United States Environmental Protection Agency

Office of Water
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2015 Drinking Water Health Advisories for Two Cyanobacterial Toxins

Summary

EPA has issued 10-Day Drinking Water Health Advisories (HAs) for the cyanobacterial toxins microcystins and cylindrospermopsin.

EPA recommends HA levels at or below 0.3 micrograms per liter for microcystins and 0.7 micrograms per liter for cylindrospermopsin in drinking water for children pre-school age and younger (less than six years old). For school-age children through adults, the recommended HA levels for drinking water are at or below 1.6 micrograms per liter for microcystins and 3.0 micrograms per liter for cylindrospermopsin. Young children are more susceptible than older children and adults as they consume more water relative to their body weight.

HAs are non-regulatory values that serve as informal technical guidance to assist federal, state and local officials, and managers of public or community water systems to protect public health from contaminants. EPA has also published health effects support documents for the cyanobacterial toxins microcystins and cylindrospermopsin. These documents contain the health effects basis for the development of HAs for the protection of human health. In addition, EPA has published a health effects support document for anatoxin-a but concluded that there was not adequate information to support a health advisory for this toxin.

Background

What are cyanobacterial toxins?

Cyanobacteria, common to freshwater and marine ecosystems, can under certain conditions (high nutrient concentrations and high light intensity) form scums or "blooms" at the surface of a water body. These blooms can produce toxic compounds (cyanobacterial toxins or "cyanotoxins") that are harmful to the environment, animals and human health. Winds and water currents can transport cyanobacterial blooms within proximity to drinking water intakes at treatment plants that, if not removed during treatment, can cause odor, taste and color problems in treated drinking water and can be harmful to human health.

What is a health advisory?

The Safe Drinking Water Act provides the authority for EPA to publish health advisories for contaminants not subject to any national primary drinking water regulation. Health advisories describe non-regulatory concentrations of drinking water contaminants at or below which adverse health effects are not anticipated to occur over specific exposure durations (e.g., one-day, 10-days, several years, and a lifetime). They serve as informal technical guidance to assist federal, state and local officials, and managers of public or community water systems by providing information on the health effects of and methods to sample and treat cyanobacterial toxins in drinking water. HAs are not legally enforceable federal standards and are subject to change as new information becomes available.

Why has EPA taken this action?

There are no U.S. federal guidelines, water quality criteria, standards or regulations for cyanobacteria or cyanotoxins in drinking water under the Safe Drinking Water Act or in surface waters under the Clean Water Act. However, EPA has listed cyanotoxins including microcystin-LR, cylindrospermopsin, and anatoxin–a on the previous and current Contaminant Candidate Lists (CCL), which identify contaminants that may need regulation under the Safe Drinking Water Act.

EPA found there are adequate health effects data to develop HAs for microcystins and cylindrospermopsin, but found the data inadequate to develop an HA for the cyanobacterial toxin anatoxin-a.

How Can I Be Exposed to Cyanobacterial Toxins?

For the cyanotoxin HAs, drinking water is the primary source of exposure. Exposure may also occur by ingestion of toxin contaminated food, including consumption of fish; by inhalation and dermal contact during bathing or showering; and during recreational activities. Effects due to these other routes of exposure cannot be quantified at this time, however, they are assumed to be less than from drinking water ingestion.

What information was used to develop the health advisories for cyanobacterial toxins?

EPA worked with Health Canada and conducted a comprehensive search of the literature from January 2013 to May 2014. The HA includes information on occurrence; environmental fate; mechanisms of toxicity; acute, short term, subchronic and chronic toxicity and cancer in humans and animals; toxicokinetics; health effects and exposure. The HA also includes information on methods for analysis and treatment techniques for removal in drinking water treatment plants.

Health Effects Information

Effects including gastroenteritis, and liver and kidney damage have been reported in humans following short-term exposure to cyanotoxins in drinking water. Recreational exposure to cyanobacterial blooms has been reported to lead to allergic reactions, including hay fever-like symptoms; skin rashes; and gastrointestinal distress. Animal studies have shown that long-term adverse effects from cyanotoxins include liver and kidney damage. However, more research is needed to quantify these effects.

Critical Studies Used

The critical study supporting the microcystins 10-day HA was conducted by Heinze (1999). This study is a 28-day study in rats, whose drinking water contained microcystin-LR. Effects observed included changes in liver weight, liver serum enzymes, and lesions in the liver. The lowest observed adverse effect level (LOAEL) based on these effects was 50 micrograms per kilogram per day, a no observed adverse effect level (NOAEL) was not identified. This dose was selected as the basis for deriving a reference dose (RfD) for microcystins. A total uncertainty factor of 1000 (10 to account for differences between humans and animals, 10 to account for variability in humans, 3 for extrapolation from a LOAEL, and 3 to address database deficiencies) was applied to determine the RfD for microcystins. These values were used along with body weight and drinking water intake for infants and adults to derive the 10-Day HA values. The 10-day HA of 0.3 µg/L is considered protective of non-carcinogenic adverse health effects for bottle-fed infants and young children of pre-school age over a ten-day exposure to microcystins in drinking water. The 10-day HA of 1.6 µg/L is considered protective of non-carcinogenic adverse health effects for children of school age through adults over a 10-day exposure to microcystins in drinking water.

The critical study supporting the cylindrospermopsin 10-day advisory was conducted by Humpage and Falconer (2002, 2003). This study is an 11-week study with cylindrospermopsin administered to male mice by gavage. Effects observed included increases in kidney weight. The NOAEL from this study was 30 micrograms per kilogram per day and the LOAEL based on kidney weight changes was 60 micrograms per kilogram per day. The NOAEL of 30 micrograms per kilogram per day was selected as the basis for the RfD. A total uncertainty factor of 300 (10 to account for differences between humans and animals, 10 to account for variability in humans, and 3 to address database deficiencies) was applied to determine the RfD for cylindrospermopsin. These values were used along with body weight and drinking water intake for infants and adults to derive the 10-Day HA values. The 10-day HA of 0.7 µg/L is considered protective of non-carcinogenic adverse health effects for bottle-fed infants and young children of pre-school age over a 10-day exposure to cylindrospermopsin in drinking water. The 10-day HA of 3 µg/L is considered protective of non-carcinogenic adverse health effects for children of

school age through adults over a 10-day exposure to cylindrospermopsin in drinking water.

As the science on the health impacts of algal toxins continues to improve, EPA will track developments and update recommendations as appropriate.

Additional EPA support document to assist states and utilities in managing cyanobacterial toxins

EPA has also published a cyanotoxin management document as a companion to the HAs. The document is designed to provide information and a framework that Public Water Systems and others can consider to inform their decisions on managing the risks from cyanotoxins to drinking water. It includes a potential stepwise approach these systems could use to inform their decisions on whether and how to monitor and treat water, and communicate with stakeholders.

Where can I find more information?

To learn more about the HAs for microcystins and cylindrospermopsin and to view the health effects support documents for these and anatoxin-a in drinking water, visit EPA's Health Advisory webpage: http://water.epa.gov/drink/standards/hascience.cfm. To learn about additional strategies Public Water Systems and others could consider in managing cyanotoxins, visit EPA's CyanoHABs website: http://www2.epa.gov/nutrient-policy-data/guidelines-and-recommendations

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Drinking Water Health Advisory for the Cyanobacterial Toxin Cylindrospermopsin

Drinking Water Health Advisory for the Cyanobacterial Toxin Cylindrospermopsin

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U.S. Department of Health and Human Services, Centers for Disease Control and Prevention

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ABBREVIATIONS AND ACRONYMS

BMD	Benchmark Dose
BMDL	Benchmark Dose Level
BW	Body Weight
CAS	Chemical Abstracts Service
CCL	Contaminant Candidate List
CWA	Clean Water Act
CYP450	Cytochrome P450
DAF	Dissolved Air Flotation
DBP	Disinfection By-Products
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DWI	Drinking Water Intake
ELISA	Enzyme Linked Immunosorbent Assay
EPA	U.S. Environmental Protection Agency
g	Gram
GAC	Granular Activated Carbon
GFR	Glomerular Filtration Rate
HA	Health Advisory
HAB	Harmful Algal Bloom
HESD	Health Effects Support Document
HPLC	High Performance Liquid Chromatography
ICR	Institute for Cancer Research
i.p.	Intraperitoneal
kg	Kilogram
K_{oc}	Organic Carbon:Water Partition Coefficient
K_{ow}	Octanol:Water Partition Coefficient
L	Liter
LC	Liquid Chromatography
LCAT	Lecithin Cholesterol Acyl Transferase
LC-ESI/MS	Liquid Chromatography Tandem Electrospray Ionization Mass Spectrometry
LCMRL	Lowest Concentration Method Reporting Limit
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LOAEL	Lowest-Observed-Adverse-Effect Level
MCH	Mean Corpuscular Hemoglobin
µg	Microgram
µm	Micromole
MNBNC	Micronucleated Binucleated Cells
mg	Milligram
mL	Milliliter
mmol	Millimole
MOA	Mode of Action
MWCO	Molecular Weight Cut-off
N	Nitrogen

N/A	Not Applicable
NARS	National Aquatic Resource Surveys
ng	Nanogram
NHANES	National Health and Nutrition Examination Survey
NLA	National Lake Assessment
NOAEL	No-Observed-Adverse-Effect Level
NOM	Natural Organic Material
OECD	Organization for Economic Cooperation and Development
P	Phosphorus
PAC	Powdered Activated Carbon
PDA	Photodiode Array
pg	Picogram
POU	Point-of-Use
RBC	Red Blood Cell
RfD	Reference Dose
SDWA	Safe Drinking Water Act
SHE	Syrian Hamster Embryo
SPE	Solid Phase Extraction
TOC	Total Organic Carbon
TOXLINE	Toxicology Literature Online
UF	Uncertainty Factor
USACE	U.S. Army Corps of Engineers
USGS	U.S. Geological Survey
UV	Ultraviolet

EXECUTIVE SUMMARY

Cylindrospermopsin is a toxin produced by a variety of cyanobacteria including: *Cylindrospermopsis raciborskii (C. raciborskii), Aphanizomenon flos-aquae, Aphanizomenon gracile, Aphanizomenon ovalisporum, Umezakia natans, Anabaena bergii, Anabaena lapponica, Anabaena planctonica, Lyngbya wollei, Rhaphidiopsis curvata,* and *Rhaphidiopsis mediterranea*.

Many environmental factors such as the ratio of nitrogen to phosphorus, temperature, organic matter availability, light attenuation and pH play an important role in the development of cylindrospermopsin blooms, both in fresh and marine water systems. These species do not tend to form visible surface scums and the highest concentrations of cells occurs below the water surface. Cylindrospermopsin may be retained within the cell, but most of the time it is found in the water (extracellular) or attached to particulates present in the water.

This Health Advisory (HA) for the cyanobacterial toxin cylindrospermopsin is focused on drinking water as the primary source of exposure. Exposure to cyanobacteria and their toxins may also occur by ingestion of toxin-contaminated food, including consumption of fish, and by inhalation and dermal contact during bathing or showering and during recreational activities in waterbodies with the toxins. While these types of exposures cannot be quantified at this time, they are assumed to contribute less to the total cyanotoxin exposures than ingestion of drinking water. Due to the seasonality of cyanobacterial blooms, exposures are not expected to be chronic.

Limited animal studies demonstrate absorption of cylindrospermopsin from the intestinal tract primarily in the liver, but also in the kidney and spleen. Limited data are available on the metabolism of cylindrospermopsin, but evidence indicates that metabolism and toxicity are mediated by the hepatic cytochrome P450 (CYP450) enzyme system. The periacinar region of the liver, an area where substantial CYP450-mediated xenobiotic metabolism occurs, appears to be the main target of cylindrospermopsin toxicity and where cylindrospermopsin and its metabolites bind to proteins. The few studies evaluating elimination suggest that cylindrospermopsin is rapidly eliminated primarily in the urine, but also in feces.

The main source of information on the toxicity of cylindrospermopsin in humans is from qualitative reports of a hepatoenteritis-like illness attributed to acute or short-term consumption of drinking water containing *C. raciborskii*. Symptoms reported include fever, headache, vomiting, bloody diarrhea, hepatomegaly, and kidney damage with loss of water, electrolytes and protein. No reliable data are available on the exposure levels of cylindrospermopsin that induced these effects.

Based on oral and intraperitoneal (i.p.) studies in mice treated with purified cylindrospermopsin or extracts of *C. raciborskii* cells, the liver and kidneys appear to be the primary target organs for cylindrospermopsin toxicity.

The U.S. Environmental Protection Agency (EPA) identified a study by Humpage and Falconer (2002, 2003) conducted on mice as the critical study used in the derivation of the reference dose (RfD) for cylindrospermopsin. The critical effects identified in the study areincreased kidney weight and decreased urinary protein. The NOAEL (No Observed Adverse

Effect Level) was determined to be 30 µg/kg/day based on kidney toxicity. The total uncertainty factor (UF) applied to the NOAEL was 300. This was based on a UF of 10 for intraspecies variability, a UF of 10 for interspecies variability, and a UF of 3 ($10^{½}$) to account for deficiencies in the database.

EPA is issuing a Ten-day HA for cylindrospermopsin based on the Humpage and Falconer (2002, 2003) 11-week study. Studies of a duration of 7 days up to 30 days are typically used to derive Ten-day HAs. In this case, a subchronic study was determined to be suitable for the derivation of the HA. Although the duration of the Humpage and Falconer (2002, 2003) study is longer (77 days) than the studies typically used for the derivation of a Ten-day HA, the short-term studies available for cylindrospermopsin (Shaw et al., 2001; Reisner et al., 2004) are not suitable for quantification; however, effects observed in these studies are the same or similar to the Humpage and Falconer study (2002, 2003) and occur at similar doses.

The short-term HA is consistent with the available data and most appropriately matches human exposure scenarios for cyanobacterial blooms in drinking water. Cyanobacterial blooms are usually seasonal, typically occurring from May through October. In the presence of algal cell pigments, photochemical degradation of cylindrospermopsin can occur rapidly, with reported half-lives of 1.5 to 3 hours. In the absence of pigments, however, there is little degradation. The biodegradation of cylindrospermopsin in natural water bodies is a complex process that can be influenced by many environmental factors, including concentration, water temperature and the presence of bacteria. Half-lives of 11 to 15 days and up to 8 weeks have been reported for cylindrospermopsin in surface waters. In addition, concentrations in finished drinking water can be reduced by drinking water treatment and management measures.

The Ten-day HA value for bottle-fed infants and young children of pre-school age is 0.7 µg/L and for school-age children through adults is 3 µg/L for cylindrospermopsin. The two advisory values use the same toxicity data (RfD) and represent differences in drinking water intake and body weight for different human life stages. The first advisory value is based on the summation of the time-weighted drinking water intake/body weight ratios for birth to < 12 months of age (U.S. EPA's Exposure Factors Handbook, 2011a). The second advisory value is based on the mean body weight and the 90th percentile drinking water consumption rate for adults age 21 and over (U.S. EPA's Exposure Factors Handbook, 2011a), which is similar to that of school-aged children. Populations such as pregnant women and nursing mothers, the elderly, and immune-compromised individuals or those receiving dialysis treatment may be more susceptible than the general adult population to the health effects of cylindrospermopsin. As a precautionary measure, individuals that fall into these susceptible groups may want to consider following the recommendations for children pre-school age and younger. This HA is not a regulation; it is not legally enforceable; and it does not confer legal rights or impose legal obligations on any party.

No epidemiological studies of the association of cylindrospermopsin and cancer are available. Also, no chronic cancer bioassays of purified cylindrospermopsin in animals were identified. Therefore, under the U.S. EPA's (2005) Guidelines for Carcinogen Risk Assessment, there is *inadequate information to assess carcinogenic potential* of cylindrospermopsin.

1.0 INTRODUCTION AND BACKGROUND

EPA developed the non-regulatory Health Advisory (HA) Program in 1978 to provide information for public health officials or other interested groups on pollutants associated with short-term contamination incidents or spills for contaminants that can affect drinking water quality, but are not regulated under the Safe Drinking Water Act (SDWA). At present, EPA lists HAs for 213 contaminants (http://water.epa.gov/drink/standards/hascience.cfm).

HAs identify the concentration of a contaminant in drinking water at which adverse health effects are not anticipated to occur over specific exposure durations (e.g., one-day, ten-days, and a lifetime). HAs serve as informal technical guidance to assist Federal, State and local officials, and managers of public or community water systems in protecting public health when emergency spills or contamination situations occur. An HA provides information on the environmental properties, health effects, analytical methodology, and treatment technologies for removal of drinking water contaminants.

The *Health Effects Support Document for Cylindrospermopsin* (U.S. EPA, 2015a) is the peer-reviewed, effects assessment that supports this HA. This document is available at http://www2.epa.gov/nutrient-policy-data/health-and-ecological-effects. The HAs are not legally enforceable Federal standards and are subject to change as new information becomes available. The structure of this Health Advisory is consistent with EPA's *Framework for Human Health Risk Assessment to Inform Decision Making* (U.S.EPA, 2014).

EPA is releasing the *Recommendations for Public Water Systems to Manage Cyanotoxins in Drinking Water* (U.S. EPA, 2015b) as a companion to the HAs for microcystins and cylindrospermopsin. The document is intended to assist public drinking water systems (PWSs) that choose to develop system-specific plans for evaluating their source waters for vulnerability to contamination by microcystins and cylindrospermopsin. It is designed to provide information and a framework that PWSs and others as appropriate may consider to inform their decisions on managing the risks from cyanotoxins in drinking water.

1.1 Current Criteria, Guidance and Standards

Currently there are no U.S. federal water quality criteria, or regulations for cyanobacteria or cyanotoxins in drinking water under the SDWA or in ambient waters under the Clean Water Act (CWA). The Safe Drinking Water Act (SDWA), as amended in 1996, requires the EPA to publish a list of unregulated contaminants every five years that are not subject to any proposed or promulgated national primary drinking water regulations, which are known or anticipated to occur in public water systems, and which may require regulation. This list is known as the Contaminant Candidate List (CCL). The EPA's Office of Water included cyanobacteria and cyanotoxins on the first and second CCL (CCL 1, 1998; CCL 2, 2005). EPA included cyanotoxins, including anatoxin-a, cylindrospermopsin, and microcystin-LR, on CCL 3 (2009) and the draft CCL 4 (April 2015 for consideration).

SDWA requires the Agency to make regulatory determinations on at least five CCL contaminants every five years. When making a positive regulatory determination, EPA determines whether a contaminant meets three criteria:

- The contaminant may have an adverse effect on the health of persons,
- The contaminant is known to occur or there is substantial likelihood the contaminant will occur in public water systems with a frequency and at levels of concern, and
- In the sole judgment of the Administrator, regulating the contaminant presents a meaningful opportunity for health risk reductions.

To make these determinations, the Agency uses data to analyze occurrence (prevalence and magnitude) and health effects. EPA continues gathering this information to inform future regulatory determinations for cyanotoxins under the SDWA. The SDWA also provides the authority for EPA to publish non-regulatory HAs or take other appropriate actions for contaminants not subject to any national primary drinking water regulation. EPA is providing this HA and the HA for microcystins to assist State and local officials in evaluating risks from these contaminants in drinking water.

Internationally, three countries and two U.S. states have developed drinking water guidelines for cylindrospermopsin, as shown in Table 1-1 and Table 1-2, respectively.

Table 1-1. International Guideline Values for Cylindrospermopsin

Country	Guideline Value	Source
Australia	1 µg/L	Australian Drinking Water Guidelines 6 (NHMRC, NRMMC, 2011)
New Zealand	1 µg/L	Drinking-water Standards for New Zealand 2005 (Ministry of Health, 2008)
Brazil	15 µg/L (recommended)	Guidelines for Drinking Water Quality, Official LA Report's, Regulation MS N 518/2004 (Brasil, 2009)

Table 1-2. State Guideline Values for Cylindrospermopsin

State	Guideline Value	Source
Ohio	1 µg/L	State of Ohio Public Water System Harmful Algal Bloom Response Strategy (OHEPA, 2014)
Oregon	1 µg/L	Public Health Advisory Guidelines, Harmful Algae Blooms in Freshwater Bodies. (OHA, 2015)

2.0 PROBLEM FORMULATION

The development of the HA begins with problem formulation, which provides a strategic framework by focusing on the most relevant cyanotoxin properties and endpoints identified in the *Health Effects Support Document for Cylindrospermopsin* (U.S. EPA, 2015a).

2.1 Cyanobacteria and Production of Cylindrospermopsin

Cyanobacteria, formerly known as blue-green algae (Cyanophyceae), are a group of bacteria with chlorophyll-a capable of photosynthesis (light and dark phases) (Castenholz and Waterbury, 1989). Most cyanobacteria are aerobic photoautotrophs, requiring only water, carbon dioxide, inorganic nutrients and light for survival, while others have heterotrophic properties and can survive long periods in complete darkness (Fay, 1965). Some species are capable of nitrogen fixation (diazotrophs) (Duy et al., 2000), producing inorganic nitrogen compounds for the synthesis of nucleic acids and proteins. Cyanobacteria can form symbiotic associations with animals and plants, such as fungi, bryophytes, pteriodophytes, gymnosperms and angiosperms (Rai, 1990), supporting their growth and reproduction (Sarma, 2013; Hudnell, 2008; Hudnell, 2010).

Under the right conditions of pH, nutrient availability, light, and temperature, cyanobacteria can reproduce quickly, forming a bloom. Although studies of the impact of environmental factors on cyanotoxin production are ongoing, nutrient (nitrogen, phosphorus and trace metals) supply rates, light, temperature, oxidative stressors, interactions with other biota (viruses, bacteria and animal grazers) and, most likely, the combined effects of these factors are all involved (Paerl and Otten 2013a, 2013b). Fulvic and humic acids reportedly encourage cyanobacteria growth (Kosakowska et al., 2007).

Cylindrospermopsin is a toxin produced by a variety of cyanobacteria including: *Cylindrospermopsis raciborskii (C. raciborskii), Aphanizomenon flos-aquae, Aphanizomenon gracile, Aphanizomenon ovalisporum, Umezakia natans, Anabaena bergii, Anabaena lapponica, Anabaena planctonica, Lyngbya wollei, Rhaphidiopsis curvata,* and *Rhaphidiopsis mediterranea.*

2.2 Physical and Chemical Properties

The cyanotoxin cylindrospermopsin is a tricyclic alkaloid with the following molecular formula $C_{15}H_{21}N_5O_7S$ (Ohtani et al., 1992) and a molecular weight of 415.43 g/mole. It is zwitterionic (i.e., a dipolar ion with localized positive and negative charges) (Ohtani et al., 1992) and is believed to be derived from a polyketide that uses an amino acid starter unit such as glycocyamine or 4-guanidino-3-oxybutyric acid (Duy et al., 2000). The chemical structure of cylindrospermopsin is presented in Figure 2-1. Two naturally occurring congeners of cylindrospermopsin have been identified (Figure 2-2): 7-epicylindro-spermopsin (the epimer of cylindrospremopsin) and 7-deoxycylindrospermopsin (Norris et al., 1999; de la Cruz et al., 2013). Recently, Wimmer et al. (2014) identified two new analogs, 7-deoxy-desulfo-

Figure 2-1. Structure of Cylindrospermopsin (de la Cruz et al., 2013)

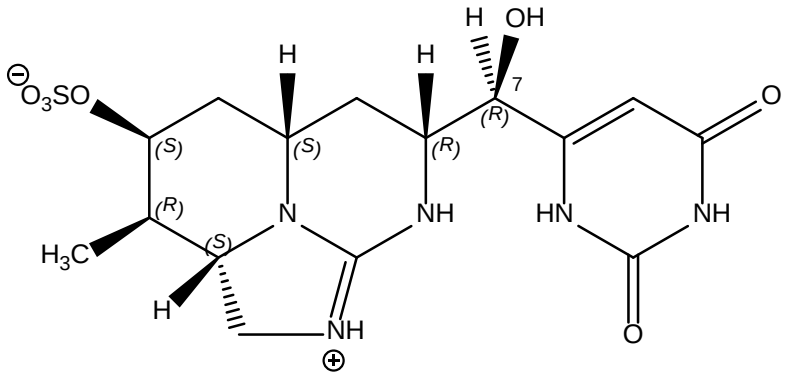
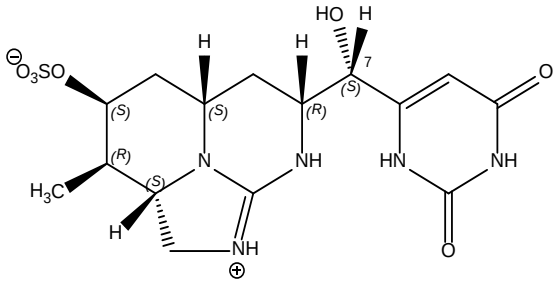
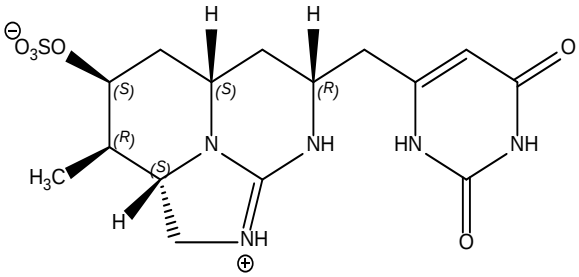


Figure 2-2. Structurally Related Cylindrospermopsins (de la Cruz et al., 2013)

7-epi-cylindrospermopsin



7-deoxycylindrospermopsin



cylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin, from the Thai strain of *C. raciborskii*. However, it is not clear if these are cylindrospermopsin congeners, precursors or degradation products. Chlorination of water containing cylindrospermopsin can produce 5-chlorocylindrospermopsin and cylindrospermic acid.

The physical and chemical properties of cylindrospermopsin are presented in Table 2-1. Cylindrospermopsin generally exists in a zwitterionic state (with both positive and negative ions) and is highly soluble in water (Moore et al., 1998, Chiswell et al., 1999). Cylindrospermopsin is isolated for commercial use mostly from *C. raciborskii.* Other physicochemical properties of cylindrospermopsin in the environment such as vapor pressure, boiling and melting points, soil organic carbon-water partition coefficient (Koc), octanol-water partition coefficient (Kow), and vapor pressure and Henry's Law constant are unknown. Available information on the chemical

Table 2-1. Chemical and Physical Properties of Cylindrospermopsin

Property	Cylindrospermopsin
Chemical Abstracts Service (CAS) Registry #	143545-90-8
Chemical Formula	$C_{15}H_{21}N_5O_7S$
Molecular Weight	415.43 g/mole
Color/Physical State	white powder
Boiling Point	N/A
Melting Point	N/A
Density	$2.03 g/cm^3$
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	Dimethylsulfoxide (DMSO) and methanol

Sources: Chemical Book, 2012; TOXLINE, 2012

breakdown, biodegradation and mobility of cylindrospermopsin in the environment is discussed in the Environmental Fate section.

2.3 Sources and Occurrence

Many environmental factors such as the ratio of nitrogen to phosphorus, temperature, organic matter availability, light attenuation and pH play an important role in the development of cylindrospermopsin blooms (Paerl and Huisman, 2008; Paerl and Otten, 2013). Although cylindrospermopsin-producing cyanobacteria (such as *C. raciborskii)* occur mostly in tropical or subtropical regions, they have also been found in warmer temperate regions, both in fresh and marine water systems. These species do not tend to form visible surface scums and the highest concentrations of cells occurs below the water surface (Falconer 2005). Cylindrospermopsin may be retained within the cell, but most of the time it is found in the water (extracellular) or attached to particulates present in the water (Chiswell et al., 2001).

2.3.1 Occurrence in Surface Water

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 algal toxins, but did not include cylindrospermopsin. The United States Geological Survey (USGS) subsequently analyzed the stored samples collected during the NLA and reported that cylindrospermopsin was present in 5% of the samples; however, concentrations of cylindrospermopsin were not reported (Loftin and Graham, 2014). Future NARS plan to include other algal toxins, including cylindrospermopsin.

Cylindrospermopsin was also detected in 9% of the blooms sampled during a 2006 USGS survey of 23 lakes in the Midwestern U.S. (Graham et al., 2010). The low concentrations of cylindrospermopsin detected (0.12 to 0.14 µg/L) in the study occurred in bloom communities dominated by *Aphanizomenon* or *Anabaena* and *Microcystis*.

Many states monitor for harmful algal blooms (HABs). State monitoring efforts are expanding with greater awareness of the toxic effects of HABs. These monitoring efforts tend to focus on priority waters used for recreation or drinking water. Sampling is seasonal or on occasions when blooms are observed.

Cylindrospermopsin has been detected in lakes throughout multiple states. In a 1999 study, cylindrospermopsin was detected in 40% of 167 water samples taken from 87 water bodies in Florida during the months of June and November (Burns, 2008). However, the actual cylindrospermopsin concentrations were not reported. In 2005, the U.S. Army Corps of Engineers (USACE) detected cylindrospermopsin at a maximum concentration of 1.6 µg/L in lake water samples from Oklahoma (Lynch and Clyde, 2009). In Grand Lake St. Marys, Ohio, cylindrospermopsin concentrations as high as 9 µg/L were reported in 2010 (OHEPA, 2012).

2.3.2 Occurrence in Drinking Water

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

EPA used information from the published literature to evaluate the potential occurrence of cylindrospermopsin in public water systems. In the single publication identified, the results of a 2000 survey of toxins in drinking water treatment plants in Florida were reported (Burns, 2008). In this survey, cylindrospermopsin was detected at concentrations ranging from 8 µg/L to 97 µg/L in nine finished drinking water samples.

2.4 Environmental Fate

Different physical and chemical processes are involved in the persistence, breakdown, and movement of cylindrospermopsin in aquatic systems.

2.4.1 Persistence

Cylindrospermopsin is relatively stable in the dark and at temperatures from 4°C to 50°C for up to five weeks (ILS, 2000). Cylindrospermopsin is also resistant to changes in pH and remains stable for up to eight weeks at pH 4, 7 and 10. In the absence of cell pigments, cylindrospermopsin tends to be relatively stable in sunlight, with a half-life of 11 to 15 days in surface waters (Funari and Testai, 2008). Cylindrospermopsin remains a potent toxin even after boiling for 15 minutes (Chiswell et al., 1999).

Degradation of cylindrospermopsin increases in the presence of cell pigments such as chlorophyll-a and phycocyanin. When exposed to both sunlight and cell pigments, cylindrospermopsin breaks down rapidly, more than 90% within 2 to 3 days (Chiswell et al., 1999). Cylindrospermopsin has been shown to be decomposed by bacteria in laboratory studies; the biodegradation is influenced by the toxin concentration, temperature and pH. Mohamed and Alamri (2012) reported that cylindrospermopsin was degraded by *Bacillus* bacteria and degradation occurred in 6 days at the highest toxin concentration (300 μg/L) and in 7 or 8 days at lower concentrations (10 and 100 μg/L, respectively). The biodegradation rate was also reported to depend on temperature and pH, with the highest rates occurring in warm waters (25 and 30°C) and neutral to slightly alkaline conditions (pH 7 and 8). Klitzke and Fastner (2012) confirmed the observations of Mohamed and Alamri (2012), noting that a decrease in temperature from 20 to 10°C slowed down degradation by a factor of 10. They also found that degradation slowed significantly under anaerobic conditions, with half-lives of 2.4 days under aerobic conditions and 23.6 days under anaerobic conditions.

2.4.2 Mobility

In sediments, cylindrospermopsin exhibits some adsorption to organic carbon, with little adsorption observed on sandy and silt sediments (Klitzke et al., 2011). The low adsorption of cylindrospermopsin reduces its residence time in sediments, thus reducing the opportunity for microbial degradation.

2.5 Nature of the Cylindrospermopsin Toxin

2.5.1 Toxicokinetics

Animal studies show that cylindrospermopsin is absorbed from the gastrointestinal tract (Humpage and Falconer, 2003; Shaw et al., 2000, 2001) and that the tissue distribution occurs

primarily to the liver, but also to the kidneys and spleen after intraperitoneal (i.p.) exposure (Norris et al., 2001).

The metabolism and toxicity of cylindrospermopsin is mediated by the hepatic cytochrome P450 (CYP450) enzyme system. The periacinar region of the liver, an area where substantial CYP450-mediated xenobiotic metabolism occurs, appears to be the main target of cylindrospermopsin toxicity and where cylindrospermopsin and its metabolites bind to proteins (Runnegar et al. 1995; Shaw et al. 2000, 2001; Norris et al., 2001).

Animal studies evaluating the elimination of cylindrospermopsin in urine and feces after i.p. exposures found a continued urinary and fecal excretion over the monitoring period (24 hours) and a mean total recovery from the urine and feces of 76.9% of the administered dose after 24 hours (Norris et al., 2001). Urinary excretion accounted for 68.4% of the 24-hour total and fecal excretion for 8.5%. There was considerable interanimal variability in this study.

2.5.2 Noncancer Health Effects Data

2.5.2.1 Human Studies

Human data on oral toxicity of cylindrospermopsin are limited, but suggest that liver and kidney are potential target organs for toxicity. Reports of a hepatoenteritis-like outbreak (mostly in children) in Palm Island, Australia in 1979 were attributed to consumption of drinking water with a bloom of *C. raciborskii*, a cyanobacteria that can produce cylindrospermopsin. No data are available on exposure levels or potential co-exposures to other cyanobacterial toxins and microorganisms. The majority of the cases, mostly children, required hospitalization. The clinical picture included fever, headache, vomiting, bloody diarrhea, hepatomegaly and kidney damage with loss of water, electrolytes and protein (Byth, 1980; Griffiths and Saker, 2003).

Dermal exposure to cylindrospermopsin was evaluated using skin-patch testing in humans (Pilotto et al., 2004; Stewart et al, 2006). Exposed individuals showed mild irritation, but no statistically significant dose-response relationship or reaction rates were found between skin reactions and increasing cell concentrations for either whole or lysed cells (Pilotto et al., 2004). No detectable skin reactions were observed in individuals exposed to lyophilized *C. raciborskii* (Stewart et al., 2006).

2.5.2.2 Animal Studies

Most of the information on the noncancer effects of cylindrospermopsin in animals is from oral and i.p. administration studies in mice exposed to purified compound or extracts of *C. raciborskii* cells. Studies conducted with purified toxin are preferred because extracts may contain other toxins or compounds with similar chemical physical properties that co-elute with the toxin. Effects on the liver and kidney, including changes in organ weights and histopathological lesions, along with increases in the hematocrit level in serum and deformation of red blood cell are observed following short-term and subchronic oral exposure to

cylindrospermopsin (Humpage and Falconer, 2002, 2003; Reisner at al., 2004; Sukenik et al., 2006). Oral and i.p. acute toxicity studies in mice also report histopathological effects in both liver and kidney. No chronic toxicity studies evaluating cylindrospermopsin are available.

No oral reproductive or developmental studies are available for cylindrospermopsin. Developmental toxicity studies following i.p. administration of cylindrospermopsin provide some evidence for maternal toxicity and decreased postnatal pup survival and body weight (Rogers et al., 2007; Chernoff et al., 2011). Sibaldo de Almeida et al. (2013) did not find any visceral or skeletal malformations in the offspring of pregnant rats receiving an oral dose of 3 mg/kg/day purified cylindrospermopsin during gestation (GD 1-20).

2.5.3 Mode of Action for Noncancer Health Effects

2.5.3.1 Liver

The occurrence of toxicity in the liver suggests a protein-synthesis inhibition mechanism of action for cylindrospermopsin. *In vitro* and *in vivo* studies have been conducted to demonstrate the ability of cylindrospermopsin to inhibit hepatic protein synthesis, which could impact mouse urinary protein production leading to decreased urinary excretion of these proteins (Froscio et al., 2008, 2009; Terao et al., 1994). Available evidence indicates that protein synthesis inhibition is not decreased by broad-spectrum CYP450 inhibitors, but they do reduce cytotoxicity (Froscio et al., 2003; Bazin et al., 2010). Hepatotoxicity appears to be CYP450-dependent, which indicates a possible involvement of oxidized and/or fragmented metabolites and mechanisms other than protein synthesis inhibition (Froscio et al., 2003; Humpage et al., 2005; Norris et al., 2001, 2002). Despite the number of studies that have been published, the mechanisms for liver and kidney toxicity by cylindrospermopsin are not completely characterized.

2.5.3.2 Red Blood Cells

There was evidence of effects on red blood cells (RBCs) in the Reisner et al. (2004) and Humpage and Falconer (2002) studies of purified cylindrospermopsin. In the Reisner et al. (2004) report, microscopic examination of blood samples showed the presence of RBCs with spiked surfaces rather than their normal biconcave-disc shape. The authors attributed the acanthocyte formation to an increase in the cholesterol to phospholipid ratio of the RBC membrane. Phospholipids constitute the matrix material of cell membranes. The authors hypothesized that this change was the consequence of decreased activity of plasma lecithin cholesterol acyl transferase (LCAT), an enzyme associated with high-density lipoproteins and the esterification of plasma cholesterol. Effects on the cholesterol content of the RBC membrane can occur with inhibition of the enzyme increasing membrane fluidity and mean corpuscular volume. Associated effects were observed in the Reisner et al. (2004) and Humpage and Falconer (2002) studies. Removal of the abnormal blood cells by the spleen increases both spleen weight and serum bilirubin as well as stimulates hematopoiesis. Additional research is

needed to examine the LCAT enzyme inhibition hypothesis in order to confirm whether it accounts for the effects on the RBC as a result of cylindrospermopsin exposure.

2.5.3.3 Kidney

No mode of action information for kidney effects was observed in the available studies of cylindrospermopsin. Since all the studies were conducted in mice, a species that excretes low molecular weight proteins in urine, there is a need to conduct a study of cylindrospermopsin in a laboratory species that does not excrete protein in the urine in order to determine whether there are comparable effects on kidney weight, protein excretion and renal cellular damage. Kidney necrosis and a decreased renal failure index at the high cylindrospermopsin doses provide support for the effects on the kidney.

2.5.4 Carcinogenicity Data

No chronic cancer bioassays of cylindrospermopsin were located in the literature. Limited data from an *in vivo* study showed no indication that the cyanobacterial extract containing cylindrospermopsin initiated tumors in mice (Falconer and Humpage, 2001). Cell transformation in Syrian hamster embryo (SHE) cells was observed using purified cylindrospermopsin (Marie et al., 2010). Transformation frequency increased at the lowest concentrations (from 1 x 10^{-2} to 1x10^{-7} ng/mL) but not at the highest concentrations (1 or 1x10^{-1} ng/mL).

Mutagenicity studies (e.g., the Ames Assay) have not observed mutagenic activity of cylindrospermopsin (Sieroslawska, 2013). A few i.p. studies investigating the *in vivo* genotoxicity (DNA damage) from exposure to cylindrospermopsin showed DNA strand breakage in the liver of Balb/c mice (Shen et al., 2002) and covalent binding between DNA and cylindrospermopsin, or a metabolite, in Quackenbush mouse liver (Shaw et al., 2000). *In vitro* mutagenic and genotoxic cell assays have shown potential damage to DNA expressed as an increase in micronucleated binucleate cells (MNBNC) in the colon adenocarcinoma line and the human hepatoma line (Bazin et al., 2010), in the human lymphoblastoid cell line (Humpage et al., 2000), in HepG2 cells (Straser et al., 2011), and in isolated human peripheral lymphocytes (Zegura et al., 2011). DNA breaks also have been observed in primary hepatocytes by comet assay (Humpage et al., 2005).

2.6 Conceptual Model

The conceptual model is intended to explore potential links of exposure to a contaminant or stressor with the adverse effects and toxicological endpoints important for management goals, including the development of HA values. The conceptual model demonstrates the relationship between exposure to cylindrospermopsin in drinking water and adverse health effects in the populations at risk.

HAs describe non-regulatory concentrations of drinking water contaminants at which adverse health effects are not anticipated to occur over specific exposure durations (e.g., one-day, ten-days, and a lifetime). HAs also contain a margin of safety to protect sensitive members of the population. They serve as informal technical guidance to assist federal, state and local officials, as well as managers of public or community water systems, in protecting public health. They are not to be construed as legally enforceable federal standards.

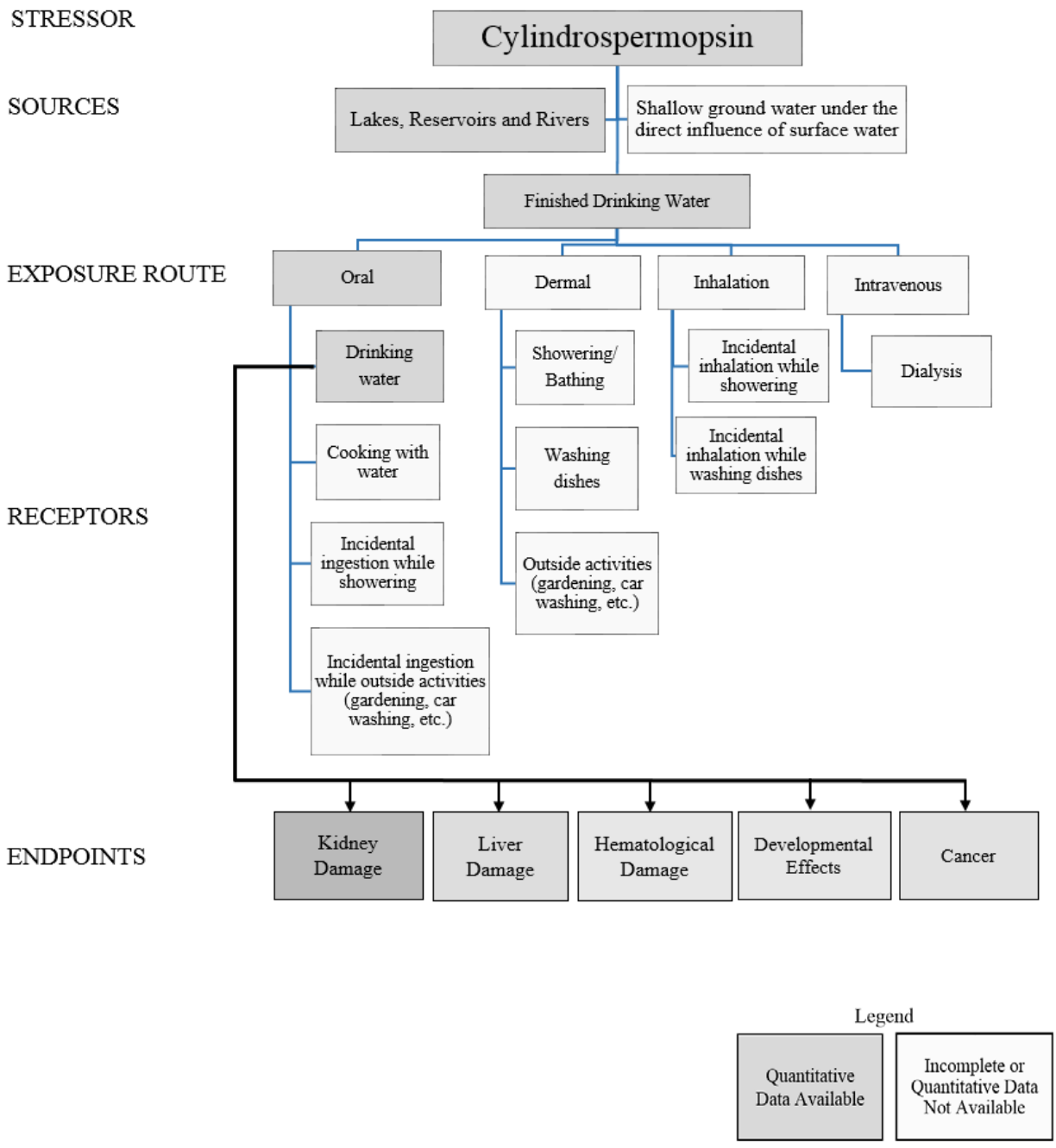
Assessment endpoints for HAs can be developed for both short-term (one-day and ten-day) and lifetime exposures periods using information on the non-carcinogenic and carcinogenic toxicological endpoints of concern. Where data are available, endpoints will reflect susceptible and/or more highly exposed populations.

- A One-day HA is typically calculated for an infant (0-12 months or 10kg child), assuming a single acute exposure to the chemical and is generally derived from a study of less than seven days' duration.
- A Ten-day HA is typically calculated for an infant (0-12 months or 10kg child), assuming a limited period of exposure of one to two weeks, and is generally derived from a study of 7 to 30-days duration.
- A Lifetime HA is derived for an adult (>21 years or 80kg adult), and assumes an exposure period over a lifetime (approximately 70 years). It is usually derived from a chronic study of two years duration, but subchronic studies may be used by adjusting the uncertainty factor employed in the calculation. For carcinogens, the HA documents typically provide the concentrations in drinking water associated with risks for one excess cancer case per ten thousand persons exposed up to one excess cancer case per million exposed for Group A and B carcinogens and those classified as known or likely carcinogens (U.S. EPA, 1986, 2005). Cancer risks are not provided for Group C carcinogens or those classified as "suggestive", unless the cancer risk has been quantified.

For each assessment endpoint EPA uses one or more measures of effect (also referred to as a point of departure), which describe the change in the attribute of the assessment endpoint in response to chemical exposure, to develop acute, short-term, longer term (subchronic) or chronic reference values when the data are available. The measures of effect selected represent impacts on survival, growth, system function, reproduction and development.

This conceptual model provides useful information to characterize and communicate the potential health risks related to exposure to cyanotoxins in drinking water. The sources of cyanotoxins in drinking water, the route of exposure for biological receptors of concern (e.g., via various human activities such as drinking, food preparation and consumption) and the potential assessment endpoints (i.e., effects such as kidney and liver toxicity, and reproductive and developmental effects) due to exposure to cylindrospermopsin are depicted in the conceptual diagram below (Figure 2-3).

Figure 2-3. Conceptual Model of Exposure Pathways to Cylindrospermopsin in Drinking Water



2.6.1 Conceptual Model Diagram

Cyanobacteria are a common part of freshwater and marine ecosystems. An increase in water column stability, high water temperatures, elevated concentrations of nutrients and low light intensity have been associated with an increase and/or dominance of cylindrospermopsin-producing cyanobacteria in surface waters (or aquatic ecosystems). The presence of detectable concentrations of cyanotoxins in the environment is closely associated with these blooms. Winds and water currents can potentially transport cyanobacterial blooms to areas within the proximity of water intakes for drinking water treatment plants. If not managed in source waters, or removed during drinking water treatment, cyanobacteria and cyanotoxins may result in exposure that could potentially affect human health.

2.6.2 Factors Considered in the Conceptual Model for Cylindrospermopsin

Stressors: For this HA, the stressor is cylindrospermopsin concentrations in finished drinking water.

Sources: Sources of cylindrospermopsin include potential sources of drinking water such as rivers, reservoirs and lakes in the U.S. where blooms producing cylindrospermopsin occur. Shallow private wells under the direct influence of surface water (in hydraulic connection to a surface water body) can also be impacted by cylindrospermopsin-producing blooms if the toxins are drawn into the well along with the water from the surface water. There is substantially less information on exposure from this source.

Routes of exposure: Exposure to cyanotoxins from contaminated drinking water sources may occur via oral exposure (drinking water, cooking with water, and incidental ingesting from showering); dermal exposure (contact of exposed parts of the body with water containing toxins during bathing or showering, washing dishes or outside activities); inhalation exposure (during bathing or showering); or intravenous exposure (e.g., via dialysis). Toxicity data are available for the oral route of exposure from drinking water, but are not available to quantify dose response for other exposure routes (inhalation, dermal, dietary and intravenous exposures).

Receptors: The general population (adults and children) could be exposed to cyanotoxins through dermal contact, inhalation and/or ingestion. Infants and pre-school age children can be at greater risk to cylindrospermopsin because they consume more water per unit body weight than adults. Other individuals of potential sensitivity include persons with kidney and/or liver disease due to the compromised detoxification mechanisms in the liver and impaired excretory mechanisms in the kidney. There are no human data to quantify risk to pregnant woman or to evaluate the transfer of cyanotoxins across the placenta. Data are also not available on the transfer of cyanotoxins through the milk from nursing mothers or on the risk to the elderly. Given this lack of information, pregnant women, nursing mothers, and the elderly may also be potentially sensitive populations. Data from the episode in a dialysis clinic in Caruaru, Brazil where microcystins, and possibly cylindrospermopsin, were not removed by treatment of dialysis water (Carmichael et al., 2011), identify dialysis patients as a population of potential concern in cases where the drinking water source for the clinic is contaminated with cyanotoxins. EPA has

data to quantify risk to infants, children, and adults based on variability in potential exposure (body weight and drinking water intake rate). However, data are not available to quantify risk to pregnant woman, nursing mothers, persons with liver or kidney disease, or dialysis patients. Data are not available to derive a one-day HA for children because studies with single oral dosing do not provide dose-response information. A lifetime HA for cylindrospermopsin is not recommended as the types of exposures being considered are short-term and episodic in nature. Although the majority of the cyanobacterial blooms in the U.S. occur seasonally, usually during late summer, some toxin-producing strains can occur early in the season and can last for days or weeks.

Endpoints: Human data on oral toxicity of cylindrospermopsin are limited, but have shown effects on the liver following potential exposure to cylindrospermopsin. Acute, short-term and subchronic studies in animals show effects on the liver, RBC and kidney. In addition, some studies suggest that cylindrospermopsin may lead to reproductive and developmental effects; however, these data are limited. *In vitro* mutagenic and genotoxic cell assays with cylindrospermopsin have shown varied results with some indications of potential damage to DNA. However, these data are limited, and there has been no long term bioassay of purified cylindrospermopsin. Thus, available data are inadequate to assess the carcinogenic potential of cylindrospermopsin at this time. Available toxicity data are described in the *Health Effects Support Document (HESD) for Cylindrospermopsin* (U.S. EPA, 2015a). Kidney effects were selected as the endpoint on which to base the measure of effect. Liver and hematological effects were not as sensitive as the reported kidney effects.

2.7 Analysis Plan

The *Health Effects Support Document (HESD) for Cylindrospermopsin* (U.S. EPA, 2015a) provides the health effects basis for development of the HA, including the science-based decisions providing the basis for estimating the point of departure. To develop the HESD for cylindrospermopsin, 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 cylindrospermopsin was included. The literature search included the following terms: cylindrospermopsin, 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 considered information from the following risk assessments during the development of the cylindrospermopsin health risk assessment:

- 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*

- ILS (2000) *Cylindrospermopsin [CASRN 143545-90-8] Review of Toxicological Literature*

The toxicity data available for an individual pollutant vary significantly. An evaluation of available data was performed by EPA to determine data acceptability. The following study quality considerations from U.S. EPA's (2002) *A Review of the Reference Dose and Reference Concentration Processes* were used in selection of the studies for inclusion in the HESD and development of the HA.

- Clearly defined and stated hypothesis.
- Adequate description of the study protocol, methods and statistical analyses.
- Evaluation of appropriate endpoints. Toxicity depends on the amount, duration, timing and pattern of exposure, and may range from frank effects (e.g., mortality) to more subtle biochemical, physiological, pathological or functional changes in multiple organs and tissues.
- Application of the appropriate statistical procedures to determine an effect.
- Establishment of dose-response relationship (i.e., no observed adverse effect level (NOAEL) and/or lowest observed adverse effect level (LOAEL) or data amenable to modeling of the dose-response in order to identify a point of departure for a change in the effect considered to be adverse (out of the range of normal biological viability). The NOAEL is the highest exposure level at which there are no biologically significant increases in the frequency or severity of adverse effect between the exposed population and its appropriate control. The LOAEL is the lowest exposure level at which there are biologically significant increases in frequency or severity of adverse effects between the exposed population and its appropriate control group.

After the available studies were evaluated for inclusion in the HESD and HA, the critical study was selected based on consideration of factors including exposure duration (comparable to the duration of the HA being derived), route of exposure (oral exposure via drinking water, gavage, or diet is preferred), species sensitivity, comparison of the point of departure with other available studies demonstrating an effect, and confidence in the study (U.S. EPA, 1999). Once, a point of departure is chosen for quantification, uncertainty factors appropriate for the study selected are then applied to the point of departure to account for variability and uncertainty in the available data.

For cylindrospermopsin, toxicity and exposure data are available to develop a Ten-day HA. EPA used measures of effect and estimates of exposure to derive the Ten-day HAs using the following equation:

$$\text{HA} = \frac{\text{NOAEL or LOAEL or BMDL}}{\text{UF} \times \text{DWI/BW}}$$

Where:

NOAEL or LOAEL = No- or Lowest-Observed-Adverse-Effect Level (mg/kg bw/day) from a study of an appropriate duration (up to 7 days and 7-30 days for the One-day and Ten-day HAs, respectively).

BMDL = When the data available are adequate, benchmark dose (BMD) modeling can be performed to determine the point of departure for the calculation of HAs. The benchmark dose (BMD) approach involves dose-response modeling to obtain dose levels corresponding to a specific response level near the low end of the observable range of the data (U.S.EPA, 2012). The lower 95% confidence limit is termed the benchmark dose level (BMDL).

UF = Uncertainty factors (UF) account for: (1) intraspecies variability (variation in susceptibility across individuals); (2) interspecies variability (uncertainty in extrapolating animal data to humans; (3) uncertainty in extrapolating from a LOAEL to a NOAEL; and (4) uncertainty associated with extrapolation when the database is incomplete. These are described in U.S. EPA, 1999 and U.S. EPA, 2002.

DWI/BW = For children, a normalized ratio of drinking water ingestion to body weight (DWI/BW) was calculated using data for infants (birth to <12 months). The estimated drinking water intake body weight ratio (L/kg/day) used for birth to < 12 months of age are the 90th percentile values of the consumers only estimates of direct and indirect water ingestion based on 1994-1996, 1998 CSFII (Continuing Survey of Food Intakes by Individuals) (community water, mL/kg/day) in Table 3-19 in the U.S. EPA (2011a) Exposure Factors Handbook. The time weighted average of DWI/BW ratios values was derived from multiplication of age-specific DWI/BW ratios (birth to <1 month, 1 to < 3 months, 3 to < 6 months, and 6 to <12 months) by the age-specific fraction of infant exposures for these time periods.

For adults (>21 years of age), EPA updated the default BW assumption to 80 kg based on National Health and Nutrition Examination Survey (NHANES) data from 1999 to 2006 as reported in Table 8.1 of EPA's Exposure Factors Handbook (U.S. EPA, 2011a). The updated BW represents the mean weight for adults ages 21 and older.

EPA updated the default DWI to 2.5 L/d, rounded from 2.546 L/d, based on NHANES data from 2003 to 2006 as reported in EPA's Exposure Factors Handbook (U.S. EPA 2011a, Table 3-33). This rate represents the consumer's only estimate of combined direct and indirect community water ingestion at the 90th percentile for adults ages 21 and older.

3.0 HEALTH EFFECTS ASSESSMENT

The health effects assessment provides the characterization of adverse effects and includes the hazard identification and dose-response assessment. The hazard identification includes consideration of available information on toxicokinetics; identification, synthesis and evaluation of studies describing the health effects of cylindrospermopsin; and the potential Mode of Action (MOAs), or toxicity pathways related to the health effects identified.

3.1 Dose-Response

3.1.1 Critical Study Selected

The critical study selected for the derivation of the reference dose (RfD) for cylindrospermopsin is Humpage and Falconer (2002, 2003). Humpage and Falconer (2002, 2003) is a comprehensive toxicity study in which male mice were exposed by gavage to purified cylindrospermopsin from cell extract for 11 weeks. The study authors used four dose groups, adequate numbers of animals per dose group (10) and evaluated a variety of endpoints. Statistically significant, dose-related effects on the kidney, liver and serum chemistry were observed. The kidney was the most sensitive target of toxicity. The Humpage and Falconer (2002) data are supported by the short-term Reisner et al. (2004) results showing exposure-duration-related increased kidney weights, liver weights and testes weights, and hematological effects (acanthocytes or abnormal red blood cells (RBCs) and changes in hematocrit) following a 21-day exposure.

Purified cylindrospermopsin in water was administered by gavage in doses of 0, 30, 60, 120 or 240 μg/kg/day to groups of male Swiss albino mice (6 to 10 mice per dose group) for 11 weeks (Humpage and Falconer, 2002, 2003). The cylindrospermopsin was from an extract of freeze-dried *C. raciborskii* cells Woloszynska (AWT 205) purified using sephadex size-exclusion gel (G-10). The individual sephadex fractions were assayed using high-performance liquid chromatography (HPLC) and concentrated to a sample that was 47% cylindrospermopsin by dry weight and 53% phenylalanine. Food and water consumption, and body weight were examined throughout the study. After 9 weeks of exposure, the study authors report conducting a clinical examination to detect physiological and behavioral signs of toxicity but do not specify the parameters evaluated. Hematology evaluations (4 to 5 per dose group, except the high dose), serum chemistry (4 to 6 per dose group), and urinalysis (6 or 10 per dose group) were conducted. All the evaluations were conducted either near or at the end of the treatment period.

Postmortem examinations were done on the following organs: liver, spleen, kidneys, adrenal glands, heart, testes, epididymis and brain, including measurement of organ weights. Comprehensive histological evaluations were conducted in accordance with the recommendations from the Organization for Economic Cooperation and Development (OECD).

No deaths or visual clinical signs of toxicity were reported in mice exposed to purified cylindrospermopsin under the study conditions. The mean final body weight was 7-15% higher in all dose groups compared to controls, but was not dose-related and was only statistically significant at 30 and 60 µg/kg/day (Humpage and Falconer, 2003). No significant changes were observed in food consumption. In all dose groups, the water intake was significantly reduced; water consumption was 53% of the control level at 30 µg/kg/day and the higher dose groups were 68-72% of the control levels.

Relative kidney weight was significantly increased in a dose-related manner at ≥60 µg/kg/day (12-23% greater than controls; see Table 3-1). Relative liver weight was significantly increased (13% greater than controls) only at the highest dose (240 µg/kg/day). Relative spleen, adrenal and testes weights were increased for doses ≥60 µg/kg/day, but the differences from control were not statistically significant (Humpage and Falconer, 2002).

Selected serum chemistry (n= 4-6), hematology (n=4-5) and urinalysis (n=6-10) results are shown in Table 3-2. The hematology and serum chemistry evaluations showed no dose-related, statistically significant changes, although serum albumin, total bilirubin and cholesterol were increased compared to controls at all doses (Humpage and Falconer, 2002). The increases in cholesterol were significant for the 30 and 60 µg/kg/day groups, but not at the higher doses. The serum urea concentration was slightly decreased at the two highest doses. A nonsignificant increase in red cell polychromasia (high number of RBCs) was indicated for all doses, but quantitative data were not presented. Packed red cell volume was slightly increased and mean corpuscular hemoglobin was slightly decreased (Table 3-2) when compared to controls, although the changes were not dose related. When combined with the bilirubin results and the increased relative spleen weight, the hematological data suggest the possibility of minor RBC effects. One of the limitations in the serum chemistry and hematology data is the small number of samples evaluated, a factor that impacts the determination of statistical significance (Humpage and Falconer, 2002).

There was a significant decrease in the urine protein-creatinine ratio (g/mmol creatinine) at 120 and 240 µg/kg/day compared to the controls (51% and 37% of controls, respectively; both $p<0.001$) (Humpage and Falconer, 2002). Also, a significant decrease in urine specific gravity normalized for creatinine was seen at 240 µg/kg/day compared to the control ($p<0.001$). The renal glomerular filtration rate (GFR) was decreased compared to controls at all doses, but the differences were not dose dependent or statistically significantly different from controls. The renal failure index[1] was decreased slightly at ≥120 µg/kg/day; the differences from control were not statistically significant (Humpage and Falconer, 2002). Tubular retention of low molecular weight urinary proteins could account for the decreased urinary protein and possibly the increased kidney weight. Although effects on kidney weight and urine protein levels were observed in male mice, the biological relevance of the latter effect and whether it would also occur in female mice needs further investigation. Mice are known to excrete a group of functional, highly-polymorphic, low-molecular-weight urinary proteins that play important roles in social recognition and mate assessment (Cheetham et al., 2009). The relevance of the urinary protein findings in mice to humans is unknown.

[1] Renal failure index= (urinary sodium concentration × plasma creatinine concentration) / urinary creatinine concentration

Table 3-1. Kidney Weight Data from Oral Toxicity Study of Cylindrospermopsin Administered Daily over Eleven Weeks (Humpage and Falconer, 2002, 2003)

Dose (µg/kg/day)	Number	Relative Kidney Weight		% Difference	Significance
		Control g/100g $_{BW}$	Exposed g/100g $_{BW}$		
0 (Control)	10	1.48	-	-	-
30	10	1.48	1.57	+6	Not significant
60	9	1.48	1.66	+12	p <0.001
120	9	1.48	1.82	+23	p <0.001
240	6	1.48	1.78	+20	P <0.001

Table 3-2. Selected Clinical Chemistry, Hematology and Urinalysis Findings (Humpage and Falconer, 2002, 2003)

Endpoint	N	Dose (µg/kg/day)				
		0	30	60	120	240
Clinical Chemistry						
Urea (mmol/L)	4-6	9.24	9.22	8.55	7.51	7.92
Albumin (g/L)	4-6	23.8	26.6	26.0	26.0	25.8
Cholesterol (mmol/L)	4-6	3.26	4.60**	4.65**	3.68	4.08
Bilirubin (mmol/L)	4-6	2.62	2.72	2.88	3.06	3.07
Hematology						
Packed Cell volume (L/L)	4-5	0.38	0.39	0.39	0.39	ND
Mean Corpuscular Hemoglobin (MCH, pg/L)	4-5	16.8	15.7	16.4	16.4	ND
Urinalysis						
Volume (mL)	6-10	9.85	11.18	10.38	11.74	6.74
Creatinine (mmol/L)	6-10	0.57	0.49	0.54	0.51	0.72**
Specific gravity/creatinine	6-10	1.79	2.04	1.91	1.99	1.44*
Protein/creatinine (g/mmol)	6-10	4.3	3.6	3.3	2.2**	1.6**
Renal Failure Index (mmol/L)	4-6	4.3	4.3	4.5	3.6	3.6

ND = not determined
Significantly different from control: *p<0.05; **p<0.01.

Serum albumin and total serum protein were not decreased in the Humpage and Falconer studies (2002, 2003). The most sensitive effects observed by Humpage and Falconer (2002, 2003) were dose-related decreases in the urinary protein: creatinine ratio at ≥120 µg/kg/day and increased relative kidney weight at ≥60 µg/kg/day. The noted decrease in urinary protein excretion could reflect an impact on excretion of mouse urinary proteins given the fact that total serum protein was not significantly increased compared to controls for all dose groups. Mouse urinary proteins are synthesized in the liver (Clissold and Bishop, 1982) and transported to the kidney for excretion. If cylindrospermopsin did reduce liver protein synthesis, a decrease in total serum protein would be expected. However, this was not the case, suggesting a lack of an effect on synthesis of the urinary proteins in the liver.

The Humpage and Falconer (2002, 2003) postmortem tissue examinations showed histopathological damage to the liver based on scores assigned for necrosis, inflammatory foci and bile duct changes at ≥120 µg/kg/day. The percent of animals with liver lesions in the 120 and 240 µg/kg/day dose groups was 60% and 90%, respectively, when compared to 10%, 10% and 20% for the 0, 30, and 60 µg/kg/day dose groups, respectively. Severity scores were not given, and the liver lesions were not further described. There was proximal renal tubular damage in kidney sections from two mice in the 240 µg/kg/day dose group (Humpage and Falconer, 2002, 2003).

The 11-week study by Humpage and Falconer (2002, 2003) provides a NOAEL (30 µg/kg/day) and a LOAEL (60 µg/kg/day) for dose-related, statistically significant increases in kidney weights along with indicators of reduced renal function effects at higher doses. Because of the similarity in the type of effects observed and the LOAELs from the Humpage and Falconer (2002, 2003) and Reisner et al. (2004) studies, the selection of the NOAEL from Humpage and Falconer was determined to be the most appropriate point of departure for ten-day exposures in infants, children and adults despite its longer exposure duration.

3.1.2 Endpoint Selection

Upon considering all effects observed by Humpage and Falconer (2002, 2003), increased relative kidney weight was considered the most appropriate basis for quantitation. Adverse effects on the kidneys were manifested by decreases in urinary protein concentration and increased relative kidney weight. The study authors reported significantly increased relative kidney weight at ≥60 µg/kg/day, decreased urinary protein and liver lesions at ≥120 µg/kg/day and renal tubular lesions at 240 µg/kg/day (Humpage and Falconer, 2002, 2003). Relative kidney weight increased significantly in a dose-related manner beginning at 60 µg/kg/day (12-23% greater than controls), and relative liver weight was significantly increased at 120 µg/kg/day (12-23% greater than controls) and at the high dose of 240 µg/kg/day (13% greater than controls). Relative spleen, adrenal and testes weights were increased for doses ≥60 µg/kg/day, but the differences from control, although dose-related, were not statistically significant. Humpage and Falconer (2002, 2003) identified the LOAEL as 60 µg/kg/day and the NOAEL as 30 µg/kg/day based on the dose-related and statistically significant increase in relative kidney weight. These adverse effects are potential indicators of suppressed hepatic protein synthesis that was not

reflected in the measurement of total serum protein and/or increased retention of low molecular weight mouse urinary proteins by the kidney because of damage to the renal tubules.

In the single dose drinking water study by Reisner et al. (2004), hematological effects (acanthocytes, increased hematocrit) and increased organ weights (liver, testicular and kidney) in young (4 week) male Institute for Cancer Research (ICR) mice were observed following a three week exposure to purified cylindrospermopsin. The 66 µg/kg/day LOAEL is comparable to that from the Humpage and Falconer (2002, 2003) study (60µg/kg/day). Humpage and Falconer (2002, 2003) evaluated 5 different doses using 6 to 10 mice per dose group; Reisner et al. (2004) used one dose with 8 male mice. Reisner et al. (2004) demonstrated effects in comparable parameters to those impacted in Humpage and Falconer at a dose of 66 µg/kg/day with a three week exposure. They also demonstrated a trend for effects on kidney weight and hematocrit across the three-week duration of exposure. Because the renal effects reported in Humpage and Falconer (2002, 2003) did not occur at 11 weeks for the 30 µg/kg dose, the point of departure from the Humpage and Falconer study was determined to be the most appropriate for the quantitative assessment. Thus, the quantification from the Humpage and Falconer NOAEL based on kidney weight changes provides the best point of departure for ten-day exposures in children and adults despite its longer exposure duration.

3.2 Ten-Day Health Advisory

The Ten-day HA is considered protective of non-carcinogenic adverse health effects over a ten-day exposure to cylindrospermopsin in drinking water.

3.2.1 Bottle-fed Infants and Young Children of Pre-school Age

The Ten-day HA for bottle-fed infants and young children of pre-school age is calculated as follows:

$$\text{Ten-day HA} = \frac{30\,\mu\text{g/kg/day}}{300 \times 0.15\,\text{L/kg/day}} = 0.7\,\mu\text{g/L}$$

Where:

30 µg/kg/day	=	The NOAEL for kidney effects in mice exposed to cylindrospermopsin in water for 11 weeks (Humpage and Falconer, 2002, 2003).
300	=	The composite uncertainty factor (UF) including a 10 for intraspecies variability (UF_H), a 10 for interspecies differences (UF_A), and a 3 for uncertainties in the database (UF_D).
0.15 L/kg/day	=	Normalized drinking water intakes per unit body weight over the first year of life based on the 90th percentile of drinking water consumption and the mean body weight (U.S. EPA, 2011a).

The Ten-day HA of 0.7 µg/L is considered protective of non-carcinogenic adverse health effects for bottle-fed infants and young children of pre-school age over a ten-day exposure to cylindrospermopsin in drinking water.

3.2.2 School-age Children through Adults

The Ten-day HA for school-age children through adults is calculated as follows:

$$\text{Ten-day HA} = \frac{30\,\mu\text{g/kg/day}}{300 \times 0.03\,\text{L/kg/day}} = 3\,\mu\text{g/L}$$

Where:

30 µg/kg/day	=	The NOAEL for kidney effects in mice exposed to cylindrospermopsin in water for 11 weeks (Humpage and Falconer, 2002, 2003).
300	=	The composite UF including a 10 for intraspecies variability (UF_H), a 10 for interspecies differences (UF_A), and a 3 for uncertainties in the database (UF_D).
0.03 L/kg/day	=	Drinking water intake per unit body weight based on adult default values of 2.5 L/day and 80 kg (U.S. EPA, 2011a).

The Ten-day HA of 3 µg/L is considered protective of non-carcinogenic adverse health effects for children of school age through adults over a ten-day exposure to cylindrospermopsin in drinking water.

3.2.3 Uncertainty Factor Application

- 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. Individuals with a low RBC count as a result of genetic or nutritional factors could be more sensitive to cylindrospermopsin exposures than the general population. Individuals with pre-existing kidney/liver problems may also be more sensitive. Pregnant woman, nursing mothers, and the elderly could also be sensitive to cylindrospermopsin exposures.

- UF_A - 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 cylindrospermopsin. Allometric scaling is not applied in the development of the Ten-Day HA values for cylindrospermopsin. The allometric scaling approach is derived from the relationship between body surface area and basal metabolic rate in adults (U.S. EPA, 2011b). This approach is not appropriate for infants and children due to the comparatively slower clearance during these ages and the limited toxicokinetic data available to assess the appropriateness of body weight scaling in early life.

- UF_D - An uncertainty factor of 3 ($10^{0.5} = 3.16$) is selected to account for deficiencies in the database for cylindrospermopsin. The database for cylindrospermopsin includes limited human studies. The database for studies in laboratory animals includes oral exposure acute, short-term and subchronic studies, but many of them lacked a comprehensive evaluation of a wide spectrum of effects. The database lacks chronic toxicity and multi-generation reproductive and developmental toxicity studies using the oral route of exposure. There is a lack of data on neurological and immunological endpoints. The RBC parameters evaluated differed between the Humpage and Falconer (2002, 2003) and Reisner et al. (2004) studies.

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., $10^{½}$) when appropriate (U.S. EPA, 2002).

4.0 RISK CHARACTERIZATION

The following topics describe important conclusions used in the derivation of the health advisory. This section characterizes each topic and its impact on the health advisory.

4.1 Studies Supporting Determination of Critical Study

Increases in kidney weight and hematological effects are detected in all three studies (Humpage and Falconer, 2002, 2003; Reisner et al., 2004; and Sukenik et al., 2006). However, the type of hematological effects varied among studies as did the statistical significance of the observed effects. Humpage and Falconer (2002, 2003) found signs indicative of hemolysis (increased bilirubin, spleen weight and polychromasia (high number of RBCs with low hemoglobin)), while Reisner et al. (2004) and Sukenik et al. (2006) found acanthocytes (abnormal RBCs). Increases in kidney weight were significant for Humpage and Falconer (2002, 2003) and Sukenik et al. (2006), but not statistically significant for Reisner et al. (2004). Humpage and Falconer (2002, 2003) and Reisner et al. (2004) used purified cylindrospermopsin, while Sukenik et al. (2006) used an extract in spent medium. Of these three studies, Humpage and Falconer (2002, 2003) provides a NOAEL (30 µg/kg/day) and a LOAEL (60 µg/kg/day) for dose-related statistically significant increases in kidney weights and indications of renal function effects at higher doses. Although the percent change in kidney weight is the same for Reisner et al. (2004), only the change observed by Humpage and Falconer (2002, 2003) was statistically significant.

4.2 Study Duration

The short-term studies with appropriate durations (typically 7 days up to 30 days) available for cylindrospermopsin (Shaw et al., 2001; Reisner et al., 2004), are not suitable for quantification, as described below. However, the Reisner study does support the use of the Humpage and Falconer (2002, 2003) study for the derivation of the Ten-day HA, despite the longer duration of the study.

The Shaw et al (2001) study reported the results from multiple experiments. These experiments each have limitations including use of extract, lack of adequate numbers of animals and monitored endpoints, and the limited number of doses tested that preclude their use in quantification. The oral data for purified extract from Shaw et al. (2001) identified fatty liver as an adverse effect in mice following a 14 day gavage exposure to 0.05 mg/kg/day. However, the only effects mentioned in the published paper are the liver effects and an absence of lymphophagocytosis in the spleen.

Reisner et al. (2004) conducted a 21 day study in mice and showed significant increases in hematocrit, acanthocytes (abnormal RBCs), and liver and testes weights effects at a 66 µg/kg/day dose and a duration-related nonsignificant increase in and kidney weight. This study was not selected for development of the Ten-day HA because this study used a single dose; however, the effects to that dose after 3-weeks were comparable to the effects seen in the

Humpage and Falconer (2002, 2003) study at a slightly lower 60 mg/kg/day dose after 11 weeks. The Humpage and Falconer (2002, 2003) study was determined to be the most appropriate for the quantitative assessment because the LOAEL at 11 weeks would be protective for the effects seen at 3-weeks in the shorter duration study.

4.3 Allometric Scaling Approach

Allometric scaling was not applied in the development of the RfD for cylindrospermopsin. In the development of short-term advisory values (One-day and Ten-day), parameters are used that reflect exposures and effects for infants up to one year of age, rather than for adults. The body weight scaling approach is derived from the relationship between body surface area and basal metabolic rate in adults. Infants/children surface area and basal metabolic rates are very different than adults with a slower metabolic rate. In addition, limited toxicokinetic data are available to assess the appropriateness of body weight scaling in early life. The body weight scaling procedure has typically been applied in the derivation of chronic oral RfDs and cancer assessments, both of which are concerned with lifetime repeated exposure scenarios (U.S. EPA, 2012). Thus, given the development of a Ten-Day HA value, and the application of the Ten-Day HA to infants and pre-school age children, the application of the body weight scaling procedure is not appropriate for this scenario.

In addition, for short-term advisories (one-day and ten-day duration), EPA assumes all exposure is derived from drinking water and, therefore, no Relative Source Contribution (RSC) term is applied. For lifetime health advisory values, EPA does include an RSC that reduces the advisory value to account for other potential sources.

4.4 Uncertainty and Variability

Uncertainty factors were applied in several areas to adjust for incomplete information. Human data on the toxic effects of cylindrospermopsin are limited. Quantification for the absorption, distribution and elimination of cylindrospermopsin in humans following oral, inhalation or dermal exposure is not well understood. The clinical significance in humans for biological changes observed in experimental animals such as increased kidney weight, decreased urinary protein levels, decrease in renal failure index and the formation of acanthocytes (abnormal RBCs) is not known. In animal studies with cylindrospermopsin, adverse effects (RBC effects) observed have not been fully characterized. No data are available to quantify the differences between humans and animals for the critical health endpoints. There is uncertainty regarding susceptibility and variability characterized in the human population following exposure to cylindrospermopsin. Additional information is needed on the potential health risks from mixtures of cylindrospermopsin with other cyanotoxins, bioactive molecules with an effect on living organisms and chemical stressors present in ambient water and/or drinking water supplies. The critical study was conducted only in male mice and therefore, any gender-specific effects of cylindrospermopsin are not understood.

4.5 Susceptibility

Available animal data are not sufficient to determine if there is a definitive difference in the response of males versus females following oral exposure to cylindrospermopsin. Based on the results from animal studies, individuals with liver and/or kidney disease might be more susceptible than the general population because of compromised detoxification mechanisms in the liver and impaired excretory mechanisms in the kidney. Data from an episode in a dialysis clinic in Caruaru, Brazil, where microcystins (and possibly cylindrospermopsin) were not removed by treatment of dialysis water, identify dialysis patients as a population of potential concern in cases where the drinking water source used by a clinic for hemodialysis is contaminated with cyanotoxins.

The data on RBC acanthocytes suggest that individuals that suffer from anemia (e.g., hemolytic or iron-deficiency) might be a potentially sensitive population. Several rare genetic defects such as abetalipoproteinemia (rare autosomal recessive disorder that interferes with the normal absorption of fat and fat-soluble vitamins from food) and hypobetalipoproteinemia are associated with abnormal RBC acanthocytes, which appears to result from a defect in expression of hepatic apoprotein B-100, a component of serum low density lipoprotein complexes (Kane and Havel, 1989). Individuals with either condition might be sensitive to exposure to cylindrospermopsin.

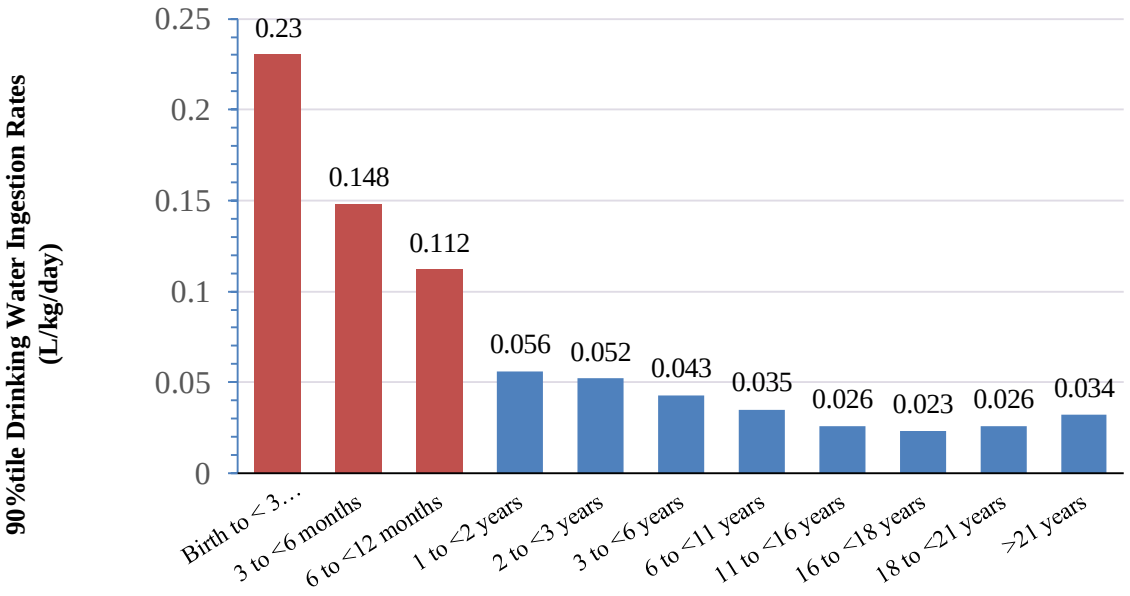
Based on the currently available science, evidence is lacking to assess differences in susceptibility between infants, children and adults. There are, however, significant differences in exposure between these life-stages that impact risk.

4.6 Distribution of Body Weight and Drinking Water Intake by Age

Both body weight and drinking water intake distributions vary with age. EPA has developed two health advisory values, a Ten-day HA of 0.7 µg/L based on exposure to infants over the first year of life, and a Ten-day HA of 3 µg/L based on exposure to adults, over 21 years of age. Section 4.7 discusses how EPA recommends application of these values to other age groups.

The U.S. EPA (2011a) Exposure Factors Handbook provides values for drinking water ingestion rate and corresponding body weight. The estimated 90th percentile of community water ingestion for the general population (males and females of all ages) has been used as the default value for water ingestion. EPA plotted the 90th percentile of drinking water intake using Table 3-19 for ages ≤ 3 years, and Table 3-38 for ages >3 years due to sample size in the respective studies. Age groups < 3 months in Table 3-19 were combined due to insufficient sample sizes. Figure 4.1 represents the 90th percentile drinking water ingestion rates (L/kg/day) for each age group (located on top of the columns). Bottle-fed ages are shown in red (first three columns on the left).

Figure 4-1. 90th Percentile Drinking Water Ingestion Rates by Age Group



Adapted from U.S. EPA 2011 Exposure Factors Handbook (U.S.EPA, 2011a).

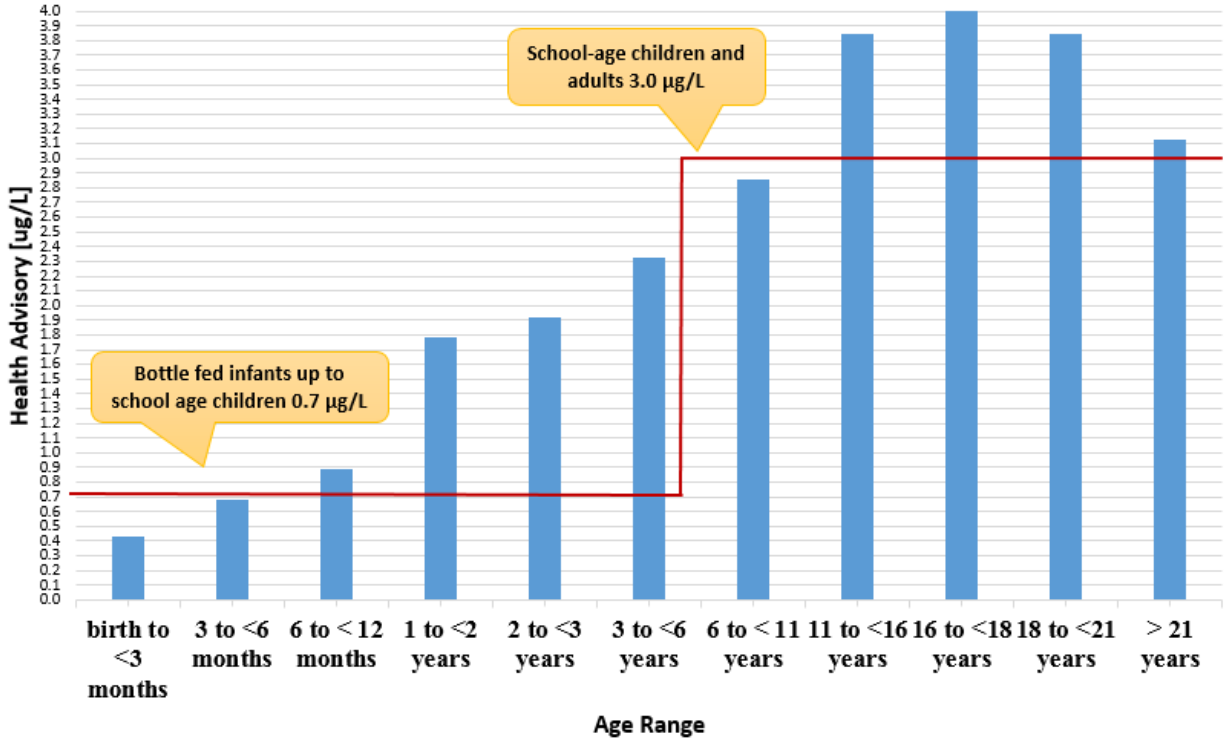
Based on the drinking water intake rates for children <12 months (0.15 L/kg-day), the exposure of children is over 4 times higher than that of adults >21 years old on a body weight basis (0.034 L/kg-day). Infants from birth to 3 months may be exclusively bottle-fed and therefore, have a higher ingestion rate. After 3 months of age, typically around 4 to 6 months of age, other food and liquids are introduced into the infant diet, lowering the ingestion rate of drinking water. Drinking water contributes the highest risk of the total cyanotoxins intake for infants to one-year-olds fed exclusively with powdered formula prepared with tap water containing cyanotoxins. At the age of 6, children's intake of drinking water relative to their body weight is approximately the same as those of an adult (>21 years). Data evaluating the transfer of cylindrospermopsin through breast milk are not available for humans.

4.7 Distribution of Potential Health Advisory Values by Age

Using the ingestion rates for each age group (from Figure 4-1), EPA estimated Ten-day HA values for cylindrospermopsin for each age group (plotted in Figure 4-2) to demonstrate the variability due to body weight and drinking water intake by age.

EPA decided to apply the Ten-day HA value calculated for infants over the first year of life (0.7 µg/L) to all bottle-fed infants and young children of pre-school age because these age groups have higher intake per body weight relative to adults. As Figure 4-2 demonstrates, when the Ten-day HA is estimated by age group, the calculated HA value for infants from birth to 3

Figure 4-2. Ten-day Health Advisories for Cylindrospermopsin by Age Group



months old is 0.4 µg/L, slightly below the infant health advisory value of 0.7 µg/L. EPA believes that infants from birth to 3 months old are not at a disproportionate risk at a 0.7 µg/L advisory value because a safety factor of 30 is built into this calculation to account for human variability and deficiencies in the database. The estimated Ten-Day HA values for infants from 3 months old through pre-school age groups (less than 6 years old), are at or above the advisory value of 0.7 µg/L. Therefore, children within these age groups are adequately protected by the advisory value for bottle-fed infants and young children of pre-school age. EPA decided to apply the adult Ten-Day HA value of 3 µg/L to school age children (children older than or equal to 6 years) through adulthood because children's intake of drinking water relative to body weight in this age group is almost the same as those of an adult (≥ 21 years).

5.0 ANALYTICAL METHODS

The primary methods used for the analysis of cylindrospermopsin are liquid chromatography (LC) and enzyme linked immunosorbent assay (ELISA). Several detection modes are generally coupled with LC including single channel ultraviolet (UV)/visible and multi-channel UV photodiode array (PDA), electrospray ionization mass spectrometry (LC-ESI/MS), and electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS). Due to the limited selectivity of UV-based detectors, the use of mass spectrometric detection is becoming more commonplace. Commercial ELISA test kits are also available for cylindrospermopsin detection. These kits are available in both semiquantitative and quantitative formats and are easily adapted to field or "screening" measurements.

EPA has recently released Method 545 (U.S. EPA, 2015c) which is a LC-ESI/MS/MS method for the determination of cylindrospermopsin and anatoxin-a in drinking water. This method requires the operation of the mass spectrometer in MS/MS mode to enhance selectivity. In this method, samples are preserved with ascorbic acid (dechlorinating agent) and sodium bisulfate (microbial inhibitor). In the laboratory, aliquots (1 mL) of sample are taken for analysis, and internal standards are added. An aliquot of the sample is injected into an LC equipped with an analytical column that is interfaced to the mass spectrometer. The analytes are separated and then identified by comparing the acquired mass spectra and retention times to reference spectra and retention times for calibration standards acquired under identical liquid chromatography tandem mass spectrometry (LC-MS/MS) conditions. The concentration of each analyte is determined using the integrated peak area and internal standard technique. A single laboratory lowest concentration method reporting limit (LCMRL) of 0.063 µg/L was determined for cylindrospermopsin along with an average value of 0.083 µg/L for all participants of a multi-lab evaluation (n=4) (Winslow et al., 2006). Method 545: Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI/MS/MS) is available at http://water.epa.gov/scitech/drinkingwater/labcert/analyticalmethods_ogwdw.cfm.

Other LC methods have generally used UV spectroscopic detection for cylindrospermopsin analysis. These methods have often incorporated solid phase extraction (SPE) to preconcentrate the target analyte, reduce matrix interferences or both. Quantitation limits have ranged from 4 µg/L to < 0.1 µg/L based on the instrumental setup and the use of preconcentration steps (Papageorgiou et al., 2012).

Commercial ELISA kits for the detection of cylindrospermopsin are available from several vendors. These kits claim a working concentration range of 0.05 and 2 µg/L.

6.0 TREATMENT TECHNOLOGIES

The information below is adapted from the draft Health Canada Guidelines for Cyanobacteria Toxins in Drinking Water, available later in 2015.

Detailed information on the operational considerations of a variety of treatment methods can be found in the *International Guidance Manual for the Management of Toxic Cyanobacteria* (GWRC, 2009) and *Management Strategies for Cyanobacteria (Blue-Green Algae): A Guide for Water Utilities* (Newcombe et al., 2010) available at: http://www.waterra.com.au/cyanobacteria-manual/PDF/GWRCGuidanceManualLevel1.pdf and http://www.researchgate.net/profile/Lionel_Ho/publication/242740698_Management_Strategies_for_Cyanobacteria_(Blue-Green_Algae)_A_Guide_for_Water_Utilities/links/02e7e52d62273e8f70000000.pdf.

For additional information on treatment strategies commonly used or being considered by water systems vulnerable to cyanotoxins, please see *Recommendations for Public Water Systems to Manage Cyanotoxins in Drinking Water* (U.S. EPA, 2015b).

6.1 Management and Mitigation of Cyanobacterial Blooms in Source Water

Algaecides can be applied to lakes and reservoirs to mitigate algal blooms, including cyanobacteria. In most cases, depending on the cyanobacteria species present, the application of algaecides has the potential to compromise cell integrity releasing cyanotoxins into the source waters. Chemical treatment to control blooms in drinking water sources in the early stages of the bloom when cyanobacterial concentrations are still relatively low (usually from 5,000 to 15,000 cells/mL) (WHO, 1999), are less likely to release significant cyanotoxin concentrations upon cell lysis and may mitigate or prevent a cyanobacterial bloom from proliferating as the season progresses. If a cyanobacteria bloom does occur, utilities may investigate alternative raw water sources, change intake locations or levels to withdraw raw water with minimal cyanotoxin concentrations, or investigate methods of destratification in the water source. Purchasing water from a neighboring interconnected water system that is unaffected by the bloom may also be an option for some systems.

Clays and commercial products such as aluminum sulfate (alum) have been used for the management of blooms in source waters. Alum treatment efficiency depends on the alum dose and the type of flocculant. Aeration and destratification have also been used to treat cyanobacterial blooms, usually in smaller water bodies (from one acre to several tens of acres). Active mixing devices, diffuse air bubblers, and other means of reducing stratification have proven to be effective in controlling outbreaks and persistence of blooms in relatively small shallow impoundments (around < 20 feet). These strategies can be applied to the entire source water body or to just a portion of the lake depending on the need, size and depth of the water body relative to the source water intake(s).

The use of ultrasonic sound waves, or sonication, to disrupt cyanobacterial cells has also been investigated as a potential source water treatment option (Rajasekhar et al., 2012). Drawbacks include that application frequencies are difficult to calculate and are system-specific;

and that applications on large scale require more powerful, and therefore, more expensive equipment. Sonication shows potential for use in cyanobacterial bloom management, but further study to determine effective operating procedures is needed before it can be considered as a feasible approach (Rajasekhar et al., 2012).

Excess nutrients are thought to be a primary driver of cyanobacterial blooms. Long-term prevention of cyanobacterial blooms likely requires reductions in nutrient pollution. Excess nitrogen and phosphorus in aquatic systems can stimulate blooms and create conditions under which harmful cyanobacteria thrive. Thus, managing nutrient pollution sources within a watershed in addition to waterbody-specific physical controls (in systems that are amenable to those controls) tends to be the most effective strategy. Nutrient pollution can be from urban, agricultural, and atmospheric sources, and therefore, reductions can be achieved through a variety of source control technologies and best management practices.

6.2 Drinking Water Treatment

Effective treatment of cyanotoxins in drinking water includes the evaluation and selection of appropriate treatment methods. The water treatment methods need to be tailored to the type(s) of cyanobacteria present, the site-specific water quality (e.g., pH, temperature, turbidity, presence of natural organic material (NOM)), the treatment processes already in place and multiple treatment goals (e.g., turbidity and total organic carbon (TOC) removal, disinfection requirements, control of disinfection by-products (DBP) formation). Utilities need to have an understanding of the type and concentration of cyanotoxins present in the source water and should conduct site-specific evaluations such as jar testings and piloting in order to determine the most effective treatment strategy. Potential target parameters include: chlorophyll-a, turbidity, cyanobacterial cells and extracellular and intracelluar toxins. Care should be taken to avoid cell lysis. A multi-barrier approach consists of conventional filtration for intracellular cylindrospermopsin removal and additional processes such as activated carbon, biodegradation, advanced oxidation, and small-pore membrane processes (e.g. nanofiltration and reverse osmosis), for the removal or oxidation of extracellular cylindrospermopsin. The most effective way to deal with cyanobacteria cells and their toxins, is to remove the cells intact, without damaging them, to prevent the release of additional extracellular toxins into the water.

When released from the cell, cylindrospermopsin can be found dissolved or attached to other materials such as particulate or soluble substances. Powdered activated carbon (PAC) has proven to be effective for removal of extracellular cylindrospermopsin. Limited information is available on the adsorption of cylindrospermopsin onto granular activated carbon (GAC).

6.2.1 Conventional Treatment for Cylindrospermopsin

In the absence of cell damage, conventional treatment employing coagulation, flocculation, clarification (sedimentation or dissolved air flotation) and rapid granular filtration can be effective at removing intact cells and the majority of intracellular toxins (cell bound) (Chow et al., 1998; Newcombe et al., 2015). However, if toxins are released into solution, a

combination of conventional treatment processes with oxidation, adsorption and/or advanced treatment needs to be considered to treat both intracellular and extracellular cyanotoxins. Rapid sand filtration without pre-treatment (i.e., direct filtration, without coagulation/clarification) is not effective for cyanobacterial cell removal.

Conventional water treatment (coagulation, flocculation, sedimentation or dissolved air flotation (DAF), and filtration) is considered effective for removal of intracellular toxins but ineffective for dissolved cyanotoxins such as cylindrospermopsin, which is partially dissolved in water under normal growth conditions (Chow et al., 1999; Rapala et al., 2006; Carrière et al., 2010). Application of a multiple barrier approach has the potential to be effective (Newcombe et al., 2015). Ho et al. (2008, 2011) conducted bench-scale studies and modeling on the use of PAC for the adsorption of cylindrospermopsin. The results demonstrated that a PAC dose of 25 mg/L and a contact time of 60 minutes would be required to reduce 5 µg/L of cylindrospermopsin to less than 1 µg/L. When concentrations of cylindrospermopsin are 1-2 µg/L or 3-4 µg/L, the recommended doses of PAC are 10-20 mg/L and 20-30 mg/L, respectively (Newcombe et al., 2010).

Dixon et al. (2011b) also conducted laboratory-scale testing of integrated membrane systems for cyanotoxin removal. The results showed that an ultrafiltration system with pre-treatment using 2.2 mg/L of alum and 20 mg/L of PAC resulted in 97% removal of intra- and extra-cellular cylindrospermopsin to achieve a treated water concentration of less than 0.1 µg/L (Dixon et al., 2011a). Nanofiltration and reverse osmosis would likely be effective in removing dissolved toxins, but only a few studies have been conducted. Dixon et al. (2011a) studied the removal of cyanobacterial toxins by nanofitration and found that average removals between 90-100% could be achieved for cylindrospermopsin using membranes with a low molecular weight cut-off (MWCO) (< 300 Daltons).

In practice, full-scale treatment plants use a combination of treatment technologies (i.e., conventional filtration and chemical oxidation) in order to remove both intracellular and extracellular cyanotoxins. Extracellular cylindrospermopsin may be removed by many treatment plants using existing treatments such as chlorination or by the addition of PAC (Carriere et al., 2010). Although it is possible to remove both intracellular and extracellular toxins effectively using a combination of treatment processes, the removal efficiency can vary considerably. Utilities need to ensure that they are using their existing treatment processes to their fullest capacity for removal of both cyanobacterial cells and extracellular toxins, and that the appropriate monitoring is being conducted to ensure that adequate removal is occurring at each step in the treatment process.

6.2.2 Chemical Oxidation

Chemical oxidation using chlorine or ozonation can be effective at oxidizing cylindrospermopsin, but can also cause the cells to lyse, resulting in an increase in concentrations of extracellular toxins in drinking water. By applying conventional filtration (or another filtration process) first to remove the majority of intact cells, the extracellular cylindrospermopsin is less likely to increase due to cell lysis when water is treated with oxidants. In cases where pre-

oxidation (oxidant applied anywhere along the treatment process prior the filter influent) is practiced, it may need to be discontinued during an algal bloom or adjustments to the oxidant type and doses may be needed to minimize cell rupture prior to filtration (Newcombe et al., 2015).

