected in all the blooms. Mixtures of all the microcystin congeners measured (LA, LF, LR, LW, LY, RR, and YR) were common, and all the congeners were present in the blooms. The 2007 EPA National Lakes Assessment found microcystin in about one third of the lakes sampled with concentrations ranging from the limit of detection (0.05 µg/L) to 225 µg/L. Sampling done in 2014 in Lake Erie by NOAA reported microcystin concentrations ranging from below detection limits to 36.7 µg/L. The U.S. Geological Survey (USGS) reported a concentration of 150,000 µg/L total microcystins, in a lake in Kansas (Graham et al., 2012).

Microcystins have been found in raw and in finished drinking water. In a study done in 2007 in 33 lakes across the U.S., microcystins exceeded 1 µg/L levels in 7% of the raw water samples. A survey conducted in 1999 in Florida found microcystins concentrations in finished water ranging from below detection levels to 12.5µg/L.

Exposure to microcystin from contaminated drinking water sources may occur mostly via oral exposure (e.g. ingestion of contaminated drinking water), dermal exposure (contact of exposed parts of the body

with water containing toxins) and inhalation exposure. Exposure to microcystins during recreational activities may occur through direct contact, inhalation and/or ingestion. Exposures are usually not chronic with the exception of regions with extensive and persistent cyanobacterial blooms. Since children consume more water per unit body weight than do adults, children may potentially receive a higher dose. Pets, livestock and wildlife are also potentially exposed to microcystin when consuming scum and mats, and drinking cyanobacteria-contaminated water.

4.0 CYANOTOXIN OCCURRENCE IN MEDIA OTHER THAN WATER

4.1 Occurrence in Soil and Edible Plants

Cyanobacteria are highly adaptable and have been found to colonize infertile substrates, such as volcanic ash and desert sand (Jaag, 1945; Dor and Danin, 1996; Metcalf et al., 2012). They also have been found in soil, at the surface or several centimeters below the surface, where they play a functional role in nutrient cycling. Cyanobacteria are known to survive on rocks or tree trunks, and in snow and ice (Adhikary, 1996). They have been reported in deeper soil layers, likely transported by percolating water or burrowing animals. Some freshwater species are halotolerant (salt tolerant) and have been found in saline environments such as salt works or salt marshes (WHO, 1999). Cyanobacterial cells can bioaccumulate in zooplankton (Watanabe et al., 1992). As a result of higher trophic level grazing, the damaged or residual cyanobacterial cells may settle out of the water column and accumulate in sediment where breakdown by sediment bacteria and protozoa can release their toxins (Watanabe et al., 1992).

Cyanobacterial cells and toxins can contaminate spray irrigation water and subsequently be associated with crop plants after spray irrigation with contaminated water (Corbel et al., 2014). Water contaminated with cyanobacterial cells and toxins used for spray irrigation of crop plants may cause food chain contamination since low levels of cyanotoxins could be absorbed by roots, migrate to shoots, and then be translocated to grains and or fruits. Cyanotoxins can be transmitted to food plants from irrigation water when cyanotoxins are deposited on the plants leaves. A study was conducted with lettuce plants grown with spray irrigation containing *M. aeruginosa* at levels ranging from 0.094 to 2.487 µg/g dw. Cyanotoxin levels detected in lettuce leaf extracts 10 days after irrigation indicated microcystin-LR equivalents up to 2.49 µg/g dw (Codd et al., 1999). Extracts from rape and rice seedlings were exposed to water with concentrations of microcystin-LR up to 3 mg/L (Chen et al., 2004a). The study found concentrations of microcystin-LR of 651 ng/g in extracts from rape and 5.4 ng/g in rice. These studies and others with high concentrations of cyanotoxins found that concentrations at these levels are able to inhibit plant growth causing visible toxic effects on the plant such as leaf withering. The microcystin concentrations detected in rice grains were very low. Studies with seedlings exposed to cyanotoxin concentrations typically found in natural surface waters (1-10 µg/L) reported microcystins at low levels in broccoli roots (0.9 to 2.4 ng microcystin-LR/g fresh weight) and mustard roots (2.5 to 2.6 ng microcystin-LR/g fresh weight) (Järvenpää et al., 2007).

Uptake of microcystins was measured in vegetables grown with irrigated contaminated groundwater in Saudi Arabia (Mohamed and Al Shehri, 2009). The concentration of total microcystins was highly variable in the plants but positively correlated with concentrations in groundwater. Radishes had the highest concentration (1.2 µg/g fresh weight) and cabbages had the lowest amount (0.07 µg/g fresh weight). Lettuce, parsley, arugula, and dill also had measurable concentrations. Generally, roots accumulated more than the leaves.

Water contaminated with cyanotoxins used for spray irrigation of crop plants will inhibit plant growth and will induce visible toxic effects such as the appearance of brown leaves (Funari and Testai, 2008). Therefore, according to the authors, affected plants and crops will most likely not be used for eating purposes. Further investigation is needed to understand the uptake and fate of microcystins and other cyanobacterial toxins by food plants.

4.2 Occurrence in Fish and Shellfish

Cyanotoxins can bioaccumulate in common aquatic vertebrates and invertebrates, including fish, snails (Carbis et al., 1997; Beattie et al., 1998; Berry et al., 2012) and mussels (Eriksson et al., 1989; Falconer et al., 1992; Prepas et al., 1997; Watanabe et al., 1997; Funari and Testai, 2008). Human exposure to

cyanotoxins may occur if fish are consumed from reservoirs with existing blooms of toxin-producing cyanobacteria (Magalhães et al., 2001).

The health risk from fish and shellfish consumption depends on the bioaccumulation of toxins in edible fish tissue compared to toxins in organs such as the liver. Numerous authors have found that microcystins accumulate to a lesser extent in the edible parts of aquatic organisms, such as muscle (Xie et al., 2005; Zimba et al., 2006; Song et al., 2009; Wilson et al., 2008; Deblois et al., 2011; Vareli et al., 2012; Gutiérrez-Praena et al., 2013). In a survey of microcystin in water and fish in two temperate Great Lakes (Erie and Ontario), the highest microcystin concentrations in fish muscle observed Lake Erie were for alewives (20.0-37.5 μg/kg) and northern pike (1.6-25.8 μg/kg); and for Lake Ontario: walleye (5.3-41.2 μg/kg), white bass (4.2-27.1 μg/kg) and smallmouth bass (1.5-43.6 μg/kg) (Poste et al., 2011). Muscle tissue microcystin concentrations in yellow perch collected during a toxic bloom were lower in comparison (0.12- 0.02 ng toxin/g dw) (Wilson et al., 2008). Nevertheless, concentrations of microcystin in edible tissues have been reported to be greater than 0.1 μg/g for fish, crab, mussels and shrimp (Magalhães et al., 2001; Mohamed et al., 2003; Xie et al., 2005; Vareli et al., 2012).

Microcystins have been shown to bioaccumulate in the liver and hepatopancreas of decapod crustaceans (Williams et al., 1997), but there was not strong evidence for biomagnification (Ibelings et al., 2005; Xie et al., 2005; Ibelings and Havens, 2008; Papadimitriou et al., 2012). Because fish are generally more tolerant of cyanobacterial toxins than mammals, they tend to accumulate them over time (ILS, 2000).

In a survey by Xie et al. (2005) microcystin-LR content in muscle was highest in carnivorous and omnivorous fish and was lowest in phytoplanktivorous and herbivorous fish. Chen et al. (2009) also found highest total microcystin levels in liver and muscle from omnivorous fish compared with other types of feeders. Berry et al. (2011) found the highest levels in phytoplanktivores and omnivores with no microcystins detected in predominantly zooplanktivorus fish. Microcystin-LR was not detected in livers from northern pike and white sucker fish collected from a lake in Canada following peak seasonal microcystin levels measured in the water (Kotak et al., 1996).

After fish are exposed, concentrations of microcystins decrease with time as a result of detoxification and depuration processes (Tencalla and Dietrich, 1997; Xie et al., 2005; Mohamed and Hussain, 2006; Wood et al., 2006; Gutiérrez-Praena et al., 2013). Researchers have also suggested that biodilution may occur given the observations of depuration and toxin elimination within organisms (Ibelings and Havens, 2008, Poste et al., 2011). It has also been raised that biotransformation of microcystin by aquatic organisms to covalently-bound forms may complicate the complete measurement of total microcystin content in tissues (Williams et al., 1997; Wilson et al., 2008; Dyble et al., 2011).

Levels of microcystins found in tissues of aquatic species potentially consumed by humans are shown in Table 5-1. Unless specified, levels are reported as microcystin-LR equivalents. Most studies have concentrated on levels in fish, although limited data show measurable amounts of microcystin-LR in mussels, shrimp, and crayfish. Recent reviews emphasize that microcystin levels in edible fish and shellfish are highly variable depending on trophic level, bloom conditions, and potential for depuration (Ibelings and Chorus, 2007; Ferrão-Filho et al., 2011, and Kozlowsky-Suzuki, 2011). Soares et al (2004) reported that microcystins could still be found in the fish muscle several days after the end of a toxic bloom. In fish, higher concentrations were consistently measured in liver compared with muscle, which is a significant dietary contribution in small fish consumed whole. Reports of deaths of marine mammals from microcystin intoxication related to trophic transfer through marine invertebrates have been reported (Miller et al., 2010). Deaths of 21 southern sea otters close to river mouths contaminated with microcystins were related to intoxication after consuming farmed and free-living marine clams, mussels and oysters in the area showing significant biomagnification (up to 107 times ambient water levels). There have been no documented cases of microcystin toxicity in humans following ingestion of fish or shellfish that have been exposed to microcystins (Mulvenna et al., 2012). Since food web exposures to

blooms can vary greatly between geographical regions, it is unlikely to have year-round exposure in humans that may consume aquatic organisms from water bodies susceptible to cyanobacterial blooms.

Data regarding microcystin elimination in fish are limited. A study of common carp (*Cyprinus carpio*) and Silver Carp (*Hypophtalmichthys molitrix*) in Europe found that microcystins were completely eliminated within one to two weeks from muscle and hepatopancreas after transferring the fish to clean water (Adamovsky et al., 2007). The mean elimination half-lives ranged from 0.7 to 2.8 days in silver carp muscle and from to 3.5 to 8.4 days in common carp liver. However, slower elimination (15 to 40 days after the end of the accumulation period), was reported in silver carp and Nile tilapia by Soares et al. (2004).

Table 4-1. Bioaccumulation Studies of Microcystins in Fish, Shellfish, and Crustaceans

Species/tissue	Tissue Concentration	Sampling Conditions	Average Water: Tissue Correlations	Reference
Fish				
Tilapia Muscle Liver Viscera	0.002-0.337 µg/g ww 0-31.1 µg/g ww 0-71.6 µg/g ww	3-year sampling from coastal lagoon; seston concentrations ranged from 0-980 µg/L during the study period	19.6 µg/L:0.02µg/g muscle 17 µg/L:0.03µg/g muscle 4.7 µg/L:0.03µg/g muscle	Magalhães et al., 2001
Tilapia Muscle Liver	0.007-0.06 µg/g 0.092-0.28 µg/g	Average levels from laboratory feeding of isolated cells	14.6 µg/fish/day (28 days):0.08 µg/g muscle (peak)	Soares et al., 2004
Fish – muscle	0.0396 µg/g ww	Peak level in samples from bay over 11 months	0.78 µg/L:0.0396 µg/g muscle	Magalhães et al., 2003
Cyprinus carpio Muscle Hepatopancreas	0.038 µg/g fresh wt 0.261 µg/g fresh wt	Laboratory feeding bloom scum at 50 µg/kg body weight for 28 days	See previous columns	Li et al., 2004
Corydoras paleathus and *Jenynsia multidentata* Muscle Liver Gill	0.04-0.11 µg/g ww 1.62-19.63 µg/g ww 0.56-1.40 µg/g ww	Laboratory exposure to 50 µg microcystin-RR/L for 24 hours	See previous columns	Cazenave et al., 2005
Odontesthes bonariensis Muscle Liver Gill	(average/maximum) 0.05/0.34 µg/g ww 0.16/1.01 µg/g ww 0.03/0.10 µg/g ww	Wild caught from cyanobacteria containing reservoir; cellular microcystin-RR = 41.59 µg/g (wet season) and 9.65 µg/g (dry season)	Maximum tissue levels correlated to wet season	Cazenave et al., 2005
8 species Muscle Liver Intestine	1.81 µg/g dw 7.77 µg/g dw 22 µg/g dw	Wild caught from lake during bloom; 240 µg/g dry weight of bloom sample	Water not sampled; ingestion by fish possible	Xie et al., 2005
Yellow perch Muscle Liver	0.00012-0.004 µg/g dw 0.017-1.182 µg/g dw	Wild caught from lake during summer months; 0.00016 - 4.28 µg/L in seston	Data presented graphically; positive correlation	Wilson et al., 2008
4 species Muscle Liver	0.002-0.027 µg/g dw 0.003-0.150 µg/g dw	Wild caught from lake during August; Total MC (-RR, -YR, -LR) in scum = 328 µg/g dry weight	See previous columns; tissue concentrations varied by species	Chen et al., 2009
2 species Muscle Liver	0.005-0.157 µg/g 0.094-0.867 µg/g	Commercial catch from lake with bloom; 0.02-0.36 µg/L in seston; 0.16-0.19 µg/L in water	Samples not matched to fish	Berry et al., 2011
Multiple Muscle Whole	0.0005-1.917 µg/g ww 0.0045-0.215 µg/g ww	Multiple temperate and tropical lakes; 0.1-57.1 µg/L in water for all lakes	See paper, multiple fish samples from all lakes	Poste et al., 2011

Species/tissue	Tissue Concentration	Sampling Conditions	Average Water: Tissue Correlations	Reference
3 species Muscle Liver	<det. limit-0.32 µg/g <det. limit-0.27 µg/g	Wild caught in lake	Multiple samples from lake and fishes, highly variable	NDEQ, 2011
Shellfish				
Mussel - several species Whole body Foot/muscle	0.064-0.188 µg/g ww 0.009-0.022 µg/g ww	Mean values from literature; water concentrations not given	Not available	Ibelings and Chorus, 2007
Crustaceans				
Crayfish – whole (not found in muscle tissue)	2.9 µg/g dw	Experimental feeding for 11 days with *M. aeruginosa* isolated from a lake; MC content not measured	Not available	Vasconcelos et al., 2001
Crab – muscle	0.103 µg/g ww	Peak level in samples from bay over 11 months	0.78 µg/L : 0.103 µg/g	Mahalhães et al., 2003
Shrimp (several species) Whole Muscle	0.051-0.114 µg/g ww 0.004-0.006 µg/g ww	Mean values from literature; water concentrations not given	Not available	Ibelings and Chorus, 2007

4.3 Occurrence in Dietary Supplements

Extracts from *Arthrospira (Spirulina spp.)* and *Aphanizomenon flos-aquae* (AFA) have been used as dietary bluegreen algae supplements (BGAS) (Funari and Testai, 2008). These supplements are reported to have beneficial health effects including supporting weight loss, and increasing alertness, energy and mood elevation for people suffering from depression (Jensen et al., 2001). In children, they have been used as an alternative, natural therapy to treat attention deficit hyperactivity disorders (ADHD).

Studies suggest that BGAS can be contaminated with microcystins ranging from 1 μg/g up to 35 μg/g (Dietrich and Hoeger, 2005). Heussner et al. (2012) analyzed 18 commercially available BGAS for the presence of toxins. Neither anatoxin-a nor cylindrospermopsin were found in any of the supplements. However, all products containing AFA tested positive for microcystins at levels ≤ 1 μg microcystin-LR equivalents/g dw. The microcystin (microcystin-LR with traces of microcystin-LA) was assumed to be the result of contamination.

The levels of algal toxins in food supplements are unregulated at the federal level in the United States. Therefore, it is difficult to appropriately evaluate the actual exposure to cyanobacterial supplements.

4.4 Summary

Microcystins have been detected in soil, at the surface or several centimeters below the surface, where they play a functional role in nutrient cycling. They have also been found in sediments, edible plants, and aquatic animals. Cyanobacterial cells and toxins can contaminate spray irrigation water and subsequently be transmitted to food plants. Since water contaminated with cyanotoxins used for spray irrigation of crop plants will inhibit plant growth and will induce visible toxic effects (e.g. brown leaves), affected plants and crops will most likely not be used for eating purposes. Further investigation is needed to understand the uptake and fate of microcystins and other cyanobacterial toxins by food plants. Bioaccumulation in aquatic animals occurs mostly in the liver of fish, shellfish and crustaceans, but microcystins have also been detected in fish tissue. After fish are exposed, concentrations of microcystins decrease with time as a result of detoxification and depuration processes. The health risk from consumption depends on the bioaccumulation of toxins in edible fish tissue compared to toxins in organs such as the liver. Currently, cases of microcystin toxicity in humans following ingestion of fish or shellfish exposed to microcystins have not been documented. Microcystin-LR has been detected in algal supplements at levels at or lower than 1μg microcystin-LR equivalents/g dw.

5.0 TOXICOKINETICS

5.1 Absorption

No data were available that quantified the intestinal, respiratory or dermal absorption of microcystin. Most of the available evidence indicates that absorption from the intestinal tract and into liver, brain, and other tissues requires facilitated transport using receptors belonging to the Organic Acid Transporter polypeptide (OATp) family. The OATp family transporters are part of a large family of membrane receptors that facilitate cellular, sodium-independent uptake and export of a wide variety of amphipathic compounds including bile salts, steroids, drugs, peptides and toxins (Cheng et al., 2005; Fischer et al., 2005; Svoboda et al., 2011). OATps are located in the liver, brain, testes, lungs, kidneys, heart, placenta and other tissues of rodents and humans (Cheng et al., 2005; Svoboda et al., 2011). Only a few of the OATps have been characterized at their functional, structural, and regulatory levels. In mice, males often express OATps in tissues to a greater extent than females (Cheng et al., 2005).

For this document the abbreviation for the Organic Acid Transporter polypeptides will be written as OATp rather than differentiating the animal versions from the human versions by using lower case letters for the animals and upper case letters for humans.

5.1.1 Oral Exposure

An *in situ* study in rats indirectly studied the oral bioavailability of microcystin-LR using isolated intestinal loops (Dahlem et al., 1989). After receiving a single 5 mg/kg infusion of microcystin-LR (>95% pure) into the ileum, the rats showed clinical signs, including labored breathing and circulatory shock, as well as evidence of liver toxicity within 6 hours. When an infusion of a similar dose was given into a jejunal loop, a lower degree of liver toxicity was observed. The authors suggested site-specificity in microcystin-LR intestinal absorption although the authors did not consider differences in absorptive surface area when their hypothesis on differences in absorptive capacity was proposed.

A study done in swine demonstrated oral absorption of ^{3}H-dihydromicrocystin (75 µg/kg) using ileal loop exposure (Stotts et al., 1997a,b). The maximum blood concentration of the toxin occurred 90 minutes after dosing.

Oral absorption of microcystin-LR (purified from an algal bloom sample) after a single gavage dose of 500 µg/kg was examined by Ito et al. (1997a) and Ito and Nagai (2000). Microcystin-LR was absorbed primarily in the small intestine, although some absorption was observed in the stomach as demonstrated by targeted immunostaining (Ito and Nagai, 2000). The authors observed an erosion of the surface epithelial cells of the small intestine villi facilitating perhaps the uptake of the toxin into the bloodstream (Ito and Nagai, 2000; Ito et al., 1997a).

5.1.2 Inhalation Exposure

Microcystins can be present as aerosols in surface waters and drinking water after they are generated by the wind or during showering or swimming providing contact with the respiratory epithelium. After an intratracheal instillation in mice of a 50 µg/kg sublethal dose or a 100 µg/kg lethal dose, pulmonary absorption of microcystin-LR (purified from an algal bloom sample) observed as immunostaining of the lung occurred within 5 minutes (Ito et al., 2001). After the lethal dose was administered, a lag period of 60 minutes occurred and staining was observed in the liver after 7 hours of the sublethal dose administration. This observation demonstrated the possibility of uptake from the lungs into systemic circulation.

Low levels of total microcystins (detection limit = 0.08 ng/m^3) were detected in air samples collected above a lake bloom, indicating that inhalation exposure was possible (Backer et al., 2008). However, recreational users of the lake at the time of the bloom had no detectable microcystin in their blood and did not report an increase in symptoms after spending time on the lake.

5.1.3 Dermal Exposure

In vivo or *in vitro* studies to determine the dermal absorption of microcystin have been identified. Skin patch testing was done on 19 human volunteers using lyophilized *M. aeruginosa* (Stewart et al., 2006b). Up to 170 ng of cyanotoxin was applied to filter paper discs applied to the back of each volunteer; patches were removed after 48 hours and the exposed skin was scored after 48 and 96 hours. No individual developed clinically detectable skin reactions.

5.2 Distribution

Facilitated transport is apparently necessary for both uptake of microcystins into organs and tissues as well as for their export. In the liver, microcystins compete with bile acid uptake such that blocking this transport system also prevents microcystin-LR uptake and toxicity in hepatocytes (Thompson and Pace, 1992). *In vitro* or *in vivo* exposures have shown that inhibition of microcystin uptake by its OATp transporter could eliminate or reduce the toxicity in the liver (Runnegar et al., 1981, 1995a; Runnegar and Falconer, 1982; Hermansky et al., 1990a,b).

In a study done by Fischer et al. (2005) human OATp1A2, OATp1B1, and OATp1B3 demonstrated the ability to mediate the transport of ^{3}H-dihydromicrocystin-LR in *Xenopus laevis* oocytes. Inhibition of the uptake was done by sulfobromophthalein and taurocholate. In addition, various *in vitro* studies have shown that cells without microcystin-competent OATp do not absorb microcystin and that the introduction of OATps to these cells will allow them to absorb microcystin (Komatsu et al., 2007, Jasionek et al., 2010, Feurstein et al., 2010, Fischer et al., 2010). Another study by Fischer et al. (2010), found that the role of OATp in microcystin uptake varies by congener and that highest uptake rates were observed in MC-LW and MC-LF in comparison with microcystin-LR and microcystin-RR.

A study done by Lu et al. (2008) used OATp1b2 null mice to demonstrate the importance of the OATp system in transporting microcystin-LR into the liver The authors found severe hepatotoxicity and death that was caused in wild-type mice after the intraperitoneal (i.p) administration of 120 µg microcystin-LR/kg. Fischer et al. (2010) used primary human hepatocytes and compared OATp-transfected HEK293 cells and control vector HEK293 cells (resistant to microcystin cytotoxicity) to show the need for microcystin-competent OATp for transporting of microcystin across the cellular membrane. The primary human hepatocytes were an order of magnitude more sensitive than the OATp-transfected HEK293 cells, probably because HEK293 cells only have OATp1b1 and 1b3, while other OATps that contribute to the uptake of the microcystin congeners may be in the primary human hepatocytes. Another study observed similar results (Komatsu et al., 2007), however, microcystin-LR accumulation in OATp-transfected HEK293 cells increased in a dose-dependent manner, which was not observed in the control vector HEK293 cells.

5.2.1 Oral Exposure

The distribution of microcystin-LR (purified from an algal bloom sample) following oral gavage administration to mice (500 µg/kg) was investigated using immunostaining methods (Ito and Nagai, 2000). Microcystin-LR was detected in large amounts in the villi of the small intestine. Erosion of the villi was observed, which may have enhanced absorption of the toxin into the bloodstream. Microcystin-LR was also present in the blood plasma, liver, lungs, and kidneys.

Once inside the cell, microcystins covalently bind to cytosolic proteins, resulting in their retention in the liver. The hepatic cytosolic proteins that bind microcystin have been identified as the protein phosphatase enzymes 1 and 2A (PP1 and PP2A). Covalent adducts of microcystin-LR, microcystin-LA, and microcystins-LL with both enzymes were identified by reverse-phase liquid chromatography. In contrast, the dihydromicrocystin-LA analog did not form covalent bonds with PP1 and PP2A which suggests a role for the double bonds of Adda in covalent binding. However, the dihydromicrocystin analog was able to inhibit the enzyme activity, supporting a role for electrostatic interactions in the mode of action (MOA) for enzyme inhibition as well as covalent binding; the IC_{50} was similar for microcystin-LR and the dihydro-analog (Craig et al., 1996).

Nishiwaki et al. (1994) demonstrated that the distribution of ^{3}H-dihydromicrocystin-LR in mice differs by route of exposure. When ^{3}H-dihydromicrocystin-LR (11.4 µCi/2.4 mmol/0.2 mL saline) is administered by intraperitoneal injection (i.p.), rapid and continuous uptake by the liver is observed, with around 72% of the dose in the liver after 1 hour of administration. Total radiolabel in small percentages was observed in various organs: 1.4% in the small intestine; 0.5% in the kidney and gallbladder; 0.4% in the lungs; 0.3% in the stomach. When ^{3}H-dihydromicrocystin-LR (22.8 µCi/2.1 µmol/0.2 mL saline) was administered orally, much lower concentrations were observed in the liver, with less than 1% of the dose in the liver at either 6 hours or 6 days after administration. Approximately 38% of the dose was found in the gastrointestinal contents.

Microcystin-LR was not detected in the milk of dairy cattle exposed to *M. aeruginosa* cells either administered by drinking water (detection limit= 2 ng/L) (Orr et al., 2001), or by ingestion of a gelatin capsule with the cells (detection limit= 0.2 ng/L) (Feitz et al., 2002). Microcystins were not detected in the blood plasma and only 10-39% of the total ingested microcystin-LR was found in the liver of beef cattle given *M. aeruginosa* cells via drinking water for 29 days (Orr et al., 2003). However, these studies were limited by study design and data reporting (e.g. lack of controls, low number of cows and exposure doses, or no concentrations of microcystin reported).

5.2.2 Inhalation Exposure

The organ distribution after intratracheal instillation of a lethal dose (100 µg/kg) of microcystin-LR purified from an algal bloom was assessed by using immunostaining methods (Ito et al., 2001). The kidney, liver, lung, and small intestine were positively stained for microcystin-LR. After 5 minutes of instillation, intense staining was observed in the lung, in the kidney after 10 minutes, in the small intestine after 45 minutes, and in the liver after an hour. Bleeding began around the hepatic central vein after 90 minutes of instillation. According to the authors, the pathological changes in the liver were the same as those seen following oral or i.p. injection exposure routes. After intratracheal instillation of a sublethal dose of 50 µg/kg, the authors observed immunostaining of the liver, kidney, lung, cecum and large intestine but no obvious pathological changes were observed (Ito et al., 2001).

5.2.3 Other Exposure Routes

Studies in female rats have investigated the organ distribution of the i.v. administration of 2 µg of ^{125}I-labeled heptapeptide toxin (MW 1019) isolated from *M. aeruginosa* (Falconer et al., 1986; Runnegar et al., 1986). High-performance liquid chromatography (HPLC) was used to purify the heptapeptide toxin prior to reaction with ^{125}I in the presence of NaI and lactoperoxidase. After 30 minutes, the liver and kidney showed the highest tissue concentrations; 21.7% in the liver and 5.6% in the kidneys. The authors reported 7% of the dose administered in the gut contents, and 0.9% cleared in the urine, with no significant accumulation in other organs or tissues (Falconer et al., 1986).

Extensive liver uptake in mice was reported by Brooks and Codd (1987) after i.p. injection of 125 µg/kg of a ^{14}C-labelled toxin extracted from *M. aeruginosa* strain 7820. After 1 minute, 70% of the radiolabel

was found in the liver, and after 3 hours increased to almost 90%. The kidneys, lungs, ileum, heart, large intestine, and spleen also showed radiolabeled accumulation.

Robinson et al. (1989) determine the distribution of ^{3}H-dihydromicrocystin-LR (>95% pure) after i.p. injection of a sublethal dose of 45 µg/kg, or a lethal dose of 101 µg/kg in mice. Similar tissue distribution of radiolabel (as % of total radioactivity) was observed after administration of both doses and after 60 minutes, accumulation in the liver from both doses reached a maximal value of 60%. For the lethal dose (101 µg/kg), the radiolabel accumulation was 56% in the liver, 7% in the intestine, and 0.9% in the kidney. Less than 1% was found in the heart, spleen, lung and skeletal muscle. In another study, Robinson et al. (1991) observed distribution of microcystin-LR in mice within one minute of a sublethal i.v. injection of 35 µg/kg to the liver, intestines, kidneys, plasma, and carcass (body minus the liver, gut, kidney, heart, lung, and spleen). After one hour, the liver had around 67% of the dose, which remained the same for the 6 days of the study even though 24% of the dose was eliminated in the urine and feces. After one hour of the administration, small percentages were found in the intestines (8.6%), the carcass (6%), the kidneys (0.8%), and trace amounts were found in the plasma. Within 3 minutes, levels in the lung were high but after 10 minutes they were not detected. There was measurable radiolabel in the spleen.

The subcellular distribution of radioactivity in the liver demonstrated that approximately 70% of the hepatic radiolabel was present in the cytosol. *In vitro* experiments showed that radiolabeled microcystin in the liver was bound to high molecular weight cytosolic proteins (Robinson et al., 1991). The nature of the binding was demonstrated to be covalent, saturable and specific for a protein with a molecular weight of approximately 40,000. Binding was inhibited by okadaic acid (a potent inhibitor of serine/threonine phosphatases [1 and 2A]), suggesting that the target protein is protein phosphatase 1 or 2A. Binding proteins for microcystin-LR were found in cytosol derived from several different organs, suggesting that liver specificity is not due to limited distribution of target proteins. Covalent binding to hepatic proteins may be responsible for the long retention of microcystin in the liver.

Rapid uptake of pure microcystin-LR into the serum was observed after i.p. injection of 35 µg/kg (sublethal dose) to 24 mice (Lin and Chu, 1994). The samples were analyzed by direct competitive ELISA and found that by 2 hours of administration, microcystin-LR reached a maximum concentration in the serum, and after 12 hours in the liver cytosol, bound to liver cytosolic proteins. The kinetics of binding was correlated by the authors with inhibition of protein phosphatase 2A activity. A maximum decrease in enzyme activity was observed after 6 to 12 hours of dose injection.

Data from humans accidentally exposed to microcystin from dialysis water indicates that a large proportion of microcystin in the serum and liver is bound to protein (Yuan et al., 2006). Three methods were compared to detect microcystin in stored sera and liver samples from the exposed dialysis patients: 1) direct competitive ELISA using a polyclonal antibody against microcystin, which detects free microcystin in a supernatant fraction; 2) liquid chromatography-mass spectrometry (LC/MS) after oxidation and solid phase extraction to detect bound microcystin in a protein pellet fraction; and 3) gas-chromatography-MS (GC/MS) after oxidation and solid phase extraction to detect total microcystin in a sera or liver homogenate.

5.2.4 Liver Tissues – in vitro

Many researchers have examined the distribution to the liver using perfused liver and hepatic cell cultures. Pace et al. (1991) demonstrated significant accumulation of ^{3}H-dihydromicrocystin-LR in isolated perfused liver despite a low overall extraction ratio (16% in liver, 79% in perfusate). In the liver, radiolabel corresponding to microcystin-LR (15%) and a more polar metabolite (85%) was primarily found in the cytosolic fraction.

Primary rat hepatocytes in suspension and isolated perfused rat liver were used to evaluate the cellular uptake of ^{3}H-dihydromicrocystin-LR (Eriksson et al., 1990; Hooser et al., 1991a). Eriksson et al. (1990) measured the uptake by scintillation counting of washed cells of a mixture of unlabeled microcystin-LR and ^{3}H-dihydromicrocystin-LR. Uptake was specific for freshly isolated rat hepatocytes and was inhibited by the bile salts cholate and taurocholate, and by bile acid transport inhibitors such as antamanide, sulfobromophthalein and rifampicin. Using both rat hepatocyte suspensions (four replicates from two rats, two from each rat), and the isolated perfused rat liver (two rats), Hooser et al. (1991a) found that for the first 5 to 10 minutes, the uptake of ^{3}H-dihydromicrocystin-LR was rapid, followed by a plateau. The uptake of ^{3}H-dihydroMCLR was measured as radioactivity in fractionated cells versus radioactivity in medium. At 0°C, the uptake was inhibited by incubation of suspended rat hepatocyte, probably by involvement of an energy-dependent process. Inhibition of uptake was also observed by preincubation of hepatocytes with rifampicin, a competitive inhibition of the bile acid transporter.