Different cyanotoxins react differently to oxidants depending on the individual characteristics of the source water such as TOC, temperature and pH (Westrick et al., 2010). While chlorination is an effective treatment for oxidizing cylindrospermopsin, its effectiveness is dependent on pH. Rodriguez et al. (2007) found that at a pH of 7 and an initial chlorine dose of 1 mg/L, oxidation of cylindrospermopsin is fast, with almost complete reaction after 30 minutes. Other chlorinated oxidants such as chloramines and chlorine dioxide have little impact on cylindrospermopsin due to a slow reaction rates. For example, the reaction of chlorine dioxide with cylindrospermopsin is relatively slow with a second-order rate constant of 0.9 $M^{-1}s^{-1}$ at pH 8. The rate constant is pH-dependent and decreases significantly under mildly acidic conditions. Chlorine dioxide may be used to inactivate *C. raciborskii*, however, in typical drinking water treatment applications, it does not appear to be practical for oxidizing cylindrospermopsin given its slow reaction rate (de la Cruz et al., 2013). Oxidation by potassium permanganate is temperature dependent and has not been shown to be effective in oxidizing cylindrospermopsin. Water treatment utilities that use chloramines or chlorine dioxide as disinfectants to reduce the formation of regulated disinfection by-products may want to reconsider oxidation efficacy for cyanotoxin inactivation during periods when algal toxins are present in source waters, while balancing these other treatment objectives. Ozone has been shown to effectively oxidize cylindrospermopsin in laboratory-scale studies (de la Cruz et al., 2013). At pH 8, approximately 95% of cylindrospermopsin (initial concentration of 415 µg/L) was oxidized using 0.38 mg/L O_3.

6.2.3 Ultraviolet Irradiation

Studies have indicated that ultraviolet (UV) irradiation may be effective for the oxidation of cylindrospermopsis cells (Westrick et al., 2010). However, exposure times and/or UV doses tested in the bench-scale experiments were greater than those typically applied in drinking water treatment.

6.3 Point-of-Use (POU) Drinking Water Treatment Units

Limited information is available on residential treatment units for the removal of cyanobacteria cells and cyanotoxins. At this time, no units have been evaluated for removal of cylindrospermopsin. Further studies need to be conducted to assess the efficacy of home filtration devices for various cyanotoxins, including cylindrospermopsin, and for other filtering conditions such as increased toxin load and the presence of other contaminants in drinking water. Third-party organizations are currently developing certification standards to test POU devices to evaluate how well they remove cyanotoxins from drinking water treatment units. Those standards are expected in the near future.

More information about treatment units and the contaminants they can remove can be found at http://www.nsf.org/Certified/DWTU/.

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Drinking Water Health Advisory for the Cyanobacterial Toxin Cylindrospermopsin

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ABBREVIATIONS AND ACRONYMS

BMD	Benchmark Dose
BMDL	Benchmark Dose Level
BW	Body Weight
CAS	Chemical Abstracts Service
CCL	Contaminant Candidate List
CWA	Clean Water Act
CYP450	Cytochrome P450
DAF	Dissolved Air Flotation
DBP	Disinfection By-Products
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DWI	Drinking Water Intake
ELISA	Enzyme Linked Immunosorbent Assay
EPA	U.S. Environmental Protection Agency
g	Gram
GAC	Granular Activated Carbon
GFR	Glomerular Filtration Rate
HA	Health Advisory
HAB	Harmful Algal Bloom
HESD	Health Effects Support Document
HPLC	High Performance Liquid Chromatography
ICR	Institute for Cancer Research
i.p.	Intraperitoneal
kg	Kilogram
K_{oc}	Organic Carbon:Water Partition Coefficient
K_{ow}	Octanol:Water Partition Coefficient
L	Liter
LC	Liquid Chromatography
LCAT	Lecithin Cholesterol Acyl Transferase
LC-ESI/MS	Liquid Chromatography Tandem Electrospray Ionization Mass Spectrometry
LCMRL	Lowest Concentration Method Reporting Limit
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LOAEL	Lowest-Observed-Adverse-Effect Level
MCH	Mean Corpuscular Hemoglobin
µg	Microgram
µm	Micromole
MNBNC	Micronucleated Binucleated Cells
mg	Milligram
mL	Milliliter
mmol	Millimole
MOA	Mode of Action
MWCO	Molecular Weight Cut-off
N	Nitrogen

N/A	Not Applicable
NARS	National Aquatic Resource Surveys
ng	Nanogram
NHANES	National Health and Nutrition Examination Survey
NLA	National Lake Assessment
NOAEL	No-Observed-Adverse-Effect Level
NOM	Natural Organic Material
OECD	Organization for Economic Cooperation and Development
P	Phosphorus
PAC	Powdered Activated Carbon
PDA	Photodiode Array
pg	Picogram
POU	Point-of-Use
RBC	Red Blood Cell
RfD	Reference Dose
SDWA	Safe Drinking Water Act
SHE	Syrian Hamster Embryo
SPE	Solid Phase Extraction
TOC	Total Organic Carbon
TOXLINE	Toxicology Literature Online
UF	Uncertainty Factor
USACE	U.S. Army Corps of Engineers
USGS	U.S. Geological Survey
UV	Ultraviolet

EXECUTIVE SUMMARY

Cylindrospermopsin is a toxin produced by a variety of cyanobacteria including: *Cylindrospermopsis raciborskii (C. raciborskii), Aphanizomenon flos-aquae, Aphanizomenon gracile, Aphanizomenon ovalisporum, Umezakia natans, Anabaena bergii, Anabaena lapponica, Anabaena planctonica, Lyngbya wollei, Rhaphidiopsis curvata,* and *Rhaphidiopsis mediterranea*.

Many environmental factors such as the ratio of nitrogen to phosphorus, temperature, organic matter availability, light attenuation and pH play an important role in the development of cylindrospermopsin blooms, both in fresh and marine water systems. These species do not tend to form visible surface scums and the highest concentrations of cells occurs below the water surface. Cylindrospermopsin may be retained within the cell, but most of the time it is found in the water (extracellular) or attached to particulates present in the water.

This Health Advisory (HA) for the cyanobacterial toxin cylindrospermopsin is focused on drinking water as the primary source of exposure. Exposure to cyanobacteria and their toxins may also occur by ingestion of toxin-contaminated food, including consumption of fish, and by inhalation and dermal contact during bathing or showering and during recreational activities in waterbodies with the toxins. While these types of exposures cannot be quantified at this time, they are assumed to contribute less to the total cyanotoxin exposures than ingestion of drinking water. Due to the seasonality of cyanobacterial blooms, exposures are not expected to be chronic.

Limited animal studies demonstrate absorption of cylindrospermopsin from the intestinal tract primarily in the liver, but also in the kidney and spleen. Limited data are available on the metabolism of cylindrospermopsin, but evidence indicates that metabolism and toxicity are mediated by the hepatic cytochrome P450 (CYP450) enzyme system. The periacinar region of the liver, an area where substantial CYP450-mediated xenobiotic metabolism occurs, appears to be the main target of cylindrospermopsin toxicity and where cylindrospermopsin and its metabolites bind to proteins. The few studies evaluating elimination suggest that cylindrospermopsin is rapidly eliminated primarily in the urine, but also in feces.

The main source of information on the toxicity of cylindrospermopsin in humans is from qualitative reports of a hepatoenteritis-like illness attributed to acute or short-term consumption of drinking water containing *C. raciborskii*. Symptoms reported include fever, headache, vomiting, bloody diarrhea, hepatomegaly, and kidney damage with loss of water, electrolytes and protein. No reliable data are available on the exposure levels of cylindrospermopsin that induced these effects.

Based on oral and intraperitoneal (i.p.) studies in mice treated with purified cylindrospermopsin or extracts of *C. raciborskii* cells, the liver and kidneys appear to be the primary target organs for cylindrospermopsin toxicity.

The U.S. Environmental Protection Agency (EPA) identified a study by Humpage and Falconer (2002, 2003) conducted on mice as the critical study used in the derivation of the reference dose (RfD) for cylindrospermopsin. The critical effects identified in the study areincreased kidney weight and decreased urinary protein. The NOAEL (No Observed Adverse

Effect Level) was determined to be 30 µg/kg/day based on kidney toxicity. The total uncertainty factor (UF) applied to the NOAEL was 300. This was based on a UF of 10 for intraspecies variability, a UF of 10 for interspecies variability, and a UF of 3 ($10^{½}$) to account for deficiencies in the database.

EPA is issuing a Ten-day HA for cylindrospermopsin based on the Humpage and Falconer (2002, 2003) 11-week study. Studies of a duration of 7 days up to 30 days are typically used to derive Ten-day HAs. In this case, a subchronic study was determined to be suitable for the derivation of the HA. Although the duration of the Humpage and Falconer (2002, 2003) study is longer (77 days) than the studies typically used for the derivation of a Ten-day HA, the short-term studies available for cylindrospermopsin (Shaw et al., 2001; Reisner et al., 2004) are not suitable for quantification; however, effects observed in these studies are the same or similar to the Humpage and Falconer study (2002, 2003) and occur at similar doses.

The short-term HA is consistent with the available data and most appropriately matches human exposure scenarios for cyanobacterial blooms in drinking water. Cyanobacterial blooms are usually seasonal, typically occurring from May through October. In the presence of algal cell pigments, photochemical degradation of cylindrospermopsin can occur rapidly, with reported half-lives of 1.5 to 3 hours. In the absence of pigments, however, there is little degradation. The biodegradation of cylindrospermopsin in natural water bodies is a complex process that can be influenced by many environmental factors, including concentration, water temperature and the presence of bacteria. Half-lives of 11 to 15 days and up to 8 weeks have been reported for cylindrospermopsin in surface waters. In addition, concentrations in finished drinking water can be reduced by drinking water treatment and management measures.

The Ten-day HA value for bottle-fed infants and young children of pre-school age is 0.7 µg/L and for school-age children through adults is 3 µg/L for cylindrospermopsin. The two advisory values use the same toxicity data (RfD) and represent differences in drinking water intake and body weight for different human life stages. The first advisory value is based on the summation of the time-weighted drinking water intake/body weight ratios for birth to < 12 months of age (U.S. EPA's Exposure Factors Handbook, 2011a). The second advisory value is based on the mean body weight and the 90th percentile drinking water consumption rate for adults age 21 and over (U.S. EPA's Exposure Factors Handbook, 2011a), which is similar to that of school-aged children. Populations such as pregnant women and nursing mothers, the elderly, and immune-compromised individuals or those receiving dialysis treatment may be more susceptible than the general adult population to the health effects of cylindrospermopsin. As a precautionary measure, individuals that fall into these susceptible groups may want to consider following the recommendations for children pre-school age and younger. This HA is not a regulation; it is not legally enforceable; and it does not confer legal rights or impose legal obligations on any party.

No epidemiological studies of the association of cylindrospermopsin and cancer are available. Also, no chronic cancer bioassays of purified cylindrospermopsin in animals were identified. Therefore, under the U.S. EPA's (2005) Guidelines for Carcinogen Risk Assessment, there is *inadequate information to assess carcinogenic potential* of cylindrospermopsin.

1.0 INTRODUCTION AND BACKGROUND

EPA developed the non-regulatory Health Advisory (HA) Program in 1978 to provide information for public health officials or other interested groups on pollutants associated with short-term contamination incidents or spills for contaminants that can affect drinking water quality, but are not regulated under the Safe Drinking Water Act (SDWA). At present, EPA lists HAs for 213 contaminants (http://water.epa.gov/drink/standards/hascience.cfm).

HAs identify the concentration of a contaminant in drinking water at which adverse health effects are not anticipated to occur over specific exposure durations (e.g., one-day, ten-days, and a lifetime). HAs serve as informal technical guidance to assist Federal, State and local officials, and managers of public or community water systems in protecting public health when emergency spills or contamination situations occur. An HA provides information on the environmental properties, health effects, analytical methodology, and treatment technologies for removal of drinking water contaminants.

The *Health Effects Support Document for Cylindrospermopsin* (U.S. EPA, 2015a) is the peer-reviewed, effects assessment that supports this HA. This document is available at http://www2.epa.gov/nutrient-policy-data/health-and-ecological-effects. The HAs are not legally enforceable Federal standards and are subject to change as new information becomes available. The structure of this Health Advisory is consistent with EPA's *Framework for Human Health Risk Assessment to Inform Decision Making* (U.S.EPA, 2014).

EPA is releasing the *Recommendations for Public Water Systems to Manage Cyanotoxins in Drinking Water* (U.S. EPA, 2015b) as a companion to the HAs for microcystins and cylindrospermopsin. The document is intended to assist public drinking water systems (PWSs) that choose to develop system-specific plans for evaluating their source waters for vulnerability to contamination by microcystins and cylindrospermopsin. It is designed to provide information and a framework that PWSs and others as appropriate may consider to inform their decisions on managing the risks from cyanotoxins in drinking water.

1.1 Current Criteria, Guidance and Standards

Currently there are no U.S. federal water quality criteria, or regulations for cyanobacteria or cyanotoxins in drinking water under the SDWA or in ambient waters under the Clean Water Act (CWA). The Safe Drinking Water Act (SDWA), as amended in 1996, requires the EPA to publish a list of unregulated contaminants every five years that are not subject to any proposed or promulgated national primary drinking water regulations, which are known or anticipated to occur in public water systems, and which may require regulation. This list is known as the Contaminant Candidate List (CCL). The EPA's Office of Water included cyanobacteria and cyanotoxins on the first and second CCL (CCL 1, 1998; CCL 2, 2005). EPA included cyanotoxins, including anatoxin-a, cylindrospermopsin, and microcystin-LR, on CCL 3 (2009) and the draft CCL 4 (April 2015 for consideration).

SDWA requires the Agency to make regulatory determinations on at least five CCL contaminants every five years. When making a positive regulatory determination, EPA determines whether a contaminant meets three criteria:

- The contaminant may have an adverse effect on the health of persons,
- The contaminant is known to occur or there is substantial likelihood the contaminant will occur in public water systems with a frequency and at levels of concern, and
- In the sole judgment of the Administrator, regulating the contaminant presents a meaningful opportunity for health risk reductions.

To make these determinations, the Agency uses data to analyze occurrence (prevalence and magnitude) and health effects. EPA continues gathering this information to inform future regulatory determinations for cyanotoxins under the SDWA. The SDWA also provides the authority for EPA to publish non-regulatory HAs or take other appropriate actions for contaminants not subject to any national primary drinking water regulation. EPA is providing this HA and the HA for microcystins to assist State and local officials in evaluating risks from these contaminants in drinking water.

Internationally, three countries and two U.S. states have developed drinking water guidelines for cylindrospermopsin, as shown in Table 1-1 and Table 1-2, respectively.

Table 1-1. International Guideline Values for Cylindrospermopsin

Country	Guideline Value	Source
Australia	1 µg/L	Australian Drinking Water Guidelines 6 (NHMRC, NRMMC, 2011)
New Zealand	1 µg/L	Drinking-water Standards for New Zealand 2005 (Ministry of Health, 2008)
Brazil	15 µg/L (recommended)	Guidelines for Drinking Water Quality, Official LA Report's, Regulation MS N 518/2004 (Brasil, 2009)

Table 1-2. State Guideline Values for Cylindrospermopsin

State	Guideline Value	Source
Ohio	1 µg/L	State of Ohio Public Water System Harmful Algal Bloom Response Strategy (OHEPA, 2014)
Oregon	1 µg/L	Public Health Advisory Guidelines, Harmful Algae Blooms in Freshwater Bodies. (OHA, 2015)

2.0 PROBLEM FORMULATION

The development of the HA begins with problem formulation, which provides a strategic framework by focusing on the most relevant cyanotoxin properties and endpoints identified in the *Health Effects Support Document for Cylindrospermopsin* (U.S. EPA, 2015a).

2.1 Cyanobacteria and Production of Cylindrospermopsin

Cyanobacteria, formerly known as blue-green algae (Cyanophyceae), are a group of bacteria with chlorophyll-a capable of photosynthesis (light and dark phases) (Castenholz and Waterbury, 1989). Most cyanobacteria are aerobic photoautotrophs, requiring only water, carbon dioxide, inorganic nutrients and light for survival, while others have heterotrophic properties and can survive long periods in complete darkness (Fay, 1965). Some species are capable of nitrogen fixation (diazotrophs) (Duy et al., 2000), producing inorganic nitrogen compounds for the synthesis of nucleic acids and proteins. Cyanobacteria can form symbiotic associations with animals and plants, such as fungi, bryophytes, pteriodophytes, gymnosperms and angiosperms (Rai, 1990), supporting their growth and reproduction (Sarma, 2013; Hudnell, 2008; Hudnell, 2010).

Under the right conditions of pH, nutrient availability, light, and temperature, cyanobacteria can reproduce quickly, forming a bloom. Although studies of the impact of environmental factors on cyanotoxin production are ongoing, nutrient (nitrogen, phosphorus and trace metals) supply rates, light, temperature, oxidative stressors, interactions with other biota (viruses, bacteria and animal grazers) and, most likely, the combined effects of these factors are all involved (Paerl and Otten 2013a, 2013b). Fulvic and humic acids reportedly encourage cyanobacteria growth (Kosakowska et al., 2007).

Cylindrospermopsin is a toxin produced by a variety of cyanobacteria including: *Cylindrospermopsis raciborskii (C. raciborskii), Aphanizomenon flos-aquae, Aphanizomenon gracile, Aphanizomenon ovalisporum, Umezakia natans, Anabaena bergii, Anabaena lapponica, Anabaena planctonica, Lyngbya wollei, Rhaphidiopsis curvata,* and *Rhaphidiopsis mediterranea.*

2.2 Physical and Chemical Properties

The cyanotoxin cylindrospermopsin is a tricyclic alkaloid with the following molecular formula $C_{15}H_{21}N_5O_7S$ (Ohtani et al., 1992) and a molecular weight of 415.43 g/mole. It is zwitterionic (i.e., a dipolar ion with localized positive and negative charges) (Ohtani et al., 1992) and is believed to be derived from a polyketide that uses an amino acid starter unit such as glycocyamine or 4-guanidino-3-oxybutyric acid (Duy et al., 2000). The chemical structure of cylindrospermopsin is presented in Figure 2-1. Two naturally occurring congeners of cylindrospermopsin have been identified (Figure 2-2): 7-epicylindro-spermopsin (the epimer of cylindrospremopsin) and 7-deoxycylindrospermopsin (Norris et al., 1999; de la Cruz et al., 2013). Recently, Wimmer et al. (2014) identified two new analogs, 7-deoxy-desulfo-

Figure 2-1. Structure of Cylindrospermopsin (de la Cruz et al., 2013)

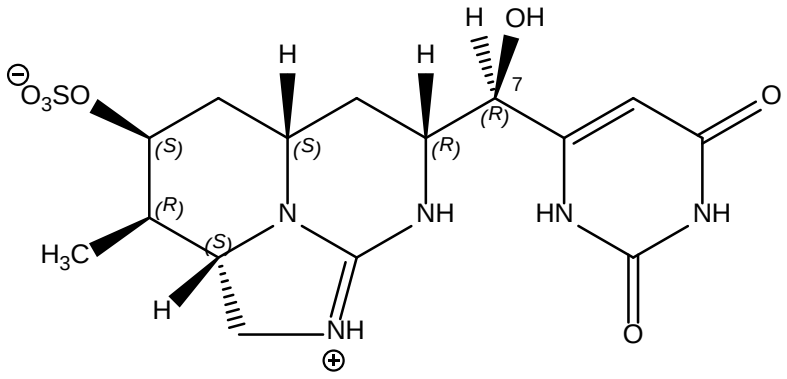
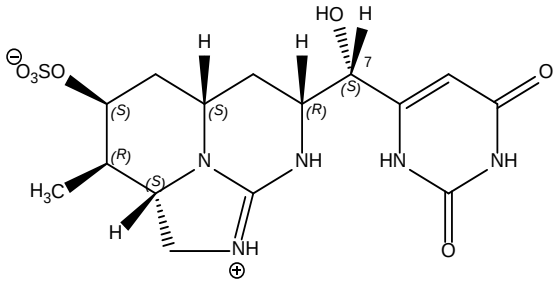
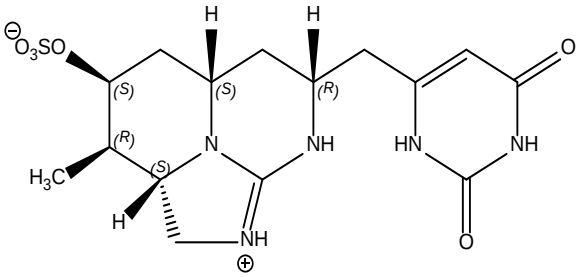


Figure 2-2. Structurally Related Cylindrospermopsins (de la Cruz et al., 2013)

7-epi-cylindrospermopsin



7-deoxycylindrospermopsin



cylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin, from the Thai strain of *C. raciborskii*. However, it is not clear if these are cylindrospermopsin congeners, precursors or degradation products. Chlorination of water containing cylindrospermopsin can produce 5-chlorocylindrospermopsin and cylindrospermic acid.

The physical and chemical properties of cylindrospermopsin are presented in Table 2-1. Cylindrospermopsin generally exists in a zwitterionic state (with both positive and negative ions) and is highly soluble in water (Moore et al., 1998, Chiswell et al., 1999). Cylindrospermopsin is isolated for commercial use mostly from *C. raciborskii.* Other physicochemical properties of cylindrospermopsin in the environment such as vapor pressure, boiling and melting points, soil organic carbon-water partition coefficient (Koc), octanol-water partition coefficient (Kow), and vapor pressure and Henry's Law constant are unknown. Available information on the chemical

Table 2-1. Chemical and Physical Properties of Cylindrospermopsin

Property	Cylindrospermopsin
Chemical Abstracts Service (CAS) Registry #	143545-90-8
Chemical Formula	$C_{15}H_{21}N_5O_7S$
Molecular Weight	415.43 g/mole
Color/Physical State	white powder
Boiling Point	N/A
Melting Point	N/A
Density	$2.03 g/cm^3$
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	Dimethylsulfoxide (DMSO) and methanol

Sources: Chemical Book, 2012; TOXLINE, 2012

breakdown, biodegradation and mobility of cylindrospermopsin in the environment is discussed in the Environmental Fate section.

2.3 Sources and Occurrence

Many environmental factors such as the ratio of nitrogen to phosphorus, temperature, organic matter availability, light attenuation and pH play an important role in the development of cylindrospermopsin blooms (Paerl and Huisman, 2008; Paerl and Otten, 2013). Although cylindrospermopsin-producing cyanobacteria (such as *C. raciborskii)* occur mostly in tropical or subtropical regions, they have also been found in warmer temperate regions, both in fresh and marine water systems. These species do not tend to form visible surface scums and the highest concentrations of cells occurs below the water surface (Falconer 2005). Cylindrospermopsin may be retained within the cell, but most of the time it is found in the water (extracellular) or attached to particulates present in the water (Chiswell et al., 2001).

2.3.1 Occurrence in Surface Water

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 algal toxins, but did not include cylindrospermopsin. The United States Geological Survey (USGS) subsequently analyzed the stored samples collected during the NLA and reported that cylindrospermopsin was present in 5% of the samples; however, concentrations of cylindrospermopsin were not reported (Loftin and Graham, 2014). Future NARS plan to include other algal toxins, including cylindrospermopsin.

Cylindrospermopsin was also detected in 9% of the blooms sampled during a 2006 USGS survey of 23 lakes in the Midwestern U.S. (Graham et al., 2010). The low concentrations of cylindrospermopsin detected (0.12 to 0.14 µg/L) in the study occurred in bloom communities dominated by *Aphanizomenon* or *Anabaena* and *Microcystis*.

Many states monitor for harmful algal blooms (HABs). State monitoring efforts are expanding with greater awareness of the toxic effects of HABs. These monitoring efforts tend to focus on priority waters used for recreation or drinking water. Sampling is seasonal or on occasions when blooms are observed.

Cylindrospermopsin has been detected in lakes throughout multiple states. In a 1999 study, cylindrospermopsin was detected in 40% of 167 water samples taken from 87 water bodies in Florida during the months of June and November (Burns, 2008). However, the actual cylindrospermopsin concentrations were not reported. In 2005, the U.S. Army Corps of Engineers (USACE) detected cylindrospermopsin at a maximum concentration of 1.6 µg/L in lake water samples from Oklahoma (Lynch and Clyde, 2009). In Grand Lake St. Marys, Ohio, cylindrospermopsin concentrations as high as 9 µg/L were reported in 2010 (OHEPA, 2012).

2.3.2 Occurrence in Drinking Water

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

EPA used information from the published literature to evaluate the potential occurrence of cylindrospermopsin in public water systems. In the single publication identified, the results of a 2000 survey of toxins in drinking water treatment plants in Florida were reported (Burns, 2008). In this survey, cylindrospermopsin was detected at concentrations ranging from 8 µg/L to 97 µg/L in nine finished drinking water samples.

2.4 Environmental Fate

Different physical and chemical processes are involved in the persistence, breakdown, and movement of cylindrospermopsin in aquatic systems.

2.4.1 Persistence

Cylindrospermopsin is relatively stable in the dark and at temperatures from 4°C to 50°C for up to five weeks (ILS, 2000). Cylindrospermopsin is also resistant to changes in pH and remains stable for up to eight weeks at pH 4, 7 and 10. In the absence of cell pigments, cylindrospermopsin tends to be relatively stable in sunlight, with a half-life of 11 to 15 days in surface waters (Funari and Testai, 2008). Cylindrospermopsin remains a potent toxin even after boiling for 15 minutes (Chiswell et al., 1999).

Degradation of cylindrospermopsin increases in the presence of cell pigments such as chlorophyll-a and phycocyanin. When exposed to both sunlight and cell pigments, cylindrospermopsin breaks down rapidly, more than 90% within 2 to 3 days (Chiswell et al., 1999). Cylindrospermopsin has been shown to be decomposed by bacteria in laboratory studies; the biodegradation is influenced by the toxin concentration, temperature and pH. Mohamed and Alamri (2012) reported that cylindrospermopsin was degraded by *Bacillus* bacteria and degradation occurred in 6 days at the highest toxin concentration (300 μg/L) and in 7 or 8 days at lower concentrations (10 and 100 μg/L, respectively). The biodegradation rate was also reported to depend on temperature and pH, with the highest rates occurring in warm waters (25 and 30°C) and neutral to slightly alkaline conditions (pH 7 and 8). Klitzke and Fastner (2012) confirmed the observations of Mohamed and Alamri (2012), noting that a decrease in temperature from 20 to 10°C slowed down degradation by a factor of 10. They also found that degradation slowed significantly under anaerobic conditions, with half-lives of 2.4 days under aerobic conditions and 23.6 days under anaerobic conditions.

2.4.2 Mobility

In sediments, cylindrospermopsin exhibits some adsorption to organic carbon, with little adsorption observed on sandy and silt sediments (Klitzke et al., 2011). The low adsorption of cylindrospermopsin reduces its residence time in sediments, thus reducing the opportunity for microbial degradation.

2.5 Nature of the Cylindrospermopsin Toxin

2.5.1 Toxicokinetics

Animal studies show that cylindrospermopsin is absorbed from the gastrointestinal tract (Humpage and Falconer, 2003; Shaw et al., 2000, 2001) and that the tissue distribution occurs

primarily to the liver, but also to the kidneys and spleen after intraperitoneal (i.p.) exposure (Norris et al., 2001).

The metabolism and toxicity of cylindrospermopsin is mediated by the hepatic cytochrome P450 (CYP450) enzyme system. The periacinar region of the liver, an area where substantial CYP450-mediated xenobiotic metabolism occurs, appears to be the main target of cylindrospermopsin toxicity and where cylindrospermopsin and its metabolites bind to proteins (Runnegar et al. 1995; Shaw et al. 2000, 2001; Norris et al., 2001).

Animal studies evaluating the elimination of cylindrospermopsin in urine and feces after i.p. exposures found a continued urinary and fecal excretion over the monitoring period (24 hours) and a mean total recovery from the urine and feces of 76.9% of the administered dose after 24 hours (Norris et al., 2001). Urinary excretion accounted for 68.4% of the 24-hour total and fecal excretion for 8.5%. There was considerable interanimal variability in this study.

2.5.2 Noncancer Health Effects Data

2.5.2.1 Human Studies

Human data on oral toxicity of cylindrospermopsin are limited, but suggest that liver and kidney are potential target organs for toxicity. Reports of a hepatoenteritis-like outbreak (mostly in children) in Palm Island, Australia in 1979 were attributed to consumption of drinking water with a bloom of *C. raciborskii*, a cyanobacteria that can produce cylindrospermopsin. No data are available on exposure levels or potential co-exposures to other cyanobacterial toxins and microorganisms. The majority of the cases, mostly children, required hospitalization. The clinical picture included fever, headache, vomiting, bloody diarrhea, hepatomegaly and kidney damage with loss of water, electrolytes and protein (Byth, 1980; Griffiths and Saker, 2003).

Dermal exposure to cylindrospermopsin was evaluated using skin-patch testing in humans (Pilotto et al., 2004; Stewart et al, 2006). Exposed individuals showed mild irritation, but no statistically significant dose-response relationship or reaction rates were found between skin reactions and increasing cell concentrations for either whole or lysed cells (Pilotto et al., 2004). No detectable skin reactions were observed in individuals exposed to lyophilized *C. raciborskii* (Stewart et al., 2006).

2.5.2.2 Animal Studies

Most of the information on the noncancer effects of cylindrospermopsin in animals is from oral and i.p. administration studies in mice exposed to purified compound or extracts of *C. raciborskii* cells. Studies conducted with purified toxin are preferred because extracts may contain other toxins or compounds with similar chemical physical properties that co-elute with the toxin. Effects on the liver and kidney, including changes in organ weights and histopathological lesions, along with increases in the hematocrit level in serum and deformation of red blood cell are observed following short-term and subchronic oral exposure to

cylindrospermopsin (Humpage and Falconer, 2002, 2003; Reisner at al., 2004; Sukenik et al., 2006). Oral and i.p. acute toxicity studies in mice also report histopathological effects in both liver and kidney. No chronic toxicity studies evaluating cylindrospermopsin are available.

No oral reproductive or developmental studies are available for cylindrospermopsin. Developmental toxicity studies following i.p. administration of cylindrospermopsin provide some evidence for maternal toxicity and decreased postnatal pup survival and body weight (Rogers et al., 2007; Chernoff et al., 2011). Sibaldo de Almeida et al. (2013) did not find any visceral or skeletal malformations in the offspring of pregnant rats receiving an oral dose of 3 mg/kg/day purified cylindrospermopsin during gestation (GD 1-20).

2.5.3 Mode of Action for Noncancer Health Effects

2.5.3.1 Liver

The occurrence of toxicity in the liver suggests a protein-synthesis inhibition mechanism of action for cylindrospermopsin. *In vitro* and *in vivo* studies have been conducted to demonstrate the ability of cylindrospermopsin to inhibit hepatic protein synthesis, which could impact mouse urinary protein production leading to decreased urinary excretion of these proteins (Froscio et al., 2008, 2009; Terao et al., 1994). Available evidence indicates that protein synthesis inhibition is not decreased by broad-spectrum CYP450 inhibitors, but they do reduce cytotoxicity (Froscio et al., 2003; Bazin et al., 2010). Hepatotoxicity appears to be CYP450-dependent, which indicates a possible involvement of oxidized and/or fragmented metabolites and mechanisms other than protein synthesis inhibition (Froscio et al., 2003; Humpage et al., 2005; Norris et al., 2001, 2002). Despite the number of studies that have been published, the mechanisms for liver and kidney toxicity by cylindrospermopsin are not completely characterized.

2.5.3.2 Red Blood Cells

There was evidence of effects on red blood cells (RBCs) in the Reisner et al. (2004) and Humpage and Falconer (2002) studies of purified cylindrospermopsin. In the Reisner et al. (2004) report, microscopic examination of blood samples showed the presence of RBCs with spiked surfaces rather than their normal biconcave-disc shape. The authors attributed the acanthocyte formation to an increase in the cholesterol to phospholipid ratio of the RBC membrane. Phospholipids constitute the matrix material of cell membranes. The authors hypothesized that this change was the consequence of decreased activity of plasma lecithin cholesterol acyl transferase (LCAT), an enzyme associated with high-density lipoproteins and the esterification of plasma cholesterol. Effects on the cholesterol content of the RBC membrane can occur with inhibition of the enzyme increasing membrane fluidity and mean corpuscular volume. Associated effects were observed in the Reisner et al. (2004) and Humpage and Falconer (2002) studies. Removal of the abnormal blood cells by the spleen increases both spleen weight and serum bilirubin as well as stimulates hematopoiesis. Additional research is

needed to examine the LCAT enzyme inhibition hypothesis in order to confirm whether it accounts for the effects on the RBC as a result of cylindrospermopsin exposure.

2.5.3.3 Kidney

No mode of action information for kidney effects was observed in the available studies of cylindrospermopsin. Since all the studies were conducted in mice, a species that excretes low molecular weight proteins in urine, there is a need to conduct a study of cylindrospermopsin in a laboratory species that does not excrete protein in the urine in order to determine whether there are comparable effects on kidney weight, protein excretion and renal cellular damage. Kidney necrosis and a decreased renal failure index at the high cylindrospermopsin doses provide support for the effects on the kidney.

2.5.4 Carcinogenicity Data

No chronic cancer bioassays of cylindrospermopsin were located in the literature. Limited data from an *in vivo* study showed no indication that the cyanobacterial extract containing cylindrospermopsin initiated tumors in mice (Falconer and Humpage, 2001). Cell transformation in Syrian hamster embryo (SHE) cells was observed using purified cylindrospermopsin (Marie et al., 2010). Transformation frequency increased at the lowest concentrations (from 1 x 10^{-2} to 1x10^{-7} ng/mL) but not at the highest concentrations (1 or 1x10^{-1} ng/mL).

Mutagenicity studies (e.g., the Ames Assay) have not observed mutagenic activity of cylindrospermopsin (Sieroslawska, 2013). A few i.p. studies investigating the *in vivo* genotoxicity (DNA damage) from exposure to cylindrospermopsin showed DNA strand breakage in the liver of Balb/c mice (Shen et al., 2002) and covalent binding between DNA and cylindrospermopsin, or a metabolite, in Quackenbush mouse liver (Shaw et al., 2000). *In vitro* mutagenic and genotoxic cell assays have shown potential damage to DNA expressed as an increase in micronucleated binucleate cells (MNBNC) in the colon adenocarcinoma line and the human hepatoma line (Bazin et al., 2010), in the human lymphoblastoid cell line (Humpage et al., 2000), in HepG2 cells (Straser et al., 2011), and in isolated human peripheral lymphocytes (Zegura et al., 2011). DNA breaks also have been observed in primary hepatocytes by comet assay (Humpage et al., 2005).

2.6 Conceptual Model

The conceptual model is intended to explore potential links of exposure to a contaminant or stressor with the adverse effects and toxicological endpoints important for management goals, including the development of HA values. The conceptual model demonstrates the relationship between exposure to cylindrospermopsin in drinking water and adverse health effects in the populations at risk.

HAs describe non-regulatory concentrations of drinking water contaminants at which adverse health effects are not anticipated to occur over specific exposure durations (e.g., one-day, ten-days, and a lifetime). HAs also contain a margin of safety to protect sensitive members of the population. They serve as informal technical guidance to assist federal, state and local officials, as well as managers of public or community water systems, in protecting public health. They are not to be construed as legally enforceable federal standards.

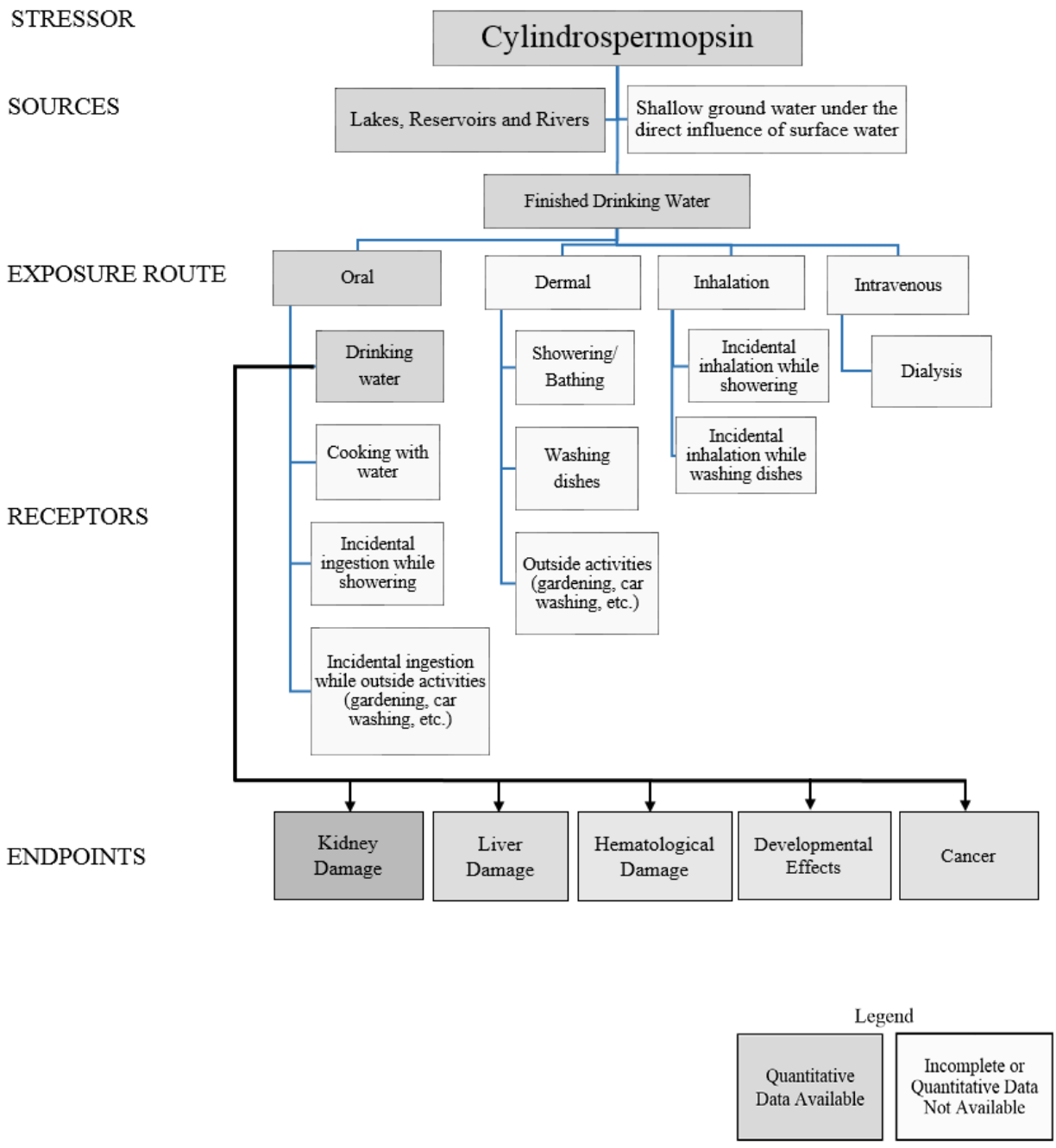
Assessment endpoints for HAs can be developed for both short-term (one-day and ten-day) and lifetime exposures periods using information on the non-carcinogenic and carcinogenic toxicological endpoints of concern. Where data are available, endpoints will reflect susceptible and/or more highly exposed populations.

- A One-day HA is typically calculated for an infant (0-12 months or 10kg child), assuming a single acute exposure to the chemical and is generally derived from a study of less than seven days' duration.
- A Ten-day HA is typically calculated for an infant (0-12 months or 10kg child), assuming a limited period of exposure of one to two weeks, and is generally derived from a study of 7 to 30-days duration.
- A Lifetime HA is derived for an adult (>21 years or 80kg adult), and assumes an exposure period over a lifetime (approximately 70 years). It is usually derived from a chronic study of two years duration, but subchronic studies may be used by adjusting the uncertainty factor employed in the calculation. For carcinogens, the HA documents typically provide the concentrations in drinking water associated with risks for one excess cancer case per ten thousand persons exposed up to one excess cancer case per million exposed for Group A and B carcinogens and those classified as known or likely carcinogens (U.S. EPA, 1986, 2005). Cancer risks are not provided for Group C carcinogens or those classified as "suggestive", unless the cancer risk has been quantified.

For each assessment endpoint EPA uses one or more measures of effect (also referred to as a point of departure), which describe the change in the attribute of the assessment endpoint in response to chemical exposure, to develop acute, short-term, longer term (subchronic) or chronic reference values when the data are available. The measures of effect selected represent impacts on survival, growth, system function, reproduction and development.

This conceptual model provides useful information to characterize and communicate the potential health risks related to exposure to cyanotoxins in drinking water. The sources of cyanotoxins in drinking water, the route of exposure for biological receptors of concern (e.g., via various human activities such as drinking, food preparation and consumption) and the potential assessment endpoints (i.e., effects such as kidney and liver toxicity, and reproductive and developmental effects) due to exposure to cylindrospermopsin are depicted in the conceptual diagram below (Figure 2-3).

Figure 2-3. Conceptual Model of Exposure Pathways to Cylindrospermopsin in Drinking Water



2.6.1 Conceptual Model Diagram

Cyanobacteria are a common part of freshwater and marine ecosystems. An increase in water column stability, high water temperatures, elevated concentrations of nutrients and low light intensity have been associated with an increase and/or dominance of cylindrospermopsin-producing cyanobacteria in surface waters (or aquatic ecosystems). The presence of detectable concentrations of cyanotoxins in the environment is closely associated with these blooms. Winds and water currents can potentially transport cyanobacterial blooms to areas within the proximity of water intakes for drinking water treatment plants. If not managed in source waters, or removed during drinking water treatment, cyanobacteria and cyanotoxins may result in exposure that could potentially affect human health.

2.6.2 Factors Considered in the Conceptual Model for Cylindrospermopsin

Stressors: For this HA, the stressor is cylindrospermopsin concentrations in finished drinking water.

Sources: Sources of cylindrospermopsin include potential sources of drinking water such as rivers, reservoirs and lakes in the U.S. where blooms producing cylindrospermopsin occur. Shallow private wells under the direct influence of surface water (in hydraulic connection to a surface water body) can also be impacted by cylindrospermopsin-producing blooms if the toxins are drawn into the well along with the water from the surface water. There is substantially less information on exposure from this source.

Routes of exposure: Exposure to cyanotoxins from contaminated drinking water sources may occur via oral exposure (drinking water, cooking with water, and incidental ingesting from showering); dermal exposure (contact of exposed parts of the body with water containing toxins during bathing or showering, washing dishes or outside activities); inhalation exposure (during bathing or showering); or intravenous exposure (e.g., via dialysis). Toxicity data are available for the oral route of exposure from drinking water, but are not available to quantify dose response for other exposure routes (inhalation, dermal, dietary and intravenous exposures).

Receptors: The general population (adults and children) could be exposed to cyanotoxins through dermal contact, inhalation and/or ingestion. Infants and pre-school age children can be at greater risk to cylindrospermopsin because they consume more water per unit body weight than adults. Other individuals of potential sensitivity include persons with kidney and/or liver disease due to the compromised detoxification mechanisms in the liver and impaired excretory mechanisms in the kidney. There are no human data to quantify risk to pregnant woman or to evaluate the transfer of cyanotoxins across the placenta. Data are also not available on the transfer of cyanotoxins through the milk from nursing mothers or on the risk to the elderly. Given this lack of information, pregnant women, nursing mothers, and the elderly may also be potentially sensitive populations. Data from the episode in a dialysis clinic in Caruaru, Brazil where microcystins, and possibly cylindrospermopsin, were not removed by treatment of dialysis water (Carmichael et al., 2011), identify dialysis patients as a population of potential concern in cases where the drinking water source for the clinic is contaminated with cyanotoxins. EPA has

data to quantify risk to infants, children, and adults based on variability in potential exposure (body weight and drinking water intake rate). However, data are not available to quantify risk to pregnant woman, nursing mothers, persons with liver or kidney disease, or dialysis patients. Data are not available to derive a one-day HA for children because studies with single oral dosing do not provide dose-response information. A lifetime HA for cylindrospermopsin is not recommended as the types of exposures being considered are short-term and episodic in nature. Although the majority of the cyanobacterial blooms in the U.S. occur seasonally, usually during late summer, some toxin-producing strains can occur early in the season and can last for days or weeks.

Endpoints: Human data on oral toxicity of cylindrospermopsin are limited, but have shown effects on the liver following potential exposure to cylindrospermopsin. Acute, short-term and subchronic studies in animals show effects on the liver, RBC and kidney. In addition, some studies suggest that cylindrospermopsin may lead to reproductive and developmental effects; however, these data are limited. *In vitro* mutagenic and genotoxic cell assays with cylindrospermopsin have shown varied results with some indications of potential damage to DNA. However, these data are limited, and there has been no long term bioassay of purified cylindrospermopsin. Thus, available data are inadequate to assess the carcinogenic potential of cylindrospermopsin at this time. Available toxicity data are described in the *Health Effects Support Document (HESD) for Cylindrospermopsin* (U.S. EPA, 2015a). Kidney effects were selected as the endpoint on which to base the measure of effect. Liver and hematological effects were not as sensitive as the reported kidney effects.

2.7 Analysis Plan

The *Health Effects Support Document (HESD) for Cylindrospermopsin* (U.S. EPA, 2015a) provides the health effects basis for development of the HA, including the science-based decisions providing the basis for estimating the point of departure. To develop the HESD for cylindrospermopsin, 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 cylindrospermopsin was included. The literature search included the following terms: cylindrospermopsin, 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 considered information from the following risk assessments during the development of the cylindrospermopsin health risk assessment:

- 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*

- ILS (2000) *Cylindrospermopsin [CASRN 143545-90-8] Review of Toxicological Literature*

The toxicity data available for an individual pollutant vary significantly. An evaluation of available data was performed by EPA to determine data acceptability. The following study quality considerations from U.S. EPA's (2002) *A Review of the Reference Dose and Reference Concentration Processes* were used in selection of the studies for inclusion in the HESD and development of the HA.

- Clearly defined and stated hypothesis.
- Adequate description of the study protocol, methods and statistical analyses.
- Evaluation of appropriate endpoints. Toxicity depends on the amount, duration, timing and pattern of exposure, and may range from frank effects (e.g., mortality) to more subtle biochemical, physiological, pathological or functional changes in multiple organs and tissues.
- Application of the appropriate statistical procedures to determine an effect.
- Establishment of dose-response relationship (i.e., no observed adverse effect level (NOAEL) and/or lowest observed adverse effect level (LOAEL) or data amenable to modeling of the dose-response in order to identify a point of departure for a change in the effect considered to be adverse (out of the range of normal biological viability). The NOAEL is the highest exposure level at which there are no biologically significant increases in the frequency or severity of adverse effect between the exposed population and its appropriate control. The LOAEL is the lowest exposure level at which there are biologically significant increases in frequency or severity of adverse effects between the exposed population and its appropriate control group.

After the available studies were evaluated for inclusion in the HESD and HA, the critical study was selected based on consideration of factors including exposure duration (comparable to the duration of the HA being derived), route of exposure (oral exposure via drinking water, gavage, or diet is preferred), species sensitivity, comparison of the point of departure with other available studies demonstrating an effect, and confidence in the study (U.S. EPA, 1999). Once, a point of departure is chosen for quantification, uncertainty factors appropriate for the study selected are then applied to the point of departure to account for variability and uncertainty in the available data.

For cylindrospermopsin, toxicity and exposure data are available to develop a Ten-day HA. EPA used measures of effect and estimates of exposure to derive the Ten-day HAs using the following equation:

$$\text{HA} = \frac{\text{NOAEL or LOAEL or BMDL}}{\text{UF} \times \text{DWI/BW}}$$

Where:

NOAEL or LOAEL = No- or Lowest-Observed-Adverse-Effect Level (mg/kg bw/day) from a study of an appropriate duration (up to 7 days and 7-30 days for the One-day and Ten-day HAs, respectively).

BMDL = When the data available are adequate, benchmark dose (BMD) modeling can be performed to determine the point of departure for the calculation of HAs. The benchmark dose (BMD) approach involves dose-response modeling to obtain dose levels corresponding to a specific response level near the low end of the observable range of the data (U.S.EPA, 2012). The lower 95% confidence limit is termed the benchmark dose level (BMDL).

UF = Uncertainty factors (UF) account for: (1) intraspecies variability (variation in susceptibility across individuals); (2) interspecies variability (uncertainty in extrapolating animal data to humans; (3) uncertainty in extrapolating from a LOAEL to a NOAEL; and (4) uncertainty associated with extrapolation when the database is incomplete. These are described in U.S. EPA, 1999 and U.S. EPA, 2002.

DWI/BW = For children, a normalized ratio of drinking water ingestion to body weight (DWI/BW) was calculated using data for infants (birth to <12 months). The estimated drinking water intake body weight ratio (L/kg/day) used for birth to < 12 months of age are the 90th percentile values of the consumers only estimates of direct and indirect water ingestion based on 1994-1996, 1998 CSFII (Continuing Survey of Food Intakes by Individuals) (community water, mL/kg/day) in Table 3-19 in the U.S. EPA (2011a) Exposure Factors Handbook. The time weighted average of DWI/BW ratios values was derived from multiplication of age-specific DWI/BW ratios (birth to <1 month, 1 to < 3 months, 3 to < 6 months, and 6 to <12 months) by the age-specific fraction of infant exposures for these time periods.

For adults (>21 years of age), EPA updated the default BW assumption to 80 kg based on National Health and Nutrition Examination Survey (NHANES) data from 1999 to 2006 as reported in Table 8.1 of EPA's Exposure Factors Handbook (U.S. EPA, 2011a). The updated BW represents the mean weight for adults ages 21 and older.

EPA updated the default DWI to 2.5 L/d, rounded from 2.546 L/d, based on NHANES data from 2003 to 2006 as reported in EPA's Exposure Factors Handbook (U.S. EPA 2011a, Table 3-33). This rate represents the consumer's only estimate of combined direct and indirect community water ingestion at the 90th percentile for adults ages 21 and older.

3.0 HEALTH EFFECTS ASSESSMENT

The health effects assessment provides the characterization of adverse effects and includes the hazard identification and dose-response assessment. The hazard identification includes consideration of available information on toxicokinetics; identification, synthesis and evaluation of studies describing the health effects of cylindrospermopsin; and the potential Mode of Action (MOAs), or toxicity pathways related to the health effects identified.

3.1 Dose-Response

3.1.1 Critical Study Selected

The critical study selected for the derivation of the reference dose (RfD) for cylindrospermopsin is Humpage and Falconer (2002, 2003). Humpage and Falconer (2002, 2003) is a comprehensive toxicity study in which male mice were exposed by gavage to purified cylindrospermopsin from cell extract for 11 weeks. The study authors used four dose groups, adequate numbers of animals per dose group (10) and evaluated a variety of endpoints. Statistically significant, dose-related effects on the kidney, liver and serum chemistry were observed. The kidney was the most sensitive target of toxicity. The Humpage and Falconer (2002) data are supported by the short-term Reisner et al. (2004) results showing exposure-duration-related increased kidney weights, liver weights and testes weights, and hematological effects (acanthocytes or abnormal red blood cells (RBCs) and changes in hematocrit) following a 21-day exposure.

Purified cylindrospermopsin in water was administered by gavage in doses of 0, 30, 60, 120 or 240 μg/kg/day to groups of male Swiss albino mice (6 to 10 mice per dose group) for 11 weeks (Humpage and Falconer, 2002, 2003). The cylindrospermopsin was from an extract of freeze-dried *C. raciborskii* cells Woloszynska (AWT 205) purified using sephadex size-exclusion gel (G-10). The individual sephadex fractions were assayed using high-performance liquid chromatography (HPLC) and concentrated to a sample that was 47% cylindrospermopsin by dry weight and 53% phenylalanine. Food and water consumption, and body weight were examined throughout the study. After 9 weeks of exposure, the study authors report conducting a clinical examination to detect physiological and behavioral signs of toxicity but do not specify the parameters evaluated. Hematology evaluations (4 to 5 per dose group, except the high dose), serum chemistry (4 to 6 per dose group), and urinalysis (6 or 10 per dose group) were conducted. All the evaluations were conducted either near or at the end of the treatment period.

Postmortem examinations were done on the following organs: liver, spleen, kidneys, adrenal glands, heart, testes, epididymis and brain, including measurement of organ weights. Comprehensive histological evaluations were conducted in accordance with the recommendations from the Organization for Economic Cooperation and Development (OECD).

No deaths or visual clinical signs of toxicity were reported in mice exposed to purified cylindrospermopsin under the study conditions. The mean final body weight was 7-15% higher in all dose groups compared to controls, but was not dose-related and was only statistically significant at 30 and 60 µg/kg/day (Humpage and Falconer, 2003). No significant changes were observed in food consumption. In all dose groups, the water intake was significantly reduced; water consumption was 53% of the control level at 30 µg/kg/day and the higher dose groups were 68-72% of the control levels.

Relative kidney weight was significantly increased in a dose-related manner at ≥60 µg/kg/day (12-23% greater than controls; see Table 3-1). Relative liver weight was significantly increased (13% greater than controls) only at the highest dose (240 µg/kg/day). Relative spleen, adrenal and testes weights were increased for doses ≥60 µg/kg/day, but the differences from control were not statistically significant (Humpage and Falconer, 2002).

Selected serum chemistry (n= 4-6), hematology (n=4-5) and urinalysis (n=6-10) results are shown in Table 3-2. The hematology and serum chemistry evaluations showed no dose-related, statistically significant changes, although serum albumin, total bilirubin and cholesterol were increased compared to controls at all doses (Humpage and Falconer, 2002). The increases in cholesterol were significant for the 30 and 60 µg/kg/day groups, but not at the higher doses. The serum urea concentration was slightly decreased at the two highest doses. A nonsignificant increase in red cell polychromasia (high number of RBCs) was indicated for all doses, but quantitative data were not presented. Packed red cell volume was slightly increased and mean corpuscular hemoglobin was slightly decreased (Table 3-2) when compared to controls, although the changes were not dose related. When combined with the bilirubin results and the increased relative spleen weight, the hematological data suggest the possibility of minor RBC effects. One of the limitations in the serum chemistry and hematology data is the small number of samples evaluated, a factor that impacts the determination of statistical significance (Humpage and Falconer, 2002).

There was a significant decrease in the urine protein-creatinine ratio (g/mmol creatinine) at 120 and 240 µg/kg/day compared to the controls (51% and 37% of controls, respectively; both $p<0.001$) (Humpage and Falconer, 2002). Also, a significant decrease in urine specific gravity normalized for creatinine was seen at 240 µg/kg/day compared to the control ($p<0.001$). The renal glomerular filtration rate (GFR) was decreased compared to controls at all doses, but the differences were not dose dependent or statistically significantly different from controls. The renal failure index[1] was decreased slightly at ≥120 µg/kg/day; the differences from control were not statistically significant (Humpage and Falconer, 2002). Tubular retention of low molecular weight urinary proteins could account for the decreased urinary protein and possibly the increased kidney weight. Although effects on kidney weight and urine protein levels were observed in male mice, the biological relevance of the latter effect and whether it would also occur in female mice needs further investigation. Mice are known to excrete a group of functional, highly-polymorphic, low-molecular-weight urinary proteins that play important roles in social recognition and mate assessment (Cheetham et al., 2009). The relevance of the urinary protein findings in mice to humans is unknown.

[1] Renal failure index= (urinary sodium concentration × plasma creatinine concentration) / urinary creatinine concentration

Table 3-1. Kidney Weight Data from Oral Toxicity Study of Cylindrospermopsin Administered Daily over Eleven Weeks (Humpage and Falconer, 2002, 2003)

Dose (µg/kg/day)	Number	Relative Kidney Weight		% Difference	Significance
		Control g/100g $_{BW}$	Exposed g/100g $_{BW}$		
0 (Control)	10	1.48	-	-	-
30	10	1.48	1.57	+6	Not significant
60	9	1.48	1.66	+12	p <0.001
120	9	1.48	1.82	+23	p <0.001
240	6	1.48	1.78	+20	P <0.001

Table 3-2. Selected Clinical Chemistry, Hematology and Urinalysis Findings (Humpage and Falconer, 2002, 2003)

Endpoint	N	Dose (µg/kg/day)				
		0	30	60	120	240
Clinical Chemistry						
Urea (mmol/L)	4-6	9.24	9.22	8.55	7.51	7.92
Albumin (g/L)	4-6	23.8	26.6	26.0	26.0	25.8
Cholesterol (mmol/L)	4-6	3.26	4.60**	4.65**	3.68	4.08
Bilirubin (mmol/L)	4-6	2.62	2.72	2.88	3.06	3.07
Hematology						
Packed Cell volume (L/L)	4-5	0.38	0.39	0.39	0.39	ND
Mean Corpuscular Hemoglobin (MCH, pg/L)	4-5	16.8	15.7	16.4	16.4	ND
Urinalysis						
Volume (mL)	6-10	9.85	11.18	10.38	11.74	6.74
Creatinine (mmol/L)	6-10	0.57	0.49	0.54	0.51	0.72**
Specific gravity/creatinine	6-10	1.79	2.04	1.91	1.99	1.44*
Protein/creatinine (g/mmol)	6-10	4.3	3.6	3.3	2.2**	1.6**
Renal Failure Index (mmol/L)	4-6	4.3	4.3	4.5	3.6	3.6

ND = not determined
Significantly different from control: *p<0.05; **p<0.01.

Serum albumin and total serum protein were not decreased in the Humpage and Falconer studies (2002, 2003). The most sensitive effects observed by Humpage and Falconer (2002, 2003) were dose-related decreases in the urinary protein: creatinine ratio at ≥120 µg/kg/day and increased relative kidney weight at ≥60 µg/kg/day. The noted decrease in urinary protein excretion could reflect an impact on excretion of mouse urinary proteins given the fact that total serum protein was not significantly increased compared to controls for all dose groups. Mouse urinary proteins are synthesized in the liver (Clissold and Bishop, 1982) and transported to the kidney for excretion. If cylindrospermopsin did reduce liver protein synthesis, a decrease in total serum protein would be expected. However, this was not the case, suggesting a lack of an effect on synthesis of the urinary proteins in the liver.

The Humpage and Falconer (2002, 2003) postmortem tissue examinations showed histopathological damage to the liver based on scores assigned for necrosis, inflammatory foci and bile duct changes at ≥120 µg/kg/day. The percent of animals with liver lesions in the 120 and 240 µg/kg/day dose groups was 60% and 90%, respectively, when compared to 10%, 10% and 20% for the 0, 30, and 60 µg/kg/day dose groups, respectively. Severity scores were not given, and the liver lesions were not further described. There was proximal renal tubular damage in kidney sections from two mice in the 240 µg/kg/day dose group (Humpage and Falconer, 2002, 2003).

The 11-week study by Humpage and Falconer (2002, 2003) provides a NOAEL (30 µg/kg/day) and a LOAEL (60 µg/kg/day) for dose-related, statistically significant increases in kidney weights along with indicators of reduced renal function effects at higher doses. Because of the similarity in the type of effects observed and the LOAELs from the Humpage and Falconer (2002, 2003) and Reisner et al. (2004) studies, the selection of the NOAEL from Humpage and Falconer was determined to be the most appropriate point of departure for ten-day exposures in infants, children and adults despite its longer exposure duration.

3.1.2 Endpoint Selection

Upon considering all effects observed by Humpage and Falconer (2002, 2003), increased relative kidney weight was considered the most appropriate basis for quantitation. Adverse effects on the kidneys were manifested by decreases in urinary protein concentration and increased relative kidney weight. The study authors reported significantly increased relative kidney weight at ≥60 µg/kg/day, decreased urinary protein and liver lesions at ≥120 µg/kg/day and renal tubular lesions at 240 µg/kg/day (Humpage and Falconer, 2002, 2003). Relative kidney weight increased significantly in a dose-related manner beginning at 60 µg/kg/day (12-23% greater than controls), and relative liver weight was significantly increased at 120 µg/kg/day (12-23% greater than controls) and at the high dose of 240 µg/kg/day (13% greater than controls). Relative spleen, adrenal and testes weights were increased for doses ≥60 µg/kg/day, but the differences from control, although dose-related, were not statistically significant. Humpage and Falconer (2002, 2003) identified the LOAEL as 60 µg/kg/day and the NOAEL as 30 µg/kg/day based on the dose-related and statistically significant increase in relative kidney weight. These adverse effects are potential indicators of suppressed hepatic protein synthesis that was not

reflected in the measurement of total serum protein and/or increased retention of low molecular weight mouse urinary proteins by the kidney because of damage to the renal tubules.

In the single dose drinking water study by Reisner et al. (2004), hematological effects (acanthocytes, increased hematocrit) and increased organ weights (liver, testicular and kidney) in young (4 week) male Institute for Cancer Research (ICR) mice were observed following a three week exposure to purified cylindrospermopsin. The 66 µg/kg/day LOAEL is comparable to that from the Humpage and Falconer (2002, 2003) study (60µg/kg/day). Humpage and Falconer (2002, 2003) evaluated 5 different doses using 6 to 10 mice per dose group; Reisner et al. (2004) used one dose with 8 male mice. Reisner et al. (2004) demonstrated effects in comparable parameters to those impacted in Humpage and Falconer at a dose of 66 µg/kg/day with a three week exposure. They also demonstrated a trend for effects on kidney weight and hematocrit across the three-week duration of exposure. Because the renal effects reported in Humpage and Falconer (2002, 2003) did not occur at 11 weeks for the 30 µg/kg dose, the point of departure from the Humpage and Falconer study was determined to be the most appropriate for the quantitative assessment. Thus, the quantification from the Humpage and Falconer NOAEL based on kidney weight changes provides the best point of departure for ten-day exposures in children and adults despite its longer exposure duration.

3.2 Ten-Day Health Advisory

The Ten-day HA is considered protective of non-carcinogenic adverse health effects over a ten-day exposure to cylindrospermopsin in drinking water.

3.2.1 Bottle-fed Infants and Young Children of Pre-school Age

The Ten-day HA for bottle-fed infants and young children of pre-school age is calculated as follows:

$$\text{Ten-day HA} = \frac{30\,\mu\text{g/kg/day}}{300 \times 0.15\,\text{L/kg/day}} = 0.7\,\mu\text{g/L}$$

Where:

30 µg/kg/day	=	The NOAEL for kidney effects in mice exposed to cylindrospermopsin in water for 11 weeks (Humpage and Falconer, 2002, 2003).
300	=	The composite uncertainty factor (UF) including a 10 for intraspecies variability (UF_H), a 10 for interspecies differences (UF_A), and a 3 for uncertainties in the database (UF_D).
0.15 L/kg/day	=	Normalized drinking water intakes per unit body weight over the first year of life based on the 90th percentile of drinking water consumption and the mean body weight (U.S. EPA, 2011a).

The Ten-day HA of 0.7 µg/L is considered protective of non-carcinogenic adverse health effects for bottle-fed infants and young children of pre-school age over a ten-day exposure to cylindrospermopsin in drinking water.

3.2.2 School-age Children through Adults

The Ten-day HA for school-age children through adults is calculated as follows:

$$\text{Ten-day HA} = \frac{30\,\mu\text{g/kg/day}}{300 \times 0.03\,\text{L/kg/day}} = 3\,\mu\text{g/L}$$

Where:

30 µg/kg/day	=	The NOAEL for kidney effects in mice exposed to cylindrospermopsin in water for 11 weeks (Humpage and Falconer, 2002, 2003).
300	=	The composite UF including a 10 for intraspecies variability (UF_H), a 10 for interspecies differences (UF_A), and a 3 for uncertainties in the database (UF_D).
0.03 L/kg/day	=	Drinking water intake per unit body weight based on adult default values of 2.5 L/day and 80 kg (U.S. EPA, 2011a).

The Ten-day HA of 3 µg/L is considered protective of non-carcinogenic adverse health effects for children of school age through adults over a ten-day exposure to cylindrospermopsin in drinking water.

3.2.3 Uncertainty Factor Application

- 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. Individuals with a low RBC count as a result of genetic or nutritional factors could be more sensitive to cylindrospermopsin exposures than the general population. Individuals with pre-existing kidney/liver problems may also be more sensitive. Pregnant woman, nursing mothers, and the elderly could also be sensitive to cylindrospermopsin exposures.

- UF_A - 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 cylindrospermopsin. Allometric scaling is not applied in the development of the Ten-Day HA values for cylindrospermopsin. The allometric scaling approach is derived from the relationship between body surface area and basal metabolic rate in adults (U.S. EPA, 2011b). This approach is not appropriate for infants and children due to the comparatively slower clearance during these ages and the limited toxicokinetic data available to assess the appropriateness of body weight scaling in early life.

- UF_D - An uncertainty factor of 3 ($10^{0.5} = 3.16$) is selected to account for deficiencies in the database for cylindrospermopsin. The database for cylindrospermopsin includes limited human studies. The database for studies in laboratory animals includes oral exposure acute, short-term and subchronic studies, but many of them lacked a comprehensive evaluation of a wide spectrum of effects. The database lacks chronic toxicity and multi-generation reproductive and developmental toxicity studies using the oral route of exposure. There is a lack of data on neurological and immunological endpoints. The RBC parameters evaluated differed between the Humpage and Falconer (2002, 2003) and Reisner et al. (2004) studies.

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., $10^{½}$) when appropriate (U.S. EPA, 2002).

4.0 RISK CHARACTERIZATION

The following topics describe important conclusions used in the derivation of the health advisory. This section characterizes each topic and its impact on the health advisory.

4.1 Studies Supporting Determination of Critical Study

Increases in kidney weight and hematological effects are detected in all three studies (Humpage and Falconer, 2002, 2003; Reisner et al., 2004; and Sukenik et al., 2006). However, the type of hematological effects varied among studies as did the statistical significance of the observed effects. Humpage and Falconer (2002, 2003) found signs indicative of hemolysis (increased bilirubin, spleen weight and polychromasia (high number of RBCs with low hemoglobin)), while Reisner et al. (2004) and Sukenik et al. (2006) found acanthocytes (abnormal RBCs). Increases in kidney weight were significant for Humpage and Falconer (2002, 2003) and Sukenik et al. (2006), but not statistically significant for Reisner et al. (2004). Humpage and Falconer (2002, 2003) and Reisner et al. (2004) used purified cylindrospermopsin, while Sukenik et al. (2006) used an extract in spent medium. Of these three studies, Humpage and Falconer (2002, 2003) provides a NOAEL (30 µg/kg/day) and a LOAEL (60 µg/kg/day) for dose-related statistically significant increases in kidney weights and indications of renal function effects at higher doses. Although the percent change in kidney weight is the same for Reisner et al. (2004), only the change observed by Humpage and Falconer (2002, 2003) was statistically significant.

4.2 Study Duration

The short-term studies with appropriate durations (typically 7 days up to 30 days) available for cylindrospermopsin (Shaw et al., 2001; Reisner et al., 2004), are not suitable for quantification, as described below. However, the Reisner study does support the use of the Humpage and Falconer (2002, 2003) study for the derivation of the Ten-day HA, despite the longer duration of the study.

The Shaw et al (2001) study reported the results from multiple experiments. These experiments each have limitations including use of extract, lack of adequate numbers of animals and monitored endpoints, and the limited number of doses tested that preclude their use in quantification. The oral data for purified extract from Shaw et al. (2001) identified fatty liver as an adverse effect in mice following a 14 day gavage exposure to 0.05 mg/kg/day. However, the only effects mentioned in the published paper are the liver effects and an absence of lymphophagocytosis in the spleen.

Reisner et al. (2004) conducted a 21 day study in mice and showed significant increases in hematocrit, acanthocytes (abnormal RBCs), and liver and testes weights effects at a 66 µg/kg/day dose and a duration-related nonsignificant increase in and kidney weight. This study was not selected for development of the Ten-day HA because this study used a single dose; however, the effects to that dose after 3-weeks were comparable to the effects seen in the

Humpage and Falconer (2002, 2003) study at a slightly lower 60 mg/kg/day dose after 11 weeks. The Humpage and Falconer (2002, 2003) study was determined to be the most appropriate for the quantitative assessment because the LOAEL at 11 weeks would be protective for the effects seen at 3-weeks in the shorter duration study.

4.3 Allometric Scaling Approach

Allometric scaling was not applied in the development of the RfD for cylindrospermopsin. In the development of short-term advisory values (One-day and Ten-day), parameters are used that reflect exposures and effects for infants up to one year of age, rather than for adults. The body weight scaling approach is derived from the relationship between body surface area and basal metabolic rate in adults. Infants/children surface area and basal metabolic rates are very different than adults with a slower metabolic rate. In addition, limited toxicokinetic data are available to assess the appropriateness of body weight scaling in early life. The body weight scaling procedure has typically been applied in the derivation of chronic oral RfDs and cancer assessments, both of which are concerned with lifetime repeated exposure scenarios (U.S. EPA, 2012). Thus, given the development of a Ten-Day HA value, and the application of the Ten-Day HA to infants and pre-school age children, the application of the body weight scaling procedure is not appropriate for this scenario.

In addition, for short-term advisories (one-day and ten-day duration), EPA assumes all exposure is derived from drinking water and, therefore, no Relative Source Contribution (RSC) term is applied. For lifetime health advisory values, EPA does include an RSC that reduces the advisory value to account for other potential sources.

4.4 Uncertainty and Variability

Uncertainty factors were applied in several areas to adjust for incomplete information. Human data on the toxic effects of cylindrospermopsin are limited. Quantification for the absorption, distribution and elimination of cylindrospermopsin in humans following oral, inhalation or dermal exposure is not well understood. The clinical significance in humans for biological changes observed in experimental animals such as increased kidney weight, decreased urinary protein levels, decrease in renal failure index and the formation of acanthocytes (abnormal RBCs) is not known. In animal studies with cylindrospermopsin, adverse effects (RBC effects) observed have not been fully characterized. No data are available to quantify the differences between humans and animals for the critical health endpoints. There is uncertainty regarding susceptibility and variability characterized in the human population following exposure to cylindrospermopsin. Additional information is needed on the potential health risks from mixtures of cylindrospermopsin with other cyanotoxins, bioactive molecules with an effect on living organisms and chemical stressors present in ambient water and/or drinking water supplies. The critical study was conducted only in male mice and therefore, any gender-specific effects of cylindrospermopsin are not understood.

4.5 Susceptibility

Available animal data are not sufficient to determine if there is a definitive difference in the response of males versus females following oral exposure to cylindrospermopsin. Based on the results from animal studies, individuals with liver and/or kidney disease might be more susceptible than the general population because of compromised detoxification mechanisms in the liver and impaired excretory mechanisms in the kidney. Data from an episode in a dialysis clinic in Caruaru, Brazil, where microcystins (and possibly cylindrospermopsin) were not removed by treatment of dialysis water, identify dialysis patients as a population of potential concern in cases where the drinking water source used by a clinic for hemodialysis is contaminated with cyanotoxins.

The data on RBC acanthocytes suggest that individuals that suffer from anemia (e.g., hemolytic or iron-deficiency) might be a potentially sensitive population. Several rare genetic defects such as abetalipoproteinemia (rare autosomal recessive disorder that interferes with the normal absorption of fat and fat-soluble vitamins from food) and hypobetalipoproteinemia are associated with abnormal RBC acanthocytes, which appears to result from a defect in expression of hepatic apoprotein B-100, a component of serum low density lipoprotein complexes (Kane and Havel, 1989). Individuals with either condition might be sensitive to exposure to cylindrospermopsin.

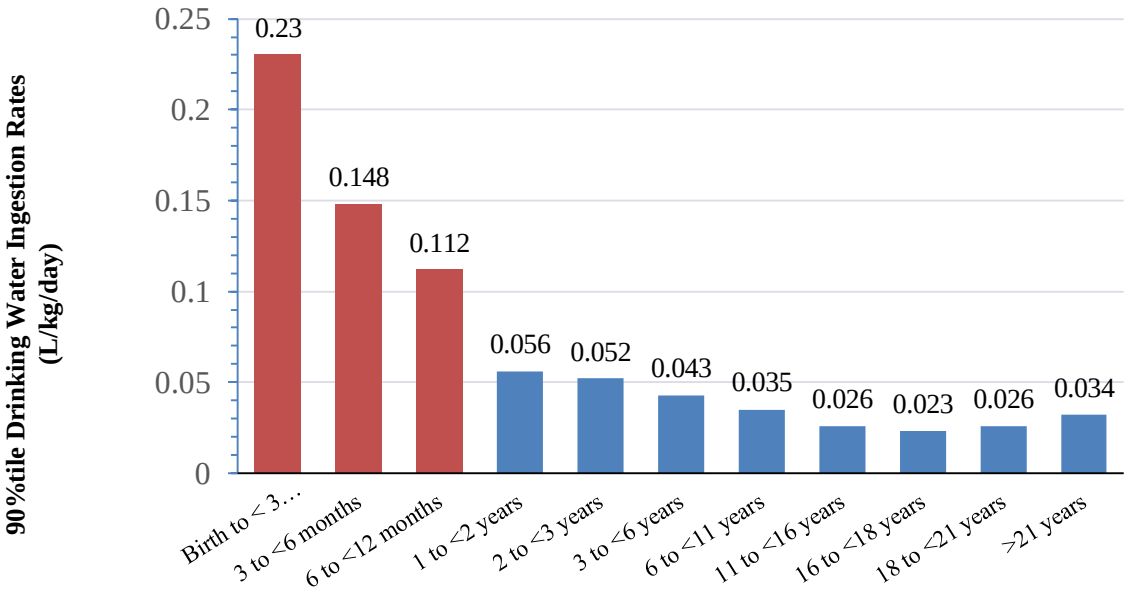
Based on the currently available science, evidence is lacking to assess differences in susceptibility between infants, children and adults. There are, however, significant differences in exposure between these life-stages that impact risk.

4.6 Distribution of Body Weight and Drinking Water Intake by Age

Both body weight and drinking water intake distributions vary with age. EPA has developed two health advisory values, a Ten-day HA of 0.7 µg/L based on exposure to infants over the first year of life, and a Ten-day HA of 3 µg/L based on exposure to adults, over 21 years of age. Section 4.7 discusses how EPA recommends application of these values to other age groups.

The U.S. EPA (2011a) Exposure Factors Handbook provides values for drinking water ingestion rate and corresponding body weight. The estimated 90th percentile of community water ingestion for the general population (males and females of all ages) has been used as the default value for water ingestion. EPA plotted the 90th percentile of drinking water intake using Table 3-19 for ages ≤ 3 years, and Table 3-38 for ages >3 years due to sample size in the respective studies. Age groups < 3 months in Table 3-19 were combined due to insufficient sample sizes. Figure 4.1 represents the 90th percentile drinking water ingestion rates (L/kg/day) for each age group (located on top of the columns). Bottle-fed ages are shown in red (first three columns on the left).

Figure 4-1. 90th Percentile Drinking Water Ingestion Rates by Age Group



Adapted from U.S. EPA 2011 Exposure Factors Handbook (U.S.EPA, 2011a).

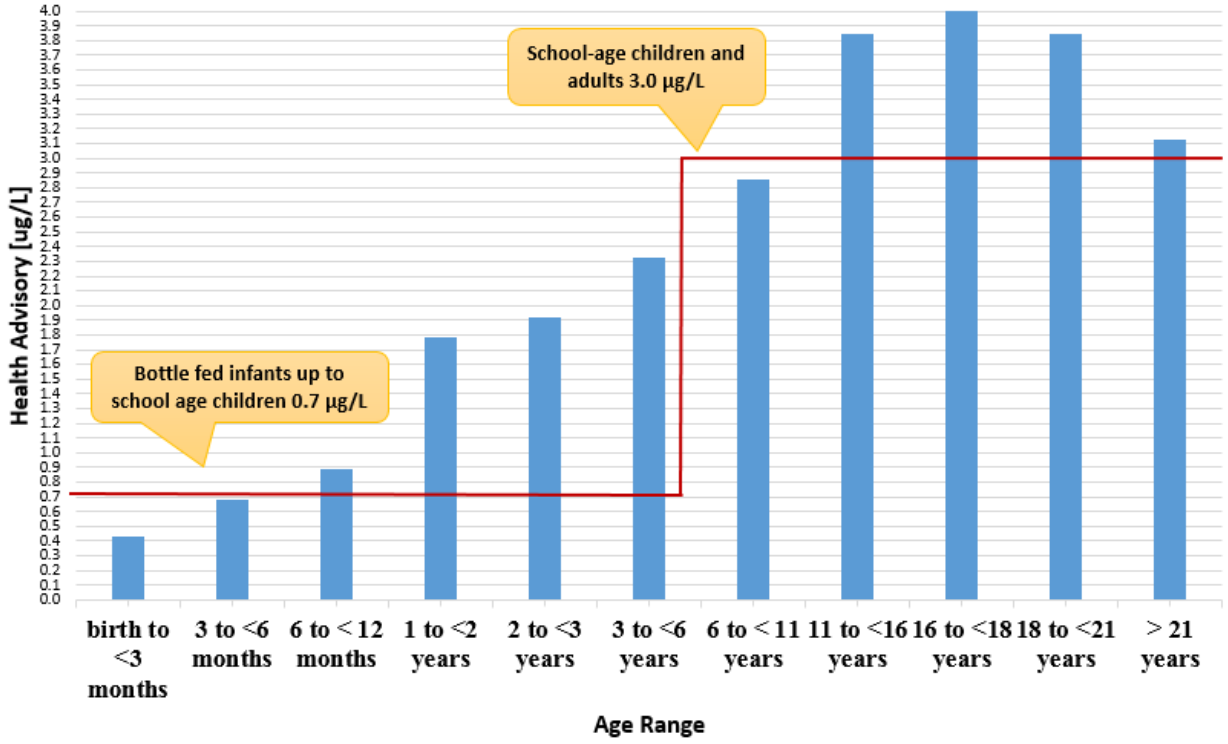
Based on the drinking water intake rates for children <12 months (0.15 L/kg-day), the exposure of children is over 4 times higher than that of adults >21 years old on a body weight basis (0.034 L/kg-day). Infants from birth to 3 months may be exclusively bottle-fed and therefore, have a higher ingestion rate. After 3 months of age, typically around 4 to 6 months of age, other food and liquids are introduced into the infant diet, lowering the ingestion rate of drinking water. Drinking water contributes the highest risk of the total cyanotoxins intake for infants to one-year-olds fed exclusively with powdered formula prepared with tap water containing cyanotoxins. At the age of 6, children's intake of drinking water relative to their body weight is approximately the same as those of an adult (>21 years). Data evaluating the transfer of cylindrospermopsin through breast milk are not available for humans.

4.7 Distribution of Potential Health Advisory Values by Age

Using the ingestion rates for each age group (from Figure 4-1), EPA estimated Ten-day HA values for cylindrospermopsin for each age group (plotted in Figure 4-2) to demonstrate the variability due to body weight and drinking water intake by age.

EPA decided to apply the Ten-day HA value calculated for infants over the first year of life (0.7 µg/L) to all bottle-fed infants and young children of pre-school age because these age groups have higher intake per body weight relative to adults. As Figure 4-2 demonstrates, when the Ten-day HA is estimated by age group, the calculated HA value for infants from birth to 3

Figure 4-2. Ten-day Health Advisories for Cylindrospermopsin by Age Group



months old is 0.4 µg/L, slightly below the infant health advisory value of 0.7 µg/L. EPA believes that infants from birth to 3 months old are not at a disproportionate risk at a 0.7 µg/L advisory value because a safety factor of 30 is built into this calculation to account for human variability and deficiencies in the database. The estimated Ten-Day HA values for infants from 3 months old through pre-school age groups (less than 6 years old), are at or above the advisory value of 0.7 µg/L. Therefore, children within these age groups are adequately protected by the advisory value for bottle-fed infants and young children of pre-school age. EPA decided to apply the adult Ten-Day HA value of 3 µg/L to school age children (children older than or equal to 6 years) through adulthood because children's intake of drinking water relative to body weight in this age group is almost the same as those of an adult (≥ 21 years).

5.0 ANALYTICAL METHODS

The primary methods used for the analysis of cylindrospermopsin are liquid chromatography (LC) and enzyme linked immunosorbent assay (ELISA). Several detection modes are generally coupled with LC including single channel ultraviolet (UV)/visible and multi-channel UV photodiode array (PDA), electrospray ionization mass spectrometry (LC-ESI/MS), and electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS). Due to the limited selectivity of UV-based detectors, the use of mass spectrometric detection is becoming more commonplace. Commercial ELISA test kits are also available for cylindrospermopsin detection. These kits are available in both semiquantitative and quantitative formats and are easily adapted to field or "screening" measurements.

EPA has recently released Method 545 (U.S. EPA, 2015c) which is a LC-ESI/MS/MS method for the determination of cylindrospermopsin and anatoxin-a in drinking water. This method requires the operation of the mass spectrometer in MS/MS mode to enhance selectivity. In this method, samples are preserved with ascorbic acid (dechlorinating agent) and sodium bisulfate (microbial inhibitor). In the laboratory, aliquots (1 mL) of sample are taken for analysis, and internal standards are added. An aliquot of the sample is injected into an LC equipped with an analytical column that is interfaced to the mass spectrometer. The analytes are separated and then identified by comparing the acquired mass spectra and retention times to reference spectra and retention times for calibration standards acquired under identical liquid chromatography tandem mass spectrometry (LC-MS/MS) conditions. The concentration of each analyte is determined using the integrated peak area and internal standard technique. A single laboratory lowest concentration method reporting limit (LCMRL) of 0.063 µg/L was determined for cylindrospermopsin along with an average value of 0.083 µg/L for all participants of a multi-lab evaluation (n=4) (Winslow et al., 2006). Method 545: Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI/MS/MS) is available at http://water.epa.gov/scitech/drinkingwater/labcert/analyticalmethods_ogwdw.cfm.

Other LC methods have generally used UV spectroscopic detection for cylindrospermopsin analysis. These methods have often incorporated solid phase extraction (SPE) to preconcentrate the target analyte, reduce matrix interferences or both. Quantitation limits have ranged from 4 µg/L to < 0.1 µg/L based on the instrumental setup and the use of preconcentration steps (Papageorgiou et al., 2012).

Commercial ELISA kits for the detection of cylindrospermopsin are available from several vendors. These kits claim a working concentration range of 0.05 and 2 µg/L.

6.0 TREATMENT TECHNOLOGIES

The information below is adapted from the draft Health Canada Guidelines for Cyanobacteria Toxins in Drinking Water, available later in 2015.

Detailed information on the operational considerations of a variety of treatment methods can be found in the *International Guidance Manual for the Management of Toxic Cyanobacteria* (GWRC, 2009) and *Management Strategies for Cyanobacteria (Blue-Green Algae): A Guide for Water Utilities* (Newcombe et al., 2010) available at: http://www.waterra.com.au/cyanobacteria-manual/PDF/GWRCGuidanceManualLevel1.pdf and http://www.researchgate.net/profile/Lionel_Ho/publication/242740698_Management_Strategies_for_Cyanobacteria_(Blue-Green_Algae)_A_Guide_for_Water_Utilities/links/02e7e52d62273e8f70000000.pdf.

For additional information on treatment strategies commonly used or being considered by water systems vulnerable to cyanotoxins, please see *Recommendations for Public Water Systems to Manage Cyanotoxins in Drinking Water* (U.S. EPA, 2015b).

6.1 Management and Mitigation of Cyanobacterial Blooms in Source Water

Algaecides can be applied to lakes and reservoirs to mitigate algal blooms, including cyanobacteria. In most cases, depending on the cyanobacteria species present, the application of algaecides has the potential to compromise cell integrity releasing cyanotoxins into the source waters. Chemical treatment to control blooms in drinking water sources in the early stages of the bloom when cyanobacterial concentrations are still relatively low (usually from 5,000 to 15,000 cells/mL) (WHO, 1999), are less likely to release significant cyanotoxin concentrations upon cell lysis and may mitigate or prevent a cyanobacterial bloom from proliferating as the season progresses. If a cyanobacteria bloom does occur, utilities may investigate alternative raw water sources, change intake locations or levels to withdraw raw water with minimal cyanotoxin concentrations, or investigate methods of destratification in the water source. Purchasing water from a neighboring interconnected water system that is unaffected by the bloom may also be an option for some systems.

Clays and commercial products such as aluminum sulfate (alum) have been used for the management of blooms in source waters. Alum treatment efficiency depends on the alum dose and the type of flocculant. Aeration and destratification have also been used to treat cyanobacterial blooms, usually in smaller water bodies (from one acre to several tens of acres). Active mixing devices, diffuse air bubblers, and other means of reducing stratification have proven to be effective in controlling outbreaks and persistence of blooms in relatively small shallow impoundments (around < 20 feet). These strategies can be applied to the entire source water body or to just a portion of the lake depending on the need, size and depth of the water body relative to the source water intake(s).

The use of ultrasonic sound waves, or sonication, to disrupt cyanobacterial cells has also been investigated as a potential source water treatment option (Rajasekhar et al., 2012). Drawbacks include that application frequencies are difficult to calculate and are system-specific;

and that applications on large scale require more powerful, and therefore, more expensive equipment. Sonication shows potential for use in cyanobacterial bloom management, but further study to determine effective operating procedures is needed before it can be considered as a feasible approach (Rajasekhar et al., 2012).

Excess nutrients are thought to be a primary driver of cyanobacterial blooms. Long-term prevention of cyanobacterial blooms likely requires reductions in nutrient pollution. Excess nitrogen and phosphorus in aquatic systems can stimulate blooms and create conditions under which harmful cyanobacteria thrive. Thus, managing nutrient pollution sources within a watershed in addition to waterbody-specific physical controls (in systems that are amenable to those controls) tends to be the most effective strategy. Nutrient pollution can be from urban, agricultural, and atmospheric sources, and therefore, reductions can be achieved through a variety of source control technologies and best management practices.

6.2 Drinking Water Treatment

Effective treatment of cyanotoxins in drinking water includes the evaluation and selection of appropriate treatment methods. The water treatment methods need to be tailored to the type(s) of cyanobacteria present, the site-specific water quality (e.g., pH, temperature, turbidity, presence of natural organic material (NOM)), the treatment processes already in place and multiple treatment goals (e.g., turbidity and total organic carbon (TOC) removal, disinfection requirements, control of disinfection by-products (DBP) formation). Utilities need to have an understanding of the type and concentration of cyanotoxins present in the source water and should conduct site-specific evaluations such as jar testings and piloting in order to determine the most effective treatment strategy. Potential target parameters include: chlorophyll-a, turbidity, cyanobacterial cells and extracellular and intracelluar toxins. Care should be taken to avoid cell lysis. A multi-barrier approach consists of conventional filtration for intracellular cylindrospermopsin removal and additional processes such as activated carbon, biodegradation, advanced oxidation, and small-pore membrane processes (e.g. nanofiltration and reverse osmosis), for the removal or oxidation of extracellular cylindrospermopsin. The most effective way to deal with cyanobacteria cells and their toxins, is to remove the cells intact, without damaging them, to prevent the release of additional extracellular toxins into the water.

When released from the cell, cylindrospermopsin can be found dissolved or attached to other materials such as particulate or soluble substances. Powdered activated carbon (PAC) has proven to be effective for removal of extracellular cylindrospermopsin. Limited information is available on the adsorption of cylindrospermopsin onto granular activated carbon (GAC).

6.2.1 Conventional Treatment for Cylindrospermopsin

In the absence of cell damage, conventional treatment employing coagulation, flocculation, clarification (sedimentation or dissolved air flotation) and rapid granular filtration can be effective at removing intact cells and the majority of intracellular toxins (cell bound) (Chow et al., 1998; Newcombe et al., 2015). However, if toxins are released into solution, a

combination of conventional treatment processes with oxidation, adsorption and/or advanced treatment needs to be considered to treat both intracellular and extracellular cyanotoxins. Rapid sand filtration without pre-treatment (i.e., direct filtration, without coagulation/clarification) is not effective for cyanobacterial cell removal.

Conventional water treatment (coagulation, flocculation, sedimentation or dissolved air flotation (DAF), and filtration) is considered effective for removal of intracellular toxins but ineffective for dissolved cyanotoxins such as cylindrospermopsin, which is partially dissolved in water under normal growth conditions (Chow et al., 1999; Rapala et al., 2006; Carrière et al., 2010). Application of a multiple barrier approach has the potential to be effective (Newcombe et al., 2015). Ho et al. (2008, 2011) conducted bench-scale studies and modeling on the use of PAC for the adsorption of cylindrospermopsin. The results demonstrated that a PAC dose of 25 mg/L and a contact time of 60 minutes would be required to reduce 5 µg/L of cylindrospermopsin to less than 1 µg/L. When concentrations of cylindrospermopsin are 1-2 µg/L or 3-4 µg/L, the recommended doses of PAC are 10-20 mg/L and 20-30 mg/L, respectively (Newcombe et al., 2010).

Dixon et al. (2011b) also conducted laboratory-scale testing of integrated membrane systems for cyanotoxin removal. The results showed that an ultrafiltration system with pre-treatment using 2.2 mg/L of alum and 20 mg/L of PAC resulted in 97% removal of intra- and extra-cellular cylindrospermopsin to achieve a treated water concentration of less than 0.1 µg/L (Dixon et al., 2011a). Nanofiltration and reverse osmosis would likely be effective in removing dissolved toxins, but only a few studies have been conducted. Dixon et al. (2011a) studied the removal of cyanobacterial toxins by nanofitration and found that average removals between 90-100% could be achieved for cylindrospermopsin using membranes with a low molecular weight cut-off (MWCO) (< 300 Daltons).

In practice, full-scale treatment plants use a combination of treatment technologies (i.e., conventional filtration and chemical oxidation) in order to remove both intracellular and extracellular cyanotoxins. Extracellular cylindrospermopsin may be removed by many treatment plants using existing treatments such as chlorination or by the addition of PAC (Carriere et al., 2010). Although it is possible to remove both intracellular and extracellular toxins effectively using a combination of treatment processes, the removal efficiency can vary considerably. Utilities need to ensure that they are using their existing treatment processes to their fullest capacity for removal of both cyanobacterial cells and extracellular toxins, and that the appropriate monitoring is being conducted to ensure that adequate removal is occurring at each step in the treatment process.

6.2.2 Chemical Oxidation

Chemical oxidation using chlorine or ozonation can be effective at oxidizing cylindrospermopsin, but can also cause the cells to lyse, resulting in an increase in concentrations of extracellular toxins in drinking water. By applying conventional filtration (or another filtration process) first to remove the majority of intact cells, the extracellular cylindrospermopsin is less likely to increase due to cell lysis when water is treated with oxidants. In cases where pre-

oxidation (oxidant applied anywhere along the treatment process prior the filter influent) is practiced, it may need to be discontinued during an algal bloom or adjustments to the oxidant type and doses may be needed to minimize cell rupture prior to filtration (Newcombe et al., 2015).

Different cyanotoxins react differently to oxidants depending on the individual characteristics of the source water such as TOC, temperature and pH (Westrick et al., 2010). While chlorination is an effective treatment for oxidizing cylindrospermopsin, its effectiveness is dependent on pH. Rodriguez et al. (2007) found that at a pH of 7 and an initial chlorine dose of 1 mg/L, oxidation of cylindrospermopsin is fast, with almost complete reaction after 30 minutes. Other chlorinated oxidants such as chloramines and chlorine dioxide have little impact on cylindrospermopsin due to a slow reaction rates. For example, the reaction of chlorine dioxide with cylindrospermopsin is relatively slow with a second-order rate constant of 0.9 $M^{-1}s^{-1}$ at pH 8. The rate constant is pH-dependent and decreases significantly under mildly acidic conditions. Chlorine dioxide may be used to inactivate *C. raciborskii*, however, in typical drinking water treatment applications, it does not appear to be practical for oxidizing cylindrospermopsin given its slow reaction rate (de la Cruz et al., 2013). Oxidation by potassium permanganate is temperature dependent and has not been shown to be effective in oxidizing cylindrospermopsin. Water treatment utilities that use chloramines or chlorine dioxide as disinfectants to reduce the formation of regulated disinfection by-products may want to reconsider oxidation efficacy for cyanotoxin inactivation during periods when algal toxins are present in source waters, while balancing these other treatment objectives. Ozone has been shown to effectively oxidize cylindrospermopsin in laboratory-scale studies (de la Cruz et al., 2013). At pH 8, approximately 95% of cylindrospermopsin (initial concentration of 415 µg/L) was oxidized using 0.38 mg/L O_3.

6.2.3 Ultraviolet Irradiation

Studies have indicated that ultraviolet (UV) irradiation may be effective for the oxidation of cylindrospermopsis cells (Westrick et al., 2010). However, exposure times and/or UV doses tested in the bench-scale experiments were greater than those typically applied in drinking water treatment.

6.3 Point-of-Use (POU) Drinking Water Treatment Units

Limited information is available on residential treatment units for the removal of cyanobacteria cells and cyanotoxins. At this time, no units have been evaluated for removal of cylindrospermopsin. Further studies need to be conducted to assess the efficacy of home filtration devices for various cyanotoxins, including cylindrospermopsin, and for other filtering conditions such as increased toxin load and the presence of other contaminants in drinking water. Third-party organizations are currently developing certification standards to test POU devices to evaluate how well they remove cyanotoxins from drinking water treatment units. Those standards are expected in the near future.

More information about treatment units and the contaminants they can remove can be found at http://www.nsf.org/Certified/DWTU/.