The dose level and exposure time in isolated rat hepatocytes on the uptake of ^{125}I- microcystin-YM was measured by Runnegar et al. (1991). Uptake was measured as radioactivity in centrifuged cell pellet. Initially, hepatocyte uptake was rapid but after 10 minutes a plateau in the uptake rate was observed. In the first minute of exposure, initial uptake rate increased with increasing concentration, however cumulative uptake stopped at a dose causing plasma membrane blebbing.

Runnegar et al. (1995a), studied the microcystin-YM uptake by isolated rat hepatocytes using cell associated radioactivity and assays for protein phosphatase inhibition in cell lysates. The authors found that uptake was temperature-dependent and inhibited around 20-60% by *in vitro* preincubation with bile acids or bile acid transport inhibitors such as trypan blue, taurocholate, cholate, cyclosporine A, sulfobromophthalein, trypan red and rifamycin. This result indicates that uptake of microcystin happens by carrier mediated transport. The pretreatment with protein phosphatase inhibitors such as okadaic acid and calyculin A, inhibited both the uptake of microcystin-YM and the protein phosphatase, suggesting that the protein phosphatase may have impacted the conformation or membrane presence of the OATp transporter. Serine phosphorylation is involved in the regulation of hepatocyte OATp1A1's transport function (Svoboda et al., 2011).

After 2 to 3 days of being maintained in culture, the primary cultures of liver cells cease to express the OATps. As a result, established liver cell lines are generally not suitable to evaluate microcystin toxicity (Eriksson and Golman, 1993; Heinze et al., 2001). This was also observed by Chong et al. (2000) who evaluated microcystin toxicity in eight rodent, primate and human permanent cell lines, and found that after microcystin-LR exposure, only two showed cytotoxicity: a human oral epidermoid carcinoma KB cells, and a rat Reuber H35 hepatoma H-4-II-E cells. Toxic response in these cells was most evident when microcystin-LR was added after cells were seeded. Those cells more resistant to microcystin toxicity were established monolayers cells.

Hooser et al. (1991a) also evaluated the subcellular distribution of ^{3}H-dihydromicrocystin-LR in primary rat hepatocytes in suspension and the isolated perfused rat liver. The authors found that after protein precipitation with trichloroacetic acid, 50% of the ^{3}H-dihydromicrocystin-LR was localized in the cytosolic fraction and bound to cytosolic proteins, and 50% was found as free toxin. The authors suggested that since ^{3}H-dihydromicrocystin-LR did not bind significantly to actin or other cytoskeletal proteins, little of the radiolabel was in the insoluble pellet containing insoluble actin and other elements (Hooser et al., 1991a).

Studies on the binding of subcellular protein of ^{3}H-dihydromicrocystin-LR in rat liver homogenates found that around 80% of the radiolabeled toxin was bound to cytosolic proteins (Toivola et al., 1994). ^{3}H-dihydromicrocystin-LR shown to bind to both PP1 and PP2A. PP2A was detected primarily in the cytosol and PP1 was found in the membrane proteins (mitochondrial and post-mitochondrial particulate fraction).

5.3 Metabolism

Limited data are available on the metabolism of microcystins. Most of the studies discussed below indicate that there is minimal if any catabolism (process of breaking down molecules into smaller units to release energy). The microcystins can be conjugated with glutathione and cysteine to increase their solubility and facilitate excretion (Kondo et al., 1996). It is not clear whether CYP450-facilitated oxidation precedes conjugation. Stotts et al. (1997a,b) found that after i.v. injection or ileal loop exposure in swine, ^{3}H-dihydromicrocystin-LR was not metabolized in the liver and was primarily present in hepatic tissues as the parent compound.

Some metabolism of microcystin-LR was shown to occur in mice and in isolated perfused rat liver (Robinson et al., 1991; Pace et al., 1991). Male CD-1 mice were administered ^{3}H-dihydromicrocystin-LR as an i.v. dose of 35 µg/kg and monitored for up to six days. Over the 6-day interval, 9.2% and 14.5% of the dose was excreted in the urine and feces, respectively, of which ~60% was parent compound. High-performance liquid chromatography analysis for urinary and fecal metabolites revealed several minor peaks of lower retention times. Analysis of liver cytosol preparations revealed that 83% of the radiolabel was bound to a high molecular weight cytosolic protein after six hours and that amount decreased to 42% by day 6 (Robinson et al., 1991). Pace et al. (1991) also demonstrated binding of both the parent toxin (^{3}H-dihydromicrocystin-LR) and a more polar metabolite to cytosolic proteins in isolated perfused rat liver. Of the hepatic cytosol radiolabeled, 60 to 85% were polar metabolites. No characterization of metabolites of microcystin-LR was done in these studies.

A decrease in the amount of cytochrome b5 and cytochrome P450 in the liver was observed after the administration of 125 µg/kg of *Microcystis* strain 7820 (primarily produces microcystin-LR) to mice (Brooks and Codd, 1987). The pretreatment of mice with microsomal enzyme (mixed function oxidase) inducers such as β-naphthoflavone, 3-methylcholanthrene and phenobarbital, eliminated this effect on hepatic cytochromes. Pretreatment also extended survival and reduced liver toxicity (i.e., changes in liver weight). However, no change in cytochrome P450 associated enzyme activity (i.e., metabolism of aminopyrene and p-nitrophenol) was found in microsomes isolated from mouse liver after animals were injected with an extract of *M. aeruginosa* (Cote et al., 1986).

Glutathione and cysteine conjugates have been identified in the liver after i.p. injection of 10 or 20 µg microcystin-RR to mice or 4µg microcystin-LR to rats (purified from blooms) (Kondo et al., 1992, 1996). The conjugates were isolated and compared to chemically prepared standards which indicated structural modification of the Adda and Mdha moieties of the microcystin toxins. The authors postulated that these moieties could be the sites of CYP oxidation and subsequent conjugation with glutathione or cysteine.

Formation of microcystin-LR glutathione conjugates occurs by glutathione S-transferase (GST) enzymes found in both liver cytosol and microsomes of rats (Takenaka, 2001). Characterization of glutathione conjugation of microcystin-LR (>95% pure isolated from *M. aeruginosa*) has been done by five recombinant human GSTs (A1-1. A3-3, M1-1, P101, and T1-1) (Buratti et al., 2011). Although with different dose-responses, all five GSTs catalyzed the conjugation. The authors also determined that the spontaneous reaction for microcystin-LR conjugation with glutathione (GSH) was dependent on GSH concentration, temperature and pH.

Based on LD_{50} estimates, Kondo et al. (1992) found that glutathione and cysteine conjugates of microcystin-LR and microcystin-YR were less toxic than the parent compounds, however, they demonstrated that these conjugates were toxic (LD_{50} values ranged from 217 to 630 µg/kg in mice). Metcalf et al. (2000) also demonstrated *in vitro* that glutathione, cysteine-glycine and cysteine conjugates were less toxic in the mouse bioassay than the parent compounds demonstrating that conjugates were also weaker inhibitors of protein phosphatases 1 and 2A. After intratracheal instillation in mice, the distribution of glutathione and cysteine conjugates of microcystin-LR start in the kidney and continue in

the intestine suggesting that *in vivo*, the lower toxicity of glutathione and cysteine conjugates may be related to the distribution through excretory organs and elimination of metabolites (Ito et al., 2002a).

Ito et al. (2002b) synthesized glutathione and cysteine conjugates of microcystin-LR and administered them by intratracheal instillation in mice. The metabolites were demonstrated to be less toxic than the parent compound as shown by lethal doses about 12-fold higher than the microcystin-LR lethal dose. The metabolites were distributed primarily to the kidney and intestine, as opposed to the liver (Ito et al., 2002b).

Several studies have investigated the role of glutathione homeostasis and lipid peroxidation in microcystin-induced liver toxicity (Ding et al., 2000a; Gehringer et al., 2004; Bouaïcha and Maatouk, 2004). Ding et al. (2000a) indicated that microcystin exposure in isolated hepatocytes resulted in an initial increase in glutathione synthesis followed by a later depletion of glutathione. Gehringer et al. (2004) suggest that increased lipid peroxidation induced by microcystins is accompanied by an increase in glutathione peroxidase, transcriptional regulation of glutathione-S-transferase and glutathione peroxidase and *de novo* synthesis of glutathione. Bouaïcha and Maatouk (2004) found that 2 ng/mL of microcystin-LR in primary rat hepatocytes caused an initial increase in ROS formation and an increase in glutathione. Additional details of the oxidative stress reaction to microcystins are given in section 6.4.5 Physiological or Mechanistic Studies.

5.4 Excretion

Biliary excretion has been shown in both *in vivo* and *in vitro* studies. Falconer et al. (1986) administered to female albino rats an i.v. dose of 2 µg of a peptide extracted from *M. aeruginosa*. The authors demonstrated a biphasic blood elimination curve, with the first component with a half-life of 2.1 minutes and a second component with a half-life of 42 minutes. After 120 minutes, the authors observed 1.9% of the administered dose in the urine and 9.4% in the intestinal contents, suggesting biliary excretion of the toxin. Pace et al. (1991) also observed biliary excretion in isolated perfused rat liver after 1.7% of radiolabeled microcystin-LR was recovered in the bile after a 60-minute perfusion. Seventy-eight percent of the radiolabel in the bile collected during the perfusion was associated with the parent toxin while the rest of the radiolabel was associated with more polar metabolites (Pace et al., 1991).

In a study by Robinson et al. (1991), male VAF/plus CD-1 mice were administered an i.v. dose of 35 µg/kg of radiolabeled microcystin-LR. A biexponential plasma elimination curve was observed with plasma half-lives of 0.8 and 6.9 minutes for the first and second phase of elimination, respectively. A total of approximately 24% of the administered dose was eliminated in the urine (9%) and feces (15%) during the 6-day study monitoring period. Around 60% of the excreted radiolabel in both urine and feces, measured at 6 and 12 hours following injection, was present as the parent compound.

Elimination in swine was evaluated following i.v. injection or ileal loop exposure (Stotts et al., 1997a,b). ^{3}H-dihydromicrocystin-LR was detected in the bile as early as 30 minutes after i.v. injection of 75 µg/kg. After ileal loop exposure to the same dose, the toxin concentration in the portal venous blood was consistently higher as compared to peripheral blood. The labeled microcystin-LR was rapidly eliminated and followed a biphasic pattern in both the i.v. and ileal loop exposures, suggesting that the liver removes the toxin rapidly from the blood. At higher dose levels, removal from the blood is slower, likely due to the liver toxicity and circulatory shock observed at high doses.

5.5 Pharmacokinetic Considerations

The blood half-life in female rats was measured following i.v. administration of a ^{125}I-labelled heptapeptide toxin extracted from *M. aeruginosa* (MW 1019, assumed to be a microcystin) (Falconer et al., 1986). A biphasic blood elimination curve was demonstrated, with a half-life of 2.1 minutes in the first component and a half-life of 42 minutes in the second component.

Microcystin-LR excretion was also evaluated in mice (Robinson et al., 1991). A biexponential plasma elimination curve was demonstrated after i.v. injection of a 35 µg/kg sublethal dose of ^{3}H-dihydromicrocystin-LR. A plasma half-life of 0.8 minutes was observed in the first phase of elimination and 6.9 minutes was reported for the second phase.

Stotts et al. (1997a,b) evaluated the toxicokinetics of ^{3}H-dihydromicrocystin in swine following i.v. injection and ileal loop exposure. Elimination of labeled microcystin-LR was rapid and followed a biphasic pattern, suggesting that the liver rapidly removes the toxin from the blood. Clearance from the blood is slower at higher dose levels, presumably due to the liver toxicity and circulatory shock observed at high doses. It is important to take into consideration that tritium radiolabeling may alter the microcystin molecule's ability to bind with protein phosphatases, thus altering microcystin protein binding and tissue distribution profile (Hilborn et al., 2007).

No physiologically based toxicokinetic models have been developed for microcystins.

6.0 HAZARD IDENTIFICATION

6.1 Human Studies

6.1.1 Epidemiology and Case Studies of Systemic Effects

Analysis of hepatic enzyme levels from a group of patients served by a public water supply contaminated with a bloom of *M. aeruginosa* were compared with levels in patients living in areas served by other water supplies not contaminated with the bloom (Falconer et al., 1983). Although 871 individual records were examined, the number of exposed and unexposed were not reported. The authors used as the study population those patients referred to a single hospital laboratory for liver function tests before, during and after a bloom of *M. aeruginosa* in the Malpas Dam reservoir of Australia. The patients were classified as those that used the reservoir for drinking water supply (Armidale residents), and residents of neighboring towns with independent water supplies. Analysis of plasma enzymes (γ-glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP)) was conducted. Liver function for each group was classified based on the liver enzyme testing date: during 5 weeks before the first signs of the bloom appeared; during the 3-weeks of the bloom or the 2 weeks after copper sulfate treatment of the bloom; and during 5 weeks after the bloom. Copper sulfate addition was identified as the high-risk time interval due to the cell lysis and subsequent toxin release. Differences in enzyme levels between comparison groups and between times within comparison groups was analyzed using analysis of variance. The authors observed a significant increase in GGT levels in residents of Armidale during the bloom period, and an increase in ALT levels in the same group, although not statistically significant.

Although the authors observed a difference in enzyme levels between the groups, the finding was attributed to the imprecise method of selecting study participants (Falconer et al., 1983). Several of the enzyme measurements for the Armidale residents were associated with one participant with chronic kidney disease requiring a repeat of the analysis. Alcoholism was reported to occur in about the same proportion, 7 to 10% in both groups assessed before and during the bloom, although in lower proportion in the post-bloom group of Armidale residents. Alcoholism has been associated with an increase in GGT levels. However, the authors concluded that these changes in GGT among Armidale residents before and during the bloom period might potentially be associated with exposure to drinking water contaminated with a *M. aeruginosa* bloom.

Turner et al. (1990) reported an outbreak among army recruits who had consumed reservoir water during canoe exercises. The reservoir contained a bloom of cyanobacteria, primarily *M. aeruginosa*. Two recruits, both 16 years old, had detailed case reports with history of malaise, sore throat, blistering around the mouth, dry cough, pleuritic pain and abdominal pain. One of them experienced vomiting and diarrhea. After physical examination, both patients presented fever, abdominal tenderness, and left basal pulmonary consolidation (pneumonia). Within 24 hours and after treatment with antibiotics, temperature returned to normal. Low platelet counts in both patients but no increases in liver enzymes was detected in blood tests. Testing of various pathogens such as *Leptospira, Legionella*, *Chlamydia*, *Coxiella*, *Mycoplasma* and influenza and adenovirus was negative. Sixteen soldiers that participated in the same canoe exercises also reported similar symptoms including diarrhea, vomiting, sore throat, dry cough, headache, abdominal pain, and blistered mouth.

Microcystins, including microcystin-LR was detected in a sample of the bloom taken the day after the patients were admitted into the hospital (Turner et al., 1990). After two weeks, high levels of *Escherichia coli* were also found in reservoir water. The authors suggested that exposure to microcystin may have been related to the pulmonary consolidation and low platelet count of the two patients, citing evidence from studies in mice. The potential role of other toxins in this event was not addressed.

A cross-sectional study was done to evaluate the relationship between liver damage in children and microcystin levels in drinking water and aquatic food (carp and duck) in China (Li et al., 2011a). Microcystin concentrations were measured in three sources of drinking water used by local residents in the Three Gorges Reservoir Region in China: a community well rarely contaminated with microcystin (unexposed), a lake with an occasional cyanobacterial bloom (Lake 1), and a lake with regular cyanobacterial blooms over the previous 5 years (Lake 2). Children from 5 schools were selected to participate and those served by water from the wells for more than 5 years and rarely ate fish or duck from the lakes (145 participants) were considered to have no exposure. Those with low exposures were the children served by Lake 1 (183 participants) and those with high exposure were the children served from Lake 2 (994 participants). A questionnaire was administered to the participants and blood samples from approximately 50 children per exposure group were obtained for analysis of ALT, AST, GGT, ALP, and mean serum microcystin levels.

Concentrations of microcystin were found to be below detection limit in the well water in all but one of the six years tested (Li et al., 2011a). Only one year detected microcystins at 0.1 µg microcystin-LR equivalents/L. The average microcystin-LR equivalents/L over the 5 years in Lake 1 was 0.24 µg/L and in Lake 2 was 2.58 µg/L. Levels of microcystin-LR (fish and ducks) were higher in the aquatic food from Lake 2 than Lake 1. Based on consumption of drinking water and aquatic food, the authors estimated that children served by Lake 1 (low exposure) consumed 0.36 µg/day of microcystin-LR, while children in Lake 2 (high-exposure) consumed 2.03 µg/day. Mean serum levels of microcystin in the groups were below detection in the unexposed group, 0.4 µg microcystin-LR equivalents/L in the low-exposure group, and 1.3 µg microcystin-LR equivalents/L in the high-exposure group. The respective serum detection rates were 1.9%, 84.2%, and 91.9% in the unexposed, low-exposed, and in the high-exposed groups, respectively.

Exposure to microcystin in drinking water was associated with increases in AST and ALP, but no increases in ALT or GGT were observed. The odds ratio (OR) for liver damage associated with microcystin exposures was 1.72 with 95% confidence intervals (95% CI) of 1.05-2.76 (dichotomous based on two or more abnormally elevated liver enzyme assays). According to the authors, Hepatitis B infection, based on serum measurements of antigens and/or antibodies, was a greater risk for liver damage than microcystin exposure among these children.

6.1.2 Other Routes of Exposures

In February 1996, there was an outbreak of acute liver failure in patients at a renal dialysis clinic in Caruaru, Brazil (Carmichael et al., 2001, Jochimsen et al., 1998). One hundred and sixteen of 130 patients who received their routine hemodialysis treatment at that time experienced headache, eye pain, blurred vision, nausea and vomiting. Subsequently, 100 of the affected patients developed acute liver failure and, of these, 76 died. A cohort study was conducted as well as an evaluation of the center water supply; patient's serum, and postmortem liver tissue were analyzed for microcystin. Analysis of the carbon, sand, and cation/anion exchange resin from in-house filters in the clinic's water treatment for microcystins and cylindrospermopsin demonstrated the presence of both cyanotoxins (Azevedo et al., 2002). Analyses of blood, sera, and liver samples from the patients revealed microcystins, but not cylindrospermopsin. The method used to extract cylindrospermopsin from the samples may have been inadequate. Based on a comparison of patient's symptoms and liver pathology with data from animal studies of microcystins and cylindrospermopsin, the authors concluded that the major contributing factor to death of the dialysis patients was intravenous exposure of microcystins.

Blood samples collected from 51 patients of the renal dialysis clinic in Caruaru, Brazil were analyzed using ELISA (Hilborn et al., 2005; 2007). Microcystin concentrations ranged from less than 0.16 µg/L (limit of detection) to 28.8 µg/L in serum samples. Additional analysis using GC/MS, in 6 serum samples

found microcystin oxidized to MMPB (2-methyl-3- methoxy-4-phenylbutyric acid) ranging from 45.7 to 112.9 ng/mL. ELISA analysis of these serum samples detected free microcystin concentrations ranging from 6.7 to 26.3 µg/L. The authors concluded that both free and protein-bound microcystins were found in human serum.

In another contamination event at a dialysis center in Rio de Janeiro, Brazil in 2001, microcystin concentrations of 0.32 µg/L were measured in the activated carbon filter used in an intermediate step for treating drinking water to prepare dialysate (Soares et al., 2005). A concentration of 0.4 µg/L was detected in the drinking water. Serum samples were collected 31 to 38 days after microcystin-LR was detected in water samples and patients were monitored for eight weeks. The presence of microcystins indicated that 44 dialysis patients were potentially exposed to microcystin from contaminated dialysate (Hilborn et al., 2013). A longitudinal study to characterize the clinicopathological outcomes among 13 dialysis patients was conducted and serum microcystin concentrations were quantified with ELISA. Although the biochemical outcomes varied among the patients, markers of hepatic cellular injury chlolestasis (elevations of AST, ALT bilirubin, ALP and GGT) in serum during weeks one to eight after treatment frequently exceeded normal values. Concentrations of microcystin-LR in the serum ranged from 0.46 to 0.96 ng/mL (Soares et al., 2005). Since microcystin was not detected during weekly monitoring after the first detection, the authors suggested that the patients were not continuously exposed to the toxin and that the toxin detected in the serum after eight weeks may have been present in the form of bound toxin in the liver (Soares et al., 2005). Results were consistent with mild to moderate mixed liver injury. Although the patients in the study had pre-existing diseases, the direct intravenous exposure to dialysate prepared from surface drinking water supplies made them at risk for cyanotoxin exposure and resultant adverse effects (Hilborn et al., 2013).

6.2 Animal Studies

6.2.1 Acute Toxicity

6.2.1.1 Oral Exposure

Fitzgeorge et al. (1994) administered microcystin-LR via gavage to newly weaned CBA/BALBc mice weighing 20±1 g. Sex of the mice and the number used per dose group were not reported. Deaths were recorded within two hours of dosing. The commercially-obtained compound was described only as "suitably purified". The LD_{50} was estimated to be 3,000 µg/kg, and increases in liver (43%) and kidney (5.9%) weights were reported. The authors reported that there was no change in lung or spleen weight; dose-response data and other endpoints were either not examined or not reported.

Acute oral toxicity of purified microcystin-LR (>95% pure by HPLC) in female BALB/c mice was evaluated by Yoshida et al. (1997). Previous studies using doses of 16.8 and 20 mg/kg resulted in death within 160 minutes in two mice. Therefore, to determine the LD_{50}, the authors administered via gavage to seven 6-week-old mice 0, 8.0, 10.0 and 12.5 mg/kg doses of microcystin-LR in saline solution. Within 24 hours, the mortality was 0/2 in controls, 0/1 at 8 mg/kg, 0/2 at 10 mg/kg and 2/2 at 12.5 mg/kg. The oral LD_{50} was identified as 10.0 mg/kg.

Light microscopy was used to examine the liver, kidneys and lung and electron microscopy was used to identify apoptotic cells in the livers of treated mice (Yoshida et al., 1997). Histopathological analysis was performed on the remaining tissues. The only effects observed were in the liver and kidneys; no effects were observed on the stomach, intestine, skin or organs after histophatological evaluation were observed. In those animals that died, liver effects included centrilobular hemorrhage and hepatocyte degeneration. In those mice administered doses greater than 12.5 mg/kg, free hepatocytes in the veins of mice were

observed. In the previous study, those mice receiving doses of 16.8 and 20 mg/kg, showed proteinaceous eosinophilic materials in the Bowman's spaces in the kidneys.

One of the surviving mice at 10.0 mg/kg was sacrificed after 24 hours. Hepatocellular necrosis was observed in the centrilobular and midzonal regions, and in the centrilobular region and surrounding necrotic areas, single cell death (possibly apoptotic) was reported. All other mice treated with 10 mg/kg and the two mice treated with 8.0 mg/kg were sacrificed one week after treatment. The authors observed livers with hypertrophic hepatocytes in the centrilobular region and fibrosis in the centrilobular and midzonal regions. In addition, a few apoptotic cells were observed in these animals. Kidney effects were not reported in those animals that survived treatment for at least 24 hours.

A comparison between the acute effects of microcystin-LR on the livers and gastrointestinal tracts of young and aged mice was done by Ito et al. (1997a). A single dose (500 μg/kg) was administered to aged (29 mice age 32 weeks) and young (12 mice age 5 weeks) male ICR mice. The microcystin-LR (purity not specified) was dissolved in ethanol and diluted in saline and administered via oral gavage. The controls were 3 aged and 3 young untreated mice. After 2 hours of treatment, 23 aged mice were sacrificed, five mice at 5 hours, and two mice at 19 hours, and 4 young mice were sacrificed at each time point. Evaluation of liver damage and gastrointestinal erosion were performed.

The authors observed that the effects in the aged mice were more severe than those in the young mice. No liver pathology or gastrointestinal changes were reported in young mice. However, 18 of 29 aged mice treated with the same dose showed pathological changes of the liver, some of them (8) showed liver injury of the highest severity (severity rating of +4), characterized as bleeding, disappearance of many hepatocytes in the whole liver and friable tissue. Other aged mice also showed liver injury of different severity: 5 of 29 mice had severity rating of +3 characterized by bleeding and disappearance of hepatocytes in centrilobular region; 4 of 29 mice had necrosis in the centrilobular region (severity rating of +2), and one mouse had eosinophilic changes in the centrilobular region (severity rating of +1).

Other effects in aged mice included gastrointestinal effects characterized by necrosis to one-third depth of the mucosa and severe duodenal damage including separation of epithelial cells from lamina propria, decreased villi density, and edema of both the submucosa and villi. Aged mice also showed thinning of gastrointestinal epithelial cells with consequent exposure of lamina propria and glands in some areas. Although details of the incidence of these effects were not reported, the authors indicated that the degree of liver injury was related to the severity of gastrointestinal effects. At 5 to 19 hours after treatment, regeneration of intestinal tissues was evident in some of the mice sacrificed. No difference was observed on the enzyme levels (AST and ALT) among untreated aged mice.

The effect of single oral gavage doses of microcystin-LR was studied by Fawell et al. (1999). Doses of 500, 1,580 and 5,000 μg/kg body weight of microcystin-LR (commercial product; purity not specified) in aqueous solution were administered to five male and five female CR1:CD-1(ICR)BR(VAF plus) mice and CR1:CD(SD)BR(VAF plus) rats. No untreated control group was included in the study. After 14 days, animals were sacrificed, necropsy was performed, and microscopic examinations of the lung and liver were conducted. The LD_{50} value in mice was estimated at 5,000 μg/kg and in rats was >5,000 μg/kg.

Signs of hypoactivity and piloerection (involuntary bristling of hairs) were observed in those animals that died (Fawell et al., 1999). However, no clinical signs were observed in survivors. Those that survived, showed no signs of body weight changes during the 14-day follow-up. Darkly discolored and distended livers, as well as pallid kidneys, spleen, and adrenals were observed at necropsy in those animals that died. The livers had moderate or marked centrilobular hemorrhage. Rats and mice of all dose groups showed diffuse hemorrhage in the liver, however the incidence was not clearly related to dose. Table 6-1 summarize the incidence and severity of liver lesions observed in the study.

Table 6-1. Incidence of Liver Lesions in Mice and Rats After Exposure to Microcystin-LR (Fawell et al., 1999)

	Mice (10 per group)			Rats (10 per group)		
	500 µg/kg	1580 µg/kg	5000 µg/kg	500 µg/kg	1580 µg/kg	5000 µg/kg
Mortality	0	1	5	0	0	1
Diffuse Hemorrhage	2	1	1	8	7	8
Moderate Centrilobular Hemorrhage	0	2	7	0	0	1
Marked Centrilobular Hemorrhage	0	1	0	0	0	1
Centrilobular Necrosis	0	0	2	0	0	1
Cytoplasmic Vacuolation	0	0	0	0	0	1

A comparison between the acute oral effects of microcystin extracts in young and aged mice was done by Rao et al. (2005). A single dose of microcystin-LR (3.5 g extract/kg from laboratory cultures of *M. aeruginosa* which corresponded to 9.625 mg microcystin-LR/kg) was administered to aged (36 weeks old) and young (6 weeks old) male Swiss albino mice. After 4 to 5 hours, mortality first occurred with the mean time to death significantly shorter in the aged mice. In comparison to the control groups, both groups of mice had an increased relative liver weight and DNA fragmentation. No difference between the age groups was observed. However, a significantly greater difference in glutathione depletion and lipid peroxidation was observed in the aged mice when compared with young mice. Although most serum enzymes were increased over controls in both groups, GGT was increased to a greater extent in aged mice than in young mice.

6.2.1.2 Inhalation Exposure

No studies of acute inhalation exposures were identified. The microcystins are not volatile; therefore inhalation exposures are likely to only occur in the form of aerosols. A brief abstract describes a study of acute microcystin-LR exposure via inhalation (Creasia, 1990). Details of study design and results were not reported. The LC_{50} for mice exposed to a microcystin-LR aerosol (nose only) for 10 minutes was reported to be 18 µg/L (mg/m^3) with a 95% confidence interval of 15.0-22.0 µg/L (mg/m^3). Based on studies of lung deposition after exposure of mice to the LC_{50} concentration, an LD_{50} of 43 µg/kg body weight was estimated. The authors reported that histological lesions in mice killed by aerosol exposure were similar to those in mice dosed intravenously with microcystin-LR.

Fitzgeorge et al. (1994) conducted experiments in newly weaned CBA/BALBc mice (20±1 g) with microcystin-LR (commercial product; purity not stated) administered either by intranasal instillation or aerosol inhalation. Few details of study design and findings were given. A single experiment with mice (number unspecified) inhaling a fine aerosol (particle size 3-5 µm) of 50 µg microcystin-LR/L for an unspecified duration of time did not result in any deaths, clinical signs of toxicity or histopathological changes. The nature of the examinations was not reported. The authors estimated the delivered dose of microcystin-LR to be very small (about 0.0005 µg/kg). The LD_{50} for intranasal instillation of microcystin-LR was equal to 250 µg/kg. All deaths occurred within two hours of dosing. Liver and kidney weights were increased by 41.6 and 7.5%, respectively, in the animals (n = 6; sex not specified) receiving the LD_{50} of microcystin-LR intranasally. The estimated LD_{50} of intranasal instillation, 250 µg/kg, is the same as the LD_{50} of i.p. exposure, which is much lower than the LD_{50} of gastric intubation (3000 µg/kg).

Fitzgeorge et al. (1994) further evaluated the relationship between dose and liver weight increase after intranasal instillation of microcystin-LR to newly weaned CBA/BALBc mice (20±1 g; assumed n = 6). At single intranasal doses of 31.3, 62.5, 125, 250 and 500 µg/kg, liver weight increased proportionally (0,

1.5, 24.4, 37.4 and 87%). Seven daily intranasal doses of 31.3 μg/kg, resulted in a liver weight increase of 75%. The authors reported histopathological findings, but failed to specify which findings resulted from single doses and which resulted from the multiple-dose experiment reported in the same publication. Findings included necrosis of respiratory and olfactory epithelium in the nasal mucosa and centrilobular necrosis with hemorrhage in the liver. Early changes in the liver included vacuolar degeneration and necrosis of hepatocytes near the central vein. The adrenal glands showed effects as well with vacuolation and necrosis of the inner cortex and congestion of medullary blood vessels. No histopathological changes were observed in the trachea, lungs, esophagus, pancreas, spleen, lymph nodes, kidneys or brain.

Several studies demonstrated the potential for uptake from the respiratory system using intratracheal or intranasal instillation. Ito et al. (2001) evaluated the distribution of purified microcystin-LR after intratracheal instillation of lethal doses in male ICR mice and included a limited description of toxic effects. Microcystin-LR in saline solution was instilled at doses of 50, 75, 100, 150 and 200 μg/kg into 34 mice; three mice were sham-exposed as controls. Mortality was 100% in 12 mice receiving doses of 100 μg/kg and greater. At 75 μg/kg, two of four mice died, while no deaths occurred in 18 mice given 50 μg/kg intratracheally.

The time course of hepatotoxicity was further evaluated in eight mice given an intratracheal dose of 100 μg/kg (Ito et al., 2001). One mouse was sacrificed at each of 5, 10, 20, 30, 45, 60, 90 and 120 minutes. Immunostaining for microcystin-LR showed the toxin in the lungs within 5 minutes and in the liver after 60 minutes. Hemorrhage in the liver was observed after 90 minutes and became severe by 120 minutes.