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ABBREVIATIONS AND ACRONYMS

Γgt	Γ-Glutamyltransferase
A	Alanine
Adda	3-Amino-9-Methoxy-2,-6,-8-,Trimethyl-10-Phenyldeca-4,-6-Dienoic Acid
ALT	Alanine Aminotransferase
ALP	Alkaline Phosphatase
AST	Aspartate Aminotransferase
AWWARF	American Water Works Association Research Foundation
BMD	Benchmark Dose
BMDL	Benchmark Dose Level
BW	Body Weight
Clo_2	Chlorine Dioxide
CAS	Chemical Abstracts Service
CASA	Computer-Assisted Sperm Analysis
CCL	Contaminant Candidate List
CWA	Clean Water Act
DBP	Disinfection By-Products
DL	Detection Limit
EBCT	Empty Bed Contact Time
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	U.S. Environmental Protection Agency
FSH	Follicle Stimulating Hormone
g	Gram
GC/MS	Gas Chromatograph/Mass Spectrometry
GAC	Granular Activated Carbon
GLERL	Great Lakes Environmental Research Laboratory
H_2O_2	Hydrogen Peroxide
HA	Health Advisory
HAB	Harmful Algal Bloom
HESD	Health Effects Support Document
HPLC	High-Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
i.p.	Intraperitoneal
Kg	Kilogram
L	Leucine
LC/MS	Liquid Chromatography/Mass Spectrometry
LDH	Lactate Dehydrogenase
LD_{50}	Lethal Dose to 50% of Organisms
LH	Luteinizing Hormone
LOAEL	Lowest-Observed-Adverse-Effect Level
MC-LA	Microcystin-LA
MC-LR	Microcystin-LR
MC-RR	Microcystin-RR
MC-YR	Microcystin-YR
MC-YM	Microcystin-YM

Mdha	Methyldehydroalanine
MERHAB-LGL	Monitoring and Event Response to Harmful Algal Blooms in the Lower Great Lakes
µg	Microgram
µm	Micromole
LOQ	Level of Quantification
Mdls	Method Detection Limit
mg	Milligram
ml	Milliliter
MMPB	2-methyl-3-methoxy-4-phenylbutyric acid
MOA	Mode of Action
MF	Microfiltration
MWCO	Molecular Weight Cut-Off
NDEA	N-Nitrosodiethylamine
NF	Nanofiltration
NLA	National Lakes Assessment
NOAA	National Oceanic and Atmospheric Administration
NOAEL	No-Observed-Adverse-Effect Level
NOD	Nodularin
NOM	Natural Organic Material
OATp	Organic Acid Transporter Polypeptides
PAC	Powdered Activated Carbon
PAS	Periodic Acid-Schiff
PBS	Phosphate-Buffered Saline
PDA	Photodiode Array Detector
P-GST	glutathione *S*-transferase placental form-positive
POD	Point of Departure
POU	Point-of-Use
PP2	Protein Phosphatase 2A
PP1	Protein Phosphatase 1
PPIA	Protein Phosphatase Inhibition Assays
RfD	Reference Dose
RO	Reverse Osmosis
ROS	Reactive Oxygen Species
SDWA	Safe Drinking Water Act
SPE	Solid-Phase Extraction
TEF	Toxicity Equivalency Factors
TOC	Total Organic Carbon
TOXLINE	Toxicology Literature Online
TUNEL	Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick End-Labeling Assay
UF	Uncertainty Factor
UF	Ultrafiltration
USGS	United States Geological Survey
UV	Ultraviolet
WHO	World Health Organization

EXECUTIVE SUMMARY

Microcystins are toxins produced by a number of cyanobacteria species, including members of *Microcystis*, *Anabaena*, *Nodularia*, *Nostoc*, *Oscillatoria*, *Fischerella*, *Planktothrix*, and *Gloeotrichia*. Approximately 100 microcystin congeners exist, which vary based on amino acid composition. Microcystin-LR is one of the most potent congeners and the majority of toxicological data on the effects of microcystins are available for this congener.

Many environmental factors such as the ratio of nitrogen to phosphorus, temperature, organic matter availability, light attenuation and pH play an important role in the development of microcystin blooms, both in fresh and marine water systems and could encourage toxin production. Microcystins are water soluble and tend to remain contained within the cyanobacterial cell (intracellular), until the cell breaks and they are released into the water (extracellular).

This Health Advisory (HA) for microcystins is focused on drinking water as the primary source of exposure. Exposure to cyanobacteria and their toxins may also occur by ingestion of toxin-contaminated food, including consumption of fish, and by inhalation and dermal contact during bathing or showering and during recreational activities in waterbodies with the toxins. While these types of exposures cannot be quantified at this time, they are assumed to contribute less to the total cyanotoxin exposures than ingestion of drinking water. Due to the seasonality of cyanobacterial blooms, exposures are not expected to be chronic.

Limited data in humans and animals demonstrate the absorption of microcystins from the intestinal tract and distribution to the liver, brain, and other tissues. Elimination from the body requires facilitated transport using receptors belonging to the Organic Acid Transporter polypeptide (OATp) family. Data for humans and other mammals suggest that the liver is a primary site for binding these proteins (i.e., increased liver weight in laboratory animals and increased 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 indicate that microcystins can be conjugated with glutathione and cysteine to increase their solubility and facilitate excretion.

The main source of human health effects data for microcystins is from acute recreational exposure to cyanobacterial blooms. Symptoms include headache, sore throat, vomiting and nausea, stomach pain, dry cough, diarrhea, blistering around the mouth, and pneumonia. However, human data on the oral toxicity of microcystins are limited and confounded by: potential co-exposure to other contaminants; a lack of quantitative information; and other confounding factors. Reports of human intravenous exposure to dialysate prepared with microcystin-contaminated water indicated acute liver failure and death in a large number of the exposed patients.

Studies in laboratory animals demonstrate liver, kidney, and reproductive effects following short-term and subchronic oral exposures to microcystin-LR. Studies evaluating the chronic toxicity of microcystins have not shown clinical signs of toxicity and are limited by study design and by the lack of quantitative data.

The U.S. Environmental Protection Agency (EPA) identified a study by Heinze (1999) conducted on rats as the critical study used in the derivation of the reference dose (RfD) for microcystins. The critical effects identified in the study are increased liver weight, slight to moderate liver lesions with hemorrhages, and increased enzyme levels as a result of exposure to microcystin-LR. The lowest-observed-adverse-effect level (LOAEL) was determined to be 50 µg/kg/day, based on these effects. The drinking water route of exposure matches potential drinking water exposure scenarios in humans. The total uncertainty factor (UF) applied to the LOAEL was 1000. This was based on a UF of 10 for intraspecies variability, a UF of 10 for interspecies variability, a UF of 3 ($10^{½}$) for extrapolation from a LOAEL to no-observed-adverse-effect level (NOAEL), and a UF of 3 ($10^{½}$) to account for deficiencies in the database. EPA is using microcystin-LR as a surrogate for other microcystin congeners. Therefore, the HA based on this critical study applies to total microcystins.

EPA is issuing a Ten-day HA for microcystins based on the Heinze (1999) short-term, 28-day study. Studies of a duration of 7 to 30 days are typically used to derive Ten-day HAs. The HA is consistent with this duration and appropriately matches human exposure scenarios for microcystins in drinking water. Cyanobacterial blooms are usually seasonal, typically occurring from May through October. Microcystins typically have a half-life of 4 days to 14 days in surface waters, (depending on the degree of sunlight, natural organic matter, and the presence of bacteria) and can be diluted via transport. In addition, concentrations in finished drinking water can be reduced by drinking water treatment and management measures.

The Ten-day HA value for bottle-fed infants and young children of pre-school age is 0.3 µg/L and for school-age children through adults is 1.6 µg/L for microcystins. The two advisory values use the same toxicity data (RfD) and represent differences in drinking water intake and body weight for different life stages. The first advisory value is based on the summation of the time-weighted drinking water intake/body weight ratios for birth to <12 months of age. The second advisory value is based on the mean body weight and 90th percentile drinking water consumption rates for adults age 21 and over (U.S. EPA's Exposure Factors Handbook (2011a)), which is similar to that of school-aged children. Populations such as pregnant women and nursing mothers, the elderly, and immune-compromised individuals or those receiving dialysis treatment may be more susceptible than the general population to the health effects of microcystins. As a precautionary measure, individuals that fall into these susceptible groups may want to consider following the recommendations for children pre-school age and younger. This HA is not a regulation, it is not legally enforceable, and it does not confer legal rights or impose legal obligations on any party.

Applying the U.S. EPA (2005) Guidelines for Carcinogen Risk Assessment, there is *inadequate information to assess carcinogenic potential* of microcystins. The few available epidemiological studies are limited by their study design, poor measures of exposure, potential co-exposure to other contaminants, and the lack of control for confounding factors. No long term animal studies were available to evaluate dose-response for the tumorigenicity of microcystins following lifetime exposures. Other studies evaluating the tumor promotion potential of microcystin found an increase in the number and/or size of GST-P positive foci observed. In two promotion studies, microcystin-LR alone showed no initiating activity.

1.0 INTRODUCTION AND BACKGROUND

EPA developed the non-regulatory Health Advisory (HA) Program in 1978 to provide information for public health officials or other interested groups on pollutants associated with short-term contamination incidents or spills for contaminants that can affect drinking water quality, but are not regulated under the Safe Drinking Water Act (SDWA). At present, EPA lists HAs for 213 contaminants (http://water.epa.gov/drink/standards/hascience.cfm).

HAs identify the concentration of a contaminant in drinking water at which adverse health effects are not anticipated to occur over specific exposure durations (e.g., one-day, ten-days, and a lifetime). HAs serve as informal technical guidance to assist Federal, State and local officials, and managers of public or community water systems in protecting public health when emergency spills or contamination situations occur. An HA provides information on the environmental properties, health effects, analytical methodology, and treatment technologies for removal of drinking water contaminants.

The *Health Effects Support Document for Microcystins* (U.S.EPA, 2015a) is the peer-reviewed, effects assessment that supports this HA. This document is available at http://www2.epa.gov/nutrient-policy-data/health-and-ecological-effects. The HAs are not legally enforceable Federal standards and are subject to change as new information becomes available. The structure of this Health Advisory is consistent with EPA's *Framework for Human Health Risk Assessment to Inform Decision Making* (U.S.EPA, 2014).

EPA is releasing the *Recommendations for Public Water Systems to Manage Cyanotoxins in Drinking Water* (U.S. EPA, 2015b) as a companion to the HAs for microcystins and cylindrospermopsin. The document is intended to assist public drinking water systems (PWSs) that choose to develop system-specific plans for evaluating their source waters for vulnerability to contamination by microcystins and cylindrospermopsin. It is designed to provide information and a framework that PWSs and others as appropriate may consider to inform their decisions on managing the risks from cyanotoxins in drinking water.

1.1 Current Criteria, Guidance and Standards

Currently there are no U.S. federal water quality criteria, or regulations for cyanobacteria or cyanotoxins in drinking water under the SDWA or in ambient waters under the Clean Water Act (CWA). The Safe Drinking Water Act (SDWA), as amended in 1996, requires the EPA to publish a list of unregulated contaminants every five years that are not subject to any proposed or promulgated national primary drinking water regulations, which are known or anticipated to occur in public water systems, and which may require regulation. This list is known as the Contaminant Candidate List (CCL). The EPA's Office of Water included cyanobacteria and cyanotoxins on the first and second CCL (CCL 1, 1998; CCL 2, 2005). EPA included cyanotoxins, including anatoxin-a, cylindrospermopsin, and microcystin-LR, on CCL 3 (2009) and the draft CCL 4 (April 2015 for consideration).

SDWA requires the Agency to make regulatory determinations on at least five CCL contaminants every five years. When making a positive regulatory determination, EPA determines whether a contaminant meets three criteria:

- The contaminant may have an adverse effect on the health of persons,
- The contaminant is known to occur or there is substantial likelihood the contaminant will occur in public water systems with a frequency and at levels of concern, and
- In the sole judgment of the Administrator, regulating the contaminant presents a meaningful opportunity for health risk reductions.

To make these determinations, the Agency uses data to analyze occurrence (prevalence and magnitude) and health effects. EPA continues gathering this information to inform future regulatory determinations for cyanotoxins under the SDWA. The SDWA also provides the authority for EPA to publish non-regulatory HAs or take other appropriate actions for contaminants not subject to any national primary drinking water regulation. EPA is providing this HA and the HA for cylindrospermopsin to assist State and local officials in evaluating risks from these contaminants in drinking water.

Internationally, eighteen countries and three U.S. states have developed drinking water guidelines for microcystins, as shown in Table 1.1 and Table 1.2, respectively, based on lifetime exposures.

Table 1-1. International Guideline Values for Microcystins

Country	Guideline Value	Source
Brazil, China, Czech Republic, Denmark, Finland, France, Germany, Italy, Japan, Korea, Netherlands, Norway, New Zealand, Poland, South Africa, and Spain	1.0 µg/L microcystin-LR	Based on the World Health Organization (WHO) Provisional Guideline Value of 1ug/L for drinking water (WHO, 1999; 2003)
Australia	1.3 µg/L microcystin-LR (toxicity equivalents)	Australian Drinking Water Guidelines 6 (NHMRC, NRMMC, 2011)
Canada	1.5 µg/L microcystin-LR	Guidelines for Canadian Drinking Water Quality: Supporting Documentation Cyanobacterial Toxins-Microcystin-LR (Health Canada, 2002)

Table 1-2. State Guideline Values for Microcystins

State	Guideline Value	Source
Minnesota	0.04 µg/L Microcystin-LR	Minnesota Department of Health (MDH, 2012)
Ohio	1 µg/L Microcystin	Public Water System Harmful Algal Bloom Response Strategy (Ohio EPA, 2014)
Oregon	1 µg/L Microcystin-LR	Public Health Advisory Guidelines, Harmful Algae Blooms in Freshwater Bodies. (OHA, 2015)

For drinking water, the provisional WHO Guideline value for microcystin-LR of 1 µg/L (or the underlying Tolerable Daily Intake (TDI) of 0.04 µg/kg) has been widely used as the basis for national standards or guideline values (WHO, 1999, 2003). Following the release of the WHO provisional guideline, drinking-water standards or national guideline values were adopted in 16 countries. Australia and Canada have used the TDI, but have adapted other factors in the calculation to reflect their national circumstances (e.g. body weight or amounts of water consumed), thus reaching somewhat higher guidance values or standards (Chorus, 2012). A few countries have issued guideline values specifically for microcystin-LR while others use microcystin-LR as a surrogate for all microcystin congeners (i.e. toxicity equivalents). Values are similar across all countries, ranging between 1.0 and 1.5 µg/L based on lifetime exposures.

2.0 PROBLEM FORMULATION

The development of the HA begins with problem formulation, which provides a strategic framework by focusing on the most relevant cyanotoxin properties and endpoints identified in the *Health Effects Support Document for Microcystins* (U.S. EPA, 2015a).

2.1 Cyanobacteria and Production of Microcystins

Cyanobacteria, formerly known as blue-green algae (Cyanophyceae), are a group of bacteria with chlorophyll-a capable of photosynthesis (light and dark phases) (Castenholz and Waterbury, 1989). Most cyanobacteria are aerobic photoautotrophs, requiring only water, carbon dioxide, inorganic nutrients and light for survival, while others have heterotrophic properties and can survive long periods in complete darkness (Fay, 1965). Some species are capable of nitrogen fixation (diazotrophs) (Duy et al., 2000), producing inorganic nitrogen compounds for the synthesis of nucleic acids and proteins. Cyanobacteria can form symbiotic associations with animals and plants, such as fungi, bryophytes, pteriodophytes, gymnosperms and angiosperms (Rai, 1990), supporting their growth and reproduction (Sarma, 2013; Hudnell, 2008; Hudnell, 2010).

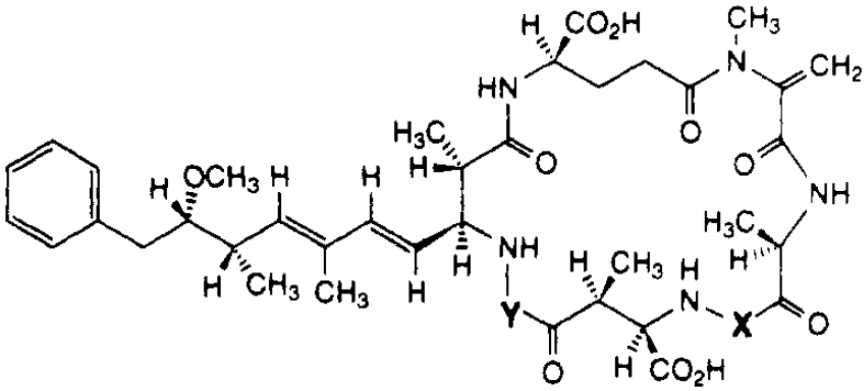
Under the right conditions of pH, nutrient availability, light, and temperature, cyanobacteria can reproduce quickly forming a bloom. Although studies of the impact of environmental factors on cyanotoxin production are ongoing, nutrient (N, P and trace metals) supply rates, light, temperature, oxidative stressors, interactions with other biota (viruses, bacteria and animal grazers), and most likely, the combined effects of these factors are all involved (Paerl and Otten 2013a; 2013b). Fulvic and humic acids reportedly encourage cyanobacteria growth (Kosakowska et al., 2007).

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

2.2 Physical and Chemical Properties

The cyclic peptides include around 100 congeners of microcystins. Table 2-1 lists only the most common microcystins congeners. Figure 2-1 provides the structure of microcystin where X and Y represent variable amino acids. 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).

Figure 2-1. Structure of Microcystin (Kondo et al., 1992)



Microcystin congeners vary based on their amino acid composition and through methylation or demethylation at selected sites within the cyclic peptide (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) (Carmichael, 1992). 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. In this HA, the term microcystin may be followed by the abbreviations for the variable amino acids. For example, microcystin-LR is for the microcystin with leucine in the X position of Figure 2-1 and arginine in the Y position. Most research has concentrated on microcystin-LR, with lesser amounts of data available for the other amino acid combinations. For the purpose of this HA, microcystin-LR is used as the surrogate for total microcystins.

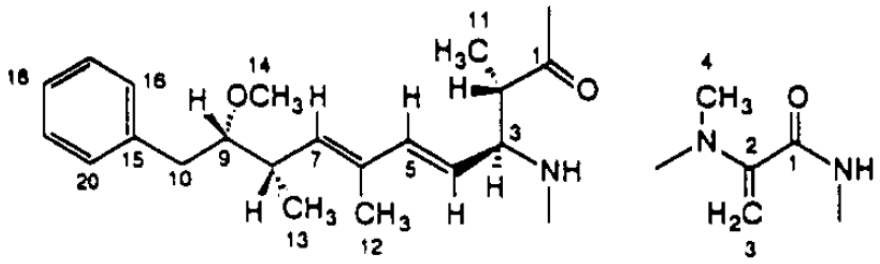
Structurally, the microcystins are monocyclic heptapeptides that contain seven amino acids: two variable L-amino acids, three common D-amino acids or their derivatives, and two novel D-amino acids. These two D-amino acids are: 3S-amino-9S-methoxy-2,6,8S,-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) and methyldehydroalanine (Mdha). Adda is characteristic of all toxic microcystin structural congeners and is essential for their biological activity (Rao et al., 2002; Funari and Testai, 2008). Mdha plays an important role in the ability of the microcystins to inhibit protein phosphatases. Figure 2-2 illustrates the structures of these 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. The microcystins are most frequently found in cyanobacterial blooms in fresh and brackish waters (WHO, 1999). Table 2-2 provides chemical and physical properties of microcystin-LR.

Table 2-1. Abbreviations for Microcystins (Yuan et al., 1999)

Microcystin Congeners	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

Figure 2-2. Structure of the amino acids Adda and Mdha (Harada et al., 1991).



Adda **Mdha**

Table 2-2. 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

2.3 Sources and Occurrence

Cyanotoxin production is strongly influenced by the environmental conditions that promote growth of particular cyanobacterial species and strain. Nutrient concentrations, light intensity, water turbidity, temperature, competing bacteria and phytoplankton, pH, turbulence, and salinity are all factors that affect cyanobacterial growth and change in cyanobacteria population dynamics. Although environmental conditions affect the formation of blooms, the numbers of cyanobacteria and toxin concentrations 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 (Hitzfeld et al., 2000; Chorus et al., 2000; WHO, 1999). Extracellular microcystins (either dissolved in water or bound to other materials) typically make up less than 30% of the total microcystin concentration in source water (Graham et al., 2010). Most of the toxin is intracellular, and released into the water when the cells rupture or die. Both intracellular and extracellular microcystins may also be present in treated water, depending on the type of treatment processes in place.

2.3.1 Occurrence in Surface Water

Microcystins are the most common cyanotoxin 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 25,000 µg/L. The concentration of extracellular microcystins ranged from 0.02 to a high of 1,800 µg/L reported following treatment of a large cyanobacteria bloom with algaecide (WHO, 1999) and 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).

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 performed using Protein Phosphatase Inhibition Assay (PPIA). Microcystins were detected in at least 65% of the 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. This 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 in cyanobacterial blooms (Graham et al., 2010). This 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 association with 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 in the NLA during the summer

of 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, which were indicators of the presence of cyanobacterial toxins. Samples were collected in open water at mid-lake. Due to the design of the survey, no samples were taken nearshore or in other areas where scums were present.

A total of 48 states were sampled in the NLA, and states with lakes reporting microcystins levels above the WHO's moderate risk[1] threshold in recreational water (>10 µg/L) are shown in Table 2-3. 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 samples 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 and 225 µg/L, respectively). EPA completed a second survey of lakes in 2012, but data have not yet been published.

Microcystins have been detected in most of the states of the U.S., and over the years many studies have been done 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 microcystin were low in the majority of the tributaries with the exception of Milford Lake, which had higher total microcystin concentrations, some exceeding the Kansas recreational guidance level of 20 µg/L. Upstream from Milford Lake, a cyanobacterial bloom was observed with a total microcystin concentration of 150,000 µg/L. When sampled a week later, total microcystin concentrations were less than 1 µg/L. The study authors indicated that this may be due to dispersion of microcystins through the water column or to other areas, or by degradation of microcystins via abiotic and biological processes. Samples taken during the same time from outflow waters contained total microcystin concentrations 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.

[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).

Table 2-3. States Surveyed as Part of the 2007 National Lakes Assessment with Water Body Microcystins 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

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 conducted in Kabetogama Lake, Minnesota which detected microcystin concentrations in association with algal blooms (Christensen et al., 2011). Microcystin concentrations were detected in 78% of bloom samples. Of these, 50% were above 1 µg/L, 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 concentration, cyanobacterial assemblages, and microcystin concentrations in 11 potable water supply reservoirs within the North Carolina Piedmont during dry summer growing seasons (Touchette et al., 2007). Microcystins concentrations were assessed using ELISA. The study found that cyanobacteria were the dominant phytoplankton community, averaging 65-95% of the total phytoplankton 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 exceeding 100 µg/L.

In 2008, NOAA began monitoring for cyanobacterial blooms in Lake Erie using high temporal resolution satellite imagery. Between 2008 and 2010, *Microcystis* cyanobacterial blooms were detected associated with water temperatures above 18°C (Wynne et al., 2013). Using the Great Lakes Coastal Forecast System (GLCFS), 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 of 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.

2.3.2 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 United States and Canada in 1996-1998 (AWWARF, 2001). Of 677 samples tested, microcystin was found in 80% (539) of the waters sampled, including source and treated waters. Only two samples of finished drinking water were above 1 µg/L. A survey conducted in 2000 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.

During the summer of 2003, a survey was conducted to test for microcystins in 33 U.S. drinking water treatment plants in the northeastern and Midwestern U.S. (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.

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.

2.4 Environmental Fate

Different physical and chemical processes are involved in the persistence, breakdown, and movement of microcystins in aquatic systems as described below.

2.4.1 Persistence

Microcystins are relatively stable and resistant to chemical hydrolysis or oxidation at or near neutral pH. Elevated or low pH or temperatures above 30°C may cause slow hydrolysis. Microcystin is not destroyed by boiling (Rao et al., 2002). In natural waters kept in the dark, microcystins have been observed to persist for 21 days to 2-3 months in solution and up to 6 months in dry scum (Rapala et al., 2006; Funari and Testai, 2008).

In the presence of full sunlight, microcystins undergo 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 in as few as two weeks to longer than six weeks, depending on the concentration of pigment and the intensity of the light (Tsuji et al., 1993; 1995). According to Tsuji et al, 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 can increase the rate of microcystin breakdown in sunlight. In deeper or turbid water, the breakdown rate is slower.

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 microcystin-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., in 1995 reported that the biotransformation of microcystin-LR followed a first-order decay with a half-life of 0.2 to 3.6 days (Lam et al., 1995). In a study done by Jones et al. (1994) with microcystin-LR in different natural surface waters, microcystin-LR persisted for 3 days to 3 weeks; however, more than 95% loss occurred within 3 to 4 days. A study by Christoffersen et al., 2002, measured half-lives in the laboratory and in the field of approximately 1 day, 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 half-lives of 4 to 14 days, with longer half-lives associated with a flowing stream and shorter half-lives associated with lakes.

2.4.2 Mobility

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). Ground water is generally not expected to be at risk of cyanotoxin contamination, however, ground water under the direct influence of surface water can be vulnerable. A study conducted by the USGS and the University of Central Florida determined that microcystin and cylindrospermopsin did not sorb in sandy aquifers and were transported along with ground water (O'Reilly et al., 2011). The authors suggested that the removal of microcystin was due to biodegradation.

2.5 Nature of the Stressor-Characteristics of the Microcystin Toxins

2.5.1 Toxicokinetics

No data were available that quantified the intestinal, respiratory or dermal absorption of microcystins. Available data indicate that the Organic Acid Transporter polypeptide (OATp) receptors facilitate the absorption of toxins from the intestinal tract into liver, brain, and other tissues. The OATp family transporters facilitate the cellular, sodium-independent uptake and export of amphipathic compounds such as bile salts, steroids, drugs, peptides and toxins (Cheng et al., 2005; Fischer et al., 2005; Svoboda et al., 2011). This facilitated transport is necessary for both uptake of microcystins into organs and tissues as well as for their export. Microcystins compete with bile acids for uptake by the liver and is limited in the presence of bile acids and other physiologically-relevant substrates for the transporter (Thompson and Pace, 1992). Other studies following *in vitro* or *in vivo* exposures have shown that inhibition of microcystin uptake by its OATp transporter reduces or eliminates the liver toxicity observed (Runnegar et al., 1981, 1995; Runnegar and Falconer, 1982; Hermansky et al., 1990a, b).

Limited information is available on the metabolism of microcystins. Some studies have found that metabolism of microcystin-LR in mice occurs in the liver (Robinson et al., 1991; Pace et al., 1991). Most of the available studies show minimal if any catabolism (process of breaking down molecules into smaller units to release energy). Microcystins can be conjugated with cysteine and glutathione to increase their solubility and facilitate excretion (Kondo et al., 1996).

However, it is not clear whether hepatic cytochromes, such as cytochrome P450-facilitated oxidation, precedes conjugation (Cote et al., 1986; Brooks and Codd, 1987). Both *in vivo* and *in vitro* studies have shown biliary excretion (Falconer et al., 1986; Pace et al., 1991; Robinson et al., 1991).

2.5.2 Noncancer Health Effects Data

2.5.2.1 Human Studies

The human data on the oral toxicity of microcystin-LR are limited by the potential co-exposure to other pathogens and toxins, by the lack of quantitative information, and by the failure to control for confounding factors.

Only a few epidemiological and case studies are available on the toxicity of microcystins in humans. An outbreak among army recruits who had consumed reservoir water with a cyanobacteria bloom with *M. aeruginosa* reported symptoms of headache, sore throat, vomiting and nausea, stomach pain, dry cough, diarrhea, blistering around the mouth, and pneumonia (Turner et al., 1990). Microcystins, including microcystin-LR, were present in bloom samples. However, high levels of *Escherichia coli* were also found in reservoir water after two weeks. The authors suggested that exposure to microcystins may have had a role in some of the clinical symptoms.

An epidemiology study done in Australia compared the hepatic enzyme levels from patients served by a public water supply contaminated with a *M. aeruginosa* bloom with enzyme levels from patients living in areas served by water supplies uncontaminated by cyanobacteria (Falconer et al., 1983). Although the authors observed significant variability in enzyme levels between the two groups, the findings were attributed by the authors to the imprecise method of study participant selection and confounding factors such as alcoholism and chronic kidney disease among some of the participants.

A cross-sectional study done in China assessed the relationship between the consumption of water and food (carp and duck) contaminated with microcystins and liver damage in children (Li et al., 2011a). The authors found that mean serum levels of microcystins ranged from below detection to 1.3 μg microcystin-LR equivalents/L. According to the authors, hepatitis B infection was a greater risk for liver damage among these children than the microcystin exposure.

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* bloom with concentrations of 48.6 μg/L. After four hours of exposure, the patient exhibited 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 alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyltransferase (γGT). The patient completely recovered within 20 days.

An outbreak of acute liver failure occurred in a dialysis clinic in 1996 in Caruaru, Brazil where dialysis water was contaminated with microcystins, and possibly cylindrospermopsin. Of the 130 patients who received their routine hemodialysis treatment (intravenously) at that time, 116 reported symptoms of headache, eye pain, blurred vision, nausea and vomiting. Subsequently, 100 of the affected patients developed acute liver failure and, of these, 76 died (Carmichael et al., 2001; Jochimsen et al., 1998). Analyses of blood, sera, and liver samples from the patients revealed microcystins. Although the patients in the study had pre-existing diseases, the direct intravenous exposure to dialysate prepared from surface drinking water supplies put them at risk for cyanotoxin exposure and resultant adverse effects (Hilborn et al., 2013).

In another contamination event at a dialysis center in Rio de Janeiro, Brazil in 2001, 44 dialysis patients were potentially exposed to microcystin concentrations of 0.32 µg/L, detected in the activated carbon filter used in an intermediate step for treating drinking water to prepare dialysate (Soares et al., 2005). Concentrations of 0.4 µg/L microcystin-LR were detected in the drinking water. Serum samples were collected from 13 dialysis patients 31 to 38 days after the detections in water samples, and patients were monitored for eight weeks. Concentrations of microcystin-LR in the serum ranged from 0.46 to 0.96 ng/mL. 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. Since microcystin-LR 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 a mild to moderate mixed liver injury.

2.5.2.2 Animal Studies

Most of the information on the noncancer effects of microcystins in animals is from oral and intraperitoneal (i.p.) administration studies in mice and rats exposed to purified microcystin-LR. Liver effects are observed following acute oral exposure to microcystin-LR (Yoshida et al., 1997; Ito et al., 1997b; Fawell et al., 1999). Effects on the liver, kidney, and male reproductive system (testicular function and sperm quality), including changes in organ weights and histopathological lesions, are observed following short-term and subchronic oral exposure to microcystin-LR (Heinze, 1999; Fawell et al., 1999; Huang et al., 2011; Chen et al., 2011). Oral and i.p. developmental toxicity studies in mice provide some evidence for fetal body weight changes and maternal mortality (Fawell et al., 1999; Chernoff et al., 2002).

According to the authors, no clinical signs of toxicity were observed in a chronic study done in mice for 18 months by Ueno et al. (1999). Although histopathology from a 280 day study in mice revealed infiltrating lymphocytes and fatty degeneration in the livers, no quantitative data were provided in the study (Zhang et al., 2012).

2.5.3 Mode of Action for Noncancer Health Effects

Mechanistic studies have shown the importance of membrane transporters for systemic uptake and tissue distribution of microcystin by all exposure routes (Fischer et al. 2005; Feurstein et al., 2010). The importance of the membrane transporters to tissue access is demonstrated when a reduction in, or lack of, liver damage happens following OATp inhibition (Hermansky et al., 1990 a,b; Thompson and Pace, 1992).

The uptake of microcystins causes protein phosphatase inhibition and a loss of coordination between kinase phosphorylation and phosphatase dephosphorylation, which results in the destabilization of the cytoskeleton. This event initiates altered cell function followed by cellular apoptosis and necrosis (Barford et al., 1998). Both cellular kinases and phosphatases keep the balance between phosphorylation and dephosphorylation of key cellular proteins controlling metabolic processes, gene regulation, cell cycle control, transport and secretory processes, organization of the cytoskeleton and cell adhesion. Each of the microcystin congeners evaluated (LR, LA, and LL) interacts with catalytic subunits of protein phosphatases PP1 and PP2A, inhibiting their functions (Craig et al., 1996).

As a consequence of the microcystin-induced changes in cytoskeleton, increases in apoptosis and reactive oxygen species (ROS) occur. In both *in vitro* and *in vivo* studies, cellular pro-apoptotic Bax and Bid proteins increased while anti-apoptotic Bcl-2 decreased (Fu et al., 2005; Weng et al., 2007; Xing et al., 2008; Takumi et al., 2010; Huang et al., 2011; Li et al., 2011b). Mitochondrial membrane potential and permeability transition pore changes (Ding and Ong, 2003; Zhou et al., 2012) lead to membrane loss of cytochrome c, a biomarker for apoptotic events. Wei et al., (2008) found a time-dependent increase in ROS production and lipid peroxidation in mice after exposure to microcystin-LR. After receiving a 55 µg/kg of body weight i.p. injection of microcystin-LR, the levels of hepatic ROS increased rapidly within 0.5 hours and continued to accumulate for up to 12 hours in a time-dependent manner.

2.5.4 Carcinogenicity Data

Several human epidemiological studies from China have reported an association between liver or colon cancer and consumption of drinking water from surface waters containing cyanobacteria and microcystins (Ueno et al., 1996; Zhou et al., 2002). In these studies, a concentrations measured in a surface drinking water supply were 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 co-occurring chemical contaminants or hepatitis infections in the population.

A study done by Flemming et al. (2002, 2004) in Florida failed to find a significant association for primary liver cancer 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, or those receiving their water from ground-water sources. The one significant association observed was between those people in the surface water service areas, versus those in their surrounding areas described as buffer zones. However, the nature of the water supply for the buffer zones were not identified.

The only longer-term oral animal study of purified microcystin-LR was conducted by Ito et al. (1997b). Ito et al. (1997b) administered 80 µg microcystin-LR/kg/day by gavage to mice for 80 or 100 days over 28 weeks (7 months). This single dose failed to induce neoplastic nodules of the liver. The lack of hyperplastic nodules at 7 months suggests that microcystins are not a mutagenic initiator of tumors, however, the fairly short duration may have been a limiting factor.

Several studies suggest that microcystin-LR is a tumor promoter. In these studies, animals were first exposed to substances known to be tumor initiators (e.g. N-methyl-N-nitroso urea or NDEA) alone, or in combination with microcystin-LR at i.p. doses known to have no significant impact on liver weight. The combination of the initiator and the microcystin-LR significantly increased the number and area of glutathione *S*-transferase placental form-positive (GST-P) foci when compared to treatment with the initiator alone. The same was true for situations where the initiator treatment was combined with a partial hepatectomy (to stimulate tissue repair) and then exposed to microcystins i.p. (Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994). GST-P foci are regarded as indicators for potential tumors formation. The results from these studies support the classification of microcystin as a tumor promoter.

2.6 Conceptual Model for Microcystins

The conceptual model is intended to explore potential links of exposure to a contaminant or stressor with the adverse effects and toxicological endpoints important for management goals, including the development of HA values. The conceptual model demonstrates the relationship between exposure to microcystins in drinking water and adverse health effects in the populations at risk.

HAs describe non-regulatory concentrations of drinking water contaminants at which adverse health effects are not anticipated to occur over specific exposure durations (e.g., one-day, ten-days, and a lifetime). HAs also contain a margin of safety to protect sensitive members of the population. They serve as informal technical guidance to assist federal, state and local officials, as well as managers of public or community water systems, in protecting public health. They are not to be construed as legally enforceable federal standards.

Assessment endpoints for HAs can be developed for both short-term (one-day and ten-day) and lifetime exposure periods using information on the non-carcinogenic and carcinogenic toxicological endpoints of concern. Where data are available, endpoints will reflect susceptible and/or more highly exposed populations.

- A One-day HA is typically calculated for an infant (0-12 months or 10kg child), assuming a single acute exposure to the chemical and is generally derived from a study of less than seven days' duration.
- A Ten-day HA is typically calculated for an infant (0-12 months or 10kg child), assuming a limited period of exposure of one to two weeks, and is generally derived from a study of 7 to 30-days duration.

- A Lifetime HA is derived for an adult (>21 years or 80kg adult), and assumes an exposure period over a lifetime (approximately 70 years). It is usually derived from a chronic study of two years duration, but subchronic studies may be used by adjusting the uncertainty factor employed in the calculation. For carcinogens, the HA documents typically provide the concentrations in drinking water associated with risks for one excess cancer case per ten thousand persons exposed up to one excess cancer case per million exposed for Group A and B carcinogens and those classified as known or likely carcinogens (U.S. EPA, 1986, 2005). Cancer risks are not provided for Group C carcinogens or those classified as "suggestive", unless the cancer risk has been quantified.

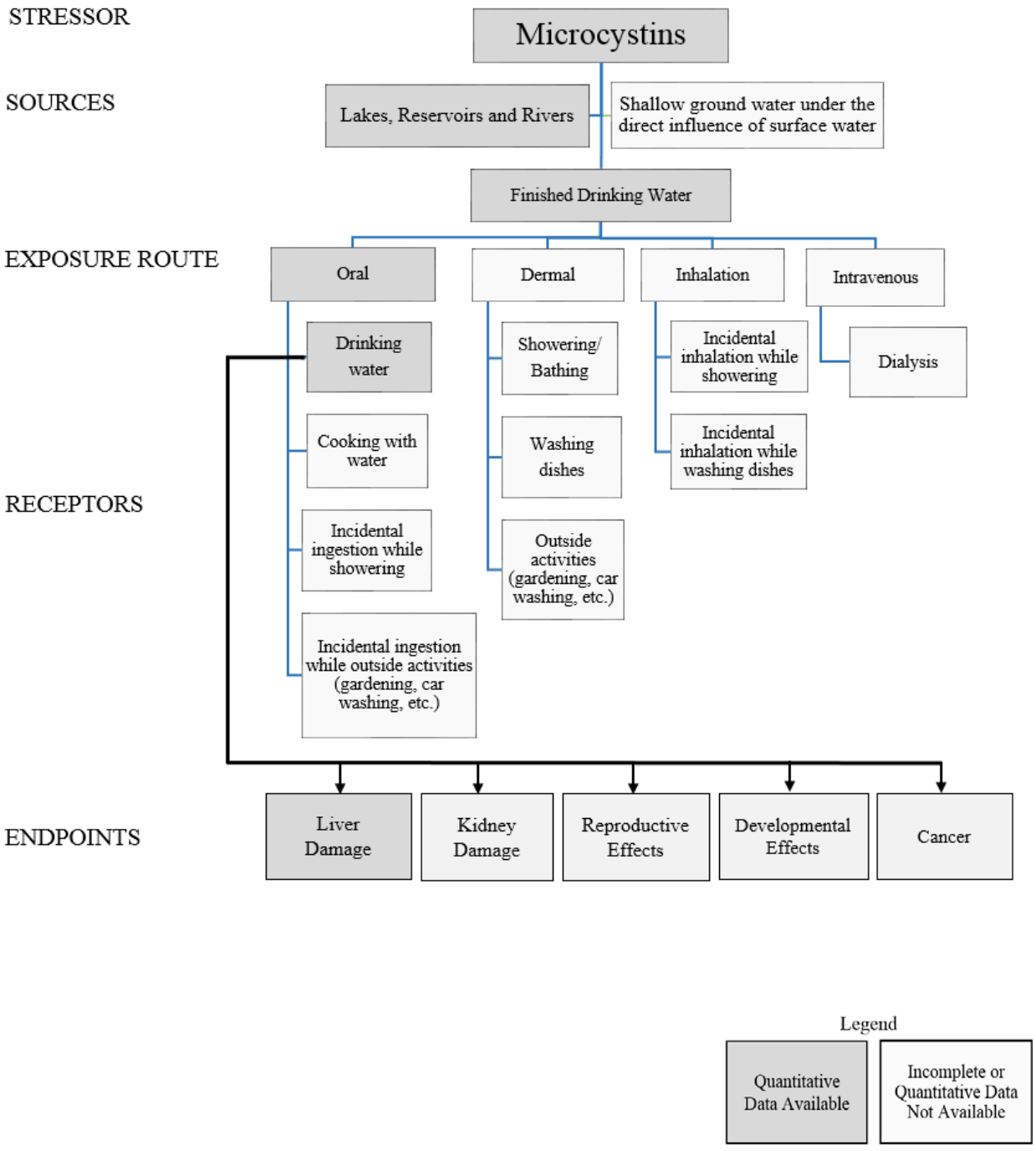
For each assessment endpoint EPA uses one or more measures of effect (also referred to as a point of departure), which describe the change in the attribute of the assessment endpoint in response to chemical exposure, to develop acute, short-term, longer term (subchronic) or chronic reference values when the data are available. The measures of effect selected represent impacts on survival, growth, system function, reproduction and development.

This conceptual model provides useful information to characterize and communicate the potential health risks related to exposure to cyanotoxins in drinking water. The sources of cyanotoxins in drinking water, the route of exposure for biological receptors of concern (e.g., via various human activities such as drinking, food preparation and consumption) and the potential assessment endpoints (i.e., effects such as kidney and liver toxicity, and reproductive and developmental effects) due to exposure to microcystins are depicted in the conceptual diagram below (Figure 2-3).

2.6.1 Conceptual Model Diagram

Cyanobacteria are a common part of freshwater and marine ecosystems. An increase in water column stability, high water temperatures, elevated concentrations of nutrients, and low light intensity have been associated with an increase and or dominance of microcystin-producing cyanobacteria in surface waters (or aquatic ecosystems). The presence of detectable concentrations of cyanotoxins in the environment is closely associated with these blooms. Winds and water currents can potentially transport cyanobacterial blooms to areas within the proximity of water intakes for drinking water treatment plants. If not managed in source waters, or removed during drinking water treatment, cyanobacteria and cyanotoxins may result in exposure that could potentially affect human health.

Figure 2-3. Conceptual Model of Exposure Pathways to Microcystins in Drinking Water



2.6.2 Factors Considered in the Conceptual Model for Microcystins

Stressors: For this HA, the stressor is microcystins concentrations in finished drinking water.

Sources: Sources of microcystins include potential sources of drinking water such as rivers, reservoirs, and lakes in the U.S. where blooms producing microcystins occur. Shallow private wells under the direct influence of surface water (in hydraulic connection to a surface water body) can also be impacted by microcystins-producing blooms, if the toxins are drawn into the well along with the water from the surface water. There is substantially less information on exposure from this source.

Routes of exposure: Exposure to cyanotoxins from contaminated drinking water sources may occur via oral exposure (drinking water, cooking with water, and incidental ingesting from showering); dermal exposure (contact of exposed parts of the body during bathing or showering, washing dishes, or outside activities); inhalation exposure (during bathing, showering or washing dishes,); or intravenous exposure (e.g. via dialysis). Toxicity data are available for the oral route of exposure from drinking water, but are not available to quantify dose response for other exposure routes (inhalation, dermal, dietary, and intravenous exposures).

Receptors: The general population (adults and children) could be exposed to cyanotoxins through dermal contact, inhalation and/or ingestion. Infants and pre-school age children can be at greater risk to microcystins because they consume more water per body weight than do adults. Other individuals of potential sensitivity are persons with kidney and/or liver disease due to the compromised detoxification mechanisms in the liver and impaired excretory mechanisms in the kidney. There are no human data to quantify risk to pregnant woman or to evaluate the transfer of cyanotoxins across the placenta. Data are also not available on the transfer of cyanotoxins through the milk from nursing mothers or regarding the risk to the elderly. Given this lack of information, pregnant women, nursing mothers, and the elderly may also be potentially sensitive populations. Data from the 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. Data are not available to derive a One-day HA for children because studies with single oral dosing do not provide dose-response information. A lifetime HA for microcystins is not recommended as the types of exposures being considered are short-term and episodic in nature. Although the majority of the cyanobacterial blooms in the U.S. occur seasonally, usually during late summer, some toxin-producing strains can occur early in the season and can last for days or weeks.

Endpoints: Human data on oral toxicity of microcystins are limited, but suggest the liver as the primary target organ. Acute, short-term, and subchronic studies in animals also demonstrate that the liver and kidney are target organs. In addition, some studies suggest that microcystins may lead to reproductive and developmental effects. Studies have suggested that microcystins have tumor promotion potential if there has been co-exposure to a carcinogen or cellular organ damage. However, these data are limited, and there has been no long term bioassay in animals to evaluate cancer. Available toxicity data are described in the *Health Effects Support Document (HESD) for Microcystins* (U.S. EPA, 2015a), and indicate that the primary target organ for microcystins is the liver. Kidney and reproductive effects in male mice were also observed, but were either not as

sensitive as the liver or lack confirmation from more than one laboratory. Data are inadequate to assess the carcinogenic potential of microcystins at this time.

2.7 Analysis Plan

The *Health Effects Support Document for Microcystins* (HESD, U.S. EPA, 2015a), provides the health effects basis for development of the HA, including the science-based decisions providing the basis for estimating the point of departure. To develop the 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. Some of the search terms included in the literature search were microcystin, microcystin 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 considered information from the following risk assessments during the development of the microcystins health risk assessment:

- 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*

The toxicity data available for an individual pollutant vary significantly. An evaluation of available data was performed by EPA to determine data acceptability. The following study quality considerations from U.S. EPA's (2002) *A Review of the Reference Dose and Reference Concentration Processes* were used in selection of the studies for inclusion in the HESD and development of the HA.

- Clearly defined and stated hypothesis.
- Adequate description of the study protocol, methods, and statistical analyses.
- Evaluation of appropriate endpoints. Toxicity depends on the amount, duration, timing, and pattern of exposure and may range from frank effects (e.g., mortality) to more subtle biochemical, physiological, pathological, or functional changes in multiple organs and tissues.
- Application of the appropriate statistical procedures to determine an effect.
- Establishment of dose-response relationship (i.e., no observed adverse effect level (NOAEL) and/or lowest observed adverse effect level (LOAEL) or data amenable to modeling of the dose-response in order to identify a point of departure for a change in the effect considered to be adverse (out of the range of normal biological viability). The NOAEL is the highest exposure level at which there are no biologically significant

increases in the frequency or severity of adverse effect between the exposed population and its appropriate control. The LOAEL is the lowest exposure level at which there are biologically significant increases in frequency or severity of adverse effects between the exposed population and its appropriate control group.

After the available studies were evaluated for inclusion in the HESD and HA, the critical study was selected based on consideration of factors including exposure duration (comparable to the duration of the HA being derived), route of exposure (oral exposure via drinking water, gavage, or diet is preferred), species sensitivity, comparison of the point of departure with other available studies demonstrating an effect, and confidence in the study (U.S. EPA, 1999). Once, a point of departure is chosen for quantification, uncertainty factors appropriate for the study selected are then applied to the point of departure to account for variability and uncertainty in the available data.

For microcystins, toxicity and exposure data are available to develop a Ten-day HA. EPA used measures of effect and estimates of exposure to derive the Ten-day HAs using the following equation:

$$HA = \frac{NOAEL \text{ or } LOAEL \text{ or } BMDL}{UF \times DWI/BW}$$

Where:

NOAEL or LOAEL = No- or Lowest-Observed-Adverse-Effect Level (mg/kg bw/day) from a study of an appropriate duration (7 to 30 days).

BMDL = When the data available are adequate, benchmark dose (BMD) modeling can be performed to determine the point of departure for the calculation of HAs. The benchmark dose approach involves dose-response modeling to obtain dose levels corresponding to a specific response level near the low end of the observable range of the data (U.S.EPA, 2012). The lower 95% confidence limit is termed the benchmark dose level (BMDL).

UF = Uncertainty factors (UF) account for: (1) intraspecies variability (variation in susceptibility across individuals); (2) interspecies variability (uncertainty in extrapolating animal data to humans; (3) uncertainty in extrapolating from a LOAEL to a NOAEL; and (4) uncertainty associated with extrapolation when the database is incomplete. These are described in U.S. EPA, 1999 and U.S. EPA, 2002.

DWI/BW = For children, a normalized ratio of drinking water ingestion to body weight (DWI/BW) was calculated using data for infants (birth to <12 months). The estimated drinking water intake body weight ratio (L/kg/day) used for birth to

<12 months of age are the 90th percentile values of the consumers only estimates of direct and indirect water ingestion based on 1994-1996, 1998 CSFII (Continuing Survey of Food Intakes by Individuals) (community water, mL/kg/day) in Table 3-19 in the U.S. EPA (2011a) Exposure Factors Handbook. The time weighted average of DWI/BW ratios values was derived from multiplication of age-specific DWI/BW ratios (birth to <1 month, 1 to <3 months, 3 to <6 months, and 6 to <12 months) by the age-specific fraction of infant exposures for these time periods.

For adults (>21 years of age), EPA updated the default BW assumption to 80 kg based on National Health and Nutrition Examination Survey (NHANES) data from 1999 to 2006 as reported in Table 8.1 of EPA's *Exposure Factors Handbook* (U.S. EPA, 2011a). The updated BW represents the mean weight for adults ages 21 and older.

EPA updated the default DWI to 2.5 L/d, rounded from 2.546 L/d, based on NHANES data from 2003 to 2006 as reported in EPA's *Exposure Factors Handbook* (U.S. EPA 2011a, Table 3-33). This rate represents the consumer's only estimate of combined direct and indirect community water ingestion at the 90th percentile for adults ages 21 and older.

3.0 HEALTH EFFECTS ASSESSMENT

The health effects assessment provides the characterization of adverse effects and includes the hazard identification and dose-response assessment. The hazard identification includes consideration of available information on toxicokinetics; identification, synthesis and evaluation of studies describing the health effects of microcystins; and the potential modes of action (MOAs), or toxicity pathways related to the health effects identified.

3.1 Dose-Response

3.1.1 Study Selection

The critical study chosen for determining the guideline value is a short-term study by Heinze (1999) in which 11-week-old male hybrid rats (F1 generation of female WELS/Fohm x male BDIX) were administered microcystin-LR via drinking water for 28 days at concentrations of 0 (n=10), 50 (n=10) or 150 (n=10) μg/kg body weight (Heinze, 1999). Water consumption was measured daily, and rats were weighed at weekly intervals. The dose estimates provided by the authors were not adjusted to account for incomplete drinking water consumption (3-7% of supplied water was not consumed over the 28-day period). Rats were sacrificed by exsanguination under ether anesthesia after 28 days of exposure, and evaluation of hematology, serum biochemistry plus histopathology of liver and kidneys, and measurement of organ weights (liver, kidneys, adrenals, spleen and thymus) was performed.

Hematological evaluation showed an increase of 38% in the number of leukocytes at the highest dose group (150 μg/kg body weight). Serum biochemistry showed a significant increase in both treatment groups in mean levels of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH); 84 and 100% increase in LDH, and 34 and 33% increase in ALP, in the low and high dose groups respectively. No changes were observed in mean levels of AST (aspartate aminotransferase), and ALT (alanine aminotransferase). An increase in relative liver weights was observed in a dose-dependent manner; 17% at 50 μg/kg body weight, and 26% at 150 μg/kg body weight. Mean enzyme levels and relative liver weights are shown in Table 3-1.

A dose-dependent increase in absolute liver weight was also reported, and data on the liver weights were provided by the author in a personal communication. A dose-dependent increase in the average absolute liver weights was also observed in all groups: 8.8 grams at the control group, 9.70 grams at the lower dose and 10.51 grams at the high dose (Table 3-1). No statistically significant changes in other organ weights or body weights were reported, and no effects on the kidneys were observed. Table 3-2 summarizes the histological observations of liver lesions. Liver lesions were considered toxic and spread diffusely throughout the parenchyma indicating cell damage expressed by an increase in cell volume, an increase in mitochondria, cell necrosis, the activation of Kupffer cells, and an increase in the amounts of periodic acid-Schiff (PAS)-positive substances. Liver lesions were observed in both treatment groups. No kidney effects were observed in either dose groups. The LOAEL was determined to be 50 μg/kg/day. The selection of Heinze (1999) as the critical study was based on the appropriateness of the study duration, the use

Table 3-1. 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

* $p \leq 0.05$ when compared with control; katal=conversion rate of 1 mole of substrate per second.
**Information provided by the author through a personal communication.

Table 3-2. 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

of multiple doses, dose-related toxicological responses, and histopathological evaluations of toxicity.

3.1.2 Endpoint Selection

The point of departure selected from the Heinze (1999) study is the LOAEL (50 μg/kg/day) for liver effects (increased liver weight, slight to moderate liver necrosis lesions, with or without hemorrhages at the low dose and increased severity at the high dose, and changes in serum enzymes indicative of liver damage). For the lesions, incidence increases from one animal impacted in the control group to ten animals impacted in the dosed groups. This dose-response is more dramatic than the difference in liver weight between the control and low dose (1.17 fold) and the differences in the ALP and LDH levels between the control and low dose group (1.34 and 1.84-fold, respectively). Therefore, the liver lesions are identified as the endpoint of greatest concern. These differences also advise against application of benchmark dose modeling for these effects. The male and female mice in the Fawell et al (1999) study displayed liver lesions, but the difference between controls and the low dose group (40 μg/kg/day) was less than two-fold. In an i.p. infusion study by Guzman and Solter (1999) with a more direct delivery of dose to the liver, necrosis was observed at doses of 32 and 48 μg/kg/day, but not at a dose of 16 μg/kg/day, thus providing support for the critical effect and dose.

3.2 Ten-day Health Advisory

This Ten-day HA is applied to total microcystins using microcystin-LR as a surrogate. The Ten-day HA is considered protective of non-carcinogenic adverse health effects over a ten-day exposure to microcystins in drinking water.

3.2.1 Bottle-fed Infants and Young Children of Pre-school Age

The Ten-day HA for bottle-fed infants and young children of pre-school age is calculated as follows:

$$\text{Ten-day HA} = \frac{50\ \mu\text{g/kg/day}}{1000 \times 0.15\ \text{L/kg/day}} = 0.3\ \mu\text{g/L}$$

Where:

50 μg/kg/day	=	The LOAEL for liver effects in 11-week-old male hybrid rats exposed to microcystin-LR in drinking water for 28 days (Heinze, 1999).
1000	=	The composite UF including a 10 for intraspecies variability (UF_H), a 10 for interspecies differences (UF_A), a 3 for LOAEL to NOAEL extrapolation (UF_L), and a 3 for uncertainties in the database (UF_D).
0.15 L/kg/day	=	Normalized drinking water intake per unit body weight over the first year of life based on the 90th percentile of drinking water consumption and the mean body weight (U.S. EPA, 2011a).

The Ten-day HA of 0.3 µg/L is considered protective of non-carcinogenic adverse health effects for bottle-fed infants and young children of pre-school age over a ten-day exposure to microcystins in drinking water.

3.2.2 School-age Children through Adults

The Ten-day HA for school-age children through adults is calculated as follows:

$$\text{Ten-day HA} = \frac{50\ \mu\text{g/kg/day}}{1000 \times 0.03\ \text{L/kg/day}} = 1.6\ \mu\text{g/L}$$

Where:

50 µg/kg/day	=	The LOAEL for liver effects in 11-week-old male hybrid rats exposed to microcystin-LR in drinking water for 28 days (Heinze, 1999).
1000	=	The composite UF including a 10 for intraspecies variability (UF_H), a 10 for interspecies differences (UF_A), a 3 for LOAEL to NOAEL extrapolation (UF_L), and a 3 for uncertainties in the database (UF_D).
0.03 L/kg/day	=	Drinking water intake per unit body weight based on adult default values of 2.5 L/day and 80 kg (U.S. EPA, 2011a).

The Ten-day HA of 1.6 µg/L is considered protective of non-carcinogenic adverse health effects for children of school age through adults over a ten-day exposure to microcystins in drinking water.

3.2.3 Uncertainty Factor Application

- 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. Individuals with pre-existing liver problems could be more sensitive to microcystins exposures than the general population. Pregnant woman, nursing mothers, and the elderly could also be sensitive to microcystins exposures.

- UF_A - 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 microcystins. Allometric scaling is not applied in the development of the Ten-day HA values for microcystins. The allometric scaling approach is derived from the relationship between body surface area and basal metabolic rate in adults (U.S. EPA, 2011b). This approach is not appropriate for infants and children due to the comparatively slower clearance during these ages and the limited toxicokinetic data available to assess the appropriateness of body weight scaling in early life.

- UF_L - An uncertainty factor of 3 ($10^{0.5}$ = 3.16) is selected to account for the extrapolation from a LOAEL to a NOAEL. The threefold factor is justified based on the evidence suggesting 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 microcystins 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 when food residues are 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 is 16 µg/kg/day and the LOAEL is 32 µg/kg/day, a two-fold difference. There is no reason to believe that the less direct delivery from the intestines to the liver following oral exposures through drinking water (as was used in Heinze, 1999) would have a more than 3-fold separation between a NOAEL and LOAEL had there been one in the Heinze (1999) study.

- UF_D - An uncertainty factor of 3 ($10^{0.5}$ = 3.16) is selected to account for deficiencies in the database for microcystins. 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, 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.

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., $10^{½}$) when appropriate (U.S. EPA, 2002).

4.0 RISK CHARACTERIZATION

The following topics describe important conclusions used in the derivation of the health advisory. This section characterizes each topic and its impact on the health advisory.

4.1 Use of microcystin-LR as a surrogate for total microcystins

Among the approximately 100 different congeners of microcystins known to exist, microcystin-LR is the most common. 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) 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 50% lethal doses (LD_{50}) of 36 ng/g-bw for -LR, 39 ng/g-bw for -LA, 91 ng/g- bw for -LY and 111 ng/g-bw for –RR. Similarly, Gupta et al., (2003) determined LD_{50} for the microcystin congeners LR, RR and YR in female mice using DNA fragmentation assay and histopathology examinations of the liver and lung. The acute LD_{50} determination showed that the most toxic congener 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 acids (-LA, -LR, -and -YM), and the least toxic are those with hydrophilic amino acids, such as microcystin-RR.

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 lipophilicity and polarity of the congeners may lead to variable absorption by non-injection routes of exposure.

The potential health risks from exposure to mixtures of microcystin congeners is unknown, and since microcystin-LR is one of the most potent congeners and has the majority of toxicological data on adverse health effects, microcystin-LR is used as a surrogate for all microcystins in the health advisory.

4.2 Consideration of Study Duration

EPA used a 28-day study conducted by Heinze (1999) to derive the Ten-day HA for microcystins. It is standard to use studies that are 7 to 30 days in duration to derive a 10-day advisory value. In the study conducted by Heinze (1999), rats were dosed daily via drinking water with microcystin and sacrificed at the conclusion of the study. No interim sacrifices were performed to evaluate effects at 10 days or any other time less than the full 28 days. At the conclusion of the 28-day study, adverse effects observed in the liver included increases in liver weight, slight to moderate liver necrosis lesions accompanying hemorrhages at the low dose with increased severity at the high dose, and changes in serum enzymes indicative of liver damage. Given the lack of interim effects data, it is not known when during the 28-day study these effects were manifested.

4.3 Consideration of Reproductive Effects as Endpoint

Upon consideration of all available studies, liver effects were 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). However, while the liver is the primary 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).

In a study conducted by 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. Because 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 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 be statistically significant. 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 mice in the study introduces uncertainty in 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), 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 testes reported by the authors did not provide sufficient detail to adequately assess the degree of damage.

The quality of the medium used for the sperm analysis, 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 could not be verified.

Based on the limitations in study design, report and methods used by Chen et al. (2011), EPA concluded that the quantitative data on decreased sperm counts and sperm motility were not appropriate for determining the point of departure for the derivation of the RfD for microcystin.

4.4 Allometric Scaling Approach

Allometric scaling was not applied in the development of the short term RfD for microcystins. In the development of short-term advisory values (One-day and Ten-day), parameters are used that reflect exposures and effects for infants up to one year of age, rather than for adults. The body weight scaling approach is derived from the relationship between body surface area and basal metabolic rate in adults. Infants/children surface area and basal metabolic rates are very different than adults with a slower metabolic rate. In addition, limited toxicokinetic data are available to assess the appropriateness of body weight scaling in early life. The body weight scaling procedure has typically been applied in the derivation of chronic oral RfDs and cancer assessments, both of which are concerned with lifetime repeated exposure scenarios (U.S. EPA, 2012). Thus, given the short term duration of the critical study and the development of a short term RfD for determination of a Ten-day HA value, and the application of the Ten-day HA to infants and pre-school age children, the application of the body weight scaling procedure is not appropriate for this scenario.

In addition, for short-term advisories (one-day and ten-day duration), EPA assumes all exposure is derived from drinking water and, therefore, no Relative Source Contribution (RSC) term is applied. For lifetime health advisory values, EPA does include an RSC that reduces the advisory value to account for other potential sources.

4.5 Benchmark Dose (BMD) Modeling Analysis

The data set reported by Heinze (1999) was evaluated for BMD modeling. Heinze (1999) demonstrated dose-related liver changes and statistically significant effects at the lowest dose (50 µg/kg/day). Histological changes were also observed in all the animals (ten) in each dose group (Table 3-2). Although differences in the degree of necrosis were observed with or without hemorrhage related to dose, all the histological effects including Kupffer cell activation and PAS staining showed no dose-response since all ten animals at the low and high doses displayed liver damage associated with each effect. Therefore, the dose-response for the sum of the incidence categories (slight, moderate, and intensive damage), are not amenable to BMD modeling. As a result, the LOAEL of 50 µg/kg/day described by Heinze (1999) was used as the POD for development of the HA.

4.6 Carcinogenicity Evaluation

While there is evidence of an association between liver and colorectal cancers in humans and microcystins exposure and some evidence that microcystin-LR is a tumor promoter in mechanistic studies, there is inadequate information to assess carcinogenic potential of microcystins in humans (U.S. EPA, 2005). The human studies are limited by lack of exposure

information and the uncertainty regarding whether or not these studies adequately controlled for confounding factors such as Hepatitis B infection. No chronic cancer bioassays for microcystins in animals are available.

The only oral study that examined the tumorgenicity of microcystin-LR failed to find preneoplastic nodules in the livers of groups of 22 mice receiving up to 100 doses of 0 or 80 µg/kg/day over 7 months. Some studies suggest that microcystin-LR is a tumor promoter. Given the potential impact on the cell cytoskeleton, necrotic effects on liver cell generation of reactive oxygen species (ROS), and other biochemical changes, this finding is not surprising. The work by Nishiwaki-Matsushima et al., 1992 that compares glutathione *S*-transferase placental form-positive (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. The results from the second part of the same study that compare P-GST foci following initiation with DEN followed by microcystin-LR (10 µg/kg), both before and after a partial hepatectomy, support this conclusion.

The International Agency for Research on Cancer (IARC) classified microcystin-LR as a Group 2B (possibly carcinogenic to humans) based on the conclusion that there was strong evidence supporting a plausible tumor promoter mechanism for these liver toxins. U.S. EPA's Cancer Guidelines (2005) state that the descriptor of *"inadequate information to assess carcinogenic potential"* is appropriate when available data are judged inadequate for applying one of the other descriptors or for situations where there is little or no pertinent information or conflicting information. The guidelines also state that (p. 2-52) "Descriptors can be selected for an agent that has not been tested in a cancer bioassay if sufficient other information, e.g., toxicokinetic and mode of action information, is available to make a strong, convincing, and logical case through scientific inference". In the case of microcystins, the data suggest that microcystin-LR may be a tumor promoter but not an initiator. Without stronger epidemiology data and a chronic bioassay of purified microcystin-LR, the data do not support classifying microcystin-LR as a carcinogen.

4.7 Uncertainty and Variability

Several uncertainty factors were applied in several areas to account for incomplete information. Human data on the toxic effects of microcystins are limited. Quantification of the absorption, distribution, and elimination of microcystins in humans following oral, inhalation or dermal exposure is not well understood. The clinical significance in humans for biological changes observed in experimental animals such as decreased sperm count and motility, and microscopic lesions in the testes needs further analysis. In animal studies with oral exposures to microcystins, some adverse effects in males such as reduced testosterone levels, as well as toxicity to the female reproductive tissues and those of offspring have not been fully characterized. No data are available to quantify the differences between humans and animals for the critical health endpoints. There is uncertainty regarding susceptibility and variability in the human population following exposure to microcystins and the relative toxicity of other microcystins congeners when compared to microcystin-LR. Additional information is needed on the potential health risks from mixtures of microcystins with other cyanotoxins, as well as biological and chemical stressors present in source water and drinking water supplies.

In addition, for short-term advisories (One-day and Ten-day duration), EPA assumes all exposure is derived from drinking water and, therefore, no Relative Source Contribution (RSC) term is applied. For lifetime health advisory values, EPA does include an RSC that reduces the advisory value to account for exposure to other potential sources.

4.8 Susceptibility

Available animal data are not sufficient to determine if there is a definitive difference in the response of males versus females following oral exposure to microcystins. 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 at 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 infants to one-year olds fed exclusively with powdered formula prepared with tap water, drinking water is the dominant route of exposure to cyanotoxins. There are significant differences in exposure between these life-stages that impact risk.

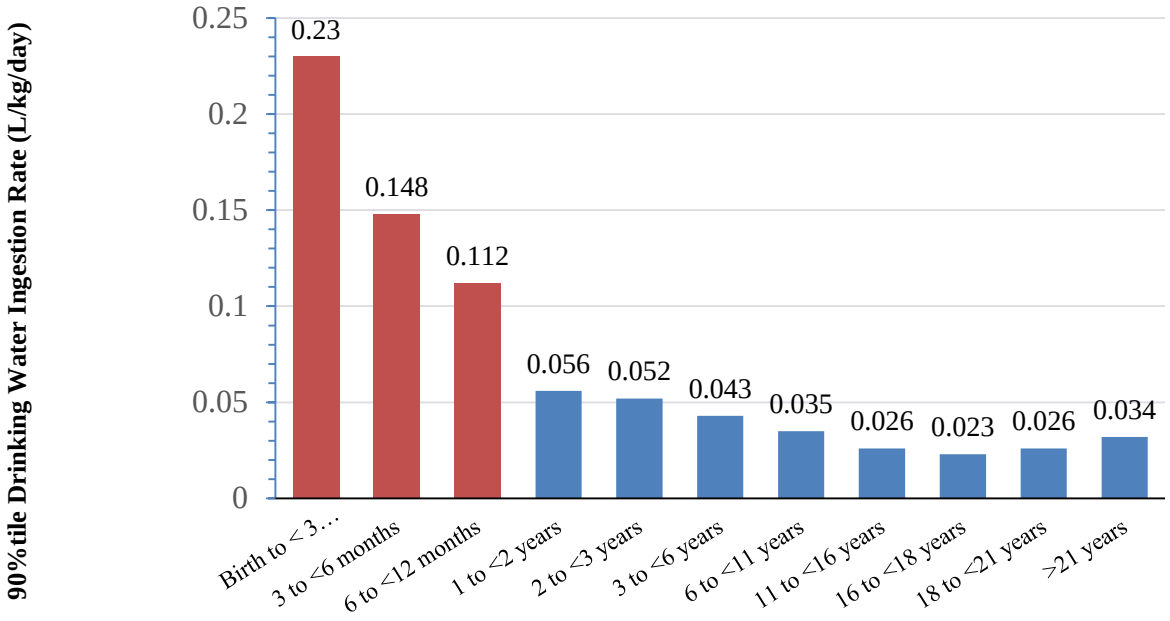
Based on the available studies in animals, individuals with liver and/or kidney disease may be more susceptible than the general population since the detoxification mechanisms in the liver and impaired excretory mechanisms in the kidney may be compromised. Data from an episode in a dialysis clinic in 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 used to prepare the dialysate is contaminated with cyanotoxins. Other potentially sensitive individuals include pregnant woman, nursing mothers, and the elderly.

4.9 Distribution of Body Weight and Drinking Water Intake by Age

Both body weight and drinking water intake are distributions that vary with age. EPA has developed two health advisory values, a Ten-day HA of 0.3 µg/L based on exposure to infants over the first year of life, and a Ten-day HA of 1.6 µg/L based on exposure to adults, over 21 years of age. Section 4.10 discusses how EPA recommends application of these values to other age groups.

The U.S. EPA (2011a) Exposure Factors Handbook provides values for drinking water ingestion rate and corresponding body weight. The estimated 90th percentile of community water ingestion for the general population (males and females of all ages) has been used as the default value for water ingestion. EPA plotted the 90th percentile of drinking water intake using Table 3-19 for ages ≤3 years, and Table 3-38 for ages >3 years due to sample size in the respective studies. Age groups <3 months in Table 3-19 were combined due to insufficient sample sizes. Figure 4.1

Figure 4-1. 90th Percentile Drinking Water Ingestion Rates by Age Group



Adapted from U.S. EPA 2011 Exposure Factors Handbook (U.S.EPA, 2011a).

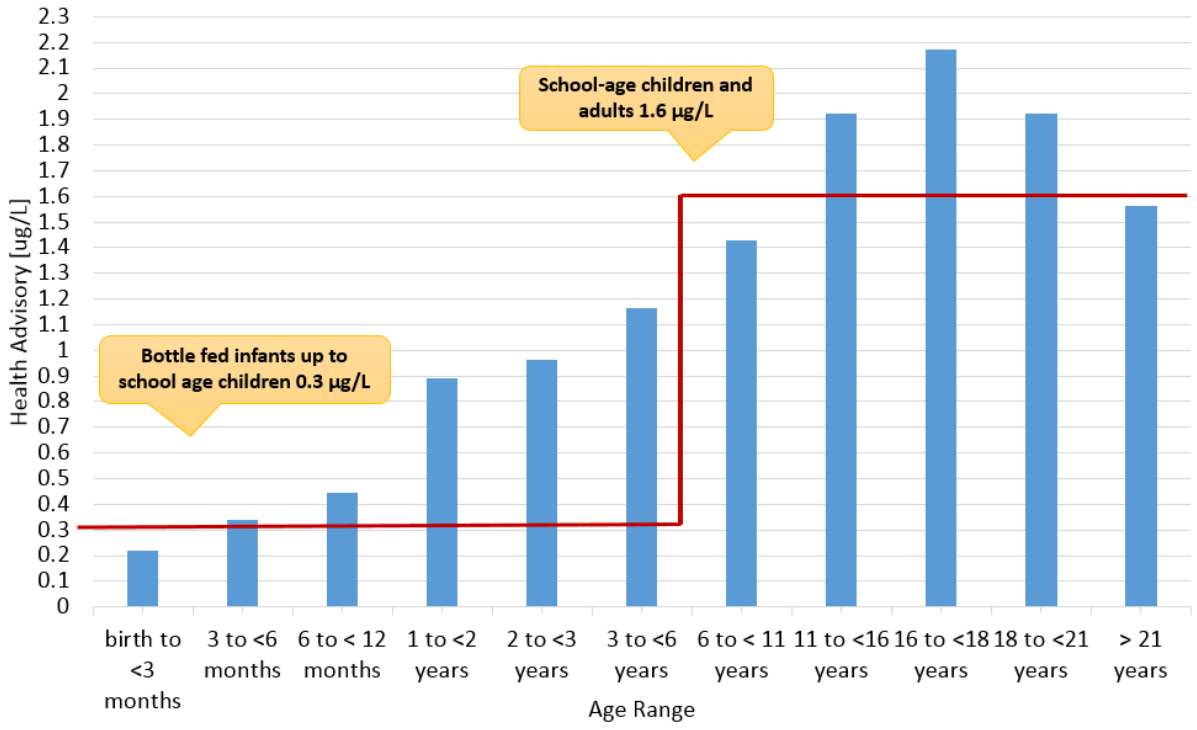
represents the 90th percentile drinking water ingestion rates (L/kg/day) for each age group (located on top of the columns). Bottle-fed ages are shown in red (first three columns on the left).

Based on the drinking water intake rates for children <12 months (0.15 L/kg-day), the exposure of children is over 4 times higher than that of adults >21 years old on a body weight basis (0.034 L/kg-day). Infants from birth to 3 months may be exclusively bottle-fed and therefore, have a higher ingestion rate. After 3 months of age, typically around 4 to 6 months of age, other food and liquids are introduced into the infant diet, lowering the ingestion rate of drinking water. Drinking water contributes the highest risk of the total cyanotoxin intake for infants to one-year-olds fed exclusively with powdered formula prepared with tap water containing cyanotoxins. At the age of 6, children's intake of drinking water relative to their body weight is approximately the same as those of an adult (>21 years). Data evaluating the transfer of microcystins through breast milk are not available for humans.

4.10 Distribution of Potential Health Advisory Values by Age

Using the ingestion rates for each age-group (from Figure 4-1), EPA estimated Ten-day HA values for microcystins for each age group (plotted on Figure 4-2) to demonstrate the variability due to body weight and drinking water intake by age.

Figure 4-2. Ten-day Health Advisories for Microcystins by Age Group



EPA decided to apply the Ten-day HA value calculated for infants over the first year of life (0.3 µg/L) to all bottle-fed infants and young children of pre-school age because these age groups have higher intake per body weight relative to adults. As Figure 4.2 demonstrates, when the Ten-day HA is estimated by age group, the calculated HA value for infants from birth to 3 months old is 0.2 µg/L, slightly below the infant health advisory value of 0.3 µg/L. EPA believes that infants from birth to 3 months old are not at a disproportionate risk at a 0.3 µg/L advisory value because a 30-fold safety factor is built into this calculation to account for human variability and deficiencies in the database. The estimated Ten-day HA values for infants from 3 months old through pre-school age groups (less than 6 years old) are at or above the advisory value of 0.3 µg/L. Therefore, children within these age groups are adequately protected by the advisory value for bottle-fed infants and young children of pre-school age. EPA decided to apply the adult Ten-day HA value of 1.6 µg/L to school age children (children older than or equal to 6 years) through adulthood because children's intake of drinking water relative to body weight in this age group is almost the same as those of an adult (>21 years).

5.0 ANALYTICAL METHODS

This Health Advisory (HA) for the Cyanobacterial Microcystin Toxins is applied to total microcystins which should include all of the measureable microcystin congeners within the cyanobacterial cells (intracellular) and outside the cell (extracellular).

Extracellular microcystins (either dissolved in water or bound to other materials) typically make up less than 30% of the total microcystin concentration in source water (Graham et al., 2010). Most of the toxin is intracellular, and released into the water when the cells rupture or die. Both intracellular and extracellular microcystins may also be present in treated water, depending on the type of treatment processes in place. Therefore, it is important to note that analysis for microcystins should account for both intracellular and extracellular toxins in samples when intact cells may be present. Release of intracellular microcystins is achieved by rupturing or lysing the cell walls in order to expose the intracellular microcystins. Cell lysis can be achieved by a variety of methods including sequential freeze-thawing, freeze drying, and mechanical or sonic homogenization. Following cell lysis, microcystins may need to be extracted for some analytical methods. At low concentrations, the direct determination of microcystins may not be feasible, and a preconcentration step may be required. Typically samples are filtered and/or centrifuged after cell lysis to remove cell fragments and particulates. This may be followed by freeze-drying or solid-phase extraction (SPE). Typical elution solvents are dilute acid, methanol, acidified methanol/water mixtures, and butanol/methanol/water mixtures.

Preconcentration is generally needed when techniques such as liquid chromatography are used in order to achieve limits of detection in the low-µg/L and ng/L range. Extraction efficiency has been shown to vary depending on the type of solvent, the hydrophobicity of the congener, the water content of the cells (freeze-dried versus frozen) and differences between field samples and laboratory cultures. Variations in extraction efficiency may impact the accurate quantitation of microcystins so the use of a surrogate compound to monitor the extraction efficiency is strongly recommended. Responsible authorities should ensure that the appropriate methods and preparation techniques (extraction, concentration and separation) are being used in the laboratory depending on the type of sample and the analytical method selected.

Analytical methods available for the detection of microcystins in drinking water include reversed phase high performance liquid chromatography (HPLC) coupled with mass spectrometric (MS, MS/MS) or ultraviolet/photodiode array detectors (UV/PDA), Enzyme Linked Immunosorbent Assays (ELISA), and Protein Phosphatase Inhibition Assays (PPIA).

EPA has developed a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for microcystins and nodularin (combined intracellular and extracellular) in drinking water (Method 544; U.S. EPA, 2015). Accuracy and precision data have been generated in reagent water, and finished ground and surface waters for the following compounds: microcystin-LA (microcystin-LA), -LF (microcystin-LF), -LR (microcystin-LR), -LY (microcystin-LY), -RR (microcystin-RR), -YR (microcystin-YR), and nodularin-R (NOD). This method is intended for use by analysts skilled in solid phase extractions, operation of LC/MS/MS instruments, and the interpretation of associated data. The single laboratory lowest concentration minimum reporting levels (LCMRLs) for this method range from 2.9 to 22 ng/L (0.0029-0.022 µg/L). The Detection Limit (DL) for analytes in this method range from 1.2 to 4.6 ng/L. In this method, a 500 mL water

sample (fortified with an extraction surrogate) is filtered, and both the filtrate and the filter are collected. The filter is placed in a solution of methanol containing 20% reagent water and held for at least one hour at -20 ºC to release the intracellular toxins from cyanobacteria cells captured on the filter. The liquid is drawn off the filter and added back to the 500-mL aqueous filtrate. The 500-mL sample (plus the intracellular toxin solution) is passed through a SPE cartridge to extract the method analytes and surrogate. Analytes are eluted from the solid phase with a small amount of methanol containing 10% reagent water. The extract is concentrated to dryness by evaporation with nitrogen in a heated water bath, and then adjusted to a 1-mL volume with methanol containing 10% reagent water. A 10-µL injection is made into an LC equipped with a C8 column that is interfaced to an MS/MS. Analytes are separated and identified by comparing the acquired mass spectra and retention times to reference spectra and retention times for calibration standards acquired under identical LC/MS/MS conditions. The concentration of each analyte is determined by external standard calibration. To download Method 544 Determination of Microcystins and Nodularin in Drinking Water by Solid Phase Extraction and Liquid Chromatography/tandem Mass Spectrometry (LC/MS/MS), please go to: http://www.epa.gov/nerlcwww/ordmeth.htm

High performance liquid chromatography (HPLC) is widely used to separate microcystin congeners. A variety of stationary phases have been used including reversed-phase C_{18} columns, amide C_{18} columns, internal surface reversed-phase columns or ion exchange columns. Optimization of chromatographic parameters is needed to ensure a good resolution of analytes. In addition to mass spectrometry, ultraviolet/visible absorbance is a commonly used detection techniques with HPLC. Most microcystin congeners have similar absorption profiles between 200 and 300 nm. The wavelength of the UV/visible detector can be set at these values to record the responses of microcystins in sample extracts separated by the HPLC. The retention time, UV spectra and peak area of commercially available or laboratory standards is the basis of identification and quantification of microcystins using HPLC-UV/visible detection. However, due to the limited number of commercially available standards, the toxins are often quantified by comparison to an microcystin-LR standard and reported in terms of microcystin-LR equivalence. HPLC-UV/visible is susceptible to interferences from natural organic materials (NOMs). Detection limits will depend partially on the sample volume extracted, the concentration of the toxins, and the presence of interfering contaminants.

A variety of antibodies have been isolated against microcystin-LR and microcystin-RR, as well as recombinant antibody fragments and antibodies against the amino acid ADDA. Commercial ELISA kits that contain all of the reagents needed for analysis have also been developed and typically provide a cross reactivity chart for some of the congeners (i.e., microcystin-LR, -RR, YR, nodularin) that are commonly found in water. These range from 50-85% for microcystin-RR, 35-181% for microcystin-YR and 10-124% for microcystin-LA. Detection of the total microcystins will be expressed as the sum of the congeners provided from ADDA ELISA. The methods detection limit (MDLs) of several commercial laboratory ELISA kits have been reported to range from 0.04 to 0.2 µg/L for microcystin-LR. Commercial ELISA kits generally have quantitation ranges from 0.2 (LOQ) to an upper limit of 5 µg/L. Two high sensitivity ELISA plate kits have become commercially available with MDLs ranging from 0.04 to 0.05 µg/L.

PPIAs are used with a variety of detection methods and substrates including radioactive detection assays using ^{32}P-radiolabelled substrates and colorimetric assays using p-nitrophenol phosphate as the substrate. The method has also been adopted for fluorescence measurements using the substrates methylumbelliferyl phosphate. The detection limit of total microcystins, reported as microcystin-LR equivalents (microcystin-LR_{equiv}) using radiometric protein phosphatase assays is approximately 0.1 µg/L or less, and using colorimetric PP1 inhibition assays range between 10 to 20 ng/mL (0.01 to 0.02 µg/L).

Rapid tests for the identification of the presence of microcystins in water have been developed for use in the field. Field test kits can be used as a presence/absence tool for determining if a bloom is toxic or if treatment plant operations need to be adjusted during a bloom event but do not currently have sufficient sensitivity at microcystin concentrations below 1 µg/L to be used for treated water analyses. Commercially-available test kits use a variety of methods including immunochromatography (test strips), ELISA, and phosphatase inhibition to estimate the level of microcystins in a water sample. In general, the results of field test kits should be considered qualitative and should only be used to conduct a preliminary assessment of microcystin levels. The applicability of test kits is between 1 and 5 µg/L of microcystins with a detection limit of approximately 0.5 µg/L. Several field test kits do not include a lysing agent and, therefore, only determine the presence of extracellular microcystins. When using these field test kits, users should consult the manufacturer regarding an appropriate lysing technique if the detection of both intracellular and extracellular microcystins is required.

A new approach using laser diode thermal desorption-atmospheric pressure chemical ionization interface coupled to tandem mass spectrometry (LDTD-APCI-MS/MS) has been developed for the analysis of total microcystins in complex environmental matrices. The method is based on oxidation of the MCs in a sample using potassium permanganate under alkaline conditions to produce 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB). MMPB is then extracted and directly injected (no chromatographic separation) into the LDTD-APCI-MS/MS system. This approach results in ultra-fast sample analysis with simple sample preparation, reducing time and material costs associated with chromatographic separation. This method does not require individual MC standards, but similar to ELISA and PPIA, the results do not provide information on the identity of the individual MC congeners. The MDL and LOQ are 0.2 and 0.9 µg/L, respectively (Roy-Lachapelle et al., 2014).

6.0 TREATMENT TECHNOLOGIES

The information below is adapted from the Health Canada Guidelines for Cyanobacteria Toxins in Drinking Water, available later in 2015.

Detailed information on the operational considerations of a variety of treatment methods can be found in the EPA *Drinking Water Treatability Database for Microcystins* (U.S. EPA, 2007); the *International Guidance Manual for the Management of Toxic Cyanobacteria* (GWRC, 2009) available at http://www.waterra.com.au/cyanobacteria-manual/PDF/GWRCGuidanceManualLevel1.pdf, and *Management Strategies for Cyanobacteria (Blue-Green Algae): A Guide for Water Utilities* (Newcombe et al., 2010) available at http://www.researchgate.net/profile/Lionel_Ho/publication/242740698_Management_Strategies_for_Cyanobacteria_(Blue-Green_Algae)_A_Guide_for_Water_Utilities/links/02e7e52d62273e8f70000000.pdf

For additional information on treatment strategies commonly used or being considered by water systems vulnerable to cyanotoxins, please see *Recommendations for Public Water Systems to Manage Cyanotoxins in Drinking Water* (U.S. EPA, 2015b).

6.1 Management and Mitigation of Cyanobacterial Blooms in Source Water

Algaecides can be applied to lakes and reservoirs to mitigate algal blooms, including cyanobacteria. In most cases, depending on the cyanobacteria species present, the application of algaecides has the potential to compromise cell integrity releasing cyanotoxins into the source waters. Chemical treatment to control blooms in drinking water sources in the early stages of the bloom when cyanobacterial concentrations are still relatively low (usually under 5,000 to 15,000 cells/mL) (WHO, 1999), are less likely to release significant cyanotoxin concentrations upon cell lysis and is able to mitigate or prevent a cyanobacterial bloom from proliferating as the season progresses. If harmful cyanobacterial blooms occur, utilities may take action to investigate alternative source water sources, change intake locations or levels to withdraw source water with minimal cyanotoxin concentrations, or investigate methods of destratification in the water source. Purchasing water from a neighboring interconnected water system that is unaffected by the bloom may also be an option for some systems.

Clays and commercial products such as aluminum sulfate (alum) have been used for the management of blooms in source waters. Alum treatment efficiency depends on the alum dose and the type of flocculant. Aeration and destratification have also been used to treat cyanobacterial blooms, usually in smaller water bodies (from one acre to several tens of acres). Active mixing devices, diffuse air bubblers, and other means of reducing stratification have proven to be effective in controlling outbreaks and persistence of blooms in relatively small shallow impoundments (around <20 feet deep). These strategies can be applied to the entire source water body or to just a portion of the lake depending on the need, and size and depth of the water body relative to the source water intake(s).

Hydrogen peroxide (H_2O_2) has been used as an algaecide in source water because of a rapid reaction time (90% of bloom collapsed in 3 days and 99% in 10 days), and environmentally safe reaction products (oxygen and water) (Wang et al., 2012; Matthjis et al; 2012). The

drawbacks (aside from cell lysis) are that oxidant breakdown is so rapid that repeated applications are needed. Further understanding of this technique is needed (Matthjis et al., 2012).

The use of ultrasonic sound waves to disrupt cyanobacterial cells has also been investigated as a potential source water treatment option (Rajasekhar et al., 2012). It is environmentally friendly compared to chemical treatment strategies. The technique has also been reported to be capable of degrading microcystin-LR (Song et al., 2005). Drawbacks include that application frequencies are difficult to calculate and are system-specific; and that applications on large scale require more powerful and, therefore, more expensive equipment. Sonication shows potential for use in cyanobacterial bloom management, but further study to determine effective operating procedures is needed before it can be considered as a feasible approach (Rajasekhar et al., 2012).

Excess nutrients are thought to be a primary driver of cyanobacterial blooms. Long-term prevention of cyanobacterial blooms likely requires reductions in nutrient pollution. Excess nitrogen and phosphorus in aquatic systems can stimulate blooms and create conditions under which harmful cyanobacteria thrive. Thus, managing nutrient pollution sources within a watershed in addition to waterbody-specific physical controls (in systems that are amenable to those controls) tends to be the most effective strategy. Nutrient pollution can be from urban, agricultural, and atmospheric sources and, therefore, reductions can be achieved through a variety of source control technologies and best management practices.

6.2 Drinking Water Treatment

Effective treatment of cyanotoxins in drinking water includes the evaluation and selection of appropriate treatment methods. Any variation in treatment methods aimed at reducing toxins concentrations need to be tailored to the type(s) of cyanobacteria present and the site-specific water quality (e.g. pH, temperature, turbidity, presence of natural organic material (NOM), etc.), the treatment processes already in place, and the utility's multiple treatment goals (e.g., turbidity and total organic carbon (TOC) removal, disinfection requirements, control of disinfection by-products (DBP) formation, etc.). Utilities need to have an understanding of the type and concentration of cyanotoxins present in the source water and should conduct site-specific evaluations such as jar testings and piloting in order to determine the most effective treatment strategy. Potential target parameters include: chlorophyll-a, turbidity, cyanobacterial cells and extracellular and intracelluar toxins.Care should be taken to avoid cell lysisTo remove both intracellular and extracellular toxins from drinking water, a multi-barrier approach is required, which may consist of conventional filtration for intracellular cylindrospermopsin removal and additional processes such as activated carbon, biodegradation, advanced oxidation, and small-pore membrane processes (e.g. nanofiltration and reverse osmosis), for the removal or oxidation of extracellular cylindrospermopsin. The most effective way to deal with cyanobacteria cells and their toxins, is to remove the cells intact, without damaging them, to prevent the release of additional extracellular toxins into the water.

6.2.1 Conventional Treatment for Microcystins

In the absence of cell damage, conventional treatment employing coagulation, flocculation, clarification (sedimentation or dissolved air flotation) and rapid granular filtration can be effective at removing intact cells and the majority of intracellular toxins (cell bound) (Chow et al., 1998; Newcombe et al., 2015). However, if toxins are released into solution, a combination of conventional treatment processes with oxidation, adsorption, and/or advanced treatment needs to be considered to treat both intracellular and extracellular cyanotoxins.

The efficiency of the conventional treatment processes to remove cyanobacterial cells and intracellular microcystins has been shown to vary from 60 to 99.9%. Factors that impact removal include the cyanobacterial species and cell density, coagulant type and dose, pH, NOM, and operational parameters such as flocculation time, frequency of filter backwashing and clarifier sludge removal (Vlaski et al., 1996; Hoeger et al., 2004; Jurczak et al., 2005; Zamyadi et al., 2012a, 2013c; Newcombe et al., 2015). Typically, 60 to 95% of cells and intracellular microcystins can be removed during sedimentation with as much as 99.9% removal achieved through filtration (Lepisto et al., 1994; Drikas et al., 2001; Hoeger et al., 2004; Newcombe et al., 2015). The efficiency of coagulation and clarification for cell removal is dependent on pH, coagulant type and dose and the morphological characteristics of the cyanobacteria. Rapid sand filtration without pre-treatment (i.e., direct filtration, without coagulation/clarification) is not effective for cyanobacterial cell removal.

If operated properly, conventional treatment (coagulation, flocculation, clarification and filtration), does not cause cell lysis or increases in the extracellular microcystin concentrations of treated water (Chow et al., 1998, 1999; Drikas, 2001; Sun et al., 2012). Drinking water treatment plants utilizing conventional treatment followed by oxidation or activated carbon may remove both intracellular and extracellular microcystins up to 99.99% of total microcystins to achieve concentrations below 0.1 µg/L in treated water (Karner et al., 2001; Lahti et al., 2001; Hoeger et al., 2005; Jurczak et al., 2005; Rapala et al., 2006; Zamyadi et al., 2013a). Conventional treatment is generally considered to have limited effectiveness for the removal of the extracellular microcystins. Therefore, additional processes such as adsorption, chemical oxidation, biodegradation or reverse osmosis, and nanofiltration are required to remove extracellular microcystins.

Although microfiltration and ultrafiltration membranes can remove both cyanobacterial cells and intracellular microcystins, removal of extracellular microcystins using ultrafiltration is variable (35 to 70%) and microfiltration is not effective (Gijsbertsen-Abrahamse et al., 2006; Dixon et al., 2011a, b). Nanofiltration and reverse osmosis membranes can achieve high removals of intracellular and extracellular microcystins, from 82% to complete removal (Westrick et al., 2010; Dixon et al., 2010). Pore size, among others, is an important factor in removal efficiency for these processes.

Successful removal of cyanobacterial cells and intracellular microcystins will depend on proper operations of the conventional treatment processes (Hoeger et al., 2004; Dugan and Williams, 2006; Ho et al., 2013; Zamyadi et al., 2012a, 2013c). Operational considerations for removing cyanobacterial cells using coagulation, flocculation and clarification are similar to

considerations for achieving effective particle removal. The appropriate coagulant and coagulation pH should be determined through jar-testing to maximize cell removal. In jar-testing, the NOM, chlorophyll-a, or cyanobacterial cell count can be used to optimize the coagulation conditions for cyanobacterial cell removal (Sklenar et al., 2014; Newcombe et al., 2015). Sufficient mixing should be provided at the point of chemical addition to ensure rapid and uniform contact, and an appropriate mixing speed should be determined to optimize the flocculation process (GWRC, 2009). It is important to minimize the potential for the accumulation of cyanobacterial cells as scums at the surface of sedimentation basins and filters (Zamyadi et al., 2012a, 2013c).

Effective sludge removal from sedimentation/clarification processes is important to minimize the release of intracellular and extracellular microcystins into the surrounding waters, as significant cell numbers can accumulate within the sludge, and cells contained within the sludge can lyse rapidly (Drikas et al., 2001; Ho et al., 2013; Zamyadi et al. 2012a). It has been reported that accumulation of cyanobacterial cells and microcystins in clarifiers can lead to their breakthrough into filter effluent. In addition, cell lysis can occur in the clarifier sludge, increasing the extracellular concentration of microcystins in the treatment plant. Therefore sludge management (decreased sludge age) in clarifiers and increased frequency of backwashing of filters is important because settled/filtered cells can remain viable and possibly multiply over a period of at least 2 to 3 weeks. Within 1 day, some cells in the sludge can lyse and release NOM and taste and odor compounds, in addition to cyanotoxins (Newcombe et al., 2015). Additionally, backwash water from the filters may contain cyanobacterial cells and/or extracellular microcystins; hence, care needs to be taken if spent backwash water is recirculated to the beginning of the treatment process to prevent the reintroduction of cells and toxins into the treatment train. Although longer filter run-times are typically desirable between backwashing, during periods of high algal concentrations, cells can accumulate in the filter, which can potentially lead to a significant amount of extracellular microcystins released into the filtered water. The optimum balance between maximizing water production and minimizing the risk of toxin breakthrough will be plant-specific.

6.2.2 Adsorption

Adsorption processes, such as granular activated carbon (GAC) or powdered activated carbon (PAC), are effective at removing extracellular microcystins but are not capable of removing intact cells and intracellular toxins (Lambert et al., 1996; Newcombe, 2002; Newcombe et al., 2003). Removal through adsorption depends on many factors including the type of activated carbon used, the microcystin congener and water quality conditions. In general, mesoporous carbons (such as chemically-activated wood-based carbons) are the most effective for the removal of microcystins (Newcombe et al., 2010). Other factors such as the type of microcystin congener present, the raw water quality (i.e., NOM and pH) and contact time affect microcystins removal efficiency when using activated carbon processes. In addition, shortened filter run times or filter overload may happen during cyanobacteria blooms. Therefore, water treatment plants should conduct jar-testing to determine the most effective activated carbon dose, type, and feed point prior to the application without affecting other water quality parameters and treatment processes (Sklenar et al., 2014).

The performance of GAC filtration for extracellular microcystin removal depends upon the empty bed contact time (EBCT), carbon age, carbon pore size, and raw water quality characteristics such as NOM and pH, as well as the microcystin variant (Newcombe, 2002; Newcombe et al., 2003; Ho and Newcombe, 2007; Wang et al., 2007). Solution chemistry can also affect microcystin-LR adsorption onto GAC. Enhanced removal of microcystin-LR has been observed at lower pH (2.5 versus 6.5) due to either precipitation or reduced solvency effect (Pendleton et al., 2001).

Removal of extracellular microcystins by PAC can be highly effective (up to 95%) depending on the microcystin congener and concentration, the PAC type and dose, the contact time and the water quality characteristics such as TOC (Newcombe et al., 2003; Cook and Newcombe; 2008; Ho et al., 2011). According to Newcombe et al. (2010), a PAC dose of 20 mg/L and a contact time of at least 45 minutes should be considered for removal of most extracellular microcystins (with the exception of microcystin-LA).

6.2.3 Chemical Oxidation

Chemical oxidation using chlorine, potassium permanganate, or ozonation can be effective at oxidizing extracellular microcystins, but can also impaired cell integrity, resulting in an increase in concentrations of extracellular microcystins in drinking water. By applying conventional filtration (or other filtration process) first to remove the majority of intact cells, the extracellular microcystin concentration is less likely to increase due to cell lysis when water is treated with oxidants. In cases where pre-oxidation (oxidant applied anywhere along the treatment process prior the filter influent) is practiced, it may need to be discontinued during an algal bloom or adjustments to the oxidant type and doses may be needed to minimize cell rupture prior to filtration (Newcombe et al., 2015).

The effectiveness of chemical oxidation of microcystins depends on the type of oxidant, dose, contact time, microcystin congener and water quality characteristics such as pH and dissolved organic carbon (DOC) (GWRC, 2009; Sharma et al., 2012). Laboratory-scale experiments have demonstrated that the general trend for the effectiveness of cyanobacterial cell and extracellular microcystin oxidation to be: ozone>permanganate>chlorine>chlorine-based oxidants (Acero et al., 2005; Rodriguez et al., 2007a, b; Ding et al., 2010; Sharma et al., 2012;). However, selection of the most appropriate oxidant for microcystins should be based on the characteristics of each water source, the disinfection requirements, and potential formation of disinfection by-products (DBPs) (Sharma et al., 2012).

It is also important to recognize that the use of oxidants may result in the formation of DBPs and should be considered when selecting a strategy for oxidizing microcystins (Merel et al., 2010; Zamyadi et al., 2012b; Wert et al., 2013). For example, ozone and chlorine dioxide can result in the formation of inorganic DBPs, such as bromate and chlorite/chlorate, respectively. Additionally, modifying pre-oxidation practices may compromise other treatment objectives (e.g., turbidity removal), and should be considered.

The oxidation of microcystins by chlorine has been found to be highly effective (>90% removal) under experimental conditions (Ho et al., 2006a; Acero et al., 2008; Merel et al., 2009; Sorlini and Collivignarelli, 2011). However, the effectiveness of chlorination on the oxidation of microcystins depends upon the chlorine dose, contact time, pH, temperature, and other water quality characteristics (Sharma et al., 2012). Several studies have found that microcystins are efficiently oxidized if pH is maintained below 8, the chlorine dose is greater than 3 mg/L and 0.5 to 1.5 mg/L of free chlorine residual is present after 30 minutes of contact time (Nicholson et al., 1994; Acero et al., 2005; Ho et al., 2006a; Xagoraraki et al., 2006; Newcombe et al., 2010). However, much higher chlorine doses (2 to 10 mg/L) are required to lyse the cyanobacterial cell and then oxidize the previously cell-bound microcystins (Zamyadi et al., 2013b).

The oxidation of microcystins in water by permanganate is one of the more effective processes for oxidizing extracellular microcystins in water (Sharma et al., 2012). Rodriguez et al. (2007a) exhibited a 90% oxidation of microcystin-LR at a dose of 1.0 mg/L, a contact time of 60 minutes, a pH of 8, and a temperature of 20°C. Complete oxidation occurred at a dose of 1.5 mg/L (Rodriguez et al., 2007a). Treatment plants considering potassium permanganate for oxidation of microcystins should be aware that permanganate can discolor water when it is present in excess of 0.05 mg/L. Therefore, dosage control and process monitoring (e.g., visual inspection of the basin effluent color, measuring permanganate residual) is important in avoiding consumer complaints (MWH, 2012).

The oxidation of microcystins in water by ozone has been shown to be highly effective (greater than 90% oxidation) in laboratory-scale studies (Rositano et al., 2001; Shawwa and Smith, 2001; Brooke et al., 2006). The efficacy depends on temperature, pH, ozone dose, contact time, and other water quality characteristics such as DOC and alkalinity (Sharma et al., 2012). Utilities should also be aware that the use of ozone may result in the formation of bromate and other DBPs.

Monochloramine is a weaker oxidant than chlorine and is not an effective treatment barrier for microcystins (Westrick et al., 2010).

Most laboratory studies have found that chlorine dioxide (ClO_2) is not effective for oxidizing extracellular microcystins (Kull et al., 2004, 2006; Ding et al., 2010; Sorlini and Collivignarelli, 2011) or cyanobacterial cells and intracellular microcystins (Ding et al., 2010; Wert et al., 2014) at dosages (1-2 mg/L) and contact times typically applied to drinking water.

6.2.4 Other Filtration Technologies

Biological filtration, using either biologically-active sand or activated carbon, has been shown to be effective for the removal of extracellular microcystins in bench- and pilot-scale studies (Keijola et al., 1998; Bourne et al., 2006; Ho et al., 2006b, 2008, 2012) and in limited full-scale studies (Grutzmacher et al., 2002, Rapala et al., 2006). The removal of intact cyanobacterial cells and their associated intracellular toxins through physical straining in slow sand filters has also been documented (Grutzmacher et al., 2002; Pereira et al., 2012). Biological filters also have the capability to remove particulate including intact cyanobacterial cells. Bank filtration may also be effective for the removal of microcystins (Lahti et al., 1998; Schijven et al., 2002). A detailed

review of biological treatment options for cyanotoxin removal conducted by Ho et al., (2012b) identified the type and concentration of microcystin-degrading bacteria, concentration of microcystins, and temperature as key factors that influence the efficiency of biological filtration for the removal of microcystins. In addition, the concentration of other organic matter within the source water may inhibit biodegradation, as microcystins may be a secondary substrate in the presence of NOM. Particle size, chemical composition and roughness or topography of the surface of the media used for filtration have also been identified as important factors for biofilm growth and ultimately the biodegradation of microcystins (Wang et al., 2007, Ho et al., 2012).

Membrane filtration including microfiltration (MF) and ultrafiltration (UF) can achieve greater than 98% removal of cyanobacterial cells and intracellular microcystins (Chow et al., 1997; Gijsbertsen-Abrahamse et al., 2006; Campinas and Rosa, 2010: Sorlini et al., 2013). Nanofiltration (NF), reverse osmosis (RO) and, to a lesser extent UF, can be used for both intracellular and extracellular microcystin removal (Neumann and Weckesser, 1998; Lee and Walker, 2008; Dixon et al., 2011a,b). The performance of membrane filtration for microcystin removal depends on characteristics of the membrane such as molecular weight cut-off (MWCO) and hydrophobicity, initial concentration, size and molecular weight of the microcystins, and operating parameters such as flux, recoveries and degree of fouling. It is recommended that cyanobacterial cells are removed prior to reverse osmosis to prevent membrane clogging and fouling.

Laboratory and pilot-scale studies have demonstrated that MF and UF can remove greater than 98% of cyanobacterial cells (Chow et al., 1997, Gijsbertsen-Abrahamse et al., 2006; Campinas and Rosa, 2010; Sorlini et al., 2013), and ultrafiltration can be moderately effective (35-70%) for removal of extracellular microcystins (Lee and Walker, 2008). Several studies have also demonstrated that the release of intracellular microcystins from the shear stress on cyanobacterial cells during MF and UF is possible, although it generally results in permeate microcystin concentration increases of less than 12 percent (Gijsbertsen-Abrahamse et al., 2006; Campinas and Rosa, 2010).

The removal of extracellular microcystins by NF and RO is very effective (greater than 90%) and depends on the MWCO, as the filtration of microcystins occurs via size exclusion (Gijsbertsen-Abrahamse et al., 2006).

6.2.5 Combined Treatment Technologies

In practice, full-scale treatment plants use a combination of treatment technologies (i.e., conventional filtration and chemical oxidation) in order to remove both intracellular and extracellular microcystins. Data indicate that utilities can effectively remove both intracellular and extracellular microcystins to achieve concentrations below 0.1 µg/L (Lahti et al. 2001; Boyd and Clevenger, 2002; Zurawell, 2002; Hoeger et al., 2005, Jurczak et al., 2005; Rapala et al., 2006; Haddix et al., 2007; Nasri et al., 2007; Zamyadi et al., 2013c). However, some studies have shown that the presence of high concentrations of cells (i.e., 10^5 cells/mL) and/or microcystins in raw water (100 µg/L) may be challenging for treatment plants to reduce concentrations to below 0.1 µg/L (Tarczyriska et al., 2001; Zamyadi et al., 2012a).

In most cases, utilities will be able to effectively remove intracellular microcystins with processes that are already in place (e.g., conventional treatment) when they are operated with a focus on cyanobacteria cell or NOM removal. Extracellular microcystins may also be removed in many treatment plants by using existing treatment such as chlorination after filtration or by the addition of PAC following conventional treatment (Carriere et al., 2010). Although it is possible to remove both intracellular and extracellular microcystins effectively using a combination of treatment processes, the removal efficiency can vary considerably. Utilities need to ensure that they are utilizing their existing treatment processes to their fullest capacity for removal of both cyanobacterial cells and extracellular microcystins and that appropriate monitoring is being conducted to ensure that adequate removal is occurring at each step in the treatment process.

6.3 Point-of-Use (POU) Drinking Water Treatment Units

Limited information is available on residential treatment units for the removal of cyanobacteria cells and microcystins. A study using common water filtration and purification systems found that the efficacy of POU filtration devices to remove microcystin (LR) varies considerably with the type of device being used (Pawlowicz et al., 2006). Microcystin-LR was successfully removed using carbon filters allowing only 0.05 to 0.3% of the toxin load to pass through the filter. However, more than 90% of microcystin-LR passed through string-wound filters and pleated paper. According to the authors, the use of carbon home filter devices tested in this study may provide additional protection beyond that provided by the drinking water treatment plant against human exposure to microcystin-LR. Additional studies are recommended to assess the efficacy of POU drinking water treatment units for other cyanotoxins and under other conditions. Third-party organizations are currently developing certification standards to test POU devices to evaluate how well they remove cyanotoxins from drinking water treatment units. Those standards are expected in the near future.

More information about treatment units and the contaminants they can remove can be found at http://www.nsf.org/Certified/DWTU/.

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Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin

Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin

U.S. Environmental Protection Agency
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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 toxin cylindrospermopsin 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 cylindrospermopsin, 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 cylindrospermopsin was included. The literature search included the following terms: cylindrospermopsin, 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 cylindrospermopsin.

- 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
- Cylindrospermopsin [CASRN 143545-90-8] Review of Toxicological Literature (ILS, 2000).

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 includes 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 cylindrospermopsin 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, 2000c)
- *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)
- *Exposure Factors Handbook 2011 Edition* (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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ABBREVIATIONS AND ACRONYMS

ADHD	Attention Deficit Hyperactivity Disorders
ALT	Alanine Aminotransferase
ALP	Alkaline Phosphatase
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
BGAS	Bluegreen Algae Supplements
BNCs	Binucleated Cells
BSO	Buthionine Sulfoximine
BUN	Blood Urea Nitrogen
BW	Body Weight
CASRN	Chemical Abstracts Service Registry Number
CCL	Contaminant Candidate List
CBMN	Cytokinesis Block Micronucleus Assay
CHO	Chinese Hamster Ovary
CI	Confidence Interval
CTA	Cell Transformation Assay
CYP450	Cytochrome P450
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DW	Dry Weight
DWHA	Drinking Water Health Advisories
ED_{50}	Median Effective Dose
ELISA	Enzyme Linked Immunosorbent Assay
EPA	United States Environmental Protection Agency
FEL	Frank Effect Level
G	Gram
GD	Gestation Day
GFR	Glomerular Filtration Rate
GSH	Glutathione
HAB	Harmful Algal Bloom
HESD	Health Effects Support Document
HPLC	High-Performance Liquid Chromatography
HSDB	Hazardous Substances Data Bank
IC_{50}	Inhibitory Concentration$_{50}$
ILS	Integrated Laboratory Systems
I.P.	Intraperitoneal
Kg	Kilogram
K_{ow}	Octanol Water Partition Coefficient
K_{oc}	Soil Organic Carbon-Water Partitioning Coefficient

L	Liter
LCAT	Lecithin-Acyl Cholesterol Transferase
LC/MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LC_{50}	Median Lethal Concentration
LD_{50}	Median Lethal Dose
LDH	Lactate Dehydrogenase
LOAEL	Lowest-Observed-Adverse-Effect Level
LPS	Lipopolysaccharides
MCH	Mean Corpuscular Hemoglobin
µg	Microgram
µm	Micromole
MN	Micronuclei
MNBNC	Micronucleated Binucleated Cells
Mg	Milligram
Ml	Milliliter
MN	Mononuclear
MRNA	Messenger RNA
N	Nitrogen
N/A	Not Applicable
NARS	National Aquatic Resource Surveys
ng	Nanogram
NLA	National Lakes Assessment
nmol	Nanomole
NOAEL	No-Observed-Adverse-Effect Level
OECD	Organization for Economic Cooperation and Development
OHEPA	Ohio Environmental Protection Agency
OR	Odds Ratio
P	Phosphorus
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear
RBC	Red Blood Cell
RfD	Reference Dose
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDH	Sorbitol Dehydrogenase
SDWA	Safe Drinking Water Act
SHE	Syrian Hamster Embryo
TEER	Trans-Epithelial Electric Resistance
TOXLINE	Toxicology Literature Online
TPA	O-Tetradecanoylphorbol-13-Acetate
Ttgase	Transglutaminase

UF	Uncertainty Factor
UMP	Uridine Monophosphate
USACOE	United States Army Corps of Engineers
USGS	United States Geological Survey
UV	Ultraviolet
WHO	World Health Organization
WSDE	Washington State Department of Ecology

EXECUTIVE SUMMARY

Cylindrospermopsin is a toxin produced by a variety of cyanobacteria including: *Cylindrospermopsis raciborskii, Aphanizomenon flos-aquae, Aphanizomenon gracile, Aphanizomenon ovalisporum, Umezakia natans, Anabaena bergii, Anabaena lapponica, Anabaena planctonica, Lyngbya wollei, Rhaphidiopsis curvata,* and *Rhaphidiopsis mediterranea.* Under the right environmental conditions, cylindrospermopsin may be produced and retained within the cell, although it is usually released outside the cell and dissolved or sorbed to other materials in water. An increase in water column stability, high water temperatures, elevated concentrations of nutrients, especially nitrogen and low light intensity have been associated with an increase or dominance of cylindrospermopsin-producing cyanobacteria in surface waters or aquatic ecosystems.

Cylindrospermopsin is relatively stable to both heat and pH in the dark. In the presence of algal cell pigments, photochemical degradation can occur rapidly, with reported half-lives of 1.5 hours and approximately 3 hours. In the absence of pigments, however, there is little decomposition. The biodegradation of cylindrospermopsin in natural water bodies is a complex process that can be influenced by many environmental factors, including its concentration, water temperature, sunlight, cell pigments, and the presence of bacteria. Half-lives of 11 to 15 days and up to 8 weeks have been reported for cylindrospermopsin in surface waters. Cylindrospermopsin is moderately mobile with low sorbtion to sediment. Sorption is well correlated with the organic carbon content of soil or sediment.

Cylindrospermopsin-producing cyanobacteria are found in brackish and marine waters, freshwater ponds, rivers, reservoirs and eutrophic lakes and have been reported in Australia, Asia, Europe, Africa and South, Central and North America. Cylindrospermopsin has been detected in agricultural soils and edible plants irrigated with cylindrospermopsin-contaminated water. In the United States, cylindrospermopsin also has been found in source water and in one case, in finished drinking water.

Human exposure to cyanotoxins can occur by ingestion of toxin contaminated water or food, by inhalation and dermal contact during bathing or showering, and during recreational activities in waterbodies containing the toxins. The main source of information on the toxicity of cylindrospermopsin in humans is from qualitative reports of a hepatoenteritis-like illness attributed to acute or short-term consumption of drinking water containing *Cylindrospermopsis raciborskii*. Symptoms reported include fever, headache, vomiting, bloody diarrhea, hepatomegaly and kidney damage with the loss of water, electrolytes and protein. No reliable data are available on exposure levels of cylindrospermopsin that induced these effects.

From limited oral toxicity studies in animals, cylindrospermopsin is likely absorbed from the gastrointestinal tract. Based on oral and intraperitoneal (i.p.) studies in mice treated with purified cylindrospermopsin or extracts of *Cylindrospermopsis raciborskii* cells, the liver and kidneys appear to be the primary target organs for cylindrospermopsin toxicity. The metabolism and toxicity of cylindrospermopsin involves the hepatic cytochrome P450 (CYP450) enzyme system. Laboratory studies have found cylindrospermopsin in the urine, feces, liver, kidney and spleen in mice. Results of *in vitro* mutagenic and genotoxic cell assays with cylindrospermopsin are varied with some indication of potential DNA damage in mouse liver. However, these data are limited and there are no long term bioassays of purified cylindrospermopsin.

The EPA reference dose (RfD) for cylindrospermopsin is 0.1 µg/kg/day based on increased relative kidney weight and decreased urinary protein from a study by Humpage and Falconer (2002, 2003). This study identified a NOAEL of 30 µg/kg/day and a LOAEL of 60 µg/kg/day based on a relative increase in kidney weight in rats. The composite uncertainty factor includes application of a 10 for intraspecies variability, 10 for interspecies differences, and a 3 for uncertainties in the database.

No epidemiological studies of the association of cylindrospermopsin and cancer are available. Also, no chronic cancer bioassays of purified cylindrospermopsin in animals were identified. Therefore, under the EPA's (2005) Guidelines for Carcinogen Risk Assessment, there is *inadequate information to assess carcinogenic potential* of cylindrospermopsin.

1.0 IDENTITY: 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, pteridophytes, 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. Compared with heterotrophic bacteria, the cyanobacterial lipopolysaccharides (LPS) have little or no 2-Keto-3-deoxy-D-manno-octonic acid, lack phosphate groups, glucosamine and L-glycero-D-mannoheptose, and have long-chain saturated and unsaturated fatty acids.

Under 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.

The cyanotoxin cylindrospermopsin is a tricyclic alkaloid with the following molecular formula $C_{15}H_{21}N_5O_7S$ (Ohtani et al., 1992) and a molecular weight of 415.43 g/mole. It is zwitterionic (i.e., a dipolar ion with localized positive and negative charges) (Ohtani et al., 1992) and is believed to be derived from a polyketide that uses an amino acid starter unit such as glycocyamine or 4-guanidino-3-oxybutyric acid (Duy et al., 2000). Two naturally occurring congeners of cylindrospermopsin (Figure 1-1) have been identified including 7-epicylindro-spermopsin (Figure 1-2) and 7-deoxycylindrospermopsin (Figure. 1-3) (Norris et al., 1999; de la Cruz et al., 2013). Recently, Wimmer et al., (2014) identified two new analogs, 7-deoxy-desulfo-cylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin, from the Thai strain of *Cylindrospermopsis raciborskii (C. raciborskii)*. The analogs were identified from a Thai strain that is very similar to strains isolated from Japan and Australia and in a genetic study by Chonudomkul et al. (2004) no differences were observed between these geographically separate strains.

Figure 1-1. Structure of cylindrospermopsin (de la Cruz et al., 2013)

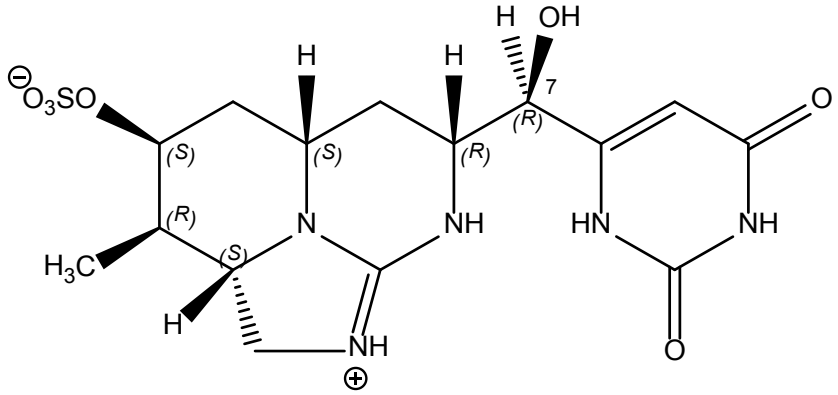


Figure 1-2. Structure of 7-epicylindrospermopsin (de la Cruz et al., 2013)

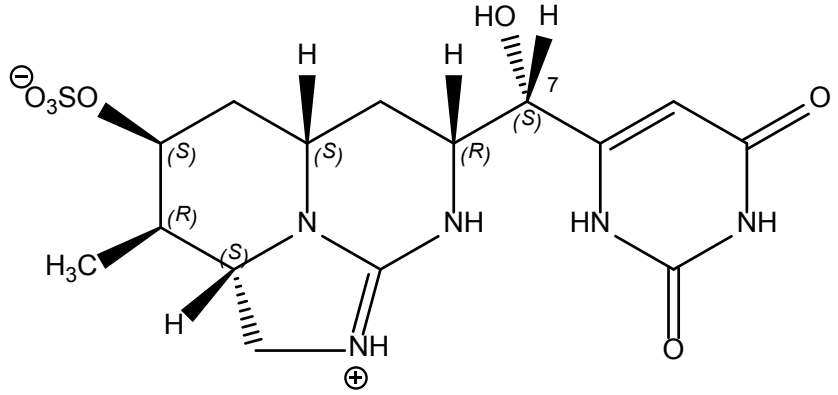
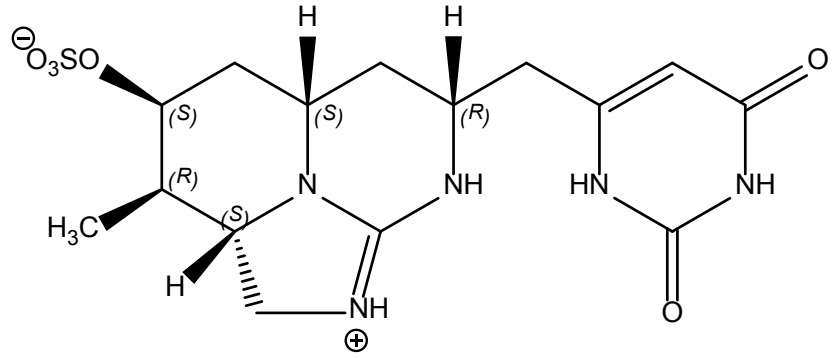


Figure 1-3. Structure of 7-deoxycylindrospermopsin (de la Cruz et al., 2013)



Cylindrospermopsin is a toxin produced by a variety of cyanobacteria including: *Cylindrospermopsis raciborskii (C. raciborskii), Aphanizomenon flos-aquae, Aphanizomenon gracile, Aphanizomenon ovalisporum, Umezakia natans, Anabaena bergii, Anabaena lapponica, Anabaena planctonica, Lyngbya wollei, Rhaphidiopsis curvata,* and *Rhaphidiopsis mediterranea.* Table 1-1 provides the chemical and physical properties of cylindrospermopsin.

Cylindrospermopsin is highly soluble in water (Moore et al., 1998, Chiswell et al., 1999). Cylindrospermopsin is isolated for commercial use mostly from *C. raciborskii* with a white powder appearance. Other physico-chemical properties of cylindrospermopsin in the environment such as vapor pressure, boiling and melting point, soil (Koc) and living organism's adsorption (Kow) coefficients, and how it volatize from water and be distributed in the atmosphere (Henry's Law constant) have not been determined. Limited information is available on the chemical breakdown, biodegradation and distribution of cylindrospermopsin in the environment (see section 2.2 on Environmental Fate).

Table 1-1. Chemical and Physical Properties of Cylindrospermopsin

Property	Cylindrospermopsin
Chemical Abstracts Registry Number (CASRN)	143545-90-8
Chemical Formula	$C_{15}H_{21}N_5O_7S$
Molecular Weight	415.43 g/mole
Color/Physical State	white powder
Boiling Point	N/A
Melting Point	N/A
Density	$2.03 g/cm^3$
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	Dimethylsulfoxide (DMSO) and methanol

Sources: Chemical Book, 2012; TOXLINE, 2012

2.0 TOXIN SYNTHESIS AND ENVIRONMENTAL FATE

2.1 Cyanotoxin Synthesis

Toxin production varies among blooms and within an individual bloom over time (Duy et al., 2000). Cyanotoxins can be produced by more than one cyanobacterial species and species can 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 determined by the mixture of species involved and their strain composition of toxic and nontoxic genotypes (WHO, 1999). Generally, cyanobacteria toxins are retained within the cell unless conditions favor cell wall lysis (ILS, 2000). Under the right environmental conditions, cylindrospermopsin may be produced and retained within the cell, although it is usually released outside the cell and dissolved or sorbed to other materials in water (Chiswell, et al. 2001). In contrast to other cyanobacteria, some species of cylindrospermopsin do not form scums (dense accumulations of cyanobacteria) and the highest cell concentrations can occur below the surface (Falconer 2005).

The synthesis of cyanotoxins is the focus of much research with evidence suggesting that the production and accumulation of toxin(s) correlates with the cyanobacterial growth rate, with the highest amount being produced during the late logarithmic growth phase (Funari and Testai, 2008). For example, Sukenik et al. (1998) found that the concentration of cylindrospermopsin within *A. ovalisporum* from Lake Kinneret increased to a plateau during the growth phase and decreased during the stationary phase. The authors attributed this decrease to cell degradation and the release of the water-soluble toxin into the medium.

Cylindrospermopsin biosynthesis starts with the production of guanidinoacetate from glycine and arginine, a natural guanidino donor, followed by successive condensations of five intact acetates, and subsequently by methylation, ketoreduction, sulfation and cyclizations (Moore et al., 1993; Looper et al., 2006). Guanidinoacetate is known to be toxic and have been found to accumulate in the cylindrospermopsin strain, contributing to the total cyanobacteria toxicity and possibly the cause of increased toxicity in crude extracts in comparison with the purified cyanotoxin (Barón-Sola et al., 2015). Enzymes encoded by two genes (*cyrA*- and *cyr0*) have been detected in cylindrospermopsin-producing strains of *C. raciborskii* and are believed to initiate toxin biosynthesis (Schembri et al., 2001; O'Neil et al., 2012). C-methylation, sulfotransfer and cyclization complete cylindrospermopsin biosynthesis. Recently, 11 genes involved in cylindrospermopsin biosynthesis in *C. raciborskii* AWT205 were identified and the biosynthesis pathway was described (Mihali et al., 2008; Mazmouz et al., 2010).

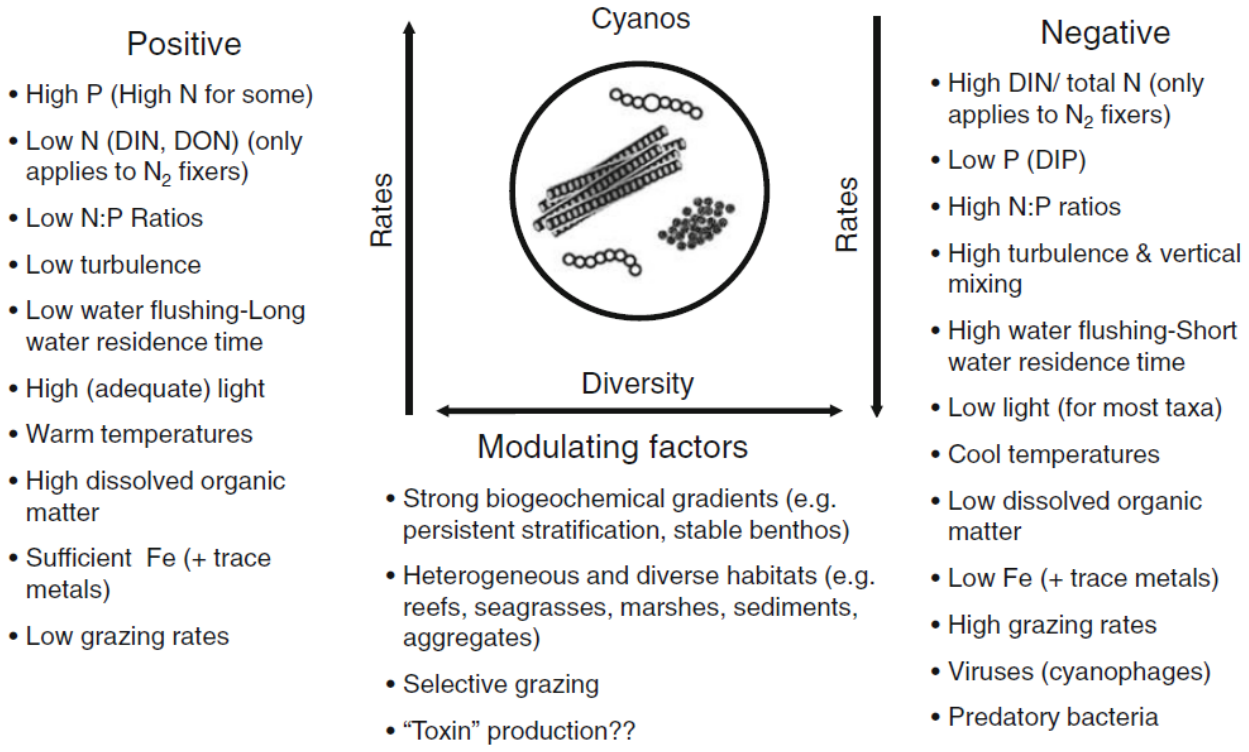
Little is known about how nitrogen affects cylindrospermopsin production. Saker and Neilan (2001) observed the highest concentration (on a dry-weight basis) of cylindrospermopsin in cultures of *C. raciborskii* in the absence of a fixed nitrogen source (Saker and Neilan, 2001). Some studies have suggested that increased intracellular cylindrospermopsin content, in the absence of fixed nitrogen, was due to *hyp* gene homologs in the *C. raciborskii* genome associated with the maturation of hydrogenases (O'Neil et al., 2012). Phosphorus appears to play an important role in cylindrospermopsin production by *C. raciborskii* due to the presence of genes that utilize inorganic and organic phosphorus, including those for high affinity phosphate binding proteins (*pstS* and *sphX*), phosphanate transport proteins (*phnC,D,E*), and enzymes for metabolism (*phnG-M,X,W* and *phoA*).

2.1.1. Environmental Factors that Affect the Fate of Cyanotoxins

Cyanotoxin production is influenced by environmental conditions that promote growth of particular cyanobacterial species and strains (Fig 2-1). Micronutrient concentrations, temperature, light intensity, water turbidity, pH, competing bacteria and phytoplankton, turbulence and salinity are all factors that affect growth and change the dynamics of a cyanobacteria population dynamics as demonstrated in

Figure 2-1. Although environmental conditions can affect the formation of blooms, the numbers of cyanobacteria and toxin concentrations produced are not always closely related. Cyanotoxin concentrations depend on the dominance and diversity of the cyanobacteria strains present within the bloom, along with environmental and ecosystem influences on bloom dynamics (Hitzfeld et al., 2000; WHO, 1999).

Figure 2-1. Environmental factors influencing cyanobacterial blooms (Reproduced from Paerl and Otten, 2013b)



Nutrients—Nutrient concentrations are key environmental drivers that influence the proportion of cyanobacteria in the phytoplankton community, the cyanobacterial biovolume, and the impact that cyanobacteria may have on ecosystem function and water quality. Cyanobacteria production and toxin concentrations are dependent on nutrient levels (Wang et al., 2002); however, different cyanobacteria species use organic and inorganic nutrient pools differently. Loading of nitrogen (N) and/or phosphorus (P) to waterbodies from agricultural, industrial, and urban sources can induce the development of cyanobacterial blooms and may be related to cyanotoxin production (Paerl et al., 2011).

Smith (1983) first described a strong relationship between the relative amounts of N and P in surface waters and cyanobacterial blooms. Smith proposed that diazotrophic cyanobacteria should be superior competitors under conditions of N-limitation because of their unique capacity for N-fixation. The hypothesis that low N:P ratios favor cyanobacteria formation has been intensely debated and challenged for its poor performance in predicting cyanobacterial dominance (Downing et al., 2001). However, 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). Eutrophic systems already subject to bloom events are prone to further expansion of these blooms due to additional N inputs, especially if sufficient P is 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 plus a range of experimental enclosures (<1 L to over 10,000 L), reveal that greater stimulation is observed in response to both N and P additions, suggesting 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.

Preußel et al. (2014) investigated the influence of nitrogen and phosphorus availability on the production and the release of cylindrospermopsin in three strains of *Aphanizomenon sp.* The authors found that cylindrospermopsin was released from cells under both nitrogen availability and phosphorus limitation. Under nitrogen-limiting conditions, the authors found a reduction in the release of cylindrospermopsin from intact cells probably due to changing metabolic activities and the efficiency of resource consumption.

Light Intensity—Sunlight availability and turbidity influence the predominance of cyanobacteria species and the depth at which they occur (Falconer et al., 2005; Carey et al., 2012). For example, *Cylindrospermopsis* forms dense layers of filaments at depths near the lower bound of the euphotic zone in deeper rivers, lakes and reservoirs. The relationship of light intensity to toxin production in blooms is somewhat unclear and continues to be investigated (Duy et al., 2000). While some researchers have found evidence that toxin production increases with high light intensity (Watanabe and Oishi, 1985), others have found little variation in toxicity at different levels of light intensity (Codd and Poon, 1988; Codd, 1995). Deep water mixing and low light have been associated with an increase in the dominance of *C. raciborskii*, a toxin producing species (O'Brien et al., 2009).

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.

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 warming may influence cyanobacterial dominance. Cyanobacteria can benefit more from warming than other phytoplankton groups due to their higher optimum growth temperatures. The increase in water column stability associated with higher temperatures also favors cyanobacteria (Wagner and Adrian, 2009; Carey et al., 2012). In their analyses of 143 lakes along a latitudinal transect from subarctic Europe to southern South America, Kosten et al. (2011) demonstrated that in shallow lakes the percentage of the total phytoplankton biovolume attributable to cyanobacteria increased steeply with temperature.

Indirectly, warming also may increase nutrient concentrations by enhancing mineralization (Gudasz et al., 2010; Kosten et al., 2009 and 2010) and by temperature- or anoxia-mediated sediment phosphorus release (Jensen and Andersen, 1992; Søndergaard et al., 2003). Thus, temperature may increase cyanobacteria biomass indirectly 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 grazers (Jeppesen et al., 2009, 2010; Teixeira-de Mello et al., 2009).

Rising global temperatures and changing precipitation patterns can 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 degree 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 favoring cyanobacteria production and release of cylindrospermopsin (Paerl and Otten, 2013b).

Other Environmental Factors—Cyanobacterial 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 well 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 molecular carbon dioxide (Shapiro, 1984; Caraco and Miller, 1998), especially when increasing pH diminishes the availability of carbon dioxide in the water column. Although this could explain the positive correlation 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. Higher pH in the water column can be a reflection of higher photosynthetic rates, which can be linked with high nutrient concentrations (Duy et al., 2000) that stimulate phytoplankton growth and bloom formation.

Most phytoplankton-cyanobacteria blooms occur in late summer and early fall and the phytoplankton community can become vertically stratified. The vertical phytoplankton biomass structure and cyanotoxin production can be influenced by seasonal changes as well as weather conditions (e.g., wind, rainfall), and also by runoff. At times, the bottom layer can have more biomass and display different population dynamics than the upper water column. Conversely, seasonal influences with increases in temperature and changes in wind patterns may favorably influence the upper water column cyanobacterial community to become dominant. 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 the cyanobacteria growth; (c) nutrient supply from organic-rich bottom sediment (even when the water body is not density-stratified), encouraging growth at or near the sediment; and, (d) species-specific factors (Drake et al., 2010). In addition, there are microbial interactions within blooms, such as competition and adaptation between toxic and nontoxic cyanobacterial strains, as well as attacks of cyanobacteria by viruses. Each of these factors can cause fluctuations in bloom development and composition. When the composition of the cyanobacterial bloom changes, the toxins present and their concentrations may change as well (Honjo et al., 2006; Paerl and Otten, 2013b). The concentration of cyanotoxins observed in the water column when a bloom collapses, such as from cell aging or from algaecide treatment, depends on dilution of the toxin in the impacted water 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 pattern 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 water and 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 on 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).

2.2 Environmental Fate of Cylindrospermopsin

Hydrolysis—Cylindrospermopsin is relatively stable to heat and pH in the dark (Moore et al., 1998). Studies have found cylindrospermopsin is stable at temperatures from 4°C to 50°C for up to five weeks in the dark (ILS, 2000).

Photolysis—Chiswell et al., 1999 reported that cylindrospermopsin in an algal extract solution decomposes rapidly (half-life of 1.5 h) when exposed to sunlight; however, no decomposition was recorded in pure cylindrospermopsin and Milli-Q water solutions. They further observed that cylindrospermopsin remains a potent toxin even after boiling for 15 minutes. Pure cylindrospermopsin is relatively stable in sunlight, but in the presence of cell pigments, photochemical degradation can occur rapidly. Researchers have noted that degradation rates are concentration-dependent. When cylindrospermopsin (1 mg/L aqueous media) was exposed to normal sunlight, 54% remained after 3 hours; at 4 mg/L, cylindrospermopsin degraded more rapidly, with 29% of the original concentration remaining after 3 hours (ILS 2000). When cell pigments are present, photolysis has been shown to degrade more than 90% of cylindrospermopsin within 2 to 3 days (Chiswell et al., 1999).

Metabolism—Toxins released from cyanobacteria into lakes are decomposed by bacteria (Falconer, 1998). A half-life of 11 to 15 days has been reported for cylindrospermopsin in surface waters (Funari and Testai, 2008). However, at pH 4, 7 and 10, cylindrospermopsin can remain stable for a period of up to eight weeks (ILS, 2000). Smith et al. (2008) concluded that the biodegradation of cylindrospermopsin in natural water bodies is a complex process that can be influenced by many environmental factors, including: concentration, temperature and the presence of copper-based algaecides. Studies by Klitzke and Fastner (2012) found that degradation of cylindrospermopsin in sediment was completely inhibited or retarded under anoxic conditions ($T_{1/2}$ oxic =2.4 days; $T_{1/2}$ anoxic =23.6 days). A decrease in temperature from 20 °C to 10 °C slowed down degradation rates by a factor of 10. Smith et al. (2008) reported an optimum degradation rate between 25°C and 30°C. Mohamed and Alamri (2012) reported that *Bacillus* strain (AMRI-03) isolated from cyanobacterial blooms degraded cylindrospermopsin in laboratory studies. Cylindrospermopsin degradation occurred rapidly, with a complete degradation based on the initial concentration of cylindrospermopsin. Degradation occurred after 6 days at the highest tested concentration (300 µg/L) compared to seven and eight days at lower concentrations (10 and 100 µg/L, respectively) and depended on temperature (25 and 30°C) and pH (7 and 8).

Transport—Klitzke et al. (2011) reported low sorption of cylindrospermopsin to sediments and moderate mobility. Sorption was non-linear and results were best fit using a Langmuir model. Organic carbon proved to be the main parameter in sediment that determines sorption of cylindrospermopsin, with little sorption observed on sandy and silt sediments. Cation exchange played only a minor role in comparison to sorption to organic carbon. Sorption of cylindrospermopsin to sediment increased at low pH (Klitzke et al., 2011). The authors suggested that the low sorption of cylindrospermopsin to sediment could be due to its high polarity and tendency to remain in solution.

2.3 Summary

Cylindrospermopsin is produced by a variety of cyanobacteria. Environmental conditions such as nutrients, pH, light intensity and temperature can influence the growth of cyanobacteria and encourage toxin production. Some species of cyanobacteria do not form scums; high cell concentrations occur below the water surface because cyanobacteria have gas vacuoles to regulate their position in the water column. Cylindrospermopsin may be retained within the cell, but is usually found dissolved or attached to other materials in water. Cylindrospermopsin is relatively stable in the dark and remains potent even after boiling for 15 minutes. In sunlight, photochemical degradation of cylindrospermopsin in water can occur rapidly, within 2 to 3 days, especially when cell pigments are present. The biodegradation of cylindrospermopsin in natural water bodies is pH and temperature dependent. The optimum degradation rate has been reported between 25°C and 30°C. Its half-life in surface water ranges from 11 to 15 days, but, cylindrospermopsin can remain stable for a period of up to eight weeks (at pH 4, 7 and 10). Cylindrospermopsin adsorbs onto sediment and is moderately mobile. Organic carbon content is a key sediment parameter determining sorption, with little sorption observed on sandy and silt sediment.

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 blooms (HAB) events. Instead, states 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 cylindrospermopsin, 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. Cylindrospermopsin may be retained within the cell, but most of the time (50/50 ratio) it is found in the water (extracellular) or attached to particulates present in the water (Chiswell et al., 2011). 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. As described above, cylindrospermopsin is not considered persistent in natural waters, thus exposure from ambient surface waters is more likely to be acute or subacute. People, particularly children, recreating close to lakes and beach shores also can be at potential risk from exposure to nearshore blooms.

Livestock and pets are potentially exposed to higher concentrations of cyanobacterial toxins than humans because they are more likely to consume scum and mats when 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 in benthic vegetation between the low- and high-tidewater marks but can grow as free-floating water blooms (Walsh et al, 2008). 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).

Gas vacuoles of *A. ovalisporum* and *C. raciborskii* can regulate the position of the cyanobacteria in the water column. These species of cyanobacteria do not form a floating scum, but concentrate (with densities up to 100,000 cells/mL) several meters below the surface. Because the cells remain suspended in the water column, potentially toxin-producing blooms of these cyanobacteria may not be readily observable. In older blooms, some cyanotoxins (including cylindrospermopsin) may be found at higher concentrations dissolved in the water column (Rucker et al., 2007).

3.2 Cylindrospermopsin Occurrence in Surface Water

C. raciborskii occurs in freshwater ponds, rivers, reservoirs and eutrophic lakes and has been found in Australia, Asia, Europe, Africa and South, Central and North America (Fuentes et al., 2010). Cylindrospermopsin-producing cyanobacteria occur in tropical or subtropical regions, but also have been detected in warmer temperate regions. Surveys conducted in Florida, the Great Lakes and the Midwest, and monitoring efforts in Ohio and Washington indicate that freshwater cyanotoxins are prevalent in the U.S., mostly during warm seasons (Hudnell, 2010; Graham et al., 2010).

According to a survey conducted in Florida in 1999 from June to November, the most frequently observed toxigenic 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. The actual cylindrospermopsin concentrations in ambient water were not reported.

Concentrations of cylindrospermopsin have been reported at concentrations between 0.05 and 0.2 mg/L in Florida since 1999 by The Harmful Algal Bloom Task Force (Pelaez et al., 2010). *C. raciborskii* have also been detected, in some cases at more than 100,000 cells per mL. Additional data collected from the Florida Department of Health found consistent cylindrospermopsin production in specific lakes at concentrations ranging from 0.5 to 1.6 mg/L during the months of July through October. Samples collected in the St. Johns River in 2008 around the same months (June through October) found cylindrospermopsin consistently present ranging from 0.05 to 0.44 mg/L.

Samples collected from 2000 to 2004 in Lake Erie and analyzed by protein phosphatase inhibition assay (PPIA) detected cylindrospermopsin in 3% of the samples at concentrations greater than 0.01 mg/L (Pelaez et al., 2010).

Between 2000 and 2004, water samples were collected for cyanotoxin analysis from 81 different New York lakes during June to October (Boyer et al., 2008). Cylindrospermopsin was measured by high performance liquid chromatography (HPLC) and detected in 8 of the 366 samples with concentrations less than 0.25µg/L.

In Oklahoma during 2005, the U.S. Army Corps of Engineers (USACE) detected cylindrospermopsin at a maximum concentration of 1.6 µg/L (Lynch and Clyde, 2009). During the same year in Wisconsin, sixty-five samples were taken in Castle Rock and Petenwell lakes for blue-green algae and toxin identification (Evans, 2011). *Cylindrospermopsis*, which is not commonly found in Wisconsin, was present in only 6% of the samples.

In 2005, Washington State Department of Ecology developed the Ecology Freshwater Algae Program, focuses on the monitoring and management of cyanobacteria in Washington lakes, ponds, and streams (WSDE, 2012). Data have been summarized in a series of reports for the Washington State Legislature (Hamel, 2009; 2012). Cylindrospermopsin was below the state recreational guidance level of 1 µg/L in 41 lakes tested in 2010, and was not detected in 46 lakes sampled in 2011.

In Florida, *C. raciborskii* was found to be the dominant cyanobacteria species in one lake all year round (Burns, 2008). Cylindrospermopsin was also detected from *Aphanizomenon ovalisporum* in levels ranging from 7.39 to 9.33 µg/mg freeze-dried cells (Yilmaz et al., 2008). This finding supports the potential of cylindrospermopsin to be produced by other cyanotoxin-producing species.

In 2006, *C. raciborskii* was detected in lakes in southern Louisiana (Fuentes et al., 2010). Conditions promoting its growth were identified as shallow, warm surface water (over 30°C) and low light intensities. The highest concentrations of *C. raciborskii* were observed from June through August with densities ranging from 37,000 cells/mL to more than 160,000 cells/mL. In a study of two lakes directly

connected to Lake Michigan, Hong et al., (2006) found low concentrations only in the late summer and these were associated with elevated bottom water temperatures and phosphorus concentrations.

In 2006, the U.S. Geological Survey (USGS) conducted a study of 23 Midwestern lakes in which cyanobacterial blooms were sampled and analyzed by enzyme-linked immunosorbent assays (ELISA) and by direct-inject multianalyte liquid chromatography/tandem (LC/MS/MS) to determine the co-occurrence of toxins and taste-and-odor compounds in cyanobacterial blooms (Graham et al., 2010). Microcystin was detected in all the blooms, anatoxin-a was detected in 30% of the blooms, and cylindrospermopsin was detected in 9% of the blooms sampled. The low concentrations of cylindrospermopsin (0.12 to 0.14 µg/L) detected in these studies were associated with algal communities dominated by *Aphanizomenon* or *Anabaena* and/or *Microcystis*, but not in those dominated by *Cylindrospermopsis*. The authors attributed the low concentration of cylindrospermopsin to either the lack of toxin production by *Cylindrospermopsis* strains in the U.S as compared to elsewhere in the world, or to the lack of favorable environmental conditions for the toxic strains and/or toxin production in the lakes sampled.

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 algal toxins in the nation's lakes. A total of 1,028 lakes were sampled for the NLA during summer 2007, representing the condition of about 50,000 lakes nationwide. The NLA looked at actual cyanobacterial cell counts and chlorophyll-a concentrations as indicators of the potential for the presence of algal toxins including microcystin and cylindrospermopsin. However, concentrations of cylindrospermopsin were not reported. The USGS subsequently analyzed the stored samples collected during the NLA and reported the presence, but not actual concentrations of cylindrospermopsin, in 5% of the samples collected (Loftin and Graham, 2014). Future NARS plan to include other algal toxins, including cylindrospermopsin.

Since 2007, Ohio EPA (OHEPA, 2012) has been monitoring inland lakes for cyanotoxins. In 2010, OHEPA sampled Grand Lake St. Marys for anatoxin-a, cylindrospermopsin, microcystin, and saxitoxin. Cylindrospermopsin concentrations ranged from below the detection limit (<0.15) to 9 µg/L.

3.3 Cylindrospermopsin Occurrence in Drinking Water

The occurrence of cyanotoxins in finished drinking water depends on their levels in the raw source water and the effectiveness of the treatment methods used for removing cyanobacteria and cyanotoxins. Currently, there is no federal or state program in place that requires monitoring for cyanotoxins at U.S. drinking water treatment plants. Therefore, data on the presence or absence of cyanotoxins in finished drinking water are limited.

A survey conducted in 2000 in Florida (Burns, 2008) found cylindrospermopsin in raw drinking water and in nine finished drinking water samples at concentrations ranging from 8 µg/L to 97 µg/L.

3.4 Summary

Cylindrospermopsin-producing cyanobacteria occur in freshwater systems in tropical or subtropical regions, but also can occur in warmer temperate regions. No national database on the occurrence of freshwater cylindrospermopsin is available, and no federal or state program is in place to monitor for cyanotoxins at U.S. drinking water treatment plants.

Exposure to cylindrospermopsin from contaminated drinking water could occur via oral exposure (e.g. ingestion of contaminated drinking), dermal exposure (contact of exposed parts of the body with water containing toxins) and inhalation exposure. Exposure to cylindrospermopsin during recreational activities could occur through direct contact, inhalation and/or ingestion. Exposures usually are not

chronic with the exception of regions with extensive and persistent cyanobacterial blooms. Since cylindrospermopsin is not expected to be persistent in surface waters, exposure will depend on the formation and persistence of the blooms and the related toxin concentration.

4.0 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 taken up by crop plants after spray irrigation (Corbel et al., 2014). Water contaminated with toxins produced by cyanobacterial cells that is then used for spray irrigation may produce food chain contamination since low levels of cyanotoxins could be absorbed by roots, migrate to shoots, and then translocated to grains and or fruits. Cyanotoxins can be accumulated in plant leaves. Kittler et al. (2012) found that crop plants irrigated with cylindrospermopsin-contaminated water showed significant cylindrospermopsin uptake in the leaves at 10% to 21% of the cylindrospermopsin concentration applied to the roots. Water contaminated with cyanotoxins used for spray irrigation of crop plants inhibited plant growth and induced visible 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 cylindrospermopsin 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 consumption depends on the bioaccumulation of cyanotoxins in edible fish tissue compared to organs such as the liver. Levels of cylindrospermopsin found in tissues of aquatic species potentially consumed by humans are shown in Table 4-1. One study (Saker and Eaglesham, 1999) determined the concentration of cylindrospermopsin in redclaw crayfish and rainbow fish from aquaculture ponds. Cylindrospermopsin concentrations were 0.9 and 4.3 μg/g freeze-dried tissue in crayfish muscle and hepatopancreas, respectively, and 1.2 μg/g freeze-dried tissue in the viscera of rainbow fish. This study also demonstrated that bioaccumulation can occur in fish that are exposed for longer periods of time to a cyanobacterial bloom. Recent reviews also included levels of cylindrospermopsin in freshwater mussels and prawns (Kinnear, 2010; Funari and Testai, 2008; Ibelings and Chorus, 2007). No cases of toxicity in humans following ingestion of fish or shellfish exposed to cylindrospermopsin have been documented.

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

Species/tissue	Concentration	Conditions	Reference
Fish			
Rainbow fish – viscera	1.2 µg/g freeze dried tissue	Aquaculture pond during bloom; 589 µg/L cylindrospermopsin	Saker and Eaglesham, 1999
Shellfish			
Alathyria pertexta	0.13-0.56 µg/g fresh tissue	Experimental exposure to reservoir water; <0.8 µg/L cylindrospermopsin	Kinnear, 2010
Swan mussel Hemolymph Viscera Whole body	 61.5 µg/g dry tissue 5.9 µg/g dry tissue 2.9 µg/g dry tissue	Experimental; 14-90 µg/L cylindrospermopsin	Kinnear, 2010
Mussel Whole body Viscera	 0.247 µg/g wet wt. 1.099 µg/g wet wt.	Experimental exposure concentration not given; secondary citation	Saker et al., 2004
Crustaceans			
Crayfish muscle tissue hepatopancreas	0.9 µg/g freeze dried tissue 4.3 µg/g freeze dried tissue	Aquaculture pond during bloom; 589 µg/L cylindrospermopsin	Saker and Eaglesham, 1999
Prawns – flesh	0.205 µg/g wet wt.	Survey; cylindrospermopsin concentrations not given	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).

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.

4.4 Summary

Cylindrospermopsin could be detected in aquatic animals, field soils and edible plants. Bioaccumulation occurs mostly in the viscera of fish, shellfish and crustaceans, but cylindrospermopsin has also been detected in fish tissue. No cases of toxicity in humans following ingestion of fish or shellfish exposed to cyanotoxins have been documented.

Cylindrospermopsin has not been found in any of the tested commercially-available blue-green algal supplements. Exposure to cylindrospermopsin for the general population is most likely through the ingestion of drinking water and incidental ingestion when recreating in a water source contaminated with cylindrospermopsin.

5.0 TOXICOKINETICS

The available toxicokinetic data for cylindrospermopsin are from studies that do not reflect environmental exposure conditions. All studies identified for this assessment were generated using intraperitoneal (i.p) exposures to mice or *in vitro* assays rather than by the oral, dermal and/or inhalation routes applicable to humans and domestic animals.

5.1 Absorption

Data on human and animal absorption of cylindrospermopsin after inhalation or dermal exposure were not located. In two oral animal studies (Humpage and Falconer, 2002, 2003; Shaw et al., 2000, 2001), mice were exposed to pure cylindrospermopsin for 14 days and 11 weeks, respectively. Systemic effects observed in these studies following oral administration of cylindrospermopsin suggest absorption from the gastrointestinal tract. The structural and conformational properties of the cylindrospermopsin molecule suggest that uptake by the intestines and other tissues likely involves facilitated transport. No data were identified relative to potential membrane receptors with properties compatible with the properties of the cylindrospermopsin ion.

5.2 Distribution

Total tissue distribution of cylindrospermopsin following oral, inhalation or dermal exposure is unknown. A series of three studies were done in six-week old male Quackenbush mice exposed to sublethal and lethal doses of ^{14}C-cylindrospermopsin (>95% pure) in normal saline by intraperitoneal (i.p.) administration (Norris et al., 2001). At 48 hours, analysis of kidney, liver, and spleen after a 0.1 mg/kg dose demonstrated 13.1% ^{14}C recovery of the dose in the liver and <1% in the rest of the tissues. In each of the four mice tested the total recovery of radiolabel cylindrospermopsin from tissues and excreta was 85-90% of the administered dose; 68% of the dose was found in urine and 15.5% in the feces.

In the second study, Norris et al. (2001) administered a single dose of 0.2 mg/kg dose of ^{14}C-cylindrospermopsin by i.p. to 12 mice. After 12 and 24 hours, urine and feces in all animals had detectable levels of ^{14}C content. Five mice euthanized after 5-6 days (due to unspecified toxicity), had ^{14}C content in the liver, kidney and spleen. The remaining 7 mice, also had ^{14}C content after 7 days with no signs of toxicity. After 5 to 7 days, the overall mean (and standard deviation) recoveries of ^{14}C were 2.1 ± 2.1 in the liver, 0.15 ± 0.14 in the kidneys and <0.1% (no standard deviation provided) of the dose in the spleen. The broad standard deviations are indicative of considerable inter-individual differences in response. There was no clear relationship between the signs of toxicity and the observed tissue distribution. However, Norris et al. (2001) proposed that the lack of toxicity could be explained by a tendency toward decreased liver retention in surviving mice.

In the third experiment, Norris et al. (2001) evaluated the excretion and tissue distribution in four mice after the administration of a 0.2 mg/kg i.p. dose of ^{14}C-cylindrospermopsin. After 6 hours, liver, kidney, heart, lung, spleen, blood and bile were examined for ^{14}C content. Detection of ^{14}C was observed in all tissues, however, mean ^{14}C content was higher in the liver (20.6% (range 14.6 to 27.9), and 4.3% (range 3.7 to 4.7) of the dose in the kidneys. After a week, around 2% of the ^{14}C content was detected in the liver.

A slow, progressive, non-energy dependent uptake of purified cylindrospermopsin was detected in a cultured African green monkey kidney cell line (Vero cells) (Froscio et al., 2009). Although, conducted *in vitro,* these results suggest facilitated transport as a mechanism for uptake by the kidney.

Studies on the distribution of cylindrospermopsin in fish using immunohistochemical (IHC) techniques have found immunopositive results in the liver, followed by the kidney, intestines, and gills (Guzman-Guillén, et al., 2014). IHC techniques were used in fish (*Oreochromis niloticus*) to determine the distribution of 200 µg pure CYN/Kg body weight (bw) administered by i.p. or by gavage and evaluated after 5 days of exposure. In addition, fish were also exposed to CYL by immersion to either 10 or 100 µg/L of lyophilized *A. ovalisporum* cells for 7 or 14 days. Results were similar in both experimental methods. Immunolabeling intensified with increasing time in both experiments, and with increasing dose, with the highest immunolabeling at the highest concentration (100 µg/L), and at the longest time of exposure (14 days). These results suggest a delay in the toxicity of cylindrospermopsin.

5.3 Metabolism

Metabolism and toxicity of cylindrospermopsin appear to be related to the hepatic CYP450 enzyme system. In a study done by Froscio et al., 2003, hepatocytes were pretreated with known inhibitors of CYP450 (50 µM proadifen or ketoconazole). A reduction in the *in vitro* cytotoxicity of cylindrospermopsin was observed. Norris et al., (2002), demonstrated that in male Quackenbush mice pretreated with the CYP450 inhibitor, piperonyl butoxide, protection against the acute lethality of cylindrospermopsin occurred. Shaw et al (2000, 2001) also noted the involvement of the CYP450s and demonstrated that cylindrospermopsin targets periacinar region of the liver, an area where xenobiotic metabolism mediated by CYP450 occurs.

In a series of studies done by Norris et al. (2001), the distribution and metabolism of ^{14}C-cylindrospermopsin (>95% pure) also was tested. A single i.p. dose of 0.1 mg/kg was administered to 4 male Quackenbush mice, and 0.2 mg/kg was given to 12 mice (Norris et al., 2001). After 12 hours of dosing, body weights were taken and urine and fecal samples were collected. The group of mice receiving the lower dose (0.1 mg/kg) were sacrificed after 48 hours of dosing and samples of urine, feces, plus liver and kidney tissues were treated with methanol to precipitate proteins. The protein precipitates were not fractionated to identify ^{14}C radiolabel. HPLC was used to fraction the ^{14}C in the methanol supernatant and to detect metabolites in urine and feces. The HPLC of the urine reveal one major, one moderate and one minor peak. The minor peak was not present in all samples. It eluted early appearing to be more hydrophilic than cylindrospermopsin. The major peak appeared to be cylindrospermopsin.

Approximately 23.5% of the urinary ^{14}C was detected in the protein precipitates, indicating the presence of a protein-bound metabolite (Norris et al., 2001). Results did not indicate whether the levels of proteins found in the urine were normal or increased. An aqueous extract of the fecal matter from one mouse indicated that a compound that elutes at the retention time for cylindrospermopsin accounted for 93% of the administered radiolabel cylindrospermopsin.

Liver tissue analysis of both the protein precipitate and the aqueous supernatant showed the presence of ^{14}C (Norris et al., 2001). After ^{14}C was fractioned by HPLC, the liver supernatant showed the same elution characteristics as the urine methanol supernatant, indicating the presence of cylindrospermopsin plus what appeared to be the minor metabolite from urine based on elution time. There were differences across the samples evaluated, with two animals showing high levels of the minor metabolite. All four animals had cylindrospermopsin present in the supernatant; for three of the animals the cylindrospermopsin accounted for less than 50% of the radiolabel present. In the case of the kidney supernatant, cylindrospermopsin accounted for about 90% of the radiolabel in the supernatant for two mice evaluated.

Evidence from Runnegar et al. (1995) and Shaw et al. (2000) studies suggests the extractable ^{14}C might be a cylindrospermopsin metabolite. Runnegar and Shaw also provided evidence of the need for the activation of cylindrospermopsin for toxicity to occur, suggesting the presence of one or more

metabolites. Although, no identification of metabolites was performed, results indicate the metabolite is either more polar than cylindrospermopsin, or that cylindrospermopsin is fragmented during metabolism.

5.4 Excretion

The excretion of cylindrospermopsin following oral, inhalation or dermal exposure has not been reported. Norris et al. (2001) reported the excretion of ^{14}C-cylindrospermopsin (>95% pure) after the i.p. administration of sublethal and lethal doses in male Quackenbush mice.

In the first study, 0.1 mg/kg was administered by i.p. to four mice and urine and feces samples were collected at 12 hour intervals for 48 hours (Norris et al., 2001). After 12 hours, the mean cumulative excretion of ^{14}C in the urine was 62.8 ± 25.3% (of the 0.1 mg/kg dose), and 15.5 ± 26.9% in the feces. One of the animals excreted a total of 15.5% of ^{14}C content in the feces (nearly 60% of the dose in this one mouse compared to less than 5% in the other mice), indicating the possibility that this single high value occurred because of injection into the upper gastrointestinal tract. However, the authors discounted this possibility due to the injection technique used. After 24 hours, little additional excretion of ^{14}C in either the urine or feces was observed. In each of the four mice, the total mean recovery of the ^{14}C in the urine, feces, liver, kidney and spleen was 85-90%.

In the second part of the study, Norris et al. (2001) administered by i.p. 0.2 mg/kg of ^{14}C-cylindrospermopsin to 12 mice and collected the urine and feces after 12 and 24 hours. In this study, continued ^{14}C excretion in urine and feces was observed over 24 hours. After 12 hours, the mean cumulative excretion of ^{14}C in the urine was 66.0 ± 27.1% and in the feces was 5.7 ± 5.6% of the dose. After 24 hours, 68.4 ± 26.7% was detected in the urine and 8.5 ± 8.1% in the feces, with a mean total recovery of 76.9% of the administered dose. There was no clear relationship between the signs of toxicity and the excretion patterns among the mice with signs of toxicity or those with no signs of toxicity. However, there was a trend in survivors towards increased urinary and decreased fecal excretion and liver retention.

In the third study, Norris et al. (2001) administered a 0.2 mg/kg i.p. dose of ^{14}C-cylindrospermopsin and collected the urine and feces after 6 hours. The mean cumulative excretion in the urine was 48.2 ± 29.3% and in the feces was 11.9 ± 21.4% of the administered dose. The authors reported that 40% of the ^{14}C dose was excreted in the feces of one of the four mice.

5.5 Pharmacokinetic Considerations

No data on half-life or other quantitative pharmacokinetic data applicable to cylindrospermopsin were identified. Gastrointestinal uptake of cylindrospermopsin is assumed based on the adverse effects observed in mice following dosing with both extract and pure cylindrospermopsin. Studies using i.p. administration of labeled compound demonstrate distribution to the liver, kidney, lung, spleen and heart in descending order. Some of the label in the liver is bound to protein. There is evidence for hepatic oxidation by the CYP450 system generating oxidized metabolites that are more toxic than the parent compound. Pretreatment with CYP450 inhibitors decreased manifestations of toxicity. The presence of labeled cylindrospermopsin in urine demonstrates the kidney is the principal excretory organ for absorbed cylindrospermopsin. In mice, a portion of labeled cylindrospermopsin in urine was bound to protein. Detection of the labeled compound in the feces after i.p. dosing likely reflects some biliary excretion.

6.0 HAZARD IDENTIFICATION

6.1 Case Reports and Epidemiology Studies

Oral Exposure—In 1979, 148 residents of aboriginal descent in Palm Island in Queensland, Australia were affected by a hepatoenteritis-like illness (Byth, 1980 and Griffiths and Saker, 2003). Although the total number of people exposed was not determined, 148 cases were reported. Of those, 138 cases were children between the ages of 2-16 years (41% boys and 59% girls), and 10 were adults (no sex or age was reported). Most of the cases required hospitalization and presented symptoms of vomiting, headache, fever and profuse, bloody diarrhea. Hepatomegaly and renal damage (represented by the presence of substances in urine such as proteinuria (89%), glycosuria (74%), ketonuria (53%), hematuria (20%), and urobilinogenuria (8%), were observed. Many (69%) of the patients received intravenous therapy for fluids and electrolyte imbalance; 12% received intravenous plasma proteins for hypovolemia (decreased volume of circulating blood) and acidosis. The prevalence of illness in children compared to adults may be due to the fact that children ingest larger amounts of tainted water compared to adults. Eighty two percent of the children developed hypokalemia (deficiency of potassium in the blood) and acidosis (Byth, 1980).

Solomon Dam reservoir, the major drinking water supply for Palm Island, was treated a few days prior to the outbreak with unreported levels of copper sulfate to control a dense algal bloom in the reservoir (Griffiths and Saker, 2003). Only people in those households connected to the reservoir were affected by the outbreak. *C. raciborskii* was identified by retrospective analyses, including epidemiological and ecological assessments, as the predominant cyanobacterial species in the reservoir and the likely source of the illness (Griffiths and Saker, 2003; Hawkins et al., 1985). Ohtani et al. (1992) later identified cylindrospermopsin as the toxin in the reservoir. Some of the reported symptoms (headache, nausea, vomiting and diarrhea) are effects that are associated with acute oral exposure to concentrations of copper as sulfate at doses ≥ 3mg/L; with a no effect level (NOAEL) of 1 mg/L (Pizzaro et al., 1999). Although the copper sulfate treatment could have accounted for reports of nausea, vomiting, headache and diarrhea, the cyanotoxins in the drinking water are the most likely cause of the observed adverse health effects in the ill people, assuming the copper sulfate was applied at the recommended 1 mg/L level. Had excess copper sulfate been added to the water or if concentrations were not uniformly distributed in the water body, copper could have contributed to the symptoms observed. No other case reports or epidemiological studies were identified for oral exposure to cylindrospermopsin.

Dermal Exposure—Skin-patch testing in humans was done by Pilotto et al., (2004) to test the potential of cylindrospermopsin to irritate the skin. Laboratory-grown *C. raciborskii* cells, both whole and lysed, were applied using adhesive patches at concentrations ranging from <5,000 to 200,000 cells/mL, to the skin of 50 adult volunteers. The cell concentrations (densities) used were similar to those that could be found in *C. raciborskii*-contaminated water bodies used for recreational activities. The patch itself and the culture media were used as the negative controls, and 1 and 5% solutions of sodium lauryl sulfate were used as the positive control. After 24 hours, patches were removed and evaluation of the erythematous reactions were graded (by a dermatologist who was not provided identifying information on the patch concentration used) using a scale of from 0 to 4: 0 = no reaction or erythema; 1= minimal or very weak spotty erythema; 2= mild diffuse erythema; 3= moderate diffuse erythema; and 4= severe diffuse erythema with edema. Logistic regression modeling and odds ratios (OR) evaluation was used to determine the distribution of clinical responses relative to patch concentration.

Analysis of volunteer reactions to patches treated with whole cells showed an OR of 2.13 and a 95% Confidence Interval (CI) of 1.79-4.21 ($p<0.001$). Lysed cells patch analysis showed an OR of 3.41 and a 95% CI of 2.00-5.84 ($p<0.001$). No statistically significant increase or dose-response between skin reactions and increasing cell concentrations for either patches (whole or lysed) was observed. Subjects had skin reactions to the cylindrospermopsin and positive control patches more frequently than to the

negative control patches. The mean percentage of subjects with a reaction was 20% (95% CI 15-31%). For subjects reacting to negative controls (39), the mean percentage was 11% (95% CI 6-18%). Evaluation of erythematous reactions showed that mild irritations (grade 2) were resolved in all cases within 24 to 72 hours. The difference in reaction rates between the whole and lysed cells was minimal and no evidence for a threshold effect (i.e., a particular concentration above which there were frequent or strong reactions) was observed.

Stewart et al. (2006) also conducted skin patch testing on 19 human volunteers using lyophilized *C. raciborskii*. Up to 160 ng of cyanotoxin was applied to filter paper discs adhered 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 a clinically detectable skin reaction.

Other Routes of Exposures—In February 1996, there was an outbreak of acute liver failure in hemodialysis patients at a clinic in Caruaru, Brazil (Carmichael et al., 2001). One hundred and sixteen of 131 patients who received their routine hemodialysis treatment at that time, experienced headache, eye pain, blurred vision, nausea and vomiting. Of the affected patients, 100 developed acute liver failure and 76 of these patients died. Analysis of the carbon, sand and cation/anion exchange resins from in-house water treatment filters from the clinic demonstrated the presence of both microcystins and cylindrospermopsin. Microcystins, but not cylindrospermopsin, were found in blood, sera and liver samples from the patients. Analysis of liver samples for cylindrospermopsin by HPLC-MS/MS did not reveal the toxin. However, the method used to detect the more polar alkaloid cylindrospermopsin may have been inadequate. Based on comparisons between liver pathology data from animal studies of microcystins and cylindrospermopsin and the symptoms observed in the outbreak, intravenous exposure to microcystins, and possibly cylindrospermopsin was most likely the cause of death of the dialysis patients.

6.2 Animal Studies

6.2.1. Acute Toxicity

Oral Exposure—Acute toxicity to cylindrospermopsin-equivalent of freeze-dried *C. raciborskii* cells (strains PHAWT/M or PHAWT/1) was tested in male MF1 mice by gavage (Seawright et al., 1999). Twelve mice were administered a single dose of 4.4, 5.3, 5.7 (to only two mice), 5.8, 6.2, 6.5, 6.7, 6.8, 6.9, 8.0 and 8.3 mg/kg by gavage and observed after 8 days. Of the 12 mice, 8 died two to six days after treatment. The lowest lethal dose was 4.4 mg/kg, and the highest non-lethal dose was 6.9 mg/kg. An average lethal dose was approximately 6 mg/kg. Histological examinations showed fatty liver effects with periacinar coagulative necrosis, acute renal tubular necrosis and atrophy of the lymphoid tissue of the spleen and thymus. Subepicardial and myocardial hemorrhages in the heart and ulceration of the esophageal section of the gastric mucosa also were observed. The authors reported thrombohemorrhagic lesions in one or both eye orbits in some of the animals.

Falconer et al. (1999) administered a single gavage dose of 1,400 mg extract/kg of a cell-free extract of freeze-dried and sonicated *C. raciborskii* Woloszynska (AWT 205) cells to an unreported number of male Swiss mice. Although not specified in this experiment, concurrent i.p. experiments stated the content of cylindrospermopsin in the extract ranged from 1.3 to 5.4 mg/g extract. This indicates that the cylindrospermopsin-equivalent gavage dose likely ranged from 1.8 to 7.6 mg/kg. Although not fatal, the authors observed severe liver and kidney pathology at this dose. No other information on the design and results of the oral study were provided (Falconer et al., 1999). In a subsequent gavage study Falconer and Humpage (2001) reported that 2,500 mg extract/kg was the minimum oral lethal dose of freeze-dried *C. raciborskii* cells (strain AWT 205) in male Swiss albino mice.

Shaw et al. (2001) administered a single gavage dose of 0, 1, 2, 4, 6 or 8 mg cylindrospermopsin/kg of cell-free extract of freeze-dried and sonicated *C. raciborskii* cells (strain AWT 205) in water to groups of four Quackenbush mice. After 7 days, all animals were evaluated for gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) changes. Different hepatic effects were observed at different doses as follows:

- 1 and 2 mg/kg showed foamy hepatocellular cytoplasmic changes;
- 4 mg/kg resulted in lipid infiltration with some hepatocyte necrosis in the periacinar region;
- 6 mg/kg resulted in uniformly pale and mottled livers with lipid infiltration throughout and cell necrosis mainly in the periacinar region;
- 6 mg/kg caused the death of two of four mice within 5 days; and
- 8 mg/kg caused the death of all of the mice within 24 to 48 hours.

In the second part of a genotoxicity study (described in Section 6.4.1), Bazin et al., (2012), administered 1, 2, and 4 mg/kg of cylindrospermopsin (98% purity) by gavage to mice (three per dose). Clinical signs and tissue sample evaluations were done 24 hours after treatment as well as histological examination. One mouse in the 2 mg/kg dose group died, and one of the three mice treated with the highest dose (4 mg/kg) was moribund. Histological evaluation found a dark red liver and intestinal hemorrhage. Another mouse manifested intestinal bleeding and liquid stools at the same dose. Apoptosis was observed in the liver and the kidneys at 2 and 4 mg/kg, involving up to 5% of hepatocytes within some sections and apoptosis of lymphocytes within the Peyer's patches in some mice within these two dose groups. Authors concluded that the liver and kidneys are target organs, but the kidneys appeared to be the most sensitive organ following gavage of cylindrospermopsin (Bazin et al., 2012)

Other Routes of Exposure—Acute i.p. lethality values have been determined for cylindrospermopsin purified from extracts of cultured *C. raciborskii* or *U. natans* cells (Ohtani et al., 1992; Shaw et al., 2000, 2001; Terao et al., 1994). In male CH3 mice, 24-hour and 5- to 6-day LD_{50} values of 2.1 and 0.2 mg/kg body weight (bw), respectively, were reported for a single i.p. dose of purified cylindrospermopsin (percent purity not reported) (Ohtani et al., 1992). Another study found that a single 0.2 mg/kg i.p. dose of purified cylindrospermopsin (percent purity not reported) caused 50% moribundity after 31 hours of exposure in Quackenbush mice (Shaw et al., 2001). The main pathological findings in the moribund animals were lipid infiltration and cell necrosis of the liver.

The results of acute i.p. studies of extracts of freeze-dried and sonicated *C. raciborskii* cells are generally similar to those of the i.p. studies of purified cylindrospermopsin. A single 0.2 mg/kg cylindrospermopsin-equivalent dose caused 50% moribundity in Quackenbush mice after 98 hours (Shaw et al., 2000, 2001). Other single-dose LD_{50} values, expressed as cylindrospermopsin-equivalent doses included 24-hour and 7-day values of 0.29 and 0.18 mg/kg, respectively, in male Swiss mice (Hawkins et al., 1997). A 24-hour LD_{50} from exposure to extract containing cylindrospermopsin was lower than the 24-hour i.p. LD_{50} of 2.1 mg/kg for purified cylindrospermopsin in mice, leading the authors to suggest that the extract contained more than one toxin (Ohtani et al., 1992). Although the liver was the main target organ in the extract studies, lesions also occurred in the kidney, adrenal gland, lung and intestine (Hawkins et al., 1985, 1997; Shaw et al., 2000, 2001).

A single dose i.p. LD_{50} value of 64 mg freeze-dried culture/kg was determined in mice observed for 24 hours (Hawkins et al., 1985). The principal tissue injury was severe centrilobular hepatic necrosis. Evidence for histological damage also was observed in the kidney, adrenal glands, lungs and intestines.

Falconer et al. (1999) assessed the acute lethality and liver and kidney effects of four different preparations of cell-free extracts of sonicated freeze-dried *C. raciborskii* cells in male Swiss albino mice treated by single i.p. injection. Reported 24-hour and 7-day LD_{50} values for the four preparations were 50 to 110 and 20 to 65 mg extract/kg, respectively. The cylindrospermopsin content in the four preparations

varied from 1.3 to 5.4 mg/g extract, indicating that the cylindrospermopsin-equivalent LD_{50} values were 0.07-0.6 mg/kg (24-hour) and 0.03-0.4 mg/kg (7-day). Centrilobular liver damage was characterized by cellular vacuolation, intercellular spaces and dark nuclear and cytoplasmic staining. In the kidney, there was a reduction in the number of erythrocytes in the glomerulus, an increase in the space around the glomerulus, proximal tubule epithelial necrosis and the presence of proteinaceous material in the distal tubules. Transmission electron microscopy suggested that the material in the distal tubules was cell debris from necrosis. The nature, location and time course of the histological damage were similar for oral and i.p. administration, with maximum damage observed from 2 to 3 days after treatment. There was no clear correlation between cylindrospermopsin preparation concentration and the LD_{50} values or the severity of liver or kidney lesions, leading the study authors to conclude that more than one toxin was present in the extract.

Terao et al. (1994) examined toxicity in 24 male ICR mice administered a single 0.2 mg/kg i.p. dose of purified cylindrospermopsin (percent purity not reported). Ultrastructural examination of the organs was conducted using electron microcopy following sacrifice of 3 mice at each of 8 time points (16 hr. to 100 hr.) after exposure. The liver cells were isolated and found to be the main toxicity target. Ribosomes were detached from the endoplasmic reticulum and there was an increase in the smooth endoplasmic reticulum plus Golgi apparatus. Nucleoli became dense and reduced in size. Severe necrosis was present in the centrilobular region. There was a dramatic increase in intracellular fat vacuoles impacting the orientation of the microorganelles. After 100 hours, the lobular hepatocytes were destroyed. Histological changes in the kidney included proliferation of the endoplasmic reticulum and fat droplet accumulation in cells along the brush borders of the tubules plus limited single cell necrosis. The thymus also was impacted as indicated by massive necrosis of lymphocytes in the cortex. Occasional single cell necrosis was identified in the heart.

Inhalation Exposure—Oliveira et al. (2012) evaluated the effects in the lung of cylindrospermopsin after intratracheal instillation of a lethal dose in BALB/c mice. Semi-purified extract of cylindrospermopsin was instilled at 70 μg/kg-bw into 52 mice. Control group (12 mice) received a single intratracheal instillation of 50 μL of saline solution. Animals were analyzed 2, 8, 24, and 96 hours after instillation for the presence of cylindrospermopsin in the lungs and liver. Pulmonary mechanics to measure airflow, lung volume, lung resistive, static elastance, viscoelastic/inhomogeneous pressures and viscoelastic component of elastance were performed. The fraction of collapsed and normal alveoli areas were determined microscopically and expressed relative to the total area examined. Polymorpho- (PMN) and mono-nuclear (MN) cells, and pulmonary tissue were determined by histological analysis. Biochemical analyses determined the total protein content, inflammation changes (myeloperoxidase activity), oxidative stress analyses, and to determine the presence of cylindrospermopsin in the liver and lungs (Oliveira et al., 2012).

No deaths occurred during the experiment by Oliveira et al. (2012). After 24 hours, the authors detected a higher concentration of cylindrospermopsin in the lung, and after 96 hours, concentration in the liver increased significantly ($p<0.05$). Histological evaluation revealed that after 24 hours, static elastance, PMN influx into lung parenchyma and myeloperoxidase activity increased. Alveolar collapse (indicative of a partial collapse of lung tissues) was apparent at 8 h and increased after 24 hours. However, the authors did not observe intra-alveolar hemorrhage. According to the authors, the increase in alveolar collapse may have occurred as a result of the production of the reactive oxygen species (ROS) from cylindrospermopsin and/or its metabolites, or by activated defense cells involved with the inflammatory process (Oliveira et al., 2012).

6.2.2. Short Term Studies

Oral Exposure—Four Quackenbush mice were administered either cell-free extract of *C. raciborskii* (strain AWT 205) or purified toxin (Shaw et al., 2001). Doses for the extract ranged from 0 to 0.3 mg/kg/day. The no-observed-adverse-effect-level (NOAEL) for cell-free extract was <0.005 mg/kg/day for lymphophagocytosis in the spleen following 14-day gavage administration. When purified cylindrospermopsin was given at doses of 0 to 0.3 mg/kg/day for 14 days, the low dose (0.05 mg/kg/day) was the lowest-observed-adverse-effect-level (LOAEL) for fatty infiltration of the liver; lymphophagocytosis did not occur at any dose (Shaw et al., 2001). One animal had a retro-orbital hematoma of one eye. The observation that lymphophagocytosis was present only in the animals that received the extract suggests the presence of an additional toxin in the extract. The authors suggested the impurity might be a lipopolysaccharide.

Shaw et al. (2001) administered drinking water containing 800 µg/L cylindrospermopsin to six Quackenbush mice and two Wistar rats for 21 days. The drinking water was "sourced" from a dammed impoundment containing cylindrospermopsin. Based on water consumption, the reported approximate daily dose for both species was 0.2 mg cylindrospermopsin/kg/day. No effects were observed in gross pathological and histological examinations of the liver, kidney, spleen, heart, lungs and thymus indicating a NOAEL of 0.2 mg/kg/day in rats and mice. No additional information on the experimental design and results was reported by the authors (Shaw et al., 2001).

Significant increases in hematocrit, acanthocytes (abnormal form of a red-blood cell that has a spiked, thorn-like cell membrane), and liver and testes weights were observed in a study of purified cylindrospermopsin from *Aphanizomenon ovalisporum* (isolated from Lake Kinneret during a 1994 bloom) by Reisner et al. (2004). Groups of eight, 4 week-old ICR mice were exposed to drinking water containing 0.6 mg/L cylindrospermopsin for 3 weeks. The dose was estimated by the authors as 66 µg/kg/day. The study was designed in order to test a hypothesis introduced by Banker et al. (2000) that the toxicity of cylindrospermopsin could be a reflection of its inhibition of one of the enzymes involved in synthesis of uridine monophosphate (UMP).

Blood was collected once per week for determination of hematocrit, red blood cells (RBC) counts and plasma cholesterol (Reisner et al., 2004). At the end of the exposure period, the animals were sacrificed; the liver, kidney, and spleen were removed, and weighed. The liver was then homogenized. A sample of the homogenate was analyzed for total cholesterol and the crude protein extract was frozen for later analysis of uracil monophosphate.

Body weight increased across the duration of the study for both the controls and treated animals and did not differ significantly between groups at 21 days (Reisner et al., 2004). Significant ($p<0.05$) increases in relative liver and testes weights were noted when compared to controls; relative kidney weight also increased, but was not statistically significant. At the end of three weeks, urinary orotic acid (a pyrimidine precursor) concentration and hematocrit were significantly increased ($p<0.05$) in the treated animals compared to the controls. There was a decrease in the urine excretion rate for both the controls and treated animals over the three week exposure period with the decrease in the treated animals being significantly greater ($p < 0.05$) in those exposed than that for controls at the end of the three week period. Acanthocyte-like RBCs were observed in Numansky light micrographs of the blood samples collected from the treated animals at the end of each exposure week. The cholesterol content of the RBC membranes and plasma were significantly ($p<0.05$) greater than the levels in controls after the three week exposure and the liver levels were significantly lower than controls (Reisner et al., 2004).

The authors attributed the acanthocyte (abnormal RBC) formation to the increase in RBC membrane cholesterol (Reisner et al., 2004). An increase in the ratio of RBC membrane cholesterol to phospholipids is believed to be a factor responsible for acanthocyte formation. The authors hypothesized that this change

is the consequence of decreased activity of plasma lecithin-acyl cholesterol transferase (LCAT), an enzyme associated with high density lipoproteins that regulates the formation of cholesterol esters (Garrett and Crisham, 1999). Effects on the cholesterol content of the RBC membrane can occur with inhibition of the enzyme increasing membrane fluidity and mean corpuscular volume. Removal of the abnormal blood cells by the spleen increases both spleen weight and serum bilirubin stimulating hematopoiesis. Additional research is needed to examine the LCAT enzyme inhibition hypothesis to confirm whether it accounts for the effects on the RBCs following cylindrospermopsin exposure. The authors proposed that there is a relationship between the cylindrospermopsin-induced liver and/or kidney damage and the decreased LCAT activity.

As stated above, the original goal of the Reisner et al. (2004) study was to investigate the role of the uracyl moiety of cylindrospermopsin as an inhibitor of uridine synthesis. Although the study revealed that the toxin was a noncompetitive inhibitor of the UMP synthase complex, there were minimal *in vitro* consequences of inhibition at the cylindrospermopsin dose evaluated (66 µg/kg/day) or *in vivo* evidence of orotic aciduria, the expected consequence from UMP synthase inhibition.

Other Routes of Exposure—In addition to the oral exposure studies discussed above, Shaw et al. (2001) also studied the effects of i.p. exposures. Four Quackenbush mice were dosed by i.p. with either cell free extract of *C. raciborskii* (strain AWT 205) or purified toxin for 14 days. The doses for the extract ranged from 0 to 0.05 mg/kg/day. The LOAEL was <0.005 mg/kg/day for slight foamy cytoplasmic changes in the liver and for lymphophagocytosis in the spleen. No NOAEL was identified. When doses of 0 to 0.025 mg/kg/day of purified cylindrospermopsin were given for 14 days, the low dose (0.005 mg/kg/day) was a LOAEL for foamy hepatocellular cytoplasm, but lymphophagocytosis did not occur at any dose (Shaw et al., 2001).

6.2.3. Subchronic Studies

Oral Exposure—Doses of 0, 30, 60, 120 or 240 µg/kg/day purified cylindrospermopsin in water was administered by gavage to groups of male Swiss albino mice (10 mice per dose for all but the highest dose group which included 6 mice) for 11 weeks (Humpage and Falconer, 2002, 2003). The cylindrospermopsin was from an extract of freeze-dried *C. raciborskii* cells (strain AWT 205) purified using Sephadex size-exclusion gel (G-10). The individual sephadex fractions were assayed using HPLC and concentrated to a sample that was 47% cylindrospermopsin by dry weight and 53% phenylalanine. Food and water consumption and body weight were examined throughout the study. After 9 weeks of exposure, a clinical examination consisting of physiological and behavioral signs of toxicity was conducted, the study authors did not report specific tests. Hematology evaluations (4 to 5 per dose group, except the high dose) was done. Serum chemistry (4 to 6 per dose group), and urinalysis (6 or 10 per dose group) were also conducted. All the evaluations were conducted either near or at the end of the treatment period.

Postmortem examinations were done on the following organ weights: liver, spleen, kidneys, adrenal glands, heart, testis, epididymis and brain. Comprehensive histological evaluations were conducted in accordance with the recommendations from the Organization for Economic Cooperation and Development (OECD).

No deaths or clinical signs of toxicity were reported in mice exposed to purified cylindrospermopsin under the study conditions. The mean final body weight was 7-15% higher in all dose groups compared to controls, but not dose-related and only statistically significant at 30 and 60 µg/kg/day (Humpage and Falconer, 2003). No significant changes were observed in food consumption. In all dose groups, the water intake was significantly reduced.

Relative kidney weight was significantly increased in a dose-related manner at ≥60 µg/kg/day (12-23% greater than controls; see Table 6-1), and only at the highest dose (240 µg/kg/day) relative liver weight was significantly increased (13% greater than controls). Relative spleen, adrenal and testes weights were increased for doses ≥60 µg/kg/day, but the differences from control were not statistically significant (Humpage and Falconer, 2002).

Selected serum chemistry (n= 4-6), hematology (n=4-5) and urinalysis (n=6-10) results are shown in Table 6-2. The hematology and serum chemistry evaluations showed no dose-related, statistically-significant changes, although serum albumin, total bilirubin, and cholesterol were increased compared to controls at all doses (Humpage and Falconer, 2002). The increases in cholesterol were significant for the 30 and 60 µg/kg/day groups, but not at the higher doses. The serum urea concentration was slightly decreased at the two highest doses. A non-significant increase in red cell polychromasia (high number of RBCs), was indicated for all doses, but quantitative data were not presented. Packed red cell volume was slightly increased and mean corpuscular hemoglobin was slightly decreased (Table 6-2) when compared to controls, although the changes were not dose related. When combined with the bilirubin results and the increased relative spleen weight, the hematological data suggest a possibility for minor red blood cell effects. One of the limitations of the serum chemistry and hematology data, is the small number of samples evaluated, a factor that impacts the determination of statistical significance (Humpage and Falconer, 2002).

Table 6-1. Kidney Weight Data from Oral Toxicity Study of Cylindrospermopsin Administered Daily over Eleven Weeks (Humpage and Falconer, 2002, 2003)

Dose (µg/kg/day)	Number	Relative Kidney Weight		% Change	Significance
		Control g/100g $_{BW}$	Exposed g/100g $_{BW}$		
30	10	1.48	1.57	+6	Not significant
60	9	1.48	1.66	+12	p <0.001
120	9	1.48	1.82	+23	p <0.001
240	6	1.48	1.78	+20	P <0.001

There was a significant decrease in the urine protein-creatinine ratio (g/mmol creatinine) at 120 and 240 µg/kg/day compared to that of controls (51% and 37% of controls, respectively; both $p<0.001$) (Humpage and Falconer, 2002). Also, a significant decrease in urine specific gravity normalized for creatinine was seen at 240 µg/kg/day compared to the control ($p<0.001$). The renal glomerular filtration rate (GFR) was decreased compared to controls at all doses, but the differences were not dose-dependent or statistically significantly different from controls. The renal failure index[1] was decreased slightly at ≥ 120 µg/kg/day; the differences from control were not statistically significant (Humpage and Falconer, 2002). Tubular retention of the low molecular weight urinary proteins could account for the decreased urinary protein and possibly the increased kidney weight. Although effects on kidney weight and urine protein levels were observed in male mice, the biological relevance of the latter effect and whether it would also occur in in female mice needs further investigation. Mice are known to excrete a group of highly polymorphic, low-molecular-weight urinary proteins that play important roles in social recognition

[1] Renal failure index= (urinary sodium concentration × plasma creatinine concentration) / urinary creatinine concentration

and mate assessment (Cheetham et al., 2009). The relevance of the urinary protein findings in mice to humans is unknown.

Table 6-2. Selected Clinical Chemistry, Hematology, and Urinalysis Findings (Humpage and Falconer, 2002, 2003)

Endpoint	N	Dose (µg/kg/day)				
		0	30	60	120	240
Clinical Chemistry						
Urea (mmol/L)	4-6	9.24	9.22	8.55	7.51	7.92
Albumin (g/L)	4-6	23.8	26.6	26.0	26.0	25.8
Cholesterol (mmol/L)	4-6	3.26	4.60**	4.65**	3.68	4.08
Bilirubin (mmol/L)	4-6	2.62	2.72	2.88	3.06	3.07
Hematology						
Packed Cell volume (L/L)	4-5	0.38	0.39	0.39	0.39	ND
Mean Corpuscular Hemoglobin (MCH, pg/L)	4-5	16.8	15.7	16.4	16.4	ND
Urinalysis						
Volume (mL)	6-10	9.85	11.18	10.38	11.74	6.74
Creatinine (mmol/L)	6-10	0.57	0.49	0.54	0.51	0.72**
Specific gravity/creatinine	6-10	1.79	2.04	1.91	1.99	1.44*
Protein/creatinine (g/mmol)	6-10	4.3	3.6	3.3	2.2**	1.6**
Renal Failure Index (mmol/L)	4-6	4.3	4.3	4.5	3.6	3.6

ND = not determined
Significantly different from control: *p<0.05; **p<0.01.

Although cylindrospermopsin appeared to inhibit protein synthesis in the liver, based on the histological evidence of ribosomal detachment from the endoplasmic reticulum after i.p. exposure to a 0.2 mg/kg dose (see previous discussion of Terao et al., 1994), serum albumin and total serum protein were not decreased in Humpage and Falconer studies (2002, 2003). The most sensitive effects observed by Humpage and Falconer (2002, 2003) were dose-related decreases in the urinary protein/creatinine ratio at ≥120 µg/kg/day and increased relative kidney weight at ≥60 µg/kg/day. The noted decrease in urinary protein excretion could reflect an impact on excretion of mouse urinary proteins given the fact that total serum protein was not significantly increased compared to controls for all dose groups. Mouse urinary proteins are synthesized in the liver (Clissold and Bishop, 1982) and transported to the kidney for excretion. If the cylindrospermopsin were to reduce liver protein synthesis a decrease in total serum protein would be expected. However, this was not the case suggesting a lack of an effect on synthesis of the urinary proteins in the liver.

The Humpage and Falconer (2002, 2003) postmortem tissue examinations showed histopathological damage to the liver based on scores assigned for necrosis, inflammatory foci and bile duct changes at ≥120 µg/kg/day. The percent of animals with liver lesions in the 120 and 240 µg/kg/day dose groups was 60% and 90% when compared to 10%, 10%, and 20% for the 0, 30 and 60 µg/kg/day dose groups, respectively. Severity scores were not given and the liver lesions were not further described. There was proximal renal tubular damage in kidney sections from two mice in the 240 µg/kg/day dose group (Humpage and Falconer, 2002, 2003). A NOAEL and LOAEL of 30 and 60 µg/kg/day, respectively, was identified based on the dose related and statistically significant increase in relative kidney weight.

Humpage and Falconer (2002, 2003) also conducted a study of the crude cylindro-spermopsin extract that was purified for the studies described above. Exposure occurred over a 10 week period in groups of 10 male Swiss albino mice given doses of 0, 216, 432 or 657 µg/kg/day cylindrospermopsin in drinking water. Significantly decreased body weight was observed at the two highest doses. Liver and kidney weights were increased in a dose-related manner at 216, 432, and 657 g/kg/day ($p<0.0001$). Serum ALP was significantly increased ($p<0.05$) for the 432 µg/kg/day dose group. There was a dose-related, significant increase in total serum bilirubin ($p<0.05$, $p<0.001$) and a decrease in serum bile acids for the two low dose groups ($p<0.001$) data were not presented for the high dose group. The urinary protein/creatine ratio was significantly decreased ($p<0.001$) compared to controls for the two high dose groups; it also was decreased for the low dose but the difference from controls was not statistically significant. The renal failure index was decreased significantly at 432 µg/kg ($p<0.01$); no data were presented for the 657 µg/kg high dose group. Glomerular filtration was increased (142% of control) for the 432 mg/kg/day dose group but the difference from controls was not significant. Glomerular filtration was increased for the 432 mg/kg/day dose group. The lowest dose tested (216 mg/kg/day) was a LOAEL.

In a follow-on study to Reisner et al. (2004), the potential effects of cylindrospermopsin were investigated in a 42-week mouse oral dose step-up protocol[2] (Sukenik et al., 2006). Food and water *ad libitum* were administered to four-week old weaned male and female ICR mice (initial body weight 24–28 g). Animals were divided into two groups of 20 males and 20 females in each group. The mice in the control group received freshly prepared cyanobacterial growth medium as their drinking water, whereas mice in the experimental group received spent medium that contained several different concentrations of cylindrospermopsin obtained from the spent medium on which cultures of *Aphanizomenon ovalisporum* had been grown. Cylindrospermopsin concentrations in the spent medium were quantified by HPLC using a UV detector set at 263 nm to identify the cylindrospermopsin peak. No other toxins were quantified in the spent medium although it is known that cyanobacterium spent medium contains an array of secondary metabolites and other compounds (A. Sukenik, personal communication, 2014).

The concentration of cylindrospermopsin in drinking water was increased gradually from 100 to 550 µg/L (Sukenik et al., 2006). The daily intake of the toxin by animals in the experimental group was ~10 µg/kg for weeks 0-8; ~15 µg/kg for males and ~17 µg/kg for females for weeks 8-16; ~30 µg/kg for males and ~34 µg/kg for females for weeks 16-24; and ~48 µg/kg for males and ~55 µg/kg for females for weeks 24-42 (these data were presented graphically). Body weight was measured weekly. Water consumption and urine excretion rates were estimated using metabolic cages every two weeks. Blood samples were obtained every 4 weeks to determine hematocrit. Ten animals were sacrificed after 20 weeks and ten animals were sacrificed after 42 weeks of treatment. At sacrifice, liver, spleen, kidney and testes were weighed and examined grossly for pathological symptoms. Cholesterol levels were measured in the liver. There were no significant changes in body weight while relative kidney weights were significantly increased ($p<0.05$) in males and females at 20 weeks and 42 weeks. Relative liver weight was increased ($p<0.05$) in males and females only at 42 weeks. Relative testes weights were increased in males at 42 weeks. Absolute organ weight data were not given.

Hematocrit levels were significantly ($p <0.05$) elevated compared to controls in both male and female mice from 16 to 32 weeks of exposure to cylindrospermopsin in drinking water, but returned to control levels by 36 weeks (Sukenik et al., 2006). The observed changes in the hematocrit level were accompanied by increased numbers of acanthocytes in the blood as observed by light microscopy. At 20 weeks "many" RBCs were present as acanthocytes (abnormal RBCs), and at 42 weeks very "few normal"

[2] In a step-up dose approach the same animals are sequentially exposed to a consecutive series of increasing doses. Each dose is given for a specified period of time. In Sukenik et al. (2006) doses were increased on weeks 8, 16, and 24. At week 20 half the animals were sacrificed for examination.

cells were present in the collected blood samples. The number of normal versus acanthocyte cells was not quantified.

As explained above, RBC conversion to acanthocytes appears to be related to increased cholesterol in the RBC membrane. The authors measured the cholesterol in the RBC membrane, plasma and liver for 8 males and 8 females at 20 and 42 weeks. Cholesterol was significantly increased in the RBC membrane and decreased in the liver for both males and females at 42 weeks. At 20 weeks, there was a significant decrease in liver cholesterol in males, but not in females. Plasma cholesterol increased slightly at 42 weeks and the difference was significant only for the females (Sukenik et al., 2006).

Based on changes in hematocrit at 16 weeks, the authors proposed a dose of 20 µg/kg/day (equivalent to 200 µg/L) as the maximal daily intake of cylindrospermopsin during the first 16 weeks that resulted in adverse effects and the proposed lowest-observed adverse-effect level for both male and female mice (Sukenik et al., 2006).

In a 90-day study, Quackenbush mice were administered a cell-free extract of freeze-dried and sonicated *C. raciborskii* cells (strain AWT 205) in drinking water (Shaw et al., 2001). Gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) examinations showed no effects at dose levels as high as 0.15 mg/kg/day. Animals were examined for mortality and other clinical signs of toxicity. Neither the number of animals per dose group nor other details of both the experimental design and results were reported.

Neurotoxicity—The published literature does not provide sufficient data to determine if cylindrospermopsin elicits neurotoxicity. Humpage and Falconer (2002) reported that they examined brain, spinal cord and peripheral nerve histopathology, but no results are given in their published report.

6.2.4. Developmental/Reproductive Toxicity

Pregnant rats (10 per dose group and control) were exposed by gavage to 0 (control group), 0.03, 0.3 and 3 mg/kg/bw purified cylindrospermopsin (purity not specified) solutions (Sibaldo de Almeida et al., 2013). The rats were exposed daily from GD 1-20 and water intake, food consumption and organ and body weight were recorded during the treatment. Histopathological evaluations were conducted to tissue portions from liver and kidney. The authors used half of the fetuses from each litter to study visceral malformations (teratogenic action), and the other half to study skeleton malformations. The authors did not find significant differences (no statistical significance was provided) between the control and treated rats in body weight gain, water and food intake or in the histopathological analysis of tissue. No visceral or skeletal malformations in the fetus were observed (Sibaldo de Almeida et al., 2013).