6.2.1.3 Dermal/Ocular Exposure

No studies evaluating the effects in animals of dermal or ocular exposure to purified microcystins were identified. Cyanobacteria bloom samples collected from five different lakes or ponds were tested for allergenic and irritative effects in guinea pigs and rabbits, respectively (Torokne et al., 2001). The microcystin content (presumed to be total LR, RR, and YR) ranged from 0.1-2.21 mg/g. To determine sensitization, guinea pigs were initiated with an intradermal injection followed seven days later by topical application at the injection site. Sensitization was moderate to strong in 30-67% of guinea pigs but did not correlate with microcystin content. All samples produced only negligible to slight skin and eye irritation on rabbits.

6.2.1.4 Other Routes

The acute toxicity of microcystins following i.p. injection has been studied in mice and rats. The LD_{50} for microcystin-LR in mice ranges between 30 and 60 μg/kg (Lovell et al., 1989; Slatkin et al., 1983; Gupta et al., 2003; Rao et al., 2005). The LD_{50} for microcystin-LR was slightly higher in fed rats (122 μg/kg) compared to fasted rats (72 μg/kg) (Miura et al., 1991) suggesting possible higher uptake by cells in the absence of competing dietary substrates.

The available studies demonstrate a very steep dose-response curve for microcystin-LR acute toxicity following i.p. administration. In female mice, the only change observed at 50 μg microcystin-LR/kg was Kupffer-cell hyperplasia, while all mice receiving 100 μg/kg died (Hermansky et al., 1991). A sublethal dose of about 25 μg/kg in male mice resulted in a significant increase in liver weight (8.7%) but no clinical signs or hepatic lesions (Lovell et al., 1989). A single injection of 60 μg/kg of microcystin-LR in male mice caused liver injury within 12 hours as indicated by increases in ALT and AST, intrahepatic hemorrhage and destruction of the liver morphology (Weng et al., 2007). Liver toxicity was also assessed in male mice administered a single dose of 55 μg/kg of microcystin-LR (Wei et al., 2008). Animals were sacrificed at various times from 0.5-12 hours after exposure. Histopathology revealed liver toxicity beginning at 6 hours including severe, intrahepatic hemorrhage and destruction of hepatic structure.

Gupta et al. (2003) determined mean LD_{50} values of 43, 235.4 and 110.6 mg/kg for microcystin-LR, microcystin-RR and microcystin-YR, respectively, using groups of 4 mice per dose (doses not specified). The microcystins were dissolved in methanol and diluted to the test concentrations with phosphate buffered saline. The time to death varied considerably with microcystin-RR being the least toxic. A significant increase in liver body weight index was induced by all of the congeners. Serum levels of AST, ALT and γ -GT increased significantly, compared with controls, as early as 30 minutes post exposure for all congeners. The acute LD_{50} determination for these three congeners showed a difference in toxicity with microcystin-LR being the most toxic followed by microcystin-YR and microcystin-RR. The findings from this study supports the hypothesis that as the hydrophilic properties of the amino acids increase, the toxicity decreases.

Both microcystin-YR and microcystin-RR have lower acute toxicity in mice than microcystin-LR with LD_{50} estimates of 111 and 171 µg/kg for microcystin-YR and 235 and 650 µg/kg for microcystin-RR (Gupta et al., 2003; Stotts et al., 1993). The difference in LD_{50} for microcystin-YR compared to microcystin-RR is consistent with the higher *in vitro* cellular toxicity of microcystin-YR using a human colon carcinoma cell line (Caco-2) (Puerto et al., 2009).

6.2.2 Short-Term Studies

6.2.2.1 Oral Exposure

The effects of microcystin-LR (commercial product; purity not stated) on 11-week-old male hybrid rats (F1 generation of female WELS/Fohm x male BDIX) after drinking water exposure was evaluated by Heinze (1999). For 28 days, three groups of 10 rats each received doses of 0, 50 or 150 µg/kg body weight of microcystin-LR in drinking water. Daily measurements of water consumption and rat weights were done at weekly intervals. Over the 28-day period, 3 to 7% of supplied water was not consumed; the dose estimates provided by the authors were not adjusted to account for the percentage of incomplete drinking water consumption. After 28 days of exposure, rats were sacrificed and organ weights (liver, kidneys, adrenals, thymus and spleen), were recorded. Hematology, serum biochemistry and histopathology of liver and kidneys were also evaluated.

A 38% increase in the number of leukocytes in rats in the highest dose group was observed after hematological evaluation (Heinze, 1999). Serum biochemistry showed significantly increased mean levels of ALP and lactate dehydrogenase (LDH) in both treatment groups; 84% in LDH and 34% in ALP in low dose group, and 100% increase in LDH and 33% increase in ALP in high dose group. No changes in mean levels of ALT or AST were observed. A dose-dependent increase in relative liver weights was observed in both dose groups: 17% at the low dose group and 26% at the high dose. Table 6-2 shows the mean enzyme levels and the relative and absolute liver weights.

A dose-dependent increase in absolute liver weight in both dose groups was also observed and provided by the author in a personal communication. The average absolute liver weights were 8.8 grams in the control group, 9.70 grams in the lower dose and 10.51 grams in the high dose. No statistically significant changes in other organ weights or body weights were reported and no effects on the kidneys were observed. The incidence of liver lesions is summarized in Table 6-3. Lesions were spread diffusely throughout the parenchyma and included increased cell volume, increased mitochondria, cell necrosis, activation of Kupffer cells and increased amounts of periodic acid-Schiff (PAS)-positive substances, indicating cell damage. Liver lesions were observed in both treatment groups, but the severity of the damage was higher in the high dose group (150 µg/kg). The low dose (50 µg/kg/day) was the LOAEL for effects on the liver.

Schaeffer et al. (1999) reported the results of a study in which *A. flos-aquae*, a cyanobacterium consumed as a food supplement, was fed to mice in the diet. The authors used recent analysis of *A. flos-aquae*, which often coexists with *Microcystis* species, to estimate the microcystin content in the material consumed by the mice. Analysis of the *A. flos-aquae* samples used in the feeding study showed an average concentration of 20±5 µg microcystin-LR per gram of *A. flos-aquae*. The authors estimated the daily exposure of microcystin-LR in the exposed mice to range from 43.3 µg/kg-day to 333.3 µg/kg-day. No clinical signs of toxicity were reported, and no effects on mortality, body weight, organ weights or histology were observed in the treated mice. In addition, no effects on reproductive parameters were reported in five treated mice from the highest dose group allowed to breed and there was no effect on growth and organ function in fetal and neonatal mice. The 333.3 µg/kg-day dose was the NOAEL under the conditions of the study.

Table 6-2. Relative Liver Weights and Serum Enzyme Levels in Rats Ingesting microcystin-LR in Drinking Water (Heinze, 1999)

	Control (Mean ± SD)	50 µg/kg (Mean ± SD)	150 µg/kg (Mean ± SD)
Serum Enzymes			
Alkaline phosphatase (ALP) (microkatals/L)	9.67 ± 2.20	13.00 ± 3.81*	12.86 ± 1.85*
Lactate dehydrogenase (LDH) (microkatals/L)	16.64 ± 4.48	30.64 ± 5.05*	33.58 ± 1.16*
Liver Weight			
Relative (g/100 g body weight)	2.75 ± 0.29	3.22 ± 0.34*	3.47 ± 0.49*
Absolute (g)**	8.28 ± 1.37	9.70 ± 1.32	10.51 ± 1.02

* $p<0.05$ when compared with control

Table 6-3. Histological Evaluation of the Rat Livers After Ingesting Microcystin-LR in drinking Water (Heinze, 1999)

	Activation of Kupffer Cells	Degenerative and Necrotic Hepatocytes with Hemorrhage	Degenerative and Necrotic Hepatocytes without Hemorrhage	PAS-positive Material
Control				
Slight	0	0	0	1
Moderate	0	0	0	0
Intensive damage	0	0	0	0
50 µg/kg				
Slight	0	4	0	5
Moderate	10	6	0	5
Intensive damage	0	0	0	0
150 µg/kg				
Slight	0	0	0	0
Moderate	10	6	1	8
Intensive damage	0	3	0	2

The effects of orally administered microcystin-RR on apoptosis in the liver of adult male ICR mice were evaluated by Huang et al. (2011) (see also section 6.4.5.1.3). For 7 days, doses of 0, 4.6, 23, 46, 93, or 186 µg/kg body weight of microcystin-RR (commercial product; purity not stated) were administered to groups of 5 mice via gavage. Animals were sacrificed after 7 days of exposure and DNA fragmentation

was evaluated with the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay. Analysis of PP2A activity was done with Western blot for B cell lymphoma/leukemia-2 (Bcl-2), Bcl-2 associated x protein (Bax), p53 expression, C/EBP homologous protein (CHOP), and glucose-related protein 78 (GRP78).

A dose-dependent increase in the percent of apoptotic cells in the liver was observed for all the dose groups: 10.46% for the 0 dose; 12.6% for the 4.6 dose; 12.7% for the 23 dose; 30.3% for the 46 dose; 28.5% for the 93 dose; and 37.5% for the 186 µg/kg dose group. The only doses with statistical significance were ≥46 µg/kg. A significant increase in Bax protein expression was observed at 46 and 93 µg/kg and in p53 protein expression was observed at 93 µg/kg. Bcl-2 was significantly decreased with doses ≥23 µg/kg but the Bax/Bcl-2 ratio was significantly increased at the same dose. No significant changes were found in CHOP protein expression. GRP78 protein expression was significantly increased only at the 93 µg/kg dose and none of the other doses were different from the control (including the high dose). No changes in PP2A activity or alterations in PP2A A subunit mRNA expression were seen for any dose groups.

6.2.2.2 Inhalation Exposure

Groups of six male BALB/c mice were exposed for 30, 60 or 120 minutes each day for seven consecutive days to monodispersed submicron aerosols of 260-265 µg/m^3 microcystin-LR via nose-only inhalation (Benson et al., 2005). The dose deposited in the respiratory tract were estimated to be 3, 6 and 12.5 µg/kg body weight/day. The control mice were exposed to 20% ethanol in water (aerosolized vehicle). Clinical signs were recorded daily and sacrifice of mice occurred the day after the last exposure. Blood and serum were collected and analysis for blood urea nitrogen [BUN], creatinine, total bilirubin, ALP, AST, ALT, total protein, albumin and globulin. Histopathological examination of the liver, kidney, spleen, thymus, respiratory tract tissues, adrenals, gastrointestinal tract and testes was conducted and organ weight (adrenals, lung, liver, kidney, spleen and thymus) were recorded. Histopathological evaluation of the epithelium lining the bone structure of the nasal passages (turbinates) from different locations was also done.

Table 6-4. Incidence and Severity of Nasal Cavity Lesions in Mice After Inhalation of Microcystin-LR

Nasal Cavity Lesions	Severity Grade	Daily Exposure Period (minutes)			
		Control	30	60	120
Turbinate 1 (immediately caudal to the upper incisors)					
Respiratory Epithelial Necrosis	Not noted	6/6	5/6	0/6	4/6
	Minimal	0/6	1/6	0/6	0/6
	Mild	0/6	0/6	6/6	0/6
	Moderate	0/6	0/6	0/6	2/6
Respiratory Epithelial Inflammation	Not noted	6/6	5/6	6/6	5/6
	Mild	0/6	1/6	0/6	1/6
Olfactory Epithelial Degeneration, Necrosis and Atrophy	Not noted	6/6	6/6	6/6	1/6
	Mild	0/6	1/6	0/6	4/6
	Moderate	0/6	0/6	0/6	1/6
Turbinate 2 (at the dose of the incisive papilla)					
Respiratory Epithelial Necrosis	Not noted	6/6	6/6	0/6	0/6
	Mild	0/6	0/6	6/6	3/6
	Moderate	0/6	0/6	0/6	3/6

Nasal Cavity Lesions	Severity Grade	Daily Exposure Period (minutes)			
		Control	30	60	120
Respiratory Epithelial Inflammation	Mild	6/6	5/6	6/6	6/6
	Moderate	0/6	1/6	0/6	0/6
Olfactory Epithelial Degeneration, Necrosis and Atrophy	Mild	6/6	6/6	0/6	0/6
	Moderate	0/6	0/6	6/6	0/6
	Marked	0/6	0/6	0/6	6/6
Turbinate 3 (at the dose of the first upper molar)					
Olfactory Epithelial Degeneration, Necrosis and Atrophy	Not noted	6/6	6/6	0/6	0/6
	Mild	0/6	0/6	6/6	0/6
	Moderate	0/6	0/6	0/6	4/6
	Marked	0/6	0/6	0/6	2/6

From Benson et al., 2005

No clinical signs or effects on body or organ weights were observed after exposure to microcystin-LR aerosol (Benson et al., 2005). Histopathological examination revealed treatment-related lesions only in the nasal cavity. Lesions were not observed in the liver or in any other organs or parts of the respiratory tract. The authors observed an increase of nasal lesions and severity with length of the daily exposure period (Table 6-4). The nasal cavity lesions observed included necrosis or inflammation of respiratory epithelial cells and degeneration, and necrosis and atrophy of olfactory epithelial cells. Necrotic lesions of olfactory epithelial cells were generally larger patches whereas few cells were involved in respiratory epithelial cell necrosis.

6.2.2.3 Other Routes

Male BALB/c mice were given 0, 40, or 50 µg/kg of microcystin-LR via i.p. injection, once a day for 10 days (Sun et al., 2011). The microcystin-LR was purified in the authors' laboratory, but the purity was not stated. No deaths were observed at 40 µg/kg, while 5/10 animals died after seven days at 50 µg/kg. Pretreatment each day with sulforaphane (an antioxidant found in cruciferous vegetables) prevented death. Groups of three male Sprague-Dawley rats were administered purified microcystin-LR (purity not stated) for 28 days via intraperitoneal implantation of osmotic pumps. The pumps were filled with microcystin-LR diluted in saline that delivered 0, 16, 32, or 48 µg/kg/day (Guzman and Solter, 1999). No significant differences were observed between the groups for body weight gain, liver-to-body weight ratio, and food consumption. Histopathology of the liver revealed necrosis, apoptosis and the presence of cytoplasmic vacuoles in mid- and high-dose animals and evidence of hepatic inflammation in high-dose animals. Livers from the mid- and high-dose animals had significantly higher levels of malondialdehyde (3-4 fold) and tissue slices in culture released greater amounts of ALT compared to controls. Hepatic ALT activity significantly decreased as its release from the liver tissues increased. There was a dose-related increase in tissue AST that reached significance for only the high doses. The fact that there were only 3 animals per dose group is a limitation of this study; the gradual infusion of microcystin-LR through the use of an osmotic pump is a positive feature of the study design.

6.2.3 Subchronic Studies

6.2.3.1 Oral Exposure

Dried bloom extract with at least seven microcystin congeners with the major peak tentatively identified as microcystin-YR (no peak could specifically be identified as microcystin-LR), was administered in the drinking water of pigs (n= 5/group) for 44 days (Falconer et al., 1994). Pigs were administered 0, 80, 227, or 374 mg of dried algae/kg body weight per day. A decrease in body weight was observed in pigs in the

highest dose group perhaps due to reduced food and/or water consumption at this dose. Dose- and time-dependent increases in GGT, ALP and total bilirubin, as well as a decrease in plasma albumin were observed in plasma samples collected over 56 days. Dose-related changes were also observed in the incidence and severity of histopathological changes of the liver, including Kupffer cell proliferation, periacinar degeneration, cytoplasmic degeneration, hepatic cord disruption, single cell necrosis, and congestion. Since exposure was via the dried algae, the study does not identify a NOAEL or LOAEL for microcystin.

Fawell et al. (1999) reported the results of a subchronic toxicity study of microcystin-LR given via gavage to Cr1:CD-1(ICR)BR (VAF plus) mice (age and body weight not specified). Microcystin-LR was obtained commercially (purity not stated) and administered in distilled water. The concentration in the dosing solution was verified by HPLC with UV detection. Groups of 15 male and 15 female mice were administered daily oral doses of 0, 40, 200 or 1000 µg/kg body weight for 13 weeks. Eye examinations were conducted prior to and at the conclusion of treatment, body weight and food consumption were recorded weekly, and clinical observations were made daily. During the final week of treatment, hematology and serum biochemistry were evaluated for seven mice of each treatment group. After 13 weeks, the authors performed gross examination of organs and microscopic evaluation of tissues. Lungs, liver and kidney were examined only in the treated animals. All other tissues were examined in the control and high dose animals.

At 1,000 µg/kg, one female was found dead during week 1 and one male was found moribund and sacrificed during week 13; a cause of death was not given and both animals appeared to be included in the histopathology analyses. No treatment-related clinical signs of toxicity were observed throughout the study. No dose-related trends were evident for body weight gain or body weight in males (data not reported). The study authors stated that mean body weight gain was decreased approximately 15% in all treated male groups and was statistically significant at 40 or 200 µg/kg-day ($p<0.05$). However, no quantitative data for these effects were presented in the published paper. Data tables obtained from the author showed that the mean body weight gain differed from controls by the same amount for all the exposed dose groups (2 g) and thus lacked a dose-response (Fawell, Personal Communication, 2015). Mean terminal body weights differed from controls by about 7% in these groups. The only body weight change observed in females was an increase in body weight gain in the 200 µg/kg-day group. No dose-response was observed for body weights or body weight gain. The only body weight change observed in females was an increase in body weight gain in the 200 µg/kg-day group.

A slight (10-12%) increase in mean hemoglobin concentration, red blood cell count and packed cell volume among females receiving 1000 µg/kg body weight was observed after hematological evaluation. In the high-dose males, ALP, ALT and AST levels were significantly elevated (2- to 6-fold higher), and only ALP and ALT were elevated (2- and 6-fold higher, respectively) in high dose females. In the mid-dose males, ALT and AST were also elevated (2-fold). All treatment groups showed a slightly decreased GGT. In males of the mid- and high-dose groups, serum albumin and protein were reduced (13%). Table 6-5 shows the clinical chemistry results.

In the males and females of the mid- and high-dose groups, a dose-related increase in incidence and severity of histopathological changes in the liver were reported (Fawell et al., 1999). The liver lesions reported were multifocal inflammation with deposits of hemosiderin and hepatocyte degeneration throughout the liver lobule. The incidence of these liver histopathological changes are summarized in Table 6-6. Chronic inflammation demonstrated the clearest dose response across all dose groups for both sexes. Hepatic degeneration showed a steep response to dose for the mid and high dose groups. Sex-related differences in liver pathology were not apparent. No lesions were found in other tissues. The NOAEL was 40 µg/kg/day and the LOAEL 200 µg/kg/day for liver histopathology and elevated serum levels of ALT and AST in males.

Table 6-5. Serum Biochemistry Results for Mice Treated with Microcystin-LR for 13 Weeks

microcystin-LR Dose (µg/kg-day)	Blood Chemistry Results (Mean ± Standard Deviation)					
	Albumin (g %)	Alkaline Phosphatase (ALP) (U/L)	Alanine Aminotransferase (ALT) (U/L)	Aspartate Aminotransferase (AST) (U/L)	Gamma Glutamyl Transaminase (GGT) (U/L)	Total Protein (g %)
Female						
Control	3.1±0.14	167±24.6	32±11.3	101±38.3	4±1.0	5.1±0.30
40	3.2±0.16	187±76.2	25±7.8	74±13.2	3±0.5	5.2±0.28
200	3.4[a]±0.14	156±33.4	27±9.4	74±22.1	3±0.0	5.3±0.31
1000	3.1±0.18	339[b]±123.7	220[b]±149.1	144±71.7	3±0.4	5.1±0.22
Male						
Control	3.2±0.19	91±22.2	27±8.0	68±27.7	6±1.0	5.5±0.32
40	3.0±0.13	95±29.2	37±17.2	64±12.2	4±0.7	5.1±0.26
200	2.8[c]±0.13	94±32.3	59[a]±28.0	121[b] ±43.7	3[c]±0.4	4.8[b]±0.29
1000	2.8[c]±0.11	232[b]±103.2	159[c]±75.0	121[b] ±26.3	4±0.4	4.8[c]±0.21

From Fawell et al., 1999; Significantly different from controls at: a p<0.05; b p<0.01; c p<0.001

Table 6-6. Liver Histopathology in Male and Female Mice Treated with Microcystin-LR for 13 Weeks

Liver Histopathology	Control (n=15)	40 µg/kg-day (n=15)	200 µg/kg-day (n=15)	1000 µg/kg-day (n=15)
Female				
Chronic inflammation	5	8	8	14
Hepatocyte vacuolation	5	5	11	8
Hemosiderin deposits	0	0	1	14
Hepatocyte degeneration	0	0	1	9
Male				
Acute inflammation	0	1	0	0
Chronic inflammation	1	2	4	15
Hepatocyte vacuolation	5	5	6	3
Hemosiderin deposits	0	0	0	15
Hepatocyte degeneration	0	0	1	14

From Fawell et al., 1999

6.2.3.2 Inhalation Exposure

No data from subchronic inhalation exposure of animals were found.

6.2.4 Neurotoxicity

Impaired long-term memory retrieval, as assessed by a step-down inhibitory avoidance task, was reported in rats after receiving an intrahippocampal injection of 0.01 or 20 µg/L of a microcystin extract from *Microcystis* strain RST 9501 (Maidana et al., 2006). Impaired spatial learning in the radial arm maze was also observed after exposure to 0.01 µg/L, but exposure at the higher concentration did not. An increase in oxidative damage, as measured by lipid peroxides and DNA damage, was observed in tissue homogenates of the hippocampus from treated animals.

Feurstein et al. (2011) examined the effects of microcystin-LR on isolated murine cerebellar granule neurons after administration of 5 μM microcystin-LR. Cell viability was significantly decreased but apoptosis was not induced by the concentrations given (up to 5 μM). Capase-3/7 activity was not increased with concentrations up to 5 μM but slight impairment of the neurite network was observed in the cells incubated for 48 hours at concentrations higher than 1 μM microcystin-LR. A significant dose-related decrease in neurite length was observed at concentrations ranging from 1 to 10 μM along with serine/threonine-specific PP inhibition and sustained hyperphoshorylation of Tau.

After intrahippocampal injection of 1 or 10 μg/L of microcystin-LR (≥98% pure), Li et al. (2012) reported impaired memory function, assessed by Morris water maze, in male rats. Rats showed an increased latency to find the platform after injection of both concentrations of microcystin-LR. Only at 10μg/L, histology of the brain revealed neuronal damage in the CA1 region of the hippocampus. In the same region (CA1) only high-dose animals showed a significant decrease in the total number of cells and the density of cells, but not in the cell volume. At both concentrations, malondialdehyde (MDA) levels and catalase activity in the hippocampal CA1 region were increased, but superoxide dismutase (SOD) and glutathione peroxidase activity were only significantly increased at 10 μg/L.

Li et al. (2014) suggested impairment of spatial learning and memory in groups of male 28 day old Sprague Dawley rats after oral exposure to microcystin-LR (95% pure) for 8 weeks. The microcystin-LR was dissolved first in methanol (1 mL/mL) and diluted with 100 mL pure water (0.001% methanol-v/v). The microcystin-LR was subsequently diluted with pure water to concentrations of 0.2, 1 and 5 μg/ml. As a result the methanol concentration of the stock solution increased with the microcystin-LR concentration. Rats were dosed with 0.2, 1.0, and 5.0 μg/kg of microcystin-LR every two days and performance in the Morris water maze test was evaluated. Pure water was used as the control. A weakness in the preparation of the dosing solution in this study is the fact that as the microcystin-LR dose increased, so did the methanol dose; thus the animals in each dose group received increasing amounts of methanol as well as microcystin.

At the conclusion of the dosing period, the animals were trained to find the platform within the water maze for 6 days. The group that received the 5 μg/kg dose took a significantly longer time to find the platform on day 3 of the training, but was comparable to the animals in the other dose groups by day 6. In the memory component of the test, after removal of the platform, the treated rats from the highest two dose groups spent less time in the platform quadrant than the controls. However, these differences were not statistically significant. At the higher dose (5 μg/kg), the treated rats showed the activation of astrocytes in the hippocampus and a dose-related increase of hippocampal nitric oxide synthase (NOS) as reflected by the number of N-20^{+} cells and detected by immunostaining of tissues from four animals from each dose group. Nitric oxide (NO) concentration was measured directly using the supernatant from the hippocampus tissue homogenate. The increase in NO concentration was also dose-related. Both NO and NOS were significantly higher ($p<0.05$) at the high dose.

A subsequent study by Li et al. (2015) examined neurological responses in pups born to dams that had been exposed to normalized doses of 0, 0.5, 2.5, or 10 μg microcystin-LR/kg/day for 8-weeks before mating, but not during gestation or lactation. A description of the developmental portion of this study is provided in Section 6.2.5. The control and microcystin-LR solutions each contained 0.002 % (v/v) methanol normalized to 0.001% methanol to account for dosing every other day over the 8 week period. The liters were culled to 4 males and 4 females per dam where possible. At specific postnatal time periods the pups were subjects to tests of motor function as follows:

- PND 7: surface righting reflex, negative geotaxis, cliff avoidance;
- PND 28: open field test, Morris water maze learning;
- PND 60 open field test and Morris water maze memory;

Twenty four hours after each behavioral test, one male and one female from each dam was sacrificed and the brain prepared for histological examination. The hippocampal tissues were analyzed for byproducts of lipid peroxidation (MDA and Total SOD).

Both the males and females had significantly ($p<0.07$) lower cliff avoidance performance than the controls on PND 7 at all doses (Li et al., 2015). There was a dose-related trend for the males but not the females. There were no significant differences from controls in the negative geotaxis, surface righting reflex tests or in the open field tests on PND 28 and PND 60. During the water maze training period (PND-28), there were no differences between groups. However, during the water maze memory tests the males in all groups scored more poorly than the females. The swimming speed for the females was significantly decreased for the mid and high dose groups. There was a significant increase in hippocampal MDA in the normalized 2.5 and 10 µg/kg/day dosed males and in both males and females at 10 µg/kg/day. Both males and females had a significant increase in measures of total hippocampal SOD at 10 µg/kg/day.

Given the neurotoxic properties of methanol, the presence of methanol in the solution in this study makes it difficult to evaluate these results as they relate to exposure to microcystin-LR in finished drinking water. A solution with a normalized 0.001% methanol (v/v) is equivalent to a concentration of 7.9 mg/L[2]. The data on intubation volumes for the dams are not provided in the published paper, thus it is not possible to quantify the methanol dose to the dams. In addition, exposure of the dams to methanol plus microcystin-LR ceased before mating and conception, making it difficult to quantify the relationship between the dosing of the dams and the exposures of the pups in the absence of data on half- life for microcystin-LR and methanol. Based on the postnatal pup responses the NOAEL was 0.2 µg/kg/day and the LOAEL was 2.5 µg/kg/day based on the results of the Morris water maze tests and the analysis of the hippocample tissues for evidence of ROS. Male pups were more sensitive than the females. The possibility of synergy between methanol and microcystin-LR cannot be eliminated. The apparent lack of direct exposure to the dams during conception, gestation and lactation, also confounds the application of the data to a risk assessment for microcystin-LR in drinking water.

6.2.5 Developmental/Reproductive Toxicity

6.2.5.1 Reproductive Effects

6.2.5.1.1 Oral Exposure

Kirpenko et al. (1981) used extracts from *M. aeruginosa* from a reservoir during the summer months to determine reproductive toxicity in rats. Male and female white rats (total of 120 rats) were intubated with 5×10^{-4} or 5×10^{-7} mg/kg of toxin extracts (no details were provided on the content of the extract) or 10 mg/kg of *M. aeruginosa* biomass for three months (dosing procedure not specified). Histopathology of the ovaries as well as estrous cyclicity and microscopic studies of the genital appendages and testes in males were conducted. After 3 months of treatment with 5×10^{-4} mg/kg of toxin extract or 10 mg/kg of biomass, and absence in the estrous cycle, (specifically an absence of estrus with prolonged diestrus) was observed. Maturation and growth of the oocytes was also affected. After 1.5 months of treatment with 5×10^{-4} mg/kg of the toxin extract, degeneration of oocytes in Graafian vesicles, decreased follicle dimensions, and increased number of involuted corpora lutea were observed. In males, there was a decrease in spermatogonia quality, spermatozoid motility, living spermatozoids (increased dead), and spermatid quality with a dose of 5×10^{-4} mg/kg of toxin extract. Spermatogonia are stem cells in the walls of the seminiferous tubules that give rise to spermatocytes, an intermediate step in the formation of

[2] 0.001% v/v = 0.001 ml/100ml x 0.79 g/ml (density of methanol) = 0.00079 g/100ml x 1000 mg/g = 0.79 mg/100 mL = 7.9 mg/L methanol

spermatozoa. Histological evaluation revealed "epithelium shelled out" (not defined) from basal membranes and greater tubule deformation. Degenerating spermatogonia and morphological abnormalities in Sertoli cells were also noted.

Falconer et al. (1988) used extract from an *M. aeruginosa* bloom sample to study the reproductive effects of microcystin in mice. Eight female mice received 1/4th dilution of the extract, estimated to contain 14 µg/mL of unspecified microcystin toxin, as drinking water since weaning. The mice were mated at age 20 weeks with similarly treated males for a premating treatment interval of approximately 17 weeks. The authors did not observe a difference in number of litters, pups per litter, sex ratio, or litter weight. Seven of 73 pups from treated parents showed reduced brain size. None of the 67 pups from controls showed reduced brain size. The authors did not report the litter distribution of the affected pups. After histological examination of one of the small brains, extensive damage to the hippocampus was observed.

Sperm quality and testicular function were assessed in male specific pathogen free mice (0.015-0.025 kg at purchase) administered microcystin-LR (commercial product; purity not stated) in the drinking water at concentrations of 0, 1, 3.2, or 10 µg/L for 3 or 6 months (Chen et al., 2011). Microcystin-LR was dissolved in 0.1% methanol and diluted to the required concentration with water; controls received water only. Although body weight and amount of water consumed were measured, these data were not presented and doses to the animals were not calculated by the study authors. Based on the subchronic reference drinking water value of 0.0078 L/day and body weight of 0.0316 kg for the male B6C3F1 mouse (U.S. EPA, 1988), doses to the animals were estimated at 0, 0.25, 0.79, and 2.5 µg/kg. Subchronic reference values were chosen to more accurately reflect status of the animals after 6 months of treatment; based on growth curves for the B6C3F1 mouse and initial body weights of the SPF mice, the B6C3F1 strain was considered reasonably similar to the strain used in this study.

No clinical signs of toxicity were observed and body weight, testes weight, and water consumption were not affected by treatment. Results of sperm and hormone analyses are shown in Table 6-7. No significant changes in any parameter were noted at 1 µg/L. At 3.2 and 10 µg/L, sperm counts were significantly decreased and sperm motility was reduced at 3 and 6 months with severity increasing with longer duration of exposure. Animals in the mid- and high-dose groups had a trend towards lower serum testosterone and higher luteinizing hormone and follicle stimulating hormone after 3 months, which was statistically significant by 6 months (except for FSH in the mid-dose group). Histopathological evaluation of the testes showed a slightly loosened appearance of the organization of the epithelium in the seminiferous tubules at 10 µg/L after 3 months. After 6 months, slight testicular atrophy associated with sparse appearance of the seminiferous tubules was found at 3.2 and 10 µg/L with dose-related increased severity. The animals given 10 µg/L also showed loss and derangement of spermatogenic cells, enlargement of the lumen of the seminiferous tubules, thinning of the spermatogenic epithelium, as well as depopulation of Leydig cells, Sertoli cells, and mature sperm. The number of apoptotic cells in the testes was increased at 10 µg/L after 3 months and at 3.2 and 10 µg/L after 6 months. The NOAEL was 0.25 µg/kg/day and the LOAEL was 0.79 µg/kg/day.

6.2.5.1.2 Other Routes

Ding et al. (2006) studied the effects of microcystin on the reproductive system of male mice administered 0, 3.33, or 6.67 µg microcystin/kg i.p. daily from an extract of *Microcystis* (99.5% microcystin-LR, 66.476 µg/mL, and 0.5% microcystin-YR, 0.361 µg/mL) for 14 days using 0.9% saline as the vehicle. A significant decrease in body weight gain in both treatment groups was observed during the course of the study. A dose-dependent decrease in absolute testes weight was observed, but a significant increase in relative testes weight was observed only in the high-dose group. The high-dose group had an increase in the percent immobile sperm and a significant decrease in absolute and relative epididymis weight. A dose-dependent decrease in sperm viability and the proportion of sperm with rapid progressive motility was observed. No increase in the percent of abnormal sperm was recorded.