A series of studies was conducted with cylindrospermopsin in pregnant CD-1 mice to investigate developmental toxicity (Rogers et al., 2007) as well as characterize maternal toxicity and recovery post-partum (Chernoff et al., 2011). Purified cylindrospermopsin (>98%) was supplied by the Australian Water Quality Centre and administered in distilled water by i.p. injection for all experiments. All controls were given distilled water. Dosages were calculated based on maternal body weight on gestation day (GD) 6 and remained constant. The first set of studies included a standard developmental toxicity study evaluating dose-response effects in dams and fetuses; this was followed by evaluation of post-natal growth and development after maternal exposure to a single dose level (Rogers et al., 2007). Dams whose offspring were used for post-natal evaluation were subsequently used to further characterize maternal effects and recovery from the single dose level during gestation (Chernoff, et al., 2011).

In a standard developmental toxicity study, groups of 20 to 25 pregnant females were administered 0, 8, 16, 32, 64, 96 or 128 µg cylindrospermopsin/kg/day on GDs 8-12 (Rogers et al., 2007). Animals were sacrificed on GD 17 and the uterine contents examined. Increased maternal mortality occurred in mice

given 32 µg/kg/day (4/20) or higher (17-19/20). Average time to death ranged from 6.5 days at 32 µg/kg/day to 4.4 days at 128 µg/kg/day. A significant ($p < 0.01$ or 0.05) dose-related increase in liver-to-body-weight ratio was observed in the dams from the 8, 16 and 32 µg/kg/day dose groups compared with control (+13, +15, +30%, respectively). Maternal body and absolute liver weights were not given. Fetal body weight and numbers of live and dead fetuses per litter were not significantly affected by maternal treatment. No treatment-related external, skeletal or visceral anomalies were observed. The LOAEL was 8 µg/kg/day based on increased relative liver weight; the 32 µg/kg/day was a frank effect level (FEL) based on maternal mortality.

The post-natal evaluation experiments from Rogers et al. (2007) were conducted using two groups per exposure period. The dams (23 to 51 mice per group) were dosed i.p. with 50 µg/kg/day on either GD 8-12 or GD 13-17 and allowed to litter. At birth, litters were examined and pups from control and treated dams were combined (total of 10/litter) and cross-fostered with control dams until post-natal day 5-6. A subset of male pups from dams treated on GDs 13-17 was weaned and their growth monitored for 15 months. Dosing on GDs 8-12 resulted in marked maternal toxicity including: vaginal bleeding, reduced activity, blood in the tail tips, and combined mortality of 49/79 animals. In contrast, dams treated on GDs 13-17 showed very low incidences of bleeding around the eyes and vaginal bleeding and only 1/71 animals died. A slight decrease in gestation length for dams treated on GDs 13-17 was noted by the authors and described as unusual. The dosing during the earlier period of gestation (GD 8-12) resulted in greater manifestations of maternal toxicity that the later dosing period.

Significant ($p < 0.01$ or 0.05) reductions in litter size at birth were observed in both the GD 8-12 and GD 13-17 treated groups (Rogers et al., 2007). No evidence of late fetal or early postnatal deaths was found; numbers of implantations were not assessed. The litters born to the dams in the GD 8-12 group were fewer than those for the controls but the differences were not statistically significant. Pup body weight and survival through post-natal day 6 were not affected by maternal treatment on GDs 8-12. In contrast, maternal treatment on GDs 13-17 resulted in significantly ($p < 0.01$) decreased survival as well as reduced pup body weight at birth and on lactation days 1 and 5-6. Necropsy of pups that died revealed blood-filled intestines. Lower body weight persisted in male pups from dams exposed on GDs 13-17 throughout the 15-month post-weaning interval.

To further characterize effects on the adult animal, 3-5 dams/group, whose litters were evaluated for post-natal growth (i.p., 50µg cylindrospermopsin/kg/day; GDs 8-12 [n=42] or GDs 13-17 [n=42]), were sacrificed the day following the last dose and on post-treatment days 7 and 14 for both exposures, days 28 and 42 for the GDs 8-12 exposure and on days 35 and 49 for the GD 13-17 exposures (Chernoff et al., 2011). Blood, liver and kidney samples were obtained at each time point for further analyses. Endpoints measured included maternal weight and clinical signs of toxicity, serum chemistries indicative of hepatic and/or renal function and general homeostasis, histopathology of liver and kidney tissues, and hepatic gene expression after the dosing period.

Dosing on GDs 8-12 resulted in maternal toxicity and death as described above (Chernoff et al., 2011). Maternal body weight gain was reduced ($p<0.05$) throughout treatment on GDs 8-12 resulting in significantly lower body weight ($p<0.01$) at termination one day after the last dose. Treatment on GDs 13-17 caused a reduced weight gain only after the second dose. Mice sacrificed the day after the last dose from either regimen had decreased albumin and numerous elevated serum enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alpha-1-antitrypsin, sorbitol dehydrogenase (SDH) and lactate dehydrogenase (only 2-5 mice per assay). Blood urea nitrogen (BUN) and creatinine (indicators of kidney damage) were also significantly increased the day after exposure ended. All clinical chemistry endpoints had returned to control levels 7 days after exposure. No significant differences in relative kidney or liver weights were observed at any time. The day after the last dose, histopathology revealed hepatocyte necrosis in 7/19 of GD 8-12 treated animals and 4/19 of GD 13-17 treated animals compared with 1/19 of both control groups. Moderate nephrosis and/or renal inflammation was found in

5/19 animals treated on GDs 8-12, but in none of the other treated and control mice. Microscopic lesions had resolved by one week post-dosing. Analysis of gene expression in liver tissue showed alterations in expression of genes involved in ribosomal biogenesis, xenobiotic and lipid metabolism, inflammatory response, and oxidative stress. The response was similar between both exposure groups, persisted for 2 weeks after treatment ended and returned to normal by 4 weeks (Chernoff et al., 2011).

6.2.5. Chronic Toxicity

No information regarding the chronic toxicity of cylindrospermopsin was located.

6.3. Carcinogenicity

***In vivo* Studies**—Falconer and Humpage (2001) tested the tumor initiating activity of cylindrospermopsin in male Swiss mice using O-tetradecanoylphorbol 13-acetate (TPA) as the promoter. Saline extract of freeze-dried *C. raciborskii* cells (strain AWT 205) of 500 or 1500 mg/kg doses were given to those treated with cylindrospermopsin, and control mice were administered saline. Three oral doses separated by a two-week recovery period between each dose were given to each control and treated group. The number of animals initially assigned to each group was not reported. However, of those that received oral doses of 1500 mg/kg, 70% died within one week of the second dose. Surviving animals were not dosed again. The cylindrospermopsin-equivalent doses in the 500 extract/kg group was 2.75 mg/kg, and in the 1500 mg extract/kg group was 8.25 mg/kg, based on the reported cylindrospermopsin content of 5.5 mg/g extract. Two weeks after the final dose, the saline and 500 mg extract/kg groups were fed liquid food containing TPA dissolved in DMSO, or food containing DMSO alone, for 24 hours two times per week for 30 weeks and divided into subgroups of 13 to 18 mice. All of the surviving mice in the 1500 mg/kg groups were similarly exposed to TPA-containing liquid food only and were not exposed to food containing DMSO alone.

At the end of the 30-week promotion period, histological examinations of the liver, kidneys, spleen and grossly abnormal organs were performed on all groups. No neoplastic changes were found in any of the 27 control mice. There were three tumors and two areas of dysplastic foci in 5 cylindrospermopsin-treated mice. No clear pattern in the neoplastic changes was observed because they occurred in different animals, target organs and treatment groups (Table 6-3). The results of the study do not indicate that the cyanobacterial extract was a tumor initiator. However, the study is limited by the number of animals tested, design of the dosing regimen, and by the 30 week observation period.

Table 6-3. *C. raciborskii* Tumor Initiating Results (Falconer and Humpage, 2001)

Oral Treatment (mg extract/kg)	Number of Mice	Histological Findings*
1 x 1500/TPA	14	2 hepatocellular dysplastic foci 1 fibroblastic osteosarcoma
2 x 1500/TPA	5	No neoplasia observed
3 x 500/DMSO	18	1 hepatocellular carcinoma, 1 lymphoma
3 x 500/TPA	16	No neoplasia observed
Saline/DMSO	14	No neoplasia observed
Saline/TPA	13	No neoplasia observed

* Findings in different animals.
* DMSO (dimethylsulphoxide); TPA (tetradecanoly phorbol acetate)

***In vitro* Studies**—The carcinogenic potential of cylindrospermopsin was assessed *in vitro* via the cell transformation assay (CTA) on Syrian hamster embryo (SHE) cells (Marie et al., 2010). This assay is

recommended by OECD Guidelines (2007) as an alternative to *in vivo* long term experiments for carcinogenic potential of chemicals because SHE cells are genetically-stable, normal diploid cells that are capable of metabolic activation. Purified cylindrospermopsin, supplied by the Australian Water Quality Centre (>98% purity; Adelaide, Australia) was dissolved in water and applied to SHE cells at cylindrospermopsin concentrations of 1×10^{-5} to 1×10^{-1} ng/mL for the evaluation of cytotoxicity and 1×10^{-7} to 1×10^{-3} ng/mL for the evaluation of cell transformation for seven days. Relative cloning efficiency was used as an indicator of cytotoxicity. Transformation frequency was determined microscopically based on the cell morphology (spindle shaped cells, the nucleoplasm to cytoplasm ratio and basophilic staining properties). Benzo(a)pyrene was used as the positive control and dimethylsulfoxide as the negative control.

Cylindrospermopsin exhibited transformation at concentrations lower than those causing cytotoxicity (Marie et al., 2010). There was no change in cloning efficiency at any concentration. However, cloning efficiency was significantly decreased at a 1×10^{-2} ng/mL concentration in a range-finding study conducted prior to the main experiment. Transformation frequency was significantly increased over the positive control at concentrations from 1×10^{-2} to 1×10^{-7} ng/mL but not at the 1 or 1×10^{-1} ng/mL. The lack of a positive response for the 1×10^{-1} and 1 ng/mL concentrations may reflect the fact that only very few colonies (3 and 4 colonies/concentration) were transformed at those concentrations compared to the colonies with the elevated transformation frequencies (34-111 transformed colonies).

6.4. Other Key Data

6.4.1. Mutagenicity and Genotoxicity

Studies investigating the *in vivo* and *in vitro* genotoxicity (evaluation of DNA damage) from exposure to cylindrospermopsin are few in number and are discussed below.

***In vivo* Studies**—Shen et al (2002) injected BALB/c mice i.p. with 0.2 mg/kg cylindrospermopsin. The animals were sacrificed after 6, 12, 24, 48 and 72 hours. The livers were removed and the DNA examined for strand breaks using alkaline gel electrophoresis. DNA strand breaks were characterized based on the median molecular lengths of the fragments. The fragment lengths were significantly shorter than those for the controls at all time points except 72 hours, when the differences in length were not statistically significant.

Covalent binding of cylindrospermopsin or a metabolite to DNA was detected in the liver of Quackenbush mice given a single i.p. injection of a cell-free extract of *C. raciborskii* (dose levels not reported). DNA was isolated from the liver using a phenol-chloroform purification technique, hydrolyzed and labeled with ^{32}P. Individual nucleotides were separated using two-dimensional thin layer chromatography and adducted nucleotides visualized by autoradiography (Shaw et al., 2000). A single adduct spot was found in each case. The authors concluded that either cylindrospermopsin or a metabolite was bound to one of the DNA nucleotides.

Based on structural characteristics (the nucleoside structure and potentially reactive guanidine group) of cylindrospermopsin, it has been speculated that cylindrospermopsin may exert its toxic effects via pathways that could include reactions with DNA and/or RNA (see Humpage et al., 2000).

Ames MPF microplate format mutagenicity assay was used to assess the mutagenic potential of cyanobacterial extracts (with different proportions of cyanobacteria) and pure microcystin-LR, (+)-anatoxin fumarate, and cylindrospermopsin (Sieroslawska, 2013). Pure toxins (purity not reported) were tested at concentrations of 0.312, 0.625, 1.25, 2.5, 5 and 10 mg/ml with four strains of *S. typhimurium* and three strains of *E.coli*. Cylindrospermopsin was detected at low concentrations (no statistical significance

reported) in only 2 of the 10 extracts. In one extract (E6), composed of *Aphanizomenon flos-aquae*, *P. agardhii*, and *D. planctonicum*, cylindrospermopsin was detected at 0.51µg/L; in the other extract (E10) composed of *D. flos-aquae*, *D. planctonicum*, *Aphanizomenon flos-aquae*, *P. agardhii*, and *M. aeruginosa* was detected at 0.89µg/L. Of all the tested extracts, four (E3, E6, E8 and E10) were mutagenic, suggesting the presence of other substances able to induce mutations and maybe synergistic interactions with cyanotoxins.

***In vitro* Studies**—The genotoxicity of cylindrospermopsin was assessed *in vitro* with two human cell lines (HepaRG and Caco-2) that represent known target organs of cylindrospermopsin (Bazin et al., 2010). The objective of this study was to investigate how changes in phenotype associated with cell differentiation affect toxic response to cylindrospermopsin exposure. In their differentiated state, HepaRG cells express metabolic enzymes at levels comparable to those found in cultured primary human hepatocytes. Therefore, HepaRG are metabolically competent cells derived from a human hepatoma that represent a suitable model to study the genotoxicity of protoxicants in the human liver. However, as the major route of human exposure to cylindrospermopsin is likely to be ingestion of contaminated water (i.e., during recreational activities or from drinking), cylindrospermopsin genotoxicity also was investigated in a human colon adenocarcinoma cell line, Caco-2. After differentiation, Caco-2 cells display morphological and biochemical characteristics of human enterocytes. Cylindrospermopsin genotoxicity was assessed using the cytokinesis-block micronucleus assay to assess various cytotoxic and genotoxic outcomes in these cells. In addition, the involvement of CYP metabolism in the cytotoxicity and genotoxicity of cylindrospermopsin was determined by the addition of the CYP3A4 inhibitor ketoconazole.

Cylindrospermopsin (>98% purity, from the Australian Water Quality Center in Adelaide, Australia) was dissolved in physiological saline. Caco-2 cells in both differentiated and undifferentiated states and undifferentiated HepaRG cells were exposed to cylindro-spermopsin at concentrations ranging from 0.5 to 2 µg/mL while differentiated HepaRG cells were exposed to 0.04 to 0.4 µg/mL for 24 hours (Bazin et al., 2010). Exposure to 0.5-1.5 µg/mL cylindrospermopsin resulted in a significant increase in micronucleated binucleate cells (MNBNC) by approximately three-fold above controls in both differentiated and undifferentiated Caco-2 cells. Above this concentration, the MNBNC frequency reached a plateau. Similarly, in differentiated HepaRG cells, MNBNC increased to a maximum of 1.8-fold over controls at 0.06 µg/mL and leveled-off above this concentration. No change in MNBNC frequency was seen in undifferentiated HepaRG cells exposed to cylindrospermopsin. The plateau in the genotoxicity results likely reflects the increase in cytotoxicity as the exposure concentrations increase. Addition of ketoconazole reduced both cytotoxicity and genotoxicity suggesting that activation by CYP450 is necessary for both cytotoxicity and genotoxicity.

Lankoff et al. (2007) examined the carcinogenic potential of cylindrospermopsin *in vitro* through the formation of chromosomal aberrations in Chinese hamster ovary (CHO)-K1 cells. Cylindrospermopsin isolated from two cultures of *C. raciborskii* in AWT 205 (Australian Water Technology Center) and Thai (from a fish pond in Thailand), was prepared in solution. CHO-K1 cells were exposed to 0, 0.05, 0.1, 0.2, 0.5, 1 and 2 µg/mL with and without metabolic activation (S9) for 3, 16 and 21 hours. No significant influence on the frequency of chromosome aberrations in cells treated with cylindrospermopsin with or without S9 compared to control groups was found. The study showed that neither cylindrospermopsin nor the S9 fraction-induced metabolites were clastogenic in CHO-K1 cells. However, significant ($p < 0.05$) decreases in the frequency of mitotic indices were observed after various exposure durations at concentrations of 0.1 µg/mL and above. Furthermore, significant ($p < 0.05$) increases in the frequency of apoptotic cells (1 µg/mL and above) and necrotic cells (0.5 µg/mL and above) after 21 hours were observed compared to the controls in a dose and time-dependent manner. The presence of metabolic activation influences susceptibility to necrotic cell death, but not apoptosis.

To confirm that cylindrospermopsin metabolism is necessary for the manifestation of genotoxicity and to characterize CYP450 involvement in activation, the micronucleus assay also was conducted with a CYP450 inhibitor (Lankoff et al., 2007). CYP3A4 is the major CYP450 form in the human small intestine, responsible for metabolizing a large number of xenobiotics (Pelkonen et al., 2008). Cells were treated with ketoconazole, widely known to inhibit CYP3A4. Results indicate ketoconazole protects undifferentiated Caco-2 cells from the induction of (micronuclei) MN induced by cylindrospermopsin. This further suggests that a CYP450-mediated metabolite is involved in the genotoxic effect at noncytotoxic concentrations in the Caco-2 cell model. This finding is in agreement with Humpage et al. (2005) who demonstrated that omeprazole, a CYP3A4 inhibitor less specific than ketoconazole, was effective in protecting mouse primary hepatocytes from cylindrospermopsin-induced genotoxicity. These results are also in accordance with Fessard and Bernard (2003) and Lankoff et al. (2007) who observed that cylindrospermopsin does not react directly with DNA in metabolically-incompetent CHO K1 cells (Table 6-2).

Humpage et al. (2000) reported that purified cylindrospermopsin caused an increase in the frequency of micronuclei in the human lymphoblastoid cell line, WIL2-NS. WIL2-NS cells were exposed to 1-10 µg/mL cylindrospermopsin for 24 hours to evaluate micronucleus frequency and cellular ploidy. Cylindrospermopsin caused a dose-dependent increase in the incidence of MN in binucleated cells (BNCs) at ≥3 µg/mL. There was an 8 fold increase in MN/1000BNCs over the control. Cylindrospermopsin also produced "multimicronucleated" cells indicating chromosomal damage, although the underlying mechanism was unclear. An increase in centromeres was observed in MNBNCs suggesting cylindrospermopsin could be a spindle poison causing changes in the centromere/kinetochore function. Two mechanisms were suggested as the cause of cytogenetic damage: the first one leading to strand breaks at the DNA level, and the other, at the level of kinetochore/spindle function, which induces loss of whole chromosomes (Humpage et al., 2000).

Fessard and Bernard (2003) examined the genotoxic potential of cylindrospermopsin in (CHO) K1 using the comet assay. Doses of 0.5 and 1 µg/mL of purified cylindrospermopsin caused cell growth inhibition and altered cell morphology linked to effects on the cytoskeleton. No apoptosis or DNA strand breaks were observed after 24 h of treatment with cylindrospermopsin. Cell mitosis was decreased at cylindrospermopsin concentrations between 0.33 and 1 µg/mL.

Humpage et al. (2005) examined the integrity of hepatocyte DNA using a comet assay following exposure to concentrations of 0.05 to 0.5 µM purified cylindrospermopsin (98% pure). Clofibrate was used as the positive control. After exposure of cultured cells to the toxin, the cells were lysed and the DNA isolated, and denatured using an alkaline pH to generate double strand breaks. The treated DNA was stained and visualized for scoring of the comet tail moment. Cylindrospermopsin produced significant DNA fragmentation at concentrations as low as 0.05 µM. The addition of CYP450 inhibitors (omeprazole and SKF525A) to the culture medium reduced the number of DNA strand breaks. The ability of cylindrospermopsin to induce DNA damage in isolated human peripheral blood lymphocytes was investigated by Zegura et al. (2011). Whole blood samples were treated with cylindrospermopsin concentrations (0, 0.05, 0.1, and 0.5 µg/mL) for the comet assay and the cytokinesis-block micronucleus (CBMN) assay at 4 and 24- hours of exposure. The number of cells containing micronuclei increased significantly following 0.5 µg/mL treatment at 4 hours incubation and after a 24-hour incubation at a concentration of 0.1 µg/mL. Nuclear buds were observed in binucleated human peripheral blood lymphocytes at 0.05 and 0.1 µg/mL after 4 hours and at 0.1 µg/mL after 24-hour exposures. This was accompanied by a significant decrease in the nuclear division index after 24 hours of exposure to the 0.1 and 0.5 µg/mL concentrations. Exposure to cylindrospermopsin was associated with a slight but significant increase in strand breaks at 24 hours. Increases in in nuclear bridges were not significant (Zegura et al., 2011).

The genotoxicity of cylindrospermopsin in a human hepatoma cell line (HepG2) was studied by Straser et al., (2011) using an alkaline comet assay and CBMN with different cylindrospermopsin concentrations (0, 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 5 µg/mL) for 4, 12 and 24-hours incubation. Cell viability was significantly decreased at concentrations of 1 and 5 µg/mL. After exposure to 0.5 µg/mL cylindrospermopsin for 24 hours, a significant decrease in the nuclear division index was observed in HepG2 cells (Straser et al., 2011). The frequency of cells with micronuclei and nuclear buds increased significantly at 0.05 and 0.5 µg/mL cylindrospermopsin. Nuclear bridges increased at both concentrations, but were only statistically significant in cells exposed to 0.05 µg/mL. These results demonstrate the occurrence of complex genomic changes including gene amplification (nuclear buds) and chromosomal rearrangements. The results of the *in vitro* genotoxicity are summarized in Table 6-4 below.

Table 6-4. Genotoxicity of Cylindrospermopsin *in vitro*

Species (test system)	End-point	Results	Reference
Human cell lines (HepaRG and Caco-2)	DNA damage	Significant increase in MNBNC in both HepaRG at 0.04-0.06 µg/mL and Caco-2 cells at 0.5-1.5 µg/mL	Bazin et al., 2010
Human lymphoblastoid WIL2-NS cells	DNA damage	Exposure to 3, 6 and 10 µg/mL increased frequency of MN in WIL2-NS cells	Humpage et al., 2000
Chinese Hamster Ovary-K1 cells	DNA damage	Comet assay showed altered cell growth and morphology but no interaction with DNA at 0.5 and 1.0 µg/mL	Fessard and Bernard, 2003
Chinese Hamster Ovary-K1 cells	DNA damage	Chromosome aberration not observed in CHO-K1 cells; apoptotic cells (1 µg/mL and above) and necrotic cells (0.5 µg/mL and above) observed	Lankoff et al., 2007
Hepatocytes from Male Albino Swiss Mouse	DNA damage	Comet assay showed concentration dependent increase in comet tail length, area, and moment in cells at 0.05 µM – 0.5 µM	Humpage et al., 2005
Human hepatoma cell lines (HepG2)	DNA damage	Significant increases in micronuclei and nuclear buds at 0.05 and 0.5 µg/mL (statistically significant) and a decrease in nuclear division at 0.5 µg/mL after 24 hours	Straser et al., 2011
Human peripheral blood lymphocytes	DNA damage	Increases in micronuclei at 0.5 µg/mL at 4 hours incubation and after a 24-hour incubation at 0.05 µg/mL. Nuclear buds were observed at 0.05 and 0.1 µg/mL after 4 hours and at 0.1 µg/mL after 24-hours and a decrease in nuclear division index after 24 hours at 0.1 µg/mL.	Zegura et al., 2011

6.4.2. Immunotoxicity

Data on the effects of cylindrospermopsin on immune function were not located. However, in single and short-term studies of high-level exposures, immune related effects were observed. A single 0.2 mg/kg dose of cylindrospermopsin purified (percent purity not reported) from cultured *U. natans* cells was administered i.p. to male ICR mice. (Terao et al., 1994).

While there was massive necrosis of lymphocytes in the cortical layer of the thymus, large lymphocytes in the medulla survived.

A single gavage dose of a suspension of freeze-dried *C. raciborskii* cells, in the lethal dose range of 4.4 to 8.3 mg/kg, was given to MF1 mice (Seawright et al., 1999). Effects observed included atrophy in the thymus (degeneration and necrosis of cortical lymphocytes) and at the lymphoid tissue of the spleen (follicular lymphocyte loss due to lymphophagocytosis). Shaw et al. (2001) administered a nonlethal dose of 0.05 mg/kg/day of a cell-free extract of freeze-dried and sonicated *C. raciborskii* cells by gavage to Quackenbush mice for 14 days. Lymphophagocytosis was observed in the spleen of exposed mice. A similar effect did not occur with purified cylindrospermopsin from the same source (Shaw et al., 2001).

In a study done by Poniedzialek et al. (2014b), cylindrospermopsin significantly inhibited ($p<0.001$) cell proliferation of cultured human T-lymphocytes. After exposing T-cell phytohaemagglutinin-L (PHA-L) to as much as 1µg/mL of purified cylindrospermopsin (>95%) isolated from *C. raciborskii*, cell viability and lymphocyte proliferation were measured after 72 hours of lymphocyte culture. The authors reported inhibition of human T-cell lymphocytes after 6 hours (91.0%), 24 hour (81.1%) and 48 hours (69.0%) of the 72 hour culture period (19.0%). At lower concentrations (0, 0.01 and 0.1µg/mL), cylindrospermopsin did not induce significant differences ($p>0.05$) in T-lymphocyte proliferation when compared to controls, regardless of the time the toxin was added to cell culture. At the highest dose (1µg/mL), the authors also observed a decrease in the viability of human T-lymphocytes in a time-dependent manner. A statistically significant ($p<0.001$) decrease in live cells was observed at 6 hours (6.8%), 24 hours (10.7%), 48 hours (11.5%) and 72 hours (2.8%) along with an increase in necrotic cells. Lower concentrations did not induce significant changes in lymphocyte viability ($p >0.05$); however, at 0.1 µg/mL of cylindrospermopsin, a statistically significant ($p<0.05$) increase in necrotic cells was observed at the beginning (1.0%) and after 6 hours of cell culture (0.9%). The greatest alterations were observed at 1µg/mL after 24 h of culturing.

Poniedzialek et al. (2014a) also studied the effect of cylindrospermopsin on the oxidative burst capacity of human neutrophils. A decline in the production of ROS in stimulated and unstimulated neutrophils from healthy donors was observed after 1 hour of exposure to purified (95% purity) cylindrospermopsin. Generation of ROS is an important step in pathogen inactivation by neutrophils. The concentrations evaluated were 0 (control), 0.01, 0.1 and 1.0 µg/mL. The decrease in ROS levels was statistically significant ($p<0.01$) for all the concentrations evaluated. Cylindrospermopsin had no effect on the neutrophils numbers in whole blood based on a stable number of apoptotic or necrotic cells in the exposed samples. There was no impact on the proportion of phagocytic neutrophils in the blood samples or in their ability to engulf bacteria at all cylindrospermopsin concentrations. However, exposure to cylindrospermopsin reduced the ability of the human neutrophils to disable the pathogens because of the decrease in their ROS production (Poniedzialek et al., 2014a).

6.5. Physiological or Mechanistic Studies

6.5.1. Noncancer Effects

Mechanistic studies have mostly assessed hepatic endpoints because the liver has been historically been regarded as the primary target of cylindrospermopsin toxicity. Although not clearly understood, the specific mechanism for liver toxicity may involve more than one mode of action. Terao et al. (1994) concluded that inhibition of protein synthesis following i.p. administration of 0.2 mg/kg could occur in various tissues because electron microscopy of liver cells revealed ribosome detachment from the endoplasmic reticulum. Hepatocyte cytotoxicity, as evidenced by lactic dehydrogenase leakage in cultured cells, co-occurred with protein synthesis inhibition, but by a mechanism that is independent of the inhibition of protein synthesis (Froscio et al., 2003).

To examine the inhibition of protein synthesis hypothesis, Terao et al. (1994) isolated liver microsomes from 6 mice (4 controls and two treated) and measured protein levels colorimetrically (Lowrey method).

Levels were lower in the treated mice than in the control, but the differences were not statistically significant (16.6 ± 1.3 mg/g liver for controls vs. 11.0 ± 1.2 mg/g liver for the treated mice). Using a rabbit cell-free reticulocyte system as a platform for globulin synthesis, there was a concentration-dependent decrease in leucine incorporation at concentrations up to 48 ng/mL cylindrospermopsin.

Cylindrospermopsin induced concentration- and time-dependent toxicity and inhibition of protein synthesis in cultured hepatocytes isolated from male Swiss mice using radiolabeled leucine uptake as a measure of protein synthesis (Froscio et al., 2003). Diminished leucine incorporation was apparent for concentrations ≥ 0.5μM over a 20-hour period, but not for a 0.1μM concentration. The authors also looked at cell leakage of lactate dehydrogenase as an indicator of cytotoxicity. A significant increase in LDH leakage at 18 hours occurred at concentrations ≥ 1μM. The broad-spectrum CYP450 inhibitors proadifen (SKF525A) and ketoconazole diminished cytotoxicity, but did not diminish the inhibition of protein synthesis. These findings suggest that the cytotoxic effects of cylindrospermopsin may be more related to oxidized metabolites than the inhibition of protein synthesis, presumably by the parent compound. The protein synthesis inhibition was not reversed by removal of the toxin or washing of the hepatocytes.

Froscio et al. (2008) extended their studies of the impact of cylindrospermopsin on protein synthesis using extracts from plant (wheat germ) and mammalian tissues (rabbit reticulocytes) as protein synthesis platforms. The template for protein synthesis was the mRNA for luciferase. They measured luminescence to determine the amount of luciferase formed. Cylindrospermopsin was able to inhibit protein synthesis with similar potency for both wheat germ and mammalian tissues. Radio labeled cylindrospermopsin binding to rabbit reticulocyte ribosomes increased as cylindrospermopsin concentration increased and was associated with the inhibition of protein synthesis. Unlabeled cylindrospermopsin displaced the radiolabeled cylindrospermopsin from ribosomes suggesting noncovalent binding. A toxin to ribosome ratio of 0.02:1 completely inhibited protein synthesis in samples with 300nM labeled toxin (Froscio et al., 2008).

The authors also examined the extent of radiolabeled cylindrospermopsin binding to detached 80S ribosomes. They concluded that the ribosome was not the target of the cylindrospermopsin because of the ease with which it could be detached from the ribosome during elution and size exclusion filtration. Eluted toxin was associated with a >100kD elution fraction, leading Froscio et al. (2008) to hypothesize that the cylindrospermopsin was bound to the elongation or initiation factors necessary for protein synthesis.

Cylindrospermopsin-induced effects on cellular protein synthesis in Vero cells (originally isolated from the kidney epithelial cells of African Green monkeys) were studied by Froscio et al. (2009). The cells were cultured to express a green fluorescent protein. Effects of cylindrospermopsin on protein synthesis were examined *in vitro* using a rabbit reticulocyte lysate as a cell-free platform and inhibition was evaluated as the decrease in green protein fluorescence over time. Fluorescence decreased after 4-hours. (IC_{50} = 5.9 μM) and 24- hour exposures to the toxin. There was a concentration-related decrease in cell viability that roughly paralleled the decrease in protein fluorescence. When the medium containing toxin was replaced with medium free of toxin, fluorescence continued to decline. The decrease was significant for the cells originally exposed to the 100 and 300 μM concentrations but not for the 30 μM concentration. The strong interaction of the toxin with its targets indicated that cylindrospermopsin remained in the intracellular environment for an extended period.

Cylindrospermopsin-induced depletion of Quackenbush mouse hepatic glutathione was demonstrated *in vivo* by Norris et al. (2002) although the study authors did not consider the effect to be of sufficient magnitude to represent the primary mechanism of cylindrospermopsin toxicity. After the mice were treated with a dose of 0.2 mg/kg toxin following pretreatment with glutathione (GSH) depleting agents (butathione and diethylmalate) the 7 day survival rate was 5/13 (38%) compared to 9/14 (64%) for the

controls yet the difference between GSH levels between the exposed and control animals was small (quantitative measures not provided). The results after treatment with piperonyl butoxide, a CYP450 inhibitor, were protective with 100% survival in for the exposed and control mice.

Cylindrospermopsin caused decreased glutathione levels, as well as decreased synthesis of glutathione and protein, in cultured rat hepatocytes (Runnegar et al., 1994, 1995, 2002). In the Runnegar et al. (1994) study, pretreatment with the CYP450 inhibitor, α-naphthoflavone, partially protected against cytotoxicity and cellular glutathione depletion, indicating involvement of the CYP450 enzyme system in cylindrospermopsin metabolism. Inhibition of glutathione synthesis was the predominant mechanism for the observed reduction in glutathione; other mechanisms, including increased utilization of glutathione, increased formation of oxidized glutathione, increased glutathione efflux, decreased glutathione precursor availability and decreased cellular adenosine triphosphate (ATP) were effectively ruled out.

Runnegar et al. (1995) investigated the decrease in cellular GSH and its role in the metabolism and toxicity of cylindrospermopsin in primary cultures of rat hepatocytes. To ascertain whether the reduction in GSH was due to decreased GSH synthesis or increased GSH consumption, total GSH was measured after treatment with 5 mM buthionine sulfoximine (BSO, an irreversible inhibitor of GSH synthesis). The rate of fall in total GSH (nmol/10^6 cells/hr.) was 8.2 ±2.5, 6.0 ±1.7, and 5.9 ±1.3 for control, 2.5 μM and 5 μM cylindrospermopsin pretreated cells, respectively. This suggests that the toxin-induced decrease in GSH induced was due to the inhibition of GSH synthesis rather than increased consumption, because, in the latter case, the rate of decrease in GSH would have been accelerated by toxin pretreatment. Furthermore, excess GSH precursor (20 mM N-acetylcysteine), which supported GSH synthesis in control cells, did not prevent the decrease in GSH or toxicity induced by cylindrospermopsin. Addition of CYP450 inhibitors α-naphthoflavone, SKF525A and cimetidine partially prevented the decrease in cell GSH induced by cylindrospermopsin. Results suggest that an oxidized and/or glutathione-conjugated derivative of cylindrospermopsin is formed and could be a more potent inhibitor of GSH synthesis than the parent cylindrospermopsin.

Humpage et al. (2005) used inhibitors of specific CYP450 isoforms, furafylline (CYP1A2) and omeprazole (CYP3A4 and CYP2C19) to determine if they would protect against cylindrospermopsin cytotoxicity in an *in vitro* mouse hepatocyte system. However, inhibitors of CYP450s 2A6, 2D6 and 2E1 (reduced glutathione (GSSG) reductase (GSSG-rd.) inhibitor 1,3-bis(chloroethyl)-l-nitrosourea (BCNU)) were not cytoprotective (Humpage et al., 2005). There was no indication that reductions in glutathione levels by cylindrospermopsin increased levels of ROS. The authors concluded that CYP450 derived metabolites were responsible for the cytotoxicity and genotoxicity of cylindrospermopsin and that ROS were not involved. In another study, the addition of the CYP3A4 inhibitor ketoconazole to cultured HepaRG cells reduced both cytotoxicity and genotoxicity of cylindrospermopsin (Bazin et al., 2010).

The CYP450 inhibitors omeprazole (100 μ*M*) and SKF525A (50 μ*M*) completely inhibited the genotoxicity induced by CYN. The toxin also inhibits production of glutathione (GSH), a finding confirmed in this study. This could potentiate cytotoxicity, and by implication genotoxicity, via reduced reactive oxygen species (ROS) quenching. The lipid peroxidation marker, malondialdehyde (MDA) was quantified in CYN-treated cells, and the effect of the reduced glutathione (GSSG) reductase (GSSG-rd.) inhibitor 1,3-bis(chloroethyl)-l-nitrosourea (BCNU) on both MDA production and lactate dehydrogenase (LDH) leakage was examined. MDA levels were not elevated by CYN treatment, and block of GSH regeneration by BCNU did not affect lipid peroxidation or cytotoxicity. It therefore seems likely that CYP450-derived metabolites are responsible for both the acute cytotoxicity and genotoxicity induced by CYN.

Shen et al. (2003) found that cylindrospermopsin induced up-regulation of the tissue transglutaminase (tTGase) gene in the liver in Balb/c mice following i.p. injection of a single 100 μg/kg dose. Tissue TGase catalyzes the post-translational modification of proteins via Ca^{2+}-dependent cross-linking reactions

as well as hydrolysis of GTP and functions as a protein kinase. It also takes part in cell adhesion processes and stabilization of the extracellular matrix (Onyekacji and Coussons, 2014). Up-regulation of tTGase can lead to cell injury and apoptosis. Using semi quantitative, real-time PCR and primer sets for mouse tTGase with enzyme separation by gel electrophoresis, Shen et al. (2003) found that toxin-exposed cells had higher levels of the enzyme at 6, 72 and 96 hours than unexposed control cells.

Zegura et al. (2011) measured gene expression in human peripheral blood lymphocytes after incubation with 0.5 µg/mL cylindrospermopsin for 24 hours. Genes for metabolism (*CYP1A1* and *CYP1A2*), DNA damage response (*P53* and downstream regulated genes), apoptosis (*BCL-2* and *BAX*) and oxidative stress response (*GPX1*, *SOD1*, *GSR* and *GCLC*) were up-regulated.

In an *in vitro* study by Fernández et al. (2014), cylindrospermopsin was analyzed for its ability to cross the intestinal epithelium and enter systemic circulation. To determine the effect of cylindrospermopsin on the Caco-2 monolayer integrity, Caco-2 monolayer, trans-epithelial electric resistance (TEER) was measured after 3, 10 and 24 hours of incubation with 1 mM, 5 mM and 10 mM of the toxin. TEER values after exposure to cylindrospermopsin did not show any significant difference when compared with controls (0), indicating that the monolayer was not disrupted or altered by cylindrospermopsin at the concentrations tested.

To test the ability of cylindrospermopsin to cross cell membranes, Fernández et al. (2014), incubated differentiated Caco-2 cells and a rat Clone 9 hepatic cell line with 0.1, 0.25, 0.5,1, 1.5, 2.5, 5 or 10 mM cylindrospermopsin. The molecular mass and hydrophilic nature of cylindrospermopsin make it a poor candidate for simple diffusion across a cell membrane without the aid of a transport channel. After first determining that the cylindrospermopsin did not increase cytotoxicity and weaken the cell membrane, the apical side of the cell monolayer was exposed to concentrations of 1, 5, or 10 µM cylindrospermopsin for 3, 10, or 24 hours. Even after 24 hours, relatively small percentages of the applied cylindrospermopsin had crossed the basolateral membrane (16.7 to 20.5%). Caco-2 cells are often used as a surrogate for the intestinal membrane. The results from this study indicate that uptake from the intestines after oral exposure is limited and is in agreement with the differences observed in LD_{50} values for oral versus i.p. exposures (Terao et al., 1994; Ohtani et al 1992).

When the Clone 9 hepatic cell monolayers were exposed to varying concentrations of cylindrospermopsin there was a time and concentration dependent decrease in viability when compared to the controls. Clone 9 cell viability decreased by more than 40% at 24 hours and more than 65% at 48 hours of exposure to 5 mM of cylindrospermopsin. Observations with a phase-contrast microscope determined that after 48 hours of exposure to 5 mM of cylindrospermopsin, Clone 9 monolayer cells showed morphological signs of cellular damage, detachment from their substrate, and decreased viability. Levels of GSH increased over time, especially 48 hours after exposure to 1 and 5 mM, probably as a means of minimizing cell damage caused by the toxin. Based on the GSH increase and increases in the proteins β-tumulin and actin, Fernández et al. (2014) concluded that the toxicity in the Cone 9 hepatocyte cultures was not related to GSH depletion or impaired protein synthesis.

6.5.2. Cancer Effects

There are no long term bioassay studies that examined the tumorigenicity of cylindrospermopsin. Thus, mechanistic data for this endpoint are lacking.

6.5.3. Interactions with Other Chemicals

No studies of mixtures of cylindrospermopsin with other specific chemicals (except those identified as being present in the growth media) were identified. Although, extracts can contain chemicals other than cylindrospermopsin, in no case were those chemicals identified other than the presence of the essential

amino acid phenylalanine in the purified cylindrospermopsin isolated by Humpage and Falconer (2002). The Caruaru outbreak (Section 6.1) involved exposure of patients at a renal dialysis clinic in Caruaru, Brazil to a mixture of microcystin and cylindrospermopsin. However, the data do not reveal any quantitative information on the toxicity of the mixture compared to its individual components.

An aquatic invertebrate study using brine shrimp (*Artemia salina, Daphnia magna* and *Daphnia galeata*) to determine the toxicity of microcystin and cylindrospermopsin in combination with cyanobacterial LPS found that pre-exposure to LPS increased the lethal concentration (LC_{50}) of cylindrospermopsin 8-fold (Lyndsay et al., 2006). The authors concluded that the decrease in susceptibility to cylindrospermopsin was due to the effects of LPS on detoxification enzyme pathways; LPS decreased toxic metabolites of cylindrospermopsin by suppressing the invertebrate cytochrome P450 system, thus decreasing toxicity.

6.5.4. Structure Activity Relationship

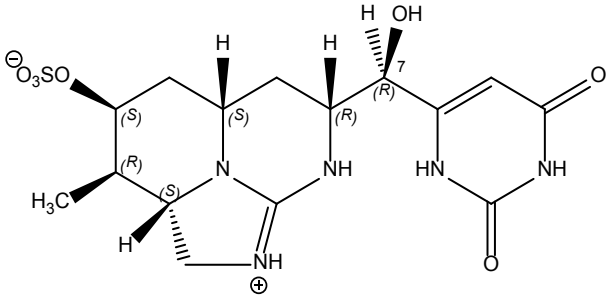
There is some evidence that the most toxic form of cylindrospermopsin is an unidentified metabolite produced by hepatic CYP450. Pretreatment of hepatocytes with known inhibitors of CYP450 diminished the *in vitro* cytotoxicity of cylindrospermopsin (Froscio et al., 2003). Similarly, Norris et al. (2002) found that pretreatment of mice with a CYP450 inhibitor protected against the acute lethality of cylindrospermopsin in male Quackenbush mice.

According to Banker et al. (2000), the uracil portion of cylindrospermopsin may play an important role in cylindrospermopsin toxicity. Banker et al. (2000) found that chlorination eliminated the acute lethality of cylindrospermopsin in mice resulting in the formation of 5-chlorocylindrospermopsin or cleavage of the pyrimidine ring to form cylindrospermic acid. This was shown by a 5-day i.p. LD_{50} value of 0.2 mg/kg for cylindrospermopsin, and 10-day i.p. LD_{50} values of >10 mg/kg for 5-chlorocylindrospermopsin, and >10 mg/kg for cylindrospermic acid.

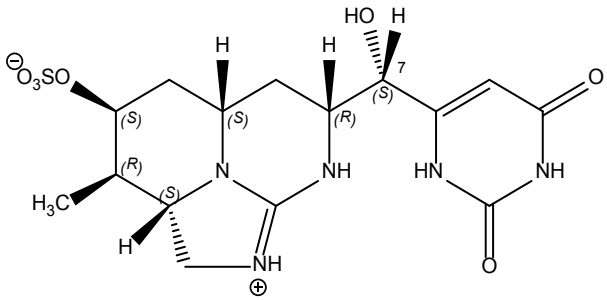
Norris et al. (1999) treated male white Quackenbush mice with a 0.8 mg/kg i.p. dose of 7-deoxycylindrospermopsin, an analogue of cylindrospermopsin isolated and purified from *C. raciborskii.* After 5 days of administration, 7-deoxycylindrospermopsin did not appear to be toxic.

Runnegar et al. (2002) conducted an analysis of natural cylindrospermopsin, synthetic (racemic) cylindrospermopsin and selected synthetically-produced cylindrospermopsin structural analogues to determine the effects on protein synthesis in the rabbit reticulocyte lysate system and in cultured rat hepatocytes. Orientation of the hydroxyl group at C7 in the carbon bridge was not important because the C7 epimer of cylindrospermopsin (Figure 6-1) and its corresponding diol showed similar inhibition of protein synthesis compared to that of synthetic (racemic) cylindrospermopsin. An analogue with a hydroxyl functional group in place of the sulfate substituent on C12 had a comparable impact on protein synthesis. Another analogue (Figure 6-2), lacking the 5-membered heterocyclic ring but retaining cationic nitrogen and without the methyl and sulfate substituents on the six membered ring (AB-MODEL), was 500 to 1000-fold less effective in the inhibition of *in vitro* protein synthesis using a rabbit reticulocyte system.

Figure 6-1. Structure of cylindrospermopsin and 7-epicylindrospermopsin (de la Cruz et al., 2013)

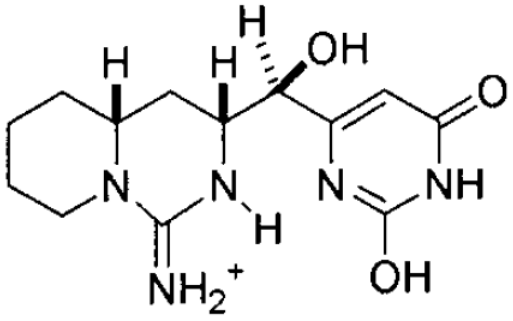


Cylindrospermopsin



7-epicylindrospermopsin

Figure 6-2. Structure of cylindrospermopsin analog AB-MODEL (Runnegar et al., 2002)



7.0 CHARACTERIZATION OF RISK

7.1 Synthesis and Evaluation of Major Noncancer Effects

Information on the human health effects of cylindrospermopsin is limited to the observations from the Australian Palm Island outbreak involving acute and/or short-term drinking water exposure to *C. raciborskii* (Byth, 1980; Griffiths and Saker, 2003). The clinical picture of the illness is well-defined and includes fever, headache, vomiting, bloody diarrhea, hepatomegaly and kidney damage with renal loss of water, electrolytes and protein, but no data are available on the exposure levels of cylindrospermopsin that induced these effects. Furthermore, the concentration of copper sulfate used to treat the lake (a source of drinking water) to control harmful algal blooms before illness was observed is not known; thus, the presence of elevated copper concentrations in the drinking water could have been a contributing factor for some of the symptoms observed.

Most of the information in animals on the non-cancer effects of cylindrospermopsin is from oral and i.p. administration studies in mice exposed to purified compound or extracts of *C. raciborskii* cells. Studies conducted with purified toxin (Riesner et al., 2004 and one component of Humpage and Falconer 2002) are preferred over other extracts which could contain other toxins or compounds with similar chemical physical properties that co-elute with the toxin. Based on available studies, effects on the liver and kidney, increases in the hematocrit level in serum and deformation of RBCs were the most sensitive endpoints observed in mice exposed to cylindrospermopsin (Humpage and Falconer, 2003; Reisner at al., 2004; Sukenik et al., 2006).

Increased relative kidney weight, observed in several studies (Humpage and Falconer, 2002, 2003; Sukenik et al., 2006; Reisner et al., 2004), was the most sensitive endpoint, co-occurring with decreased urinary protein excretion (Humpage and Falconer, 2002, 2003), and RBC changes (Humpage and Falconer, 2002, 2003; Sukenik et al., 2006; Reisner et al., 2004). The mode of action for the decreased urinary protein levels observed in male mice in both drinking water and gavage studies need further investigation but are consistent with either an impact on hepatic mouse urinary protein synthesis and/or retention of mouse urinary proteins by the kidney. The relevance of kidney effects in mice to humans is supported by the results obtained from the Palm Island water poisoning incident. The most severe impacts observed in the exposed population reflected impairment of kidney function, with decreased serum electrolytes, glycosuria, proteinurea, ketonurea and hematourea that led to hospitalization. Treatment included intravenous electrolyte and replacement of serum proteins. Without this support for kidney malfunction, several would have most likely died. Children were particularly sensitive to hypokalemia and acidosis as evidenced by the fact that 82% displayed these conditions when hospitalized. It is important to note that in mice the effect observed was diminished excretion of protein. In mice, decreased protein excretion is as much as a reflection of altered kidney function as is increased protein loss in humans because urinary proteins in mice have distinct physiological functions.

The long-term extract study by Sukenik et al. (2006) and the shorter duration study of purified cylindrospermopsin by Reisner et al. (2004) showed structural changes in the RBC wherein the cells acquired a spiked external membrane (acanthocytes) rather than their normal appearance. The acanthocytes (abnormal RBCs) were associated with an increased hematocrit value as an indicator for adverse changes along with increased kidney and liver weights. Humpage and Falconer (2002, 2003) did not find significant changes in RBC membrane parameters in their 11-week mouse subchronic study. They did observed a trend towards increased serum bilirubin and spleen weight across the 30 µg/kg/day to 120 µg/kg/day range plus non-significant increases in polychromasia. These observations are consistent with removal of abnormal or hemolysized RBCs by the spleen. Sample sizes were small (4-5 per dose group) which is a limiting factor for determining statistical significance.

An increase in the ratio of RBC membrane cholesterol to phospholipids could be responsible for acanthocyte formation according to Reisner et al. (2004). Studies of the effects of cylindrospermopsin on LCAT structure and/or function in altering the cholesterol content of the red cell membrane could shed light on the mode of action for this effect. There are no data from human epidemiology studies of cylindrospermopsin that have examined RBC morphology.

No information was located regarding the chronic toxicity or neurotoxicity of cylindrospermopsin. Effects on the liver/body weight ratio were seen in maternal CD-1 mice exposed to 8 µg/kg/day and a 32 µg/kg/day dose was identified as a FEL (Rogers et al., 2007). There was no significant difference in the number of live fetuses/per litter at either dose. In separate studies by the same authors, there was some evidence for an impact on postnatal growth and survival after a maternal i.p. dose of 50 µg/kg/day on GDs 13-17, but not on GDs 8-12 (Rogers et al., 2007). Sibaldo de Almeida et al. (2013) did not find any visceral or skeletal malformations in the offspring of pregnant rats receiving an oral dose of 3 mg/kg/day purified cylindrospermopsin during gestation (GD 1-20).

7.1.1. Mode of Action for Noncancer Effects

Liver—The occurrence of toxicity in the liver suggests a protein-synthesis inhibition mechanism of action for cylindrospermopsin. *In vitro* and *in vivo* studies have been conducted to demonstrate the ability of cylindrospermopsin to inhibit hepatic protein synthesis, which could impact mouse urinary protein production leading to decreased urinary excretion of these proteins (Froscio et al., 2008, 2009; Terao et al., 1994). Available evidence indicates that protein synthesis inhibition is not decreased by broad-spectrum CYP450 inhibitors, but they do reduce cytotoxicity (Froscio et al., 2003; Bazin et al., 2010). Hepatotoxicity appears to be CYP450-dependent which indicates a possible involvement of oxidized and or fragmented metabolites and mechanisms other than protein synthesis inhibition (Froscio et al., 2003; Humpage et al., 2005; Norris et al., 2001, 2002). Despite the number of studies that have been published, the mechanism for liver and kidney toxicity by cylindrospermopsin are not completely characterized.

Red Blood Cells—There was evidence of effects on RBCs in the Reisner et al. (2004) and Humpage and Falconer (2002) studies of purified cylindrospermopsin. In the Reisner et al. (2004) report, microscopic examination of blood samples showed the presence of RBCs with spiked surfaces rather than their normal biconcave-disc shape. The authors attributed the acanthocyte formation to an increase in the RBC membrane cholesterol to phospholipid ratio. Phospholipids constitute the matrix material of cell membranes. The authors hypothesized that this change was the consequence of decreased activity of plasma lecithin-acyl cholesterol transferase (LCAT), an enzyme associated with high density lipoproteins and the esterification of plasma cholesterol. Effects on the cholesterol content of the RBC membrane can occur with inhibition of the enzyme increasing membrane fluidity and mean corpuscular volume. Associated effects were observed in the Reisner et al. (2004) and Humpage and Falconer (2002) studies. Removal of the abnormal blood cells by the spleen increases both spleen weight and serum bilirubin plus stimulates hematopoiesis. Additional research is needed to examine the LCAT enzyme inhibition hypothesis in order to confirm whether it accounts for the effects on the RBC as a result of cylindrospermopsin exposure.

Kidney—No information on the mode of action for the kidney effects observed in the studies of cylindrospermopsin was provided by the researchers. Since all of the studies were conducted using mice, a species that excretes low molecular weight proteins in urine, there is a need to conduct a study of cylindrospermopsin in a laboratory species that does not excrete protein in the urine in order to determine whether there are comparable effects on kidney weight, protein excretion and renal cellular damage. Kidney necrosis and the decreased renal failure index at the high cylindrospermopsin doses provides support for the effects on the kidney. Numerous signs of renal damage including proteinuria, glycosuria,

and hematuria were observed after the Palm Island incident, all of which are associated with impaired kidney function (Byth, 1980).

7.1.2. Dose Response Characterization for Noncancer Effects

Human Data—The limited information on the toxicity of cylindrospermopsin in humans is from qualitative reports of a hepatoenteritis-like illness attributed to the acute or short-term consumption of drinking water containing *C. raciborskii* (Byth, 1980; Griffiths and Saker, 2003). The clinical picture of the illness includes fever, headache, vomiting, bloody diarrhea, hepatomegaly and kidney damage with loss of serum electrolytes, proteinuria, glycosuria, and hematuria, but no data are available on exposure levels of cylindrospermopsin that can induce these effects. Thus, human data on the oral toxicity of cylindrospermopsin are limited by lack of quantitative information and by potential co-exposures to other cyanobacterial toxins and microorganisms.

Animal Data—The information on non-cancer effects of cylindrospermopsin in animals is available from oral and i.p. administration studies in mice exposed to purified compound or extracts of *C. raciborskii* cells. Based on available studies, the liver, kidneys and RBCs appear to be the main targets of cylindrospermopsin toxicity.

Studies conducted with purified toxin are preferred over those of extracts, which may contain other toxins or compounds with similar chemical physical properties that co-elute with the toxin. Effects on the liver and kidney, including changes in organ weights and histopathological lesions, along with increases in the hematocrit level in serum and deformation of RBC are observed following short term and subchronic oral exposure to cylindrospermopsin (Humpage and Falconer, 2002, 2003; Reisner at al., 2004; Sukenik et al., 2006). Oral and i.p. acute toxicity studies in mice also report histopathological effects in both liver and kidney.

No oral reproductive or developmental and chronic toxicity studies are available for cylindrospermopsin. Developmental toxicity studies following i.p. administration of cylindrospermopsin provide some evidence for maternal toxicity and decreased post-natal pup survival and body weight (Rogers et al., 2007; Chernoff et al., 2011).

7.2 Synthesis and Evaluation of Major Carcinogenic Effects

Studies investigating the *in vitro* and *in vivo* genotoxicity (evaluation of DNA damage) from exposure to cylindrospermopsin are relatively few in number. *In vitro* mutagenic and genotoxic cell assays with cylindrospermopsin show varied results with some indications of potential damage to DNA. The human hepatocytic and enterocytic models for HepaRG and Caco-2 cells showed increased MNBNC (Bazin et al., 2010). Micronucleated cells were observed in a study with human lymphoblastoid WIL2-NS cells (Humpage et al., 2002). DNA breaks have been observed in primary hepatocytes by the comet assay indicating that DNA strand breakage could be a mechanism for cylindrospermopsin-induced cytogenetic damage (Humpage et al., 2005). Following i.p. exposure, DNA strand breakage was observed in the liver of Balb/c mice (Shen et al., 2002) and covalent binding between DNA and cylindrospermopsin, or a metabolite, occurred in a study on Quackenbush mouse liver (Shaw et al., 2000). However, these data are limited and there has been no long term bioassay of purified cylindrospermopsin. The study by Falconer and Humpage (2001) on initiation with TPA promotion did not support classification of cylindrospermopsin as a tumor initiator.

7.2.1. Mode of Action and Implications in Cancer Assessment

There is minimal information available to inform a cancer mode of action hypothesis for cylindrospermopsin. The study by Falconer and Humpage (2001) noted only one tumor and two areas of dysplastic foci in a study of two doses of *C. raciborskii* extracts or three doses of freeze-dried cells combined in treatment with TPA as a promoter. Genotoxicity studies indicate a potential for cylindrospermopsin to cause DNA strand breaks in cell lines with activated CYP450s as well as provide evidence for possible gene amplification and chromosomal alterations form *in vitro* (Straser et al., 2011; Zegura et al., 2011) and *in vivo* studies (Shen et al., 2002). The data from the Marie et al. (2010) study in SHE cells, the clastogenicity seen in the comet assays (Humpage et al. 2000, 2005), and micronuclei observed by Bazin et al. (2010) and Lankoff et al. (2007) support the need for additional research.

7.2.2. Weight of Evidence Evaluation for Carcinogenicity

There are no studies in humans evaluating cancer and no chronic cancer bioassays in animals available for cylindrospermopsin. In accordance with the Guidelines for Carcinogen Risk Assessment (U.S. EPA, 2005a), there is *inadequate information to assess carcinogenic potential* of cylindrospermopsin.

7.2.3. Dose Response Characterization for Cancer Effects

Dose-response data regarding the carcinogenicity of cylindrospermopsin are not available.

7.3. Potentially Sensitive Populations

A review of the available animal data does not support a definitive difference in the response of males versus females following oral exposure to cylindrospermopsin. Based on animal study results, individuals with liver and/or kidney disease might be more susceptible than the general population because of compromised detoxification mechanisms in the liver and impaired excretory mechanisms in the kidney. Results of an episode in a dialysis clinic in Caruaru, Brazil, where microcystins (and possibly cylindrospermopsin) were not removed by treatment of dialysis water, suggest that dialysis patients are a population of potential concern in cases where the drinking water source is contaminated with cyanotoxins.

The data on RBC acanthocytes (abnormal RBCs) identifies individuals that suffer from anemia (e.g. hemolytic or iron-deficiency) as a potentially sensitive population. Several rare genetic defects such as abetalipoproteinemia and hypobetalipoproteinemia are associated with RBCs acanthocytes and appears to result from a defect in expression of hepatic apoprotein B-100, a component of serum low density lipoprotein complexes (Kane and Havel, 1989). Individuals with either condition might be sensitive to cylindrospermopsin.

Based on the currently available science, evidence is lacking to assess differences in susceptibility between infants and children and adults. However, for cyanotoxins including cylindrospermopsin, 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 average drinking water intake rates for children <12 months (0.15 L/kg-day), the exposure of children is 5 times higher than those of adults >21 years old on a body weight basis (0.03 L/kg-day).

7.4. Characterization of Health Risk

7.4.1. Choice of Key Study

Human data on the toxicity of cylindrospermopsin are limited by the lack of quantitative information and by potential co-exposures to other cyanobacterial toxins and microorganisms. The limited information on the toxicity of cylindrospermopsin in humans is from qualitative reports of a hepatoenteritis-like illness that is attributed to the acute or short-term consumption of drinking water containing *C. raciborskii* (Byth, 1980; Griffiths and Saker, 2003). Although clinical symptoms of the illness from the Australian Palm Island poisoning incident are well-defined and documented, no data are available on the exposure levels for cylindrospermopsin that induced these effects.

Observed health effects following single, oral exposures included mortality and toxicity in the liver and kidneys (previously described in Section 6.2.1). Although these effects were observed, they are inadequate to support the derivation of an RfD due to limitations such as: testing inadequate numbers of animals, incomplete reporting, failure to measure clinical and pathological endpoints and exposure to a single dose. Additionally, these studies include exposure to *C. raciborskii* cells or cell extracts (not purified cylindrospermopsin).

Several short term and developmental toxicity studies that evaluated the effects of cylindrospermopsin are also available (Shaw et al., 2001, Rogers et al., 2007, Chernoff et al, 2011). These studies were not selected for the derivation of the RfD due to limitations including use of extract (Shaw et al, 2001), i.p. route of administration (Rogers et al., 2007, Chernoff et al., 2011), lack of adequate numbers of animals and monitored endpoints (Shaw et al., 2001), and the limited number of doses tested (Shaw et al., 2001). The oral data for purified extract from Shaw et al. (2001) identified fatty liver as an adverse effect in mice following a 14 day gavage exposure to 0.05 mg/kg/day. The only effects mentioned in the published paper are the liver effects and an absence of lymphophagocytosis in the spleen.

The critical study selected for the derivation of the RfD is Humpage and Falconer (2002, 2003). Humpage and Falconer (2002, 2003) is a comprehensive toxicity study in which mice were exposed by gavage to purified cylindrospermopsin from cell extract for 11weeks. The study authors also utilized four dose groups, adequate numbers of animals per dose group and evaluated a variety of endpoints. Statistically significant, dose-related effects on the kidney, liver and serum chemistry were observed. The kidney was the most sensitive target of toxicity. The Humpage and Falconer (2002) data are supported by the Riesner et al. (2004) results showing increased kidney weights and hematological effects (acanthocytes) following a 30 day exposure.

7.4.2. Selection of the Principal Study

The subchronic study by Humpage and Falconer (2002, 2003) and the short term studies by Sukenik et al. (2006) and Reisner et al. (2004) all identified increases in kidney weight and hematological effects as the result of exposure to cylindrospermopsin. Humpage and Falconer (2002, 2003) found signs indicative of hemolysis (increased bilirubin, spleen weight and polychromasia), while Reisner et al. (2004) and Sukenik et al. (2006) found acanthocytes and increased hematocrit. Increases in kidney weight were significant for Humpage and Falconer (2002, 2003) and Sukenik et al. (2006), but not significant for Reisner et al. (2004). Humpage and Falconer (2002, 2003) and Reisner et al. (2004) used purified cylindrospermopsin, while Sukenik et al. (2006) used an extract in spent medium.

Sukenik et al., (2006) was a step up dose study. The authors used an extract that was not purified and identified 20 µg/L as the LOAEL at about 20 weeks. Because it was an extract study using spent medium dissolved in water as the exposure vehicle the study was not selected to derive the RfD.

The results from the Reisner et al. (2004) single dose study demonstrated that hematological and kidney weight effects were present in young (4 week) male ICR mice (8 mice) after a three week exposure with a LOAEL of 66 µg/kg/day. Humpage and Falconer (2002, 2003) used 10 male Swiss mice per dose group and evaluated 5 doses. Because of the similarity in the LOAELs from Humpage and Falconer (2002, 2003) and Reisner et al. (2004) and the type of effects observed, the selection of Humpage and Falconer (2002, 2003) was determined to be the most appropriate study for derivation of the RfD, despite its subchronic duration of exposure.

7.4.3. Selection of the Critical Endpoint

Upon considering all effects observed by Humpage and Falconer (2002, 2003), increased relative kidney weight was considered the most appropriate basis for quantitation. Adverse effects on the kidney were manifested by decreased urinary protein concentration and increased relative kidney weight. The study authors reported significant increased relative kidney weight at ≥60 µg/kg/day, decreased urinary protein and liver lesions at ≥120 µg/kg/day and renal tubular lesions at 240 µg/kg/day (Humpage and Falconer, 2002, 2003). Relative kidney weight was increased in a significant, dose-related manner beginning at 60 µg/kg/day (12-23% greater than controls) and relative liver weight was significantly increased at the high dose of 240 µg/kg/day (13% greater than controls). Relative spleen, adrenal and testes weights were increased for doses ≥60 µg/kg/day, but the differences from control, although dose-related, were not statistically significant. Humpage and Falconer (2002, 2003) identified the LOAEL as 60 µg/kg/day and a NOAEL of 30 µg/kg/day based on the dose related and statistically significant increase in relative kidney weight. These adverse effects are potential indicators of suppressed hepatic protein synthesis and/or increased retention of low molecular weight of mouse urinary proteins by the kidney because of damage to the renal tubules.

7.4.4. RfD Determination

The NOAEL from the Humpage and Falconer studies (2002, 2003) was the 30 µg/kg/day dose based on increased relative kidney weight. The RfD is calculated as follows:

$$\text{RfD} = \frac{30\ \mu\text{g/kg/day}}{300} = 0.1\ \mu\text{g/kg/day}$$

where:

30 µg/kg/day	=	The NOAEL for kidney effects in mice exposed to cylindrospermopsin in water for 11 weeks (Humpage and Falconer, 2002, 2003).
300	=	Composite uncertainty factor including a 10 for intraspecies variability (UF_H), a 10 for interspecies differences (UF_A), and 3 for uncertainties in database (UF_D).

Uncertainty Factor Application:

- UF_H. A Ten-fold value is applied to account for variability in the human population. No information was available to characterize inter-individual and age-related variability in the toxicokinetics or toxicodynamics among humans. Individuals with a low-red-cell count as a result of genetic or nutritional factors could be more sensitive to cylindrospermopsin exposures than the general population. Individuals with pre-existing kidney problems may also be more sensitive.
- UF_A. 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

cylindrospermopsin. 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.

- UF_D. An uncertainty factor of 3 ($10^{0.5} = 3.16$) is selected to account for deficiencies in the database for cylindrospermopsin. The database for cylindrospermopsin includes limited human studies. The database for studies in laboratory animals includes oral exposure acute, short-term and subchronic studies, but many of them lacked a comprehensive evaluation of a wide spectrum of effects. The database lacks chronic toxicity and multi-generation reproductive and developmental toxicity studies using the oral route of exposure. There is a lack of data on neurological and immunological endpoints. The RBC parameters evaluated differed between the Humpage and Falconer (2002, 2003) and Reisner et al. (2004) studies.

8.0 RESEARCH GAPS

The deficiencies in the toxicological database for cylindrospermopsin are many. The nature of the problem limits research in humans to outbreak reports and case studies. Both are retrospective scenarios with confounding variables related to the composition of the toxins in the water source, the timing of exposure and the dose. Controlled animal systemic studies have been conducted only in male mice making it difficult to determine whether the critical effects (increase in kidney weight and decreased protein excretion) are relevant to species that do not normally excrete urinary protein for functional reasons. Mode of action information is lacking for the liver, kidney and hematological.

Research is needed to improve the quantitative assessment for human health consequences from exposure to cylindrospermopsin in drinking water. Key research gaps were identified during the development of this document and are not intended to be an exhaustive list. Additional research efforts are needed on:

- Quantification for the absorption, distribution, and elimination of cylindrospermopsin in humans or animals following oral, inhalation and/or dermal exposure.
- The clinical significance in humans for biological changes observed in experimental animals such as increased kidney weight, decreased urinary protein levels, decrease in renal failure index, and the formation of acanthocytes.
- Health risks posed by repeated, low-level exposures to cylindrospermopsin in a second species.
- The chronic toxicity of cylindrospermopsin. Whole-lifetime toxicity studies showing cumulative detrimental effects.
- The immunotoxic, neurotoxic and developmental/reproductive toxicity of cylindrospermopsin following oral exposure.
- The in *vivo* genotoxicity of cylindrospermopsin exposure.
- The carcinogenic potential of cylindrospermopsin, including lifetime carcinogenicity studies.
- Health risks from exposure to mixtures of cylindrospermopsin with other cyanotoxins, bioactives, and chemical stressors present in ambient and or drinking water supplies.
- Populations that might be sensitive to cylindrospermopsin exposure via the oral, dermal, and/or inhalation routes.

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Appendix A: Studies Used in Support of Reference Value Derivation for Cylindrospermopsin

	Humpage and Falconer (2002)		Reisner et al., 2004	Sukenik et al., 2006
	Study 1	Study 2		
Test Substance	**Extract**	**Compound**	**Compound**	**Spent medium with: 95% CYL 5% 7-epiCYL**
Species	Male Swiss Albino mice	Male Swiss Albino mice	Male ICR mice	ICR mice
Number	10	10 (4-5 for hematology)	8	20/sex
Route	Drinking Water	Gavage	Drinking Water	Drinking Water
Duration	10 weeks	11 weeks	3 weeks	42 weeks (step up dose design
Doses	0.216, 423, 657 µg/kg/day	1, 30, 60, 120, 240 µg/kg/day	0, 66 µg/kg/day	10-50 µg/kg/day
Histopathology	Liver injury (432)	Liver injury (≥120)	ND	ND
Liver weight*	↑	ND	↑	↑ (42 weeks male)
Kidney weight*	↑	↑	↑	↑ 20 and 42 weeks
Testes weight*	ND	↑	↑	↑ (42 weeks)
Spleen weight*		↑ trend	↔	↔
Hematocrit or paced cell volume	ND	↑ PCV trend (N=5)	↑ with duration	↑ (32 weeks) ↓ 42 weeks
Acanthocytes	ND	ND	Detected	Detected
Polychromasia		↑		
Serum bilirubin	↑	↑ trend (N=5)	ND	ND
Cholesterol	ND	↑30 and 60 µg/kg/day ↑ higher doses but <60 (N=5)	↑	↑
Bile acids			ND	ND
Urinary protein	↓	↓	ND	ND
NOAEL µg/kg/day	-	30	-	-
LOAEL µg/kg/day	216	60	66	20 (based on 20 week data)

ND = Not Determined; * Relative organ weight

-Shaded cell entries signify that the study provides numeric values for the effects with or without accompanying graphic representation.

-From Reisner et al. (2004) and Sukenik et al., (2006) the data on statistical significance represent a relationship between exposure duration and response, not a relationship between dose and response.

-The term trend does not denote a statistical test for trend, it indicates a uniform direction for the change as reported in the Humpage and Falconer, 2002.

-Reisner et al. (2004) hypothesis for acanthocyte formation: Studies in humans and rats indicate that acanthocytes form due to alterations in RBC membrane lipoproteins that increase the ratio of cholesterol to phospholipids.

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Health Effects Support Document for the Cyanobacterial Toxin Microcystins

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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)
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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.

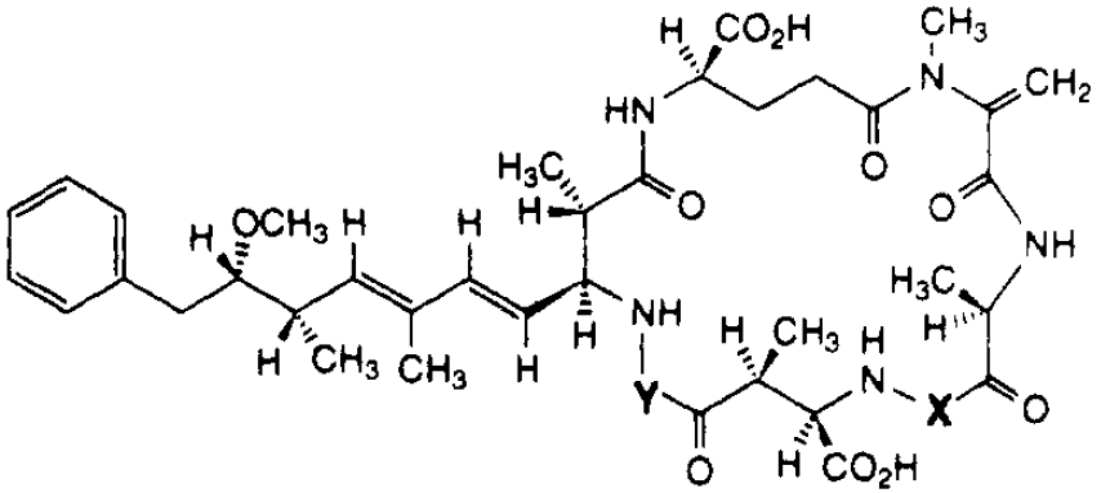


Figure 1-1. Structure of Microcystin (Kondo et al., 1992).

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.

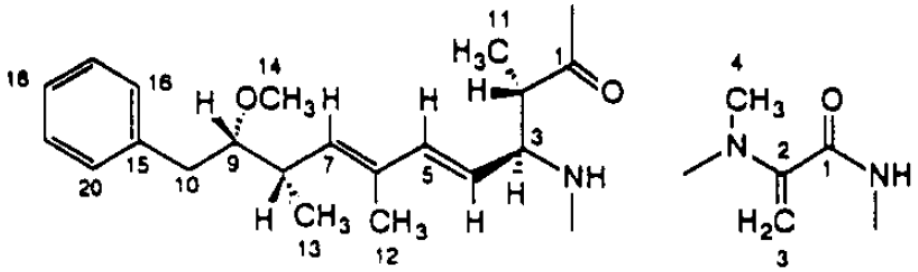
Microcystin congeners vary based on their amino acid composition and through methylation or demethylation at selected sites within the cyclicpeptide (Table 1-1; Duy et al., 2000). The variations in composition and methylation account for the large number of toxin congeners. The microcystins are named 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

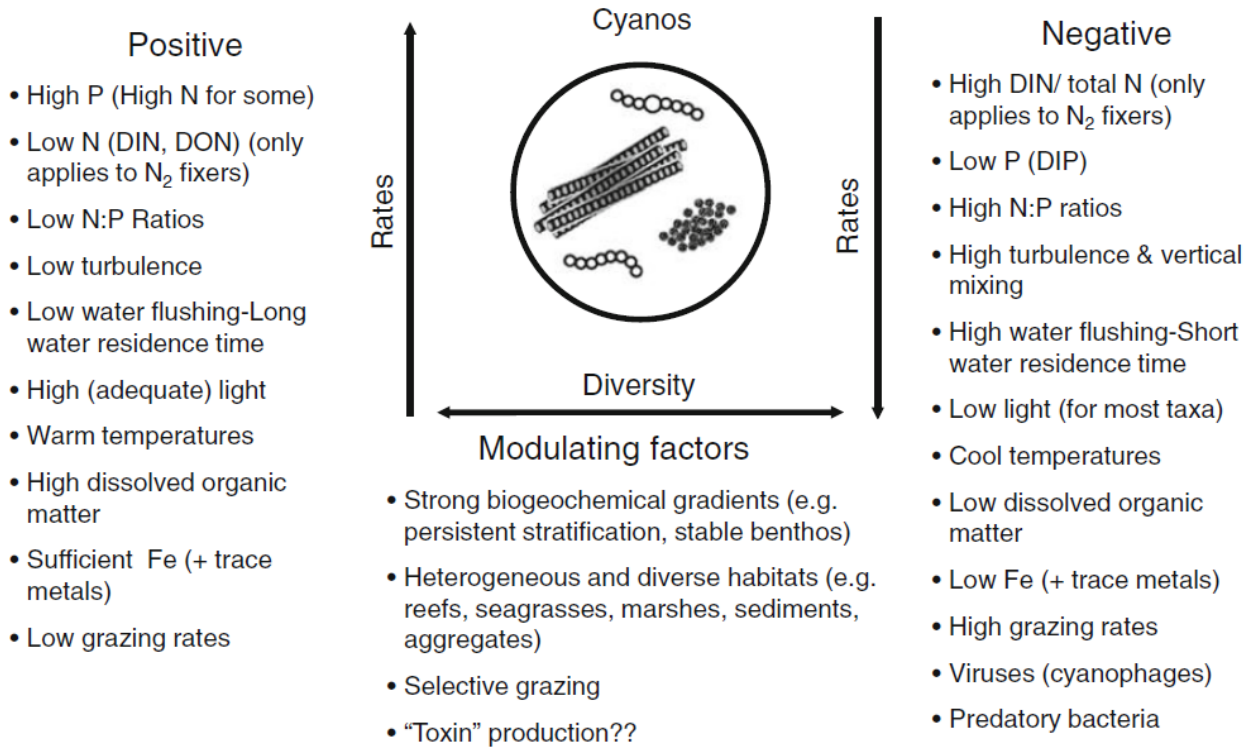


Figure 2-1. Environmental Factors Influencing Cyanobacterial Blooms.

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.

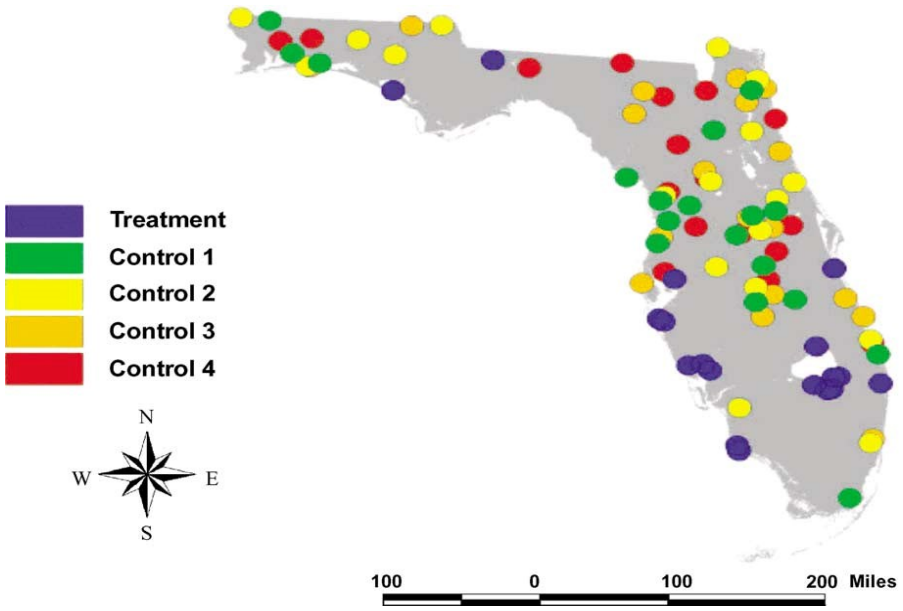


Figure 6-1. Sites of Surface Water Treatment Service Areas and Control Ground Water Treatment Service Areas.
From: Fleming et al., 2002

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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Human Health Recreational Ambient Water Quality Criteria or Swimming Advisories for Microcystins and Cylindrospermopsin

Draft

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Draft

Prepared by:

U.S. Environmental Protection Agency
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Health and Ecological Criteria Division
Washington, DC

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NOTICES

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FOREWORD

Section 304(a) of the Clean Water Act requires the Administrator of the Environmental Protection Agency to publish water quality criteria that accurately reflect the latest scientific knowledge on the kind and extent of all identifiable effects on health and welfare that might be expected from the presence of pollutants in any body of water, including ground water.

U.S. Environmental Protection Agency (EPA) is publishing these recommended values under Clean Water Act (CWA) 304(a) for states to consider as the basis for swimming advisories for notification purposes in recreational waters to protect the public. Alternatively, states may consider using these same values when adopting new or revised water quality standards (WQS). If adopted as WQS and approved by EPA under CWA 303(c), the WQS could be used for all CWA purposes. States may also wish to consider using these values as both swimming advisory values and WQS. EPA envisions that if states decide to use the values as swimming advisory values they might do so in a manner similar to their current recreational water advisory programs.

This draft document has undergone an EPA intra-agency peer review process. Final review by the Health and Ecological Criteria Division, Office of Science and Technology, Office of Water, U.S. Environmental Protection Agency has been completed and the document is approved for publication. These values were derived using the existing peer-reviewed and published science on the adverse human health effects of these toxins, established criteria methodologies, and recreation-specific exposure parameters from EPA's Exposure Factors Handbook.

The term “water quality criteria” is used in two sections of the CWA—§304(a)(1) and §303(c)(2). The term has a different program impact in each section. In section 304, the term represents a non-regulatory, scientific assessment of effects on human health or aquatic life. The criteria recommendations presented in this document are such a scientific assessment. If water quality criteria associated with specific designated uses are adopted by a state or authorized tribe as water quality standards under section 303, and approved by EPA, they become applicable Clean Water Act water quality standards in ambient waters within that state or tribe. Water quality criteria adopted in state or tribal water quality standards could have the same numerical values as criteria developed under section 304. Alternatively, states and authorized tribes may derive numeric criteria based on other scientifically defensible methods but the criteria must be protective of designated uses. It is not until their adoption as part of state or tribal water quality standards, and subsequent approval by EPA, that criteria become Clean Water Act applicable water quality standards. Guidelines to assist in modifying the criteria recommendations presented in this document are contained in the Water Quality Standards Handbook (U.S. EPA 2012b). This handbook and additional guidance on the development of WQS and other water-related programs of this agency have been developed by EPA which along with additional guidance on the development of water quality standards and other water-related programs of this Agency have been developed by the Office of Water.

This document provides recommendations only. It does not establish or affect legal rights or obligations. It does not establish a binding norm and cannot be finally determinative of the issues addressed. Agency decisions in any particular situation will be made by applying the Clean Water Act and EPA regulations on the basis of specific facts presented and scientific information then available.

/signed/

Elizabeth Southerland
Director
Office of Science and Technology

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ACRONYMS AND ABBREVIATIONS

AWQC	Ambient Water Quality Criteria
BGAS	blue-green algae supplements
bw	body weight
CalEPA	California Environmental Protection Agency
CDC	U.S. Centers for Disease Control and Prevention
CI	confidence interval
CWA	Clean Water Act
CYP450	Cytochrome P450
ELISA	Enzyme Linked Immunosorbent Assays
EPA	U.S. Environmental Protection Agency
GI	gastrointestinal
HAB	harmful algal bloom
HESD	Health Effects Support Document
HPLC	high performance liquid chromatography
IARC	International Agency for Research on Cancer
i.p.	intraperitoneal
kg	kilograms
K_{oc}	soil organic carbon-water partition coefficient
K_{ow}	octanol-water partition coefficient
L	liter
LOAEL	lowest-observed-adverse-effect-level
LOD	level of detection
LPS	lipopolysaccharide
mL	milliliters
NLA	National Lakes Assessment
NOAEL	no-observed-adverse-effect-level
OATp	organic acid transporter polypeptide
OPP	EPA Office of Pesticide Programs
OR	odds ratio
pg	picogram
RfD	reference dose
ROS	reactive oxygen species
RSC	relative source contribution
SWIMODEL	Swimmers Exposure Assessment Model
TMDL	Total Maximum Daily Load
UF	uncertainty factor
UF_A	uncertainty factor for interspecies variability
UF_D	database uncertainty factor
UF_H	uncertainty factor for intraspecies variability

UF_L	uncertainty factor for LOAEL to NOAEL extrapolation
µg	microgram
USGS	U.S. Geological Survey
WHO	World Health Organization
WQBEL	Water Quality-Based Effluent Limits
WQS	water quality standards

1.0 EXECUTIVE SUMMARY

Cyanobacteria, also commonly referred to as blue-green algae, are photosynthetic bacteria that are ubiquitous in nature, including surface waters. Environmental conditions that promote excessive growth of cyanobacteria in surface waters can lead to situations in which cyanobacterial cell density is high, known as blooms. Environmental factors that play an important role in the development of cyanobacterial blooms and their production of cyanotoxins include the levels of nitrogen and phosphorus, the ratio of nitrogen to phosphorus, temperature, organic matter availability, light attenuation, and pH.

Microcystins can be produced by a variety of cyanobacteria genera including *Microcystis*, *Anabaena*, *Nostoc*, *Oscillatoria*, *Fischerella*, *Planktothrix*, and *Gloeotrichia*. Some of these species can be distributed through the water column, concentrate in the upper layers, or form surface scums depending on environmental conditions. More than 100 microcystin congeners exist, which vary based on amino acid composition. The majority of toxicological data on the effects of microcystins are available for microcystin-LR, which is also a frequently monitored congener. Microcystins are water-soluble and tend to remain contained within the cyanobacterial cell, until the cell breaks, and they are released into the water. Microcystins typically have a half-life of 4 to 14 days in surface waters or may persist longer, depending on such factors as the degree of natural degradation owing to sunlight, organic matter, and the presence of bacteria. Microcystins can persist even after a cyanobacterial bloom is no longer visible.

Cylindrospermopsin can be produced by a variety of cyanobacteria species including *Cylindrospermopsis raciborskii*, *Aphanizomenon* species, *Anabaena* species, *Lyngbya wollei*, and *Rhaphidiopsis* species. Some of these species tend not to form visible surface scums, and the highest concentrations of cyanobacterial cells typically occur below the water surface. Cylindrospermopsin may be retained within the cell or released into the water. The biodegradation of cylindrospermopsin in natural water bodies is a complex process that can be influenced by many environmental factors, including toxin concentration, water temperature, sunlight, and the presence of cell pigments and bacteria. Half-lives of 11 to 15 days and up to 8 weeks have been reported for cylindrospermopsin in surface waters.

This document for microcystins and cylindrospermopsin focuses on the human health risks associated with recreational exposures in waters containing these cyanotoxins. Exposure to cyanobacteria and their toxins can also occur through non-recreational pathways such as consumption of cyanotoxin-contaminated drinking water and food (including fish), and during bathing or showering. The non-recreational exposures were not quantified in the recreational exposure scenario described herein. Given that cyanobacterial blooms typically are seasonal events, recreational exposures are likely to be episodic, and may be short-term in nature.

U.S. Environmental Protection Agency (EPA) is publishing these recommended values for microcystins and cylindrospermopsin under Clean Water Act (CWA) 304(a) for states to consider as the basis for swimming advisories for notification purposes in recreational waters to protect the public. Additionally, states may consider using these same values when adopting new or revised water quality standards (WQS). If adopted as WQS and approved by EPA under CWA 303(c), the WQS could be used for all CWA purposes. States may also wish to consider using these values as both swimming advisory values and/or WQS. EPA envisions that if states decide to use the values as swimming advisory values they would do so in a manner similar to their

current recreational water advisory programs. The recommended values for use as swimming advisories and/or WQS leverage the information collected and evaluated in EPA's *Drinking Water Health Advisory for the Cyanobacterial Microcystin Toxins* and *Drinking Water Health Advisory for the Cyanobacterial Toxin Cylindrospermopsin* (Drinking Water Health Advisories) for these cyanotoxins.

At this time, available data are insufficient to develop quantitative recreational values for cyanobacterial cell density related to inflammatory health endpoints. The reported epidemiological relationships in the literature are not consistent for specific health outcomes (e.g., dermal symptoms, eye/ear irritation, fever, gastrointestinal (GI) illness, and respiratory symptoms) or for those health outcomes associated with specific cyanobacterial cell densities. The uncertainties related to the epidemiological study differences, such as study size, species and strains of cyanobacteria present, and the cyanobacterial cell densities associated with significant health effects, do not provide sufficient information to determine a consistent association between cyanobacterial densities associated with adverse inflammatory health effects.

EPA evaluated the health effects of microcystins and derived a Reference Dose (RfD) in its 2015 *Health Effects Support Document for the Cyanobacterial Toxin Microcystins.* Exposure to higher-levels of microcystins can lead to liver damage and renal failure. The critical study for the derivation of the microcystins RfD was conducted by Heinze et al. (1999) based on rat exposure to microcystin-LR in drinking water. The critical effect from this study was liver damage, including increased liver weight, slight to moderate liver lesions with hemorrhages, and increased liver enzyme levels. EPA established an RfD for microcystin-LR and used it as a surrogate for other microcystin congeners.

EPA evaluated the health effects of cylindrospermopsin and derived an RfD in its 2015 *Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin.* The kidneys and liver appear to be the primary target organs for cylindrospermopsin toxicity. The critical study for the derivation of the cylindrospermopsin RfD was conducted by Humpage and Falconer (2002, 2003) based on drinking water exposure to mice. The critical effect was kidney damage, including increased kidney weight and decreased urinary protein.

Based on available noncancer health effects information, EPA is recommending values protective of primary contact recreation for two cyanotoxins as follows:

- For microcystins, the recreational value is 4 micrograms (µg)/liter (L).
- For cylindrospermopsin, the recreational value is 8 µg/L.

These values are based on overall exposure to children at the 90th percentile. If used as a swimming advisory to protect swimmers at a beach, these values are not to be exceeded on any single day. If used as a water quality criterion for assessment and listing purposes, EPA recommends that states consider the number of exceedances of no more than 10 percent of days per recreational season up to one year. These criteria are based on noncancer health effects because EPA concluded in its Health Effects Support Documents (HESDs) for microcystins and cylindrospermopsin that there is inadequate information to assess carcinogenic potential of these cyanotoxins (U.S. EPA 2015c; U.S. EPA 2015d). Should additional information become available in the future, EPA can review and revise these recommendations, as appropriate.

2.0 INTRODUCTION AND BACKGROUND

This section provides background information about cyanobacteria and cyanobacterial blooms, the source of the stressors, microcystins and cylindrospermopsin. It discusses briefly the occurrence of cyanobacterial blooms and these cyanotoxins in the United States. Section 2.1 describes Clean Water Act provisions relevant to these recreational ambient water quality criteria or swimming advisories. Section 2.2 summarizes international and state recreational water guidelines for microcystins, cylindrospermopsin, and cyanobacteria to provide context regarding how other jurisdictions are addressing the human health concerns.

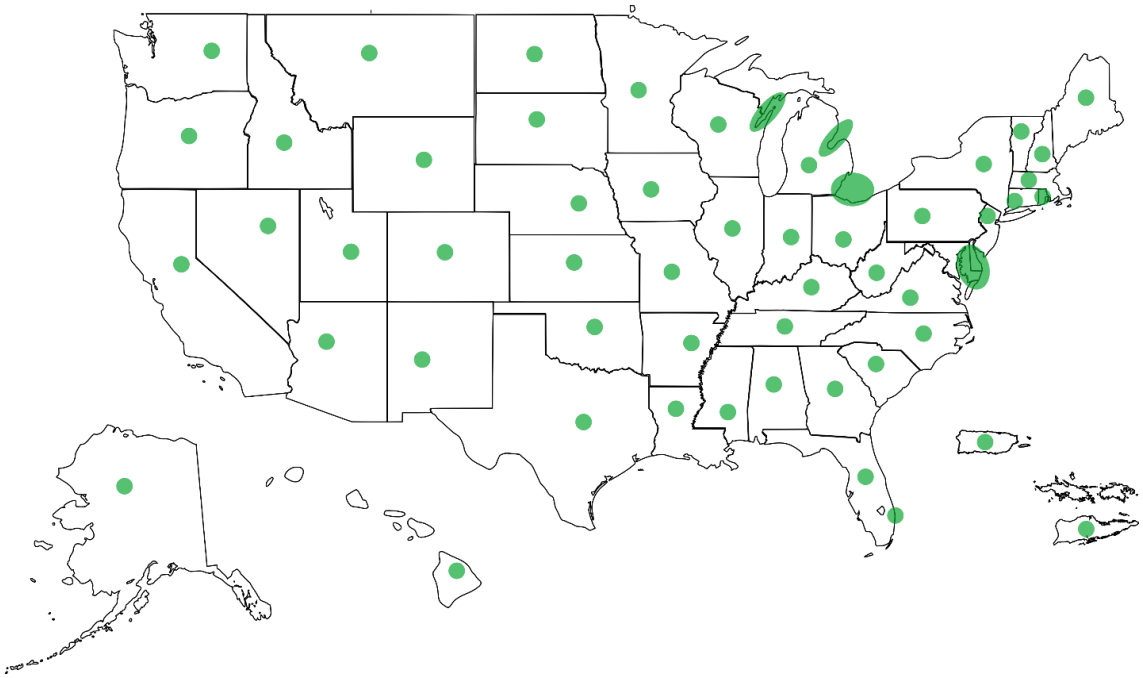
Cyanobacteria are a group of microorganisms that naturally occur in freshwater and marine environments and can be found at higher densities in eutrophic or nutrient-enriched water bodies. Many cyanobacteria are capable of producing toxins, generally referred to as cyanotoxins, which can impact human health. Under the right conditions of temperature, light, pH, nutrient availability, etc., cyanobacteria can reproduce rapidly to high densities in water, forming what are commonly referred to as cyanobacterial harmful algal blooms (HABs). Other microorganisms can form HABs, but for the purpose of this document, which addresses cyanotoxins, usage of "HABs" will be in reference to cyanobacterial HABs unless otherwise specified. A variety of factors can influence both cyanobacteria proliferation and toxin production, including nutrient (e.g., nitrogen and phosphorus) concentrations, light levels, temperature, pH, oxidative stressors, and interactions with other biota (viruses, bacteria, and animal grazers), and others, as well as their combined effects (Paerl & Otten 2013a; Paerl & Otten 2013b).

Because they are a natural part of algal communities, cyanobacteria are commonly observed in freshwater systems. The occurrence of HABs has been documented in surface waters of all 50 states as well as U.S. territories between 2006 and 2015 as shown in Figure 2-1 (Richlen 2016; WHOI 2016). Figure 2-1 also identifies areas where more widespread HAB problems have occurred, e.g., parts of the Great Lakes.

In 2007, the EPA's National Lakes Assessment (NLA) conducted a national probability-based survey of the nation's lakes, ponds, and reservoirs (Loftin et al. 2016b; U.S. EPA 2009). These surveys covered a total of 1,028 lakes, which represented nearly 50,000 lakes larger than 4 hectares (10 acres) in the conterminous United States. This assessment found that cyanobacteria were detected in almost all lakes (U.S. EPA 2009). Cyanobacteria were the dominant member of the phytoplankton community in 76 percent of lake samples. Subsequent analysis indicated that potential microcystin- and cylindrospermopsin- producing species occurred in 95 and 67 percent of samples, respectively (Loftin et al. 2016b).

Microcystins are the most commonly detected class of cyanotoxin and have been found in lakes in the contiguous United States (U.S. EPA 2009) and streams in the Southeastern United States (Loftin et al. 2016a). The NLA 2007 reported that 30 percent of lakes in the conterminous United States had detectable microcystin. In a separate study, Graham et al. (2010) sampled cyanobacterial blooms in 23 Midwestern lakes and detected microcystins in all blooms sampled. The researchers also found that cylindrospermopsin co-occurred with microcystins in 9 percent of samples (Graham et al. 2010). In an expanded analysis of NLA samples, Loftin et al. (2016b) identified cylindrospermopsin in 4 percent of samples with a mean concentration of 0.56 µg/L.

Figure 2-1. Generalized Distribution of Cyanobacterial HABs in the United States and Territories[a]



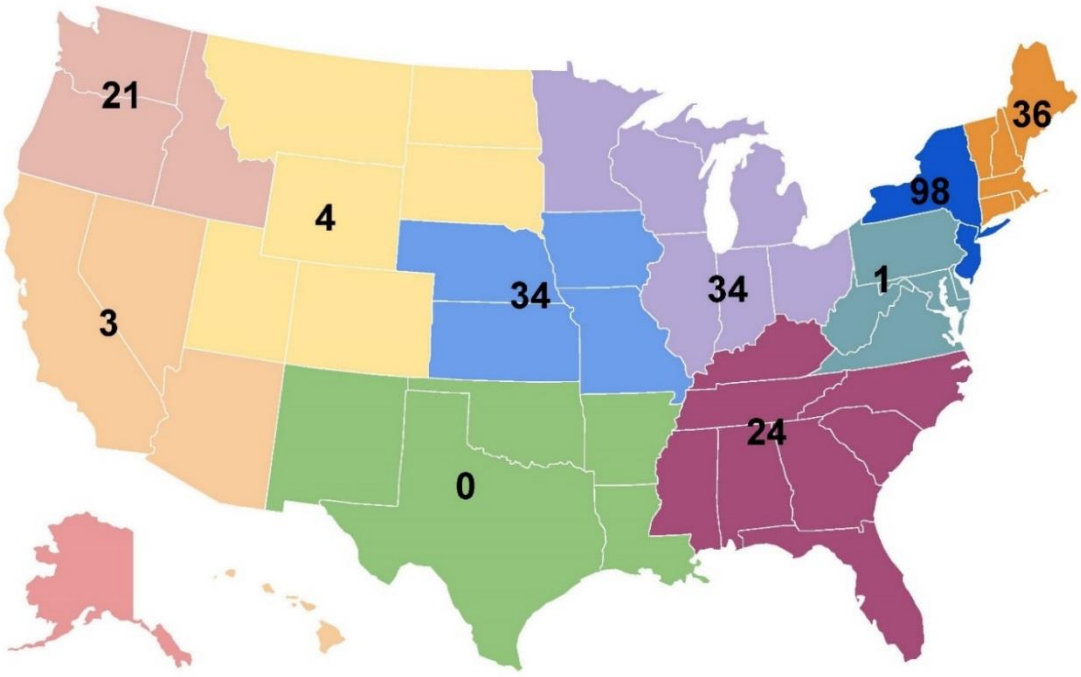
[a] Graphic adapted from a Woods Hole Oceanographic Institute (WHOI) map of HABs that occurred between 2006 and 2015. It reflects input from HAB experts with broad experience in HAB events and reports to the U.S. National Office for Harmful Algal Blooms (Richlen 2016; WHOI 2016). Each state that has experienced one or more cyanobacterial HABs is indicated with a single green dot. Larger green ovals mark areas where more widespread cyanobacterial HAB problems occurred.