Histological evaluation of both treatment groups showed atrophy of the seminiferous tubules with increased spacing between the seminiferous tubule cells and the effect increased with increasing dose. The high-dose group also exhibited a decreased number of interstitial cells, Sertoli cells, and mature sperm in the seminiferous tubules, and a deformation of Leydig and Sertoli cells.

Table 6-7. Serum Hormone Levels and Sperm Analyses From Mice Given Microcystin-LR in the Drinking Water for 3 or 6 Months

Endpoint	0 µg/kg/day	0.25 µg/kg/day	0.79 µg/kg/day	2.5 µg/kg/day
3 months				
Testosterone (ng/mL)	2.23 ± 1.15	2.77 ± 0.93	2.34 ± 1.11	1.07 ± 0.27
LH (mIU/mL)	7.03 ± 0.41	7.28 ± 0.66	8.05 ± 0.37	7.71 ± 0.27
FSH (mIU/mL)	3.05 ± 0.14	3.12 ± 0.36	3.37 ± 0.32	3.49 ± 0.47
Sperm count (×10^6/mL)	27.0 ± 1.5	23.5 ± 0.8	17.8 ± 1.5**	13.3 ± 1.3**
Sperm motility (%)	71.7 ± 3.3	57.6 ± 5.5	54.0 ± 6.4*	34.6 ± 3.3**
Abnormal sperm (%)	5.9 ± 1.0	5.9 ± 1.0	6.1 ± 0.9	6.5 ± 1.0
6 months				
Testosterone (ng/mL)	3.33 ± 0.98	2.03 ± 0.73	1.08 ± 0.17**	0.89 ± 0.29**
LH (mIU/mL)	4.89 ± 0.25	4.84 ± 0.25	5.88 ± 0.25*	5.66 ± 0.17**
FSH (mIU/mL)	2.36 ± 0.35	2.59 ± 0.37	3.16 ± 0.32	4.27 ± 0.52**
Sperm count (×10^6/mL)	21.5 ± 0.7	19.7 ± 0.9	13.6 ± 1.1**	6.6 ± 0.9**
Sperm motility (%)	60.6 ± 5.1	46.8 ± 6.7	23.1 ± 3.2**	17.4 ± 5.0**
Abnormal sperm (%)	6.5 ± 1.0	9.0 ± 1.0	13.8 ± 1.8**	14.5 ± 1.1**

From Chen et al., 2011 Data are mean±S.E.; n = 10; Significantly different from control: *p<0.05; **p<0.01.

Li et al. (2008) also observed reproductive effects in male Sprague-Dawley rats after i.p. injection of 0, 5, 10, or 15 µg microcystin-LR/kg-day by for 28 days. The microcystin-LR was dissolved in a minimal amount of 0.1% methanol and diluted with saline for injection. In all treatment groups, body weight gain and sperm motility were decreased. The percent of abnormal sperm was increased in all dose groups. The high-dose group had decreased absolute and relative testes weights and epididymal sperm concentrations. In both 10 and 15 µg/kg-day dose groups, serum testosterone levels were significantly decreased. At 5 µg/kg-day, both FSH and LH were significantly increased, but significantly decreased at 15 µg/kg-day. Histopathological change, including atrophied and obstructed seminiferous tubules in the testes occurred in all treated groups, but were more pronounced in the high-dose group.

Cellular damage was observed in the testes of male mice administered a single i.p. dose of 55-110 µg microcystin-LR/kg prepared from a crude extract of a lyophilized cyanobacterial bloom (Li et al., 2011b). The effects of a single i.p. injection of microcystin extracts from a surface bloom containing 167.7 µg microcystin-RR/mL and 47.0 µg microcystin-LR/mL or 80.5 µg microcystin-LR equivalents/mL was found to have an effect on male rabbit testes. Lesions, including a variety of histological changes to both spermatogonia and Sertoli cells, were seen in immature male Japanese white rabbits (1.6±0.2 kg) treated with 12.5 µg microcystin-LR equivalents/kg; recovery occurred by 48 hours with the tissue resembling the control (Liu et al., 2010).

In a study by Chen et al. (2013), male rats (10 per group) were i.p. injected with microcystin-LR (purity ≥ 98%) in saline for 50 days at doses of 1 or 10 µg/kg/day; a control group (n =10) was injected with the same volume of 0.9% saline solution. Animals were sacrificed twelve hours following the final injection and the testes removed. The relative testes weight was significantly decreased ($p < 0.01$) at 10 µg/kg/day, however, body weight and absolute organ weight data were not given. Light microscopic observations indicated that the space between the seminiferous tubules and lumen size increased with increasing dose; blockages in the seminiferous tubules were also reported at 10 µg/kg/day. Ultrastructural observations in

spermatogonia showed some abnormal histopathological characteristics, including cytoplasmic shrinkage, cell membrane blebbing, swollen mitochondria and deformed nucleus; these changes became more pronounced with increasing dose. Using qPCR methods, the transcriptional levels of select cytoskeletal and mitochondrial genes were determined. Microcystin-LR exposure affected the homeostasis of the expression of cytoskeletal genes, causing possible dysfunction of cytoskeleton assembly. Transcription of β-actin, β-tubulin, and stathmin were significantly decreased while ezrin and moesin were increased. In both microcystin-LR treated groups, all 8 mitochondrial genes related to oxidative phosphorylation (OXPHOS) were significantly increased. The levels of reactive oxygen species (ROS) were significantly increased ($p < 0.01$) at 10 µg/kg/day as was mitochondrial swelling and DNA damage. Changes in testicular hormone levels included increased FSH levels at 10 µg/kg/day, significantly increased LH levels in both treated groups ($p < 0.05$ or 0.01), and decreased testosterone levels in both dose groups ($p < 0.01$) compared to those of the controls. The authors concluded that this study provides evidence that both cytoskeleton structural disruption and mitochondrial dysfunction interact through induction of reactive oxygen species and oxidative phosphorylation resulting in testis impairment following exposure to microcystin-LR.

Wu et al. (2014) studied the effect of i.p. injection of microcystin-LR (commercial product; purity not specified) on the female reproductive tract of both rats and mice. Female Sprague-Dawley rats (n = 6) were given 0 or 200 µg/kg/day for six days and the ovaries removed for Western blot analysis of microcystin-LR-protein phosphatase 1 and 2A (PP1/2A) adducts. Female BALB/c mice (n = 20) were given 0, 5, or 20 µg/kg/day for 28 days. A subset of six mice per group was maintained for 28 days for estrous cyclicity monitoring. The remaining mice were sacrificed 24 hours after the last injection for serum hormone analysis and histopathology of the ovaries. In rats, microcystin-LR-PP1/2A adducts were detected in liver and ovary with the band from the ovarian extract being much weaker than that of liver. At 20 µg/kg/day, mice had significantly lower ovarian weight and a significantly decreased number of primordial follicles compared with those of controls. Estrous cyclicity was not affected by treatment with microcystin-LR and no differences in serum FSH, LH, and estradiol were seen. Serum progesterone levels were significantly reduced in both groups of treated mice compared with that of controls.

Bu et al. (2006) evaluated the potential embryotoxicity of microcystis cell extracts from water samples from the Nanwan reservoir in China in pregnant Kunming mice. HPLC analysis showed that the main components of these samples were microcystin-LR and –YR, with the majority being microcystin-LR. The study authors indicated that the LD50 for was much lower compared to previous i.p. studies and noted that it was possible that other substances that can increase the toxicity of microcystin may have been present in the extracts. Bu et al. (2006) exposed pregnant mice to 3, 6, or 12 µg/kg microcystin (12/dose group) on GD 6-15 via i.p. injection. Control mice were injected with saline on the same GDs. Mice were sacrificed on GD 18 and number of dead and resorbed fetuses and viable fetuses was recorded.

Additionally, the study authors evaluated the body weight, body length, tail length, skeletal development, and external anomalies of viable fetuses. The number of viable embryos was statistically significantly decreased and the number of dead or resorbed embryos was statistically significantly increased at the high dose. In the fetuses, body weight, body length, and tail length were also statistically significantly decreased at the high dose. The study authors noted that petechial hemorrhage and hydropic degeneration were observed in the livers of fetuses at the 6 and 12 µg/kg doses.

6.2.5.2 Developmental Effects

Fawell et al. (1999) reported the results of a developmental toxicity study of microcystin-LR (commercial product; purity not stated) given via gavage to Cr1:CD-1(ICR)BR (VAF plus) mice. Microcystin-LR (0, 200, 600 or 2,000 µg/kg/day) was administered to groups of 26 mice on days 6-15 of pregnancy. On day 18, the mice were sacrificed and necropsied. External, visceral and skeletal examinations were performed, and weight and sex of the fetuses were recorded. Of the 26 dams receiving 2,000 µg/kg/day, seven died

and 2 others were sacrificed moribund. An altered liver appearance was observed during gross examination. The surviving dams in this group did not express any clinical signs of toxicity or differences in body weight or food consumption. According to the authors, fetal body weight was significantly lower than controls and delayed skeletal ossification was observed at the highest dose. However, data were not included in the publication. No effects on litter size or resorptions were observed in any treatment group, nor were there increases in external, visceral or skeletal abnormalities in fetuses. The 600 µg/kg/day dose is the apparent NOAEL with a Frank effect level (FEL) of 2,000 µg/kg/day for decreased skeletal ossification and lethality low fetal body weight.

Groups of 6-8 timed-pregnant CD-1 mice were administered microcystin-LR (commercial product; 95% purity) in sterile saline by i.p. injection at doses of 0, 32, 64, or 128 µg/kg. Animals were treated on gestation days 7-8, 9-10, or 11-12 followed by sacrifice on day 17. Fetuses were examined for gross and skeletal malformations (Chernoff et al., 2002). Maternal weight change, pregnancy rate, litter size, fetal deaths, and fetal body weight were similar between control and treated groups. No treatment-related malformations were found in fetal examination.

In another part of the Chernoff et al. (2002) study, pregnant CD-1 mice were administered microcystin-LR (commercial product; 95% purity) in sterile saline by i.p. injection at doses of 0, 32, 64, 96, or 128 µg/kg. Animals were treated on gestation days 7-8, 9-10, or 11-12, and allowed to give birth. The growth and viability of pups was monitored for 5 days. A different lot of microcystin-LR from the same supplier was used in this part of the study and was much more toxic than the lot used in the developmental toxicity study. Maternal deaths were observed at all doses independent of days of dosing. In the control and treated groups, 0/25, 3/27, 19/35, 33/34, and 33/34 animals died, respectively. For surviving dams, numbers of pups born, and offspring survival and body weight through postnatal day 5 were not affected by treatment.

An *in vitro* study to determine the effect of microcystin-LR in the syncytiotrophoblast using villous cytotrophoblast isolated from term human placentas was done by Douglas et al. (2014). Cells were exposed to 0, 0.5, 1.25, 2.5, 5, 10, 20, and 25 µM of microcystin-LR and analysis of trophoblast morphology, detachment, differentiation and apoptosis was performed. Measurement of secretion of human chorionic gonadotropin (hCG), the pregnancy hormone secreted by the syncytiotrophoblast was also done. The authors observed round cells and significant cell loss at 25 µM microcystin-LR, but no change in the spreading and general morphology of trophoblasts at concentrations lower than 25 µM. No detachment, apoptosis, or differentiation of cytotrophoblasts to multinucleated syncytiotrophoblast were observed. However, the secretion of the pregnancy hormone hCG was increased in a dose-dependent manner. However, the cause of the increase in the hCG secretion remains undetermined.

Li et al. (2015) conducted a developmental neurotoxicity study in female Sprague Dawley Rats. The neurotoxicity portion of the study is presented in Section 6.2.4 above. Groups of 7 and 28 day old rats were dosed intragastrically every other day for 8 weeks at doses of 0, 1, 5 or 20 µg microcystin-LR/kg/days in a solution that contained 0.002% methanol (v/v). The microcystin-LR was identified as 95% pure. These doses normalize to values of 0, 0.5, 2.5 or 10 µg/kg/day. At the end of the exposure period the females were mated with unexposed males. No dosing occurred during the gestation period. After conception, gestation and delivery, the litters were culled to 4 males and 4 females, where possible, with subsequent evaluation of the pups for developmental neurotoxicity. At the end of the gestation period the only significant change observed for the dams was decreased body weight gain ($p<0.05$). The number of pregnant dams decreased across dose groups (7, 6, 5, and 5, respectively) while the number of dead pups increased (0.4 ±0.2, 0.7 ±03, 1.3 ±0.8, and 1.6 ±0.9, respectively) but the differences were not statistically significant. Other parameters evaluated were live pups/litter, fetal weight and sex-ratio; no differences across dose groups were noted. Developmental milestones (e.g. incisor eruption, hair appearance and eye opening) did not differ significantly from those for the controls. The NOAEL based

on maternal gestational weight gain is 2.5 µg/kg/day and the LOAEL is 10 µg/kg/day. There was no direct exposure to the dams during the gestation and lactation periods.

6.2.6 Chronic Toxicity

Falconer et al. (1988) conducted a chronic exposure experiment (up to 1 year) using an extract of a *M. aeruginosa* water bloom in Swiss Albino mice. A concentration-dependent increase in mortality, reduced body weight and a concentration-dependent increase in serum alanine aminotransferase levels were observed among groups of mice receiving serial dilutions of the extract as their drinking water for a year. The incidence of bronchopneumonia observed in the treated animals was directly related to the microcystin concentration. No significant differences in liver histopathology were observed when compared to the control, although the observed liver changes (neutrophil infiltration, hepatocyte necrosis) were slightly more prevalent in treated animals. The data showed some indication of sex differences in susceptibility; male mice showed effects (including mortality and serum enzyme level increases) at lower concentrations than females.

Thiel (1994) reported the results of a chronic toxicity study of microcystin-LA in velvet monkeys as an expanded abstract in the proceedings of an international workshop; a published version of this study was not located. A group of six monkeys was divided into two treatments: three controls and three monkeys that were given increasing intragastric doses of microcystin-LA for 47 weeks. At the beginning of the study, the dose was 20 µg/kg/day and increased to 80 µg/kg/day at study termination. The intervals of the dosage were not reported. No body weight or clinical signs such as respiration, pulse, or temperature were observed. No statistically significant changes in hematological parameters (hematocrit, bilirubin, hemoglobin, erythrocyte and leukocyte, and platelet count) were observed. No changes were observed in serum biochemistry analyses including albumin, globulins and electrolytes, as well as serum AST, LDH, ALP, ALT and GGT. Histopathological examination of the liver and other organs, not specified in the expanded abstract, did not show any differences in treated monkeys when compared with controls.

A chronic study done by Ueno et al. (1999) evaluated the toxicity of microcystin-LR in mice via drinking water. Two hundred 6-week-old female BALB/c mice were randomly assigned to receive either drinking water (*ad libitum*) containing 20 µg/L of microcystin-LR (95% pure) or no treatment for 7 days/week for up to 18 months. After 3, 6 and 12 months, 20 animals from each group were sacrificed, and the remaining 40 animals in each group were retained for chronic toxicity evaluation and sacrificed at 18 months.

The authors recorded daily observations for clinical signs of toxicity, morbidity and mortality, and weekly estimates of food and water consumption. The body weights were recorded weekly for the first 2 months, biweekly up until the first year and monthly until 18 months. Blood was obtained from 20 animals from each group at 3, 6, 12 and 18 months. Hematological evaluations were done in 10 animals per group, and samples from 10 additional animals were used for serum biochemistry evaluation. Complete necropsy of 10 animals per group was conducted and necropsy was also done in those animals in the chronic study when moribund or found dead prior to scheduled sacrifice or upon sacrifice at 18 months. Record of relative and absolute organ weights including liver, kidneys, spleen, thymus, adrenal, ovaries, brain, heart and uterus were done for 9-10 animals per group at each scheduled sacrifice, and histopathological evaluation of these and numerous other organs was conducted. Immunohistochemistry of the liver was also examined upon sacrifice of three to five animals per group to determine the distribution of microcystin-LR in the liver.

The calculated cumulative intake of microcystin-LR over 18 months was 35.5 µg/mouse (based on weekly estimates of water consumption) (Ueno et al., 1999). This is equivalent to an exposure of 2.3 µg/kg/day based on the reported average adult body weight of 26.68 g/mouse and the reported 567 day exposure. No clinical signs of toxicity and no statistically significant differences in body weight, food

consumption, water consumption or hematology were observed. However, hematology data were lost due to sampling errors from the 3-month sacrifice. Survival in the control and chronic treatment groups was similar. After 12 months, the treated mice had a statistically significant decrease in serum ALP (13%) and at month 18, a significant increase in cholesterol (22%). None of these effects were considered by the authors to be toxicologically significant in the absence of other treatment-related effects. However, according to the authors, the increase in cholesterol could be related to interference of microcystin-LR with bile acid transport from the liver.

Treated mice showed sporadic changes in absolute and relative thymus weight, but histological and morphometric evaluation revealed no abnormalities attributable to exposure (Ueno et al., 1999). Treated mice sacrificed after 12 months showed a decrease in heart weight that was not considered treatment-related in the absence of histopathological changes. In contrast to other studies, no difference in the incidence of liver histopathology between treated and control mice was observed. No accumulation of microcystin-LR was observed after immunohistochemistry of the liver.

Microcystin-LR (commercial product; ≥95% purity) was administered for 180 days to 8-week old male C57bl/6 mice (10/treatment group) via drinking water at the following concentrations: 0, 1, 40, or 80 µg/L (Zhang et al., 2010). The doses were reported as 0, 0.2, 8.0, and 16 µg/kg/day, but the method of calculation was not given by the authors. Body weight was measured at the beginning and at the end of the study. Livers were removed at sacrifice and processed for routine (hematoxylin-eosin) or immunohistochemical staining to measure matrix metalloproteinase (MMP[3]) expression. Measurement of MMP protein and mRNA levels were measured in other liver portions.

A significant ($p < 0.01$) decrease in body weight, and an increase in relative liver weight, was reported at 8.0 and 16.0 µg/kg/day (Zhang et al., 2010). Histopathology of mice treated with 8.0 and 16.0 µg/kg/day, revealed infiltrating lymphocytes and fatty degeneration in the liver, but incidence and severity data were not provided. There was a significant increase in the area stained positive for MMP2 at 8.0 and 16.0 µg/kg/day and for MMP9 in all treatment groups. Only in the high-dose group, the MMP2 protein concentration was significantly increased. The concentrations of MMP9 protein were increased at all doses. In the mid- and high-dose groups, messenger RNA expression for both MMPs was significantly increased. The phosphorylation extracellular signal-regulated protein kinase (ERK) 1/2 and p38 (members of the mammalian of the mitogen-activated protein kinase (MAPK) family were also increased.

In a subsequent study by Zhang et al. (2012), microcystin-LR (commercial product; ≥95% purity) at concentrations of 0, 1, 40, or 80 µg/L (0, 0.2, 8.0, and 16 µg/kg/day) was administered to 8-week old male C57bl/6 mice (10/treatment group) via drinking water for 270 days. . Body weight was measured at the beginning and at the end of the study, and livers were removed and processed for routine or immunohistochemical staining at sacrifice to measure MMP expression. MMP protein and mRNA analyses were done in other liver portions from five randomly selected mice. Body weight results were not included in the main publication but the data was reported in supplemental information. No differences in water consumption were observed between the groups. Histopathology showed infiltrating lymphocytes and fatty degeneration in the livers of mice (doses not specified in main publication). In all dose groups, MMP expression and protein levels for both MMP2 and MMP9 were significantly increased. MMP mRNA levels were also increased in all dose groups for MMP2 and in the mid- and high-dose groups for MMP-9.

[3] Matrix metalloproteinases are a family of zinc requiring matrix-degrading enzymes, which include the collagenases, gelatinases, and the stromelysins, all of which have been implicated in invasive cell behavior (Brooks et al. 1996).

6.2.7 Immunotoxicity

Shirai et al. (1986) reported that C3H/HeJ mice, immunized i.p. with either sonicated or live cells from a *Microcystis* water bloom, developed delayed-type hypersensitivity when challenged 2 weeks later with a subcutaneous injection of sonicated *Microcystis* cells. A positive reaction, as assessed by footpad swelling, was seen in mice immunized with either live cells or sonicated cells. Because this strain of mouse is unresponsive to LPS, the footpad delayed-type hypersensitivity was not related to LPS, thus, the antigenic component of the sonicated cells is not known, but might have been microcystin.

Shen et al. (2003) studied the effect of cyanobacterial cell extract on immune function. Mice received 14 daily i.p. injections containing a cell-free extract from a water bloom dominated by *M. aeruginosa* at 16, 32 and 64 mg lyophilized cells/kg body weight doses or as 4.97, 9.94 and 19.88 μg/kg of microcystin equivalents. Analysis by HPLC indicated that the microcystin content of the extract was 79.53%, although specific congeners in the extract were not reported. Immunotoxicity endpoints examined were: phagocytosis, lymphocyte proliferation and antibody production in response to sheep red blood cells.

Phagocytic capacity was reduced at the two highest doses, but percentage phagocytosis was not affected. B-lymphocyte proliferation was significantly reduced (33%), compared to controls (at 32 mg/kg). Body weight was significantly reduced in all treatment groups. Relative spleen weight was significantly increased at 9.94 μg/kg, and significantly decreased at 19.88 μg/kg. In the high-dose group, relative thymus weight was significantly decreased, and relative liver weight was significantly increased in all treatment groups, although not related to dose. However, changes in T-lymphocyte proliferation were mild, and deemed biologically insignificant. In the treated mice, humoral immune response, as measured by antibody-forming plaques, was reduced in a dose-dependent manner.

Shi et al. (2004) reported a study where mice received a single i.p. injection containing a cell-free extract from a water bloom dominated by *M. aeruginosa* processed in the same manner as the Shen et al. (2003) study. Although specific congeners in the extract were not reported, it was stated that microcystin-LR was the predominant component. Doses were reported as 0, 23, 38, 77 and 115 mg lyophilized cells/kg body weight or as 0, 7, 12, 24 and 36 μg/kg of microcystin equivalents. Animals were sacrificed 8 hours after exposure. Messenger RNA levels of TNF-α, IL-1β, IL-2, and IL-4 were significantly decreased, IL-6 was unaffected, and IL-10 was increased at the lowest dose and decreased at higher doses. None of the changes were dose-related.

Chen et al. (2004b, 2005b) evaluated the role of nitric oxide generation and macrophage related cytokines on the reduced phagocytic capacity induced by pure microcystin-LR. A dose-dependent inhibition of nitric oxide production was observed in activated macrophages, and a repressive effect was seen in cytokine formation at the mRNA level (e.g., IL-1β, TNF-α, GM-CSF, IFN-γ) after either a 24-hour (Chen et al., 2004b) or a 6-hour treatment (Chen et al., 2005b). Hernandez et al. (2000) showed that microcystin-LR enhanced the early spontaneous adherence of polymorphonuclear leukocytes (PMNs) to substrate; no effects were found on late adherence (steady state) or with stimulated PMNs.

Several studies evaluated the effects of microcystin-LR on immune system components *in vitro* (Lankoff et al., 2004b; Teneva et al., 2005; Chen et al., 2004b; Kujbida et al., 2006). Lankoff et al. (2004b) reported that microcystin-LR inhibited B-cell proliferation in human and chicken peripheral blood lymphocytes at all concentrations tested and decreased T-cell proliferation only at the highest concentration. Apoptosis was enhanced in both human and chicken lymphocytes (Lankoff et al., 2004b). Similarly, microcystin-LR was cytotoxic to mouse splenocytes, and caused apoptosis in B-cells but not in T-cells (Teneva et al., 2005).

Kujbida et al. (2006) assessed the effects of microcystin-LR and [Asp3]-microcystin-LR on human polymorphonuclear lymphocytes (PMNs) *in vitro*. Both compounds caused migration of neutrophils in a chemotaxis chamber, suggesting that PMNs may migrate from the blood stream to the organs such as the

liver that concentrate microcystins. In addition, both caused a dose-related increase in reactive oxygen species (ROS) production as measured by chemiluminescence of PMN degranulation products that accompany ROS production. The phagocytosis of *Candida albicans* by PMNs was increased after exposure to either compound, but only microcystin-LR increased the intracellular killing of *C. albicans*. These findings suggest the possibility that PMNs may mediate some of the toxic effects of microcystins.

Kujbida et al. (2008) found that microcystin-LR, microcystin-LA, and microcystin-YR increased interleukin-8 levels and extracellular ROS in human neutrophils, and chemoattractant-2αβ in rat neutrophils, but had no effect on tumor necrosis factor-α in either rat or human neutrophils. *In vitro* all three microcystins caused neutrophil chemotaxis by increased intracellular calcium levels (Kujbida et al., 2009). *In vivo,* topical application of microcystin-LR to male rats caused an enhancement of the number of rolling and adhered leukocytes in the endothelium of postcapillary mesenteric venules, but microcystin-LA and microcystin-YR had no effect (Kujbida et al., 2009).

Yuan et al. (2012) evaluated the immunotoxicity in rabbits using extracts of microcystins isolated from a surface bloom in China. The extracts contained 0.84 mg/g dry weight of microcystin-RR, 0.50, mg/g dry weight of microcystin-LR, and 0.07 mg/g dry weight of microcystin-YR. Four rabbits per treatment group received single i.p. injections of 0, 12.5, or 50 µg microcystin-LR equivalents/kg. After administration of the 50 µg/kg dose, blood was collected from the heart at 0, 1, and 3 hours, and at 0, 1, 3, 12, 24, 48, and 168 hours after administration for the 12.5 µg/kg dose. A significant increase in plasma white blood cells was observed after microcystin-LR treatment with both doses. The peak increase was observed 1 hour after treatment with 50 µg/kg and 12 hours after treatment with 12.5 µg/kg. IFN-γ, INF-α, IL-3, IL-4, and IL-6 production was decreased at all time points measured after the 50 µg/kg treatment. However, at the 12.5 µg/kg dose, production of IFN-γ, INF-α, IL-3, IL-4, and IL-6 was increased through the first 12 hours after exposure, but decreased or was the same as the controls from 24 to 168 hours.

Bernstein et al. (2011) studied skin sensitization to non-toxic extracts of *M. aeruginosa* in 259 patients with chronic rhinitis over 2 years. Patients were evaluated with aeroallergen skin testing and skin-prick testing (SPT). The authors found that 86% of the clinical subjects had positive skin prick tests to *Microcystis aeruginosa*, and that patients with existing allergic rhinitis were more likely to have reactions and sensitization to cyanobacteria than the controls (non-atopic health subjects). This study indicates that cyanobacterial allergenicity is associated with the non-toxic portion of the cyanobacteria.

Geh et al., (2015) studied the immunogenicity of *M. aeruginosa* toxic and non-toxic extracts in patient sera (18 patients with chronic rhinitis and 3 non-atopic healthy subjects collected from the study done by Bernstein et al., in 2011). ELISA test was used to test IgE-specific reactivity, and 2D gel electrophoresis, followed by immunoblot and mass spectrometry (MS), was done to identify the relevant sensitizing peptides. The authors found an increase in specific IgE in those patients tested with the non-toxic microcystin extract than the toxic extract. After pre-incubation of the non-toxic extract with various concentrations of microcystin, the authors found that phycocyanin and the core-membrane linker peptide were responsible for the release of β- hexosaminidase in rat basophil leukemia cells. The authors concluded that non-toxic strains of cyanobacteria are more allergenic than toxic-producing strains in allergic patients, and that the toxin may have an inhibitory effect on the allergenicity.

6.2.8 Hematological Effects

Several studies have noted thrombocytopenia (platelet deficiency) in laboratory animals treated with microcystins or bloom extracts purportedly containing microcystins (Slatkin et al., 1983; Takahashi et al., 1995). Early investigations with parenteral injection into mice of microcystins found thrombocytopenia, pulmonary thrombi, and hepatic congestion (Slatkin et al., 1983). However, *in vitro* studies have shown that microcystin-LR neither induces nor impedes the aggregation of platelets (Adams et al., 1985). Pulmonary thrombi apparently consist of necrotic hepatocytes circulating in the blood. Subsequent

research supports the hypothesis that hematological effects observed in animals acutely exposed to microcystins are secondary effects of liver hemorrhage (Takahashi et al., 1995).

Takahashi et al. (1995) reported dose-dependent reductions in erythrocyte count, leukocyte count, hemoglobin concentration, hematocrit and coagulation parameters one hour after rats were exposed to microcystin-LR (100 and 200 μg/kg i.p). None of these parameters changed until after massive liver hemorrhage commenced. Further, hematological changes such as increased prothrombin time and fibrin deposition in the renal glomeruli were not observed. The authors concluded that the depletion of blood components occurred as a result of liver hemorrhage.

Sicińska et al. (2006) evaluated the effects of microcystin-LR on human erythrocytes *in vitro.* Microcystin-LR exposure resulted in the formation of echinocytes, hemolysis, conversion of oxyhemoglobin to methemoglobin, and a decrease in membrane fluidity. In addition, measures of oxidative stress were affected in treated erythrocytes; glutathione reductase and superoxide dismutase activities were decreased, while ROS and lipid peroxidation were increased

6.3 Carcinogenicity

6.3.1 Cancer Epidemiology Studies

A survey of microcystin content in drinking water supplies was conducted in Haimen City, China to determine if microcystins in drinking water supplies could contribute to the higher incidence of liver cancer (Ueno et al., 1996). Samples were taken in ponds/ditches and river waters as well as shallow and deep wells and analyzed by ELISA. Microcystin concentrations were higher in pond/ditch water (17% reported as positive with concentration >50 pg/mL), followed by river water (32% positive), shallow wells (4% positive), and deep wells (no detections >50 pg/mL). The averages of microcystin concentrations across the drinking water types differed with an average of 101 in pond/ditch, 160 in river, and 68 pg/mL in shallow well samples. The authors used the average microcystin concentration in the ponds/ditches (101 pg/mL) and in river water (160 pg/mL), and the average adult consumption over June to September (1.5 L) to calculate the exposure levels to microcystin in Haimen city. The authors determined that over a period of 4 months the levels to which people would have been exposed was 0.19 pg of microcystin per day and the average adult was exposed to these levels over a period of 40 to 50 years. The authors did not collect any data that would support a correlation between consumption of the different water sources showing seasonal contamination with microcystins, and local cases of hepatocellular carcinoma. Therefore, this study generates a hypothesis of a possible association to exposure to microcystins, but does not investigate that relationship. Haimen City, like Haining City discussed below is on the Yangtze River. It was once largely an agricultural area but is now also noted for its production of textiles and more recently electronics (http://www.ccpittex.com/eng/tbases/49302.html). Thus there are likely multiple exposures to possible carcinogens that could account for the high cancer incidence.

Zhou et al. (2002) conducted a retrospective cohort study to analyze a previously reported association by Jiao et al. (1985) and Chen et al. (1994) between colorectal cancer and exposure to microcystins in drinking water in a Chinese province. Between 1977 and 1996, a total of 408 cases of primary colorectal adenocarcinoma (245 rectums and 163 colons) obtained from the Cancer Registry of Haining Cancer Research Institute, were diagnosed in eight randomly selected towns within Haining City of Zhejiang Province. The local cancer registry was used to identify the cases and verified independently by two pathologists. The drinking water source used during the lifetime was used as a surrogate of oral exposure to microcystins. Interviews of patients or family members of deceased cases were performed to obtain information on drinking water source. Ten water sources including 3 rivers, 3 ponds, 2 wells and 2 taps, were randomly selected and sampled twice per month for microcystins from June through September

(total of eight samples from each source) and analyzed by ELISA. The authors did not provide information on the congeners tested or a complete description of the "tap" water samples. However, the study description implies that samples were collected from various treatment plants.

To determine the incidence rate of colorectal cancer, the authors compared the rates among the four different water sources with well water users serving as the reference population. The authors determined an average incidence rate of colorectal cancer of 8.37/100,000 per year across all of the study areas. The colorectal cancer incidence rates among users of the tap, pond, and river water sources were significantly increased compared with the incidence among well water users. Relative risks (RR) are listed in Table 6-8 and differed by water source; 1.88 for tap water, while river and pond water use both had a RR greater than 7.0. Very little difference in colorectal cancer incidence between river and pond water users was observed. The authors suggested that exposure to trihalomethanes in tap water could contribute to the risk for those users.

Microcystins were detected, only in river and pond water, at concentrations exceeding 50 pg/mL, which was considered by the authors to be the limit for positive detection (Zhou et al., 2002). Average concentrations in river and pond water were 30-50 fold higher than those for well or tap water. Since about 25% of the residents in each of the eight towns used river and pond water for drinking water, a comparison between the average microcystin concentration in river and pond water in each town with the incidence rate by town was performed. Their results showed a strong correlation between colorectal cancer incidence rate and concentration of microcystin (Spearman correlation coefficient = 0.88, $p<0.01$). This comparison is limited by failure to test for chemical carcinogens that could have also been present in the untreated surface water sources. For example, Haining City borders the Yangtze River and is the site of industries specializing in leather products and textiles and electronics among others http://en.haining.gov.cn/

The study by Zhou et al. (2002) provides suggestive evidence for an association between colorectal cancer and exposure to microcystin in drinking water, which is consistent with earlier reports of an association between drinking water from the river or pond and incidence of colorectal cancer in the Zhejiang Province of China (Jiao et al., 1985; Chen et al., 1994). Since demographic information was not provided in the study, it is not clear which factors, including diet, genetics, and lifestyle, and chemical contaminants associated with colorectal cancer, were adequately controlled in the analysis.

A number of epidemiological studies have been conducted in an area of Southeast China with high rates of hepatocellular carcinoma. These studies are summarized by the International Agency for Research on Cancer (IARC, 2010) and Health Canada (2002). Overall a positive association was found between the risk for hepatocellular carcinoma and surface waters as the drinking water source. In an analysis of pooled data from six case-control studies, RR was 1.59 (confidence limits not given); estimates of RR from other individual studies ranged from 1.5-4 (IARC, 2010). Consumption of pond or ditch water was associated with a higher risk of liver cancer incidence when compared with well water consumption. Confounding factors such as hepatitis B infection and aflatoxin exposure, were not generally considered in most studies. The presence of cyanobacteria in the water source was not a component of the study. Thus, the only relationship between these estimates of risk and cyanotoxins is the fact that cyanobacteria are primarily surface water contaminants.

Table 6-8. Relative Risk of Colorectal Cancer By Drinking Water Source

	Water Source			
	Well	Tap	River	Pond
Colorectal Cancer Incidence Rate per 100,000	3.61	6.77	28.50	27.76
Relative Risk of Colorectal Cancer (p<0.01)	-	1.88	7.94	7.7
95% CI	-	1.39-2.54	6.11-10.31	5.75-10.30
Number of Microcystin Samples >50 pg/mL	0/12	0/17	25/69	6/35
Mean Microcystin Concentration (pg/mL)	3.73	4.85	141.08	106.19
Maximum Microcystin Concentration (pg/mL)	9.13	11.34	1083.43	1937.94

From Zhou et al., 2002

A case-control study was done to evaluate the relationship between liver cancer in Haimen City, China and microcystin in drinking water (Yu et al., 2002). Participants were selected from a pool of 248 patients with hepatocellular carcinoma and 248 age-, sex- and residence-matched controls. Of those, 134 paired cases and controls agreed to blood samples for virus infection and ALDH2 (Aldehyde dehydrogenase 2) and CYP2E1 gene polymorphism analyses. The authors evaluated a variety of risk factors for liver cancer including hepatitis B and C virus infection, aflatoxin B1 or microcystin exposure along with genetic polymorphisms, smoking, drinking, and diet. Questionnaire information on possible lifestyle and dietary risk factors for liver cancer was also conducted. Exposure to microcystin was assessed based on type of drinking water supplied (tap, deep or shallow well, river, ditch, or pond water). No association between consumption of river, pond, or ditch water and hepatocellular carcinoma was determined by either univariate or multivariate analysis. The authors identified hepatitis B virus infection and history of i.v. injection as factors strongly associated with primary liver cancer (Yu et al., 2002).

An ecological epidemiological study was conducted to investigate the relationship between drinking water source and incidence of primary liver cancer in Florida (Fleming et al., 2002). Data on cyanobacteria and toxins, especially microcystins in surface drinking water sources in Florida were used to measure the exposure. All cases of primary hepatocellular carcinoma reported to the Florida State cancer registry between 1981 and 1988 were the study population. The study population was placed in two groups depending on the residence location at the time of diagnosis: those served by18 surface drinking water supplies, and a second group using other sources. Deep groundwater treatment plants and surface water treatment plants and their service areas were geocoded (Figure 6-1). The following comparisons were made:

- Comparison between cases residing in the service area of a surface water treatment plant with those residing in the service area of a deep groundwater treatment plant. Several referent groups were identified (one randomly sampled from the available groundwater service areas, one matched on median income and rent, one matched on ethnic makeup and one matched on income, rent and ethnicity).
- Comparison between cases in the surface water service area with equally-sized buffer areas surrounding the surface water service area, but not served by the treatment plant (GIS was used to delineate a buffer area assuming a population living contiguous to, but outside of, surface water treatment service areas).
- Comparison between cases and the primary liver cancer incidence in the general population of Florida.

There was no statistically significant difference among the individual incidence rates between the controls (four sets of 18 groundwater service areas) and the individual rates from the 18 individual surface water service. A statistically significant age-adjusted cancer rate for hepatocellular carcinoma (1.13) was

associated with residence in a surface water service area for 1981-1998, when the 18 ground water system areas were pooled. This rate was lower than the age-adjusted rates for the four comparison ground water areas and the state of Florida in general. The Standard Rate Ratio (SRR) for the surface water areas compared to the four ground-water area controls was 0.95. 0.84, 0.81, and 0.98. Compared with the state, the SRR was 0.8 (1.13-1.41). It should be noted that the measure of exposure was residence within a circular surface water service area derived using a diameter based on the average size of the service area plus two standard deviations, with the treatment plant theoretically but not physically located at the center of the circle. The dimensions of the ground water areas were determined in a similar fashion.

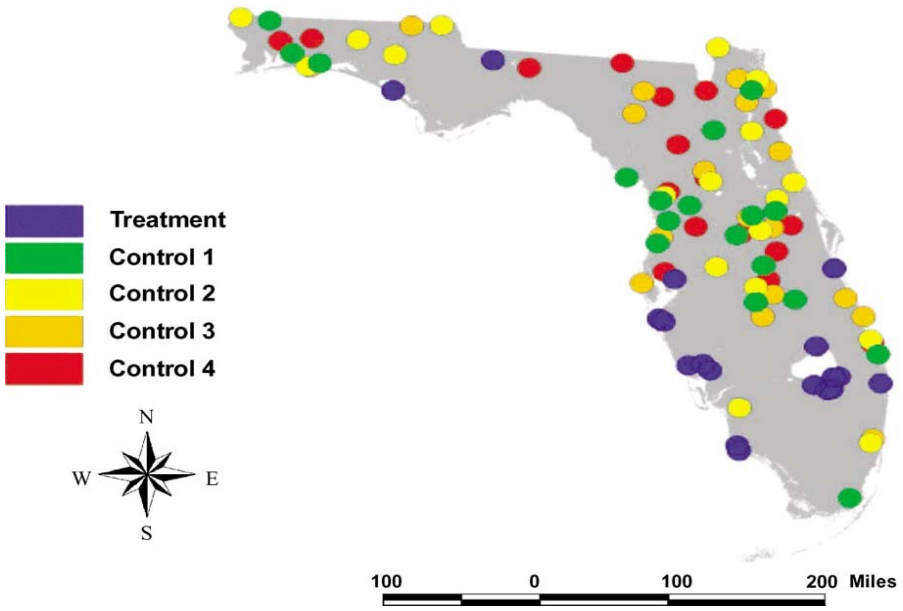


Figure 6-1. Sites of Surface Water Treatment Service Areas and Control Ground Water Treatment Service Areas.
From: Fleming et al., 2002

A statistically significant increase in the incidence of hepatocellular carcinoma was observed for those residing within the surface water service area (SRR=1.39, CI=1.38-1.4; average age adjusted cancer rate 1.15 versus 0.83) when compared with residence in the actual (i.e., not estimated as above) surface water service areas and residence in the buffer areas surrounding the service areas. According to the 1990 census data, ethnic and socioeconomic backgrounds of the service areas and buffer areas were similar (data not reported by the authors). When compared to the incidence of hepatocellular carcinoma in the general Florida population, the incidence of hepatocellular carcinoma in the buffer areas was also significantly lower (SRR=0.59 average age-adjusted state cancer rate = 1.41).

Due to the ecological design of the study by Fleming et al. (2002) establishing an exposure-response relationship is not possible because of the lack of exposure data on individuals and the strong possibility of misclassifying the exposure. Given residential mobility and likely latency time for cancer development, residence in a surface water service area at the time of diagnosis of hepatocellular carcinoma a poor measure of potential exposure to cyanobacterial toxins. In addition, not using the actual service areas but instead GIS-generated estimates of surface water service areas with which may be to make the initial comparisons with groundwater service areas could increase the misclassification of exposure.

Another ecological study by Fleming et al. (2004) evaluated the relationship between incidence of colorectal cancer and exposure to cyanobacteria using the proximity to a surface drinking water treatment plant as a surrogate for exposure. The authors used the same methods as those described above for

Fleming et al. (2002). However, the colorectal cancer data was obtained from the Florida Cancer Data System from 1981 to 1999. The following referent groups were formed:

- A random group of groundwater treatment service areas.
- A group of groundwater treatment service areas matched on median income and rent.
- A group of groundwater treatment service areas matched on ethnic makeup,
- a group of groundwater treatment service areas matched on both median income and ethnicity.
- Groups residing in an equally-sized buffer areas surrounding the surface water service area.
- General Florida population.

No association between colorectal cancer and residence at time of diagnosis in a surface water treatment area was observed based on results of the Mann Whitney rank sum test, however details of the tests were not provided.

6.3.2 Animal Studies

6.3.2.1 Oral Exposure

Falconer and Buckley (1989) and Falconer (1991) reported evidence of skin tumor promotion by extracts of *Microcystis spp*. The extract was administered at a concentration of 40 µg microcystin/mL via drinking water to mice pretreated topically with an initiating dose of dimethylbenzanthracene (DMBA). Details of the incidence of tumors in the control mice were not provided by the authors. The total skin tumor weight in mice drinking *Microcystis* extract was significantly higher than that of initiated mice receiving only water after initiation after 52 days. In mice receiving the extract, only the number of tumors per mouse was slightly increased due to the weight of individual tumors (Falconer and Buckley, 1989). The total weight of tumors in the mice receiving extract also exceeded that of mice pretreated with DMBA and subsequently treated with topical croton oil, with or without concurrent consumption of *Microcystis* extract.

No evidence of promotion of lymphoid or duodenal adenomas and adenocarcinomas was observed when *Microcystis* extract was provided in the drinking water (0, 10, or 40 µg/mL) of mice pretreated with two oral doses of N-methyl-N-nitroso-urea. No primary liver tumors were observed as well. (Falconer and Humpage, 1996).

No full oral cancer bioassay was found in which animals were administered microcystins or an extract. Ito et al. (1997b) evaluated the carcinogenicity and liver toxicity of 80 or 100 gavage doses of 80 µg microcystin-LR/kg/day (purity not specified) administered to twenty-two ICR mice (13 weeks old; sex not stated) over the course of 28 weeks (196 days). Microcystin-LR was isolated and dissolved in ethanol and saline for dosing from a water bloom from Lake Suwa, Japan. After 80 treatments, ten mice were sacrificed, five were sacrificed after 100 treatments, and seven were withdrawn from treatment and sacrificed after 2 months of receiving 100 doses. There were three control mice. Although the authors did not specify the nature of the postmortem examinations, apparently the liver was the only organ examined. When compared to controls, no change in mean liver weight was observed in the microcystin-LR-treated animals. The authors reported light injuries to hepatocytes in the vicinity of the central vein in 8 of 15 mice sacrificed immediately after treatment, and in 5 of 7 mice that were withdrawn 2 months after exposure from treatment. None of the treated animals showed fibrous changes or neoplastic nodules. Analysis by immunohistochemistry for microcystin-LR and its metabolites failed to detect either the parent compound or any metabolites in the livers of mice sacrificed immediately after treatment.

Humpage et al. (2000) administered *M. aeruginosa* extract in drinking water to mice pretreated with azoxymethane (an extract only control group was not included). The content of microcystins in the

drinking water was determined by mouse bioassay, HPLC, capillary electrophoresis, and protein phosphatase inhibition. The estimated doses of total microcystins were 0, 382, and 693 µg/kg/day at the midpoint of the trial. Mice were sacrificed at intervals up to 31 weeks after commencement of extract exposure. Enzyme analysis in mice treated with extract showed a concentration-dependent increase in ALP and decrease in albumin. A concentration-dependent increase in the mean area of aberrant crypt foci of the colon was observed. However, the number of foci per colon and the number of crypts per focus were not different among the groups. Two colon tumors were found, one each in a low- and high-dose animal treated with extract. The authors proposed that the increase in cell proliferation caused the increase in size of foci. An increase in leukocyte infiltration in animals treated with the highest concentration of extract was higher after histological examination of the livers of mice treated with extract compared to those receiving a low concentration.

6.3.2.2 Other Routes of Exposure

Groups of 9-16 male Fischer 344 rats, 7 weeks of age, were given a single i.p. injection of 0 or 200 mg/kg of *N*-nitrosodiethylamine (NDEA) in saline followed 3 weeks later by i.p. injections of 0, 1 or 10 µg microcystin-LR/kg twice a week for 5 weeks in a study by Nishiwaki-Matsushima et al. (1992). The doses of microcystin used did not appear to cause liver damage based on the absence of an increase in hepatic AST. Phenobarbital (0.05%) in the diet was used as a positive control. At the end of week 8, the rats were sacrificed, the livers removed and evaluated for GSTP-Foci (both the number of lesion and the foci area). GST-P foci are considered to be biomarkers for early stage development of potential liver tumors. All animals receiving DEN had foci; those receiving 10 mg/kg microcystin had significantly ($p < 0.01$) more foci than the control receiving saline. At the low microcystin dose the differences from control were not significant. The two groups receiving microcystin alone (1 or 10 µg/kg) had no GST-P foci. The group receiving DEN with phenobarbital as a promoter had the largest number and area of foci. Accordingly, microcystin showed the properties of a promoter but not an initiator.

In a second part of the study, 4 groups of animals were given NDEA injections as above (Nishiwaki-Matsushima et al., 1992). One of the groups received no microcystin; the other three received 10 µg/kg by i.p. injection. After 3 weeks the animals received a partial hepatectomy to stimulate tissue repair and received injections of 0, 10, 25, or 50 µg microcystin-LR/kg twice a week for 5 weeks. After the partial hepatectomy, there was a significant dose-related increase in the number and area of foci compared to the control not treated with microcystin ($p < 0.01$ or 0.001). The last group of rats received initial 10 µg/kg microcystin injections followed by a 50 µg/kg dose after the post partial hepatectomy. Those animals had a mean number of 0.4 ± 0.3 foci/cm^2 and an area of 0.1 ± 0.02 % compared with the NDEA control of 13.4 ± 44.2 foci/cm^2 with and area of 2.6 ± 3.1%. The evidence from this part of the study also indicates that microcystin has little if any initiating potential but can promote the formation of preneoplastistic foci in the liver of exposed rats (Nishiwaki-Matsushima et al., 1992).

Groups of male Fischer 344 rats (n = 5 to 20), 7 weeks of age, received a single i.p. injection of 0 or 200 mg NDEA/kg in saline followed 2 weeks later by 20 i.p. injections of 0 or 25 µg microcystin-LR/kg (Ohta et al., 1994). The study design resembled that of Nishiwaki-Matsushima et al. (1992) discussed above. Animals treated with NDEA plus microcystin-LR had significant ($p<0.005$) increases in the number, area, and volume of GST-P-positive foci per liver compared to NDEA-treated rats. The number of foci from the animals treated with microcystin-LR alone were six-fold lower than those treated with NDEA alone. The area and volume of the foci were a tenth of those with NDEA alone. The authors concluded that microcystin was a tumor promoter rather than a carcinogen.

In a study by Ito et al. (1997b), thirteen male ICR mice, 5 weeks of age, received 100 i.p. injections of 20 µg/kg-bw of microcystin-LR (five times a week) over 20 weeks and were sacrificed after the end of the treatment (five mice) or after a 2-month withdrawal period (eight mice). Three non-treated mice were

used as controls. Using the 1980 Guidelines on the Histology Typing of Liver Tumors in Rats by the National Research Council, neoplastic nodules were found in the liver of all 13 treated mice. These guidelines have since changed in that some types of nodules once considered as preneoplastic no longer indicated an increased cancer risk (Wolf and Mann, 2005). Re-examination of the original histopathology records is required to determine if the original findings can be confirmed.

Sekijima et al. (1999) used a similar approach in evaluating whether microcystin-LR is a tumor initiator, promoter or both. In their study DEN or Aflatoxin B1 served as initiators. Groups of 5-15 male Fischer 344 rats, 6 weeks of age, received an i.p. injection of 0, 200 mg DEN/kg, or 0.5 mg aflatoxin B_1/kg two weeks before i.p. injections of 0, 1 or 10 µg microcystin-LR/kg twice a week for 6 weeks. Other groups were also treated with aflatoxin B_1 plus DEN before microcystin-LR treatment. A subset of each treatment scenario was given partial hepatectomy one week after initiation of microcystin-LR administration. There was no statistically significant difference in the number of GST-P positive foci and their area for the DEN Control and those that received both 1 µg/kg, and 10 µg/kg microcystin-LR without the hepatectomy. For those that received 10 µg/kg and the hepatectomy, the number and area of foci increased, but were not significantly higher than the DEN control. Combining Aflatoxin B1 with DEN resulted in foci numbers and areas significantly greater that the DEN control. With addition of microcystin-LR at 1 or 10 µg/kg, the number and area of foci increased but the increase was not significant. No foci were observed in livers of animals treated with only microcystin-LR at 10 µg/kg. A combination of Alflatoxin B1 with microcystin-LR µg/kg and no hepatectomy resulted in a small number of foci (0.31 /cm^2 and an area of 0.05 mm^2/cm^2) as compared to the DEN alone control (2.46 /cm^2 and an area pf 13.6 mm^2/cm^2).

6.4 Other Key Data

6.4.1 Mutagenicity and Genotoxicity

The available data on mutagenicity and genotoxicity of cyanobacterial toxins, including microcystins, has been recently reviewed (Žegura et al., 2011). These authors concluded that current evidence indicates that the microcystins are not bacterial mutagens and that discrepancies in results from cyanobacterial extracts are likely due to differences in source of the cyanobacteria and composition of the complex extract mixtures. Both *in vitro* and *in vivo* genotoxicity studies have shown positive results with DNA damage induced by formation of reactive oxygen species as well as inhibition of repair pathways. These studies are summarized below and listed in Tables 6-9, 6-10, and 6-11.

6.4.1.1 Mutagenicity

Ding et al. (1999), and Huang et al. (2007), did not find that pure microcystin-LR induced mutations in the Ames assay (strains TA97, TA98, TA100, and TA102), either with or without metabolic activation. Extracts from *Microcystis* exhibited mutagenic activity in the absence of activation, which was decreased slightly in TA98 with activation. A crude toxin extracted from *M. aeruginosa* did not induce mutations in the Ames assay (strains TA98 and TA100) with and without activation (Grabow et al., 1982). Wu et al. (2006) used three assays (ara test in *E. coli* UC1121, Ames test in *S. typhimurium* strains TA98 and TA100, and SOS/umu test in *S. typhimurium* TA1535/pSK1002) to test the mutagenicity of microcystin-LR extracted from a *M. aeruginosa* bloom. All tests were negative with and without metabolic activation. Repavich et al. (1990) reported that Ames assays (using strains TA98, TA100 and TA102) of a purified hepatotoxin (supplied by Wright State University and presumed to be microcystin) were negative with and without metabolic activation, as were *Bacillus subtilis* multigene sporulation assays.

In contrast, Suzuki et al. (1998) reported increased ouabain resistance mutation frequency in human embryo fibroblast cells treated with microcystin-LR (purity not specified). Similarly, Zhan et al. (2004)

observed a 5-fold increase in the frequency of thymidine kinase mutations when human lymphoblastoid TK6 cells were treated with commercially-obtained microcystin-LR over control. More slow-growing mutants were observed than fast-growing mutants, suggesting that microcystin-LR induced large deletions, recombinations or rearrangements and that the mutation damage was larger than the TK locus.

The differences in mutagenicity response between bacteria and human cell lines may be related to differences in the cell uptake of microcystin-LR. For example, the failure of microcystin-LR to induce mutations in bacterial cells may be related to poor uptake. Zhan et al. (2004) observed that microcystin-LR is not taken up by many cell types, including bacteria. However, no references to support this assertion were provided by the authors. While hepatocytes take up microcystin-LR at a significant rate, other cell types show limited or no uptake unless measures are taken to enhance the penetration of the cells by microcystin-LR.

Table 6-9. Mutagenicity Assays with Microcystins

Species (test system)	End-point	With metabolic activation	Without metabolic activation	Reference
Ames assay	Gene mutation; Pure microcystin-LR; extracts containing microcystins	-	-	Ding et al., 1999; Huang et al., 2007
Ames assay	Gene mutation; Crude extract	-	-	Grabow et al., 1982
Ames assay; ara test; SOS/umu test	Gene mutation; microcystin-LR extract	-	-	Wu et al., 2006
Ames assay	Gene mutation; Purified hepatotoxin assumed to be microcystin	-	-	Repavich et al., 1990
Human embryo fibroblast cells	Gene mutation; microcystin-LR (purity not specified)	Not applicable	+	Suzuki et al., 1998
Human lymphoblastoid TK6 cells	Gene mutation; 5x increased frequency of thymidine kinase mutations; induction of micronuclei	Not applicable	+	Zhan et al., 2004

Shi et al. (2011) showed that microcystin-LR could interact with isolated plasmid DNA (4361 base pairs) using atomic force microscopy combined with UV and fluorescence quenching in the presence of ethidium bromide. The results eliminated the potential for intercalation binding and electrostatic interactions with the DNA phosphate backbone and are most consistent with electrostatic interactions between the microcystin-LR and exposed bases in the minor groove. In the presence of microcystin-LR, the plasmid DNA aggregated into rod-like structures. The authors hypothesized that this might be the result of electrostatic repulsion between the DNA double helix strands because of the interactions with microcystin-LR.

6.4.1.2 Genotoxicity – in vitro studies

Recent studies suggest that apoptosis may be intimately linked to observations of DNA damage in cells treated with microcystin-LR. Lankoff et al. (2004a) showed a strong correlation between DNA damage, as measured by the comet assay, and the induction of apoptosis, as measured by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay, in human lymphocytes. Other evidence has suggested that the comet assay can give a false positive measure of DNA damage when apoptosis is induced, as DNA fragmentation occurs during the process of apoptosis (Lankoff et al., 2004a). The authors postulated that earlier reports of DNA damage measured by the

comet assay may have been related to early stages of apoptosis due to cytotoxicity rather than a direct effect on DNA. The induction of apoptosis appears to be dose-related. Humpage and Falconer (1999) showed that low (picomolar) concentrations of commercially-obtained microcystin-LR induced cytokinesis and inhibited apoptosis in primary mouse hepatocytes, while higher (nanomolar) concentrations resulted in opposite effects. Ding et al. (1999) showed DNA damage in primary rat hepatocytes by the Comet assay at 1 µg microcystin-LR/mL.

Nong et al. (2007) observed a dose-dependent increase in test tail DNA using the Comet assay in HepG2 cells incubated with 1-100 µM microcystin-LR (purity not reported) for 24 hours; the 30 and 100 µM concentrations yielded statistically significant results . Žegura et al. (2006) also found a significant increase in the proportion of tail DNA (indicating DNA damage in the Comet assay) in HepG2 cells incubated with microcystin-LR (purity not reported) for up to 16 hours. Buthionine sulfoximine (BSO) pretreatment increased the susceptibility to microcystin-LR induced DNA damage, while pretreatment with the glutathione precursor N-acetylcysteine protected against the microcystin-LR induced DNA damage.

In a study with a similar design using HepG2 cells, Žegura et al. (2008a) observed elevation of p53 and the down regulated genes p21 and gadd45a, which are responsible for cell cycle arrest and DNA repair, as well as mdm2, which is a feedback regulator for p53 expression and activity. The study authors concluded that these findings indicate that microcystin-LR has genotoxic potential. Žegura et al. (2008b) evaluated the genotoxic effects of microcystin-LR (purity not reported) on different cell types using the Comet assay. Three human cell lines were used: CaCo-2, which is a human colon adenocarcinoma cell line; IPDDC-A2, which is a human astrocytoma cell line; and NCNC, which is a human B-lymphoblastoid cell line. A significant increase in DNA damage was only observed in CaCo-2 cells. Žegura et al. (2011) observed DNA damage using the Comet assay in human peripheral blood lymphocytes at concentrations of 0.1 to 10 µg/mL of microcystin-LR (purity not reported). As was previously observed in HepG2 cells, DNA damage-responsive gene p53 was upregulated along with its downstream-regulated genes involved in DNA repair and cell cycle regulation, mdm2, gadd45a, and p21. DNA fragmentation was significantly increased in rat neutrophils with microcystin-LA and microcystin-YR, but not in human neutrophils (Kujbida et al., 2008).

Bouaïcha et al. (2005) reported that noncytotoxic concentrations of microcystin-LR slightly decreased the amount of endogenously formed DNA adducts compared with controls in cultured hepatocytes. Microcystin-LR was shown to cause a dose- and time-dependent increase in the formation of 8-oxo-7, 8-dihydro-2'-deoxyguanosine (a measure of oxidative DNA damage) in cultured hepatocytes (Maatouk et al., 2004; Bouaïcha et al., 2005).

Lankoff et al. (2004a) observed no effect of microcystin-LR on the incidence of chromosomal aberrations in human peripheral blood lymphocytes. In a separate study by Lankoff et al. (2006a) microcystin-LR inhibited repair of gamma-induced DNA damage in human lymphocytes and a human glioblastoma cell line.

Observations of polyploidy in microcystin-LR-treated cells (Humpage and Falconer, 1999; Lankoff et al., 2003) may be related to its effects on cytokinesis. Lankoff et al. (2003) showed that microcystin-LR, through its effect on microtubules, damages the mitotic spindle, leading to the formation of polyploid cells. Repavich et al. (1990) reported a dose-related increase in chromosome breakage in human lymphocytes exposed to a purified hepatotoxin (presumed to be a microcystin). Microcystin-LR disrupted chromatin condensation in Chinese hamster ovary cells at the end of interphase and the beginning of metaphase (Gácsi et al., 2009).

Neither microcystin-LR nor cyanobacterial extracts resulted in an increase in micronucleus formation in cultured human lymphocytes (Abramsson-Zetterberg et al., 2010).

6.4.1.3 Genotoxicity – in vivo studies

A number of studies have reported DNA damage after microcystin-LR treatment *in vivo*. microcystin-LR was shown to cause a dose- and time-dependent increase in the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (a measure of oxidative DNA damage) in rat liver cells after *in vivo* treatment via i.p. injection (Maatouk et al., 2004; Bouaïcha et al., 2005).

Gaudin et al. (2008) observed DNA damage in female mice administered microcystin-LR (>95% pure) via either oral or i.p. injection. Groups of three female Swiss albino mice were administered a single gavage dose of 0, 2, or 4 mg/kg or a single i.p. dose of 10, 25, 40, or 50 µg/kg and sacrificed 3 or 24 hours after treatment. DNA damage was assessed in whole blood, bone marrow, liver, kidney, colon, and intestine using the comet assay. Clinical observations were not reported. After oral administration, a statistically significant dose-dependent increase in DNA damage was observed in blood from both dose groups at three hours, but not at 24 hours; no effects were seen in the other tissues assayed. After i.p. exposure, DNA damage was found at doses ≥40 µg/kg only in bone marrow after 3 hours; after 24 hours DNA damage was found in kidney, intestine and colon at ≥25 µg/kg with the most pronounced effect being a dose-related increase in the liver at all doses. In contrast, Gaudin et al. (2009) did not find any DNA damage as assessed by the Comet assay and unscheduled DNA synthesis in the livers of female rats administered 12.5-50 µg microcystin-LR/kg (commercial product; purity not reported) via intravenous injection.

Dong et al. (2008) evaluated the genotoxicity of microcystin-LR (source and purity not provided) in mouse testes. Male KM mice were administered 0, 3, 6, or 12 µg/kg of microcystin-LR daily for seven days. Five mice/treatment were sacrificed on day 8 and their testes were removed for analysis. Fourteen days after injection, five mice per treatment were also sacrificed to evaluate the micronuclei in the sperm cell early stage. An increase in micronuclei and DNA-protein crosslinks was observed with all doses (highest dose lower than mid dose, no dose response), but only the 6 and 12 µg/kg treatments were statistically different from controls.

Li et al. (2011b) administered (i.p.) crude extracts from a cyanobacterial bloom containing 244.26 µg microcystin-LR per gram of lyophilized algae to male mice and observed a dose-dependent increase in olive tail moment from the Comet assay in the liver and testes. microcystin-YR has also been found to induce DNA damage measured by the Comet assay in the blood (lymphocytes), liver, kidney, lung, spleen, and brain of mice administered 10 µg/kg of microcystin-YR via i.p. injection every other day for 30 days (Filipič et al., 2007).

Neither microcystin-LR nor cyanobacterial extracts resulted in an increase in micronucleus formation in erythrocytes from peripheral blood of mice given up to 55 µg/kg (Abramsson-Zetterberg et al., 2010). However, Zhang et al. (2011a) observed a significant increase in the frequency of micronuclei in polychromatic erythrocytes (PCEs) in the bone marrow of rabbits (6/treatment group) administered 6 µg/kg-day microcystin from an extract of *M. aeruginosa* via i.p. injection for 7 or 14 days. The microcystin extracts contained ≥80% total microcystin, with 0.84 mg/g dry weight microcystin-RR, 0.50 mg/g dry weight microcystin-LR, and 0.07 mg/g dry weight microcystin-YR. There was also a significant decrease in PCEs/total erythrocytes. Similarly, dose-related increased micronuclei formation were seen in bone marrow from male mice given 1-100 mg extract/kg (Ding et al., 1999).