Exposure to HABs can result in adverse human health outcomes such as gastrointestinal, dermatologic, respiratory, neurologic, and other symptoms. The Centers for Disease Control and Prevention (CDC) collected information on outbreaks of illness related to HABs reported via the National Outbreak Reporting System (NORS) and the Harmful Algal Bloom-Related Illness Surveillance System (HABISS). During 2009 and 2010 in the United States, 11 waterborne disease outbreaks associated with HABs were reported to CDC, all occurring in freshwater lakes: eight of these investigations evaluated the presence of cyanotoxins; eight detected microcystins; and two detected cylindrospermopsin (Hilborn et al. 2014). The 11 outbreaks associated with HABs affected at least 61 persons, resulting in 2 hospitalizations; 66 percent of the affected persons overall were aged 18 or younger, and 35 percent were aged 9 years or younger. Hilborn et al. (2014) reported that microcystins were present during all eight outbreak investigations in which cyanotoxin testing was performed. In four of the outbreaks, microcystin concentrations exceeded 20 µg/L. During investigations of these outbreaks, cylindrospermopsin, and anatoxin-a also were detected. The researchers concluded that the disease outbreak data suggest that the time to onset of effects might be rapid, that children might be at higher risk for illness, and that HAB–associated outbreaks occur during the warmer months. Hilborn et al. (2014) noted that recognizing HAB-associated illness from recreational exposure might be underreported due to multiple possible exposure routes and the non-specific nature of potential health effects. In addition, Graham et al. (2009) counted 36 states with anecdotal reports of acute cyanotoxin poisonings of animals, humans, or both as reported in journal articles and newspaper articles (Chorus & Bartram 1999; Huisman et al. 2005; Yoo et al. 1995).

As a result of potential adverse health effects associated with recreational exposure to HABs, many states have developed guidelines and/or health advisories related to HABs. For the summer of 2008, Graham et al. (2009) identified at least 13 states that posted recreational health advisories because the concentrations of cyanobacteria or cyanotoxins were large enough to be considered a risk to animals and people by the state (Graham et al. 2009). These included cautions, warnings, public health advisories, and public health warnings, due to the presence of cyanobacteria, cyanotoxins, or both.

Figure 2-2 shows the number of 2016 freshwater HAB recreational advisories states publicly reported in each EPA region between January 1 and August 12, 2016. To develop this regional summary map, EPA researched and compiled publically available reports posted on states' websites between these dates. During that time, states reported at least 255 notices for freshwater HABs with reported microcystins concentrations ranging from not detected (i.e., below the limit of detection) to 392 µg/L. These notices included cautions, warnings, public health advisories, and public health warnings due to the presence of cyanobacteria, cyanotoxins, or both. Advisories can last for multiple days. The review was not exhaustive and might not reflect all of the monitoring, beach, or general health advisories (e.g., some advisories at local or county-level may not be posted on the state website). Thus, the number of actual HAB advisories during this time might be higher. In addition, many states have only recently begun to monitor HABs, thus monitoring may be limited.

Figure 2-2. State-reported HAB Advisories by EPA Region, January 1 to August 12, 2016



The presence of detectable concentrations of cyanotoxins in the environment is closely associated with HAB occurrences. Cyanotoxin concentrations in surface waters can be higher after the initial bloom fades, so potential exists for human exposures even after the visible signs of a bloom are gone. Thus, high densities of cyanobacteria and high cyanotoxin concentrations are capable of affecting the health of humans, domestic animals, and wildlife in contact with

affected waters. These events are not always independent; animal health effects associated with harmful cyanobacteria have served as sentinel events to warn of potential human health risks (Hilborn & Beasley 2015). Cyanotoxin production by cyanobacteria can vary spatially and temporally, and studies of the impacts of environmental factors on cyanotoxin production are ongoing.

Nutrients are key environmental drivers that influence the proportion of cyanobacteria in the phytoplankton community, the cyanobacterial biovolume, cyanotoxin production, and the impact that cyanobacteria may have on ecosystem function and water quality (Paerl et al. 2011). Cyanobacteria production and cyanotoxin concentrations are dependent on nutrient levels (Wang et al. 2002); however, nutrient uptake rates and the utilization of organic and inorganic nutrient forms of nitrogen and phosphorus vary considerably by cyanobacteria species. In addition to nutrient concentrations, factors such as the nitrogen:phosphorus ratio and organic matter availability, as well as other physico-chemical processes, can play a role in determining HAB composition and cyanotoxin production (Paerl & Huisman 2008; Paerl & Otten 2013b).

2.1 Clean Water Act

Section 304(a) of the Clean Water Act (CWA) requires the Administrator of EPA to publish water quality criteria that accurately reflect the latest scientific knowledge on the kind and extent of all identifiable effects on health and welfare that might be expected from the presence of pollutants in any body of water, including ground water.

EPA is publishing these recommended values under CWA 304(a) for states to consider as the basis for swimming advisories for notification purposes in recreational waters to protect the public. Additionally, states may consider using these same values as criteria when adopting new or revised WQS. If adopted as WQS and approved by EPA under CWA 303(c), the WQS could be used for CWA purposes. States may also wish to consider using these values as both swimming advisory values and WQS. EPA envisions that if states decide to use the values as swimming advisory values they might do so in a manner similar to their current recreational water advisory programs.

This document recommends values for cyanotoxins that are protective of human health given a primary contact recreational exposure scenario. The cyanotoxins included in this document have been demonstrated to occur in nutrient-enriched waters affected by cyanobacterial HABs.

The term “water quality criteria” is used in two sections of the CWA§304(a)(1) and §303(c)(2). The term has a different program impact in each section. In section 304, the term represents a non-regulatory, scientific assessment of effects on human health or aquatic life. The criteria recommendations presented in this document are such a scientific assessment. If water quality criteria associated with specific designated uses are adopted by a state or authorized tribe as water quality standards under section 303, and approved by EPA, they become applicable CWA water quality standards in ambient waters within that state or tribe. Water quality criteria adopted in state or tribal water quality standards could have the same numerical values as criteria developed under section 304. Alternatively, states and authorized tribes may derive numeric criteria based on other scientifically defensible methods, but the criteria must be protective of designated uses. It is not until their adoption as part of state or tribal water quality standards, and subsequent approval by EPA, that criteria become CWA applicable water quality standards.

Guidelines to assist in modifying the criteria recommendations presented in this document are contained in the Water Quality Standards Handbook (U.S. EPA 2012b). This handbook and additional guidance on the development of WQS and other water-related programs of this Agency have been developed by EPA, which along with additional guidance on the development of water quality standards and other water-related programs of this Agency have been developed by the Office of Water.

2.2 International and State Guidelines

In 2003, World Health Organization (WHO) derived a series of guideline values for recreational exposure to cyanobacteria associated with incremental severity and probability of adverse health effects (WHO 2003b); see Table 2-1. For these guidelines, WHO recommended values that included the potential health effects from exposure to cyanobacteria because it was "unclear whether all important cyanotoxins had been identified and that the health outcomes observed after recreational exposure could be related to cyanobacterial substances other than the well-known cyanotoxins (WHO 2003b)." They also considered the potential for liver damage by microcystins. WHO highlighted that there are multiple potential health endpoints related to recreational exposure to cyanobacteria and their toxins and developed a series of guidelines associated with incremental severity and probability of health effects at increasing densities of cyanobacteria and corresponding concentrations of chlorophyll *a* (if cyanobacteria dominate). The different levels were an effort to distinguish between irritative or inflammatory-response symptoms associated with cyanobacterial cells and the more severe hazard of exposure to elevated concentrations of cyanotoxins, particularly microcystins. The WHO guidelines combine the potential for both sets of endpoints (i.e., cyanotoxins and cyanobacterial cells) across three categories of increasing probability of risk. The cell-associated inflammatory responses are represented by the low probability of adverse health effects category of < 20,000 cells/milliliter (mL), corresponding to less than 10 µg/L chlorophyll *a* if cyanobacteria dominate and estimated microcystin levels of less than 10 µg/L. According to the WHO, as the density of cyanobacteria increase above that level, the probability of inflammatory responses increases, and the potential for more severe adverse health effects associated with exposure to the cyanotoxins also increases. The high-risk category identified by WHO related > 100,000 cells/mL (corresponding to 50 µg/L of chlorophyll *a*, if cyanobacteria dominate) and > 20 µg/L microcystin levels, was primarily due to the toxic effects of microcystins.

Table 2-1. WHO (2003b) Recreational Guidance/Action Levels for Cyanobacteria, Chlorophyll *a,* and Microcystin

Relative Probability of Acute Health Effects	Cyanobacteria (cells/mL)	Chlorophyll *a* (µg/L)	Estimated Microcystin Levels (µg/L)[a]
Low	< 20,000	< 10	< 10
Moderate	20,000–100,000	10–50	10–20
High	>100,000–10,000,000	50–5,000	20–2,000
Very High	> 10,000,000	> 5,000	> 2,000

[a] WHO (2003b) derived the microcystin concentrations from the cyanobacterial cell density levels.

The WHO guideline value development was supported by results from a review conducted by Chorus and Bartram (1999). A primary study identified in this review was a prospective epidemiological study by Pilotto et al. (1997), which evaluated health effects after recreational exposure to cyanobacteria and reported associations between cyanobacterial cell densities and health. Pilotto et al. (1997) found a significant association among recreators exposed to > 5,000 cells/mL for > 1 hour and one or more symptoms and similar significant associations for symptoms in people exposed to 5,000–20,000 cells/mL. WHO chose a guideline level of 20,000 cells/mL to represent the upper bound of the low probability of adverse health effects category (WHO 2003b). The low category includes irritative or inflammatory health effects associated with exposure to cyanobacterial cells (WHO 2003b). While the association among recreators exposed to > 5,000 cells/mL for > 1 hour and one or more symptoms reported in Pilotto et al. (1997) were statistically significant, WHO states that they represented less than 30 percent of the individuals exposed (Chorus & Bartram 1999). Therefore, the level of health effect and the small number of people affected at 5,000 cells/mL were not considered by WHO to be a basis to justify action (WHO 2003b).

WHO (2003b) also made the connection between cyanobacterial cell densities and microcystin concentrations. It assumed microcystin-producing cyanobacteria dominate the population of cyanobacteria present and that the average microcystin content of *Microcystis* sp. cells averages 0.2 pg/cell. Thus, WHO estimated that 20,000 cells/mL could potentially equate to approximately 2-4 μg/L of microcystin. Similarly, using the same assumptions at a cyanobacterial cell density of 100,000 cells/mL, they estimated approximately 20 μg/L. WHO pointed out that the potential concentration of microcystins could vary based on the composition of the community of cyanobacteria present. WHO states that, at the same cyanobacterial cell density, cyanotoxin levels may approximately double if *Planktothrix agardhii* is the dominant member of the community. Several states have adopted the estimated microcystins concentrations as their guideline values rather than the cell density or chlorophyll *a* values from the WHO guidelines, as discussed later in this section.

Many countries have adopted the WHO recommendations for recreational waters including multiple parameters (e.g., cell density, biovolume, and cyanotoxin concentration). Table 2-2 provides international recreational water guideline or action levels for cyanotoxins or cyanobacteria for several countries. Table 2-2 lists the lowest concentrations of cyanotoxins or densities of cyanobacteria that prompt a health protective action. For a more complete list of guideline or action levels and recommended actions for international jurisdictions, see Appendix A. EPA did not identify any recreational guideline levels for cylindrospermopsin established by other international regulatory authorities. Some international authorities have multiple action levels; for brevity, Table 2-2 that follows presents the guideline reflecting *the lowest concentration* of microcystin or density of cyanobacterial cells that recommended or triggered a health protective action. More details are in Appendix A.

Table 2-2. International Recreational Water Guideline or Action Levels for Cyanobacteria and Microcystins

Jurisdiction	Lowest Recreational Water Guideline/Action Level[a]	Reference
Australia[b]	microcystins (total): ≥ 10 µg/L or *Microcystis aeruginosa* (total): ≥ 500 to < 5,000 cells/mL or cyanobacteria (total): ≥ 0.4 to < 4 mm^3/L (where a known toxin producer is dominant in the total biovolume)	Australian Government National Health and Medical Research Council (2008)
Canada	microcystins (total): ≥ 20 µg/L (expressed as microcystin-LR) or cyanobacteria (total): ≥ 100,000 cells/mL	Health Canada (2012)
Cuba	cyanobacteria: > 1 of the species known as potentially toxic or phytoplankton cells: > 20,000 – to < 100,000 cells/mL, > 50 percent of cells cyanobacteria	German Federal Environment Agency (2012)[c]
Czech Republic	cells: >20,000 cells/mL	German Federal Environment Agency (2012)[c]
Denmark	chlorophyll *a*: >50 µg/L, dominated by cyanobacteria or visible surface scum	German Federal Environment Agency (2012)[c]
European Union	Appropriate monitoring must be implemented if there is a risk of proliferation of algae. Member state authorities responsible must take management measures and provide information immediately if a proliferation of cyanobacteria (or blue algae) occurs.	European Parliament and the Council of the European Union (2006)
Finland	algae (includes cyanobacteria): detected	German Federal Environment Agency (2012)[c]
France[b]	microcystins: > 25 µg/L or cyanobacteria: > 20,000 to < 100,000 cells/mL (±20 percent)	German Federal Environment Agency (2012)[c]
Germany	Secchi Disk reading > 1 m AND (microcystins: ≥ 10 µg/L or chlorophyll *a* (with dominance by cyanobacteria): ≥ 40 µg/L or biovolume: ≥ $1mm^3/L$)	German Federal Environment Agency (2012)[c]
Hungary	microcystins: ≥ 4 to < 10 µg/L or cell count: ≥ 20,000 to < 50,000 cells/mL or chlorophyll *a* (with dominance by cyanobacteria): ≥ 10 to < 25 µg/L	German Federal Environment Agency (2012)[c]
Italy[b]	microcystins: > 25 µg/L or cyanobacterial cell count (combined with identification of genus and, if possible, species): > 20,000 cells/mL	German Federal Environment Agency (2012)[c]
Netherlands	chlorophyll *a*: ≥12.5 to ≤ 75 µg/L or biovolume (cyanobacterial cell count): ≥ 2.5 to ≤ 15 mm^3/L	German Federal Environment Agency (2012)[c]
New Zealand[b]	microcystins (total): ≥ 12 µg/L or cyanobacteria (benthic): 20−50 percent coverage of potentially toxigenic cyanobacteria attached to substrate or cyanobacteria (total): > 0.5 to < 1.8 mm^3/L (biovolume equivalent of potentially toxic cyanobacteria) or cyanobacteria (total): > 0.5 to < 10 mm^3/L (biovolume equivalent of the combined total of all cyanobacteria)	Wood et al. (2008)

Jurisdiction	Lowest Recreational Water Guideline/Action Level[a]	Reference
Poland	visible blooms	German Federal Environment Agency (2012)[c]
Scotland[b]	chlorophyll *a*: ≥ 10 µg/L with dominance of cyanobacteria or cyanobacteria: ≥ 20,000 cells/mL	Scottish Government Health and Social Care Directorates Blue-Green Algae Working Group (2012)
Spain	cyanobacteria proliferation potential (Low)	German Federal Environment Agency (2012)[c]
Turkey	microcystin-LR: > 25 µg/L equivalents or cells: ≥ 20,000 to 100,000 cells/mL	German Federal Environment Agency (2012)[c]
World Health Organization (WHO)	cyanobacteria: 20,000 cells/mL or chlorophyll *a*: 10 µg/L (approximately 2–4 µg microcystin/L, assuming cyanobacteria dominance)	Chorus and Bartram (1999); WHO (2003b)

[a] More details are provided in Appendix A.

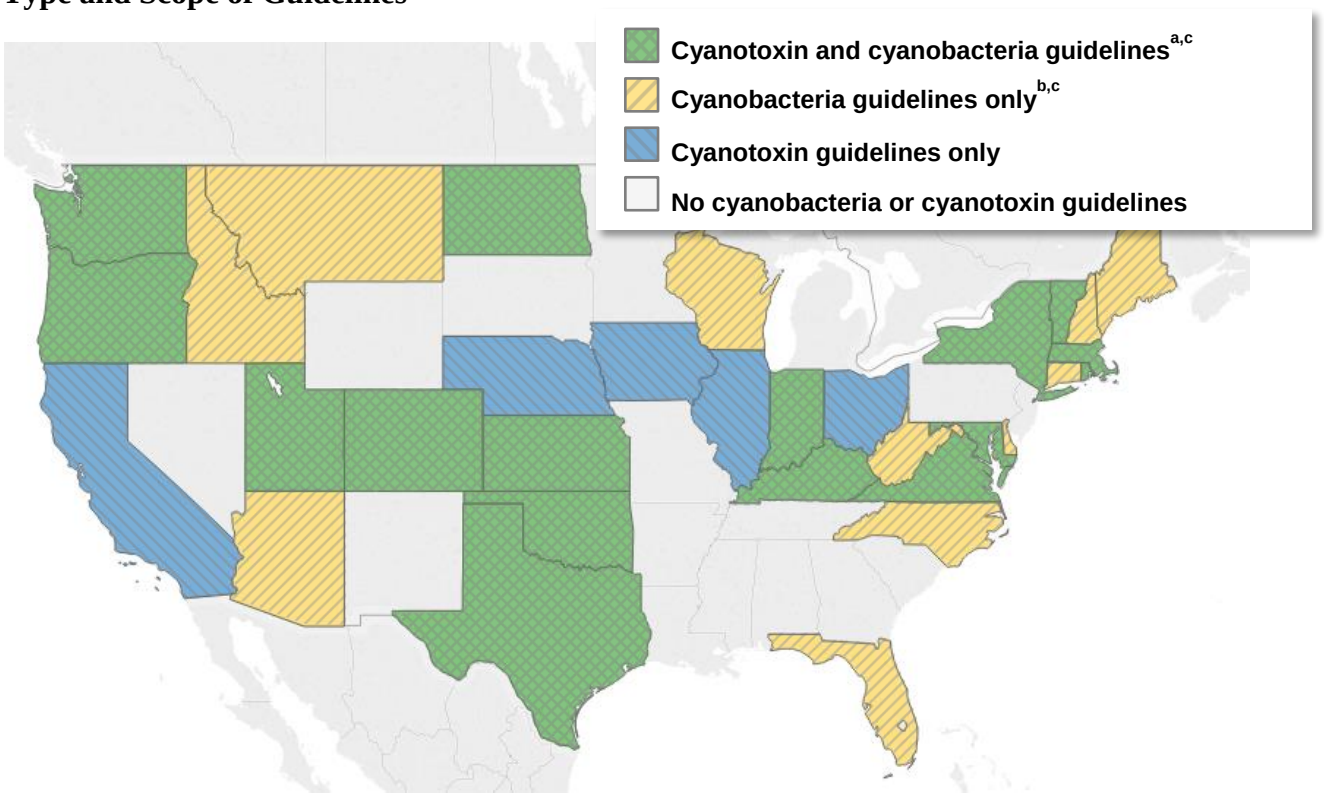
[b] The lowest guideline values for each quantitative parameter (i.e., cyanobacterial cell density, biovolume, cyanotoxin concentration) are not associated with the same action level. For example, for Australia, the lowest cyanobacterial cell density and biovolume criteria trigger the green level surveillance mode, and the lowest cyanotoxin concentration triggered the red level action mode.

[c] Following the VIIIth International Conference on Toxic Cyanobacteria, the German Federal Environmental Agency compiled and published in 2012 regulatory approaches to the assessment and management of cyanotoxin risks based on contributions by member countries.

Approximately 30 U.S. states have implemented cyanobacterial HAB guidelines for recreational waterways as of November 2015. As shown in Figure 2-3, five states had quantitative cyanotoxin guidelines only, and fourteen states had quantitative guidelines for cyanotoxins, as well as either quantitative or qualitative guidelines for cyanobacterial cell density. Generally, qualitative guidelines use visual inspection and not quantitative detection methods. In addition, twelve states had quantitative guidelines for cyanobacterial cell density only or had qualitative guideline values only.

For brevity, Table 2-3 lists the lowest recreational water guideline or action levels for microcystins, cylindrospermopsin, or cyanobacteria that trigger or recommend a health protective action for U.S. states. For a more complete list of state guideline/action levels and recommended actions see Appendix B. Parameters and values used as the basis for guidelines varied across states, as does the methodology for developing the values. Similar to international authorities, many states used a tiered approach, which evaluates multiple parameters including cyanobacterial cell density, chlorophyll *a* concentration, cyanotoxin concentration, and visual appearance. New York, for example, considered all four of these parameters at lower tier guideline levels, but only considered cyanotoxin concentrations at the highest advisory level. Other states had only one response guideline level and only consider cyanotoxin concentration (e.g., California) or had only one response guideline level, but considered cyanobacterial cell density, cyanotoxin concentration, and visual appearance (e.g., Oregon). In contrast, other states, like Connecticut, used a tiered approach and did not consider cyanotoxin concentrations at any tier but rather consider visual inspection and cyanobacterial cell density.

Figure 2-3. State Guidelines for Cyanotoxins and Cyanobacteria in Recreational Water by Type and Scope of Guidelines



[a] Includes states with quantitative cyanotoxin guidelines as well as either quantitative or qualitative cyanobacteria guidelines.

[b] Includes states that either have quantitative cyanobacteria guidelines only or qualitative guidelines only.

[c] EPA found that Texas and North Carolina published guidelines in the past, but the guidelines were no longer on their websites.

Table 2-3. State Guideline or Action Levels for Microcystin, Cylindrospermopsin, and Cyanobacterial Cells in Recreational Water

State	Lowest Recreational Water Guideline or Action Level[a]	Reference
Arizona	blue-green algae (mean value based on a minimum of two sample events within one peak season): 20,000 cells/mL and chlorophyll *a* result (mean value based on a minimum of two sample events within one peak season) in target range	Arizona Department of Environmental Quality (2008)
California	microcystins: 0.8 µg/L	Butler et al. (2012)
	cylindrospermopsin: 4 µg/L	
Colorado	microcystin-LR: ≥ 10 µg/L and < 20 µg/L	Colorado Department of Public Health & Environment (2016)
	cylindrospermopsin: ≥ 7 µg/L	
Connecticut	visual rank category 2: cyanobacteria present in low numbers; there are visible small accumulations but	Connecticut Department of Public Health: Connecticut Energy

State	Lowest Recreational Water Guideline or Action Level[a]	Reference
	water is generally clear; OR blue-green algae cells > 20,000 cells/mL and < 100,000 cells/mL	Environment (2013)
Delaware	thick green, white, or red scum on surface of pond	Delaware Department of Natural Resources and Environmental Control: Division of Water (2016)
Florida	cyanobacteria bloom	Florida Department of Environmental Protection (2016); Florida Department of Health (2016)
Idaho	*Microcystis* or *Planktothrix*: > 40,000 cells/mL	IDEQ (2015)
	sum of all potentially toxigenic taxa: ≥ 100,000 cells/mL	
Illinois	microcystin-LR: > 10 µg/L	Illinois Environmental Protection Agency (2015)
Indiana	blue-green algae: 100,000 cells/mL	Indiana Department of Environmental Management (2015)
	microcystin-LR: 6 µg/L	
	cylindrospermopsin: 5 µg/L	
Iowa	microcystin: ≥ 20 µg/L	Iowa Environmental Council (2015)
Kansas	cyanobacteria: ≥ 80,000 and < 250,000 cells/mL	Kansas Department of Health & Environment (2015)
	microcystin: ≥ 4 and < 20 µg/L	
Kentucky	blue-green algae: > 100,000 cells/mL	Kentucky Department for Environmental Protection (2014)
	microcystins: > 20 µg/L	Commonwealth of Kentucky: Department for Environmental Protection Division of Water (2015)
Maine	Secchi disk reading < 2 meters caused by algae	Maine Department of Environmental Protection (2013)
Maryland	*Microcystis aeruginosa* or other potential microcystin producing blue green algae > 40,000 cells/mL, and samples contain microcystins: > 10 ppb	Maryland Department of Natural Resources (2010)
Massachusetts	blue-green algae: > 50,000 cells/mL	Massachusetts Bureau of Environmental Health (2015)
	microcystins: > 14 µg/L	
Montana	reservoirs that seem stagnated and harbor large quantities of algae	State of Montana Newsroom (2015)
Nebraska	microcystin: ≥ 20 µg/L	Nebraska Department of Environmental Quality and Nebraska Department of Health and Human Services: Division of Public Health (2016)
New Hampshire	cyanobacteria: > 50 percent of total cell counts from toxigenic cyanobacteria	New Hampshire Department of Environmental Services (2014)

State	Lowest Recreational Water Guideline or Action Level[a]	Reference
New York	bloom: credible report or digital imagery of a bloom determined as likely to be potentially toxic cyanobacteria by DEC or DOH staff	Gorney (2016)
	blue green chlorophyll *a*: > 25–30 µg/L	
	potential toxin-producing cyanobacteria taxa: > 50 percent of algae present	
	microcystin-LR: 4 µg/L	
North Carolina	visible discoloration or surface scum	North Carolina Health and Human Services: Division of Public Health (2014)
North Dakota	blue-green algae bloom is present over a significant portion of the lake AND microcystin-LR: ≥ 10 µg/L	North Dakota Department of Health: Division of Water Quality (2016)
Ohio	microcystin-LR: 6 µg/L	Kasich et al. (2015)
	cylindrospermopsin: 5 µg/L	
Oklahoma	cyanobacteria: 100,000 cell/mL	Oklahoma Legislature (2012)
	microcystin: > 20 µg/L	
Oregon	cylindrospermopsin: ≥ 20 µg/L	Oregon Health Authority (2016)
	microcystin: ≥ 10 µg/L	
	Microcystis: > 40,000 cells/mL	
	Planktothrix: > 40,000 cells/mL	
	toxigenic species: > 100,000 cells/mL	
	visible scum with documentation and testing	
Rhode Island	cyanobacteria: > 70,000 cells/mL	Rhode Island Department of Environmental Management and Rhode Island Department of Health (2013)
	microcystin-LR: ≥ 14 µg/L	
	visible cyanobacteria scum or mat	
Texas	> 100,000 cell/mL of cyanobacteria cell counts and > 20µg/L microcystin	U.S. EPA (2016)
Utah	blue-green algae: 20,000–100,000 cells/mL	Utah Department of Environmental Quality and Department of Health (2015)
	microcystin: 4–20 µg/L	

State	Lowest Recreational Water Guideline or Action Level[a]	Reference
Vermont	cylindrospermopsin: ≥ 10 µg/L	Vermont Department of Health (2015)
	microcystin-LR (equivalents): ≥ 6 µg/L	
	visible known blue-green algae bloom/scum or an unknown, potentially blue-green algae (i.e., not pollen), bloom/scum	
Virginia	blue-green algal "scum" or "mats" on water surface	Virginia Department of Health (2012)
	microcystin: > 6 µg/L	
	Microcystis: 5,000 to < 20,000 cells/mL	
Washington	bloom is forming or a bloom scum is visible (toxic algae may be present); cyanotoxin levels do not exceed thresholds	Hardy and Washington State Department of Health (2011)
	microcystins: 6 µg/L	
	cylindrospermopsin: 4.5 µg/L	
West Virginia	blue-green algal blooms observed and monitored	West Virginia Department of Health & Human Resources (2015)
Wisconsin	cyanobacteria: > 100,000 cells/mL	Wisconsin Department of Natural Resources (2012)
	visible scum layer	Werner and Masnado (2014)

[a] More details are provided in Appendix B.

Among states that consider the same parameters, there is considerable variation in guideline levels and associated responses. As shown in Table 2-3, the state recreational guidelines featuring the lowest microcystins or cylindrospermopsin concentrations that recommended or triggered a health protective action ranged from 0.8 to 20 µg/L and 4 to 20 µg/L, respectively. The guidelines reflecting the lowest densities of cyanobacterial cells that triggered a health protective action ranged from 5,000 to 100,000 cells/mL. Some of the variation in guideline levels is attributable to variations in exposure parameters, as well as variations in the basis for guideline values. Ten states base at least one guideline value on either WHO guidance or a modified version of WHO guidance (e.g., Indiana, Oregon, Utah). Eleven states, including California, Massachusetts, and Ohio, base at least one guideline value on jurisdiction-specific risk assessments or monitoring information, or studies or guidelines other than those from WHO. For more information on individual state guidelines, see Appendix B.

3.0 NATURE OF THE STRESSORS

This section discusses cyanobacteria and cyanobacterial blooms that have the potential to produce microcystins and cylindrospermopsin. It also describes these toxins' chemical and physical properties, sources and occurrence information in different media, environmental fate, and toxicokinetics.

3.1 Cyanobacteria and Cyanobacterial Blooms

Cyanobacteria are photosynthetic prokaryotes (Seckbach & Oren 2007) and are ubiquitous in the environment. The chloroplast, found in photosynthetic eukaryotes like algae and plants, evolved from an endosymbiotic relationship with cyanobacteria (Kutschera & Niklas 2005). Ecologists historically grouped cyanobacteria, often referred to as "blue-green algae," with eukaryotic algae because they contain chlorophyll *a* and their ability to perform oxygenic photosynthesis. However, cyanobacteria are prokaryotes (i.e., no discrete membrane-bound nucleus or membrane-bound subcellular organelles) and are genetically related to other bacteria in the eubacteria domain. Taxonomically, they are classified in the phylum Cyanobacteria or Cyanophyceae (Carmichael 2008; O'Neil et al. 2012).

Cyanobacteria can produce bioactive compounds including toxins, which may be harmful. These biomolecules include hepatotoxic, neurotoxic, and cytotoxic compounds and compounds that can result in allergic reactions (Carmichael 1994; Jaiswal et al. 2008; Volk & Mundt 2007). Studies have also shown that exposure to cyanobacterial cells independent of cyanotoxins can cause health effects; this information is detailed in Appendix D.

Members of *Microcystis*, *Dolichospermum* (*Anabaena*), *Nostoc*, *Oscillatoria*, *Fischerella*, *Planktothrix*, and *Gloeotrichia* can produce microcystins (Carey et al. 2012b; Codd et al. 2005; Duy et al. 2000; Stewart et al. 2006c). *Microcystis* sp. have been documented to occur in blooms on all continents except Antarctica and often dominate phytoplankton assemblages in the summer (O'Neil et al. 2012). Along the margins of Antarctica, other genera of cyanobacteria occur in exposed soils, glaciers, ice shelves, frozen lakes, and stream beds, including *Nostoc*, *Oscillatoria*, *Lyngbya*, or *Synechococcus* (Paerl et al. 2016; Vincent 2007). *Microcystis* sp. have been documented throughout the United States (Carmichael 2001; Jacoby et al. 2000).

Several environmental factors, including nutrient load, increased water temperature, salinity, pH, light intensity, and reduced mixing, provide competitive advantages to *Microcystis* relative to other phytoplankton (Jacoby et al. 2000; Marmen et al. 2016). There is evidence that these environmental factors also affect the relative abundance of microcystin-producing strains and non-microcystin-producing strains (Marmen et al. 2016). *Microcystis* thrives in warmer temperatures, with optimal growth and photosynthesis occurring above 25°C (O'Neil et al. 2012). A Japanese study between May and November 2006 found that the toxin-producing species, *M. aeruginosa*, dominated in months with relatively higher water temperatures, while the non-toxin-producing species, *M. wesenbergii*, dominated in months with lower water temperatures (Imai et al. 2009). Elevated nitrogen and phosphorus inputs may both stimulate *Microcystis* cell growth and biomass accumulation, and can favor microcystin-producing strains (Marmen et al. 2016; O'Neil et al. 2012). During the summer of 1994, *M. aeruginosa* was observed as the dominant species in a toxic bloom in Washington, associated with elevated nitrogen inputs resulting in low nitrogen to phosphorus ratios (Jacoby et al. 2000). The genetic

composition of the bloom can also influence the degree of toxicity associated with an algal bloom. Lee et al. (2015) found that, although *Microcystis* sp. was rarely detected in a shallow lake bloom, most of this population contained the toxin-producing gene. They observed intracellular microcystins at concentrations two to three orders of magnitude greater than extracellular microcystins (Lee et al. 2015).

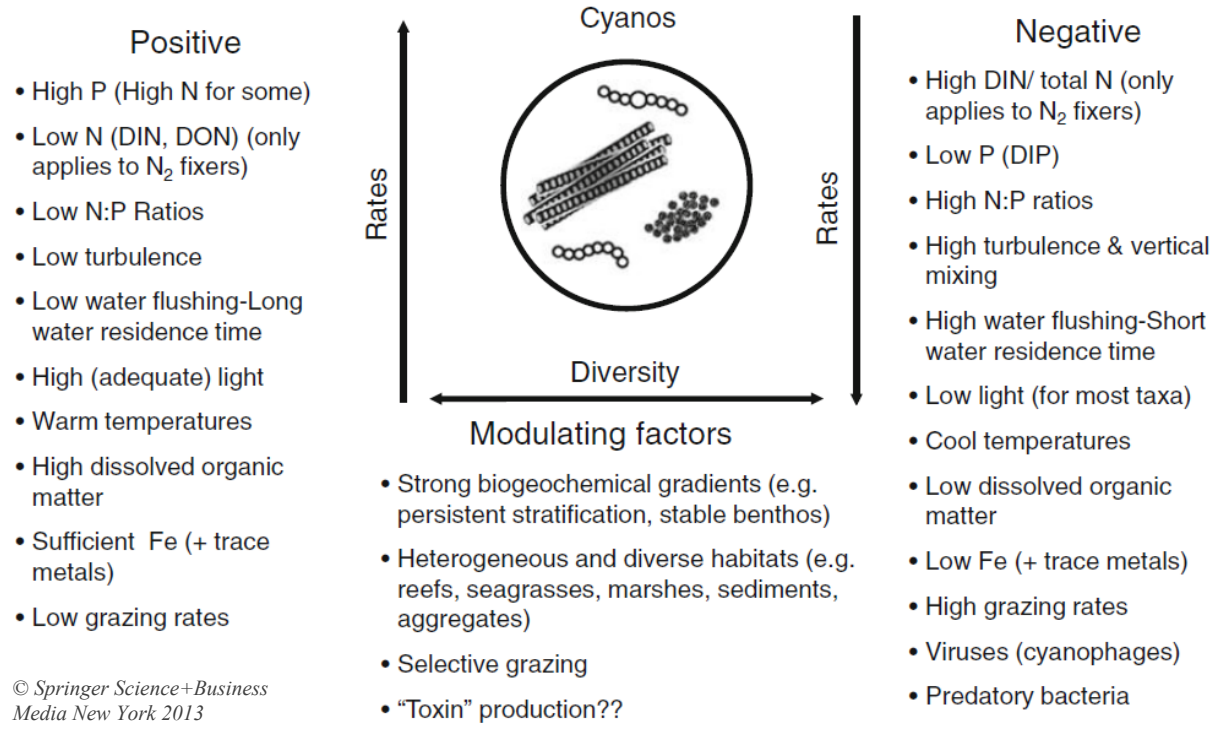
A number of cyanobacterial species including *Cylindrospermopsis raciborskii* (*C. raciborskii*), *Aphanizomenon flos-aquae, Aphanizomenon gracile, Aphanizomenon ovalisporum, Umezakia natans, Anabaena bergii, Anabaena lapponica, Anabaena planctonica, Lyngbya wollei, Rhaphidiopsis curvata,* and *Rhaphidiopsis mediterranea* can produce cylindrospermopsin (B-Béres et al. 2015; Kokocinski et al. 2013; McGregor et al. 2011; Moreira et al. 2013). Cylindrospermopsin-producing cyanobacteria occur in tropical or subtropical regions, but also have been detected in warmer temperate regions. These species do not tend to form visible surface scums and the highest concentrations of cyanobacterial cells occurs below the water surface (Falconer 2005). *C. raciborskii* occurs in freshwater ponds, rivers, reservoirs and eutrophic lakes and has been found in Australia, Asia, Europe, Africa and South, Central and North America (Fuentes et al. 2010). According to a survey conducted in Florida in 1999 from June to November, the most frequently observed toxigenic cyanobacteria were *Microcystis* (43.1 percent), *Cylindrospermopsis* (39.5 percent), and *Anabaena spp.* (28.7 percent) (Burns 2008). In Florida, *C. raciborskii* was found to be the dominant cyanobacteria species in one lake all year round (Burns 2008). In 2006, *C. raciborskii* was detected in lakes in southern Louisiana (Fuentes et al. 2010). Conditions promoting its growth were shallow, warm surface water (over 30°C) and low light intensities. The highest concentrations of *C. raciborskii* were observed from June through August with densities ranging from 37,000 cells/mL to more than 160,000 cells/mL. In a study of two lakes directly connected to Lake Michigan, Hong et al. (2006) found low concentrations only in the late summer, and these were associated with elevated bottom water temperatures and phosphorus concentrations.

Research indicates that cyanotoxins are associated with physiological functions of cyanobacterial cell signaling, nutrient uptake, iron scavenging, maintenance of homeostasis, and protection against oxidative stress and can confer a competitive advantage (Holland & Kinnear 2013). Cylindrospermopsin provides a competitive advantage to cyanobacteria when phosphorus becomes scarce. Bar-Yosef et al. (2010) observed that, when phosphorus is scarce, the cyanobacterium *Aphanizomenon ovalisporum* releases cylindrospermopsin, which causes other microorganisms to release alkaline phosphatase, a compound which will increase available phosphorus. Subsequently, *Aphanizomenon* can gain access to phosphorus made available by other microorganisms while simultaneously conserving the energy and resources required to express and excrete alkaline phosphatase (Bar-Yosef et al. 2010). The precise biological function of microcystin has not been conclusively determined (Zurawell et al. 2005). Studies comparing wild-types and mutants of a microcystin species, examining the genes involved in microcystin biosynthesis, and evaluating *Microcystis* colony size have suggested that microcystins play important physiological roles in cyanobacteria, including colony formation (Kaplan et al. 2012; Zurawell et al. 2005). Although cyanotoxins can negatively affect humans and other animals, research suggests that the primary functions of cyanotoxins are in cyanobacterial physiology and microbial ecology.

A variety of physical, chemical, and environmental factors affect the growth and population dynamics of cyanobacteria, including light intensity, temperature, nutrient

concentrations, biological interactions, and other environmental factors (see summary in Figure 3-1). When the rate of cyanobacterial cell growth exceeds the loss rate for a population, positively buoyant, floating cyanobacterial cells can form a visibly colored scum on the water surface, which can contain more than 10,000 cells/mL (Falconer 1998). The floating scum, as in the case of *Microcystis* species, can be concentrated by prevailing winds in certain surface water areas, especially at the shore. In larger freshwater bodies, such as Lake Erie, these areas of high *Microcystis* concentrations are readily detected by satellite (Stumpf 2014; Wynne et al. 2010). Although these blooms can occur naturally, increasing consensus among scientists is that these blooms have been increasing in recent decades (Carmichael 2008; Hallegraeff 1993; Hudnell 2010).

Figure 3-1. Environmental Factors Influencing Cyanobacterial Bloom Potential in Aquatic Ecosystems, Reproduced from Paerl and Otten (2013b) with Permission of Springer



Nutrients, particularly nutrient over-enrichment, are key environmental drivers that influence the proportion of cyanobacteria in the phytoplankton community, the cyanobacterial biovolume, cyanotoxin production, and the impact that cyanobacteria may have on ecosystem function and water quality (Beaulieu et al. 2013; Paerl et al. 2011). Cyanobacterial toxin concentrations are associated with nutrient levels (Wang et al. 2002); however, different cyanobacteria species use organic and inorganic nutrient forms differently. Loading of nitrogen and/or phosphorus to water bodies from agricultural, industrial, and urban sources influences the development of cyanobacterial blooms and are associated with cyanotoxin production (Paerl et al. 2011). Nitrogen loading can enhance the growth and cyanotoxin levels of *Microcystis* sp. blooms and microcystin synthetase gene expression (Gobler et al. 2007; O'Neil et al. 2012). Gobler et al. (2007) suggested that dominance of toxic *Microcystis* sp. blooms during summer is linked to nitrogen loading, which stimulates growth and cyanotoxin synthesis. This may cause

the inhibition of grazing by mesozooplankton and further accumulation of cyanobacterial cells. Optimal concentrations of total and dissolved phosphorus (Wang et al. 2002) and soluble phosphates and nitrates (ILS 2000; O'Neil et al. 2012; Paerl & Scott 2010; Wang et al. 2010) may also result in the increased production of microcystins. Smith (1983) was the first to describe a strong relationship between the relative amounts of nitrogen and phosphorus in surface waters and toxic cyanobacterial blooms. Smith proposed that cyanobacteria should be superior competitors under conditions of nitrogen-limitation because of their unique capacity for nitrogen fixation, although many cyanobacteria like *Microcystis* species that produce toxins do not fix nitrogen. While the dominance of nitrogen-fixing cyanobacteria at low nitrogen to phosphorus ratios has been demonstrated in mesocosm- and ecosystem-scale experiments in prairie and boreal lakes (Schindler et al. 2008), the hypothesis that low nitrogen to phosphorus 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 nitrogen inputs, especially if these nutrients are available from internal sources. As the trophic state increases, aquatic systems absorb higher concentrations of nitrogen (Paerl & Huisman 2008; Paerl & 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 nitrogen and phosphorus additions. Further, this evidence suggests that nutrient co-limitation is widespread (Elser et al. 2007; Lewis et al. 2011; Paerl et al. 2011). These results strongly suggest that reductions in nutrient pollution are needed to stem eutrophication and cyanobacterial bloom expansion. For example, analysis of observational data collected at larger spatial scales support the idea that controlling total phosphorus and total nitrogen could reduce the frequency of high microcystin events by reducing the biomass of cyanobacteria in the system (Orihel et al. 2012; Scott et al. 2013; Yuan et al. 2014).

The increasing body of laboratory and field data (Carey et al. 2012a; De Senerpont Domis et al. 2007; Huisman et al. 2005; Jeppesen et al. 2009; Kosten et al. 2012; Reynolds 2006; Wagner & Adrian 2009; Weyhenmeyer 2001) suggest that an increase in temperature may influence cyanobacterial dominance in phytoplankton communities. 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 2003a). The increase in water column stability associated with higher temperatures also may favor cyanobacteria (Carey et al. 2012a; Wagner & Adrian 2009). Kosten et al. (2012) 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 non-toxic ecotypes (Dziallas & Grossart 2011; Paerl & Otten 2013b). Indirectly, warming also may increase nutrient concentrations by enhancing mineralization (Gudasz et al. 2010; Kosten et al. 2009; Kosten et al. 2010) by temperature- or anoxia-mediated sediment phosphorus release (Jensen & Andersen 1992; Søndergaard et al. 2003). Thus, temperature may indirectly increase cyanobacterial 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 cyanobacterial blooms (Jeppesen et al. 2009; Jeppesen et al. 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 (Carey et al. 2012a; Kosten et al. 2012). Rising global temperatures and changing precipitation patterns may stimulate cyanobacterial 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 (Reynolds 2006; Robarts & Zohary 1987). 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 the 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 & 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 (Carey et al. 2012a; Wagner & Adrian 2009).

Sunlight availability and turbidity can have a strong influence on the cyanobacteria species that predominate, as well as the depth at which they occur (Carey et al. 2012a; Falconer 2005). For example, *Microcystis aeruginosa* occurs mostly at the surface with higher light intensities and in shallow lakes. Kosten et al. (2012) 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. (2012) 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 (Scheffer et al. 1997; Smith 1986). Overall, results from Kosten et al. (2012) 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.

Cyanobacterial blooms have been shown to intensify and persist at pH levels between six and nine (WHO 2003a). When these blooms are massive or persist for a prolonged period, they can become harmful. Kosten et al. (2012) 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 (Caraco & Miller 1998; Shapiro 1984). 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 cyanobacterial cell density and chlorophyll content in *Microcystis aeruginosa* (Kosakowska et al. 2007).

Cyanobacterial blooms commonly occur from spring to early fall in various regions of the United States (Wynne & Stumpf 2015). Cyanobacteria take advantage of conditions that can occur in late summer and early fall such as elevated water temperatures and increased vertical stratification in lakes and reservoirs (Paerl & Huisman 2008). Some blooms occur later in summer and early fall. Vertical 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 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 cyanobacterial 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 to occurrence in lakes and reservoirs, cyanobacteria and cyanotoxins have been detected in flowing rivers and streams (Chaney 2016; Commonwealth of Kentucky: Energy and Environment Cabinet 2015; Florida Department of Environmental Protection 2016; Loftin et al. 2016a; Otten et al. 2015; Paerl & Otten 2013b; Parker 2016). In some cases, the source of the cyanobacteria can be traced to an upstream water body such as a lake or reservoir. In 2016, a bloom in Lake Okeechobee impacted the St. Lucie River and estuary and the Caloosahatchee River and estuary in Florida (Florida Department of Environmental Protection 2016). Otten et al. (2015) used microbial source tracking techniques to trace the source of a toxic *Microcystis* bloom in the Klamath River in Oregon to a single upstream reservoir. Their results showed that large quantities of cyanobacterial cells can withstand passage through hydroelectric installations and transport over 300 kilometers. Cyanobacterial bloom development has been documented near dams and man-made reservoirs (Chaney 2016; Giannuzzi et al. 2011; Otten et al. 2015; Sierosławska et al. 2010). Environmental characteristics including nutrients and flow rate can affect phytoplankton dynamics (Paerl & Otten 2013b). Zhang et al. (2015) observed that low flow conditions favored cyanobacteria and higher flow conditions favored green algae.

Cyanobacterial blooms can also occur in rivers and streams without a known lake or reservoir source in the water column or as part of the benthic community (Commonwealth of Kentucky: Energy and Environment Cabinet 2015; Loftin et al. 2016a). Loftin et al. (2016a) suggest that low stream flow, shallow depth, and high water column light penetration in Piedmont streams favored periphyton occurrence (mixture of algae, cyanobacteria, heterotrophic bacteria, and detritus). A review by Quiblier et al. (2013) of benthic freshwater cyanobacterial

ecology found that nutrients, flow regime, wave action, climate, and geology can influence benthic cyanobacterial community composition and suggest that due to a high desiccation tolerance, cyanobacteria occurrence in benthic mat communities is also a concern in ephemeral streams.

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 and zooplankton grazers like *Daphnia* (large generalist grazers), copepods, and cladocerans (Ger et al. 2014). Each of these microbial-related factors can cause fluctuations in bloom development and composition.

In summary, there is a complex interplay of environmental factors that dictates the spatial and temporal changes in the concentration of cyanobacterial cells and their toxins with respect to the dominant species as illustrated in Figure 3-1 (Paerl & Otten 2013b). Factors such as the amount and timing of nutrient supply (i.e., nutrient concentration and nutrient loading), the relative proportions of nutrients (i.e., nitrogen to phosphorus ratio), dissolved organic matter availability, temperature, and light attenuation, as well as other physico-chemical processes, can play a role in shaping cyanobacterial bloom composition and cyanotoxin production (Paerl & Huisman 2008; Paerl & Otten 2013b). Phytoplankton competition and food web interactions that occur as blooms develop, persist, and decline 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 & Huisman 2008; Paerl & Otten 2013b; Paerl et al. 2011).

3.2 Cyanotoxins

Much of the information and the studies summarized in this section for microcystin and cylindrospermopsin are described in detail in EPA's *Health Effects Support Document for the Cyanobacterial Toxin Microcystins* and *Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin* (HESDs), and EPA's *Drinking Water Health Advisory for the Cyanobacterial Microcystin Toxins* and *Drinking Water Health Advisory for the Cyanobacterial Toxin Cylindrospermopsin* (Drinking Water Health Advisories) (U.S. EPA 2015a; U.S. EPA 2015b; U.S. EPA 2015c; U.S. EPA 2015d).

3.2.1 Chemical and Physical Properties

Summary information for chemical and physical properties is provided in this section. Additional information can be found in the EPA's HESDs for microcystins and cylindrospermopsin (U.S. EPA 2015c; U.S. EPA 2015d).

Structurally, 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 range from approximately 800 to 1,100 g/mole. The cyclic peptides include more than 100 congeners of microcystins (Niedermeyer 2014). Figure 3-2 provides the structure of microcystin where X and Y represent variable amino acids. 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 microcystins are named based on their two variable amino acids (Carmichael et al. 1988). For example, microcystin-LR, the most common congener, contains leucine (L) and arginine (R) (Carmichael 1992). 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. For example, microcystin-LR is for the microcystin with leucine in the X position of Figure 3-2 and arginine in the Y position in Figure 3-2. Table 3-1 lists the most common microcystins congeners.

Figure 3-2. Structure of Microcystin (Kondo et al. 1992)

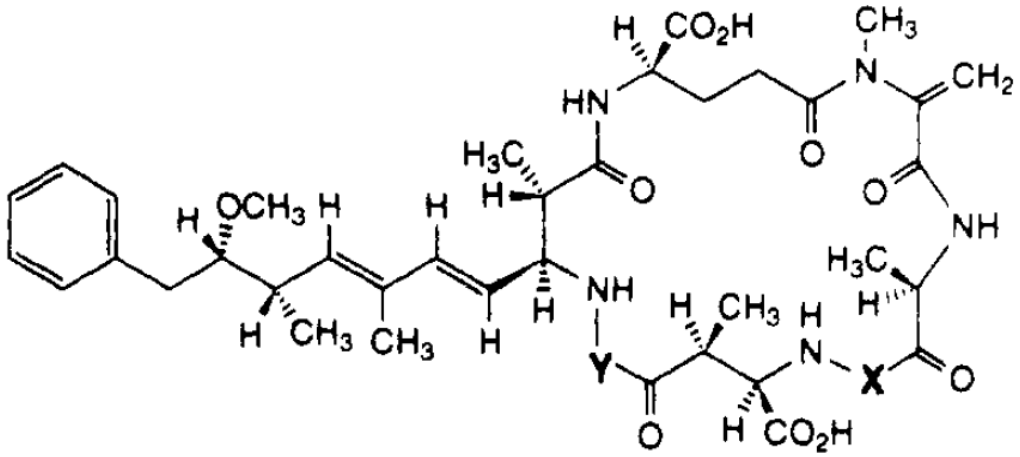


Table 3-1. Abbreviations for Microcystins (Yuan et al. 1999)

Microcystin Congeners	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

The preponderance of toxicological data on the effects of microcystins is restricted to the microcystin-LR congener. Toxicity data suggest that microcystin-LR is as potent as or more potent than other studied microcystins and that the most toxic microcystins are those with the more hydrophobic L-amino acids (-LA, -LR, -YR, and -YM); the least toxic are those with hydrophilic amino acids, such as microcystin-RR. Data on the -RR, -YR, and -LA congeners, however, are limited, and toxicity values cannot be derived for them. Values developed from data specific to microcystin-LR are considered applicable to and appropriate for individual and mixtures of microcystin congeners.

Table 3-2 provides chemical and physical properties of microcystin-LR. 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 cyanobacterial cell lysis.

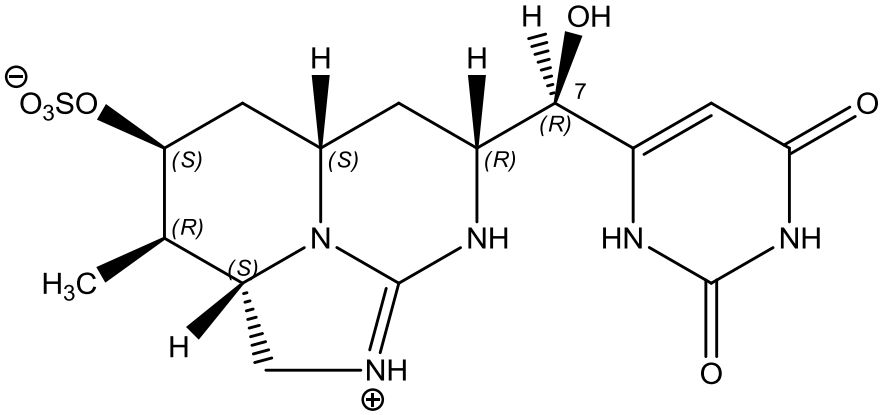
Table 3-2. Chemical and Physical Properites of Microcystin-LR

Property	Microcystin-LR
Chemical Abstracts Registry (CAS) Number	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
Log K_{ow}	2.16
K_{oc}	N/A
Solubility in Water	Highly
Other Solvents	Ethanol and methanol

Sources: Chemical Book (2012); TOXLINE (2012); Ward and Codd (1999) for log K_{ow}.

Cylindrospermopsin is a tricyclic alkaloid with the following molecular formula $C_{15}H_{21}N_5O_7S$ (Ohtani et al. 1992) and a molecular weight of 415.43 g/mole. It is zwitterionic (i.e., a dipolar ion with localized positive and negative charges) (Ohtani et al. 1992). The chemical structure of cylindrospermopsin is presented in Figure 3-3. Additional congeners and analogs have been identified; see U.S. EPA (2015b; 2015c) for more information.

Figure 3-3. Structure of Cylindrospermopsin (de la Cruz et al. 2013)



The physical and chemical properties of cylindrospermopsin are presented in Table 3-3. Cylindrospermopsin is highly soluble in water (Chiswell et al. 1999; Moore et al. 1998). Cylindrospermopsin is isolated for commercial use mostly from *C. raciborskii*. Many of the physicochemical properties of cylindrospermopsin in the environment such as vapor pressure and boiling and melting points are unknown.

Table 3-3. Chemical and Physical Properties of Cylindrospermopsin

Property	Cylindrospermopsin
Chemical Abstracts Service (CAS) Registry Number	143545-90-8
Chemical Formula	$C_{15}H_{21}N_5O_7S$
Molecular Weight	415.43 g/mole
Color/Physical State	white powder
Boiling Point	N/A
Melting Point	N/A
Density	2.03 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	Dimethyl sulfoxide (DMSO) and methanol

Sources: Chemical Book (2012); TOXLINE (2012).

3.2.2 Sources and Occurrence

Cyanobacterial density in a bloom and cyanotoxin concentration are not always closely related. Cyanotoxin concentrations depend on the dominance and diversity of species and strains within the bloom along with environmental and ecosystem influences on bloom dynamics (Chorus et al. 2000; Hitzfeld et al. 2000; WHO 1999). Cyanotoxin production by cyanobacteria is highly variable and strongly influenced by the environmental conditions. It can vary among strains and clones of a single species (Carmichael 1994; Utkilen & Gjølme 1992) and within and between blooms (Codd & Bell 1985). Growth phase also can influence cyanotoxin production (Jaiswal et al. 2008). Although studies of the impact of environmental factors on cyanobacteria bloom are ongoing, a variety of factors can influence cyanotoxin production, including nutrient (nitrogen, phosphorus) concentrations, light levels, temperature, pH, oxidative stressors, and interactions with other biota (viruses, bacteria, and animal grazers), and the combined effects of these factors (Paerl & Otten 2013a; Paerl & Otten 2013b). Factors discussed previously that influence cyanobacterial growth can also influence cyanotoxin production, however, growth and toxin production do not necessarily coincide. Recent research by Francy et al. (2016) on modeling the relationship of environmental variables and cyanotoxin levels has shown that

certain environmental factors may be useful to estimating microcystin concentrations above a threshold level.

The proportion of intracellular versus extracellular cyanotoxin can also vary. Extracellular microcystins (either dissolved in water or bound to other materials) typically make up less than 30 percent of the total microcystin concentration in source water (Graham et al. 2010). Most of the microcystins are intracellular and released into the water when the cyanobacterial cells rupture or die. Cylindrospermopsin may be retained within the cyanobacterial cell or released. The ratio of intracellular to extracellular cyanotoxin can change depending on the growth phase with as much as 50 percent of cylindrospermopsin produced by *C. raciborskii* released extracellularly (Griffiths & Saker 2003).

3.2.2.1 Surface Water

Microcystins

Microcystins are the most common cyanotoxins found worldwide and have been reported in surface waters in most of the states in the United States (Funari & 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 25,000 μg/L. The concentration of extracellular microcystins ranged from 0.02 to a high of 1,800 μg/L reported following treatment of a large cyanobacteria bloom with algaecide (WHO 1999), and 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 detected in most states, and over the years, many studies have been done to determine their occurrence in surface water. The remainder of this section provides examples of microcystin occurrence observations throughout the United States.

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 percent), *Cylindrospermopsis* (39.5 percent), and *Anabaena* spp. (28.7 percent) (Burns 2008). Of 167 surface water samples taken from 75 waterbodies, 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 project evaluated the occurrence and distribution of cyanotoxins in the lower Great Lakes region (Boyer 2007). Analysis for total microcystins was performed using Protein Phosphatase Inhibition Assay. Microcystins were detected in at least 65 percent of the samples, mostly in Lake Erie, Lake Ontario, and Lake Champlain. The National Oceanic and Atmospheric Administration Center of Excellence for Great Lakes and Human Health continues to monitor the Great Lakes and regularly samples cyanobacterial 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 protein phosphatase inhibition assay (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. This study reported higher levels of microcystin-LR (1.6 to 10.7 μg/L) in smaller lakes in the Lake Ontario watershed.

In 2006, USGS conducted a study of 23 lakes in the Midwestern United States in which cyanobacterial blooms were sampled to determine the co-occurrence of cyanotoxins in cyanobacterial blooms (Graham et al. 2010). This study reported that microcystins were detected in 91 percent of the lakes sampled with 17 percent of microcystin-positive samples exceeding 20 µg/L. Mixtures of all the microcystin congeners measured (-LA, -LF, -LR, -LW, -LY, -RR, and -YR) were common, and all the congeners were present in association with the blooms. Microcystin-LR and -RR were the dominant congeners detected with mean concentrations of 104 and 910 µg/L respectively.

In 2007, the NLA conducted the first national probability-based survey of the condition of the nations' lakes, ponds, and reservoirs (U.S. EPA 2009). This baseline study provided estimates of the condition of natural and man-made freshwater lakes, ponds, and reservoirs greater than 4 hectares (10 acres) and at least one meter deep. 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, which were indicators of the presence of cyanotoxins. Samples were collected in open water at mid-lake. Due to the design of the survey, no samples were taken nearshore or in other areas where scums were present. These surveys covered a total of 1,028 lakes, which represented nearly 50,000 lakes in the conterminous United States. This assessment found that cyanobacteria were detected in almost all lakes (U.S. EPA 2009). Cyanobacteria were the dominant member of the phytoplankton community in 76 percent of lake samples. Subsequent analysis indicated that potential microcystin-producing species occurred in 95 percent of samples (Loftin et al. 2016b).

Microcystins are the most commonly detected class of cyanotoxin and have been found in lakes in the contiguous United States (U.S. EPA 2009) and streams in the Southeastern United States (Loftin et al. 2016b). Microcystins were present in 30 percent of the lakes sampled nationally by the NLA, with sample concentrations that ranged from the limit of detection (0.1 µg/L) to 225 µg/L (U.S. EPA 2009). Microcystins were detected in in 32 percent of lake water samples with a mean concentration of 3.0 µg/L (based on detections only) and microcystin concentrations above the WHO thresholds of concern of 10 and 20 µg/L were present in 1.1 percent of samples nationally (Loftin et al. 2016b). States with lakes reporting microcystins levels above > 10 µg/L are shown in Table 3-4. NLA data show two states (North Dakota and Nebraska) had 9 percent of samples above 10 µg/L. Other states including Iowa, Texas, South Dakota, and Utah also had samples that exceeded 10 µg/L. Several NLA samples in North Dakota, Nebraska, and Ohio exceeded 20 µg/L (192 and 225 µg/L respectively). EPA completed a second survey of lakes in 2012, however, those data have not yet been released.

USGS 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 microcystin in 16 percent of samples and at concentrations less than or equal to 0.2 µg/L (Beussink & Graham 2011). In 2011, USGS conducted a study on the upstream reservoirs of the Kansas River to characterize the transport of cyanobacteria and associated compounds (Graham et al. 2012). Concentrations of total microcystin were low in the majority of the tributaries with the exception of Milford Lake, which had higher total microcystin concentrations, some exceeding the Kansas

Table 3-4. States Surveyed as Part of the 2007 National Lakes Assessment with Water Body Microcystins Concentrations above 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 percent	192 µg/L
Nebraska	42	9.1 percent	225 µg/L
South Dakota	40	4.9 percent	33 µg/L
Ohio	21	4.5 percent	78 µg/L*
Iowa	20	4.5 percent	38 µg/L*
Utah	26	3.6 percent	15 µg/L*
Texas	51	1.8 percent	28 µg/L*

*Single Sample

recreational guideline level of 20 µg/L. Upstream from Milford Lake, a cyanobacterial bloom was observed with a total microcystin concentration of 150,000 µg/L. When sampled a week later, total microcystin concentrations were less than 1 µg/L. The study authors indicated that this may be due to dispersion of microcystins through the water column or to other areas, or by degradation of microcystins via abiotic and biological processes. Samples taken during the same time from outflow waters contained total microcystin concentrations 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; Hamel 2012). Microcystin levels ranged from the detection limit (0.05 µg/L) to 4,620 µg/L in 2008, to 18,700 µg/L in 2009, to 853 µg/L in 2010, and to 26,400 µg/L in 2011.

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 & 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).

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

In 2008, NOAA began monitoring for cyanobacterial blooms in Lake Erie using high temporal resolution satellite imagery. Between 2008 and 2010, *Microcystis* cyanobacterial blooms were associated with water temperatures above 18°C (Wynne et al. 2013). Using the

Great Lakes Coastal Forecast System, 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 collected samples at 6 to 8 stations each week for 24 weeks, measuring cyanotoxin concentrations as well as chlorophyll biomass and an additional 18 parameters (e.g., nutrients) to improve future forecasts of these blooms. Microcystins can be separated into particulate (cell-bound) and dissolved (extracellular) phases, which can be measured by testing concentrations in the filter and filtrate fractions of the sampled water (Graham & Jones 2007; Zastepa et al. 2014). In 2014, particulate microcystin concentrations ranged from below detection to 36.7 μg/L. Samples taken in 2015 and 2016 showed particulate microcystin concentration ranges from below detection to 9.19 μg/L and from below detection to 21.26 μg/L, respectively. Particulate microcystin concentrations peaked in August 2014 at all sites, with the Maumee Bay site yielding the highest concentration of the entire three-year sampling period. Dissolved microcystin concentrations were also collected at each site in 2014 from September until the end of the sampling period in November, as well as during the field sampling seasons in 2015 and 2016. During the final months of sampling in 2014 (October to November), dissolved microcystin concentrations were detected with peak concentrations of 0.8 μg/L (mean: 0.28 +/- 0.2 μg/L) whereas particulate microcystin concentrations were below detection limits on many dates, indicating that a majority of the microcystin (mean: 72 percent +/- 37 percent) were in the dissolved form, as the bloom declined in intensity. Measured dissolved microcystin concentrations in the following two years ranged from levels below detection to peaks of 0.69 μg/L in September 2015 and 1.76 μg/L in July 2016 (NOAA 2014). Note that the health-protective value for microcystins recommended in this document should be compared with the total microcystins detected and not delineated between intracellular or extracellular microcystin. Cells containing microcystin can be swallowed while recreating and contribute to the overall exposure to the toxin.

Two notable cyanobacterial blooms occurred in Florida and Utah in 2016, resulting in microcystin detections. From July 14 to September 14, an extensive cyanobacterial bloom covering 100 square miles occurred in Utah Lake, Jordan River, and nearby canals and included the cyanobacterial genera *Geitlerinema, Oscillatoria*, and *Pseudanabaena* (Utah Department of Environmental Quality 2016). Microcystin concentrations ranged from < 0.5–176 μg/L. The Utah Department of Environmental Quality reported over 500 human exposures with 30 percent of these cases reporting symptoms such as gastrointestinal distress, headache, and eye and skin irritation. In addition, 27 animal exposures were reported (Utah Department of Environmental Quality 2016).

In 2016, a 239-square mile cyanobacterial bloom in Lake Okeechobee, Florida, and downstream waterways resulted in a state of emergency in four counties on the Gulf and Atlantic coasts of Florida (Chaney 2016; Parker 2016). From May 4 to August 4, the Florida Department of Environmental Protection took approximately 200 water samples from the St. Lucie River and estuary, Caloosahatchee River and estuary, Lake Okeechobee, Indian River Lagoon, and other nearshore marine locations (Florida Department of Environmental Protection 2016). Microcystin concentrations ranged from below the detection limit to 414.3 μg/L. Among the species identified were *Microcystis aeruginosa*, *Scrippsiella trochoidea*, *Planktolyngbya limnetica*, *Dolichospermum circinalis*, and *Plectonema wollei* (Florida Department of Environmental Protection 2016). Lake Okeechobee, located north of the Everglades, is the largest freshwater lake in Florida. It is subject to agricultural runoff from adjacent cattle farms and sugar cane

fields, which contributed to the formation of this massive cyanobacterial bloom (Parker 2016). Water may be pumped out of the lake to the coast through the St. Lucie River and the Caloosahatchee River to prevent the lake level from rising too high after periods of heavy rain, (Parker 2016). As a result of the microcystin levels and visible cyanobacterial scum from water discharged from the lake that flowed downstream to coastal areas, beaches along the Atlantic were closed, and a state of emergency was declared in the counties of Martin, St. Lucie, Palm Beach, and Lee (Chaney 2016; Florida Department of Environmental Protection 2016).

Cylindrospermopsin

As noted above, EPA's NLA conducted the first national probability-based survey of lakes (U.S. EPA 2009) and published results in 2007. USGS subsequently analyzed the stored samples collected and detected cylindrospermopsin in 4 percent of samples, with a mean concentration 0.56 µg/L and a range from the limit of detection, 0.01 µg/L, to a maximum of 4.4 µg/L (Loftin et al. 2016b). Potential cylindrospermopsin-producing species occurred in 67 percent of samples (Loftin et al. 2016b). In general, fewer surface water occurrence data are available for cylindrospermopsin compared to microcystin. This is likely because during blooms, testing for microcystin is much more common than testing for cylindrospermopsin.

USGS also detected cylindrospermopsin in 9 percent of blooms sampled during a 2006 USGS survey of 23 lakes in the Midwestern United States (Graham et al. 2010). The low concentrations of cylindrospermopsin detected (0.12 to 0.14 µg/L) in the study occurred in bloom communities dominated by *Aphanizomenon* or *Anabaena* and *Microcystis*.

Cylindrospermopsin has been detected in lakes throughout multiple states. In a 1999 study, cylindrospermopsin was detected in 40 percent of 167 water samples taken from 87 water bodies in Florida during the months of June and November (Burns 2008). However, the actual cylindrospermopsin concentrations were not reported. In 2005, the U.S. Army Corps of Engineers detected cylindrospermopsin at a maximum concentration of 1.6 µg/L in lake water samples from Oklahoma (Lynch & Clyde 2009). In Grand Lake in St. Marys, Ohio, cylindrospermopsin concentrations as high as 9 µg/L were reported in 2010 (Ohio EPA 2012).

3.2.2.2 Ambient Air

According to Wood and Dietrich (2011), waterborne cyanotoxins can be aerosolized through a bubble-bursting process, in which the cyanobacteria and cyanotoxins are ejected and carried into the air by the resulting droplets from the bubble bursting. Microcystin that is free or bound to particles can be deposited into the deepest bronchiolar or alveolar cavities; the cyanobacterial cells can be likely deposited in the upper respiratory tract (Wood & Dietrich 2011).

Four studies provide air concentration data indicating that recreational surface waters with cyanotoxin-producing cyanobacterial blooms can result in aerosolized cyanotoxins. Backer et al. (2008) used personal air samplers in a 3-day study of recreational activities in a lake with a cyanobacterial bloom, either carried by the study participant or placed on the participant's boat. The microcystin concentrations in air ranged from below the limit of detection (0.0037 ng/m^3) to 0.456 ng/m^3. Backer et al. (2010) also detected microcystins in ambient air for one day, at one lake, and only from the shoreline sampler. The average air concentration was 0.052 ng/m^3. They also collected 44 personal air samples, which ranged from the limit of detection (0.1 ng/m^3) to

0.4 ng/m^3. The authors noted that the daily mean concentrations of microcystin in personal air samples did not correlate with the concentrations of *Microcystis* cells, dissolved microcystin, or total microcystin in the sampled lake water.

Wood and Dietrich (2011) studied Lake Rotorua (New Zealand) when it was experiencing a dense bloom of microcystin-producing *Microcystis* species. They measured a maximum microcystin concentration in the water of (2,140 µg/L) and air concentrations from 0.0003 to 0.0018 ng/m^3.

Cheng et al. (2007) used high volume and personal air samplers to measure microcystins in the air in at a lake with a cyanobacterial bloom. They measured low concentrations of microcystin in the water (approximately 1 µg/L) and air concentrations ranging from below the detection limit (0.02 ng/m^3) to 0.08 ng/m^3.

3.2.2.3 Other Sources of Microcystins and Cylindrospermopsin

Extracts from *Arthrospira* (*Spirulina* spp.) and *Aphanizomenon flos-aquae* have been used as dietary blue-green algae supplements (BGAS) (Funari & 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). A study suggested that BGAS can be contaminated with microcystins ranging from 1 µg/g up to 35 µg/g (Dietrich & Hoeger 2005). Heussner et al. (2012) analyzed 18 commercially available BGAS for the presence of cyanotoxins. All products containing *Aphanizomenon flos-aquae* tested positive for microcystins at levels ≤ 1 µg microcystin-LR equivalents/g dry weight. Cylindrospermopsin was not found in any of the supplements.

3.2.3 Environmental Fate

Different physical and chemical processes are involved in the persistence, breakdown, and movement of microcystins and cylindrospermopsin in aquatic systems as described below.

3.2.3.1 Mobility

Cyanotoxins can move within water systems or they can be transported between systems. Mechanisms concentrating cyanobacterial cells can also act to concentrate their cyanotoxins, leading to negative human health impacts including impacts on surface waters and direct contact and aerosol exposure (Bláha et al. 2009; Carmichael 2001; Cheung et al. 2013; Codd et al. 2005).

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 (Falconer 1998; Han et al. 2012). Ground water is generally not expected to be at risk of cyanotoxin contamination, however, ground water under the direct influence of surface water can be vulnerable. A study conducted by the USGS and the University of Central Florida determined that microcystin and cylindrospermopsin did not sorb in sandy aquifers and were transported along with ground water (O'Reilly et al. 2011). The authors suggested that the removal of microcystin was due to biodegradation.

In sediments, cylindrospermopsin exhibits some adsorption to organic carbon, with little adsorption observed on sandy and silt sediments (Klitzke et al. 2011). The low adsorption of

cylindrospermopsin reduces its residence time in sediments, thus reducing the opportunity for microbial degradation.

3.2.3.2 Persistence

Microcystins

Microcystins are relatively stable and resistant to chemical hydrolysis or oxidation at or near neutral pH. Elevated or low pH or temperatures above 30°C may cause slow hydrolysis. Microcystins have been observed to persist for 21 days to 2–3 months in solution and up to 6 months in dry scum (Funari & Testai 2008; Rapala et al. 2006). Environmental conditions such as temperature, pH, presence of light, salinity, and presence of certain aquatic bacteria, can influence the rate of microcystin degradation (Schmidt et al. 2014). Microcystins can persist even after a cyanobacterial bloom is no longer visible (Lahti et al. 1997b; Zastepa et al. 2014). In a study by Zastepa (2014), dissolved microcystin-LA was present at a concentration of 20 μg/L or greater for 9.5 weeks even though the *Microcystis* bloom was not visible after 5 weeks.

In the presence of full sunlight, microcystins undergo photochemical breakdown, but this varies by microcystin congener (Chorus et al. 2000; WHO 1999). Zastepa et al. (2014) suggest that microcystin-LA degrades at a slower rate than microcystin-LR, -RR, and -YR congeners. The presence of water-soluble cyanobacterial cell pigments, in particular phycobiliproteins, enhances this breakdown. Breakdown can occur in as few as 2 weeks to longer than 6 weeks, depending on the concentration of pigment and the intensity of the light (Tsuji et al. 1994; Tsuji et al. 1995). Several other factors, including photosensitizer concentration, pH, wavelength of light (Schmidt et al. 2014), and whether microcystins are dissolved or present in particulate matter (Lahti et al. 1997b) can affect the rate of transformation or photodegradation. According to Tsuji et al. (1994) and Tsuji et al. (1995), microcystin-LR was photodegraded with a half-life of about 5 days in the presence of 5 mg/L of extractable cyanobacterial pigment. Humic substances can also act as photosensitizers and can increase the rate of microcystin breakdown in sunlight. Others have found that high concentrations of humic acids can slow the rate of microcystin transformation by sunlight (Schmidt et al. 2014). In deeper or turbid water, the breakdown rate is slower. Welker and Steinberg (2000) estimated the maximum rate of microcystin-LR degradation in the presence of humic substance photosensitizers. Extrapolating results from their small experimental tubes to a water column of 1 meter, Schmidt et al. (2014) estimated the half-life of microcystin-LR to be 90 to 120 days per meter of water depth in surface waters. The researchers also demonstrated that the wavelength of light can also affect degradation rates; complete microcystin degradation has been observed within 1 hour when exposed to 254-nm light and within 5 days using 365-nm light. According to Lahti et al. (1997b), microcystin-LR follows first-order decay kinetics, with a decimal reduction time of 30 days for dissolved microcystins compared with 15 days for microcystins found in particulate matter. Zastepa et al. (2014) also found that dissolved microcystin-LA persists longer than microcystin-LA in particulates, with *in situ* half-lives of 15.8 days and 6.5 days, respectively.

Microcystins are susceptible to degradation by aquatic bacteria found naturally in surface waters (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 microcystin-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

(Cousins et al. 1996; Jones et al. 1994; Lahti et al. 1997b), and lake sediment (Lahti et al. 1997a; Rapala et al. 1994; U.S. EPA 2015a). Lam et al. (1995) reported that the biotransformation of microcystin-LR followed a first-order decay with a half-life of 0.2 to 3.6 days. In a study done by Jones et al. (1994) with microcystin-LR in different natural surface waters, microcystin-LR persisted for 3 days to 3 weeks; however, more than 95 percent loss occurred within 3 to 4 days. A study by Christoffersen et al. (2002) measured half-lives in the laboratory and in the field of approximately 1 day, driven largely by bacterial aerobic metabolism. These researchers found that approximately 90 percent 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 half-lives of 4 to 14 days, with longer half-lives associated with a flowing stream and shorter half-lives associated with lakes. Microcystin-LR degradation by *Sphingopyxis* species has been observed with an optimal degradation rate at a pH between 6.5 and 8.5 (Schmidt et al. 2014). Several studies have demonstrated bacterial degradation of microcystin-LR, but other congeners, such as microcystin-LF or -LA, are not significantly degraded (Zastepa 2014; Zastepa et al. 2014). Although microcystin-degrading bacteria might be present, initial degradation could be slow as the bacteria need time to become active (Hyenstrand et al. 2003), and microcystins can accumulate in the water column if these bacteria are not present at the time of a toxic bloom (Schmidt et al. 2014).

Where rivers discharge to the ocean, freshwater cyanobacteria, cyanotoxins, or both can enter the marine environment and this may impact aquatic life in marine environments (Andersen et al. 1993; Miller et al. 2010). Miller et al. (2010) confirmed the transfer of freshwater microcystins to the marine environment. The researchers found that after introducing *Microcystis* cyanobacteria to a saline environment, cyanobacteria can survive for 48 hours before lysing and releasing microcystins. Microcystins concentrations decreased to 29 to 56 percent of the initial concentration after 1 hour in the saline environment, but continued to be detected in the seawater for at least 21 days, based on a detection limit of 0.02 µg/L (Miller et al. 2010). Gibble and Kudela (2014) made additional observations of microcystins at the interface of freshwater and seawater, in the Monterey Bay area, California. In the first year of a 3-year study, microcystin was detected in 15 of 21 fresh-water, estuarine, and marine locations. In the two subsequent years, monitoring focused on four major watersheds that feed into Monterey Bay. The authors observed high concentrations of microcystin in both autumn and spring seasons and concluded that microcystins are likely present throughout the year and transfer to the coastal environment, with the potential to be a persistent issue in the Monterey Bay area. The authors also correlated anthropogenic nutrient loadings with microcystin.

Cylindrospermopsin

Cylindrospermopsin is relatively stable in the dark and at temperatures from 4°C to 50°C for up to 5 weeks (ILS 2000). Cylindrospermopsin is also resistant to changes in pH and remains stable for up to 8 weeks at pH 4, 7, and 10. In the absence of cyanobacterial cell pigments, cylindrospermopsin tends to be relatively stable in sunlight, with a half-life of 11 to 15 days in surface waters (Funari & Testai 2008).

Like microcystin, degradation of cylindrospermopsin increases in the presence of cell pigments such as chlorophyll *a* and phycocyanin, a blue photosynthetic pigment found in cyanobacteria. When exposed to both sunlight and cell pigments, cylindrospermopsin breaks down rapidly, more than 90 percent within 2 to 3 days (Chiswell et al. 1999).

Cylindrospermopsin has been shown to be decomposed by bacteria in laboratory studies; the biodegradation is influenced by the cyanotoxin concentration, temperature and pH. Mohamed and Alamri (2012) reported that cylindrospermopsin was degraded by *Bacillus* bacteria and degradation occurred in 6 days at the highest toxin concentration (300 μg/L) and in 7 or 8 days at lower concentrations (10 and 100 μg/L, respectively). The biodegradation rate was also reported to depend on temperature and pH, with the highest rates occurring in warm waters (25 and 30°C) and neutral to slightly alkaline conditions (pH 7 and 8). Klitzke and Fastner (2012) confirmed the observations of Mohamed and Alamri (2012), noting that a decrease in temperature from 20 to 10°C slowed down degradation by a factor of 10. They also found that degradation slowed significantly under anaerobic conditions, with half-lives of 2.4 days under aerobic conditions and 23.6 days under anaerobic conditions.

3.2.4 Toxicokinetics

Limited data are available regarding the toxicokinetics of microcystins in environmental exposure conditions (U.S. EPA 2015d). Available intestinal data indicate that the organic acid transporter polypeptide (OATp) family transporters facilitate the absorption of microcystins from the intestinal tract into liver, brain, and other tissues, as well as their export out of organs and tissues (Cheng et al. 2005; Fischer et al. 2005; Svoboda et al. 2011). However, bile acids and other physiologically-relevant substrates compete with microcystins for transporter uptake by the liver (Thompson & Pace 1992); reduction or elimination of liver toxicity has been observed during *in vivo* or *in vitro* exposures when microcystin uptake by OATp transporters is limited or inhibited (Hermansky et al. 1990a; Hermansky et al. 1990b; Runnegar et al. 1995; Runnegar & Falconer 1982; Runnegar et al. 1981). Both *in vivo* and *in vitro* studies have shown biliary excretion of microcystins (Falconer et al. 1986; Pace et al. 1991; Robinson et al. 1991), possibly via conjugation with cysteine and glutathione (Kondo et al. 1996). Additional details of microcystin toxicokinetics can be found in U.S. EPA's Drinking Water Health Advisory and HESD for microcystins (U.S. EPA 2015a; U.S. EPA 2015d).

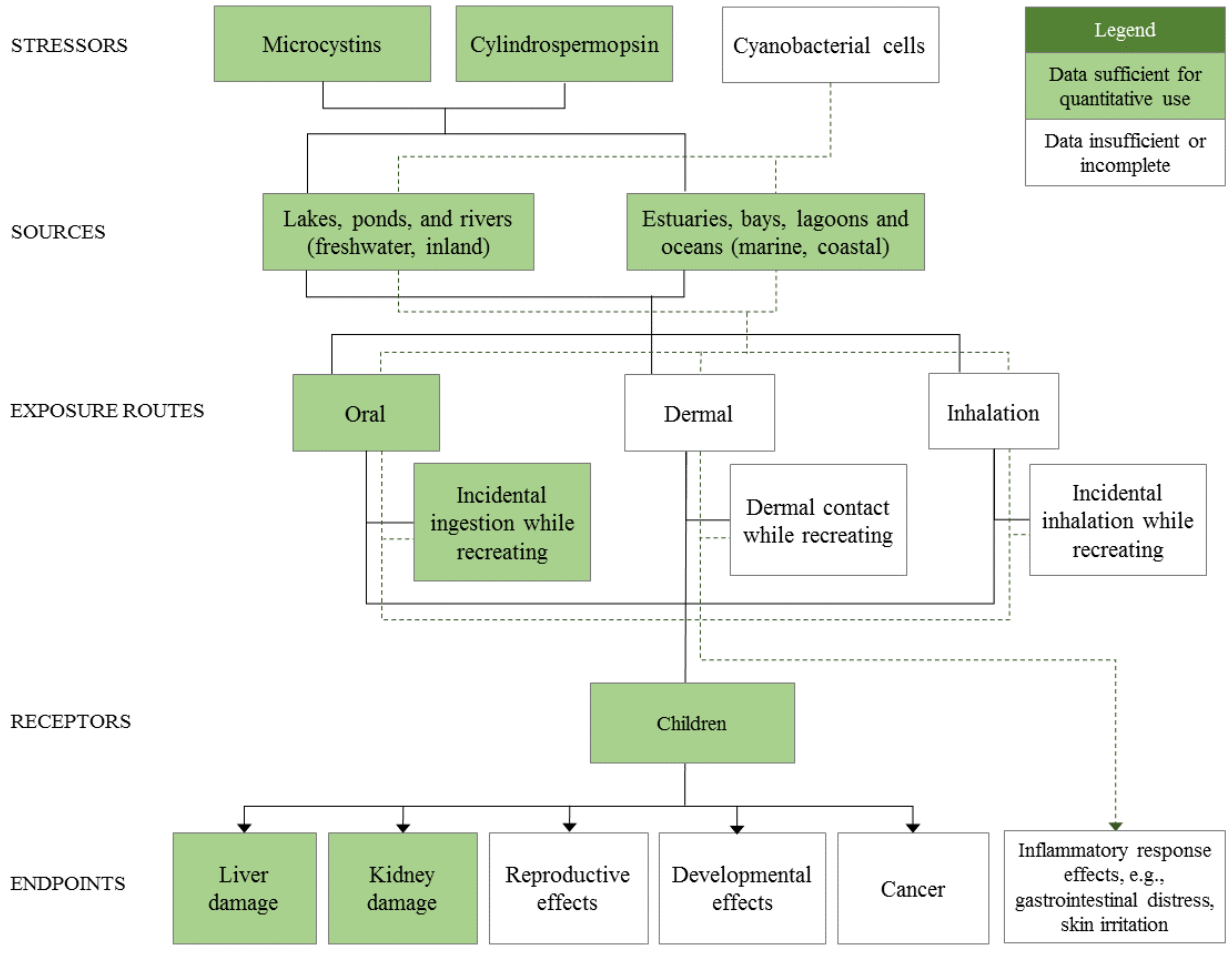
Limited toxicokinetic data for cylindrospermopsin are available, and are derived from mice intraperitoneal studies and *in vivo* studies that do not necessarily reflect environmental exposure conditions (U.S. EPA 2015c). Cylindrospermopsin is absorbed from the GI tract (Humpage & Falconer 2003; Shaw et al. 2001; Shaw et al. 2000) and is distributed primarily to the liver but also to the kidneys and spleen (Norris et al. 2001). The metabolism and toxicity of cylindrospermopsin is mediated by hepatic cytochrome P450 (CYP450) enzymes, and the periacinar region of the liver appears to be the main target of toxicity where cylindrospermopsin and its metabolites bind to proteins (Norris et al. 2001; Runnegar et al. 1995; Shaw et al. 2001; Shaw et al. 2000). Elimination of cylindrospermopsin was continuous over a monitoring period of 24 hours, with a large mean total recovery primarily from urine, and to a smaller extent, feces, after 24 hours (Norris et al. 2001). Additional details of cylindrospermopsin toxicokinetics can be found in EPA's Drinking Water Health Advisory and HESD for cylindrospermopsin (U.S. EPA 2015b; U.S. EPA 2015c).

4.0 PROBLEM FORMULATION

4.1 Conceptual Model

This conceptual model provides useful information to characterize and communicate the potential health risks related to exposure to microcystins and cylindrospermopsin in recreational waters. The sources of cyanotoxins in these waters, the recreational route of exposure for biological receptors of concern, and the potential assessment endpoints (e.g., effects such as kidney and liver toxicity) are depicted in the conceptual diagram below (Figure 4-1).

Figure 4-1. Conceptual Model of Exposure Pathways to the Cyanotoxins, Microcystins and Cylindrospermopsin, and Cyanobacteria in Surface Waters while Recreating



Conceptual Model Diagram for Exposure via Recreational Exposures

The conceptual model is intended to explore potential links of exposure to a contaminant or stressor with the adverse effects and toxicological endpoints important for management goals, including the development of recreational ambient water quality criteria. Boxes that are shaded indicate pathways that EPA considered quantitatively in estimating the advisory level, whereas

the white boxes did not have sufficient data for EPA evaluate quantitatively. The solid lines are for the cyanotoxins and the dotted lines are for the cyanobacterial cells.

Factors Considered in the Conceptual Model for Microcystins and Cylindrospermopsin

Stressors. The stressors are microcystins and cylindrospermopsin concentrations in water. These toxins can be produced by cyanobacteria occurring in freshwater. Once produced, the toxins have the potential to affect downstream waters, including coastal areas. The values recommended in this document could be applied to coastal waters affected by toxins produced by upstream freshwater cyanobacteria. Cyanobacterial cells as direct stressors in recreational surface waters are discussed in Appendix D.

Sources. Cyanobacteria occur naturally in surface waters, such as lakes, ponds, rivers, estuaries, bays, lagoons, and oceans in or surrounding the United States. Some genera of the cyanobacteria, including *Microcystis*, *Cylindrospermopsis*, *Anabaena*, *Planktothrix*, and *Nostoc*, can produce the cyanotoxins microcystins and cylindrospermopsin. Once these toxins are produced, they can be stable in the environment for weeks (Funari & Testai 2008; Zastepa et al. 2014).

Routes of exposure. Exposure to cyanotoxins from recreational water sources can occur via oral exposure (incidental ingestion while recreating); dermal exposure (contact of exposed parts of the body with water containing cyanotoxins during recreational activities such as swimming, wading, surfing); and inhalation exposure to contaminated aerosols (while recreating). The route of exposure considered quantitatively is oral exposure to microcystin and cylindrospermopsin via incidental ingestion while swimming. Dermal exposure happens during swimming; however, significant dermal absorption of microcystins and cylindrospermopsin is not expected due to the large size and charged nature of these molecules (Butler et al. 2012; U.S. EPA 2004; U.S. EPA 2007). EPA estimated that ingestion from inhalation is likely negligible compared to incidental ingestion while recreating (see section 7.5.1.1). Routes of exposure other than ingestion of drinking water are taken into account by the application of a relative source contribution value (U.S. EPA 2000a). Routes of exposure other than incidental oral ingestion while swimming are discussed further in the Effects Characterization, section 7.5.1.

Receptors. Anyone who recreates in a water body where cyanotoxins are present could be exposed to cyanotoxins through ingestion, dermal contact, and inhalation of aerosols while recreating in contaminated surface waters. Childhood is considered a vulnerable lifestage due to children's potential increased exposure while recreating. Recreating children can be at greater risk from exposure to microcystins or cylindrospermopsin because they have smaller body mass compared to adults, they spend more time in contact with the water compared to adults, and they incidentally ingest more water than adults while recreating. Thus, EPA is specifically considering the recreational exposures children experience in this assessment. EPA evaluates and discusses differences between lifestages in section 7.4 of the Effects Characterization. While there are many examples in the literature and reports of animal poisonings and death from exposure to cyanotoxins, values protective of animals such as dogs and livestock are not generated in this document. However, section 7.6 discusses some animal specific issues, including a summary of guidelines several states have developed for animals.

Endpoints. Available microcystin toxicity data indicate that the primary target organ for microcystins is the liver as described in EPA's *Health Effects Support Document for the Cyanobacterial Toxin Microcystins* (U.S. EPA 2015d). Available cylindrospermopsin toxicity

data are described in EPA's *Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin* (U.S. EPA 2015c). For cylindrospermopsin, EPA selected kidney effects as the endpoint on which to base the measure of effect. Clinical, epidemiological, and outbreak study results (see Appendix D) suggest a link between an increase in adverse inflammatory symptoms among recreators and elevated cyanobacterial cell densities. However, there is considerable uncertainty and variability associated with the epidemiological results, which did not identify consistent effects at similar cyanobacterial densities. Specifically, significant associations occur across a wide range of cell densities; associations vary with different specific health endpoints or combined symptom categories; and differences in cyanobacterial community composition are largely uncharacterized. These endpoints are not considered quantitatively in this assessment, but potential health effects are described in the Effects Characterization section 7.1 along with a discussion of the uncertainties related to the data for cyanobacterial cells.

4.2 Analysis Plan

EPA's 2000 *Methodology for Deriving Ambient Water Quality Criteria for the Protection of Human Health* (2000 Human Health Methodology) outlines EPA's process for deriving Ambient Water Quality Criteria (AWQC) and guides the development of these recreational criteria and swimming advisories (U.S. EPA 2000a).

The 2000 Human Health Methodology includes identifying the population subgroup that should be protected, evaluation of cancer and non-cancer endpoints, measures of effect, measures of exposure, relative source contribution (RSC), and evaluation of bioaccumulation. In this analysis plan, EPA: (1) describes the RfD previously derived for microcystin and cylindrospermopsin (measure of effect); (2) describes the calculation for the recreational criteria; (3) discusses incidental ingestion exposure in terms of volume ingested, duration of exposure, and body weight (measure of exposure) described in EPA's *Exposure Factors Handbook* and; (4) discusses the RSC. These criteria focus on human exposure as a result of primary contact recreation activities such as swimming where immersion and incidental ingestion of ambient water are likely.

EPA's *Health Effects Support Document for the Cyanobacterial Toxin Microcystins* and *Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin* (U.S. EPA 2015c; U.S. EPA 2015d) provide the health effects basis for the development of the Drinking Water Health Advisories for microcystins and cylindrospermopsin (U.S. EPA 2015a; U.S. EPA 2015b), including the science-based decisions providing the basis for estimating the point of departure. To develop the HESDs for microcystins and cylindrospermopsin, EPA assembled available information on toxicokinetics, acute, short-term, subchronic and chronic toxicity along with developmental and reproductive toxicity, neurotoxicity, immunotoxicity, genotoxicity and cancer in humans and animals. For detailed descriptions of the literature search strategies, see the HESDs for microcystins and cylindrospermopsin (U.S. EPA 2015c; U.S. EPA 2015d). This document was subject to rigorous internal and external peer review before it was finalized in 2015. The information evaluated for these documents also supports the development of the recreational criteria and swimming advisories for microcystins and cylindrospermopsin, which evaluate exposure via recreational water ingestion. EPA conducted supplemental literature searches in September 2015 to capture new references, including effects related to recreational exposure to cells. For detailed information search terms, see Appendix C.

4.2.1 Approach for Recreational AWQC Derivation

The Recreational AWQC for microcystins and cylindrospermopsin are calculated as described in the 2000 Human Health Methodology and presented in the equation below:

$$\text{Recreational AWQC } (\mu g/L) = \frac{RfD \times RSC \times BW}{IR}$$

Where:

RfD	=	Reference dose (μg/kilograms [kg] body weight [bw]/day [d])
RSC	=	Relative source contribution (RSC is discussed in section 4.2.5).
BW	=	Mean body weight (kg)
IR	=	Ingestion rate (L/d) at approximately the 90th percentile (discussed in section 4.2.3)

4.2.1.1 Magnitude, Duration and Frequency

EPA recommends that recreational criteria consist of a magnitude, duration, and frequency. Magnitude is the numeric expression of the maximum amount of the contaminant that may be present in a waterbody that supports the designated use. Duration is the period of time over which the magnitude is calculated. Frequency of excursion describes the number of times the pollutant may be present above the magnitude over the specified time period (duration). A criterion is derived such that the combination of magnitude, duration, and frequency protect the designated use (e.g., primary contact recreation). For microcystins and cylindrospermopsin, the magnitude of the criteria is based on the data used to derive the toxicity (in this case the RfDs for microcystins and cylindrospermopsin) values developed to support the Drinking Water Health Advisories (U.S. EPA 2015a,b). The duration and frequency components of the criteria are consistent with the approach discussed in previous recreational criteria, including the application of the recommended magnitudes using different durations for beach management and waterbody assessment.

4.2.2 Measures of Effect

A reference dose or RfD is an estimate (with uncertainties spanning perhaps an order of magnitude) of the daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. EPA's HESDs for microcystins and cylindrospermopsin (U.S. EPA 2015c,d), provide the health effects basis for development of the reference dose or RfD, including the science-based decisions (i.e., selection of the critical study and endpoints) providing the basis for estimating the point of departure and application of uncertainty factors. EPA uses the RfD values for oral exposure previously peer reviewed and documented in the HESDs for microcystins and cylindrospermopsin (U.S. EPA 2015c; U.S. EPA 2015d) in derivation of the recreational criteria and swimming advisories. Dermal exposure happens during swimming; however, significant dermal absorption of microcystins and cylindrospermopsin is not expected due to the large size and charged nature of these molecules (Butler et al. 2012; U.S. EPA 2004; U.S. EPA 2007). Because available data are

not sufficient, EPA is not quantifying effects resulting from dermal exposure to cyanotoxins. See section 7.5.1.2 for a characterization of dermal exposure to these cyanotoxins.

Inhalation exposure occurs during swimming; however, data are not sufficient to quantify health effects resulting from inhalation exposure to cyanotoxins at this time. See section 7.5.1.1 for a characterization of potential effects from inhalation exposure.

Dermal exposure to cyanobacterial cells can also result in adverse health effects, such skin rashes, eye irritation, and ear irritation. Because adequate data are not available, EPA is not quantifying effects resulting from exposure to cells at this time. Available epidemiological study results do not provide consistent associations between cell densities and the inflammatory health endpoints. Some of the studies have been limited in size, which could affect the ability to detect an association if one exists. Differences in the cyanobacterial communities present at the study sites may have affected the detection of associations. Characterization of confounders, such as the presence of pathogens, was not consistent among the studies. See section 7.1.1 for a characterization of potential effects from recreational exposure to cyanobacterial cells.

4.2.3 Measures of Exposure

The exposure parameters selected for use in calculating recreational criteria and swimming advisories for microcystins and cylindrospermopsin include an ingestion rate (volume of surface water incidentally ingested per day) and body weight (kg). Both body weight and incidental ingestion while recreating are parameters that vary with age. The key study and other data supporting these exposure factors are described in the sections that follow.