Table 6-10. Genotoxicity of Microcystins *In vitro*

Species (test system)	End-point	Results	Reference
Primary rat hepatocytes	Liver DNA	DNA damage with microcystin extract containing microcystin-LR.	Ding et al., 1999
Rat hepatocytes	DNA adducts	Noncytotoxic concentrations of microcystin-LR slightly decreased endogenously formed DNA adducts	Bouaïcha et al., 2005
Rat hepatocytes	DNA adducts	microcystin-LR caused oxidative DNA adducts	Maatouk et al., 2004; Bouaïcha et al., 2005
Primary mouse hepatocytes	DNA damage	Commercial microcystin-LR induced cytokinesis and inhibited apoptosis at picomolar concentrations; nanomolar concentrations resulted in inverse effects	Humpage and Falconer, 1999
HepG2 cells	DNA damage	Microcystin-LR increased comet test tail moment.	Nong et al., 2007; Žegura et al., 2006
Human hepatoma cells	Liver DNA and repair	DNA damage with microcystin-LR; elevated p53 and down regulated p21 and gadd45a	Žegura et al., 2003; 2004; 2008a
CaCo-2, IPDDC-A2, and NCNC human cell lines	DNA damage	Microcystin-LR increased DNA damage only in CaCo-2 cells.	Žegura et al., 2008b
Human and rat neutrophils	DNA damage	Microcystin-LA and microcystin-YR increased DNA fragmentation in rat, but not human, neutrophils.	Kujbida et al., 2008
Human lymphocytes	DNA damage	Microcystin-LR caused DNA damage and induction of apoptosis but no chromosome aberrations.	Lankoff et al., 2004a
Human lymphocytes and glioblastoma cell line	DNA damage and repair	No micronuclei formation in lymphocytes; inhibited repair of gamma-induced DNA damage.	Lankoff et al., 2006a
Human lymphocytes	DNA damage	Microcystin-LR caused DNA damage and up regulation of damage-responsive genes	Žegura et al., 2011
Human lymphocytes	DNA damage	Dose-related chromosome breakage.	Repavich et al., 1990
Chinese hamster ovary cells	Cell cycle	Microcystin-LR disrupted chromatin condensation.	Gácsi et al., 2009
Human lymphocytes	Micronucleus formation	No increase with microcystin-LR or extract.	Abramsson-Zetterberg et al., 2010

Table 6-11. Genotoxicity of Microcystins *In vivo*

Species (test system)	End-point	Results	Reference
Mouse	Liver DNA	DNA damage after treatment with microcystin-LR	Rao and Bhattacharya, 1996
Mouse	DNA damage	microcystin-LR caused damage in blood cells after oral; in liver, kidney, intestine, and colon after i.p.; none in liver after i.v.	Gaudin et al., 2008; 2009
Mouse	DNA damage	Microcystin-LR extract i.p. caused dose-dependent olive tail moment in liver and testes.	Li et al., 2011b
Mouse	DNA damage	Microcystin-YR given i.p. induced damage in multiple organs.	Filipič et al., 2007
Mouse	DNA damage	Increased DNA-protein crosslinks and micronuclei in testes with microcystin-LR.	Dong et al., 2008
Rat	Liver DNA	Oxidative damage after i.p. injection of microcystin-LR	Maatouk et al., 2004; Bouaïcha et al., 2005
Mouse bone marrow erythrocytes	DNA damage	Induction of micronuclei with microcystin extract	Ding et al., 1999
Mouse erythrocytes; peripheral blood	DNA damage	No induction of micronuclei with microcystin-LR or extract.	Abramsson-Zetterberg et al., 2010
Rabbit bone marrow	DNA damage	Extract containing microcystin-RR, -LR, and -YR increased frequency of micronuclei in PCEs	Zhang et al., 2011a

6.4.2 Physiological or Mechanistic Studies

6.4.2.1 Noncancer Effects

Mechanistic studies, including *in vivo* investigations in laboratory animals, *in situ* studies in isolated perfused organ systems and *in vitro* assays in isolated cell preparations have been conducted to characterize the toxicology of microcystins. These studies have evaluated many aspects of microcystin toxicity, including: 1) interaction with serine and threonine protein phosphatases (i.e., PP1 and PP2A) as the molecular target for microcystins, 2) the role of cytoskeletal effects, 3) apoptosis, 4) the importance of oxidative stress as a mode of toxic action, and 5) the reasons for target organ and cell type specificity of microcystins. Each of these topics is discussed in further detail below.

6.4.2.1.1 Protein Phosphatase Inhibition

Protein phosphatase enzymes PP1 and PP2A has been identified as the primary molecular target of microcystins. Protein phosphatases function in the post-translational modification of phosphorylated cellular polypeptides or proteins. PP1 and PP2A groupings belong to the PPP family of protein phosphatases, which hydrolyze the ester linkage of serine and threonine phosphate esters. Both enzyme groupings have a single catalytic unit which is joined to a variety of regulatory and targeting subunits. There are approximately 1,000 protein phosphatase genes in higher eukaryotes which confer considerable regulatory diversity to the individual super families (Barford et al., 1998).

The actions of members of the protein kinase family of enzymes precede that of the protein phosphatases because they esterify phosphates to the hydroxyl functional groups of serine, threonine and tyrosine in proteins. Together, kinases and phosphatases maintain the balance of phosphorylation and dephosphorylation for key cellular proteins involved in a variety of activities including transport and secretory processes, metabolic processes, cell cycle control, gene regulation, the organization of the cytoskeleton, and cell adhesion (Barford et al., 1998).

Immunoprecipitation, X-ray crystallography, autoradiography, nuclear magnetic resonance (NMR) solution structures, reverse phase liquid chromatography, and molecular dynamics simulation have been used to evaluate the molecular interaction between microcystins and protein phosphatases (Runnegar et al., 1995b; MacKintosh et al., 1995; Goldberg et al., 1995; Craig et al., 1996; Bagu et al., 1997; Mattila et al., 2000; Mikhailov et al., 2003; Maynes et al., 2004, 2006). Molecular modeling and molecular dynamics simulations have reported that microcystins bind in a Y-shaped groove containing the catalytic site on the surface of PP1 (Mattila et al., 2000). Studies with PP1 suggest that the C-terminal β12-β13 loop of PP1 (with residues 268-281) is important for microcystin-protein phosphatase interactions as well as for substrate recognition (Maynes et al., 2004, 2006). Current information indicates that the binding process primarily involves the amino acids Adda, leucine, Mdha and glutamate of microcystins.

According to Craig et al. (1996), microcystins LR, LA and LL interact with the catalytic subunits of PP1 and PP2A in two phases: the first phase, a rapid inactivation of the phosphatase occurs within minutes; the second, a slower phase represented by a covalent interaction that takes place within several hours. The initial binding and inactivation of protein phosphatases appears to result from several non-covalent interactions that are still under investigation. Mattila et al. (2000) showed an interaction of the glutamate-free carboxylate of microcystin-LR with a metal ion, either iron or manganese, in the PP1 catalytic site. Glutamate appears to be an important component since the esterification of the carboxylate functional group eliminates toxicity (Namikoshi et al., 1993; Rinehart et al., 1994). A review of the mechanisms of microcystin toxicity demonstrated that the Adda side chain may be involved in a hydrophobic interaction between the tryptophan 206 and isoleucine 130 residues in the hydrophobic groove of PP1 (Herfindal and Selheim, 2006).

Microcystins Adda amino acid residue plays an important role in the inhibition of protein phosphatase activity (Nishiwaki-Matsushima et al., 1991; Gulledge et al., 2002, 2003a,b). Mattila et al. (2000) suggested that the long hydrophobic side chain of the Adda residue may guide the toxin into the hydrophobic groove of the catalytic site. The toxic activity of microcystins is eliminated by the isomerization of the diene from 4E,6E to 4E,6Z on the Adda chain (Harada et al., 1990; Nishiwaki-Matsushima et al., 1991; Stotts et al., 1993). Those microcystin analogues with only Adda and one additional amino acid are capable of substantial inhibition of PP1 and PP2A, while modifications to the Adda structure abolished the inhibition (Gulledge et al., 2003b). Herfindal and Selheim (2006) indicated that the L-Leucine of microcystin-LR plays an important role in the hydrophobic interaction with Tyrosine 272 of PP1 (on the β12-β13 loop).

During the second phase of interaction between microcystins and protein phosphatase, covalent bonding occurs (Craig et al., 1996). Immunoprecipitation and autoradiography methods indicate that a covalent bond results from the interaction between the thiol of Cys273 residue of PP1 and the methylene of the Mdha residue of microcystins. X-ray crystallography data on the microcystin-LR/PP1 complex and NMR solution structures illustrate the covalent linkage at Cys273 (Goldberg et al., 1995; Bagu et al., 1997). Site-directed mutagenesis replacing Cys273 in PP1 results in a loss of microcystin binding (MacKintosh et al., 1995; Maynes et al., 2004). Based on sequence similarity between PP1 and PP2A, Craig et al. (1996) suggested that Cys-266 is the site of a covalent linkage between PP2A and microcystins.

Microcystin analogues with a reduced Mdha residue are not able to covalently bind to protein phosphatases. MacKintosh et al. (1995) indicated that a modification of the Mdha residue of microcystin-

YR by reaction with ethanethiol, abolished covalent binding to PP1. Similarly, Craig et al. (1996) showed that a decrease of the Mdha residue of microcystin-LA with $NaBH_2$ abolished the covalent binding phase with PP2A. Maynes et al. (2006) confirmed the lack of covalent interaction by showing the crystal structure of dihydromicrocystin-LA bound to PP1. Their work demonstrated that the β12-β13 loop of PP1 has a different conformation when the covalent bond is absent, and that other interactions (including hydrogen bonding) are responsible for the bond between dihydromicrocystin-LA and PP1.

The relevance of covalent bonding between microcystins and protein phosphatases to enzyme inhibition is unknown because other interactions are apparently responsible for the rapid inactivation of the enzymes (Herfindal and Selheim, 2006). The modifications to either molecule (microcystin or protein phosphatase) to prevent covalent bonding, usually decrease but do not eliminate the toxic action (Meriluoto et al., 1990; MacKintosh et al., 1995; Hastie et al., 2005).

Under both *in vivo* and *in vitro* conditions, microcystins bind to the phosphatase enzymes, resulting in an inhibition of enzyme activity and leading to a decrease in protein dephosphorylation. Microcystins have been shown to directly inhibit the activity of PP1 and PP2A derived from different species such as fish, mammals, plants, and different cell types (cultured cell lines as well as isolated tissue cells) (Honkanen et al., 1990; MacKintosh et al., 1990; Matshushima et al., 1990; Yoshizawa et al., 1990; Sim and Mudge, 1993; Xu et al., 2000; Leiers et al., 2000; Becchetti et al., 2002). Microcystin has also been related to binding to PP4, another member of the protein phosphatase family (Imanishi and Harada, 2004).

Ito et al. (2002b) observed a similar degree of inhibition of protein phosphatases 1 and 2A *in vitro* with microcystin-LR and its glutathione and cysteine conjugates. However, Metcalf et al. (2000) demonstrated weaker inhibition of PP1 and PP2A *in vitro* by microcystin glutathione, cysteine-glycine, and cysteine conjugates than by parent microcystins; these conjugates also are less toxic in the mouse bioassay than parent microcystin. As noted in Section 5.3, Kondo et al. (1992, 1996) postulated that the Adda and Mdha moieties could be the sites of CYP oxidation and subsequent conjugation with glutathione or cysteine.

Microcystins have been used as a tool to investigate the importance of serine and threonine phosphorylation in specific cellular functions. The regulatory effects of phosphorylation on the sodium channel proteins increases the probability of the channel being open in renal cells (Becchetti et al., 2002). Phosphorylation appears to inhibit ATP-dependent actin and myosin interaction in smooth and skeletal muscle contraction (Hayakawa and Kohama, 1995; Knapp et al., 2002) and increase insulin secretion (Leiers et al., 2000).

Several *in vitro* studies indicate that low levels of microcystins can upregulate protein phosphatase mRNA expression such that protein phosphatase activity is increased rather than decreased. Liang et al. (2011) used the FL amniotic epithelial cell line to test the effects of microcystin-LR and found that incubation for 6 hours with low concentrations of microcystin-LR (0.5 or 1 μM) caused increases in PP2A activity. However, incubation for 24 hours with higher concentrations (i.e., 5 or 10 μM) caused a decrease in PP2A activity. The authors reported that the increases in PP2A activity were due to the up-regulation of mRNA and protein levels of the C subunit. Fu et al. (2009) and Xing et al. (2008) found comparable up regulation of PP2A in FL cells at comparable concentrations and incubation times. However, no change in PP2A activity or in PP2A subunit expression was observed by Huang et al. (2011) in the livers of male mice after 7 days of oral exposure with doses up to 186 μg microcystin-RR/kg.

Li et al. (2011d) tested the effects of microcystin-LR on PP2A in human embryonic kidney (HEK) 293 cells. PP2A activity was inhibited with concentrations of 5-10 μM (only significantly inhibited with 7.5 and 10 μM), and increased at concentrations ≤2.5 μM (only statistically significant with 1 and 2 μM). Treatment with microcystin-LR caused a disassociation between PP2A and its α4 regulatory subunit, at all concentrations tested. The study authors suggested that disassociation of α4, a PP2A subunit that regulates activity of PP2A leading to an increase in active PP2A catalytic subunit in the cell, could

explain the higher activity at low concentrations. At higher concentrations the increase in the PP2A catalytic unit is unable to compete with the inhibitory effects of microcystin-LR.

Not all microcystins are equipotent inhibitors of protein phosphatases. Table 6-12 provides comparative data of the IC_{50} values for inhibition of protein phosphatases (IC_{50}s) by microcystin-LR, microcystin-YR, microcystin-RR and microcystin-LA as reported by several different authors. The table demonstrates that there is not much consistency in the results. Differences across studies are likely due to variations in methodology of the individual studies. There is also a lack of consistency in the relative potencies of individual microcystins across the individual studies.

Table 6-12. Protein Phosphatase Inhibition Activity Among Microcystin Congeners

Reference	IC_{50} (nM)			
	MC-LR	MC-LA	MC-YR	MC-RR
PP2A Inhibition				
Craig et al., 1996	0.15	0.16		
Nishiwaki-Matsushima et al., 1991	0.28			0.78
Matsushima et al., 1990	7.6		4.5	5.8
PP1 Inhibition				
MacKintosh et al., 1995	0.2		0.2	
Mixture of PPs				
Yoshizawa et al., 1990	1.6		1.4	3.4

6.4.2.1.2 Cytoskeletal Disruption

Protein phosphatase inhibition by microcystins relates to changes in cytoskeletal structure and cell morphology (Eriksson and Golman, 1993). The cytoskeleton is comprised of a variety of polymeric, proteinaceous filaments that form a flexible framework for the cell. The cytoskeleton provides attachment points for organelles within cells, and makes possible communication between parts of the cell and between cells (Sun et al., 2011). The major cytoskeletal proteins can be broadly categorized (Hao et al., 2010) as microfilament proteins (e.g. actins and myosin; 7 nm diameter), intermediate filaments (e.g. keratins, desmins; 10 nm diameter), and microtubules (e.g. dyneins, tubulin; 25 nm diameter). In addition, there are a broad number of individual proteins that are associated with the microtubules and microfilaments. Serine-threonine proteases are of critical importance in maintaining cytoskeletal integrity (Eriksson et al., 1992 a,b) because of their dephosphorylating impact on phosphoprotein-cytoskeletal precursors.

Several studies using light, electron and fluorescent microscopy have demonstrated the cytoskeletal effects of microcystins in the liver (Runnegar and Falconer, 1986; Eriksson et al., 1989b; Hooser et al., 1989, 1991b; Falconer and Yeung, 1992). Ultrastructural changes in rats given a lethal dose of microcystin include:

- breakdown of the endothelium;
- loss of microvilli in the space between the hepatocytes and sinusoids (known as the Space of Disse);
- progressive cell-cell disassociation followed by rounding, blebbing and invagination of hepatocytes;
- a widening of intracellular spaces;
- hemorrhage; and
- loss of lobular architecture (Hooser et al., 1989).

No toxicity effects were noted in liver endothelial cells or Kupffer cells. Other studies of isolated hepatocytes, actin aggregates were seen at the base of the membrane blebs following microcystin exposure. As membrane blebs grew larger and were drawn toward one pole of the cell, the microfilaments were organized toward the same pole, resulting in rosette formation with a condensed band of microfilaments at the center.

Similar histopathological changes in the rat testes have been described by Chen et al. (2013). Repeated i.p. dosing showed an increased space between the seminiferous tubules, cell membrane blebbing, cytoplasmic shrinkage, swollen mitochondria, and deformed nuclei. The transcriptional levels of β-actin and β-tubulin were also significantly decreased.

Studies in primary isolated hepatocytes have demonstrated the morphological and histopathological changes induced by microcystins that relate to loss of sinusoidal architecture and cytotoxicity (Runnegar et al., 1981; Runnegar and Falconer, 1982; Aune and Berg, 1986; Ding et al., 2000a). Exposure of microcystin to hepatocytes in suspension or cultured in a monolayer results in membrane blebbing that becomes more pronounced and localized in one region of the cell surface. Cells are rounded in appearance and become dissociated from one another. Microfilaments are reorganized as a compact spherical body in the vicinity of the blebbing, and the rest of cell is depleted of filamentous actin. Microcystin-LR disrupts hepatocellular morphology within minutes, leading to loss of sinusoidal architecture and hemorrhage. Morphological changes in hepatocytes (i.e., blebbing, rounding) occurred prior to any effect on cell viability (generally measured as decreased trypan blue exclusion) or cell membrane integrity (measured as LDH leakage or release of radiolabeled adenine nucleotides).

Thompson et al. (1988) described the time course of cellular effects of microcystins (type not specified) on primary cultures of rat hepatocytes. The cells were isolated, attached in a monolayer, treated with 0.001-10 μg/mL of microcystin, and then monitored for 24 hours. After 15 minutes, disintegration of the attachment matrix occurred at the highest microcystin concentration. After one hour, cells clustered in groups with no extracellular material. Between 2 and 4 hours, cells began to release from the plates. After these visual effects occurred, LDH was released and was concentration-related.

Several studies have demonstrated that the observed reorganization of microfilaments leading to alteration of hepatocyte morphology does not appear to be due to effects on actin polymerization (Runnegar and Falconer, 1986; Eriksson et al., 1989b; Falconer and Yeung, 1992). Instead, microcystins caused a decrease in the dephosphorylation of cytokeratin intermediate filament proteins (Falconer and Yeung, 1992; Ohta et al., 1992; Wickstrom et al., 1995; Blankson et al., 2000). Toivola et al. (1997) studied the effects of microcystin-LR on hepatic keratin intermediate filaments in primary hepatocyte cultures. The authors observed a disruption of the desmoplakin, a cytoskeletal linker protein that connects an intermediate filament to the plasma membrane, followed by a dramatic reorganization of the intermediate filament and microfilament networks, resulting in intermediate filaments being organized around a condensed actin core.

The authors observed that the major target proteins for microcystin-induced hyperphosphorylation include keratins 8 and 18 and desmoplakin I/II. Keratins 8 and 18 are the major proteins of intermediate filaments in hepatocytes; desmoplakin I and II attach keratin filaments in epithelial cells to desmosomes, (complexes of adhesion proteins that function in cell to cell adhesion). Hyperphosphorylation of desmoplankin I/II leads to loosening of cell junction and loss of interactions with cytoplasmic intermediate filaments. The hyperphosphorylation of keratin proteins prevents subunit polymerization resulting in the observed morphological changes. A Ca^{2+}/ calmodulin-dependent kinase may be involved in regulating the serine-specific phosphorylation of keratin proteins 8 and 18. Kinase-induced phosphorylation in the absence of phosphatase dephosphorylation leads to the disassembly of the microfilaments, breakdown of the cytoskeleton and it's anchoring to desmoplankin I and II (Toivola et al., 1998).

An *in vitro* study investigated the cell-type specificity of the effects caused by microcystin using isolated rat hepatocytes, rat skin fibroblasts (ATCC 1213) and rat renal epithelial cells (ATCC 1571) (Khan et al., 1995; Wickstrom et al., 1995). After exposure to microcystin-LR, the time course of light microscopic and ultrastructural effects was examined (Khan et al., 1995). After 4 minutes, effects were noted in hepatocytes, in renal cells after 1 hour, and in fibroblasts after 8 hours. Similar lesions observed in all cell types included cytoplasmic vacuolization, blebbing, clumping and rounding, loss of cell-cell contact, and redistribution of cellular organelles. Effects that were seen only in hepatocytes include whirling of rough ER, dense staining, loss of microvilli, and dilated cristae of mitochondria plus pinching off of membrane blebs.

Meng et al. (2011) demonstrated that microcystin-LR causes reorganization of the cytoskeletal structure in the neuroendocrine PC12 cell line. Pretreatment with a p38 MAPK inhibitor blocked the cytoskeletal alterations as well as the hyperphosphorylation of tau and HSP27. According to the study authors, direct PP2A inhibition by microcystin-LR and indirect p38 MAPK activation may be responsible for the hyperphosphorylation of tau and HSP27 causing cytoskeletal disorganization.

In addition, Sun et al. (2011) evaluated the effects of microcystin-LR on cultures of a human liver cell line (HL7702). As was the case for the PC12 cell line, hyperphosphorylation of Heat Shock Protein 27 in the presence of microcystin as a phosphatase inhibitor was accompanied by increased activity of several kinases (p38 MAPK, JNK and ERK1/2) leading to cytoskeleton reorganization. Treatment with kinase inhibitors reduced the cytoskeletal changes (Sun et al., 2011). Taken together these studies implicate kinase-induced phosphorylation combined with inhibition of phosphatase removal of key phosphate moieties from serine or threonine esters as the cause of the cytoskeletal changes. When microcystins are present *in vitro*, balance can be partially restored by inhibiting the activity kinases.

The effects of exposure intravenously to microcystin-LR (purified from a bloom) on transcription of cytoskeletal genes of rats were reported by Hao et al. (2010). The authors observed alterations in transcription of genes for tubulin, actin, an intermediate filament (vimentin), and six associated proteins (ezrin, radixin, moesin, MAP1b, tau and stathmin) in the liver, kidney, and spleen. Ezrin, moesin, and stathmin are tumor-associated genes which may contribute to tumor promotion by microcystins.

The direction and degree of the cytoskeletal protein change depended on time of measurement after exposure and the organ examined. The effects were most pronounced in the liver. Although there were numerous changes that occurred in the transcription of the nine cytoskeletal genes, only a few of the changes were directly correlated with the levels of microcystin in the tissue. Alterations in the transcription such as an increase of actin, ezrin, and radixin, and a decrease of tau in the liver were correlated with tissue microcystin levels (microcystin-LR, microcystin-RR, and total levels). Other apparent trends included a steady increase in vimentin and MAP1b in the liver over time, followed by progressively lower levels. The levels of tubulin and stathmin in the liver were below control levels by the end of the experiment.

As explained above, the responses in the liver differed from those in other tissues. In the kidney, increased transcription of stathmin was significantly correlated with levels of microcystin-RR. In the spleen, a decrease in transcription of radixin was significantly correlated with the levels of microcystin-RR or total microcystin. The levels of actin at the time of the final measurement were lower than the control in both the kidney and the spleen.

6.4.2.1.3 Apoptosis

The ultrastructural changes in hepatocytes observed after exposure to microcystin suggest that cell death is related to apoptosis and not necrosis. Changes include cell shrinkage (decreased volume and increased density), condensation of chromatin and segregation of organelles separated by apoptotic microbodies. As discussed in the previous section, the cytoskeletal damage may be related to these changes (Boe et al., 1991; Fladmark et al., 1998; McDermott et al., 1998; Ding et al., 2000b; Mankiewicz et al., 2001). Several studies have investigated the effects of microcystins on signaling pathways involved in rapid apoptosis (Ding et al., 1998a,b, 2000b, 2001, 2002; Ding and Ong, 2003; Huang et al., 2011; Feng et al., 2011; Ji et al., 2011).

In an abstract of a non-English publication by Lei et al. (2006), rates of apoptosis were approximately 22-29% in L-02 cells (a hepatic cell line) after incubation with different concentrations of microcystin-LR for 36 hours. However, after 60 hours of treatment with 50 μg/mL of microcystin-LR, the rates of apoptosis increased to 80%. ROS levels also increased in a time-dependent manner (from 0.5-12 hours) in male mice after a single i.p. injection of 55 μg microcystin-LR/kg (purity not reported). After exposure of male mice orally to microcystin-RR, apoptosis occurred in the liver (Huang et al., 2011). Reported changes in protein expression including decreased Bcl-2 (an antiapoptotic regulator) and increased Bax (a proapoptotic regulator), lead to a significant increase in the ratio of Bax/Bcl-2. These changes are suggestive of altered regulation of the outer mitochondrial membrane apoptosis channel proteins (Campos and Vasconcelos, 2010).

Botha et al. (2004) indicated that apoptosis and oxidative stress can be induced in nonhepatic cells by microcystins. Microcystin-RR changed the concentration of several proteins associated with apoptosis in FL human amniotic epithelial cells (Fu et al., 2009). LDH leakage and increased apoptotic indices were observed in the human colon carcinoma cell line (CaCo2) and MCF-7 cells (deficient in pro-caspase-3), accompanied by increased hydrogen peroxide formation and increased calpain activity. Apoptosis was also observed in testes cells by Chen et al. (2011) in male mice orally administered low doses of microcystin-LR. Wang et al. (2013) showed apoptosis in testes of mice given ≥7.5 μg microcystin-LR/kg by i.p. injection; mRNA expression for Bax, capsase 3 and capsase 8 were upregulated and increased phosphorylation of p53 and Bcl-2 was noted. Zhang et al. (2011b) also observed apoptosis in isolated rat Sertoli cells incubated with 10 μg/mL of microcystin-LR for 24 hours. Accompanying this were increases in p53, Bax, and caspase-3, and a decrease in Bcl-2. After 48 hours of exposure to microcystin-LR, Gácsi et al. (2009) observed a dose-dependent increase in apoptosis in Chinese hamster ovary cells. Ji et al. (2011) also observed apoptosis *in vitro* with a rat insulinoma cell line exposed to microcystin-LR for 72 hours.

Microcystins have been shown, both *in vitro* and *in vivo* studies, to increase the pro-apoptotic Bax and Bid proteins, and the expression of p53, and to decrease expression of the anti-apoptotic Bcl-2 protein in rats (Fu et al., 2005; Weng et al., 2007; Xing et al., 2008; Takumi et al., 2010; Huang et al., 2011; Li et al., 2011c) as well as change mRNA levels (Lei et al., 2006; Žegura et al., 2008a; Qin et al., 2010; Žegura et al., 2011; Li et al., 2011c). The same concentrations of microcystin-LR that induced Fas receptor and Fas ligand expression (a critical step in inducing apoptosis), were found to induce apoptosis in HepG2 cells, at both the protein and mRNA level (Feng et al., 2011). Also, microcystin-LR induced nuclear translocation and activation of the p65 subunit of NF-κB, a signal transduction protein that controls a number of cellular processes, many linked to inflammation and apoptosis (Feng et al., 2011). The knock-down of p65 in HepG2 cells resulted in a reduction in microcystin-LR-induced Fas receptor and Fas ligand expression and reduced apoptosis, suggesting that microcystin-LR-induced apoptosis is a complex process involving many cellular signaling proteins.

Opening of the mitochondrial permeability transition (MPT) pores, thereby increasing permeability, is considered to be a critical rate-limiting event in apoptosis. Ding and Ong (2003) observed an early surge of mitochondrial Ca^{2+} in cultured hepatocytes prior to MPT and cell death. Prevention of this Ca^{2+} surge by either chelation of intracellular Ca^{2+}, blockage of the mitochondrial Ca^{2+} uniporter or use of a mitochondrial uncoupler, prevented MPT and cell death. Electron transport chain inhibitors including rotenone, actinomycin A, oligomycin or carbonyl cyanide m-chlorophenylhydrazone, also inhibited the onset of MPT. Microcystin-LR caused the release of cytochrome c through MPT, considered as a universal step in mitochondrial apoptosis. However, caspases-9 and -3, which are also linked to apoptosis, were not activated. After exposure to microcystins, the increase in intracellular Ca^{2+} may instead facilitate the activation of calpain, a calcium- dependent protease (Ding and Ong, 2003).

In an English abstract from a Chinese-language publication by Liu et al. (2011), i.p. administration of 50 µg microcystin-LR/kg to mice caused an increase in ALT, AST, Bcl-2 protein, and liver ROS levels; a decrease in mitochondria membrane potential; and a significant DNA ladder indicative of apoptosis. Administration of a MPT inhibitor, cyclosporin A, 1 hour before injection of microcystin-LR blocked the effects. The study authors concluded that inhibiting MPT inhibited microcystin-LR-induced apoptosis. Mitochondrial respiration was decreased in primary hepatocytes and isolated kidney mitochondria incubated with microcystin-LR (Jasionik et al., 2010; La-Salete et al., 2008). An uncoupling effect on the mitochondria was observed in both studies, as well as an indication of mitochondrial generated ROS.

In a study by Qin et al. (2010), the role for the endoplasmic reticulum stress pathway is also implicated in microcystin-LR-induced liver apoptosis in male ICR mice treated i.p. with 20 µg/kg (≥ 95% pure). After measuring mRNA and protein levels of endoplasmic reticulum stress-specific molecules in the liver and kidney, the authors found an increase in mRNA and protein expression of CHOP (an apoptosis linked protein) and cleaved capase-12 in the liver where apoptotic cells also were noted. In the kidney, only a slight inhibition of these proteins and no apoptosis was observed. The authors concluded that Bcl-2 was down-regulated in the liver and slightly up-regulated in the kidney. Xing et al. (2008) also observed regulation of CHOP in cells incubated for 24 hours with microcystin-LR.

6.4.2.1.4 Reactive Oxygen Generation Cellular Response

Oxidative stress may play a role in the induction of MPT and the onset of apoptosis. In cultured hepatocytes exposed to microcystins, an increase in the generation of ROS preceded the onset of MPT, mitochondrial depolarization, and apoptosis. A dose- and time-dependent increase in ROS and lipid peroxidation, measured as malondialdehyde formation, was shown to precede morphological changes in hepatocytes and release of LDH. The addition of deferoxamine or cyclosporine A inhibited the formation of ROS and delayed the onset of MPT and cell death. Addition of superoxide dismutase prevented collapse of the cytoskeleton and release of LDH from isolated hepatocytes. Ding et al. (2001) showed that generation of superoxide and hydrogen peroxide radicals preceded microfilament disorganization and cytotoxicity. Hepatocellular glutathione levels were affected by microcystins, and administration of N-acetylcysteine was shown to protect against cytoskeletal alterations (Ding et al., 2000a).

Lipid peroxidation in the liver of male mice was observed after 2 hours of exposure to a single i.p. injection of 55 µg microcystin-LR/kg (purity not stated) (Wei et al., 2008). The effects of microcystin-LR on ROS and enzyme activities indicated that microcystin-LR-induced liver injury in mice begins with the production of ROS, which stimulated the sustained activation of c-Jun N-terminal protein kinase (JNK) as well as AP-1 and Bid, changes that lead to mitochondrial dysfunction followed by apoptosis and oxidative liver injury.

The role of glutathione homeostasis and lipid peroxidation in microcystin-induced liver toxicity have been examined in several studies (Runnegar et al., 1987; Eriksson et al., 1989b; Bhattacharya et al., 1996; Ding et al., 2000a; Towner et al., 2002; Gehringer et al., 2003a,b, 2004; Bouaïcha and Maatouk, 2004).

Ding et al. (2000a) indicated that exposure to microcystin in isolated hepatocytes resulted in an initial increase in glutathione synthesis followed by a later depletion of glutathione. Gehringer et al. (2004) suggest that increased lipid peroxidation induced by microcystins is accompanied by an increase in glutathione peroxidase, transcriptional regulation of glutathione-S-transferase and glutathione peroxidase and *de novo* synthesis of glutathione. An intravenous LD_{50} (87 µg microcystin-LR equivalents/kg) of a crude microcystin extract resulted in a general suppression of GSTs (14 GST isoforms were measured) in both liver and testes of male rats (Li et al., 2011e). Bouaïcha and Maatouk (2004) found that 2 ng/mL of microcystin-LR in primary rat hepatocytes caused an initial increase in ROS formation and an increase in glutathione. The antioxidants, vitamin E, selenium, silymarin, and glutathione provided some protection against liver toxicity and lethality from microcystins in mice (Hermansky et al., 1991; Gehringer et al., 2003a,b).

Moreno et al. (2005) reported, in both the liver and kidney of rats treated intraperitoneally with single doses of microcystin-LR, significant reductions in glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase, along with increases in lipid peroxidation. Glutathione reductase, SOD, glutathione peroxidase, and catalase were significantly decreased while nitric oxide synthetase activity was significantly increased in both the liver and kidney of male mice administered i.p. injections of 25 µg microcystin-LR/kg (purified from a bloom of *M . aeruginosa*) every other day for a month (Sedan et al., 2010). Increases in MDA (a measure of lipid peroxidation) in the livers of mice administered crude extracts containing microcystin-LR (estimated dose 2.9 µg microcystin-LR/kg) by i.p. injection for 21 days were reported by Li et al. (2011b). The lower doses applied in the study (0.73 and 1.5 µg/kg) did not significantly increase MDA levels. There was also no change in SOD in these animals, but there was a significant decrease in catalase.

Some studies report the absence of lipid peroxidation during microcystin-induced hepatotoxicity. In liver slices exposed to a cell extract (concentration not given), a time-dependent leakage of LDH, ALT and AST was observed with no change seen in glutathione content or lipid peroxidation (Bhattacharya et al., 1996). In addition, Runnegar et al. (1987) suggested that glutathione depletion did not occur until after morphological changes (i.e., blebbing) were observed suggesting that ROS may not be the initiating factor for the cytoskeletal changes. This suggestion is supported by Eriksson et al. (1989b) who concluded that rapid deformation of isolated rat hepatocytes by microcystin-LR was not associated with alterations in glutathione homeostasis.

Liu et al. (2010) demonstrated that lipid peroxidation was induced in the testes of immature male rabbits with a single i.p. injection of 12.5 µg microcystin-LR equivalents/kg of a crude extract. Other indicators of oxidative stress identified were increased hydrogen peroxide, increased catalase, SOD, glutathione peroxidase, GST, and GSH.

6.4.2.1.5 Target Organ/Cell Type Specificity

Liver

Most oral and injection studies in laboratory animals have demonstrated that the liver is a primary target organ for microcystin toxicity. Mechanistic studies suggest that the target organ specificity is directly related to the limited ability of microcystins to cross cell membranes in the absence of an active transport system (see section 6.2). Liver toxicity produced by *in vitro* or *in vivo* exposures to microcystins was reduced or eliminated by inhibition of hepatocellular uptake using OATp transport inhibitors (e.g., antamanide, sulfobromophthalein and rifampicin) and bile salts (i.e., cholate and taurocholate). Lu et al. (2008) used OATp1b2 null mice to demonstrate the importance of the OATp system for transporting microcystin-LR into the liver.

Toxicological effects of microcystins in the isolated perfused rat liver were similar to those demonstrated following *in vivo* exposure (Pace et al., 1991). During a 60-minute exposure, microcystin-LR caused liver

engorgement and cessation of bile flow. Electron microscopy revealed loss of sinusoidal architecture, dilation of bile canaliculi and the space of Disse and decreased intracellular contact. Mitochondrial swelling, disruption of endoplasmic reticulum and formation of whorls and loss of desmosomal intermediate filaments were also observed. Mitochondrial function was impaired, with inhibition of stage 3 respiration and a decrease in the respiratory control index.

Runnegar et al. (1995b) demonstrated cessation of bile flow, increased perfusion pressure, decreased protein secretion and decreased glucose secretion following exposure to microcystins. Histological changes included hepatocyte swelling, loss of sinusoidal architecture, pyknotic nuclei and extensive necrosis. Exposure to high concentrations of toxin extracts in the isolated perfused liver produced loss of cord architecture due to hepatocyte disassociation, membrane damage, cytolysis and nuclear effects (pyknosis, karyokinesis, and karyolysis) (Berg et al., 1988). Ultrastructural effects included swollen mitochondria, vacuoles, necrosis, abnormal nuclei, bile canaliculi lacking microvilli, and whorls of rough endoplasmic reticulum.

Studies (Runnegar et al., 1981; Runnegar and Falconer, 1982; Aune and Berg, 1986; Ding et al., 2000a) show that microcystin exposure to hepatocytes in suspension or cultured in a monolayer results in membrane blebbing that becomes more pronounced and localized in one region of the cell surface. Morphological changes in hepatocytes (i.e., blebbing, rounding) have been shown to occur prior to any effect on cell membrane integrity (measured as LDH leakage or release of radiolabeled adenine nucleotides) or cell viability (generally measured as decreased trypan blue exclusion).

Similar toxicological effects were observed in isolated human hepatocytes (Yea et al., 2001; Batista et al., 2003; Thompson et al., 1988). Microcystin-LR produced blebbing, fragmentation and hepatocyte disassociation. Cytotoxicity, as measured by LDH leakage, occurred after morphological changes were evident. Yea et al. (2001) indicated that cytotoxicity in human hepatocytes was observed at a concentration (1 μM) that did not affect rat hepatocytes. Batista et al. (2003) also reported a slightly higher susceptibility to microcystin-induced morphological change in human hepatocytes as compared to rat hepatocytes. Thompson et al. (1988) described the disintegration of the attachment matrix after 15 minutes, followed by cells clustered in groups with no extracellular material at 1 hour and release of cells from plates between 2 and 4 hours. LDH release did not occur until after these visual effects and was dose-related when measured.

After incubation with microcystin-LR in the range of 0.1-50 nM, inhibition of mitochondrial respiration occurred in primary hepatocytes (Jasionek et al., 2010). The authors indicated changes in ATP levels and mitochondrial uncoupling, suggesting that microcystin-LR may target electron transport chain (ETC) complex I function. At noncytotoxic concentrations in HepG2 cells, microcystins interfered with the metabolism of amino acids, lipids, carbohydrates, and nucleic acids (Birungi and Li, 2011).

Kidney

Nobre et al. (1999, 2001) used an isolated perfused kidney model to evaluate the kidney toxicity of 1 μg microcystin-LR/mL. The authors found that microcystin-LR produced vascular, glomerular and tubular effects in the exposed kidney. An increase in perfusion pressure was followed by an increase in the glomerular filtration rate (GFR), increased urinary flow rate and a reduction in tubular transport at the proximal tubules. Protein in the urinary spaces, although not further described, was observed after histopathological evaluation. Dexamethazone and indomethacin antagonize the effects of microcystin-LR, possibly by blocking the microcystin-LR-induced activation of phospholipase A_2 and cyclooxygenase. Nobre et al. (2003) used rat peritoneal macrophages exposed to microcystin-LR in the isolated perfused kidney model to further investigate the role of inflammatory mediators. The authors observed that macrophage supernatants from exposed rats caused an increase in renal vascular resistance, GFR and urinary flow and reduced Na^+ transport. These effects were reduced by cyclohexamide, dexamethasone

and quinacrine, indicating the involvement of phospholipase A_2 and other inflammatory mediators in microcystin-induced kidney toxicity.

A chronic study performed in male Wister rats with low doses of microcystin-LR and microcystin-YR reported damage to the kidney cortex and medulla (Milutinovik et al., 2002, 2003). For 8 months, the authors injected 10 mg/kg i.p. of microcystin-LR and microcystin-YR every second day and found numerous glomeruli collapsed and the renal tubules filled with eosinophilic protein casts. The tubuli of the outer and inner medulla were dilated and the lumens filled with eosinophilic proteinaceous casts, which were described and likely composed of congregated actin filaments. The authors concluded that their results were consistent with microcystin impact on the cytoskeleton as result on PP2 inhibition. Tubular cells displayed evidence of both apoptosis and necrosis. A TUNEL assay showed DNA damage in both the kidney cortex and medulla. Microcystin-LR induced more sever pathological changes than those induced by microcystin-YR. The authors concluded that long-term microcystin exposures presented a risk for kidney damage with functional consequences.

Alverca et al. (2009) evaluated the effects of microcystin-LR (>85% pure, extracted from *M. aeruginosa* isolated from a bloom) on a kidney cell line (Vero-E6). The viability of the cell line decreased in a time and dose-dependent manner affecting the cell morphology, with enlarged lysosomes, lysosomal leakage, damage to mitochondrial structure, disassembly of actin filaments, reduction in the number of intact lysosomes, and shortening or disappearance of stress fibers observed. Swelling of the endoplasmic reticulum cisterna, Golgi apparatus vacuolization and a dose- and time related increase in apoptotic cells were also observed.

Testes

The testes are another target organ for microcystin in *in vivo* studies on male mice or rats (Li et al., 2008; Liu et al., 2010; Chen et al., 2011; Wang et al., 2012; Ding et al., 2006; Li et al., 2011b). With the exception of the Chen et al. (2011) study, dosing was by i.p. administration. The effects of a single i.p. injection of microcystin extracts from a surface bloom containing 167.7 µg microcystin-RR/mL and 47.0 µg microcystin-LR/mL or 80.5 µg microcystin-LR equivalents/mL was found to have an effect on male rabbit testes. Lesions, including a variety of histological changes to both spermatogonia and Sertoli cells, were seen in animals treated with 12.5 µg microcystin-LR equivalents/kg; recovery occurred by 48 hours with the tissue resembling the control (Liu et al., 2010). Apoptosis has been observed in the testes of rats and mice given microcystin-LR (Chen et al., 2011; Wang et al., 2013) accompanied by changes in expression of apoptosis-related genes (Zhang et al., 2011b; Wang et al., 2013).

The *in vitro* toxicity of microcystins to Leydig cells and Sertoli cells, demonstrated by decreased cell viability (Li et al., 2008; Li & Han, 2012; Zhang et al., 2011b), suggests that microcystin uptake by the testes may be similar to that by the liver. OATps (OATp 1A4, 1A5, 2A1, 2B1, 3A1, 6A1, 6B1, 6C1, and 6D1) are active in the testes (Klaassen and Aleksunes, 2010; Svoboda et al., 2011) although no studies have been located addressing their specific contribution to the testicular toxicity of microcystins. Augustine et al. (2005) found that OAT3 expression in Sertoli cells and the testes was similar to, or exceeded, that found in the liver.

Zhou et al. (2012) investigated whether the target membrane transporters could deliver microcystin-LR to the spermatogonia. The authors isolated mRNA from rat spermatogonia using PCR expansion of the mRNA pool and primer sequences for OATp 1A4, 1A5, 2B1, 3A1, 6B1, 6C1, and 6 D1. Cultured cells were exposed to concentrations of 0, 0.5, 5, 50, or 500 nmol microcystin-LR/L and spermatogonia were isolated from the testes of 9-10 day old male rats and cultured for examination. A significant concentration-related decline in cell viability at concentrations ≥ 5 nmol/L was observed. Microcystin entry into the cell was demonstrated using gel electrophoreses to separate the proteins combined with targeted Western Blot analysis. Five OATps (1A5, 3A1, 6B1, 6C1, and 6D1) were identified in the spermatogonia. Microcystin-LR affected the testicular and spermatogonia expression of all the identified

OATps, especially that for OATp131. Cellular apoptosis, as determined using flow cytometry, increased at concentrations ≥ 50 nmol/L. After a 6 hour exposure to microcystin-LR, a decrease in total antioxidant capacity as reflected in increased mitochondrial membrane potential, ROS, and free Ca^{2+} was observed. The authors hypothesized that the microcystin-LR inhibited PP1 and PP2 causing oxidative stress and cytotoxicity, thus impacting sperm production. Members of the protein phosphatase PP1 and PP2 families along with PP-associated proteins have been identified in testes and sperm, some localized to the sperm head and others to the tail (Mishra et al., 2003; Fardilha et al., 2013).

To analyze the acute effects of microcystin-LR on gene expression and reproductive hormone levels in male BALB/c mice, Wang et al. (2012) administered by i.p. 0, 3.75, 7.5, 15, or 30 μg/kg of microcystin-LR (purity not reported) for 1, 4, 7, or 14 days. Over the 14 days, the animals in the 15 and 30 μg/kg groups lost weight resulting in significantly lower body weight by the end of treatment. No effect on the expression of Kisspeptin-1 (Kiss-1 which stimulates the reproductive system), GPR54 (a Kisspeptin receptor), gonadotropin releasing hormone receptor (GnRHR), FSH receptor (Fshr), or luteinizing hormone receptor (Lhr) was observed. However, after 1, 4, 7, and 14 days, a significant decrease of GnRH expression at all doses was reported. Fshβ was upregulated at 7.5 and 15 μg/kg, but after 14 days was significantly decreased at 30 μg/kg. At all doses, Lhβ expression was significantly decreased. Through the 7 days of treatment, changes in gene expression corresponded to increases in FSH, LH, and testosterone levels followed by decreases in LH and testosterone levels at all doses after 14 days of treatment. At 15 μg/kg, FSH levels were significantly increased, but significantly decreased at 30 μg/kg after 14 days.

Chen et al. (2013) found that repeated i.p. dosing of rats with 10 μg microcystin-LR/kg affected expression of cytoskeletal genes and mitochondrial dysfunction in the testes. Levels of FSH and LH increased while testosterone levels decreased. Male reproductive effects were consistently observed after single and repeated parenteral exposures. These studies are described in more detail in section 7.2.5. Histological damage to the testes was observed in mice, rabbits, and rats administered microcystin-LR or a cellular extract (Chen et al., 2013; Li et al., 2008; 2011b; Liu et al., 2010; Ding et al., 2006). Sertoli cells were shown to be affected in rabbits and mice, testes and epididymal weights were decreased in mice and rats, and sperm motility and viability were affected in mice and rats.

6.4.2.1.6 Other Tissues

Soares et al. (2007), Carvalho et al. (2010), and Casquilho et al. (2011) all observed lung damage after a single i.p. administration of microcystin-LR at a sublethal dose (i.e., 40 μg/kg). None of the studies detected microcystin-LR in the lungs but damage was evident within 2 hours of exposure. Lung effects include an increase in the proportion of areas with alveolar collapse accompanied by an increase in the percentage of PMN cells; increased impedance; increased oxidative stress in the lung as measured by decreased SOD, and increased catalase, thiobarbituric acid reactive substances, and myeloperoxidase; elevated pulmonary mechanical parameters; and increases in TNFα, IL-1β, and IL-6.

Milutinovic et al. (2006) demonstrated that 10 μg/kg of microcystin-LR administered i.p. every other day for 8 months to male rats caused microscopic lesions to the heart including disarray and short runs of myocardial fibers interrupted by connective tissue, increased volume density of interstitial tissue with a few lymphocyte infiltrations, enlarged cardiomyocytes with enlarged and often "bizarre-shaped" nuclei; some cells also demonstrated loss of cell cross-striations and degenerative muscle fibers with myocytolysis. A similar study by the same group using microcystin-YR (Šuput et al., 2010) also found similar histopathological results, but less prominent effects on the heart with microcystin-YR compared to microcystin-LR. Neither microcystin-LR nor microcystin-YR induced apoptosis in the heart.

Zhao et al. (2015) revealed thyroid dysfunction in mice after i.p. injection of microcystin-LR for 4 weeks. Mice exposed to either 5 or 20 μg/kg of microcystin-LR showed an increase in the circulating thyroid

hormone (TH) levels and the free triiodothyronine (FT3). The authors also observed a decreased free thyroxin (FT4), presumably responsible for the changes observed after exposure. An increased expression of TH receptor (Trα) and mTOR expression in the brain was also observed and related to a consequence of the increased FT3. In addition, disrupted glucose, triglyceride and cholesterol metabolism with obvious symptoms of hyperphagia, polydipsia, and weight loss were also observed.

6.4.2.2 Cancer Effects

Mechanistic evidence provides support for the hypothesis that microcystin-LR can act as a promoter at low doses due to increased cell proliferation and decreased apoptosis, as well as inhibition of repair. Data related to cancer and cell proliferation indicate that at low doses, microcystin-LR may increase cell proliferation. microcystin-LR has been shown to increase the expression of the bcl-2 protein (that inhibits apoptosis) and decrease the expression of the bax protein (that induces apoptosis) (Hu et al., 2002; Lei et al., 2006; Weng et al., 2007; Li et al., 2011c). Further, microcystin-LR upregulates the transcription factors c-fos and c-jun, leading to abnormal proliferation (Zhao and Zhu, 2003). Gehringer (2004), in a review of the molecular mechanisms leading to promotion by microcystin-LR and the related tumor promoter okadaic acid, reported that microcystin-LR inhibits protein phosphatase PP2A, which regulates several MAPKs. The MAPK cascade regulates transcription of genes required for cell proliferation, including c-jun and c-fos. In addition, activation of the MAPK cascade has been postulated to inhibit apoptosis and thus increase cell proliferation. In addition, microcystin-LR has been reported to increase phosphorylation of p53 (Gehringer, 2004; Fu et al., 2005; Li et al., 2009; Hu et al., 2008; Xing et al., 2008; Žegura et al., 2008a; Li et al., 2011c), which is involved in regulation of the cell cycle and apoptosis.

Clark et al. (2007, 2008) found microcystin-LR administered i.p. at a sublethal dose caused changes in gene transcription related to actin organization, cell cycle, apoptotic, cellular redox status, cell signaling, albumin metabolism, and glucose homeostasis pathways, as well as the OATp system in the livers of p53 knockout mice. The gene expression analysis found increases in genes related to cell-cycle regulation and cellular proliferation in microcystin-LR treated mice livers was greater compared to the p53-deficient mice, control livers and that observed in the livers of microcystin-LR treated wild type mice (Clark et al., 2008). Ki-67 (a marker of cell proliferation) and phospho-histone H3 (a mitotic marker) for immunoreactivity were also increased in microcystin-LR-treated knockout mice. The study authors concluded that p53 may play an important role in tumor promotion by microcystin-LR.

Changes in MMP levels have been linked to cancer and tumor promotion. Zhang et al. (2010; 2012) found increased levels of MMP2 and MMP9 in the livers of male mice orally administered microcystin-LR for at least 180 days (subchronic and chronic results of these studies were described in Sections 6.2.3 and 6.2.6, respectively). To study further possible effects of microcystin-LR on tumor metastasis, Zhang et al. (2012) cultured breast cancer cells with different concentrations of microcystin-LR for different lengths of time. Acceleration of cell migration was found to be dependent on both the concentration and length of microcystin-LR incubation time. The levels of MMP2 and MMP9 were also increased with microcystin-LR concentration in breast cancer cells.

Birungi and Li (2011) tested the effects on noncytotoxic concentrations (1-100 ng/mL) of microcystin-LR, microcystin-YR, and microcystin-RR on HepG2 cells. While higher concentrations (1000 ng/mL) are known to cause cell death, cells continued to proliferate at the noncytotoxic concentrations used in this study. The study authors suggested this could lead to uncontrolled growth and possibly tumors. Microcystin-LR (10 µg/L) incubated with WRL-68 cells, a human cell line, for 25 passages had an increased growth rate compared to controls (Xu et al., 2012). Gan et al. (2010) also found that microcystin-LR enhanced cell proliferation in the liver cancer cell lines HepG2 and Hep3B. Microcystin-

LR was also found to activate nuclear factor erythroid-2 (Nrf2) in a dose-dependent manner. Inhibiting Nrf2 also inhibited microcystin-LR-induced cell proliferation.

Nong et al. (2007) incubated HepG2 cells with 100 μM microcystin-LR for 24 or 48 hours. After both time periods there was an increase in the number of cells in G0/G1 phase of the cell cycle with less in the S phase of the cell cycle. ROS scavengers (catalase, SOD, or deferoxamine) did not affect the blockage in the cell cycle induced by microcystin-LR. The opposite was observed in a kidney cell line.

Dias et al. (2010) studied the effects of microcystin-LR on the proliferation of nonhepatic cells using a kidney epithelial cell line (Vero-E6). Previous studies (Dias et al., 2009; Alverca et al., 2009) had found microcystin-LR cytotoxic to this cell line, at doses as low as 11 μM. Therefore, Dias et al. (2010) used commercial microcystin-LR (purity ≥95%) or extracted microcystin-LR (purity not reported, but stated to have been tested) in the range of 5-5000 nM. Even the lowest concentration caused an increase in ERK1/2 activity, suggesting that microcystin-LR stimulates the G1/S transition and activates the ERK1/2 pathway (as noted by increases in p38, JNK, and ERK1/2 activity) in kidney cells.

Zhu et al. (2005) reported that microcystin-LR can transform immortalized colorectal crypt cells, resulting in anchorage-independent growth and enhanced proliferation. Lankoff et al. (2006b) did not find any DNA damage in CHO-K1 cells incubated with microcystin-LR (10 or 24 μg/mL), but microcystin-LR did inhibit the repair of DNA damage induced by ultraviolet light. The study authors suggested that microcystin-LR inhibited the nucleotide excision repair through inhibition of the inclusion/exclusion phase as well as the rejoining phase. In a different study, microcystin-LR inhibited DNA repair by gamma radiation in human lymphocytes and a human glioblastoma cell line (Lankoff et al., 2006a). Microcystin-LR and microcystin-RR have been shown to increase the expression of the Bcl-2 protein (that inhibits apoptosis) and decrease the expression of the bax protein (that induces apoptosis) (Hu et al., 2002; Hu et al., 2010; Huang et al., 2011; Li et al., 2011c). However, one study found decreased expression of Bax, Bcl-2, and bad (pro-apoptotic) proteins (Billam et al., 2008). In addition, microcystin-LR upregulates the transcription factors c-fos and c-jun, leading to abnormal proliferation (Zhao and Zhu, 2003; Li et al., 2009).

Xing et al. (2008) observed increases in p53 expression and decreased PP2A expression in FL human amniotic epithelial cells incubated with 10-1000 nM microcystin-LR for 24 hours. Hu et al. (2008) observed a significant increase in p53 expression in livers of rats exposed to pure microcystin-LR (purity not reported) via i.p. injection twice a week for 6 weeks, but did not observe a significant increase in p53 expression with cyanobacterial extracts containing microcystin-LR at a concentration of 529.656 ng/L administered via the drinking water. Neither treatment altered p16 expression. Takumi et al. (2010) studied the role of p53 on cell fate in HEK293-OATP1B3 cells exposed to microcystin-LR. The data suggested that when p53 is inactivated, chronic low exposure to microcystin-LR could lead to cell proliferation through activation of Akt signaling. Akt is a general mediator of growth factor induced survival and has been shown to suppress the apoptotic death of a number of cell types induced by a variety of stimuli, including growth factor withdrawal, cell-cycle discordance, loss of cell adhesion, and DNA damage. Fu et al. (2009) also found changes in proteins associated with the cell cycle in human amniotic epithelial cells exposed to microcystin-RR.

6.4.2.3 Structure-Activity Relationships

With a few exceptions, microcystin congeners exhibit i.p. LD_{50} values between 50 and 300 μg/kg in mice (Rinehart et al., 1994; WHO, 1999). Microcystin-LR is one of the most potent congeners (i.p. LD_{50} approximately 50 μg/kg). Pharmacokinetic differences among the various microcystin congeners may be at least partially responsible for observed variations in lethal potency (Ito et al., 2002b). Microcystin congeners of varying hydrophobicity were shown to interact differently with lipid monolayers

(Vesterkvist and Meriluoto, 2003). Effects on membrane fluidity could alter the cellular uptake of these toxins.

Wolf and Frank (2002) proposed toxicity equivalency factors (TEFs) for the four major microcystin congeners based on LD_{50} values obtained after i.p. administration. The proposed TEFs, using microcystin-LR as the index compound (TEF=1.0) were 1.0 for microcystin-LA and microcystin-YR and 0.1 for microcystin-RR. The application of TEFs based on i.p. LD_{50} values to assessment of risk from oral or dermal exposure is questionable given that differences in liphophilicity and polarity of the congeners may lead to variable absorption by non-injection routes of exposure.

7.0 CHARACTERIZATION OF RISK

7.1 Synthesis and Evaluation of Major Noncancer Effects

Although, some studies have shown differences in toxicity and in uptake rates (Zeller et al., 2011; Zurawell et al., 2005), the preponderance of toxicological data on the effects of microcystins are restricted to the microcystin-LR congener. As a result, this section largely describes the available information on the toxic effects of microcystin-LR.

Elevated liver enzymes have been measured in humans served by a public water supply contaminated with a bloom of *M. aeruginosa* (Falconer et al., 1983) and in children consuming high levels of microcystin through water and food (Li et al., 2011a). One study of human exposure to drinking water before, during and after a bloom of *M. aeruginosa* reported a significant increase in GGT levels during the bloom compared with levels before the bloom and compared to the levels in patients living in areas served by other water supplies (Falconer et al., 1983). The study population consisted of all persons subjected to liver function tests in the area served by the affected drinking water supply; as such, it is not fully representative of the general population. A study in China evaluated liver damage in children in relation to the microcystin levels in the drinking water and select aquatic foods (e.g. carp and duck) (Li et al., 2011a). Microcystin levels were associated with increasing levels of AST and ALP, but not ALT and GGT. The Odds Ratio for liver damage, as defined by increased serum enzyme levels in exposed children, was 1.72 (95% CI: 1.05-2.76).

A major noncancer health effect of exposure to microcystin-LR in animal studies is liver damage. Oral exposure to single 500 μg/kg gavage doses of microcystin-LR caused diffuse hemorrhage in the liver of mice and rats; more pronounced liver damage occurred at higher doses (Ito et al., 1997a; Fawell et al., 1999). Young mice (5 weeks old) did not develop signs of hepatotoxicity at 500 μg/kg of microcystin-LR, while aged mice (32 weeks old) developed clear signs (Ito et al., 1997a). This difference may result in part from differences in the ontology of the intestinal transporters responsible for gastrointestinal absorption of microcystins, but cannot be entirely explained by absorption differences, because similar age-dependent effects were reported after i.p. exposure (Adams et al., 1985; Rao et al., 2005). However, liver transporters may also show age-related differences in expression.

A 28-day study of oral exposure to 50 or 150 μg/kg of microcystin-LR in drinking water showed increased liver weight, slight to moderate liver lesions with necrosis (with and without hemorrhage) and increased ALP and LDH in rats exposed at 50 μg/kg-day (Heinze, 1999). A subchronic gavage study in mice using a similar dose range identified a LOAEL of 200 μg/kg (Fawell et al., 1999). At this dose, mild liver lesions, including chronic inflammation and hepatocyte vacuolization were observed. Two animals had hepatocyte degradation and there were hemosiderin deposits in one liver. Mean serum ALT and AST were significantly increased in male animals. No adverse effects were identified at a dose of 40 μg/kg, although mean bodyweight gains were uniformly reduced to the same extent for all treated animals. The authors expressed that these reductions were within the normal range for this strain of mice and because no dose response was observed, they considered these findings coincidental. Mild hepatocyte injury in the area of the central vein was reported in mice given 80 or 100 gavage doses of 80 μg/kg each over 28 weeks, corresponding to time-weighted average doses of 33-41 μg/kg-day (Ito et al., 1997b). No liver or other toxicity was reported after a mean cumulative microcystin-LR drinking water intake of 35.5 μg per mouse for 18 months (Ueno et al., 1999).

It is important to consider the route of administration in conjunction with the effects observed after oral exposures to microcystins. It is known that organic anion transporting polypeptides control uptake of microcystin from serum into the liver and other organs (Fischer et al., 2005). Less is known about uptake from the gastrointestinal tract. Given the resistance of microcystins to digestion and their molecular

composition, some form of facilitated transport is likely. Two *in vitro* studies using human Caco-2 intestinal cells demonstrated that microcystin-LR cellular apical uptake with efflux from the cell apparently required active transport (Zeller et al., 2011; Henri et al., 2014). Henri et al. (2014) concluded that basolateral efflux and not apical uptake was the limiting factor for transfer to portal circulation. Under such circumstances, dosing by drinking water is the preferred route for delivering the dosed material to serum and subsequently to organs. Dosing from drinking water, wherein exposure occurs relatively consistently across a day, can deliver a larger portion of the dosed compound to circulation than gavage dosing. The opportunity for absorption with a bolus dose is limited by the dosing medium, concentration, and the small intestine transit time.

Delivery to target tissues is also transport controlled and impacted by serum concentration. Uptake as a proportion of dose by an organ such as the liver, is greater when the serum level is low and constant than when the level in serum is high and of short duration. These factors become important when contrasting the results from drinking water studies such as the 28 day study by Heinze (1999) with gavage studies such as the 90 day study done by Fawell et al. (1999). The same factors must also be considered when comparing these results with those of Guzman and Solter (1999), wherein an osmotic pump was used to slowly deliver microcystin-LR directly into the intraperitoneal membrane. Conceptually, one would expect that the risk for hepatic damage would be greatest in the Guzman and Solter study and lowest in the Fawell et al. study (1999) under the situation where the total daily doses were the same or similar and there was a difference in dose delivery (gradual versus bolus).

In the Guzman and Solter (1999) study, the 32 and 48 µg/kg/day dose caused histological damage to the liver of male Sprague Dawley rats (3 per dose group) as manifested by inflammation, fibrous tissue, cell death and apoptosis. Infiltrates of macrocytes, lymphocytes, and neutrophils were seen in the centrilobular area and round lipid staining vacuoles in the pericentral region. Hepatocellular damage was more severe in the high dose group than the mid-dose group. Changes in liver enzymes and the concentration of malondialdehyde increased in a dose related manner for the mid and high doses. The 16 µg/kg/day dose did not display any histological damage. The malondialdehyde concentrations suggest that oxidative stress is part of the pathological changes from exposure to microcystin-LR after 28 days. The leakage of liver enzymes suggests the inability of the hepatocytes to maintain membrane integrity due to toxin induced injury. The intraperitoneal infusion route of exposure can account for the fact that the rats in this study, were vulnerable to liver effects at a lower dose than the animals in Heinze (1999) and Fawell et al. (1999).

The Fawell et al. (1999) study in groups of 15 male and 15 female Cr1:CD-1(ICR)BR (VAF plus) mice used gavage dosing of microcystin-LR in aqueous solution over a 90 day period. Conceptually gavage would deliver a lower daily dose to the liver than a drinking water dose, given that the time for serum uptake will be limited by small intestinal transit time and transporter kinetics. Fawell et al. (1999) used doses of 0, 40, 200, or 1000 µg/kg/day. There were no signs of liver damage at the lowest dose and mild evidence at the 200 µg/kg/day dose. Chronic inflammation was present in animals from all dose groups as was hepatocyte vacuolization. There was a dose related increase across all dose groups for chronic inflammation with 1, 2, 8, and 14 males impacted in the control, low, mid and high dose groups respectively, and 5, 8, 8, and 14, respectively in females. The hepatic vacuolization did not exhibit a clear dose-response in females. Hepatocyte degeneration was first reported at 200 µg/kg/day in two animals and increased to 23/30 animals at the high dose. Serum ALT and AST were significantly increased at doses ≥ 200 µg/kg/day in males and females. Accordingly, the results are consistent with the concept that once per day gavage dosing allows less of the microcystin-LR to be delivered from the intestines to the liver.

Considering that the kinetics of i.p. infusion differs from that of drinking water and oral gavage routes of administration, it is difficult to compare the effects across the three studies. In addition, the Fawell et al.

(1999) study was conducted using mice while those by Heinze (1999) and Guzman and Solter (1999) used male rats, which further limits comparisons.

The Heinze (1999) 28 day drinking water study of groups of 10 male F1 hybrid rats (WELS/Fohm-BD1X,) with doses of 0, 50 or 150 µg/kg/day, has a broader dose range than the Guzman and Solter (1999) study. It uses the most relevant route of exposure and has more animals/dose group (n=10) than the Guzman and Solter (1999) study where there were only 3 per dose group. Only males were studied in the Heinz et al (1999) study. Liver histopathology was seen in both dose groups. There was degeneration and necrosis of hepatocytes and PAS positive staining (indicative of cell membrane damage) that increased in severity with dose. Serum levels of LDH and ALP were significantly ($p=0.05$) increased above controls for both doses. All animals displayed Kupffer cell activation in response to hepatic cell injury. Each of these biomarkers for liver damage was increased at both doses in all ten animals. The severity scores increased with dose but not the number of animals affected. The doses used by Heinze (1999) in the rat study also falls between the exposures used in the Fawell et al. mice study, however fewer animals (10) were used by Heinze (1999) in comparison to Fawell et al. (1999) (30).

While the liver is the primary target of microcystin toxicity, there have been some reports of effects in other systems, including hematological, kidney, cardiac, reproductive, and gastrointestinal effects. It has been suggested that some effects in other organs observed after high doses of microcystin-LR may result from ischemia or hypoxia caused by hepatic hemorrhage. However, effects outside the liver have been observed in the absence of hemorrhage. In most cases effect levels are at doses greater than those impacting the liver.