All recreational exposure studies that included both children and adults found that age could influence incidental ingestion exposure while recreating. More specifically, children tend to ingest more water and spend more time in the water compared to adults (Dufour et al. 2006; Schets et al. 2011; U.S. EPA 1997). EPA's *Exposure Factors Handbook* (U.S. EPA 2011) provides recommended values for body weights and incidental ingestion volumes and rates for children and adults on an event basis. The *Handbook* recommends using the 97th percentile ingestion rate for children and the maximum reported value for adults because the dataset is limited (U.S. EPA 2011).

EPA's *Exposure Factors Handbook* (2011) edition and (1997) edition provided values for time spent swimming per month and time spent in a pool/spa per day, respectively. EPA (2011) compiled mean and 95th percentile swimming durations for different children's age groups in minutes/month (e.g., mean swimming duration value for children 1 to < 2 years was 105 minutes/month, for children 3 to < 6 years was 137 minutes/month, and for children 6 to 11 years was 151 minutes/month). EPA needed a duration parameter expressed as time exposed per day to calculate a daily ingestion rate. Converting the monthly values to daily durations (e.g., dividing the monthly value by 30 days per month) resulted in very short daily exposures that do not seem reasonable given the other duration estimates available. Therefore, EPA used the duration of recreational event per day reported in U.S. EPA (1997) of 2.7 hours per day for children 5 to 11 years old.

4.2.3.1 Incidental Ingestion

Primary Contact Exposure Scenario

EPA selected incidental ingestion during primary contact activities such as swimming for the criteria derivation because data suggest that incidental ingestion can be considered the highest potential exposure pathway for cyanotoxins while recreating. In a combined analysis of 2,705 individuals recreating in the Chicago Area Waterway System and 662 individuals recreating at a public outdoor swimming pool, Dorevitch et al. (2011) studied the volume of water ingested during a range of recreational activities. Study subjects took part in one of the following activities: canoeing, fishing, kayaking, motor boating, rowing, wading/splashing, head immersion (i.e., immersed one's head three times over a 10-minute interval), or swimming. At the end of their exposure, participants self-reported whether they ingested water, and how much, during their recreational experience. The results indicate that the odds of ingesting a teaspoon or more of water are significantly higher among swimmers than among those who just immersed their head in a swimming pool or those who participated in the other, more limited contact activities on surface waters. More specifically, rowing, motor boating, fishing, wading/splashing, and non-capsizing kayaking and canoeing were found to be low-ingestion activities, resulting in 95th percentile ingestion volumes between 0.01 and 0.012 L/hr. The study authors considered those who capsized during canoeing or kayaking a "middle ingestion category," with a 95th percentile ingestion volume of about 0.017 to 0.02 L/hr. Swimmers were the highest ingestion category, with a 95th percentile ingestion volume of approximately 0.035 L/hr. Evaluations of inhalation (see section 7.5.1.1) and dermal (see section 7.5.1.2) exposures suggest that those two routes are minor compared to the oral exposure route. Thus, EPA determined that using a swimmer scenario for exposure as the basis for the criteria is protective of these other aquatic activities.

Incidental Ingestion per Day

To calculate the recreational incidental ingestion rate in units of volume per day, EPA combined a distribution from EPA's (2011) *Exposure Factors Handbook* on incidental ingestion volumes (volume per event normalized to volume per hour) and a distribution of exposure durations (hours per day) from EPA's (1997) *Exposure Factors Handbook*. The recommended 97th percentile incidental ingestion volume for children combined with the mean exposure duration represented the 90th percentile of this combined distribution to represent incidental ingestion per day. These data are discussed in the following sections.

Ingestion Volume Studies

EPA's *Exposure Factors Handbook* (2011) cites Dufour et al. (2006) as the basis for its default recreational ingestion values. Dufour et al. (2006) measured the incidental ingestion of water while participants were swimming in a pool and found that children under the age of 18 years ingested higher volumes of water while swimming than adults and that males ingested more than females. This small-scale pilot study (n = 53) used cyanuric acid as an indicator of amount of pool water ingested while swimming in an outdoor pool. Participants were instructed

to stay in the pool and actively swim for at least 45 minutes. Pool-water samples were collected before the start of swimming activities, and participants' urine was collected for 24 hours after the swimming event ended; pool-water and urine samples were analyzed for cyanuric acid. The combined study population had a mean incidental ingestion volume of 0.019 L per swimming event. Because sample size for the Dufour et al. (2006) study was small (i.e., 41 children and 12 adults), the authors reported results for children under the age of 18 years and adults. In addition, children younger than 6 years were not included in the study design. One study by Schets et al. (2011) reported surveyed parents' estimates of incidental ingestion for children younger than 6 years old. The Schets et al. (2011) reported ingestion values for children ages 0 to < 15 years were similar to the Dufour et al. (2006) findings; see section 7.3 for more detail.

The values presented in EPA's *Exposure Factors Handbook* (2011) adjusted the Dufour et al. (2006) data from a per event (e.g., 45 minutes) basis to an hourly ingestion rate. The distribution of Dufour et al. (2006) measured incidental ingestion rates are graphically presented in Figure 4-2. Based on these data, the *Exposure Factors Handbook* recommended assessments use a 97th percentile (0.12 L/hr) for children and a maximum value (0.071 L/hr) for adults as "upper percentile" values due to the limited sample size of the Dufour et al. (2006) study. Several other studies (Dufour et al. 2006; Schets et al. 2011; Suppes et al. 2014; U.S. EPA 2000a) characterizing incidental ingestion while swimming are available and described in the effects characterization section (section 7.3). These other studies reported similar results to Dufour et al. (2006).

Duration of Recreational Exposure

Duration of recreational exposure quantifies the length of time people might be exposed to cyanotoxins during their primary contact recreational use of surface waters contaminated with cyanotoxins. Duration of recreational exposure is needed to convert recreational ingestion rates in units of volume per hour to an amount incidentally ingested per day, which is the exposure parameter needed for the recreational AWQC derivation.

EPA's *Exposure Factors Handbook* (1997) lists, for different age groups, time spent per 24 hours in an outdoor spa or pool, which is interpreted for purposes of this calculation as time spent in direct contact with water, for example, swimming. The data are based on analysis of the National Human Activity Pattern Survey by Tsang and Klepeis (1996). Figure 4-3 compares the recreational duration data for different age groups and shows that recreators ages 5 to 11 years tend to spend more time in the water than other child age groups and adults, although the 90th percentile values are similar. A duration was not provided for children younger than 1 year. EPA evaluated both the mean duration for the various age groups and available exposure parameters for children younger than 6 years old; see section 7.4.

Other data show a similar trend of longer recreational durations for children. Schets et al. (2011) investigated swimming durations in freshwater, marine water, and pools. They surveyed 8,000 adults, 1,924 of whom also provided estimates for their eldest child (< 15 years of age) and found that children spend, on average, 25 minutes longer swimming in freshwaters compared to adults. The mean duration of swimming events for children ages 0–14 years in freshwater and marine water were 79 minutes (1.3 hours, 95 percent CI: 12–270 minutes) and 65 minutes (1.1 hours, 95 percent CI: 8–240 minutes), respectively. Adult averages were all less than 1 hour.

Figure 4-2. Incidental Ingestion Rates Measured for Adults and Children (Dufour et al. 2006)

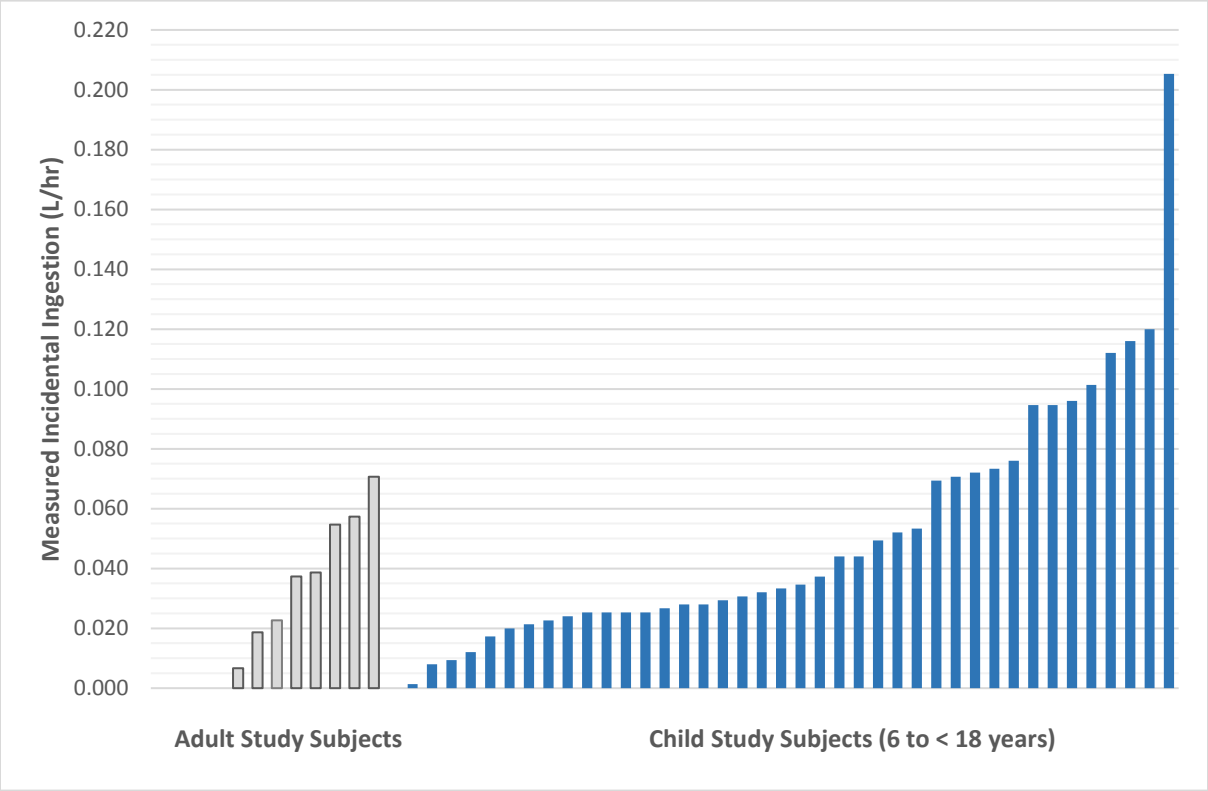
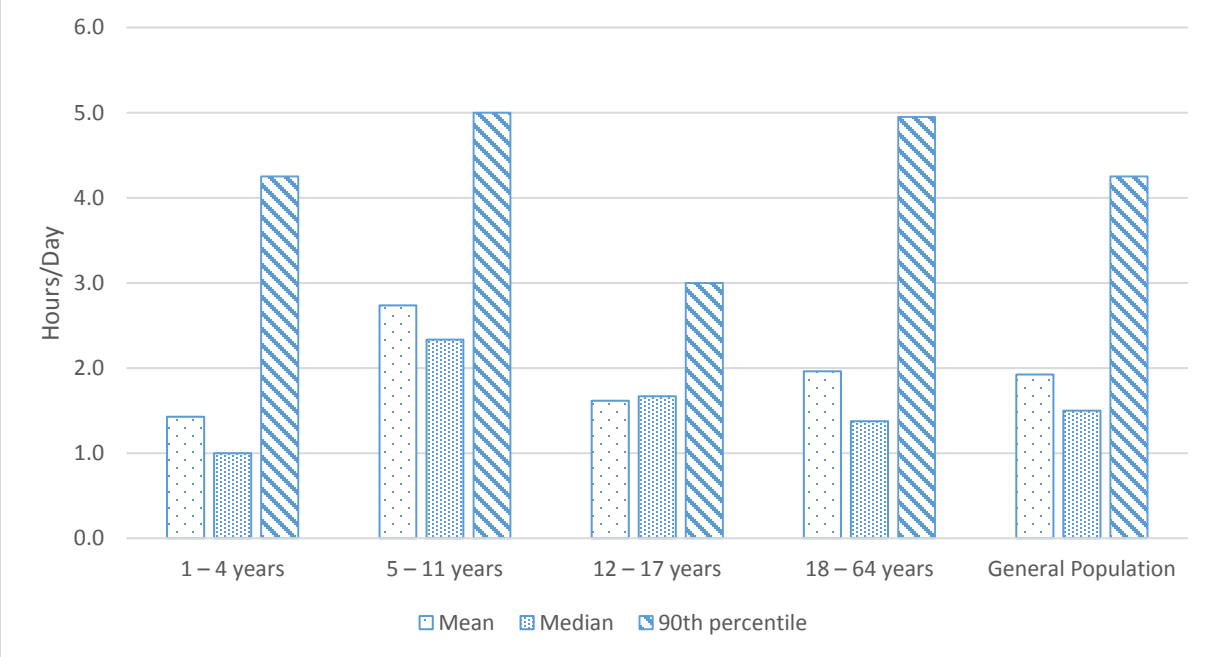


Figure 4-3. Direct Contact Recreational Exposure Duration by Age Group, Based on Table 15-119 in U.S. EPA (1997)



Additional duration estimates of children's pool swimming have been identified by EPA's Office of Pesticide Programs for use in its Swimmers Exposure Assessment Model (SWIMODEL) for estimating chemical exposures during pool swimming, including direct contact for competitive swimmers (U.S. EPA 2003). EPA's SWIMODEL considers short-term exposure (using a high-end estimate of exposure time per event in order to represent a maximum, one-time exposure) and intermediate/long-term exposure (using a shorter event duration to represent an average of maximum and minimum exposures overtime). Among competitive children swimmers, the longest short-term exposure duration used by the SWIMODEL is 2 hours/day for children ages 11–15 years (U.S. EPA 2003). Competitive swimming practice durations, however, are less relevant for recreational scenarios in lakes and rivers than for exposure in a pool. EPA's *Exposure Factors Handbook* (1997) lists the mean exposure duration for children 5 to 11 years as 2.7 hours/day, which is longer than the maximum value for children 11 to 15 years used in the SWIMODEL.

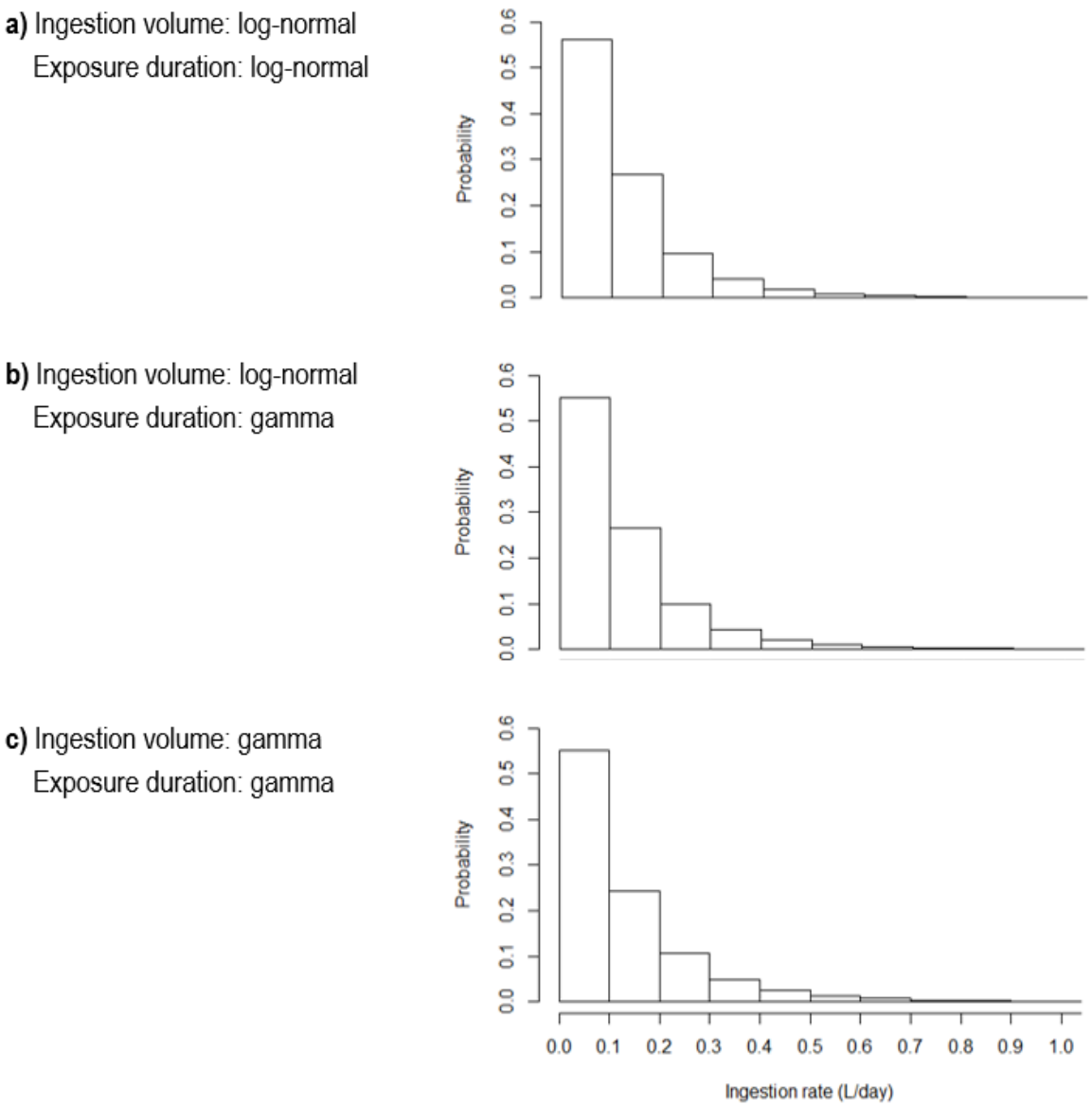
Determination of Incidental Ingestion per Day

The incidental ingestion volume per day EPA used to calculate the recreational criteria or swimming advisory is the product of the 97th percentile children's incidental ingestion rate (0.12 L/hr) and mean exposure duration (2.74 hr/day) for children ages 5 to 11 years. EPA evaluated the effect these multiple parameters had on the level of protection by analyzing the combined distributions of ingestion volume per hour and duration of recreational exposure. EPA compiled the published statistical parameters (i.e., mean, standard deviation, and minimum and maximum data values) and evaluated the resulting distributions for both parameters compared to a normal, log-normal, and gamma function. For both parameters, the log-normal or gamma functions better described the distributions. Log-normal and gamma functions are strictly positive distributions and reflect the apparent skewness in the data. Describing a distribution with a normal function can result in negative values that are not representative. In the analysis, both distributions were limited to their respective minimum and maximum data values. Table 4-1 shows the statistics of the combined distributions: (a) a lognormal distribution for both parameters, (b) a lognormal distribution for the ingestion rate and a gamma distribution for the exposure duration, and (c) a gamma distribution for both parameters. The combinations of the two distributions assuming log-normal, gamma, or both, are shown in Figure 4-4 as hybrid distributions. For all three combined distributions using combinations of log-normal and/or gamma functions, the incidental ingestion rate per day (0.33 L/d) represents approximately the 90th percentile of the hybrid distributions (range 92nd to 94th percentile). Additional details including the methodology for this analysis are provided in Appendix E.

Table 4-1. Summary Statistics of Combined Ingestion Volume and Exposure Duration Distributions

Combined Distribution	Ingestion Volume (L/hr) Distribution	Exposure Duration (hr/d) Distribution	Summary Statistics for Ingestion Rate (L/d)				
			Minimum	Median	Mean	Maximum	Percentile Associated with 0.33 L/d
a	Log-normal	Log-normal	0.0023	0.0873	0.12	1.47	94
b	Log-normal	Gamma	0.0018	0.0888	0.13	1.46	93
c	Gamma	Gamma	0.0000	0.0871	0.13	1.40	92

Figure 4-4. Hybrid Distributions for Incidental Ingestion per Day (L/d)



4.2.3.2 Body Weight

Table 8-1 in EPA's *Exposure Factors Handbook* (U.S. EPA 2011) reported recommended and other body weight statistics based on the National Health and Nutrition Examination Survey. A range of age groups is included. Mean body weight for children aged 6 to < 11 years was 31.8 kg. EPA selected this body weight because it reflected the age group with higher ingestion volumes (U.S. EPA 2011; Evans et al. 2006) and exposure duration (U.S. EPA 1997). A discussion of younger children's exposure factors can be found in section 7.4.2.

4.2.4 Relative Source Contribution

EPA's 2000 Human Health Methodology (2000a) outlines EPA's process for deriving AWQC and guides the development of these recreational criteria. The 2000 Human Health Methodology recommends the application of a RSC in the AWQC derivation to ensure that an individual's total exposure from all routes of exposure to a contaminant does not exceed the RfD. EPA considered the 2000 Human Health Methodology's Exposure Decision Tree Approach to determine the RSC used in deriving the recreational values for microcystins and cylindrospermopsin (Figure 4-1 in the 2000 Human Health Methodology document).

The RSC component of the AWQC calculation allows a percentage of the RfDs exposure to be attributed to the consumption of ambient water and fish and shellfish from inland and nearshore waters when there are other potential exposure sources. The RSC describes the portion of the RfD available for AWQC-related sources (USEPA 2000a); the remainder of the RfD is allocated to other sources of the pollutant. The rationale for this approach is that for pollutants exhibiting threshold effects, the objective of the AWQC is to ensure that an individual's total exposure from all sources does not exceed that threshold level. Exposures outside the RSC include, but are not limited to, exposure to a particular pollutant from fish and shellfish consumption, non-fish food consumption (e.g., fruits, vegetables, grains, meats, poultry, dietary supplements), dermal exposure, and respiratory exposure.

Cyanotoxins are produced by cyanobacteria. As discussed previously, certain environmental factors can lead to rapid growth of cyanobacteria in ambient water. Because environmental factors are not always favorable for cyanobacterial growth, blooms and the production of cyanotoxins are episodic in nature; therefore, determination of background levels is not relevant for cyanotoxins in determining the RSC.

EPA determined that an RSC of 80 percent, as recommended in EPA's 2000 Human Health Methodology, is appropriate for microcystins and cylindrospermopsin. The use of this RSC means that 80 percent of recreators' exposure to cyanotoxins is from incidental ingestion of ambient water during recreational activities. The application of an RSC 80 percent takes into account the uncertainty associated with effects from dermal and inhalation exposures, exposure to contaminated fish and shellfish or drinking water (i.e., 20 percent is set aside for these other exposure routes). An RSC of 80 percent represents the ceiling for setting an RSC, and provides a margin of safety for individuals, given currently available data on exposure to different sources and via other routes.

5.0 EFFECTS ASSESSMENT

The health effects studies summarized below for microcystin and cylindrospermopsin are described in detail in EPA's HESDs and Drinking Water Health Advisories for these two cyanotoxins (U.S. EPA 2015a,b,c,d).

5.1 Hazard Identification

5.1.1 Noncancer Health Effects

5.1.1.1 Animal Toxicity Studies

Microcystins

Studies in laboratory animals demonstrate liver, kidney, and reproductive effects following short-term and subchronic oral exposures to microcystin-LR. Studies evaluating the chronic toxicity of microcystins have not shown clinical signs of toxicity and are limited by study design and by the lack of quantitative data. Observed effects in animals exposed orally or via intraperitoneal (i.p.) to microcystin-LR include liver, reproductive, developmental, kidney, and GI effects.

The preponderance of animal toxicity data on the noncancer effects of microcystins is restricted to the microcystin-LR congener. Studies evaluating the chronic toxicity of microcystins have not shown clinical signs of toxicity and are limited by study design and by the lack of quantitative data. Available data on the RR, YR, and LA congeners did not provide dose-response information sufficient for quantification. EPA is using data on effects of microcystin-LR to represent other microcystin congeners (U.S. EPA 2015d).

For details see the *Health Effects Support Document for the Cyanobacterial Toxin Microcystins* (U.S. EPA 2015d).

Cylindrospermopsin

Based on oral and i.p. studies in mice treated with purified cylindrospermopsin or extracts of C. raciborskii cells, the liver and kidneys appear to be the primary target organs for cylindrospermopsin toxicity.

No oral reproductive or developmental studies are available for cylindrospermopsin. Developmental toxicity studies following i.p. administration of cylindrospermopsin provide some evidence for maternal toxicity and decreased postnatal pup survival and body weight (Chernoff et al. 2011; Rogers et al. 2007).

For details, see EPA's *Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin* (U.S. EPA 2015c).

5.1.1.2 Human Studies

Microcystins

Limited human studies examining microcystin effects on humans are available; however, no dose-response data are available from ambient exposures to microcystins. The scant human data on the oral toxicity of microcystin-LR are limited by the potential co-exposure to other pathogens, cyanotoxins, and microorganisms; by the lack of quantitative information; and by the failure to control for confounding factors. Available human studies evidence is supportive of the liver as a target organ for toxicity (Carmichael 2001; Falconer et al. 1983; Giannuzzi et al. 2011; Hilborn et al. 2013; Jochimsen et al. 1998; Li et al. 2011b).

More detailed information on the human health effects of microcystins based on epidemiological studies related to drinking water outbreaks, clinical studies, and cases studies are discussed in the *Health Effects Support Document for the Cyanobacterial Toxin Microcystins* (U.S. EPA 2015d). Of the epidemiological studies EPA identified, three studies evaluated human health effects associated with recreational exposures to cyanobacteria and microcystins. These studies are also summarized in the microcystins HESD and are summarized below.

- Backer et al. (2008) conducted an epidemiological study in a small lake in the United States and compared microcystin concentrations in blood and reported symptoms in people recreating in a lake with a *M. aeruginosa* bloom to those of people recreating in a nearby bloom-free lake. Low levels of total microcystins (detection limit = 0.08 ng/m^3) were detected in air samples collected above a lake bloom. Phytoplankton counts ranged from 175,000 to 688,000 cells per mL with > 95 percent of those cells being cyanobacteria. Cell densities of potentially toxigenic cyanobacteria ranged from approximately 54,000 to 144,000 cells/mL. Although a visible bloom was present and contained cyanobacterial species capable of producing microcystin, microcystin concentrations in water during the study ranged from 2 to 5 μg/L. 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 GI, dermal, respiratory, or neurological symptoms after spending time on the lake. Adenoviruses (level of detection [LOD] = 1,250 gene copy equivalents) and enteroviruses (LOD = 200 plaque forming units/10 L) were not detected in any water sample. This study was limited in the number of participants and included a limited number of exposure days in the analysis. Given a small number of recreators exposed to low levels of microcystin over the course of 3 study days, the lack of significant associations is not surprising.
- In a similar study conducted by this same author at three lakes in California, microcystin concentrations from personal air samples ranged from the limit of detection (0.1 ng/m^3) to 0.4 ng/m^3, and extracellular microcystin concentrations in water ranged from < 2 μg/L to > 10 μg/L (Backer et al. 2010). No statistically significant differences were noted in the frequency of reported GI, dermal, or respiratory symptoms between participants immediately after they engaged in direct- or indirect-contact recreational activities in the lake with a cyanobacterial bloom and those in a lake without a cyanobacterial bloom. The study design characterized the potential inhalation of aerosolized microcystin among people who recreated at the lakes and included blood assays for microcystin among the study participants. Adenoviruses or enteroviruses were not detected at the study

locations. This study contained a limited number of participants over the course of 3 exposure days.

- Lévesque et al. (2014) conducted a prospective study of residents living in proximity to three lakes in Canada affected by cyanobacteria to investigate the relationship between recreational exposure, specifying full contact and limited contact with lake water and the incidence of GI, dermal, respiratory, and other (e.g., ear pain, muscle pain) symptoms. Full contact included swimming, waterskiing, windsurfing, use of watercraft involving launching, accidental falls, and similar activities, and limited contact included fishing, use of watercraft not involving launching, and other activities. No associations were observed between any symptoms and recreational exposures to microcystins. The maximum microcystin concentrations for which recreational-related GI symptoms were reported was 7.65 μg/L. The authors did observe a relationship between cyanobacterial cell counts and gastrointestinal illness with a significant association above 20,000 cells/mL.
- Additional outbreak and case reports document health effects following exposure to cyanotoxins. In a case report, 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* bloom containing 33,680 and 35,740 cells/mL. A level of 48.6 μg/L of microcystin-LR concentrations was detected in water samples associated with the bloom. After 4 hours of exposure, the patient exhibited fever, nausea, and abdominal pain, and 3 days later, presented dyspnea and respiratory distress and was diagnosed with an atypical pneumonia. One week after the exposure, the patient developed a hepatotoxicosis with a significant increase of alanine aminotransferase, aspartate aminotransferase, and γ-glutamyltransferase. The patient completely recovered within 20 days.
- Dziuban et al. (2006) and Hilborn et al. (2014) reported nine outbreaks associated with recreational exposure to HABs in which microcystins were detected, one in 2004 and eight in 2009 and 2010. In the one outbreak in which microcystin was measured at 20.8 μg/L and other cyanotoxins were either not detected or measured, 9 cases reported symptoms, which included abdominal cramps (3 cases), diarrhea (3), nausea (3) vomiting (2), fever (2), headache (2), rash (8), eye irritation (1), earache (1), neurologic symptoms (2), tingling (2), confusion (1), and respiratory symptoms (1) (Hilborn et al. 2014). Cyanobacterial cells were present. The results reported from the outbreaks should not be interpreted as cause and effect. Rather, the stressors and health endpoints discussed can be considered a co-occurrence due to the nature of the data collated in the outbreak reports.

Cylindrospermopsin

No epidemiological studies were found for recreational exposure to cylindrospermopsin.

Hilborn et al. (2014) reported two outbreaks associated with recreational exposure to HABs in which cylindrospermopsin was detected between 2009 and 2010. Cyanobacteria, microcystins, and other cyanotoxins, however, also were detected in these two outbreaks. As mentioned above, the results reported from the outbreaks should not be interpreted as cause and

effect, only that two or more parameters were demonstrated to co-occur spatially and/or temporally.

5.1.1.3 Noncancer Mode of Action

Microcystins

Mechanistic studies have shown the importance of membrane transporters for systemic uptake and tissue distribution of microcystin by all exposure routes (Feurstein et al. 2010; Fischer et al. 2005). The importance of the membrane transporters to tissue access is demonstrated when a reduction in, or lack of, liver damage happens following OATp inhibition (Hermansky et al. 1990a; Hermansky et al. 1990b; Thompson & Pace 1992).

The uptake of microcystins causes protein phosphatase inhibition and a loss of coordination between kinase phosphorylation and phosphatase dephosphorylation, which results in the destabilization of the cytoskeleton. This event initiates altered cell function followed by cellular apoptosis and necrosis (Barford et al. 1998). Both cellular kinases and phosphatases keep the balance between phosphorylation and dephosphorylation of key cellular proteins controlling metabolic processes, gene regulation, cell cycle control, transport and secretory processes, organization of the cytoskeleton, and cell adhesion. Each of the microcystin congeners evaluated (LR, LA, and LL) interacts with catalytic subunits of protein phosphatases PP1 and PP2A, inhibiting their functions (Craig et al. 1996).

As a consequence of the microcystin-induced changes in cytoskeleton, increases in apoptosis and reactive oxygen species (ROS) occur. In both *in vitro* and *in vivo* studies, cellular pro-apoptotic Bax and Bid proteins increased while anti-apoptotic Bcl-2 decreased (Fu et al. 2005; Huang et al. 2011; Li et al. 2011a; Takumi et al. 2010; Weng et al. 2007; Xing et al. 2008). Mitochondrial membrane potential and permeability transition pore changes (Ding & Nam Ong 2003; Zhou et al. 2012) lead to membrane loss of cytochrome c, a biomarker for apoptotic events. Wei et al. (2008) identified a time-dependent increase in ROS production and lipid peroxidation in mice after exposure to microcystin-LR. After receiving a 55 μg/kg of body weight i.p. injection of microcystin-LR, the levels of hepatic ROS increased rapidly within 0.5 hours and continued to accumulate for up to 12 hours in a time-dependent manner.

Cylindrospermopsin

Despite the number of studies that have been published, the mechanisms for liver and kidney toxicity by cylindrospermopsin are not completely characterized.

The occurrence of toxicity in the liver suggests a protein-synthesis inhibition mechanism of action for cylindrospermopsin. *In vitro* and *in vivo* studies have been conducted to demonstrate the ability of cylindrospermopsin to inhibit hepatic protein synthesis, which could impact mouse urinary protein production leading to decreased urinary excretion of these proteins (Froscio et al. 2009; Froscio et al. 2008; Terao et al. 1994). Available evidence indicates that protein synthesis inhibition is not decreased by broad-spectrum CYP450 inhibitors, but they do reduce cytotoxicity (Bazin et al. 2010; Froscio et al. 2003). Hepatotoxicity appears to be CYP450-dependent, which indicates a possible involvement of oxidized and/or fragmented metabolites and mechanisms other than protein synthesis inhibition (Froscio et al. 2003; Humpage et al. 2005; Norris et al. 2002; Norris et al. 2001).

In the Reisner et al. (2004) report, microscopic examination of blood samples showed the presence of red blood cells with spiked surfaces rather than their normal biconcave-disc shape. The authors attributed the acanthocyte formation to an increase in the cholesterol to phospholipid ratio of the red blood cell membrane. Phospholipids constitute the matrix material of cell membranes. The authors hypothesized that this change was the consequence of decreased activity of plasma lecithin cholesterol acyl transferase, an enzyme associated with high-density lipoproteins and the esterification of plasma cholesterol. Effects on the cholesterol content of the red blood cell membrane can occur with inhibition of the enzyme increasing membrane fluidity and mean corpuscular volume. Removal of the abnormal blood cells by the spleen increases both spleen weight and serum bilirubin as well as stimulates hematopoiesis. Additional research is needed to examine the lecithin cholesterol acyl transferase enzyme inhibition hypothesis in order to confirm whether it accounts for the effects on the red blood cell as a result of cylindrospermopsin exposure.

Kidney necrosis and a decreased renal failure index at the high cylindrospermopsin doses provide support for the effects on the kidney. Numerous signs of renal damage including proteinuria, glycosuria, and hematuria were observed after a hepatoenteritis-like outbreak in Palm Island, Australia in 1979 (Byth 1980). The outbreak was attributed to consumption of drinking water with a bloom of *C. raciborskii*, a cyanobacteria that can produce cylindrospermopsin. These effects are associated with impaired kidney function (Byth 1980); however, no mode of action information for kidney effects was observed in the available animal studies of cylindrospermopsin. Since all the studies were conducted in mice, a species that excretes low molecular weight proteins in urine, there is a need to conduct a study of cylindrospermopsin in a laboratory species that does not excrete protein in the urine in order to determine whether there are comparable effects on kidney weight, protein excretion, and renal cellular damage.

5.1.2 Cancer

5.1.2.1 Weight of Evidence Classification

While there is evidence of an association between liver and colorectal cancers in humans and microcystins exposure and some evidence that microcystin-LR is a tumor promoter in mechanistic studies, there is inadequate information to assess carcinogenic potential of microcystins in humans (U.S. EPA 2005). The human studies are limited by lack of exposure information and the uncertainty regarding whether or not these studies adequately controlled for confounding factors such as hepatitis B infection. No chronic cancer bioassays for microcystins in animals are available. U.S. EPA (2005) states that the descriptor of "*inadequate information to assess carcinogenic potential*" is appropriate when available data are judged inadequate for applying one of the other descriptors or for situations where there is little or no pertinent information or conflicting information. The guidelines also state that (p. 2-52) "Descriptors can be selected for an agent that has not been tested in a cancer bioassay if sufficient other information, e.g., toxicokinetic and mode of action information, is available to make a strong, convincing, and logical case through scientific inference." In the case of microcystins, the data suggest that microcystin-LR may be a tumor promoter but not an initiator. Without stronger epidemiological data and a chronic bioassay of purified microcystin-LR, the data do not support classifying microcystin-LR as a carcinogen. The International Agency for Research on Cancer

(IARC) classified microcystin-LR as a Group 2B (possibly carcinogenic to humans) based on the conclusion that there was strong evidence supporting a plausible tumor promoter mechanism for these liver toxins (IARC 2010).

No chronic cancer bioassays of cylindrospermopsin were located in the literature. Limited data from an *in vivo* study showed no indication that the cyanobacterial extract containing cylindrospermopsin-initiated tumors in mice (Falconer & Humpage 2001).

5.2 Dose-Response Assessment

The RfD value for microcystin for this recreational AWQC is from EPA's *Health Effects Support Document for the Cyanobacterial Toxin Microcystins* (U.S. EPA 2015d), where additional details are available. EPA identified a study by Heinze (1999) as the critical study in which male hybrid rats were administered microcystin-LR in drinking water at doses of 0 (n = 10), 50 (n = 10) or 150 (n = 10) µg/kg body weight for 28 days (Heinze 1999). The RfD of 0.05 µg/kg/d derived for microcystins was based on observed liver effects that included increased liver weight, slight to moderate liver necrosis lesions (with or without hemorrhages at the low dose and increased severity at the high dose), and changes in serum enzymes indicative of liver damage.

The RfD value for cylindrospermopsin for this recreational AWQC is from EPA's *Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin* (U.S. EPA 2015c), where additional details are available. EPA identified a study by Humpage and Falconer (2002; 2003) as the critical study in which male Swiss albino mice were administered purified cylindrospermopsin in water via gavage at doses of 0, 30, 60, 120, or 240 µg/kg/d for 11 weeks. The RfD of 0.1 µg/kg/d derived for cylindrospermopsin was based on increases in relative kidney weights along with indicators of reduced renal function effects at higher doses and decreased urinary protein.

6.0 SWIMMING ADVISORY AND RECREATIONAL CRITERIA DERIVATION

This section summarizes the inputs and shows the calculation for the recreational criteria and swimming advisory for microcystins and cylindrospermopsin.

6.1 Microcystins Magnitude

The magnitude of the swimming advisory and recreational criteria for microcystin toxins is calculated as follows:

$$\text{Recreational value (µg/L)} = \text{RfD} \times \frac{\text{RSC} \times \text{BW}}{\text{Ingestion Rate}}$$

Where:

RfD (µg/kg/d)	=	0.05 µg/kg/d (U.S. EPA 2015d)
RSC	=	0.8 (U.S. EPA 2000a)
BW (kg)	=	mean body weight of children 6 to < 11 years (31.8 kg) (U.S. EPA 2011)
Ingestion rate (L/d)	=	recreational water incidental ingestion rate for children (0.33 L/d) at approximately the 90th percentile (U.S. EPA 2011; U.S. EPA 1997)

$$\text{Microcystins recreational value} = 0.05 \text{ µg/kg/d} \times \frac{0.8 \times 31.8 \text{ kg}}{0.33 \text{ L/d}} = 4 \text{ µg/L}$$

6.2 Cylindrospermopsin Magnitude

The magnitude of the recreational criteria and swimming advisory values for cylindrospermopsin is calculated as follows:

$$\text{Recreational value (µg/L)} = \text{RfD} \times \frac{\text{RSC} \times \text{BW}}{\text{Ingestion Rate}}$$

Where:

RfD (µg/kg/d)	=	0.1 (U.S. EPA 2015c)
RSC	=	0.8 (U.S. EPA 2000a)
BW (kg)	=	mean body weight of children 6 to < 11 years (31.8 kg) (U.S. EPA 2011)
Ingestion rate (L/d)	=	recreational water incidental ingestion rate for children (0.33 L/d), at approximately the 90th percentile (U.S. EPA 2011; U.S. EPA 1997)

$$\text{Cylindrospermopsin recreational value} = 0.1 \text{ µg/kg/d} \times \frac{0.8 \times 31.8 \text{ kg}}{0.33 \text{ L/d}} = 8 \text{ µg/L}$$

6.3 Recommended Swimming Advisory and Recreational Criteria for Microcystins and Cylindrospermopsin

Recreational criteria and the swimming advisory include a magnitude, duration, and frequency. Magnitude is the numeric expression of the maximum amount of the contaminant that may be present in a waterbody that supports the designated use, in this case protecting public health of recreators. Duration is the period of time over which the magnitude is calculated. Frequency of excursion describes the maximum number of times the pollutant may be present above the magnitude over the specified time period (duration). The magnitude, duration, and frequency in combination protect the designated use (in this case primary contact recreation). EPA requests public comment on all three of these recommendations.

The magnitude values are based on body weight and intake in children and are considered protective of adverse health effects for adults. To protect public health of swimmers at a beach, EPA recommends that the magnitude of the advisory value not be exceeded on any single day. For adoption as a recreational water quality criterion, EPA recommends using an excursion frequency of no greater than 10 percent of days per recreational season (up to one year), which is similar to recommendations for other recreational criteria (U.S. EPA 2012a). The 10 percent exceedance rate can help inform decisions on identifying impaired and threatened waters. The seasonal assessment period can take into consideration the temporal variability of HABs in the waterbody. For example, HABs can occur in some waterbodies earlier and later in the recreational season, while in other waterbodies HABs can occur and persist as long as conditions are conducive to their growth. HABs that produce toxins that last for extended periods or that reoccur across years when conditions are conducive to cyanobacterial growth can signify waterbodies with excessive nutrient loadings. EPA does not anticipate states using these cyanotoxin recommendations alone for developing load allocations for Total Maximum Daily Loads (TMDLs) or for Water Quality-Based Effluent Limits (WQBELs). For permitting purposes, cyanobacteria or their toxins are not typically present in permitted discharges. Permits are more likely to be written to address point source discharges of the causal pollutants, such as nutrients, on a waterbody-specific or watershed basis, where the permit writer has determined there is a reasonable potential for the causal pollutants in the discharge to cause or contribute to an exceedance of the cyanotoxin standards.

The recommended recreational criteria or swimming advisory values for the cyanotoxins microcystins and cylindrospermopsin are presented in Table 6-1.

Table 6-1. Recreational Criteria or Swimming Advisory Recommendations for Microcystins and Cylindrospermopsin

Application of Recommended Values	Microcystins			Cylindrospermopsin		
	Magnitude (µg/L)	Frequency	Duration	Magnitude (µg/L)	Frequency	Duration
Swimming Advisory	4	Not to be exceeded	One day	8	Not to be exceeded	One day
Recreational Water Quality Criteria		No more than 10 percent of days	Recreational season (up to one calendar year)		No more than 10 percent of days	Recreational season (up to one calendar year)

As an example:

- To protect swimmers, the concentration of total microcystins shall not exceed 4 micrograms per liter in a day.
- To protect the recreational use, the concentration of total microcystins shall not exceed 4 micrograms per liter more than 10 percent of days in a recreational season.

7.0 EFFECTS CHARACTERIZATION

7.1 Cyanobacterial Cells

Cyanobacterial cell densities can indicate the eutrophic status of a water body, especially when considering the frequency and severity of HAB occurrence (Yuan & Pollard 2015). Thus, cyanobacterial cell densities, especially the extent, severity, and frequency of blooms under environmental conditions conducive to cyanobacterial cell growth, are an indicator of the ecological health of a water body.

Cyanobacterial cells are associated with two distinct sets of health endpoints. First, cyanobacteria are associated with toxin-related endpoints. The second set of health effects associated with cyanobacterial cells are the inflammatory health endpoints including rashes, respiratory and gastrointestinal distress, and ear and eye irritation, which may be instigated by direct contact with the cells, bioactive compounds in the cyanobacteria not currently classified as toxins, or by contact with cyanobacteria-associated microbial commensals via dermal, oral and/or inhalation exposure routes (Eiler and Bertilsson 2004; Gademann and Portman 2008). Such effects have been observed in various health studies, including epidemiological and clinical studies and outbreak reports (Bernstein et al. 2011; Geh et al. 2015; Lévesque et al. 2014; Lin et al. 2015; Pilotto et al. 1997; Pilotto et al. 2004; Stewart et al. 2006a,b). Also, while not all cyanobacteria produce cyanotoxins, scientists have observed a relationship between cyanobacteria density and cyanotoxin concentration (Loftin et al. 2016b). Environmental conditions and ecosystem interactions also affect the production and release of cyanotoxins into ambient waters. Cyanobacterial cell densities can be an indicator of the potential of a bloom to produce cyanotoxins. While there is uncertainty and variability associated with the propensity of a bloom to produce cyanotoxins, cell densities can be used to estimate the potential for cyanotoxin concentrations to exceed the recommended values presented in section 6.

7.1.1 Cyanobacterial Cells Related to Inflammatory Health Effects

Various health studies, described in more detail in Appendix D, relate recreational exposure to cyanobacterial cells with specific health endpoints that can be described as acute inflammatory or allergenic reactions. It is possible that these endpoints could be related to other biological or biochemical mechanisms that are not yet understood. Studies have (1) examined the epidemiological relationships of recreational exposure to cyanobacteria in the water to recreator-reported symptomologies, (2) characterized the allergenic and dermal reactions to exposed animals and humans in clinical and *in vitro* studies, and (3) collated information on illness outbreaks associated with recreational exposure to HABs. However, the reported health endpoints and cyanobacterial density associated with the inflammatory response health outcomes are not consistent. Empirical study differences, such as study size, species, strains of cyanobacteria present, and measurement of possible co-exposures and the cyanobacterial densities associated with significant health effects, lead to uncertainties in determining what level of cyanobacteria result in a specific level of inflammatory responses in these studies. The lack of a described dose-response characterizing cell-related inflammatory health effects could suggest a “threshold” rather than a specific dose-response relationship (Cochrane et al. 2015; Stewart et al. 2006b). Allergy is an example of a threshold mechanism, meaning that there is a level of exposure (i.e., a threshold value) below which the development of sensitization and the elicitation of an allergic reaction will not occur. Defining accurate numerical values for threshold

exposure levels is difficult due to lack of validated methods and uncertainties about the mechanism of sensitization (Cochrane et al. 2015).

WHO recommends the use of cyanobacterial cell densities related to an increasing scale of the probability of adverse health effects in the *Guidelines for Safe Recreational Water Environments* (WHO 2003b). They also estimated microcystin concentrations that could be associated with these cell density levels. WHO used this approach to differentiate "*between the chiefly irritative symptoms caused by unknown cyanobacterial substances and the potentially more severe hazard of exposure to high concentrations of cyanotoxins, particularly microcystins.*" Therefore, WHO recommended a series of three guideline values associated with incremental severity and probability of health effects rather than a single guideline value. The WHO guideline values are:

- Low probabilities of adverse health effects (20,000 cells per mL) can be associated with irritative or allergenic effects from exposure to cyanobacterial cells (corresponding to 10 µg chlorophyll *a* per liter, under conditions of cyanobacterial dominance). The Pilotto et al. (1997) epidemiological study directly informed the derivation of this cut point. They also estimated that microcystin concentrations of 2–4 µg/L can be expected at this level.
- Moderate probability of the adverse health effects (i.e., 100,000 cells per mL) (equivalent to approximately 50 µg chlorophyll *a* per liter, if cyanobacteria dominate) is associated with an increased potential for irritative health outcomes and the potential for negative health impacts associated with exposure to higher cyanotoxin concentrations. The 100,000-cell cut point was informed by (1) modifying the value for the WHO drinking-water guideline for microcystin-LR for a recreational exposure scenario and (2) translating microcystin concentrations to cell densities based on the average microcystin content of *Microcystis* cells (equivalent to 20 µg microcystin/L). The WHO estimated that "*at a cell density of 100,000 cells per mL, there is the potential for some frequently occurring species (i.e., microcystis) to form scums,"* which can "*increase risks for bathers and others involved in body-contact water sports."*
- The high probability of adverse health effects category is associated with the elevated potential for exposure to cyanotoxins and the potential for severe health outcomes. "*The presence of cyanobacterial scum in swimming areas represents the highest risk of adverse health effects due to abundant evidence for potentially severe health outcomes associated with these scums"* (estimated at 50–100 µg microcystin/L).

Epidemiological studies, clinical studies, and recreational water outbreak reports were identified during searches of the publicly available and peer-reviewed scientific literature that characterized the human health effects associated with recreating in surface waters where cyanobacteria were present (see Appendix D). Although these epidemiological studies provide evidence for statistically significant associations between cyanobacterial cell densities and possible inflammatory or allergenic health endpoints (Lévesque et al. 2014; Lévesque et al. 2016; Lin et al. 2015; Pilotto et al. 1997; Stewart et al. 2006b), they do not provide consistent evidence of associations either at similar densities of cyanobacterial cells or with the associated health endpoints. The wide range in cyanobacterial cell densities associated with various health outcomes, either with specific health endpoints or with combined symptom categories, implies potential variability in the stressor occurrence. Differing cyanobacterial community composition

and proportions of the more allergenic, non-cyanotoxin-producing strains relative to the cyanotoxin-producing strains at each site is a factor. Additionally, potential variability in sensitivity in the study populations, differences among the specific sites studied (e.g., fresh versus marine beaches), and uncertainty with the potential confounding effects of other microbes that can co-occur with cyanobacteria were some uncertainties associated with these data. Additional uncertainties are described below.

The limited size of some studies could have affected the ability to detect significantly increased rates of illness in individual symptom categories (Pilotto et al. 1997; Stewart et al. 2006b). Small sample size can diminish the statistical power of the study and the ability to detect an association if one exists (Rothman et al. 2008). The incomplete characterization or consideration of frank or opportunistic pathogens that could co-occur with cyanobacteria in ambient waters also could complicate conclusions related to the etiologic agent of the reported symptoms (Lévesque et al. 2014; Lin et al. 2015; Pilotto et al. 1997; Stewart et al. 2006b).

Variability in the reported associations, including with the range of cyanobacterial cell densities reported and with specific symptom categories, affected the ability to identify a discrete cyanobacterial cell density value that would provide a consistent level of protection across different waters. Pilotto et al. (1997) reported a significant association with the occurrence of one or more symptoms, such as skin rashes, eye irritation, ear irritation, gastrointestinal distress, fever and respiratory symptoms, and exposure to > 5,000 cells/mL for > 1 hour. Lévesque et al. (2014) observed a significant increase in GI symptoms associated with recreational contact. The increase in GI symptoms was significant in the > 20,000-cells/mL and > 100,000-cells/mL categories, and the positive trend for increasing illness with increased cyanobacterial cell densities also was significant at $p = 0.001$. Pilotto et al. (1997), however, in discussing the significance of the trend of increasing symptom occurrence and with the 5,000 cells/mL cut point, specifically suggested that the 20,000 cell/mL threshold might be too high to be adequately protective of recreators (Pilotto et al. 1997). Lin et al. (2015) reported significant associations between respiratory symptoms and exposure to the 25th to 75th percentile range of cyanobacterial cells excluding picocyanobacteria (range 37–237 cells/mL) and between reported respiratory, rash, and earache symptoms and exposure to the highest quartile (range 237–1,461 cells/mL). The 1,461-cells/mL value was the highest cell density observed in that study (Lin et al. 2015).

Cyanobacterial cell densities reported in the literature are used by states to provide "safe to swim" decisions by state and local health departments (see Table 2-3 for a list of states with cyanobacterial cell density guidelines; see Appendix B for state guidelines and associated actions). Due to the uncertainties associated with delineating discrete cyanobacterial densities associated with a specified level of protection for recreators, EPA is not recommending CWA 304(a) criteria that include quantitative cyanobacterial cell densities predictive of the inflammatory or allergenic health outcomes because available data do not support a consistent quantitative dose-response relationship at this time. However, EPA recognizes that studies examining the potential health effects associated with exposure to cyanobacterial cells demonstrate that exposure to the cells—particularly via dermal and inhalation exposure—can be associated with numerous health endpoints potentially characterized as inflammatory responses.

7.1.2 Cyanobacterial Cells as Indicators for Potential Toxin Production

Available information suggests that cyanobacterial cell density could be used as an indicator of the potential for a cyanobacterial HAB to produce cyanotoxins at the concentrations discussed in section 6. Although EPA is not recommending criteria at this time that address inflammatory health effects based on cyanobacterial cell density, many states already use cell-based guidelines based on recommendations from the World Health Organization (WHO 2003a). States use the cell density information gleaned from their monitoring efforts to inform decision-making. Also, remote sensing techniques using satellite-based imagery to observe cyanobacterial blooms are of increasing interest to states (Schaeffer et al. 2012; 2013). This approach detects the level of chlorophyll *a* or phycocyanins in the water and converts that to a cell density estimate. Therefore, a cell density value corresponding to the cyanotoxin criteria value is needed to interpret the remote sensing data. Below, EPA has used a similar approach as WHO to calculate a cyanobacterial cell density with the potential to produce the cyanotoxin at the criteria concentration.

The WHO guidelines were developed for microcystin and cyanobacterial cell density at different probabilities of adverse health effects to support management of recreational waters. The WHO designated a low probability of adverse health effects category associated with the cyanobacterial cell-related inflammatory response health endpoints (see section 7.1.1). The probability of adverse health effects increased to moderate and high levels based on the risk associated with the potential of the cyanobacterial cells to produce microcystin. For example, at a level of 100,000 cyanobacterial cells per mL, WHO estimated that a concentration of 20 μg microcystin per L is possible if those cells were predominantly *Microcystis* sp. and each cell contained an average of 0.2 pg microcystin per cell (WHO 2003).

Using this approach, EPA calculates a cyanobacterial density associated with the recommended microcystins criteria/ swimming advisory concentration as follows:

$$\text{Cyanobacterial Cell Density (CCD)} = \frac{\text{Ambient cyanotoxin concentration (ACC)}}{\text{Cell toxin amount (CTA)}}$$

Where:

CCD = calculated cell density associated with a specific toxin concentration

ACC = specific toxin concentration target in ambient water (e.g., AWQC value)

CTA = amount of toxin produced in a cyanobacterial cell

For the microcystin produced by *Microcystis* sp.:

ACC = 4 μg/L; recommended recreational criteria value for microcystins

CTA = 0.2 pg/cell; reported mean concentration of microcystin in a cell of *Microcystis* species

Adding in the conversion factors to convert units, the equation is:

$$CCD = \frac{ACC\ (\mu g/L) \times 10^6\ pg/\mu g}{CTA\ (0.2\ pg/cell)} \times \frac{L}{1{,}000\ mL}$$

Adding in the values,

$$CCD = \frac{4\ \mu g/L \times 10^6\ pg/\mu g}{0.2\ pg/cell} \times \frac{1\ L}{1{,}000\ mL} = 20{,}000\ cells/mL$$

Thus, a *Microcystis* sp. cell density of 20,000 cells/mL has the potential to result in a microcystin concentration of 4 μg/L.

There is variability in the estimate of cyanotoxin concentrations associated with cell density. WHO acknowledged that various cyanobacterial species could contain more or less microcystin per cell. Species that contain more microcystin could result in much higher water-column concentrations of the cyanotoxin at a similar cyanobacterial cell density. Cyanobacterial community differences between locations could affect the level of cyanotoxin that is present. For example, WHO discussed that a bloom dominated by *Planktothrix* could result in 10 to 20 times higher water-column cyanotoxin concentrations given the same cell density (WHO 2003b). The same cell density applied at different locations could result in inconsistent levels of health protection for recreators at those locations.

EPA surveyed the published peer-reviewed scientific literature for information on the amount of microcystin and cylindrospermopsin produced by or contained in a cell from a variety of freshwater blooms reported around the world. Laboratory-based culture studies with numerous clones of *Microcystis aeruginosa*, *Cylindrospermopsis raciborskii*, *Planktothrix agardhii*, and *Planktothrix rubescens* were also found. Many of these references also included either biomass-toxin conversions or graphic data which would support conversion factors from cyanobacterial cell density (expressed in a variety of units including cells L^{-1}, biovolume (μm^3) L^{-1}, chlorophyll *a* L^{-1}) to toxin concentrations for these species. Cyanotoxin concentration is generally related to cyanobacterial cell abundance, which is determined by nutrient availability (Welker 2008), so nutrient concentration is often correlated to cyanotoxin concentration. Information gleaned from this literature search also suggests that cyanotoxin amounts can vary with genetic factors (i.e., some isolates lack the genes involved in toxin production, physiological factors (e.g., growth rate, growth stage, photosynthetic rate, and allelopathic factors), trophic factors (e.g., grazing interactions), and environmental factors (e.g., temperature, salinity, carbon dioxide concentration, light intensity, macronutrient [i.e., nitrogen, phosphorus] and micronutrient [e.g., trace metal concentrations]). Most data available are for microcystins rather than for cylindrospermopsin. Please refer to Appendix F for additional information on cyanotoxin amounts per cell and conversion factors found in the literature survey.

States that currently have guidelines for HABs in recreational waters consider cell densities, cyanotoxin concentrations, or both. Decisions to issue recreational water warnings/advisories, or initiate monitoring for cyanotoxins based on the cyanobacterial cell density only once a bloom is observed (i.e., green, discolored water and/or scum formation/accumulation associated with high densities of cells) may overlook situations where extracellular toxins are present. Cells may accumulate in locations different from where the bloom originated (e.g., by wind and/or wave action, or transport downstream). A cell density of

20,000 cells/mL (corresponding to the recommended AWQC value) is lower than that typically associated with a bloom (WHO 2003b). Decision points contingent on visually confirmed blooms may miss or delay the identification of the hazardous condition associated with exposure to elevated cyanotoxins. States may wish to consider using visual identification of blooms preferentially for waterbodies with a previous history of HAB events and/or microcystin detections.

7.2 Enhanced Risk or Susceptibility

Children recreating are likely to spend more time in direct contact with waters and measured incidental ingestion data while swimming indicate that children between 6 and 11 years ingest on average more water than older children and adults (Evans et al. 2006). No measured incidental ingestion data are available for children younger than 6 years old. A study by Schets et al. (2011), described in more detail in section 7.3, provides incidental ingestion volumes for children ages 0 to 14 years, but this study relied on surveyed parents' estimates of the amount their children incidentally ingested. Although this study used a qualitative approach that is less certain than the studies that used analytical methods, Schets et al. (2011) identified an average incidental ingestion volume for children aged 0 to 14 years that was the same as the mean ingestion volume reported by Dufour et al. (2006) for children aged 6 to 18 years (37 mL). Children ages 5 to 11 years also tend to spend more time in the water compared to younger and older life stages (U.S. EPA 1997). The significant differences between life-stages in the volume of water ingested while recreating and duration of exposure can translate to increased risk of exposure to cyanotoxins for children compared to adults.

Based on the available studies in animals, individuals with liver and/or kidney disease may be more susceptible than the general population since the detoxification mechanisms in the liver and impaired excretory mechanisms in the kidney may be compromised. Data from an episode in a dialysis clinic in Caruaru, Brazil where microcystins (and possibly cylindrospermopsin) were not removed by treatment of dialysis water, identify dialysis patients as a population of potential concern in cases where the drinking water source was contaminated with cyanotoxins.

The data on red blood cell acanthocytes suggest that individuals that suffer from anemia (e.g., hemolytic or iron-deficiency) might be a potentially sensitive population. Several rare genetic defects such as abetalipoproteinemia (rare autosomal recessive disorder that interferes with the normal absorption of fat and fat-soluble vitamins from food) and hypobetalipoproteinemia are associated with abnormal red blood cell acanthocytes, which appears to result from a defect in expression of hepatic apoprotein B-100, a component of serum low density lipoprotein complexes (Kane & Havel 1989). Individuals with either condition might be sensitive to exposure to cylindrospermopsin.

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

7.3 Other Studies of Ingestion While Swimming

EPA used the recommended incidental ingestion while recreating values discussed in the *Exposure Factors Handbook* (2011), which cites Dufour et al. (2006) as the basis for its default recreational ingestion values. Dufour et al. (2006) measured the incidental ingestion of water while participants were swimming in a pool and found that children under the age of 18 ingested higher volumes of water while swimming than adults. The values presented in EPA's *Exposure Factors Handbook* (2011) adjusted the Dufour et al. (2006) data from a per event basis to an hourly ingestion rate.

In addition to Dufour et al. (2006), five other studies (Dorevitch et al. 2011; Evans et al. 2006; Schets et al. 2011; Schijven & de Roda Husman 2006; Suppes et al. 2014) evaluated recreation-associated incidental ingestion. See Table 7-1 for a summary overview of the available studies of incidental ingestion while recreating.

Evans et al. (2006) presented results from an observational study of incidental water ingestion during recreational swimming activities using the same methodology as the Dufour et al. (2006) pilot study. They cited the methods published in the Dufour et al. (2006) pilot study, which involved using cyanuric acid as an indicator of pool water ingestion to estimate the amount of water ingested by boys (n = 107) and girls (n = 80) ages 6–18 years who were directed to stay in the pool and actively swim for 45 to 60 minutes. Evans et al. (2006) reported that children ages 6–18 years incidentally ingested a mean volume of 47 mL per swimming event (boys: 48 mL/event; girls: 47 mL/event). Consistent with Dufour et al. (2006), Evans et al. (2006) found that children ingested higher volumes of water than both adults and the entire study population combined. Adults (both genders combined) incidentally ingested a mean volume of 24 mL. Adult men and adult women incidentally ingested 30 mL and 19 mL, respectively. The entire study population had a mean incidental ingestion volume of 32 mL. The Evans et al. (2006) reported study has not been peer reviewed.

Suppes et al. (2014) evaluated incidental water ingestion rates using cyanuric acid as an indicator of pool-water ingestion, and found that children on average ingested pool water at a higher rate than adult participants. Total time in water, quantified by viewing videos, was used to adjust pool-water ingestion volumes to obtain rates. After adjustments for false-positive measurements were applied, the mean rate at which adults ingested water was 0.0035 L/hr with range 0–0.051 L. The mean rate at which children ingested water was 0.026 L/hr with range 0.0009–0.106 L/hr.

Taking a different approach, a study in the Netherlands by Schets et al. (2011) used questionnaires to collect estimates of water swallowed while swimming/bathing in freshwater, marine water, and swimming pools and found children had higher ingestion volumes. Two rounds of surveys were conducted, one in 2007 and another in 2009. Of the 8,000 adults who completed the questionnaire, 1,924 additionally provided estimates for their eldest child (< 15 years of age). The participants estimated the amount of water they or their children swallowed while swimming. Schets et al. (2011) also conducted a series of experiments to measure the amount of water that corresponded to a mouthful of water and converted the data in the four response categories to volumes of water ingested. Depending on the water type, adult men swallowed, on average, 0.027–0.034 L per swimming event and women swallowed 0.018–0.023 L. Children swallowed more than adults on average, 0.031–0.051 L per swimming event (Schets et al. 2011). Although the incidental ingestion data reported by Schets et al. (2011) were

based primarily on participant-reported estimates, the mean values were similar to those reported in Dufour et al. (2006).

Schijven and de Roda Husman (2006) studied sport and occupational diver incidental ingestion. The types of water studied for occupational divers (n = 37 divers) were open sea and coastal marine water, and freshwater. For sport divers (n = 483 divers), the types of water considered were open sea and coastal marine water, fresh recreational water, canals and rivers, city canals, and swimming pools. The divers were asked to estimate how much water they swallowed in terms of: none, few drops, shot glass, coffee cup, or soda glass. The authors translated the description of volumes from the questionnaires into average volumes. Occupational divers reported incidentally ingesting more water per dive in marine water (mean: 0.0098 L/dive; maximum: 0.1 L/dive) compared to freshwater (mean: 0.0057 L/dive; maximum: 0.025 L/dive). Sports divers wearing an ordinary diving mask reported incidentally ingesting the most water per dive in swimming pools (mean: 0.02 L/dive; maximum: 0.19 L/dive), followed by recreational freshwater (mean: 0.013 L/dive; maximum: 0.19 L/dive) and coastal marine water (mean: 0.0099 L/dive; maximum: 0.19 L/dive). Sports divers wearing a full face mask reported incidentally ingesting less water than sports divers wearing an ordinary diving mask. The age of the divers was not included in the study report. Duration of dives was also not reported.

Dorevitch et al. (2011) evaluated incidental ingestion associated with multiple types of water contact activities in both surface water and in pools. Volume of ingestion was self-reported via interviews (3,367 participants), and the authors used a subset of the pool exposures to assess cyanuric acid in urine to determine the accuracy of the self-reported ingestion volumes. There was strong agreement between self-reported results and cyanuric acid measurement (none = 0.0014±0.008 L; drop to teaspoon = 0.0094±0.011 L; mouthful = 0.026±0.037 L). In surface water, participants ages 6 and above incidentally ingested the most water while canoeing and capsizing compared to any other activity assessed (median = 0.0036 L; mean = 0.006 L; Upper 95 percent CI = 0.0199 L). In swimming pool water, participants ages 6 and above incidentally ingested the most water while swimming compared to any other activity assessed (median = 0.006 L; mean = 0.01 L; Upper 95 percent CI = 0.0348 L). Swimmers in a pool were more than 50 times as likely to report swallowing a teaspoon of water compared to people who canoed or kayaked in surface waters. Duration of activities was not reported, so the ingestion volumes are on a per event basis.

Additional estimates of incidental water ingestion rates while swimming in pools have been identified by EPA's Office of Pesticide Programs (OPP). OPP calculates people's exposures to pool chemicals while they swim using its SWIMODEL (U.S. EPA 2003). SWIMODEL uses incidental ingestion values for children that are twice the values used for adults. Incidental ingestion rates among adults while swimming competitively and noncompetitively are 0.0125 L/hr and 0.025 L/hr, respectively. The model assumes an incidental ingestion rate of 0.050 L/hr for children ages 7–10 and 11–14 years while swimming noncompetitively. The 0.050-L/hr value is the value used in EPA OPP's Standard Operating Procedures (2000b) and is based on recommendations from EPA's *Risk Assessment Guidance for Superfund*, Part A (ACC 2002; U.S. EPA 1989; U.S. EPA 1997; U.S. EPA 2000b; U.S. EPA 2003). SWIMODEL assumes that noncompetitive swimmers incidentally ingest water at twice the rate as competitive swimmers, which is based on recommendations from ACC (2002), which is unpublished.

Table 7-1. Studies of Incidental Ingestion Volumes or Rates While Recreating

Reference	Number of Participants, Water Type	Recreational Activity	Measurement Methodology	Measure-ment Parameter	Parameter Provided	Age Group(s)	Value	Mean Duration of Event	Mean Rate of Ingestion (mL/hr)
Dufour et al. (2006)	n = 53 Swimming pool	Swimming	Cyanuric acid was measured in pool water and urine samples	Ingestion volume per event	Mean	Children (6–<18 years old)[a]	37 mL	≥ 45 min	49
						Adults	16 mL		21
						All ages	32 mL		43
Evans et al. (2006)	n = >500 Swimming pool	Swimming	Cyanuric acid was measured in pool water and urine samples, and ingestion rate was calculated based on duration of swimming event	Ingestion volume per event	Mean (upper 95 percent CI)	Children (6–18 years old)[a]	47 mL (142 mL)	≥ 45 min	63
					Mean (95 percent CI)	Adults	24 mL (2–84 mL)		32
				Ingestion rate	Mean	6–15 years	42 mL/hr		42
						16+ years	28 mL/hr		28
						Children and adults	33 mL/hr		33
Dorevitch et al. (2011)	n = 3,367 Surface water	Canoeing and capsizing	Estimates of amount of water swallowed were self-reported	Ingestion volume per event	Median; Mean (upper 95 percent CI)	6+years[b]	3.6 mL; 6 mL (19.9 mL)	No duration constraints	-
		Kayaking and capsizing					2.9 mL; 5 mL (16.5 mL)		-
	Swimming pool	Swimming	Estimates of amount of water swallowed were self-reported; cyanuric acid was measured in urine in a subset of participants				6.0 mL; 10 mL (34.8 mL)	60 min	10
		Kayaking and capsizing					4.8 mL; 7.9 mL (26.8 mL)		7.9
		Canoeing and capsizing					3.9 mL; 6.6 mL (22.4 mL)		6.6
Schets et al. (2011)	n = 8,000 adults, 1,924 children Freshwater	Swimming	Descriptive estimates of the amount of water swallowed were self-reported by participants or parents of participants, and estimates were converted to volumes	Ingestion volume per event	Mean (95 percent CI)	0–14 years[a]	37 mL (0.14–170 mL)	79 min	28
						Adults, males	27 mL (0.016–140 mL)	54 min	30
						Adults, females	18 mL (0.022–86 mL)		20
	Marine water					0–14 years[a]	31 mL (0.08–140 mL)	65 min	29
						Adults, males	27 mL (0.016–140 mL)	45 min	36

Reference	Number of Participants, Water Type	Recreational Activity	Measurement Methodology	Measure-ment Parameter	Parameter Provided	Age Group(s)	Value	Mean Duration of Event	Mean Rate of Ingestion (mL/hr)
	Swimming pool					Adults, females	18 mL (0.022–90 mL)	41 min	26
						0–14 years[a]	51 mL (0.62–200 mL)	81 min	38
						Adults, males	34 mL (0.022–170 mL)	68 min	30
						Adults, females	23 mL (0.033–110 mL)	67 min	21
Suppes et al. (2014)	n = 38 Swimming pool	Swimming	Cyanuric acid was measured and total time in water was quantified using videos to adjust ingestion volumes to rates; authors adjusted Ingestion volumes to correct for potential false positive measurements from cyanuric acid carry-over between sample injections	Ingestion rate, adjusted	Mean (Standard deviation); Range	Children (5–17 years old)[a]	26 mL/hr (29 mL/hr); 0.9–106 mL/hr	≥ 45 min	26
						Adults	3.5 mL/hr (11.7 mL/hr); 0–51 mL/hr		3.5
						Children and adults	14 mL/hr (24 mL/hr); 0–106 mL/hr		14
				Ingestion rate, unadjusted	Mean; Maximum	Children (5–17 years old)[a]	59 mL/hr; 225 mL/hr		59
					Mean	Adults	9 mL/hr		9
						Children and adults	32 mL/hr		32
Schijven and de Roda Husman (2006)	n = 37 Freshwater	Diving, occupational	Descriptive estimates of the amount of water swallowed were self-reported, and estimates were converted to volumes	Ingestion volume per event	Mean; Maximum	Adults	5.7 mL; 25 mL	60–95 min	3.6–5.7
	Marine water						9.8 mL; 100 mL		6.2–9.8
	Coastal marine water[4]						12 mL; 100 mL		7.6–12
	n = 483 Swimming pool	Diving, recreational with ordinary diving mask					20 mL; 190 mL	42–52 min	23–29
	Recreational freshwater						13 mL; 190 mL		15–19
	Coastal marine water						9.9 mL; 190 mL		11–14
	Swimming pool						13 mL; 190 mL		15–19

Reference	Number of Participants, Water Type	Recreational Activity	Measurement Methodology	Measure-ment Parameter	Parameter Provided	Age Group(s)	Value	Mean Duration of Event	Mean Rate of Ingestion (mL/hr)
	Coastal and open marine water	Diving, recreational with full face mask					1.3 mL; 15 mL		1.5–1.9

[a] Data cannot be separated by different age groups among children.
[b] Results were not reported in children and adult categories.

Although these studies used different methodologies and have limitations with respect to reporting information for different age group categories, their results corroborate the Dufour et al. (2006) data. Similar to Dufour et al. (2006), the studies that included children confirmed that children ingested more than adults. The freshwater ingestion results reported by Schets et al. (2011) included parent estimates of children's ingestion of water while swimming in freshwater that are most similar to the Dufour et al. (2006) findings. Schets et al. (2011) found a mean ingestion volume for children aged 0 to 14 years of 37 mL, which is the same as the mean ingestion volume reported by Dufour et al. (2006) for children. The adult self-reported ingestion volumes in Schets et al. (2011) were also similar to the Dufour et al. (2006) adult value. Schets et al. (2011) reported adult values ranging from 18 and 27 mL for females and males, respectively, while Dufour et al.'s adult ingestion volume was 16 mL. Schijven and de Roda Husman (2006) found adult divers mean ingestion volumes while diving recreationally in a swimming pool or in freshwater ranged between 13 and 20 mL, varying depending on mask type used. Dorevitch et al. (2011) also evaluated self or parent estimates of ingestion volumes while swimming and found a mean ingestion volume for all ages of 10 mL. Suppes et al. (2014) used a similar measurement method as Dufour et al. (2006), i.e., measuring cyanuric acid as an indicator of pool water ingestion, to estimate the amount of water ingested by 16 children ages 5 to 17 years. After adjustment for false positives, the mean rate at which child participants ingested water was 26 mL/hr, just about half of the Dufour et al. (2006) normalized ingestion rate of 50 mL/hr.

7.4 Distribution of Potential Recreational Health Protective Values by Age

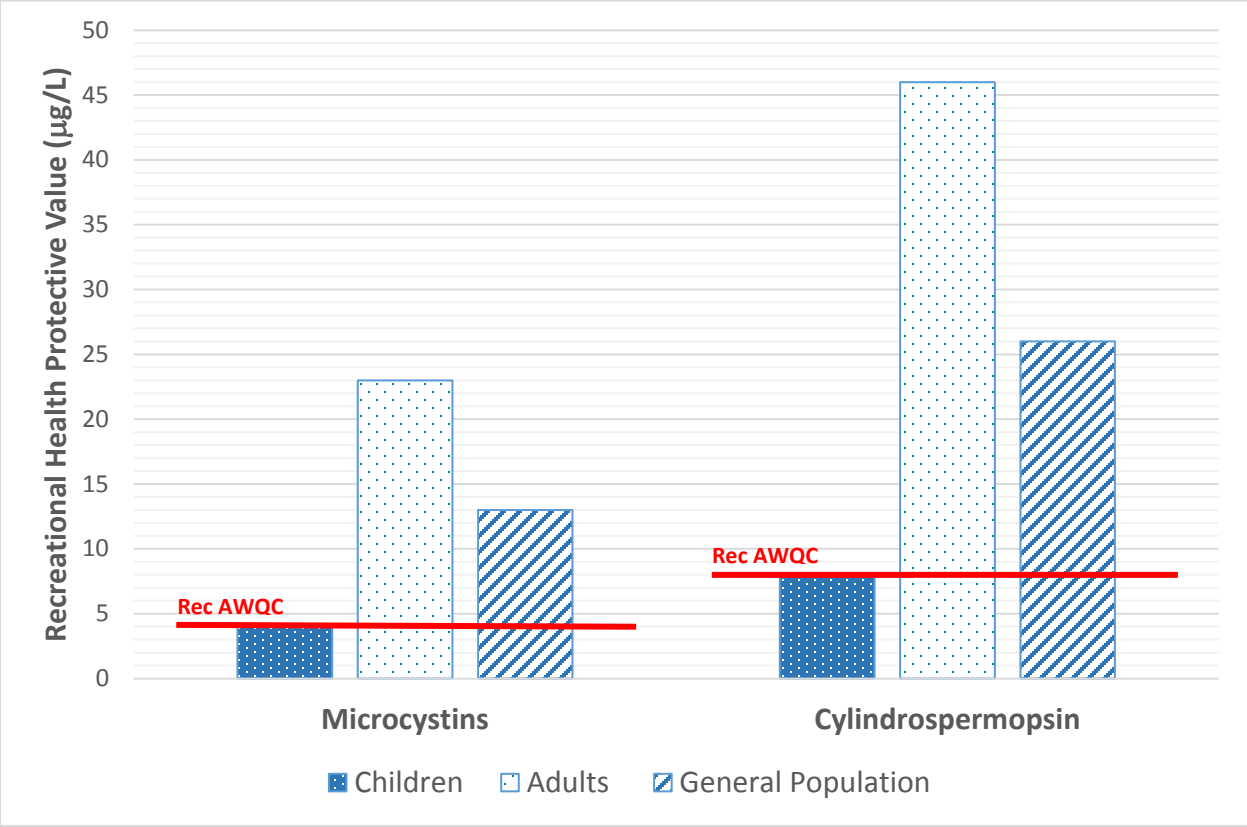
To evaluate the parameters used to calculate the cyanotoxin recreational AWQC, EPA compiled and evaluated available information for various lifestages. This section discusses potential health protective values for children and adults based on alternative data sets (section 7.4.1) and considers younger children's exposure parameters (section 7.4.2).

7.4.1 Evaluation of Criteria Related Lifestages

Using the ingestion rates for each age-group from EPA's *Exposure Factors Handbook* (U.S. EPA 2011), EPA estimated recreational health protective values for microcystins and cylindrospermopsin (plotted on Figure 7-1) to demonstrate the variability due to body weight, recreational water incidental ingestion, and exposure duration by lifestage.

EPA derived the recreational AWQC based on children's recreational exposures because this life stage has higher recreational exposures relative to adult recreators and the general population as a whole (i.e., all ages). As Figure 7-1 demonstrates, the calculated values for children (4 µg/L for microcystins and 8 µg/L for cylindrospermopsin) are protective of adults and the general population. EPA calculated for comparison recreational health protective values for adults using (1) 80 kg as the body weight (U.S. EPA 2011), (2) the maximum observed incidental ingestion value for adults (0.07 L/h) which EPA's *Exposure Factors Handbook* (2011) recommended due to the limited size of the data set, and (3) the mean recreational exposure duration for the 18- to 64-year age group (2.0 hr/d) (U.S. EPA 1997). The estimated recreational health protective values for adults are 23 µg/L for microcystins and 46 µg/L for cylindrospermopsin, Therefore, the recreational criteria and swimming advisories EPA calculated to be protective of children are protective of adults.

Figure 7-1. Comparison of Recreational Health Protective Values for Microcystins and Cylindrospermopsin for Children, Adults, and General Population



The parameters used to calculate health protective values for children include incidental ingestion values for children less than 18 years, mean body weight for children ages 6 to 11 years, and recreational exposure duration for children ages 5 to 11 years. The Dufour et al. (2006) incidental ingestion data are limited to children older than 6 years but less than 18 years. Schets et al. (2011) surveyed individuals 15 years and older to estimate their incidental ingestion of freshwater while recreating and asked those who had children to estimate incidental ingestion of their oldest child aged 0 to < 15 years. The ingestion volumes were initially binned into exposure classes and then translated into volumes using the results of a second study that quantified the distribution of volumes associated with ingested mouthfuls of water (Schets et al. 2011). The Schets et al. (2011) results for ages 0 to < 15 years were similar to estimates for 6 to < 18 years in Dufour et al. (2006). In both studies, children ingested more than adults on average and in the range of volumes (see Table 7-1). Based on the qualitative nature of the data available for the youngest children and given that the mean values were similar, EPA concludes that the values reported in the *Exposure Factors Handbook* are protective of children of all ages, including those younger than 6 years.

Evans et al. (2006) reported results of a full-scale study using the same methodology reported in Dufour et al. (2006); results of this study were presented by Evans et al. (2006) at an EPA recreational waters conference in 2006. The full-scale study included a study population sufficient to break out age categories that included younger children (6 to 10 years old), older children (11 to 17 years old), and adults. The number of study participants in the Dufour et al.

(2006) pilot study was 53, while their full-scale study evaluated more than 500 participants (Evans et al. 2006). Similar to the results reported in the pilot study, children (< 18 years old) ingested significantly more than adults. Additionally, Evans et al. (2006) reported that younger children (6 to 10 years old) ingest significantly more than older children (11 to 17 years old), or adults. Data quality standards require EPA to use independently peer reviewed and published data within our recommendations. Until this data set is published, EPA cannot include it in its analysis.

Table 7-2 presents a comparison of the daily ingestion rates (i.e., hourly ingestion rate times the exposure duration in hours) for the Dufour et al. (2006) study compared to Evans et al. (2006). While rates for "children" (< 18 years old) are similar between the studies, the ingestion rates using information from the newer study and duration rates from the EPA's *Exposure Factors Handbook* (2011) indicate a significantly higher exposure for younger children compared to older children or adults. A Kruskal-Wallis statistical test indicated that ingestion rates differed significantly between groups (p-value < 0.001). The pairwise Wilcoxon test with Bonferroni correction also indicated that ingestion rates in younger children (aged 6 to 10) were significantly different from ingestion rates in older children (p-value < 0.001). However, there is no difference between ingestion rates between older children (aged 11 to 17) and adults (p-value > 0.05).

Table 7-2. Comparison of Daily Ingestion Rates While Recreating between Dufour et al. (2006) and Evans et al. (2006)[a]

Age Group	Parameter Type[b]	Dufour et al. (2006) Based Daily Ingestion Rate (L/d)		Evans et al. (2006) Based Daily Ingestion Rate (L/d)		
Children	Mean	6 to < 18 years	0.13	0.14	6 to 10 years	0.22
					11 to 17 years	0.09
	Upper percentile		0.33	0.34	6 to 10 years	0.50
					11 to 17 years	0.23
Adults	Mean	18+ years	0.04	0.06		
	Upper percentile		0.14	0.12		

[a] The results reported in Evans et al. (2006) are not yet published. It is EPA's policy to use peer-reviewed study results to inform its regulatory efforts. The Evans et al. (2006) results are included within this effects characterization to provide context to the parameter values EPA used in the criteria derivation and because these results were presented publicly at the National Recreational Water Conference in 2006.

[b] The calculations of daily ingestion rate all used the mean exposure duration; the parameter type refers to the hourly ingestion rate.

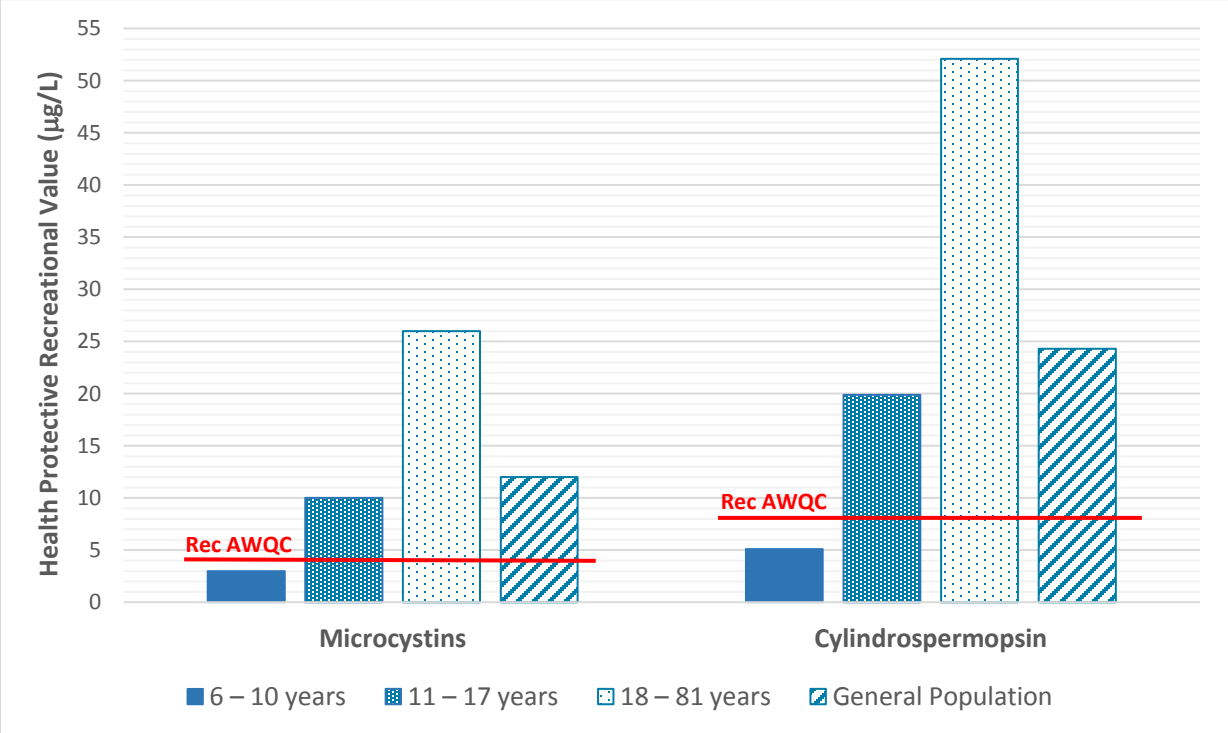
Table 7-3 presents alternative recreational values derived based on the more specific children's age groups available from Evans et al. (2006). Figure 7-2 provides a chart of this information for comparison purposes.

Table 7-3. Alternative Recreational Criteria Values for Microcystins and Cylindrospermopsin Calculated based on Alternative Ingestion Data from Evans et al. (2006)

Age Group	Body Weight (kg)	Ingestion Rate (L/d)[a]	Alternative Health Protective Recreational Cyanotoxin Value (µg/L)	
			Microcystins	Cylindrospermopsin
Children 6 to 10 years	31.8	0.50	3	5
Children 11 to 17 years	56.8	0.23	10	20
Adults 18 to 64	80	0.12	26	52
General Population	60	0.20	12	24

[a] Ingestion rate is the product of incidental ingestion volume normalized to one hour (L/hr) and the recreational duration (hr/d). Scenario uses mean body weight and ingestion rate based on upper percentile or maximum value for ingestion rate from EPA's *Exposure Factors Handbook* (U.S. EPA 2011) and mean value for recreational exposure duration in the older version of EPA's *Exposure Factors Handbook* (U.S. EPA 1997).

Figure 7-2. Comparison of Alternative Health Protective Recreational Values and Recreational AWQC for Microcystins and Cylindrospermopsin Calculated based on Evans et al. (2006)[a]



[a] The results reported in Evans et al. (2006) are not yet published. It is EPA's policy to use peer-reviewed study results to inform its regulatory efforts. The Evans et al. (2006) results are included within this effects characterization to provide context to the parameter values EPA used in the criteria derivation and because these results were presented publicly at the National Recreational Water Conference in 2006.

7.4.2 Evaluation of Younger Children's Exposure Factors

In the calculation of the cyanotoxin values reported in section 6, EPA utilized exposure parameters reported in the *Exposure Factors Handbook* (U.S. EPA 1997; U.S. EPA 2011). Information on children's mean body weights were available for children's age groups including 0 to 1 year, as well as 1 to < 2 year, 2 to < 3 years, etc. Using the body weight data provided in U.S. EPA (2011), weighted mean for the age groups 0 to < 6 years and 1 to < 6 years were calculated.

The available values from the *Exposure Factors Handbook* (1997, 2011) for incidental ingestion volume and exposure duration, however, were limited to specific age ranges. For incidental ingestion, the data reported were limited to children 6 years old and older. U.S. EPA (2011) recommends using the 97th percentile ingestion volume for children < 18 years based on the Dufour et al. (2006) measured incidental ingestion volume normalized to 1 hour. The Dufour et al. (2006) study did not include children younger than 6 years. The 97th percentile is recommended because the study had a small number of participants. U.S. EPA (1997) provided a recreational exposure duration for children ages 1 to 4 years (1.4 hr/d). This duration is shorter than the duration for children ages 5 to < 11 years (2.7 hr/d). Values for exposure duration were not available for children younger than 1 year.

To evaluate potential health-protective water quality values specifically for children younger than 6 years, EPA searched for additional exposure parameter information in the peer-reviewed and published scientific literature. Table 7-4 shows data availability and differences between the exposure parameters used in the microcystin and cylindrospermopsin recreational AWQC calculation (ages 6 to < 11 years) and estimates for younger lifestages. The younger lifestages include children aged 0 to 6 years, children aged 1 to 6 years, and children younger than 1 year.

EPA found one other study that characterized incidental ingestion for children. Schets et al. (2011) reported incidental ingestion volumes for children ages 0 to < 15 years. However, the study did not further divide this cohort into younger children and older children. These data for children represent parental estimates of volumes of freshwater incidentally ingested by their children. The ingestion volumes were initially binned into exposure classes and then translated into volumes using the results of a second study that quantified the distribution of volumes associated with ingested mouthfuls of water (Schets et al. 2011). Because of the initial binning and then the translation step to arrive at a distribution of ingestion volume for each exposure class, there is some uncertainty associated with the estimates. However, these estimates represent a different methodological approach compared to the approach used by Dufour et al. (2006). To facilitate comparing the results between the studies, EPA calculated an hourly incidental ingestion volume based on the Schets et al. (2011) data using the mean freshwater recreational durations reported in the same study. Because this study was not limited in size, as was the case with Dufour et al. (2006), EPA calculated both the 90th percentile and 97th percentile hourly ingestion volume. The 97th percentile was calculated to facilitate a more direct comparison with the results from Dufour et al. (2006) and the 90th percentile was calculated to provide a value that would typically be used to calculate health-protective values for a pollutant (U.S. EPA 2000a).

Table 7-4. Comparison of Younger Children's Exposure Factors and Incidental Ingestion Data Sets

Exposure Parameter	Used in Recreational AWQC Calculation	Dufour et al. (2006) Incidental Ingestion Data			Schets et al. (2011) Incidental Ingestion Data		
		Children 1 to < 6 years	Children 0 to < 6 years	Children < 1 year	Children 1 to < 6 years	Children 0 to < 6 years	Children < 1 year
Body weight (U.S. EPA 2011)	31.8 kg = mean body weight of children 6 to < 11 years	15.6 kg = weighted mean body weight of children 1 to < 6 years	13.4 kg = weighted mean body weight of children 0 to < 6 years	7.8 kg = weighted mean body weight of children 0 to < 1 year	15.6 kg = weighted mean body weight of children 1 to < 6 years	13.4 kg = weighted mean body weight of children 0 to < 6 years	7.8 kg = weighted mean body weight of children 0 to < 1 year
Incidental ingestion volume normalized to incidental ingestion per hour	0.12 L/hr = upper 97th percentile calculated based on study that included children 6 to < 18 years[a]				0.07 L/hr (90th percentile ingestion volume) 0.12 L/hr (97th percentile ingestion volume) calculated based on parent surveys for children 0 to < 15 years[b]		
Recreational exposure duration (U.S. EPA 1997)	2.7 hr/d = mean recreational exposure duration for children ages 5 to 11 years	1.4 hr/d = mean recreational exposure duration for children ages 1 to 4 years[c]					
Ingestion rate	0.33 L/d	0.17 L/d			0.10 L/d (90th percentile ingestion volume) 0.17 L/d (97th percentile ingestion volume)		

[a] Hourly ingestion rate for children is from EPA *Exposure Factors Handbook* (2011) Table 3-5: Ingestion of Water and Other Select Liquids from Dufour et al. (2006). The Dufour et al. (2006) pilot study measured incidental ingestion of water of participants who spent time swimming or playing in a swimming pool for at least a 45-minute period (n = 53; 41 children ages 6 to < 18 years; 12 adults > 18 years). This study did not include children younger than 6 years. U.S. EPA (2011) reported an hourly ingestion rate, which EPA calculated by normalizing the Dufour et al. (2006) ingestion volume per 45 minutes to an ingestion volume per hour (hourly ingestion rate) and also recommended using the 97th percentile as the "upper percentile" for children.

[b] Hourly ingestion rate for children is based on Schets et al. (2011) survey of Dutch parents' estimates of recreational duration and incidental ingestion volume while recreating in surface water; 486 of the survey respondents reported their children recreated in fresh water. The incidental ingestion volume 90th and 97th percentiles for children, 0.10 and 0.16 L/event, respectively, were calculated based on the distribution parameter reported in the paper. These volumes per event were normalized to an hourly ingestion rate by dividing these values by the mean duration per recreational event reported for children by Schets et al. (2011), which was 79 minutes or 1.3 hours.

[c] Recreational exposure duration values reported in EPA's *Exposure Factors Handbook* (1997) are limited to children > 1 year old. Children aged 1 to 4 years are the youngest life stage for which duration data are reported in Table 15-119 of U.S. EPA (1997).

When comparing the values for the normalized mean hourly ingestion volume (Table 7-4), both studies estimated a similar incidental ingestion volume of 0.12 L/hr. This is notable because Dufour et al. (2006) characterized the 6 to < 18-year age group and Schets et al. characterized the 0 to < 15-year age group. The similar volumes between the cohorts could indicate that the children younger than 6 years old were not contributing substantially to the distribution of incidental ingestion volume. Likewise, the same could be said for the 15 to 18-year age group. The results in Table 7-2 provide evidence that 6 to 10-year-old children incidentally ingest significantly more than 11 to < 18-year-old children or adults. The 90th percentile for incidental ingestion by 0 to < 15-year-old children reported by Schets et al. (2011) is approximately 40 percent lower than the 97th percentile volume for 6 to < 18-year-old children reported by Dufour et al. (2006).

EPA relied on the incidental ingestion volume recommended in the *Exposure Factors Handbook* (2011), which discusses the use of the 97th percentile ingestion volume reported in Dufour et al. (2006), because that study directly quantifies the water incidentally ingested while recreating. EPA included the Schets study in this discussion because it provides valuable context for characterizing children's incidental ingestion while recreating.

For children younger than 1 year, specific information is only available for body weights. Combining this parameter with ingestion volumes and exposure duration times reported for older age groups creates an exposure profile that would not seem to be representative of this early life stage. This combination of factors is presented in Table 7-4 for comparison purposes only. The available information is a better fit for children 1 to < 6 years.

Children 1 to 4 years are exposed for less time compared to children 5 to 11 years old, 1.4 hr/d compared to 2.7 hr/d, respectively (U.S. EPA 1997). Calculating the mean incidental ingestion rate per day for children younger than 6 years old based on results from Dufour et al. (2006) (0.17 L/d) or Schets et al. (2011) (0.10 L/d) results in lower estimated mean incidental ingestion rates compared to children ages 6 to < 11 years (Table 7-4). However, these estimates have large uncertainties given the lack of measured incidental ingestion data specifically for children younger than 6 years. Information on exposure durations for children < 1-year-old is also lacking. Because ingestion rates are greatest for 5 to 11 year olds, EPA concluded that calculating the ingestion rate using a higher duration was protective of children younger than 6 years old as indicated in Table 7-4 (Dufour et al. 2006; Schets et al. 2011).

7.5 Other Recreational Exposures

This section compares primary and secondary contact exposures and discusses tribal considerations for cyanotoxin and cyanobacterial cell exposure.

7.5.1 Other Recreational Exposure Pathways

EPA selected primary contact activities and incidental ingestion of water as the primary exposure pathway for derivation of the recreational criteria and swimming advisories. Alternative exposure parameter data could be considered in this approach as described in section 7.4. In this section, EPA evaluated potential cyanotoxin exposures via inhalation of aerosols and dermal contact. Inhalation and dermal toxicity data were not available; however, there are limited available data to estimate inhalation and dermal exposure. EPA conducted analyses to estimate inhalation and dermal exposure and compared those estimates to incidental ingestion of the

cyanotoxins while recreating. Section 7.5.1.1 compares recreational ingestion and inhalation exposures to microcystins. Similarly, section 7.5.1.2 compares recreational ingestion and dermal exposure.

7.5.1.1 Inhalation of Cyanotoxins

Volatilization of microcystins and cylindrospermopsin from water to air is not expected due to their size and charges. Both cyanotoxins are rather large compared to volatile chemicals. Microcystins' acid groups are charged at the pH of normal surface waters. Cylindrospermopsin features both negative and positive changes and like other zwitterions, do not volatilize significantly into the air from water (Butler et al. 2012).

EPA did an analysis to determine if the criteria/swimming advisory values based on incidental ingestion are protective of recreational inhalation exposures. Although the recreational use is primary contact recreation, such as swimming, data are available for secondary contact activities such as jet skiing or boating and white-capped wave, bubble-bursting action, which can result in cyanotoxins becoming aerosols (microscopic liquid or solid particles suspended in air). Cheng et al. (2007) collected via personal samples and found that volunteers recreating on a lake with a 1 µg/L concentration in water were exposed to air concentrations of microcystin-LR of approximately 0.08 ng/m^3 in their breathing zone.