Gastrointestinal effects (necrosis, duodenal damage) were observed in aged mice exposed orally to single 500 µg/kg doses of microcystin-LR (Ito et al., 1997a). Female mice exposed subchronically to 1000 µg/kg had slight increases in hemoglobin concentration, erythrocyte count and packed cell volume (Fawell et al., 1999).

Kidney effects, including eosinophilic materials in the Bowman's spaces, were observed in two mice exposed to lethal doses of microcystin-LR (Yoshida et al., 1997). Fawell et al. (1999) described the appearance of the kidneys as pallid. Milutinovic et al. (2002, 2003) exposed Wistar rats to i.p. doses of microcystin-LR and microcystin-YR (10 µg/kg for 8 months) finding evidence for necrotic and apoptotic damage to both the glomerulus and the tubular epithelium. Evidence of action aggregation implicated damage to the cytoskeleton as a result of PP2A inhibition as a factor contributing to the damage. The effects associated with microcystin-LR were greater than those caused by microcystin-YR.

Mechanistic studies by Alverca et al. (2009) in cultured Vero cells and Nobre et al. (1999) examining perfused kidneys observed changes in cellular morphology such as enlarged lysosomes, reduction in the number of intact lysosomes, and lysosomal leakage in the former study, and urinary flow rate in the latter study.

Studies by Li et al. (2014, 2015) found an impact of microcystin-LR on learning the Morris water maze and subsequent retention of the learning. In the memory phase of the trial, the male rats did not do as well at remembering the quadrant where the platform had been located during the learning component of the study. The 2014 study evaluated mature male rats directly exposed to microcystin-LR in solutions with methanol. The 2015 study exposed the tested animals (pups PND7-60) through their dams. The dams were dosed during an 8-week period prior to mating but not during gestation and lactation. The results of both studies are confounded by the presence of methanol in the dosed solutions.

A single oral study of developmental toxicity in mice reported maternal toxicity, liver effects and deaths in some dams treated at the highest dose of microcystin-LR (2000 µg/kg during GD 6-15), along with reduced fetal body weight and delayed skeletal ossification in offspring (Fawell et al., 1999). Li et al.,

(2015) identified a NOAEL of 2.5 µg/kg/day and a LOAEL of 10 µg/kg/day based on maternal gestational weight gain in Sprague Dawley rats exposed to microcystin-LR for 8 weeks prior to conception, but not during mating and gestation. The study is confounded by the presence of methanol in all the tested solutions, resulting in uncertainty regarding whether synergy between the microcystin-LR and methanol could have influenced the results. There were no significant differences in the developmental parameters in the pups, although neurological effects were observed in postnatal testing as described above. In an i.p. study by Chernoff et al., (2002) there were maternal deaths at doses ≥32 µg/kg but no observed effects on number of pups and pup body weight up to PND 5 for the dams that survived.

Effects observed in the male reproductive system include decreased absolute and relative testes weights; decreased absolute and relative epididymis weight; decreased epididymal sperm concentration, decreased sperm viability, decreased sperm motility, increased percent immobile sperm and sperm abnormalities. Histological examination of the testes revealed atrophy of the seminiferous tubules, obstructed seminiferous tubules, deformation of androgonial and sperm mother cells; decreased number of interstitial cells, Sertoli cells, and mature sperm in the seminiferous tubule; lipid peroxidation; and apoptosis (Chen et al., 2011, 2013; Li et al., 2011b; Liu et al., 2010; Li et al., 2008; Zhang et al., 2011b). *In vitro* studies of rat spermatogonia, the precursor cells from which spermatocytes arise, demonstrate uptake of microcystin-LR with resultant cellular apoptosis and oxidative stress (Zhou et al., 2012).

Male mice administered microcystin-LR via their drinking water for 3 or 6 months at low concentrations (3.2g/L) had decreased sperm counts and sperm motility (Chen et al., 2011). By 6 months there was also an increase in sperm abnormalities, decreased serum testosterone and increased serum LH levels. Testes weights, however, were not affected. The LOAEL for these effects was 0.79 µg/kg with a NOAEL of 0.25 µg/kg. The observed effects suggest a need to confirm the reported results.

Data from a number of mechanistic studies involving the male reproductive system support the need for additional research. *In vitro* cell viability of Sertoli and Leydig cells was decreased by exposure to microcystin-LR (Li et al., 2008; Zhang et al., 2011b; Li and Han, 2012). Changes in morphology were marked by cell shrinkage and loss of membrane integrity. Wang et al. (2012), found that microcystin-LR was not able to enter Leydig cells reflected by the lack of Leydig cell cytotoxicity. Testosterone production was also decreased *in vitro* in Leydig cells incubated with microcystin-LR (Li et al., 2008).

Male hormone levels were affected by microcystin-LR in both *in vitro* and *in vivo* studies. *In vivo* studies in male mice found that microcystin-LR induced decreases in serum testosterone and increases in serum LH and FSH (Chen et al., 2011; Li et al., 2008). Microcystin-LR also affected hormone levels in male mice by damaging the hypothalamic-pituitary axis as measured by decreased mRNA expression for GnRH (Wang et al., 2012; Xiong et al., 2014).

7.1.1 Mode of Action of Noncancer Effects

Mechanistic studies of microcystin cellular effects shed light on the mode of action for noncancer effects. One important feature appears to be the need for membrane transporters for systemic uptake and tissue distribution of microcystin by all exposure routes (Fischer et al., 2005; Feurstein et al., 2010). Members of the OATp transporter family regulate uptake and efflux from the intestines, liver, kidney, testes, brain, lung, heart, and placenta (Augustine et al., 2005; Cheng et al., 2005; Svoboda et al., 2011). The importance of the transporters to tissue access is demonstrated by the data that indicate a reduction in, or lack of, liver damage when OATp is inhibited (Hermansky et al., 1990 a,b; Thompson and Pace, 1992).

Uptake of microcystins causes protein phosphatase inhibition and loss of coordination between kinase phosphorylation and phosphatase dephosphorylation resulting in destabilization of the cytoskeleton; this event initiates altered cell function followed by cellular apoptosis and necrosis (Barford et al., 1998). Together cellular kinases and phosphatases maintain the balance between phosphorylation and

dephosphorylation of key cellular proteins that control metabolic processes, gene regulation, cell cycle control, transport and secretory processes, organization of the cytoskeleton and cell adhesion. Microcystins LR, LA and LL each interact with catalytic subunits of PP1 and PP2 inhibiting their functions (Craig et al., 1996).

The consequences of the microcystin induced changes in cytoskeleton appear to be increases in apoptosis and ROS. Cellular pro-apoptotic Bax and Bid proteins increased and anti-apoptotic Bcl-2 decreased in both *in vitro* and *in vivo* studies (Fu et al., 2005; Weng et al., 2007; Xing et al., 2008; Takumi et al., 2010; Huang et al., 2011; Li et al., 2011d). Mitochondrial permeability transition pore, and mitochondrial membrane potential changes (Ding and Ong, 2003; Zhou et al., 2012) led to membrane loss of cytochrome c, a biomarker for apoptotic events. Wei et al., (2008) found that microcystin-LR induces a time-dependent increase in ROS production and lipid peroxidation in mice. The levels of hepatic ROS increased rapidly within 0.5 hours of receiving a 55 μg/kg body weight i.p. injection of microcystin-LR, and continued to accumulate in a time-dependent manner for up to 12 hrs.

7.1.2 Dose-Response Characterization for Noncancer Effects

7.1.2.1 Human Data

There are no dose response data from the epidemiology case studies of microcystin. Acute intoxication with microcystin-producing cyanobacteria blooms in recreational water was reported in Argentina in 2007 (Giannuzzi et al., 2011). A single person was immersed in a *Microcystis* blooms with a concentration of 48.6 μg/L. After four hours of exposure, the patient showed fever, nausea, and abdominal pain and three days later, presented dyspnea and respiratory distress and was diagnosed with an atypical pneumonia. A week after the exposure, the patient developed a hepatotoxicosis with a significant increase of ALT, AST and γGT. The patient was completely recovered within 20 days.

The scant human data on the oral toxicity of microcystin-LR are limited by the potential co-exposure to other toxins and microorganisms and by the lack of quantitative information. Symptoms reported after acute recreational exposure to cyanobacterial blooms (including microcystin-producing genera) included headache; sore throat; vomiting and nausea; stomach pain; dry cough; diarrhea; blistering around the mouth; and pneumonia (Turner et al., 1990). Elevated liver enzymes have been measured in humans served by a public water supply contaminated with a bloom of *M. aeruginosa* (Falconer et al., 1983) and in children consuming high levels of microcystin through water and food (Li et al., 2011a). Symptoms occurring after exposure to cyanobacteria cannot be directly attributed to microcystin toxins or other endotoxins; some effects may result from exposure to the cyanobacterial cells themselves, or from exposure to multiple toxins in the bloom.

7.1.2.2 Animal Data

A major noncancer health effect of exposure to microcystin-LR in animal studies is liver damage. Oral exposure to single 500 μg/kg doses of microcystin-LR resulted in diffuse hemorrhage in the liver of mice and rats; more pronounced liver damage occurred at higher doses (Ito et al., 1997a; Fawell et al., 1999). Oral LD_{50} values ranged from 3000 μg/kg to >5000 μg/kg in rats and mice (Fawell et al., 1999; Yoshida et al., 1997; Fitzgeorge et al., 1994). Studies which utilized parenteral administration of microcystin-LR show a steep dose-response with rapid onset of liver damage.

The dose-response database for microcystins is almost exclusively limited to data on a single congener, microcystin-LR. Data on the RR, YR, and LA do not provide useful dose-response information suitable for quantification. With consideration of the seasonal episodic nature of algal blooms and resultant

potential exposures to microcystins from public water supplies, the following studies summarized in Table 7-1 were those selected as most suitable for derivation of guideline values.

Table 7-1. Adverse Effects By Route of Exposure to Microcystins

Dose µg/kg/day	Severity Finding	Description of Effect	Study
Intraperitoneal infusion			
32	++	Fibrous tissue, cell death, necrosis, lipid vacuoles, Kupffer cell activation	Guzman and Solter, 1999
48	+++	Fibrous tissue, cell death, necrosis, lipid vacuoles, Kupffer cell activation	
Gavage			
40	10/30	Chronic inflamation[1]	Fawell (1994)
200	15/30	Chronic inflamation[1] hepatocyte degeneration (2/30)	
Drinking Water			
50	++	Hepatocyte degeneration and necrosis; and PAS positive staining, Kupffer cell activation	Heinze (1999)
150	+++	Hepatocyte degeneration and necrosis; and PAS positive staining Kupffer cell activation	

[1]Lesion with the best response to dose; the effect was seen in 6/30 controls, 10 at the low does, 12 at the mid dose and 29 at the high dose
++ moderate severity
+++ high severity

7.2 Synthesis and Evaluation of Carcinogenic Effects

Several human epidemiological studies have reported a possible association between consumption of surface waters containing cyanobacteria and microcystins that served as drinking water sources and liver or colon cancer in certain areas of China (Ueno et al., 1996; Zhou et al., 2002). In these studies, the use of a surface drinking water supply was used as a surrogate for exposure to microcystins. Individual exposure to microcystins was not estimated and there was no examination of numerous possible confounding factors such as hepatitis infection, industrial discharges and/or waste water discharges to the same surface water sources.

Flemming et al. (2002, 2004) failed to find a significant association for primary liver cancers between populations living in areas receiving their drinking water from a surface water treatment plant (with the potential for microcystin exposures) and the Florida general population plus those receiving their water from ground water sources. The strongest association observed was that between those receiving their water from a surface water service area and those receiving their water from the surroundings of the buffer zones with socioeconomic factors assumed to be similar for the residents of the 18 surface water sources evaluated. The origin of the water supplies for the buffer zones was not identified. The age-adjusted cancer rate for the surface-water area was 1.15 cases versus 0.83 cases for the buffer zone, yielding a SRR of 1.39 cases in the surface water zone to 1 case in the buffer zone area. The buffers zone also had a lower age adjusted cancer rate than the state of Florida (SRR = 0.59). A major weakness of this study is the fact that it examined the cancer rate based on location of residence at the time of diagnosis without any data on the duration of residence. Florida is known to be a state with population residence turnover because of its appeal to retirees and winter-only residents.

Ito et al. (1997b) conducted the only longer-term oral animal study of a purified microcystin. In this study, chronic gavage doses of 80 µg microcystin-LR/kg/day for 80 or 100 days over 28 weeks (7 months) failed to induce neoplastic nodules of the liver in mice. Despite the study duration problem, the

lack of hyperplastic nodules and limited liver damage at 7 months suggests that microcystin is not a mutagenic initiator of tumors. The i.p. studies of microcystin-LR as an initiator found low levels of GST-P foci when up to 10 µg/kg of microcystin-LR was injected in rats with liver weights that were statistically equivalent to those of the initiated controls (Nishiwaki- Matsushima et al., 1992; Ohta et al., 1994; Sekijima et al., 1999), but significantly increased after a partial hepatectomy stimulated tissue repair. The lack of an increase in liver weight was used as a marker for lack of liver damage and protein phosphatase inhibition. All three studies concluded that microcystin was a promoter of tumorigenesis rather than an initiator of the process.

7.2.1 Mode of Action and Implications in Cancer Assessment

Protein phosphatase inhibition and its impact on the cytoskeleton increases the risk for DNA replication errors during cell division. Microcystin-LR can promote tumorigenesis because it perpetuates existing DNA damage in cases where a cell divides before replication errors can be repaired. Once a cell had been damaged by microcystin-LR and the repair process has begun, ROS, spindle problems, the presence of alkylating agents such as DEN, and other factors generate a high risk for uncontrolled cell proliferation resulting in tumors.

Genotoxicity studies of microcystin-LR provide conflicting results. Two microcystin-containing extracts gave positive results in the Ames assay (Ding et al., 1999; Huang et al., 2007), while negative results were observed using *M. aeruginosa* extracts as well as purified microcystin (Grabow et al., 1982; Wu et al., 2006; Repavich et al., 1990).

Positive genotoxicity results were observed in mammalian cell lines (Suzuki et al., 1998; Zhan et al., 2004; Nong et al., 2007; Žegura et al., 2006, 2008a,b, 2011; Li et al., 2011b) but *in vivo* animal studies yielded conflicting results (Gaudin et al., 2008, 2009; Abramsson-Zetterberg et al., 2010; Zhang et al., 2011a; Dong et al., 2008). Evidence for microcystin-LR-induced DNA damage as measured by the comet assay has been called into question by the finding that apoptosis can lead to false positive findings in this assay (Lankoff et al., 2004a). Some evidence exists for a clastogenic effect of microcystin-LR (Ding et al., 1999; Zhan et al., 2004; Lankoff et al., 2006a; Repavich et al., 1990). Metabolic activation has been found to decrease microcystin-LR mutagenicity. The inconsistent outcomes from the mutagenicity studies may be related to differences in the cell uptake of microcystin-LR, the metabolism of microcystin-LR in the test system, or the amount of damage to the cytoskeleton and its impact on DNA and cell replication.

DNA fragmentation was significantly increased in rat neutrophils with microcystin-LA and microcystin-YR, but not in human neutrophils (Kujbida et al., 2008). Microcystin-YR has also been found to induce DNA damage in the blood (lymphocytes), liver, kidney, lung, spleen, and brain of mice administered 10 µg microcystin-YR/kg via i.p. injection every other day for 30 days (Filipič et al., 2007). Lankoff et al. (2003) showed that microcystin-LR, through its effect on microtubules, damages the mitotic spindle, leading to the formation of polyploid cells.

7.2.2 Weight of Evidence Evaluation for Carcinogenicity

Applying the Guidelines for Carcinogen Risk Assessment (U.S. EPA, 2005a), there is *inadequate information to assess carcinogenic potential* for microcystins. The epidemiology studies are limited by their ecological study design, poor measures of exposure, potential co-exposure to microbial and/or chemical contaminants and, in most cases, failure to control for known liver and colorectal risk factors.

Oral exposure, dose-response data from animal studies of the carcinogen potential of microcystins are not available. Several studies suggest that microcystin-LR is a promoter of tumors initiated by known mutagens or during tissue damage repair. However, there are no clear data that demonstrate heritable, structural changes to genes or chromosomes as a consequence of the direct interaction of microcystin-LR

with the genome to justify classifying it as a direct carcinogen. Given microcystin-LR potential impact on the cell cytoskeleton, liver necrosis, generation of ROS, and cell biochemistry, it is not surprising that studies support the concept that microcystin-LR plays a secondary role in the tumorigenic process. The work by Nishiwaki-Matsushima et al. (1992) and others, which compared liver P-GST foci from 10 µg/L microcystin-LR in combination with initiation, indicate that microcystin-LR can be a promoter, especially when accompanied by tissue damage requiring repair. The results from the second part of the Nishiwaki-Matsushima et al. (1992) study that compared P-GST foci following initiation with NDEA followed by microcystin-LR (10 µg/kg) treatment, both before and after a partial hepatectomy, support this conclusion. The number of foci/cm^2 with NDEA alone was 13.4 ± 4.2 foci/cm^2; with DEN, microcystin-LR and a partial hepatectomy it was 17.4 ± 3.8 foci/cm^2. The results for microcystin exposure alone (10 µg/kg before the hepatectomy and 50 µg/kg after) in the absence of the NDEA was 0.4 ±0.3 foci/ cm^2

The International Agency for Research on Cancer (IARC) classified microcystin as a Group 2B (possibly carcinogenic to humans) largely based on its ability to disrupt cellular architecture, along with cell division and repair as supported by the i.p. tumor promotion data. The EPA 2005 cancer guidelines support selecting a descriptor for an agent that has not been tested in a cancer bioassay if sufficient other information is available to make a strong, convincing, and logical case through scientific inference. In the case of Microcystin-LR, strong information to support classifying microcystin as a carcinogen is not available, even though mechanistic data support a role for its contribution to the progression of tumors initiated by other compounds.

7.2.3 Dose Response Characterization for Cancer Effects

Dose-response data regarding the carcinogenicity of microcystins from animal studies are not available. Some studies suggest that microcystin is a promoter for tumors initiated by known mutagens or through complication of tissue repair such as that caused by a partial hepatectomy. Given the potential impact on the cell cytoskeleton, necrotic effects on liver cells, generation of ROS and other biochemical changes, this is not surprising. Tissue damage requires cell division in the repair process. The cytoskeleton plays an integral role in reparative cell division and ROS are capable of changing the altered DNA structure. Thus, there are multiple opportunities for changes that lead to loss of control of the cell division process and clonal explosion of the impacted cells. The work by Nishiwaki-Matsushima et al., (1992),which compares P-GST foci from 10 µg/L microcystin LR to that from the phenobarbital (0.05% in the diet) as a positive control, suggests that it is at best a weak promoter because the combination of NDEA with phenobarbital resulted in more GSTP foci than the combination of NDEA with microcystin. The results from the second part of the same study, which compared GST-P foci following initiation with NDEA followed by microcystin (10 µg/kg) both before and after a partial hepatectomy, support this conclusion. The number of foci/cm^2 with NDEA alone was 13.4 ± 4.2 foci/cm^2 and with microcystin-LR and a partial hepatectomy the number of foci/cm^2 was 17.4 ± 3.8. The impacted foci area was lower for the initiated promoted rats (0.1 ±0.2 %) than the initiated group in the absence of microcystin-LR promotion (2.7 ±3.1%). Therefore, although there were slightly more foci in the initiated/promoted rats, the area of the liver impacted was smaller.

7.3 Potentially Sensitive Populations

Available animal data is not sufficient to determine if there is a difference in the response of males versus females following oral exposure to microcystin. Fawell et al. (1999) observed a slight difference between male and female mice in body weight and serum proteins (ALT and AST), but no sex-related differences in liver pathology.

Studies in laboratory rodents suggest that the acute effects of microcystin-LR may be more pronounced in adult or aged animals than in juvenile animals (Adams et al., 1985; Ito et al., 1997a; Rao et al., 2005). In

these studies, young animals showed little or no effect with microcystin-LR doses found to be lethal to adult animals. Age-dependent differences in toxicity were observed after both oral and i.p. exposure, suggesting that differences in gastrointestinal uptake were not entirely responsible for the effect of age. The relevance of these age-related differences to acute toxicity in humans is unknown. However, for cyanotoxins including microcystins, drinking water contributes the highest risk of the total cyanotoxin intake for infants to one-year old fed exclusively with powdered formula prepared with tap water containing cyanotoxins. Based on the average drinking water intake rates for infants (< 12 months; 0.15 L/kg/day), the exposure of infants is 5 times higher than those of adults (>21 yrs. old) on a body weight basis.

Based on the available studies in animals, individuals with liver and/or kidney disease might be more susceptible than the general population since the detoxification mechanisms in the liver are compromised and excretory mechanisms in the kidney are impaired. Data from an episode in a dialysis clinic in Caruaru, Brazil, where microcystins were not removed by treatment of dialysis water, identify dialysis patients as a population of potential concern in cases where the drinking water source for the clinic is contaminated with cyanotoxins. Other individuals of potentially sensitivity are pregnant woman, nursing mothers, and the elderly population.

7.4 Characterization of Health Risk

7.4.1 Choice of Key Study

The critical study chosen for determining the guideline value is the short-term study by Heinze (1999) in which rats were administered microcystin-LR via drinking water for 28 days at concentrations of 0, 50 or 150 μg/kg body weight (Heinze, 1999). The LOAEL was determined to be 50 μg/kg/day based on increased liver weight, slight to moderate liver lesions with hemorrhages, and increased enzyme levels. The selection of the study by Heinze (1999) was based on the appropriateness of the study duration, the use of multiple doses, dose-related toxicological responses, and histopathological evaluations of toxicity. After 28 days of exposure, rat organ weights (liver, kidneys, adrenals, thymus and spleen) were measured, and hematology, serum biochemistry plus histopathology of liver and kidneys were evaluated.

The route of exposure was another important factor for the selection of this study. Although the studies discussed above used different species and strains of laboratory animal and differed in dose, duration, route of exposure, and description of liver histopathology, they all reported effects to the liver in the 30-50 μg/kg dose range. The results reported are consistent with the hypothesis that the risk for liver damage is proportional to the exposure route as predicted because of the requirement for intestinal facilitated or active transport (i.p. infusion > drinking water > gavage). Although the biomarkers for liver damage differ, the results are consistent with the hypothesis that the route of exposure needs to be considered as an important variable. The cell necrosis following a 28 day exposure reported by Heinze (1999) is supported by the Guzman and Solter (1999) findings using slow osmotic infusion to the peritoneum. The fact that the oral drinking water dose in Heinze (1999) caused similar signs of liver damage as did i.p. infusion in Guzman and Solter (1999), is consistent with the conclusion that the intestinal barrier limits flow to serum for distribution to the liver. Although the Fawell et al. (1999) study had a longer duration, dosing occurred as a bolus to the intestine once per day, thereby limiting most absorption to the period of small intestinal transit. It may also be that mice of the strain used by Fawell et al. (1999) are less sensitive to microcystin-LR hepatic injury than rats. The increase in hepatic chronic inflammation in the 40 μg/kg/day group (10/30) compared to the control group (4/30) is supportive of the conclusion that a smaller amount of the chemical reached the liver in the Fawell et al. (1999) experiment than it did in the drinking water and i.p infusion studies based on the manifestation of necrosis in the latter at similar doses.

7.4.2 Endpoint Selection

Upon considering all available studies, liver damage was considered the most appropriate basis for quantitation as it was a common finding among oral toxicology studies (Falconer et al., 1994; Fawell et al., 1999; Ito et al., 1997b). While the liver is the usual target of microcystin toxicity, there have been reports of effects of microcystin-LR on the male reproductive system and sperm development following oral exposures (Chen et al., 2011).

Oral exposures to low concentrations of microcystin-LR for 3 to 6 months showed reproductive toxicity including decreased sperm counts and sperm motility, as well as an increase in sperm abnormalities, decreased serum testosterone and increased serum luteinizing hormone (LH) levels (Chen et al., 2011). Since these effects were observed at doses lower (0.79 μg/kg/day) than those observed for liver effects in Heinze (1999), EPA evaluated Chen et al. (2011) and the lesions in the testes and effects on sperm motility as the potential critical study and points of departure for the derivation of the RfD for microcystins.

The Chen et al. (2011) study has several limitations in the experimental design and reporting. There was a lack of data reported on testis weights and sperm motility. The authors only reported "no significant differences in testis weights", but no information was provided on the weights of the testis or whether there was a trend toward decreasing weights that failed to reach significance. Also, no information was given on the methodology used for sperm motility evaluation. No information was provided on how samples were handled and what measurements were made to determine the percentage of sperm motility. Although body weight and amount of water consumed were measured, these data were not presented and doses to the animals were not calculated by the study authors. In addition, the purity of microcystin-LR and the species and age of the mouse used were not reported. Male sperm characteristics such as volume, motility, and structure of sperm differ developmentally by age; therefore, not knowing the age of the mouse in the study introduces uncertainty to the quantification of the reproductive effects.

The fixation and staining of the testes used for microscopic examination (paraformaldehyde in phosphate-buffered saline (PBS) and paraffin) in Chen et al. (2011) could result in the generation of artifacts, such as disruption of the testicular tubes. Cytoplasmic shrinkage and chromatin aggregations were observed in both control and experimental groups. In order to preserve the microstructure of the testis, dual fixation such as Davidson's or Bouin's fixation followed by PAS staining should have been done. In addition, the histopathology analysis of the testis reported by the authors did not provided sufficient detail to adequately assess the degree of damage.

The quality of the medium used for the sperm analysis in Chen et al. (2011), and the lack of additional data from the sperm analysis measurements carried out through the computer-assisted sperm analysis (CASA) are additional limitations in experimental design for this study. Very few details of the serum hormone assay protocol and the quantitative parameters of sperm motility from the CASA analysis were provided. Therefore, the calculation for the motility of the sperm was unclear and couldn't be verified.

Based on the limitations in study design, report and methods used by Chen et al. (2011), it was concluded that the quantitative data on decreased sperm counts and sperm motility were not appropriate for use as the effect to determine the point of departure for the derivation of the RfD for microcystin-LR.

7.4.3 RfD Determination

The LOAEL from the Heinze (1999) study was the 50 μg/kg/day dose based on liver effects (increased liver weight, slight to moderate liver necrosis lesions, with or without hemorrhages at the low dose and increased in severity at the high dose, and changes in serum enzymes indicative of liver damage). The RfD for microcystin-LR is calculated as follows:

$$\text{RfD} = \frac{50\,\mu\text{g/kg/day}}{1000} = 0.05\,\mu\text{g/kg/day}$$

where:

50 µg/kg/day	=	The LOAEL for liver effects in 11-week-old male hybrid rats exposed to MC-LR in drinking water for 28 days (Heinze, 1999).
1000	=	The composite UF including 10 for intraspecies variability (UF_H), 10 for interspecies differences (UF_A), 3 ($10^{0.5}$) for LOAEL to NOAEL extrapolation (UF_L), and 3 ($10^{0.5}$) for uncertainties in the database (UF_D).

- UF_H. A Ten-fold value is applied to account for variability in the human population. No information was available to characterize interindividual and age -related variability in the toxicokinetics or toxicodynamics among humans.
- UFA. A Ten-fold value is applied to account for uncertainty in extrapolating from laboratory animals to humans (i.e., interspecies variability). Information to quantitatively assess toxicokinetic or toxicodynamic differences between animals and humans is unavailable for microcystin. Allometric scaling is not applied in the development of the Ten-Day HA values for microcystin. The allometric scaling approach is derived from the relationship between body surface area and basal metabolic rate in adults (U.S. EPA, 2011). For infants and children, surface area and basal metabolic rates are very different than adults and are not appropriate for infants and children.
- UF_L. An uncertainty factor of 3 ($10^{0.5}$ = 3.16, rounded to 3) to adjust the LOAEL to a NOAEL was applied. The threefold factor is justified based on the evidence that suggests that the uptake of microcystins by tissues requires membrane transporters. Uptake from the intestines involves both apical and basolateral transporters, uptake by the microvilli capillaries and portal transport to the liver. Transporters are again necessary for hepatic uptake. When there is slow infusion into the peritoneum and into the portal intraperitoneal capillaries, uptake is described as rapid because of the rich blood supply and large surface area of the peritoneal cavity (Klassen, 1996). Delivery of the microcystin to the intraperitoneum increases the amount of the dose that reaches the liver for three additional reasons: 1) the apical and basolateral intestinal barriers to uptake are eliminated with the i.p. infusion; 2) there is no dilution of dose by the gastric plus intestinal fluids as well as with food residues in the gastrointestinal track; and 3) there is no delay in reaching the site of absorption because of gastric emptying time (Klassen, 1996). In addition, facilitated transporter kinetics are similar to Michaelis Menton enzyme kinetics in that there are Km and Vmax components that are defined by the affinity of the transported substance for the transporter.

  In the Guzman and Solter (1999) intraperitoneal infusion study in rats, the NOAEL was 16 µg/kg/day and the LOAEL was 32 µg/kg/day. Given the 2-fold difference between the NOAEL and LOAEL in this study, there is no reason to believe that the less direct delivery from the intestines to the liver expected following oral exposures through drinking water (as was used in Heinze) would have a more than 3-fold separation between a NOAEL and LOAEL had there been one.

- UF_D. An uncertainty factor of 3 ($10^{0.5}$ = 3.16 rounded to 3) is selected to account for deficiencies in the database for microcystin. The database includes limited human data, including studies evaluating the association between microcystin exposure and cancers in liver and colon, and systemic effects including liver endpoints such as elevated liver enzymes. Oral and i.p. acute and short-term studies on mice and rats, and subchronic studies done in mice are available. Chronic data are also available for microcystin, however, they are limited by the lack of quantitative data provided in the study. Additionally, there are limited neurotoxicity studies (including a recent publication on developmental neurotoxicity) and several i.p. reproductive and developmental toxicity studies. The database lacks a multi-generation reproductive toxicity study.

It should be noted that, the default factors typically used cover a single order of magnitude (i.e., 10^1). By convention, in the Agency, a value of 3 is used in place of one-half power (i.e., 100.5) when appropriate (U.S. EPA, 2002).

8.0 RESEARCH GAPS

Microcystin-LR has the most comprehensive database among the cyanotoxins produced by cyanobacteria and among the microcystin congeners yet much remains to be done. As anthropogenic activities and climate change continue to stress lakes, rivers, ponds, and streams that serve as sources of drinking water, irrigation water and sites for recreation, research to fill existing data gaps on health effects in humans, wildlife and domestic animals becomes increasingly important. This chapter provides a summary of gaps in knowledge identified during the development of this document. The key research gaps listed below are not intended to be an exhaustive list. Additional research efforts are needed on:

- The absorption, distribution, and elimination of microcystins in humans and animals following oral, inhalation or dermal exposure that can be used to support extrapolation of the oral exposure data across species and to other exposure routes.
- The toxicity of microcystin-LR to the male reproductive system after sub-acute to chronic oral exposure. Special attention should be given to the potential clinical significance of the decreased sperm count and motility; reduced testosterone levels; and microscopic lesions in the testes observed in mice by Chen et al. (2011).
- The toxicity of microcystin-LR to the female reproductive tissues and those of offspring following oral exposure.
- The relative potencies of other microcystin congeners when compared to microcystin-LR.
- Health risks posed by repeated, low-level exposures of microcystins.
- The adverse effects of chronic exposures to microcystins.
- The immunotoxic, neurotoxic and developmental/reproductive toxicity of microcystins following oral exposure.
- The carcinogenic potential of microcystin-LR.
- Potential health risks from exposure to mixtures of microcystins with other cyanotoxins and chemical stressors present in ambient and or drinking water supplies.
- Populations that might be sensitive to microcystins exposure via the oral, dermal and/or inhalation routes.
- Bioconcentration and bioaccumulation of microcystins in aquatic vertebrates and invertebrates and the transfer in the food web.
- Bioavailability of microcystins in seafood and crops to humans consuming fish, shellfish and edible plants that have been exposed to microcystins contaminated water.

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