Using the information from Cheng et al. (2007) and inhalation exposure parameters provided in EPA's *Exposure Factors Handbook* (2011), EPA compared the microcystin inhaled dose (ng/d) to the ingested dose. The parameters and calculations for this analysis are presented in Table 7-5. Using conservative assumptions for inhalation rates (i.e., moderate intensity and 95th percentile) and inhalation exposure duration (i.e., 5 hr/d) and comparing with mean incidental ingestion rates, the estimated ingested dose is 151 times higher than the estimated inhaled dose for children and 43 times higher than the estimated inhaled dose for adults.

This analysis supports the conclusion that inhalation exposure is negligible compared to incidental ingestion while recreating. The inhalation *toxicity* is unknown for microcystin, but if it is equal to ingestion toxicity, the values based on oral ingestion should be protective of recreational inhalation exposures. EPA did not conduct a similar analysis for cylindrospermopsin because published measured air concentration data for this cyanotoxin were not available.

The California Environmental Protection Agency (CalEPA) came to a similar conclusion for water skiers (Butler et al. 2012). They cited Cheng et al. (2007) and noted that their results showed that a liter of water contains 700,000 to 800,000 times the amount of cyanotoxins as in a cubic meter of air. CalEPA calculated that this concentration is equivalent to 1.3 to 1.4 µL aerosolized microcystin/m^3. Compared to the ingestion assumptions used for swimmers in the calculation of their recreational guideline (i.e., 50 mL/hr), CalEPA calculated that a water-skier would have to inhale at least 35,000 m^3/hr while skiing to achieve a dose equal to the swimmer, which is 17,000 times the inhalation rate of a marathon runner. CalEPA concluded that a water skier would not inhale enough aerosol to receive a dose similar to what a swimmer gets from ingestion.

Table 7-5. Comparison of Recreational Exposure Ingested Dose to Inhaled Dose of Microcystin

Age Group	Inhalation Rate (m^3/min)[a]	Inhalation Rate per Hour (m^3/hr) [volume per min × 60 min/hr]	Duration of Inhalation Exposure per Day (hr/d)	Daily Inhalation Rate Adjusted for Duration of Exposure (m^3/d)	Concen-tration in Air (ng/m^3)[b]	Inhaled Dose (ng/day) [daily rate × conc. in air]	Inhaled Dose (μg/day)	Ingestion Rate (L/d)	Concen-tration in Water (μg/L)	Ingestion Dose (μg/day) [water conc. × daily ingestion rate]	Ratio of Ingested Dose to Inhalation Dose [ingested dose/inhaled dose]
Assumed 95th percentile short-term inhalation exposure, moderate intensity activity level inhalation rate (U.S. EPA 2011), a 24-hour inhalation exposure duration, and mean ingestion rate (U.S. EPA 2011), mean recreational exposure duration (U.S. EPA 1997), and water concentration of 1 μg/L (Cheng et al. 2007)											
Children	0.037	2.2	5.0	53	0.08	4.3	0.004	0.13	1	0.13	151
Adults	0.040	2.4	5.0	58	0.08	4.6	0.005	0.04	1	0.04	43

[a] EPA's *Exposure Factors Handbook* (2011) did not report recommended short term, moderate intensity activity level inhalation rate values for children or adults in aggregate; used highest inhalation rate listed for children and adult age groups for this conservative screen. For children, it was the age group 16 to < 21 years, and for adults, it was 51 to < 61 years.

[b] Cheng et al. (2007) measured 0.08 ng/m^3 in air near surface waters with a concentration of 1 μg/L microcystins. Assuming a linear relationship of water concentration to air concentration based on Cheng et al. (2007), the concentration in air at the recreational AWQC concentration for microcystins (i.e., 4 μg/L) is calculated by multiplying 4 μg/L by the ratio of (0.08 ng/m^3)/(1 μg/L), which equals 0.32 ng/m^3.

Another comparison considers spray exposures from jet-ski and boat spray. Sinclair et al. (2016) modeled a water-spray exposure scenario and observed much lower exposures than those resulting from swimming or limited-contact recreational activities reported in the previous study. Thus, EPA expects that the comparison above based on exposure from secondary contact recreation is protective of primary contact recreation. Sinclair et al. (2016) also measured urinary concentrations of cyanuric acid after 26 participants' exposure to spray in a simulated 10-minute car wash situation. Each subject wore a protective coverall with hood, vinyl gloves, waterproof footwear, and safety glasses to ensure that only their face and mouths were exposed. The estimated median and 90th percentile ingestion volumes were 0.18 and 1.89 mL, respectively. Converted to a duration of 1 hour, the amounts would be 1.08 mL and 11.3 mL, which are much lower than the incidental ingestion intakes per hour.

7.5.1.2 Dermal Absorption

EPA did not find any peer reviewed measured data for microcystin or cylindrospermopsin dermal absorption. EPA's *Dermal Exposure Assessment: A Summary of EPA Approaches* (U.S. EPA 2007) states that to get through the skin, a chemical must dissolve into the stratum corneum, which is a stabilized lipid barrier; therefore, lipid solubility is required initially (U.S. EPA 2007).

EPA used the dermal exposure equations in its *Risk Assessment Guidance for Superfund* (U.S. EPA 2004) to estimate the potential absorbed dose of microcystins and compare it to the incidentally ingested dose. Octanol-water partition coefficients required by these equations are available for four microcystins, including microcystin-LR. Ward and Codd (1999) estimated the log octanol-water partition coefficients of microcystin-LR, -LY, -LW and -LF using high performance liquid chromatography (HPLC) as 2.16, 2.92, 3.46, and 3.56, respectively. Cylindrospermopsin dermal absorption could not be predicted due to the lack of these lipophilicity parameters.

The equation to estimate skin permeability coefficient from U.S. EPA (2004) is

$$\text{Log } K_p = -2.80 + 0.66 \times \log K_{ow} - 0.0056 \times MW$$

Where:

K_p	=	Dermal permeability coefficient of compound in water (cm/hr)
K_{ow}	=	Octanol-water partition coefficient (dimensionless)
MW	=	molecular weight (g/mole)

The equation to estimate dermal absorbed dose for highly ionized organic chemicals from U.S. EPA (2004) is:

$$DA = K_p \times Cw \times t$$

Where:

DA	=	Absorbed dose per event (mg/cm^2-event)
K_p	=	Dermal permeability coefficient of compound in water (cm/hr)

Cw	=	Chemical concentration in water (mg/cm^3)
t	=	Event duration (hr/event)

The estimated microcystins absorbed dose based on these calculations and the exposure parameters used for microcystins are presented in Table 7-6. Although this analysis is based on very limited data, it supports the hypothesis that the dermal absorbed dose of microcystins is likely to be negligible compared to incidentally ingested doses during recreational activities.

CalEPA also concluded dermal absorption of microcystins and cylindrospermopsin while swimming is not expected to be significant due to the large size and charged nature of these molecules (Butler et al. 2012). CalEPA eliminated the dermal absorption pathway from its risk assessment of microcystins and cylindrospermopsin citing evidence that similarly large molecules such as antibiotics have not been able to be formulated in a way to penetrate the skin (Butler et al. 2012). A U.S. Army-contracted *in vitro* study by Kemppainen et al. (1990) measured microcystin dermal penetration in 48 hours through excised human abdominal skin and found 0.9 (±0.3) percent of the total dose in water penetrated through the skin; however, this study has not been peer reviewed.

7.5.2 Tribal Considerations

EPA considered alternative exposure scenarios tribal communities might have, given their cultural practices. Native American food foraging customs or cultural or religious ceremonies can put them into primary or secondary contact with cyanotoxins. Primary contact ceremonial use may include the use of a surface water body for religious or traditional purposes by members of a tribe, involving immersion and intentional or incidental ingestion of water (Eastman 2007).

It is uncertain whether these activities would lead to cyanotoxin exposures higher than the primary recreational contact assumptions for incidental ingestion and exposure duration used in this assessment.

7.6 Livestock and Pet Concerns

The world's first scientific report of adverse effects to animals from cyanobacteria was written by George Francis, who described in 1878 the rapid death of stock animals at Lake Alexandrina, a freshwater lake at the mouth of the Murray River in South Australia (Francis 1878). Since then, there have been numerous descriptions of mammal and bird mortalities associated with exposure to cyanobacteria (Backer et al. 2015; Hilborn & Beasley 2015). The literature throughout the 20th century includes reports from all inhabited continents (Stewart et al. 2008). However, the impacts of cyanotoxins on domestic and companion animals are likely under-recognized because many cases are misdiagnosed, few cases are biochemically confirmed, and even fewer are reported in the scientific literature or to animal health systems (Zaias et al. 2010).

Table 7-6. Comparison of Recreational Exposure Ingested Dose to Dermal Absorbed Dose of Microcystins

Microcystin	Log K_{ow} (Ward & Codd 1999)	Molecular Weight	Log Skin Perm-eability Coefficient (Log Kp)	Skin Perm-eability Coefficient (K_p) (cm/hr)	Chemical Conc. in Water (mg/cm^3) Assuming Rec AWQC Level	Event Duration[a] (hr/event) (mean for 5- to 11-year-olds)	Dermal Absorbed Dose per Event (mg/cm^2-event)	Total Body Surface Area (cm^2) (U.S. EPA 2011) 95th percentile Children 6 to < 11 Years	Dermal Absorbed Dose per Event (mg/event)	Dermal Absorbed Dose per Event (mg/event)	Ratio of Ingested Dose to Dermal Absorbed Dose
Microcystin-LR	2.16	995.17	-6.95	1.1E-07	4.00E-06	2.7	1.2E-12	1.48E+04	2E-08	2E-05	71,824
Microcystin-LY	2.92	1002.16	-6.48	3.3E-07	4.00E-06	2.7	3.6E-12	1.48E+04	5E-08	5E-05	24,764
Microcystin-LW	3.46	1025.2	-6.26	5.5E-07	4.00E-06	2.7	6.1E-12	1.48E+04	9E-08	9E-05	14,670
Microcystin-LF	3.56	986.16	-5.97	1.1E-06	4.00E-06	2.7	1.2E-11	1.48E+04	2E-07	2E-04	7,618

[a] Event duration is defined as 24-hour cumulative time spent at home in outdoor pool or spa as reported in EPA's *Exposure Factors Handbook* (U.S. EPA 1997).

Livestock and pets can potentially be exposed to higher concentrations of, or have increased exposure to, cyanotoxins than humans because they are known to consume cyanobacterial scum and mats and drink cyanobacteria-contaminated water (Backer et al. 2013). Dogs are additionally at risk, as they may lick cyanobacterial cells from their fur after swimming in a water body with an ongoing bloom. Mats and scums can represent thousand-fold to million-fold concentrations of cyanobacterial cell populations, and published microcystin concentrations have ranged up to 24 mg microcystin/L from scum material (Chorus & Bartram 1999). Common signs of HAB cyanotoxin poisonings in pets include repeated vomiting, diarrhea, loss of appetite, abdominal swelling, stumbling, seizures, convulsions, disorientation, inactivity, or skin rashes and hives (New York Sea Grant 2014; Trevino-Garrison et al. 2015). Although reports of livestock deaths are relatively rare, in extreme cases death can occur minutes after drinking from a contaminated water source. Acute symptoms of cyanotoxin poisoning can include loss of appetite, weakness, staggering, or inflammation of the muzzle, ear, or udder. Higher levels of cyanotoxins can lead to severe liver damage, the development of jaundice, and severe photosensitization. Often livestock or pets that recover from these ailments can then suffer from chronic failure to thrive (Australia Department of Economic Development Jobs Transport and Resources 2013; Robinson & Alex 1987).

7.6.1 States and Animal HAB Guidelines

A few states have guideline levels specific to the protection of animals from cyanotoxin poisoning (Appendix G). California has dog and cattle action levels for the cyanotoxins microcystin, anatoxin-a, and cylindrospermopsin (Butler et al. 2012). For both dogs and cattle, California estimated drinking water ingestion rates based on two publications by the National Research Council, Nutrient Requirements for Beef Cattle and Nutrient Requirements for Dogs and Cats. The animal specific RfD for each cyanotoxin was divided by the final water and cyanobacterial biomass intake exposure levels, providing a cyanotoxin concentration that would result in exposure at the RfD level or below. These calculations were performed for an acute (lethal) and a subchronic scenario. Oregon has dog-specific guideline values for the cyanotoxins anatoxin-a, cylindrospermopsin, microcystin, and saxitoxin based on the CalEPA method. However the dog-specific guideline value for saxitoxins was modified by applying an uncertainty factor for interspecies differences in sensitivity between humans (the species in the critical study) and dogs (Oregon Health Authority 2016). Grayson County in Texas gives information for domestic animals at current advisory levels for microcystin and cylindrospermopsin. Advisories levels of 20 ppb for microcystin and cylindrospermopsin are calculated as gallons of water that can be consumed for 10 and 80 pound dogs that will cause a lethal or near-lethal dose. This does not include additional dose amounts that could be ingested by a dog while self-grooming cyanobacteria scum off its fur (Lillis et al. 2012).

Other states mention animal poisoning in their guideline documents but do not give guideline values specific to livestock or companion animals. For example, Utah and Washington report that animal illness or death can be reason to issue or accelerate a HAB advisory warning (Hardy & Washington State Department of Health 2008; Utah Department of Environmental Quality and Department of Health 2015). However, Ohio issues the disclaimer that thresholds used are protective of human exposure and may or may not be protective of animals such as dogs or livestock (Kasich et al. 2015). Several other states including Connecticut, Idaho, Kansas, Massachusetts, Nebraska, Vermont, and Virginia provide informational pamphlets, warn about

harm to pets or other animals, or post about harm to animals in their beach warnings and advisory signage (Connecticut Department of Public Health: Connecticut Energy Environment 2013; IDEQ 2015; Kansas Department of Health and Environment 2016; Massachusetts Bureau of Environmental Health 2015; Nebraska Department of Environmental Quality and Nebraska Department of Health and Human Services: Division of Public Health 2016; Vermont Department of Health 2015; Virginia Department of Health 2012).

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APPENDIX A. INTERNATIONAL RECREATIONAL WATER GUIDELINES FOR CYANOTOXINS AND CYANOBACTERIA

Jurisdiction	Recreational Water Guideline Level	Recommended Action
Australia[a]	cyanobacteria (total): $\geq$ 10 mm^3/L (where known toxins are not present)	red level action mode; level 2 guideline: • Immediately notify health authorities for advice on health risk. • Make toxicity assessment or toxin measurement of water if this has not already been done. • Health authorities warn of risk to public health (i.e., the authorities make a health risk assessment considering toxin monitoring data, sample type and variability).
	cyanobacteria (total): $\geq$ 4 mm^3/L (where a known toxin producer is dominant in the total biovolume)	red level action mode; level 1 guideline: • Immediately notify health authorities for advice on health risk. • Make toxicity assessment or toxin measurement of water if this has not already been done. • Health authorities warn of risk to public health (i.e., the authorities make a health risk assessment considering toxin monitoring data, sample type and variability).
	cyanobacteria (total): $\geq$ 0.4 to < 10 mm^3/L (where known toxin producers are not present)	amber level alert mode: • Increase sampling frequency to twice weekly where toxigenic species are dominant within the alert level definition (i.e., total biovolume). • Monitor weekly or fortnightly where other types are dominant. • Make regular visual inspections of water surface for scums. • Decide on requirement for toxicity assessment or toxin monitoring.
	cyanobacteria (total): $\geq$ 0.4 to <4 mm^3/L (where a known toxin producer is dominant in the total biovolume)	amber level alert mode: • Increase sampling frequency to twice weekly where toxigenic species are dominant within the alert level definition (i.e., total biovolume). • Monitor weekly or fortnightly where other types are dominant. • Make regular visual inspections of water surface for scums. • Decide on requirement for toxicity assessment or toxin monitoring.
	cyanobacteria (total): $\geq$ 0.04 to <0.4 mm^3/L	green level surveillance mode: • Weekly sampling and cell counts at representative locations in the water body where known toxigenic species are present; or • Fortnightly for other types including regular visual inspection of water surface for scums.
	cyanobacterial scums consistently present	red level action mode; level 2 guideline: • Immediately notify health authorities for advice on health risk.

Jurisdiction	Recreational Water Guideline Level	Recommended Action
		• Make toxicity assessment or toxin measurement of water if this has not already been done. • Health authorities warn of risk to public health (i.e., the authorities make a health risk assessment considering toxin monitoring data, sample type and variability).
	microcystins (total): ≥ 10 µg/L	red level action mode; level 1 guideline: • Immediately notify health authorities for advice on health risk. • Make toxicity assessment or toxin measurement of water if this has not already been done. • Health authorities warn of risk to public health (i.e., the authorities make a health risk assessment considering toxin monitoring data, sample type and variability).
	Microcystis aeruginosa (total): ≥ 50,000 cells/mL	red level action mode; level 1 guideline: • Immediately notify health authorities for advice on health risk. • Make toxicity assessment or toxin measurement of water if this has not already been done. • Health authorities warn of risk to public health (i.e., the authorities make a health risk assessment considering toxin monitoring data, sample type and variability).
	Microcystis aeruginosa (total): ≥ 5,000 to < 50,000 cells/mL	amber level alert mode: • Increase sampling frequency to twice weekly where toxigenic species are dominant within the alert level definition (i.e., total biovolume). • Monitor weekly or fortnightly where other types are dominant. • Make regular visual inspections of water surface for scums. • Decide on requirement for toxicity assessment or toxin monitoring
	Microcystis aeruginosa (total): ≥ 500 to < 5,000 cells/mL	green level surveillance mode: • Weekly sampling and cell counts at representative locations in the water body where known toxigenic species are present; or • Fortnightly for other types including regular visual inspection of water surface for scums.
Canada[d]	cyanobacteria (total): ≥ 100,000 cells/mL	issue swimming advisory
	detection of a cyanobacterial bloom	issue beach closure
	microcystins (total): ≥ 20 µg/L (expressed as microcystin-LR)	issue swimming advisory

Jurisdiction	Recreational Water Guideline Level	Recommended Action
Cuba[c]	any report of toxic effect in humans or animals	action (in red): as for "Alert", but with increased actions for public communication
	benthic mats: < 40 percent coverage of surfaces with any cyanobacteria; > 20 percent with toxicogenic cyanobacteria; > 50 percent with potentially toxicogenic cyanobacteria (particularly where they are visibly detaching and accumulating in scum)	alert: increased sampling (weekly and more sites); daily inspection; notification to public health unit and local managers; report to local government; warning of the public
	cyanobacteria: < 500 cells/mL	monthly visual inspection
	cyanobacteria: ≥ 1 of the species known as potentially toxic	alert: increased sampling (weekly and more sites); daily inspection; notification to public health unit and local managers; report to local government; warning of the public
	phytoplankton cells: ≥ 20,000 to < 100,000 cells/mL, > 50 percent of cells cyanobacteria	alert: increased sampling (weekly and more sites); daily inspection; notification to public health unit and local managers; report to local government; warning of the public
	phytoplankton: > 0 to < 1,500 cells/mL	monthly visual inspection and sampling at least four months per year
	scum consistently present; confirmed bloom persistence	action (in red): as for "Alert", but with increased actions for public communication
Czech Republic[c]	cells: > 100,000 cells/mL	2nd warning level: closure for public recreation
	cells: > 20,000 cells/mL	1st warning level (not otherwise specified)
Denmark[c]	chlorophyll *a*: > 50 μg/L, dominated by cyanobacteria	relevant authorities are informed and decide when and how the public should be informed; warnings include signs, media and contact to local user groups such as kindergardens, scouts, water sports clubs
	visible surface scum	relevant authorities are informed and decide when and how the public should be informed; warnings include signs, media and contact to local user groups such as kindergardens, scouts, water sports clubs
European Union[f]	cyanobacterial proliferation (occurrence)	when cyanobacterial proliferation occurs and a health risk has been identified or presumed, adequate management measures shall be taken immediately to prevent exposure, including information to the public
	cyanobacterial proliferation (potential for)	appropriate monitoring shall be carried out to enable timely identification of health risks.

Jurisdiction	Recreational Water Guideline Level	Recommended Action
Finland[c]	algae (includes cyanobacteria): detected	level 1: Possibly microscopic examination and even toxin analysis if there is a specific cause such as very popular beach or reports of adverse health effects or animal deaths
	algae (includes cyanobacteria): high amount	level 2: Preferably microscopical examination; toxin analysis; warning of the public is compulsory
	algae (includes cyanobacteria): very high amount	level 3: Preferably microscopical examination; toxin analysis; warning of the public is compulsory
France[c]	bloom, scum, change in water color	microscopy examination. If cyanobacteria are absent: no further action. If present: counting and genus identification
	cyanobacteria: < 20,000 cells/mL (±20 percent)	active daily monitoring. Counting at least on a weekly basis. Normal recreational activity at the site
	cyanobacteria: > 100,000 cells/mL (±20 percent)	bathing and recreational activities are restricted. Public is informed.
	cyanobacteria: $\geq$ 20,000 to < 100,000 cells/mL (±20 percent)	active daily monitoring. Counting on a weekly basis. Recreationalactivities are still allowed; the public is informed by posters on site.
	microcystins (MC): 25 µg/L (±5 percent)	if MC < 25 µg/L bathing and recreational activities are restricted. If MC > 25 µg/L bathing is banned and recreational activities are restricted. In either case, public is informed.
	visible scum or foam in recreational or bathing area	all water activities in this area are prohibited. Restrictions do not necessarily apply to the whole recreational site. Other areas without scum may still be open.
Germany[c]	Secchi Disk reading > 1 m AND biovolume: < 1 mm^3/L	monitor further cyanobacterial development
	Secchi Disk reading > 1 m AND biovolume: $\geq$ 1 mm^3/L	publish warnings, discourage bathing, consider temporary closure
	Secchi Disk reading > 1 m AND chlorophyll *a* (with dominance by cyanobacteria): < 40 µg/L	monitor further cyanobacterial development
	Secchi Disk reading > 1 m AND chlorophyll *a* (with dominance by cyanobacteria): $\geq$ 40 µg/L	publish warnings, discourage bathing, consider temporary closure
	Secchi Disk reading > 1 m AND microcystins: < 10 µg/L	monitor further cyanobacterial development

Jurisdiction	Recreational Water Guideline Level	Recommended Action
	Secchi Disk reading > 1 m AND microcystins: ≥ 10 µg/L	publish warnings, discourage bathing, consider temporary closure
	visible heavy scums and/or microcystins: > 100 µg/L	publish warnings, discourage bathing, temporary closure is recommended
Hungary[c]	cell count: ≥ 50,000 to < 100,000 cells/mL	no recommended actions listed, water body classification: Acceptable
	cell count: < 20,000 cells/mL	no recommended actions listed, water body classification: Excellent
	cell count: ≥ 20,000 to < 50,000 cells/mL	no recommended actions listed, water body classification: Good
	cell count: ≥ 100,000 cells/mL	no recommended actions listed, water body classification: Unacceptable
	chlorophyll *a* (with dominance by cyanobacteria): < 10 µg/L	no recommended actions listed, water body classification: Excellent
	chlorophyll *a* (with dominance by cyanobacteria): ≥ 10 to < 25 µg/L	no recommended actions listed, water body classification: Good
	chlorophyll *a* (with dominance by cyanobacteria): ≥ 25 to < 50 µg/L	no recommended actions listed, water body classification: Acceptable
	chlorophyll *a* (with dominance by cyanobacteria): ≥ 50 µg/L	no recommended actions listed, water body classification: Unacceptable
	microcystins: ≥ 4 to < 10 µg/L	no recommended actions listed, water body classification: Good
	microcystins: ≥ 10 to < 20 µg/L	no recommended actions listed, water body classification: Acceptable
	microcystins: < 4 µg/L	no recommended actions listed, water body classification: Excellent
	microcystins: ≥ 20 µg/L	no recommended actions listed, water body classification: Unacceptable
Italy[c]	cyanobacterial cell count (combined with identification of genus and, if possible, species): < 20,000 cells/mL	if possible, daily visual observation; weekly counting
	cyanobacterial cell count (combined with identification of genus and, if possible, species): > 100,000 cells/mL	bathing prohibited until quantification of microcystins; information to the public; at least weekly counting

Jurisdiction	Recreational Water Guideline Level	Recommended Action
	cyanobacterial cell count (combined with identification of genus and, if possible, species): ≥ 20,000 to ≤ 100,000 cells/mL	daily visual observation; at least weekly counting; information to the public; quantification of microcystins
	microcystins: >25 µg/L	bathing prohibited
	visible scums	bathing prohibited until quantification of microcystins; warning notice; scum drift monitoring
Netherlands[c]	biovolume (cyanobacterial cell count): >0 to < 2.5 mm^3/L	surveillance level: continue fortnightly monitoring
	biovolume (cyanobacterial cell count): > 15 mm^3/L (if 80 percent dominance of microcystin-producers and microcystin < 20 µg/L, revert to Alert Level 1).	alert level 2: weekly monitoring and advice against bathing (by public authority): "You are advised not to bathe in this water;" prohibition by local authority is possible.
	biovolume (cyanobacterial cell count): ≥ 2.5 to ≤ 15 mm^3/L	alert level 1: weekly monitoring and issue warning (by site operator) for duration of that week: "Toxic blue-green algae. Risk of skin irritation or intestinal problems." In case of daily site inspection, reevaluate the warning on a daily basis.
	chlorophyll *a*: > 0 to < 12.5 µg/L	surveillance level: continue fortnightly monitoring
	chlorophyll *a*: > 75 µg/L	alert level 2: weekly monitoring and advice against bathing (by public authority): "You are advised not to bathe in this water;" prohibition by local authority is possible.
	chlorophyll *a*: ≥ 12.5 to ≤ 75 µg/L	alert level 1: weekly monitoring and issue warning (by site operator) for duration of that week: "Toxic blue-green algae. Risk of skin irritation or intestinal problems." In case of daily site inspection, reevaluate the warning on a daily basis.
	surface scum: category 1	surveillance level: continue fortnightly monitoring
	surface scum: category 2	alert level 1: weekly monitoring and issue warning (by site operator) for duration of that week: "Toxic blue-green algae. Risk of skin irritation or intestinal problems". In case of daily site inspection, reevaluate the warning on a daily basis.
	surface scum: category 3	alert level 2: weekly monitoring and advice against bathing (by public authority): "You are advised not to bathe in this water"; prohibition by local authority is possible.
New Zealand[h]	cyanobacteria (benthic): 20−50 percent coverage of potentially toxigenic cyanobacteria attached to substrate	alert (amber mode): • Notify the public health unit. • Increase sampling to weekly.

Jurisdiction	Recreational Water Guideline Level	Recommended Action
		• Recommend erecting an information sign. • Consider increasing the number of survey sites. • If toxigenic cyanobacteria dominate the samples, testing for cyanotoxins is advised. If cyanotoxins are detected in mats or water samples, consult the testing laboratory to determine if levels are hazardous.
	cyanobacteria (benthic): greater than 50 percent coverage of potentially toxigenic cyanobacteria attached to substrate	action (red mode) situation 1: • Immediately notify the public health unit • If potentially toxic taxa are present (see Table 2) then consider testing samples for cyanotoxins • Notify the public of the potential risk to health
	cyanobacteria (benthic): Up to 20 percent coverage of potentially toxigenic cyanobacteria attached to substrate	surveillance (green mode): • Undertake fortnightly surveys between spring and autumn at representative locations in the water body where known mat proliferations occur and where there is recreational use
	cyanobacteria (benthic): up to 50 percent where potentially toxigenic cyanobacteria are visibly detaching from the substrate, accumulating as scums along the river's edge or becoming exposed on the river's edge as the river level drops.	action (red mode) situation 2: • Immediately notify the public health unit • If potentially toxic taxa are present (see Table 2) then consider testing samples for cyanotoxins. • Notify the public of the potential risk to health
	cyanobacteria (total): < 0.5 mm^3/L (biovolume equivalent of the combined total of all cyanobacteria)	surveillance (green mode): • Undertake weekly or fortnightly visual inspection and sampling of water bodies where cyanobacteria are known to proliferate between spring and autumn
	cyanobacteria (total): $\leq$ 500 cells/mL	surveillance (green mode): • Undertake weekly or fortnightly visual inspection and sampling of water bodies where cyanobacteria are known to proliferate between spring and autumn
	cyanobacteria (total): $\geq$ 1.8 mm^3/L (biovolume equivalent of potentially toxic cyanobacteria)	action (red mode) situation 1: • Continue monitoring as for alert (amber mode) • If potentially toxic taxa are present (see Table 1), then consider testing samples for cyanotoxins • Notify the public of a potential risk to health
	cyanobacteria (total): $\geq$ 0.5 to < 1.8 mm^3/L (biovolume equivalent of potentially toxic cyanobacteria)	alert (amber mode): • Increase sampling frequency to at least weekly

Jurisdiction	Recreational Water Guideline Level	Recommended Action
		• Notify the public health unit • Multiple sites should be inspected and sampled
	cyanobacteria (total): ≥ 0.5 to < 10 mm^3/L (total biovolume of all cyanobacterial material where the cyanobacterial population has been tested and shown not to contain known toxins)	alert (amber mode): • Increase sampling frequency to at least weekly. • Notify the public health unit. • Multiple sites should be inspected and sampled.
	cyanobacteria (total): ≥ 10 mm^3/L (total biovolume of all cyanobacterial material where the cyanobacterial population has been tested and shown not to contain known toxins)	action (red mode) situation 2: • Continue monitoring as for alert (amber mode) • If potentially toxic taxa are present (see Table 1), then consider testing samples for cyanotoxins • Notify the public of a potential risk to health
	cyanobacterial scums consistently present for more than several days in a row	action (red mode) situation 3: • Continue monitoring as for alert (amber mode) • If potentially toxic taxa are present (see Table 1), then consider testing samples for cyanotoxins • Notify the public of a potential risk to health
	microcystins (total): ≥ 12 µg/L	action (red mode) situation 1: • Continue monitoring as for alert (amber mode) • If potentially toxic taxa are present (see Table 1), then consider testing samples for cyanotoxins • Notify the public of a potential risk to health
Poland[c]	visible blooms	sampling of bathing sites not less than 4 times per season (the interval between sampling does not exceed one month), including responses to cyanobacteria if blooms are observed.
Scotland[e]	chlorophyll *a*: ≥ 10 µg/L with dominance of cyanobacteria	1. watch for scum or conditions conducive to scums. 2. discourage bathing and further investigate hazard. 3. post on-site risk advisory signs. 4. inform relevant authorities.
	cyanobacteria: ≥ 20,000 cells /mL	1. watch for scum or conditions conducive to scums. 2. discourage bathing and further investigate hazard. 3. post on-site risk advisory signs. 4. inform relevant authorities.

Jurisdiction	Recreational Water Guideline Level	Recommended Action
	cyanobacterial scum formation in bathing areas	1. immediate action to control contact with scums; possible prohibition of swimming and other water-contact activities. 2. public health follow-up investigation. 3. inform public and relevant authorities.
Singapore[c]	chlorophyll *a*: ≤ 50 µg/L (of 95 percent of a 3-year rolling period)	status of the sites reviewed annually. If the assessment is that the water body is unsuitable for primary water contact activities, the public is notified.
Spain[c]	cyanobacteria proliferation potential (High, Medium, Low)	criteria for assessment of health risk and response are set locally; some health authorities use WHO scheme, others include further risk parameters (such as number of users, type of use); temporary closure has occasionally occurred based on the abundance of cyanobacteria.
Turkey[c]	cells: < 20,000 cells/mL	level 1: recreational activities are allowed to continue and users are informed by posters on site. Monitoring (sampling, counting and species identification) should be done fortnightly.
	cells: 20,000–100,000 cells/mL	level 2: At > 20 000 cells/mL, microcystins are analyzed. If microcystin-LR equivalents >25 µg/L, immediate action to inform relevant authorities and public. Discourage users from swimming and other water-contact activities by advisory signs on site.
	chlorophyll *a* (if dominated by cyanobacteria): < 10 µg/L	level 1: recreational activities are allowed to continue and users are informed by posters on site. Monitoring (sampling, counting and species identification) should be done fortnightly.
	microcystin-LR: < 10 µg/L equivalents	level 1: recreational activities are allowed to continue and users are informed by posters on site. Monitoring (sampling, counting and species identification) should be done fortnightly.
	microcystin-LR: > 25 µg/L equivalents	level 2: At > 20,000 cells/mL, microcystins are analyzed. If microcystin-LR equivalents >25 µg/L, immediate action to inform relevant authorities and public. Discourage users from swimming and other water-contact activities by advisory signs on site.
	visible scum in bathing area	level 3: all activities in the water may be prohibited
World Health Organization (WHO)[b,g]	chlorophyll *a*: 10 µg/L with dominance of cyanobacteria	low risk: post on-site advisory signs, inform relevant authorities
	chlorophyll *a*: 50 µg/L with dominance of cyanobacteria	moderate risk: watch for scums or conditions conducive to scums, discourages swimming and further investigate hazard, post on-site risk advisory signs, inform relevant authorities
	cyanobacteria: 100,000 cells/mL	moderate risk: watch for scums or conditions conducive to scums, discourages swimming and further investigate hazard, post on-site risk advisory signs, inform relevant authorities
	cyanobacteria: 20,000 cells/mL	low risk: post on-site advisory signs, inform relevant authorities

Jurisdiction	Recreational Water Guideline Level	Recommended Action
	cyanobacterial scum formation in areas where whole-body contact and/or risk of ingestions/aspiration occur	high risk: immediate action to control contact with scums, possible prohibition of swimming and other water contact activities, public health follow-up investigation, inform public and relevant authorities

[a] Australian Government National Health and Medical Research Council (2008). Guidelines for Managing Risk in Recreational Water.

[b] Chorus, I. and Bartram, J. (eds.) (1999). Toxic cyanobacteria in water: A guide to public health significance, monitoring and management. E. & F.N. Spon / Chapman & Hall, London, United Kingdom.

[c] Federal Environment Agency (Germany) (2012). Current approaches to Cyanotoxin risk assessment, risk management and regulations in different countries.

[d] Health Canada (2012). Guidelines for Canadian Recreational Water Quality, Third Edition. Water, Air and Climate Change Bureau, Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa, Ontario. (Catalogue No H129-15/2012E).

[e] Scottish Government Health and Social Care Directorates Blue-Green Algae Working Group (2012). Cyanobacteria (Blue-Green Algae) in Inland and Inshore Waters: Assessment and Minimization of Risks to Public Health.

[f] European Parliament and the Council of the European Union (2006). Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC.

[g] WHO (World Health Organization) (2003). Guidelines for Safe Recreational Water Environments: Volume 1: Coastal and Fresh Waters. World Health Organization.

[h] Wood, S; Hamilton, D; Safi, K; Williamson, W. (2008). New Zealand Guidelines for Cyanobacteria in Recreational Fresh Waters: Interim Guidelines. New Zealand Ministry for the Environment and Ministry of Health.

APPENDIX B. STATE RECREATIONAL WATER GUIDELINES FOR CYANOTOXINS AND CYANOBACTERIA

EPA compiled the information presented in this appendix based on searches of state websites for publicly available information regarding guidelines or action levels for cyanotoxins and cyanobacteria. The website research was completed in November 2015. Subsequent direct personal communication of state guidelines revealed some updates for a few states later in 2015 and in early 2016.

Table B-1. Summary Counts of State Recreational Water Guidelines for Cyanotoxins and Cyanobacteria by Type and Scope of Guidelines

Recreational Water Guideline Type and Scope	Number of States and List of States	Additional Information
Quantitative guidelines for cyanobacteria only	6 states Arizona, Connecticut, Idaho, Maine, New Hampshire, Wisconsin	Measurements for these criteria include cyanobacterial cell densities, proportion of toxigenic cyanobacteria, chlorophyll concentration, and Secchi disk depth measurements.
Quantitative guidelines for cyanotoxins only	7 states California, Colorado, Illinois, Iowa, Nebraska, North Dakota, Ohio	State guidelines address four cyanotoxins in order from most to least common: microcystins (20 states) anatoxin-a (9 states) cylindrospermopsin (7 states) saxitoxin (4 states)
Quantitative guidelines for cyanotoxins and either quantitative or qualitative guidelines for cyanobacteria	14 states Indiana, Kansas, Kentucky, Maryland, Massachusetts, New York, Oklahoma, Oregon, Rhode Island, Texas, Utah, Vermont, Virginia, Washington	
Qualitative guidelines only	6 states Delaware, Florida, Montana, North Carolina, North Dakota, West Virginia	Examples include: presence of surface scum visible discoloration presence of potentially toxic algae

Note: EPA found that Texas and North Carolina published guidelines in the past, but the guidelines are no longer found on their websites.

Table B-2. Summary Counts of State Recreational Water Guidelines for Cyanotoxins and Cyanobacteria by Basis of Guidelines

Recreational Water Guideline Basis of Guideline Category	Number of States and List of States
Based on WHO	6 states Colorado, Indiana, Kentucky, Oklahoma, Utah, Wisconsin
Modified WHO	8 states Illinois, Indiana, Iowa, Kentucky, Oklahoma, Oregon, Utah, Virginia
Jurisdiction-specific (i.e., based on risk assessments or site-specific monitoring information)	8 states California, Colorado, Kansas, Massachusetts, Ohio, Oregon, Vermont, Washington
Based on studies or guidelines other than WHO	3 states Idaho, Indiana, Utah
Qualitative evaluations or narrative criteria application (includes states with insufficient documentation to categorize the source of their guideline)	21 states Arizona, Connecticut, Delaware, Florida, Maine, Maryland, Massachusetts, Montana, Nebraska, New Hampshire, New York, North Carolina, North Dakota, Oregon, Rhode Island, Texas, Utah, Virginia, Washington, West Virginia, Wisconsin

Note: Some states are listed in more than one category because they had more than one guideline (e.g., both cyanobacterial cell and cyanotoxin guidelines), and these guidelines fit into different categories.

Table B-3. State Recreational Water Quality Guideline for Cyanotoxins and Cyanobacteria Sorted by Type

State	Recreational Water Guideline Level	Recommended Action	Reference
States with Guidelines Based on Cyanobacteria Only			
Arizona	blue-green algae (mean value based on a minimum of two sample events within one peak season): 20,000 cells/mL and chlorophyll *a* result (mean value based on a minimum of two sample events within one peak season) in target range	violation of the Narrative Nutrient Standard	Arizona Department of Environmental Quality (2008). Narrative Nutrient Standard Implementation Procedures for Lakes and Reservoirs. http://www.azdeq.gov/environ/water/standards/download/draft_nutrient.pdf. Last Accessed: 08/03/2016.
Connecticut	visual rank category 1: visible material is not likely cyanobacteria or water is generally clear	no action	Connecticut Department of Public Health: Connecticut Energy Environment (2013). Guidance to Local Health Departments For Blue–Green Algae Blooms in Recreational Freshwaters. http://www.ct.gov/deep/lib/deep/water/water_quality_standards/guidance_lhd__bga_blooms_7_2013.pdf. Last Accessed: 08/03/2016.
	visual rank category 2: cyanobacteria present in low numbers; there are visible small accumulations but water is generally clear; OR blue-green algae cells > 20,000 cells/mL and < 100,000 cells/mL	notify Connecticut Department of Public Health (CT DPH), Connecticut Department of Energy and Environmental Protection (CT DEEP)	
	visual rank category 3: cyanobacteria present in high numbers; scums may or may not be present; water is discolored throughout; large areas affected; color assists to rule out sediment and other algae; OR blue-green algae cells > 100,000 cells/mL	update/inform CT DPH & CT DEEP and expand risk communication efforts; POSTED BEACH CLOSURE: if public has beach access, alert water users that a blue-green algae bloom is present; POSTED ADVISORY: at other impacted access points	
Idaho	*Microcystis* or *Planktothrix*: >40,000 cells/mL	public health advisory posting by Public Health District in conjunction with water body operator	IDEQ (Idaho Department of Environmental Quality) (2015). Blue-Green Algae Bloom Response Plan: Final. http://www.epa.illinois.gov/topics/water-quality/monitoring/algal-bloom/2013-program/index . Last Accessed: 08/03/2016.
	sum of all potentially toxigenic taxa: ≥ 100,000 cells/mL	public health advisory posting by Public Health District in conjunction with water body operator	
	visible surface scum that is associated with toxigenic species	public health advisory posting by Public Health District in conjunction with water body management agency	

State	Recreational Water Guideline Level	Recommended Action	Reference
Maine	Secchi disk reading < 2 meters caused by algae	body of water considered impaired, but still safe to swim	Maine Department of Environmental Protection (2013). Reports of Algal Blooms. http://www.maine.gov/dep/water/lakes/repbloom.html. Last Accessed: 08/03/2016.
New Hampshire	cyanobacteria: > 50 percent of total cell counts from toxigenic cyanobacteria	post beach advisory	New Hampshire Department of Environmental Services (2014). Beach Advisories. http://des.nh.gov/organization/divisions/water/wmb/beaches/advisories.htm. Last Accessed: 08/03/2016.
Wisconsin	cyanobacteria: > 100,000 cells/mL	post health advisory and possible beach closure	Wisconsin Department of Natural Resources (2012). Draft Blue-Green Algae Section of 303 (d) Report-7/3/2012: Harmful Algal Blooms. http://dnr.wi.gov/lakes/bluegreenalgae/documents/HarmfulAlgalBloomsvs2.pdf. Last Accessed: 08/03/2016.
	visible scum layer	post health advisory and possible beach closure	Werner M, & Masnado R (2014). Guidance for Local Health Departments: Cyanobacteria and Human Health. http://city.milwaukee.gov/ImageLibrary/Groups/healthAuthors/DCP/PDFs/CyanobacteriaLHD.pdf. Last Accessed: 08/03/2016.
States with Guidelines Based on Cyanotoxin(s) Only			
California	anatoxin-a: 90 µg/L	Unclear	Butler N, Carlisle J, Kaley KB, & Linville R (2012). Toxicological Summary and Suggested Action Levels to Reduce Potential Adverse Health Effects of Six Cyanotoxins.
	cylindrospermopsin: 4 µg/L	Unclear	
	microcystins: 0.8 µg/L	Unclear	

State	Recreational Water Guideline Level	Recommended Action	Reference
			http://www.waterboards.ca.gov/water_issues/programs/peer_review/docs/calif_cyanotoxins/cyanotoxins053112.pdf. Last Accessed: 08/03/2016.
Colorado	anatoxin-a: ≥ 7 µg/L	issue toxic algae caution: a. post sign with "caution" language. b. perform routine testing for toxin levels. bi. if test results are below caution thresholds, test at least once per week until algae visually subsides. bii. if test results are above caution thresholds, test at least twice per week until toxin levels are below caution thresholds for two consecutive tests. c. notify drinking water providers and county health department if toxin levels exceed the caution thresholds. d. toxic algae caution ends when there is no visual evidence of algae and toxin levels are non-detectable for two consecutive weeks. di. notify drinking water providers and county health department that bloom has ended. dii. remove "caution" sign.	Colorado Department of Public Health & Environment. Algae bloom risk-management toolkit for recreational waters. https://drive.google.com/file/d/0B0tmPQ67k3NVN2U4VHZBcWxPN0E/view. Last Accessed: 10/21/2016
	cylindrospermopsin: ≥ 7 µg/L	issue toxic algae caution: a. post sign with "caution" language. b. perform routine testing for toxin levels. bi. if test results are below caution thresholds, test at least once per week until algae visually subsides. bii. if test results are above caution thresholds, test at least twice per week until toxin levels are below caution thresholds for two consecutive tests. c. notify drinking water providers and county health department if toxin levels exceed the caution thresholds. d. toxic algae caution ends when there is no visual evidence of algae and toxin levels are non-	

State	Recreational Water Guideline Level	Recommended Action	Reference
		detectable for two consecutive weeks. di. notify drinking water providers and county health department that bloom has ended. dii. remove "caution" sign.	
	microcystin-LR: ≥ 10 µg/L and < 20 µg/L	issue toxic algae caution: a. post sign with "caution" language. b. perform routine testing for toxin levels. bi. if test results are below caution thresholds, test at least once per week until algae visually subsides. bii. if test results are above caution thresholds, test at least twice per week until toxin levels are below caution thresholds for two consecutive tests. c. notify drinking water providers and county health department if toxin levels exceed the caution thresholds. d. toxic algae caution ends when there is no visual evidence of algae and toxin levels are non-detectable for two consecutive weeks. di. notify drinking water providers and county health department that bloom has ended. dii. remove "caution" sign.	
	microcystin-LR: ≥ 20 µg/L	issue toxic algae warning: a. immediately post sign with "warning" language. b. take necessary steps to prevent contact with water in affected area for humans and pets. c. notify drinking water providers and county health department if toxin levels exceed warning thresholds. d. test at least twice per week until toxin levels are below warning thresholds for two consecutive tests. e. posting can be reduced to "caution" language when microcystin test results drop below the warning threshold and no new human illness or pet	

State	Recreational Water Guideline Level	Recommended Action	Reference
		deaths have been reported for two consecutive weeks.	
	saxitoxin: ≥ 4 µg/L	issue toxic algae caution: a. post sign with "caution" language. b. perform routine testing for toxin levels. bi. if test results are below caution thresholds, test at least once per week until algae visually subsides. bii. if test results are above caution thresholds, test at least twice per week until toxin levels are below caution thresholds for two consecutive tests. c. notify drinking water providers and county health department if toxin levels exceed the caution thresholds. d. toxic algae caution ends when there is no visual evidence of algae and toxin levels are non-detectable for two consecutive weeks. di. notify drinking water providers and county health department that bloom has ended. dii. remove "caution" sign.	
	potentially toxic algae are visible	issue toxic algae caution: a. post sign with "caution" language. b. perform routine testing for toxin levels. bi. if test results are below caution thresholds, test at least once per week until algae visually subsides. bii. if test results are above caution thresholds, test at least twice per week until toxin levels are below caution thresholds for two consecutive tests. c. notify drinking water providers and county health department if toxin levels exceed the caution thresholds. d. toxic algae caution ends when there is no visual evidence of algae and toxin levels are non-detectable for two consecutive weeks. di. notify drinking water providers and county	

State	Recreational Water Guideline Level	Recommended Action	Reference
		health department that bloom has ended. dii. remove "caution" sign.	
Illinois	microcystin-LR: > 10 µg/L	appropriate lake management personnel and Illinois EPA staff will be notified; follow-up monitoring by the Illinois EPA may occur as professional judgment dictates and staff, laboratory, and financial resources allow	Illinois Environmental Protection Agency (2015). 2013 Statewide Harmful Algal Bloom Program. http://epa.illinois.gov/topics/water-quality/monitoring/algal-bloom/2013-program/index. Last Accessed: 08/03/2016.
California	anatoxin-a: 90 µg/L	unclear	Butler N, Carlisle J, Kaley KB, & Linville R (2012). Toxicological Summary and Suggested Action Levels to Reduce Potential Adverse Health Effects of Six Cyanotoxins. http://www.waterboards.ca.gov/water_issues/programs/peer_review/docs/calif_cyanotoxins/cyanotoxins053112.pdf. Last Accessed: 08/03/2016.
	cylindrospermopsin: 4 µg/L	unclear	
	microcystins: 0.8 µg/L	unclear	
Illinois	microcystin-LR: > 10 µg/L	appropriate lake management personnel and Illinois EPA staff will be notified; follow-up monitoring by the Illinois EPA may occur as professional judgment dictates and staff, laboratory, and financial resources allow	Illinois Environmental Protection Agency (2015). 2013 Statewide Harmful Algal Bloom Program. http://epa.illinois.gov/topics/water-quality/monitoring/algal-bloom/2013-program/index. Last Accessed: 08/03/2016.
Iowa	microcystin: ≥ 20 µg/L	warnings are posted at state park beaches	Iowa Environmental Council (2015). State Park Beach Advisories Report. Updated September 3, 2015. http://www.iaenvironment.org/webres/File/Program%20Publications/2015%20State%20Park%20Beach%20Advisories%20Report.pdf. Last Accessed: 08/03/2016.

State	Recreational Water Guideline Level	Recommended Action	Reference
Nebraska	microcystin: ≥ 20 µg/L	health alert; signs posted advising public to use caution; affected swimming beaches will be closed; boating and other recreational activities will be allowed, but public advised to use caution and avoid prolonged exposure to the water	Nebraska Department of Environmental Quality and Nebraska Department of Health and Human Services: Division of Public Health (2016). Fact Sheet: Precautions and facts regarding toxic algae at Nebraska Lakes. http://deq.ne.gov/NDEQProg.nsf/OnWeb/ENV042607. Last Accessed: 08/03/2016.
North Dakota	blue-green algae bloom is present AND microcystin-LR: < 10 µg/L	issue advisory	North Dakota Department of Health: Division of Water Quality (2016). Blue-green algae advisories and warnings. http://www.ndhealth.gov/WQ/SW/HABs/HABs_Information/Blue-greenLakeListings_20160808.pdf. Last Accessed: 10/18/2016.
	blue-green algae bloom is present over a significant portion of the lake AND microcystin-LR: ≥ 10 µg/L	issue warning	
Ohio	anatoxin-a: 300 µg/L	issue no contact advisory	Kasich JR, Taylor M, Butler CW, Zehringer J, & Hodges R (2016). State of Ohio Harmful Algal Bloom Response Strategy For Recreational Waters. http://epa.ohio.gov/portals/35/hab/HABResponseStrategy.pdf. Last Accessed: 08/03/2016.
	anatoxin-a: 80 µg/L	issue recreational public health advisory	
	cylindrospermopsin: 20 µg/L	issue no contact advisory	
	cylindrospermopsin: 5 µg/L	issue recreational public health advisory	
	microcystin-LR: 20 µg/L	issue no contact advisory	
	microcystin-LR: 6 µg/L	issue recreational public health advisory	
	saxitoxin: 0.8 µg/L	issue recreational public health advisory	
	saxitoxin: 3 µg/L	issue no contact advisory	
Nebraska	microcystin: ≥ 20 µg/L	health alert; signs posted advising public to use caution; affected swimming beaches will be closed; boating and other recreational activities will be	Nebraska Department of Environmental Quality and Nebraska Department of Health and Human Services: Division of Public Health

State	Recreational Water Guideline Level	Recommended Action	Reference
		allowed, but public advised to use caution and avoid prolonged exposure to the water	(2016). Fact Sheet: Precautions and facts regarding toxic algae at Nebraska Lakes. http://deq.ne.gov/NDEQProg.nsf/OnWeb/ENV042607. Last Accessed: 08/03/2016.
Ohio	anatoxin-a: 300 µg/L	issue no contact advisory	Kasich JR, Taylor M, Butler CW, Zehringer J, & Hodges R (2015). State of Ohio Harmful Algal Bloom Response Strategy For Recreational Waters. http://epa.ohio.gov/portals/35/hab/HABResponseStrategy.pdf. Last Accessed: 08/03/2016.
	anatoxin-a: 80 µg/L	issue recreational public health advisory	
	cylindrospermopsin: 20 µg/L	issue no contact advisory	
	cylindrospermopsin: 5 µg/L	issue recreational public health advisory	
	microcystin-LR: 20 µg/L	issue no contact advisory	
	microcystin-LR: 6 µg/L	issue recreational public health advisory	
	saxitoxin: 0.8 µg/L	issue recreational public health advisory	
	saxitoxin: 3 µg/L	issue no contact advisory	
Vermont	anatoxin-a: ≥ 10 µg/L	close recreational beaches	Vermont Department of Health (2015). Cyanobacteria (Blue-green Algae) Guidance for Vermont Communities. http://healthvermont.gov/enviro/bg_algae/documents/BGA_guide.pdf. Last Accessed: 08/03/2016.
	cylindrospermopsin: ≥ 10 µg/L	close recreational beaches	
	microcystin-LR (equivalents): ≥ 6 µg/L	close recreational beaches	
	visible known blue-green algae bloom/scum or an unknown, potentially blue-green algae (i.e., not pollen), bloom/scum	close recreational beaches	
Washington	anatoxin-a: 1 µg/L	tier 2: local health posts WARNING sign; local health takes additional site-specific steps; minimum weekly sampling. In addition, if history of high toxicity, or reports of illness, pet death than tier 3: local health posts DANGER sign; lake closed	Hardy J, & Washington State Department of Health (2008). Washington State Recreational Guidance for Microcystins (Provisional) and Anatoxin-a (Interim/Provisional). http://www.doh.wa.gov/Portals/1/Doc

State	Recreational Water Guideline Level	Recommended Action	Reference
			uments/4400/334-177-recguide.pdf. Last Accessed: 08/03/2016.
	bloom is forming or a bloom scum is visible (toxic algae may be present); toxin levels do not exceed thresholds	tier 1: local health posts CAUTION sign; samples taken and sent for toxicity tests; weekly sampling until bloom dissipates	Hardy J, & Washington State Department of Health (2011). Washington State Provisional Recreational Guidance for Cylindrospermopsin and Saxitoxin. http://www.doh.wa.gov/portals/1/documents/4400/332-118-cylindrosax%20report.pdf. Last Accessed: 08/03/2016.
	cylindrospermopsin: 4.5 µg/L	tier 2: local health posts WARNING sign; local health takes additional site-specific steps; minimum weekly sampling. In addition, if history of high toxicity, or reports of illness, pet death than tier 3: local health posts DANGER sign; lake closed.	
	microcystins: 6 µg/L	tier 2: local health posts WARNING sign; local health takes additional site-specific steps; minimum weekly sampling. In addition, if history of high toxicity, or reports of illness, pet death than tier 3: local health posts DANGER sign; lake closed.	Hardy J, & Washington State Department of Health (2008). Washington State Recreational Guidance for Microcystins (Provisional) and Anatoxin-a (Interim/Provisional). http://www.doh.wa.gov/Portals/1/Documents/4400/334-177-recguide.pdf. Last Accessed: 08/03/2016.
Washington (continued)	saxitoxin: 75 µg/L	tier 2: local health posts WARNING sign; local health takes additional site-specific steps; minimum weekly sampling. In addition, if history of high toxicity, or reports of illness, pet death than tier 3: local health posts DANGER sign; lake closed.	Hardy J, & Washington State Department of Health (2011). Washington State Provisional Recreational Guidance for Cylindrospermopsin and Saxitoxin. http://www.doh.wa.gov/portals/1/documents/4400/332-118-cylindrosax%20report.pdf. Last Accessed: 08/03/2016.
States with Guidelines Based on Cyanobacteria and Cyanotoxin(s)			
Indiana	anatoxin-a: 80 µg/L	issue recreation advisory	Indiana Department of Environmental Management (2015). Addressing
	blue-green algae: 100,000 cells/mL	issue recreation advisory	

State	Recreational Water Guideline Level	Recommended Action	Reference
	cylindrospermopsin: 5 µg/L	issue recreation advisory	Concerns About Blue-Green Algae: Indiana Reservoir and Lake Update. http://www.in.gov/idem/algae/2310.htm. Last Accessed: 08/03/2016.
	microcystin-LR: 20 µg/L	close beaches	
	microcystin-LR: 6 µg/L	issue recreation advisory	
Kansas	cyanobacteria: ≥ 10,000,000 cells/mL	recommended that all in-lake recreation cease and that picnic, camping and other public land activities adjacent to affected waters be closed	Kansas Department of Health & Environment (2015). Guidelines for Addressing Harmful Algal Blooms in Kansas Recreational Waters. http://www.kdheks.gov/algae-illness/download/HAB_policy.pdf. Last Accessed: 08/03/2016.
	cyanobacteria: ≥ 250,000 cells/mL	issue public health warning	
	cyanobacteria: ≥ 80,000 and < 250,000 cells/mL	issue public health watch	
	microcystin: ≥ 2,000 µg/L	recommended that all in-lake recreation cease and that picnic, camping and other public land activities adjacent to affected waters be closed	
	microcystin: ≥ 20 µg/L	issue public health warning	
	microcystin: ≥ 4 and < 20 µg/L	issue public health watch	
Kentucky	blue-green algae: > 100,000 cells/mL	issue an harmful algal bloom (HAB) advisory	Kentucky Department for Environmental Protection (2014). Harmful Algal Blooms: Background. http://water.ky.gov/waterquality/Documents/HAB_FACTs/HAB%20Background%20Fact%20Sheet.pdf. Last Accessed: 08/03/2016.
	microcystins: > 20 µg/L	issue recreational use advisory	Commonwealth of Kentucky: Department for Environmental Protection Division of Water (2015). Harmful Algal Blooms. http://water.ky.gov/waterquality/pages/HABS.aspx. Last Accessed: 08/03/2016.

State	Recreational Water Guideline Level	Recommended Action	Reference
Maryland	*Microcystis aeruginosa* or other potential microcystin producing blue-green algae > 40,000 cells/mL, and samples contain microcystins: > 10 ppb	put up signs advising public of health risk, notify local press (through joint DHMH, DNR, MDE press release) and coordinate with local health department, place advisory information on DNR web site (Eyes on the Bay), Maryland Healthy Beaches web site if a swimming beach is affected, or other local web site. MDE will initiate emergency closure to shellfish harvesting if warranted, and coordinate with DNR Natural Resource Police	Maryland Department of Natural Resources (2010). Harmful Algal Bloom (HAB) Monitoring and Management SOP. SOP document (with edits) sent via email correspondence with Catherine Wazniak, Program Manager at the MD DNR, on February 22, 2016.
	presence of potentially toxic algae	issue algae bloom beach alert	Maryland Department of Natural Resources (2010). Harmful Algal Bloom (HAB) Monitoring and Management SOP. SOP document (with edits) sent via email correspondence with Catherine Wazniak, Program Manager at the MD DNR, on February 22, 2016.
Massachusetts	blue-green algae: > 50,000 cells/mL	toxin testing of lysed cells should be done to ensure that guideline of 14 ppb is not exceeded	Massachusetts Bureau of Environmental Health (2015). MDPH Guidelines for Cyanobacteria in Freshwater Recreational Water Bodies in Massachusetts. Boston, Massachusetts. http://www.mass.gov/eohhs/docs/dph/environmental/exposure/protocol-cyanobacteria.pdf. Last Accessed: 08/03/2016.
	blue-green algae: > 70,000 cells/mL	post an advisory against contact with the water	
	microcystins: > 14 µg/L	post an advisory against contact with the water	
	visible cyanobacteria scum or mat is evident	MDPH recommends an immediate posting by the local health department, state agency, or relevant authority to advise against contact with the water body	
New York	visible HAB	prohibit wading, swimming, diving and any water contact activities in the swimming area; post beach closure and advisory signs at the beach and other shoreline access areas; contact local health department	June, Stephanie. Senior Sanitarian at the New York State Department of Health. Email correspondence on Feb. 23, 2016.

State	Recreational Water Guideline Level	Recommended Action	Reference
	microcystins: 10 µg/L	prohibit wading, swimming, diving and any water contact activities in the swimming area; post beach closure and advisory signs at the beach and other shoreline access areas; contact local health department; to reopen swim areas: • water must be visibly clear of HABs or associated material for one day • at that time, a water sample is to be collected and tested for microcystins • if the sample indicates toxin levels <10 µg/L and the HAB has not returned to the swim area, the signs may be removed and the beach may be reopened	June, Stephanie. Senior Sanitarian at the New York State Department of Health. Email correspondence on Feb. 23, 2016.
	bloom: credible report or digital imagery of a bloom determined as likely to be potentially toxic cyanobacteria by the Department of Environmental Conservation (DEC) or Department of Health (DOH) staff; a descriptive field report from professional staff or trained volunteer may be used as a report in absence of digital images; for all other surveillance reports received from the general public, lay monitors, etc., DEC HABs Program staff will determine if a bloom is suspicious and whether collection of a sample is feasible or warranted	post DEC blue-green algal bloom notice: suspicious bloom	Gorney, Rebecca. Research Scientist at New York State Department of Environmental Conservation. Email correspondence on Feb. 23, 2016.
	blue green chlorophyll *a*: >25-30 µg/L	post DEC blue-green algal bloom notice: confirmed bloom	Gorney, Rebecca. Research Scientist at New York State Department of Environmental Conservation. Email correspondence on Feb. 23, 2016.
	potential toxin-producing cyanobacteria taxa: >50% of algae present	post DEC blue-green algal bloom notice: confirmed bloom	Gorney, Rebecca. Research Scientist at New York State Department of Environmental Conservation. Email correspondence on Feb. 23, 2016.

State	Recreational Water Guideline Level	Recommended Action	Reference
	microcystin-LR: 4 µg/L	post DEC blue-green algal bloom notice: confirmed bloom	Gorney, Rebecca. Research Scientist at New York State Department of Environmental Conservation. Email correspondence on Feb. 23, 2016.
	anatoxin-a or other cyanotoxins: high risk of exposure based on consult among DEC or DOH staff	post DEC blue-green algal bloom notice: confirmed bloom	Gorney, Rebecca. Research Scientist at New York State Department of Environmental Conservation. Email correspondence on Feb. 23, 2016.
	microcystin-LR: 10 µg/L in open water sample	post DEC blue-green algal bloom notice: confirmed with high toxins	Gorney, Rebecca. Research Scientist at New York State Department of Environmental Conservation. Email correspondence on Feb. 23, 2016.
	microcystin-LR: 20 µg/L in shoreline sample	post DEC blue-green algal bloom notice: confirmed with high toxins	New York State Department of Environmental Conservation. Water Clarity Fact Sheet. http://www.dec.ny.gov/docs/water_pdf/cslaplkpara.pdf. Last Accessed: 10/23/2015.
Oklahoma	cyanobacteria: 100,000 cell/mL	issue advisory	Oklahoma Legislature (2012). SB 259 Bill Summary. http://webserver1.lsb.state.ok.us/CF/2011-12%20SUPPORT%20DOCUMENTS/BILLSUM/House/SB259%20ccr%20a%20billsum.doc. Last Accessed: 08/03/2016.
	microcystin: > 20 µg/L	issue advisory	
Oregon	anatoxin-a: ≥ 20 µg/L	issue public health advisory	Oregon Health Authority (2016). Oregon Harmful Algae Bloom Surveillance (HABS) Program Public Health Advisory Guidelines: Harmful Algae Blooms in Freshwater Bodies. https://public.health.oregon.gov/HealthyEnvironments/Recreation/HarmfulA
	cylindrospermopsin: ≥ 20 µg/L	issue public health advisory	
	microcystin: ≥ 10 µg/L	issue public health advisory	
	Microcystis: > 40,000 cells/mL	issue public health advisory	
	Planktothrix: > 40,000 cells/mL	issue public health advisory	

State	Recreational Water Guideline Level	Recommended Action	Reference
	saxitoxin: ≥ 10 µg/L	issue public health advisory	lgaeBlooms/Documents/HABPublicHealthAdvisoryGuidelines.pdf. Last Accessed: 08/03/2016.
	toxigenic species: > 100,000 cells/mL	issue public health advisory	
	visible scum with documentation and testing	issue public health advisory	
Rhode Island	cyanobacteria: > 70,000 cells/mL	issue health advisory	Rhode Island Department of Environmental Management, & Rhode Island Department of Health (2013). Cyanobacteria Related Public Health Advisories in Rhode Island. http://www.health.ri.gov/publications/datareports/2013CyanobacteriaBloomsInRhodeIsland.pdf. Last Accessed: 08/03/2016.
	microcystin-LR: ≥ 14 µg/L	issue health advisory	
	visible cyanobacteria scum or mat	issue health advisory	
Texas	>100,000 cell/mL of cyanobacterial cell counts and >20µg/L microcystin	blue-green algae awareness level advisory	U.S. EPA (United States Environmental Protection Agency) (2016). What are the Standards or Guidelines for Cyanobacteria/Cyanotoxin in Recreational Water. https://www.epa.gov/nutrient-policy-data/guidelines-and-recommendations#what3. Last Accessed: 08/03/2016.
Utah	anatoxin-a: > 20 µg/L	issue caution advisory; post CAUTION sign; weekly sampling recommended	Utah Department of Environmental Quality and Department of Health (2015). Utah Guidance for Local Health Departments: Harmful Algal Blooms and Human Health. http://www.deq.utah.gov/Topics/Water/HealthAdvisoryPanel/docs/07Jul/HarmfulAlgalBlooms.pdf. Last Accessed: 08/03/2016.
	blue-green algae: >10,000,000 cells/mL	issue danger advisory; post DANGER sign; weekly sampling recommended; consider closure	
	blue-green algae: 100,000-10,000,000 cells/mL	issue warning advisory; post WARNING sign; weekly sampling recommended	
	blue-green algae: 20,000-100,000 cells/mL	issue caution advisory; post CAUTION sign; weekly sampling recommended	

State	Recreational Water Guideline Level	Recommended Action	Reference
	large scum mat layer	issue danger advisory; post DANGER sign; weekly sampling recommended; consider closure	
	microcystin: > 2,000 µg/L	issue danger advisory; post DANGER sign; weekly sampling recommended; consider closure	
	microcystin: 20-2,000 µg/L	issue warning advisory; post WARNING sign; weekly sampling recommended	
	microcystin: 4-20 µg/L	issue caution advisory; post CAUTION sign; weekly sampling recommended	
	reports of animal illnesses or death	issue warning advisory; post WARNING sign; weekly sampling recommended	
	reports of human illness	issue danger advisory; post DANGER sign; weekly sampling recommended; consider closure	
Virginia	blue-green algal "scum" or "mats" on water surface	immediate public notification to avoid all recreational water contact where bloom is present; continue weekly sampling	Virginia Department of Health (Division of Environmental Epidemiology) (2012). Virginia Recreational Water Guidance for Microcystin and *Microcystis* Blooms: Provisional Guidance. https://www.vdh.virginia.gov/epidemiology/dee/HABS/documents/VDHMicrocystisGuidance.pdf. Last Accessed: 08/03/2016.
	microcystin: > 6 µg/L	immediate public notification to avoid all recreational water contact where bloom is present; continue weekly sampling	
	Microcystis: > 100,000 cells /mL	immediate public notification to avoid all recreational water contact where bloom is present; continue weekly sampling	
	Microcystis: 20,000 to 100,000 cells/mL	notify public through press release and/or signage; advise people and pet-owners that harmful algae are present; initiate weekly water sampling	
	Microcystis: 5,000 to < 20,000 cells/mL	local agency notification; initiate bi-weekly water sampling	
States with Qualitative Guidelines Only			
Delaware	thick green, white, or red scum on surface of pond	post water advisory signs	Delaware Department of Natural Resources and Environmental Control:

State	Recreational Water Guideline Level	Recommended Action	Reference
			Division of Water. Blue-Green Algae in Delaware. http://www.dnrec.delaware.gov/wr/INFORMATION/OTHERINFO/Pages/Blue-GreenAlgae.aspx. Last Accessed: 08/03/2016.
Florida	cyanobacteria bloom	health advisory	Florida Department of Environmental Protection (2016). South Florida Algal Bloom Monitoring and Response. https://depnewsroom.wordpress.com/south-florida-algal-bloom-monitoring-and-response/. Last Accessed: 08/16/2016. Florida Department of Health (2016). Blue-Green Algae (Cyanobacteria). http://www.floridahealth.gov/environmental-health/aquatic-toxins/cyanobacteria.html. Last Accessed: 08/16/2016.
Montana	reservoirs that seem stagnated and harbor large quantities of algae	the Montana Department of Environmental Quality advises people to avoid swimming in ponds, lakes, or reservoirs	State of Montana Newsroom (2015). DEQ Issues Advisory on Blue-Green Algae Blooms: Ponds, Lakes, and Reservoirs Most Often Affected. http://news.mt.gov/Home/ArtMID/24469/ArticleID/1564/DEQ-Issues-Advisory-on-Blue-Green-Algae-Blooms. Last Accessed: 08/03/2016.
North Carolina	visible discoloration or surface scum	Microcystin testing	North Carolina Health and Human Services: Division of Public Health (2014). Occupational & Environmental Epidemiology: Cyanobacteria (Blue-green Algae). http://epi.publichealth.nc.gov/oee/a_z/

State	Recreational Water Guideline Level	Recommended Action	Reference
			algae.html. Last Accessed: 08/03/2016.
West Virginia	blue-green algal blooms observed and monitored	issue public health advisory	West Virginia Department of Health & Human Resources (2015). DHHR Continuing to Monitor Blue-Green Algal Blooms on the Ohio River: Residents Advised to Adhere to Public Health Advisory. http://www.dhhr.wv.gov/News/2015/Pages/DHHR-Continuing-to-Monitor-Blue-Green-Algal-Blooms-on-the-Ohio-River%3B-Residents-Advised-to-Adhere-to-Public-Health-Advisory.aspx. Last Accessed: 08/03/2016.

APPENDIX C. LITERATURE SEARCH DOCUMENTATION

The recreational ambient water quality criteria document for microcystins, cylindrospermopsin, and cyanobacteria relied significantly on information identified, reviewed, and synthesized in U.S. EPA's *Health Effects Support Document for the Cyanobacterial Toxin Microcystins, Heath Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin, Drinking Water Health Advisory for the Cyanobacterial Microcystin Toxins*, and *Drinking Water Health Advisory for the Cyanobacterial Toxin Cylindrospermopsin* ((U.S. EPA (2015c); U.S. EPA (2015d)); (U.S. EPA 2015a; U.S. EPA 2015b). EPA conducted supplemental literature searches to answer additional questions related to recreational exposures, exposure factors, and to identify new health data.

For the Health Effects Support Documents, EPA conducted a comprehensive literature search from January 2013 to May 2014 using Toxicology Literature Online (TOXLINE), PubMed, and Google Scholar. EPA assembled available information on occurrence; environmental fate; mechanisms of toxicity; acute, short-term, subchronic, and chronic toxicity and cancer in humans and animals; and toxicokinetics and exposure. For a detailed description of the literature review search and strategy, see the Health Effects Support Documents for microcystins and cylindrospermopsin (U.S. EPA 2015c; U.S. EPA 2015d).

EPA conducted supplemental literature searches in September 2015 to capture references published since the completion of the Health Effects Support Documents literature searches and to account for the recreational exposure scenario. The specific questions investigated include:

1. What levels of anatoxin-a, cylindrospermopsin, or microcystins are humans—of all ages, including children—exposed to through recreational use (activities) in freshwaters or marine waters from incidental ingestion, inhalation, and dermal exposure routes?
2. What health effects information for humans or animals exposed to cylindrospermopsin or microcystins (through ingestion, inhalation, and dermal exposure routes) has been published since the health effects literature searches were conducted for EPA's 2015 Health Effects Support Documents for cylindrospermopsin and microcystins?
3. What recreational water use safety levels or criteria have been set for microcystins or cylindrospermopsin by states or international governments, and how did they derive them?
4. What new information, if any, is available regarding how aquatic recreational exposure ingestion rates in children differ among age groups between 0 and 18 years?
5. What incidents of companion animal (e.g., dogs, horses) or livestock poisonings, including mortality or adverse health effects, due to exposure to cyanotoxins in freshwaters, marine waters, or beaches have occurred in the past 15 years? Specifically, when and where did these incidents occur, to which cyanotoxin were the animals exposed, how were they exposed, and what were the weights and breeds of the affected animal(s)?

EPA implemented a unique literature search strategy to address each research question. Trial searches were conducted, and results were evaluated to refine the search strategies (e.g., to reduce retrieval of citations unrelated to the research questions). The search strings were refined to improve the relevancy of the results. The literature search strategies implemented for each research question are subsequently detailed.

Research Question 1: What levels of anatoxin-a, cylindrospermopsin, or microcystins are humans—of all ages, including children—exposed to through recreational use (activities) in freshwaters or marine waters, from incidental ingestion, inhalation, and dermal exposure routes?

EPA searched the bibliographic databases, PubMed and Web of Science (WoS), to identify candidate journal article literature relevant to human exposure to anatoxin-a, cylindrospermopsin, or microcystins through recreational activities. PubMed and WoS contain peer-reviewed journal abstracts and articles on various biological, medical, public health, and chemical topics. The WoS search string differs slightly from the PubMed search string due to how the search engines treat search terms with more than one word. Both search strings are presented below.

Results

The searches returned 321 journal articles after removing duplicates between PubMed and WoS results. Based on a screening review of each article's title and abstract, EPA retrieved 9 articles that appeared to be studies that measured, reviewed, or estimated human recreational exposure to cyanotoxins.

PubMed Search:

("*A. lemmermannii Raphidiopsis mediterranea*" OR *Anabaena flos-aquae* OR *flos-aquae* OR anatoxin-a OR *Aphanizomenon* OR cylindrospermopsin OR "*C. raciborskii*" OR *Cuspidothrix* OR *Cylindrospermopsis* OR *Cylindrospermum* OR "*Cylindrospermopsis raciborskii*" OR *Dolichospermum* OR "*M. aeruginosa*" OR *Microcystis* OR microcystin OR microcystins OR *Oscillatoria* OR *Planktothrix* OR *Phormidium* OR *Tychonema* OR *Woronichinia*)

AND

("boogie board" OR "boogie boarding" OR "jet ski" OR "jet skier" OR "jet skiers" OR "jet skiing" OR "water ski" OR "water skier" OR "water skiers" OR "water skiing" OR aerosol OR boat OR boating OR boats OR bodyboard OR bodyboarding OR canoe OR canoeing OR canoes OR capsize OR capsized OR dermal OR inhalation OR inhale OR kayak OR kayaker OR kayaking OR kayaks OR kneeboard OR kneeboarding OR paddle OR paddling OR raft OR rafting OR rafts OR recreation OR recreational OR rowing OR skin OR surf OR surfer OR surfing OR swim OR swimmer OR swimmers OR swimming OR tubing OR wading OR wakeboarding OR wakeboard)

AND

("marine water" OR "surface water" OR beach OR beaches OR estuaries OR estuarine OR estuary OR "fresh water" OR freshwater OR lake OR lakes OR ocean OR oceans OR pond OR ponds OR reservoir OR reservoirs OR river OR rivers OR sea OR stream OR streams OR water)

Filters: English
Date search was conducted: 10/9/2015
Publication dates searched: 1/1/1995 – 10/9/2015

Web of Science Search:

("*lemmermannii Raphidiopsis mediterranea*" OR *Anabaena flos-aquae* OR *flos-aquae* OR anatoxin OR *Aphanizomenon* OR cylindrospermopsin OR "*C. raciborskii*" OR *Cuspidothrix* OR *Cylindrospermopsis* OR *Cylindrospermum* OR "*Cylindrospermopsis raciborskii*" OR *Dolichospermum* OR "*M. aeruginosa*" OR *Microcystis* OR microcystin OR microcystins OR *Oscillatoria* OR *Planktothrix* OR *Phormidium* OR *Tychonema* OR *Woronichinia*)

AND

("boogie board" OR "boogie boarding" OR "jet ski" OR "jet skier" OR "jet skiers" OR "jet skiing" OR "water ski" OR "water skier" OR "water skiers" OR "water skiing" OR aerosol OR boat OR boating OR boats OR bodyboard OR bodyboarding OR canoe OR canoeing OR canoes OR capsize OR capsized OR dermal OR inhalation OR inhale OR kayak OR kayaker OR kayaking OR kayaks OR kneeboard OR kneeboarding OR paddle OR paddling OR raft OR rafting OR rafts OR recreation OR recreational OR rowing OR skin OR surf OR surfer OR surfing OR swim OR swimmer OR swimmers OR swimming OR tubing OR wading OR wakeboarding OR wakeboard)

AND

("marine water" OR "surface water" OR beach OR Beaches OR estuaries OR estuarine OR estuary OR "fresh water" OR freshwater OR lake OR lakes OR ocean OR oceans OR pond OR ponds OR reservoir OR reservoirs OR river OR rivers OR sea OR stream OR streams OR water)

Filters: English
Date search was conducted: 10/9/2015
Publication dates searched: 1/1/1995–10/9/2015

Research Question 2: What health effects information for humans or animals exposed to microcystins, cylindrospermopsin, or anatoxin-a (through ingestion, inhalation, and dermal exposure routes) has been published since the health effects literature searches were conducted for EPA's 2015 *Health Effects Support Documents for Cylindrospermopsin and Microcystins*?

EPA searched PubMed and WoS to identify candidate journal article literature relevant to health effects associated with exposure to anatoxin-a, cylindrospermopsin, or microcystins. The WoS search string differs slightly from the PubMed search string due to how the search engines treat search terms with more than one word. Both search strings are presented below.

Results

The searches returned 1,000 journal articles after removing duplicates between PubMed and WoS results. Based on a screening review of each article's title and abstract, EPA retrieved 40 articles that appeared to be prospective human epidemiological studies (n = 1), ecological human epidemiologic studies (n = 2), reviews of human health effects (n = 4), *in vivo* animal studies (n = 30), or reviews of *in vivo* animal studies (n = 3).

PubMed Search:

("*A. lemmermannii Raphidiopsis mediterranea*" OR *Anabaena flos-aquae* OR *flos-aquae* OR anatoxin-a OR *Aphanizomenon* OR cylindrospermopsin OR "*C. raciborskii*" OR *Cuspidothrix* OR *Cylindrospermopsis* OR *Cylindrospermum* OR "*Cylindrospermopsis raciborskii*" OR *Dolichospermum* OR "*M. aeruginosa*" OR *Microcystis* OR microcystin OR microcystins OR *Oscillatoria* OR *Planktothrix* OR *Phormidium* OR *Tychonema* OR *Woronichinia*)

AND

("non cancer" OR "blurred vision" OR "cell damage" OR "cellular damage" OR "health effect" OR "health endpoint" OR "health outcome" OR "health risk" OR "loss of protein" OR "loss of water" OR "micronucleated binucleate cell" OR abdominal pain OR ache OR acute OR alanine aminotransferase OR allergic OR allergies OR allergy OR aspartate aminotransferase OR blister OR blistered OR blisters OR cancer OR carcinogen OR carcinogenic OR carcinogens OR chronic OR clinical OR cough OR dermal OR detoxification OR detoxify OR develop OR development OR developmental OR dialysis OR diarrhea OR disease OR DNA OR dyspnea OR electrolyte OR emergency room OR enzyme OR enzymes OR epidemiologic OR epidemiological OR epidemiology OR epilepsy OR epileptic OR epithelium OR eye OR failure OR fever OR gastrointestinal OR genetox OR genotoxic OR glutamyltransferase OR head OR hematologic OR hematological OR hepatic OR histopathologic OR histopathological OR histpathology OR hospital OR hospitalizations OR hospitals OR hospitalization OR ill OR illness OR illnesses OR intoxicate OR intoxicated OR irritate OR irritated OR kidney OR larynx OR lesion OR lesions OR liver OR lung OR lymph OR lymph nodes OR lymphatic OR metabolic OR metabolism OR mucosa OR mutate OR mutated OR mutation OR mutations OR nausea OR necrosis OR neonatal OR neonate OR neonates OR neoplasm OR neurologic OR neurological OR noncancer OR oral OR organ OR pain OR placenta OR pneumonia OR polymorphism OR polymorphisms OR prenatal OR red blood cell OR renal OR reproduction OR respiratory OR seizure OR sick OR sickness OR skin OR stomach OR subacute OR subchronic OR symptom OR symptoms OR teratogen OR teratogenic OR teratogens OR throat OR toxic OR toxicity OR trachea OR tumor OR tumors OR urinary OR urine OR vomit OR vomiting OR conjugate OR conjugated OR diagnose OR diagnosis OR diagnosed OR diagnoses)

Filters: English
Date search was conducted: 10/9/2015
Publication dates searched: 1/1/2014–10/9/2015

Web of Science Search:

("*lemmermannii Raphidiopsis mediterranea*" OR *Anabaena flos-aquae* OR *flos-aquae* OR anatoxin OR *Aphanizomenon* OR cylindrospermopsin OR "*C. raciborskii*" OR *Cuspidothrix* OR *Cylindrospermopsis* OR *Cylindrospermum* OR "*Cylindrospermopsis raciborskii*" OR *Dolichospermum* OR "*M. aeruginosa*" OR *Microcystis* OR microcystin OR microcystins OR *Oscillatoria* OR *Planktothrix* OR *Phormidium* OR *Tychonema* OR *Woronichinia*)

AND

("non cancer" OR "blurred vision" OR "cell damage" OR "cellular damage" OR "health effect" OR "health endpoint" OR "health outcome" OR "health risk" OR "micronucleated binucleate cell" OR abdominal pain OR ache OR acute OR alanine aminotransferase OR allergic OR allergies OR allergy OR aspartate aminotransferase OR blister OR blistered OR blisters OR cancer OR carcinogen OR carcinogenic OR carcinogens OR chronic OR clinical OR cough OR

dermal OR detoxification OR detoxify OR develop OR development OR developmental OR dialysis OR diarrhea OR disease OR DNA OR dyspnea OR electrolyte OR emergency room OR enzyme OR enzymes OR epidemiologic OR epidemiological OR epidemiology OR epilepsy OR epileptic OR epithelium OR eye OR failure OR fever OR gastrointestinal OR genetox OR genotoxic OR glutamyltransferase OR head OR hematologic OR hematological OR hepatic OR histopathologic OR histopathological OR histpathology OR hospital OR hospitalizations OR hospitals OR hospitalization OR ill OR illness OR illnesses OR intoxicate OR intoxicated OR irritate OR irritated OR kidney OR larynx OR lesion OR lesions OR liver OR lung OR lymph OR lymph nodes OR lymphatic OR metabolic OR metabolism OR mucosa OR mutate OR mutated OR mutation OR mutations OR nausea OR necrosis OR neonatal OR neonate OR neonates OR neoplasm OR neurologic OR neurological OR noncancer OR oral OR organ OR pain OR placenta OR pneumonia OR polymorphism OR polymorphisms OR prenatal OR red blood cell OR renal OR reproduction OR respiratory OR seizure OR sick OR sickness OR skin OR stomach OR subacute OR subchronic OR symptom OR symptoms OR teratogen OR teratogenic OR teratogens OR throat OR toxic OR toxicity OR trachea OR tumor OR tumors OR urinary OR urine OR vomit OR vomiting OR conjugate OR conjugated OR diagnose OR diagnosis OR diagnosed OR diagnoses)

Filters: English
Date search was conducted: 10/9/2015
Publication dates searched: 1/1/2014–10/9/2015

WoS research areas searched: Environmental Sciences Ecology OR Marine Freshwater Biology OR Toxicology OR Pharmacology Pharmacy OR Public Environmental Occupational Health OR Microbiology OR Immunology OR Biotechnology Applied Microbiology OR Biochemistry Molecular Biology OR Research Experimental Medicine OR Water Resources OR Infectious Disease OR Science Technology Other Topics OR Life Sciences Biomedicine Other Topics OR Gastroenterology Hepatology OR Pediatrics.

Research Question 3: What recreational water use safety levels or criteria have been set for microcystins or cylindrospermopsin by states or international governments and how did they derive them?

To identify state-level recreational guidelines for cyanobacteria and cyanotoxins, EPA searched the websites of state-level departments of public health, environmental health, and natural resources for all 50 U.S. states. If relevant recreational guidelines were not found by searching state-level websites, EPA conducted Google searches of the internet using state names, key terms for cyanobacteria and cyanotoxins (e.g., harmful algal bloom, blue green algae, microcystin, cylindrospermopsin), and key terms for guidelines (e.g., advisory, guidance, guideline, standard, regulation). For international governments, EPA used the 2012 report, *Current Approaches to Cyanotoxin Risk Assessment, Risk Management and Regulations in Different Countries*, by Dr. Ingrid Chorus, Federal Environment Agency, Germany, to identify international government recreational safety levels for cyanobacteria and cyanotoxins. In addition, EPA implemented the same search strategy as used for U.S. states to identify updated international recreational guidelines or guideline levels not featured in the 2012 report by Dr. Ingrid Chorus.

Research Question 4: What new information, if any, is available regarding how aquatic recreational exposure ingestion rates in children differ among age groups between 0 and 18 years?

Search of Bibliographic Databases

EPA searched PubMed, WoS, and Google Scholar to identify literature that has cited, or is similar (based on terms identified in the titles and abstracts) to, the studies that provide water ingestion data for swimmers or during water recreational activities in EPA's (2011) *Exposure Factors Handbook* (i.e.,(Dorevitch et al. 2011; Dufour et al. (2006); Schets et al. 2011). The PubMed and WoS searches were conducted on 10/9/2015, the publication dates searched were 1/1/2011 to 10/9/2015, and an English filter was applied. The Google Scholar search was conducted on 10/9/2015 and could not be limited by year or language.

Results

Together all three searches returned 341 journal articles. Duplicates were removed between PubMed and Wos, but this total might include duplicates between Google Scholar results and WoS/PubMed results. Based on a screening review of each article's title and abstract, EPA retrieved 5 articles, 4 of which were published between 2013–2015 and appeared to measure or estimate incidental water ingestion. EPA also retrieved one 2012 study that assessed duration of non-swimming recreational water exposure by using novel time lapse photography technology.

Google Search of Internet:

In addition, EPA conducted a Google search of the internet focused on specified URL domains (listed in Table C-1) to identify candidate gray literature (e.g., state, federal, or international government reports or guidance). The Google search string is presented below. The Google search of the internet could not be limited by year or language.

Table C-1. Internet URL Domains Searched for Research Question 4

Organization	URL Domain
U.S. Government	.gov .us
All U.S. States	Google Custom Search Engine
Centers for Disease Control and Prevention, including Agency for Toxic Substances and Disease Registry	cdc.gov
Australia, including Australian Department of Health	gov.au
Canada, including Health Canada	gc.ca
European Union, including • European Chemicals Agency • European Commissions on Environment, Public Health, Food, and Health and Consumers	europa.eu
Public Health England	hpa.org.uk
United Kingdom	gov.uk

Organization	URL Domain
Germany	.de
Education websites	.edu
HERA (Human and Environmental Risk Assessment) Project	heraproject.com
World Health Organization	who.int

Results:

The Google search returned 390 results after removing duplicates. Based on a preliminary screen of each result, EPA retrieved two documents which appeared to either derive or cited an incidental ingestion rate while recreating which had not previously been identified during the literature search process.

Google Search of Internet (conducted separately for each URL domain listed in Table C-1)

(pool OR swim OR swimmer OR swimmers OR swimming OR recreation OR recreational)

AND

(adolescents OR boys OR child OR children OR girls OR kids OR teenagers)

AND

(“activity-related ingestion” OR “incidental ingestion” OR “activity-related ingestion” OR “ingestion of water” OR “water ingestion”)

AND

rate

AND

inurl:.

Filters: None
Date search was conducted: 10/9/2015
Dates searched: Not specified
Web browser: Internet Explorer

Research Question 5: What incidents of companion animal (e.g., dogs, horses) or livestock poisonings, including mortality or adverse health effects, due to exposure to cyanotoxins in freshwaters, marine water, or beaches have occurred in the past 15 years? Specifically, when and where did these incidents occur, to which cyanotoxin were the animals exposed, how were they exposed, and what were the weights and breeds of the affected animal(s)?

EPA searched PubMed, WoS, and Agricola to identify candidate journal article literature relevant to companion animal or livestock poisoning due to exposures to cyanobacterial cells, anatoxin-a, cylindrospermopsin, or microcystins. EPA first searched PubMed and WoS with a focus on dogs. EPA conducted two additional searches in PubMed, WoS, and Agricola focused on livestock, and on cats and birds. The search strings for each search iteration are presented below.

Results

The number of journal articles returned by the three searches is provided in Table C-2. Based on a screening review of the article's title and abstract, EPA retrieved 5 of the 35 journal articles retrieved during the search focused on dogs. These 5 articles appeared to provide information about an incident of cyanotoxin exposure to an animal where the authors confirm that the animal was exposed to a cyanotoxin by either measuring the concentration of cyanotoxin found in the animal and/or by sampling the body of water to which the animal had contact.

Table C-2. Number of Journal Articles Returned by Three Search Strategies for Research Question 5

Search Strategy Focus	Number of Results Returned from PubMed, WoS, and Agricola Searches
Dogs	35[a]
Livestock	100
Cats and birds	169[b]

[a] Search conducted in PubMed and WoS only.

[b] Duplicates between PubMed/WoS results and Agricola results were not removed. Therefore, the cats and birds search might include duplicates between Agricola results and PubMed/WoS results.

C.1 Search strategy focused on dogs

PubMed Search:

("*A. lemmermannii Raphidiopsis mediterranea*" OR *flos-aquae* OR anatoxin-a OR *Aphanizomenon* OR cylindrospermopsin OR "*C. raciborskii*" OR *Cuspidothrix* OR *Cylindrospermopsis* OR *Cylindrospermum* OR "*Cylindrospermopsis raciborskii*" OR *Dolichospermum* OR "*M. aeruginosa*" OR *Microcystis* OR microcystin OR microcystins OR *Oscillatoria* OR *Planktothrix* OR *Phormidium* OR *Tychonema* OR *Woronichinia* OR Cyanobacteria OR cyanotoxin OR Cyanotoxins OR "harmful algae" OR "harmful algal bloom" OR blue green algae)

AND

("health effect" OR "health endpoint" OR "health outcome" OR dead OR death OR deaths OR died OR disease OR diseased OR diseases OR exposed OR exposure OR ill OR illness OR illnesses OR infect OR infected OR infection OR infections OR morbidity OR mortality OR poison OR poisoned OR poisoning OR poisonings OR sick OR sickness OR toxic OR toxicity OR diagnose OR diagnosis OR diagnosed OR diagnoses)

AND

(canine OR canines OR dog OR dogs OR "*Canis lupus familiaris*" OR "*Canis familiaris*")

Filters: English
Date search was conducted: 10/5/2015
Publication dates searched: 1/1/2012–10/5/2015

Web of Science Search:

(“*lemmermannii Raphidiopsis mediterranea*” OR *flos-aquae* OR anatoxin OR *Aphanizomenon* OR cylindrospermopsin OR “*C. raciborskii*” OR *Cuspidothrix* OR *Cylindrospermopsis* OR *Cylindrospermum* OR “*Cylindrospermopsis raciborskii*” OR *Dolichospermum* OR “*M. aeruginosa*” OR *Microcystis* OR microcystin OR microcystins OR *Oscillatoria* OR *Planktothrix* OR *Phormidium* OR *Tychonema* OR *Woronichinia* OR Cyanobacteria OR cyanotoxin OR Cyanotoxins OR “harmful algae” OR “harmful algal bloom” OR blue green algae)

AND

(“health effect” OR “health endpoint” OR “health outcome” OR dead OR death OR deaths OR died OR disease OR diseased OR diseases OR exposed OR exposure OR ill OR illness OR illnesses OR infect OR infected OR infection OR infections OR morbidity OR mortality OR poison OR poisoned OR poisoning OR poisonings OR sick OR sickness OR toxic OR toxicity OR diagnose OR diagnosis OR diagnosed OR diagnoses)

AND

(canine OR canines OR dog OR dogs OR “*Canis lupus familiaris*” OR “*Canis familiaris*”)

Filters: English
Date search was conducted: 10/5/2015
Publication dates searched: 1/1/2012–10/5/2015

C.2 Search strategy focused on livestock

PubMed and Agricola Searches:

(“*A. lemmermannii Raphidiopsis mediterranea*” OR *flos-aquae* OR anatoxin-a OR *Aphanizomenon* OR cylindrospermopsin OR “*C. raciborskii*” OR *Cuspidothrix* OR *Cylindrospermopsis* OR *Cylindrospermum* OR “*Cylindrospermopsis raciborski*i” OR *Dolichospermum* OR “*M. aeruginosa*” OR *Microcystis* OR microcystin OR microcystins OR *Oscillatori*a OR *Planktothrix* OR *Phormidium* OR *Tychonema* OR *Woronichinia* OR *Cyanobacteria* OR *cyanotoxin* OR *Cyanotoxins* OR “harmful algae” OR “harmful algal bloom” OR blue green algae)

AND

(“health effect” OR “health endpoint” OR “health outcome” OR dead OR death OR deaths OR died OR disease OR diseased OR diseases OR exposed OR exposure OR ill OR illness OR illnesses OR infect OR infected OR infection OR infections OR morbidity OR mortality OR poison OR poisoned OR poisoning OR poisonings OR sick OR sickness OR toxic OR toxicity OR diagnose OR diagnosis OR diagnosed OR diagnoses)

AND

(alpaca OR alpacas OR bronco OR broncos OR buffalo OR bull OR bulls OR cattle OR colt OR colts OR cow OR cows OR bovine OR bison OR oxen OR donkey OR donkeys OR duck OR ducks OR equine OR ewe OR ewes OR fillies OR filly OR foal OR foals OR gelding OR geldings OR heifer OR heifers OR horse OR horses OR lamb OR lambs OR livestock OR llama OR llamas OR mare OR mares OR mule OR mules OR mustang OR mustangs OR ponies OR pony OR ram OR rams OR sheep OR stallion OR stallions OR steer OR pig OR pigs OR piglet OR piglets)

Filters: English
Date search was conducted: 11/25/2015
Publication dates searched: 1/1/2012–11/25/2015

Web of Science Search:

("*lemmermannii Raphidiopsis mediterranea*" OR *flos-aquae* OR anatoxin OR *Aphanizomenon* OR cylindrospermopsin OR "*C. raciborskii*" OR *Cuspidothrix* OR *Cylindrospermopsis* OR *Cylindrospermum* OR "*Cylindrospermopsis raciborskii*" OR *Dolichospermum* OR "*M. aeruginosa*" OR *Microcystis* OR microcystin OR microcystins OR *Oscillatoria* OR *Planktothrix* OR *Phormidium* OR *Tychonema* OR *Woronichinia* OR Cyanobacteria OR cyanotoxin OR Cyanotoxins OR "harmful algae" OR "harmful algal bloom" OR blue green algae)

AND

("health effect" OR "health endpoint" OR "health outcome" OR dead OR death OR deaths OR died OR disease OR diseased OR diseases OR exposed OR exposure OR ill OR illness OR illnesses OR infect OR infected OR infection OR infections OR morbidity OR mortality OR poison OR poisoned OR poisoning OR poisonings OR sick OR sickness OR toxic OR toxicity OR diagnose OR diagnosis OR diagnosed OR diagnoses)

AND

(alpaca OR alpacas OR bronco OR broncos OR buffalo OR bull OR bulls OR cattle OR colt OR colts OR cow OR cows OR bovine OR bison OR oxen OR donkey OR donkeys OR duck OR ducks OR equine OR ewe OR ewes OR fillies OR filly OR foal OR foals OR gelding OR geldings OR heifer OR heifers OR horse OR horses OR lamb OR lambs OR livestock OR llama OR llamas OR mare OR mares OR mule OR mules OR mustang OR mustangs OR ponies OR pony OR ram OR rams OR sheep OR stallion OR stallions OR steer OR pig OR pigs OR piglet OR piglets)

Filters: English
Date search was conducted: 11/25/2015
Publication dates searched: 1/1/2012–11/25/2015

C.3 Search strategy focused on cats and birds

PubMed and Agricola Searches:

("*A. lemmermannii Raphidiopsis mediterranea*" OR *flos-aquae* OR anatoxin-a OR *Aphanizomenon* OR cylindrospermopsin OR "*C. raciborskii*" OR *Cuspidothrix* OR *Cylindrospermopsis* OR *Cylindrospermum* OR "*Cylindrospermopsis raciborskii*" OR *Dolichospermum* OR "*M. aeruginosa*" OR *Microcystis* OR microcystin OR microcystins OR *Oscillatoria* OR *Planktothrix* OR *Phormidium* OR *Tychonema* OR *Woronichinia* OR *Cyanobacteria* OR cyanotoxin OR Cyanotoxins OR "harmful algae" OR "harmful algal bloom" OR blue green algae)

AND

("health effect" OR "health endpoint" OR "health outcome" OR dead OR death OR deaths OR died OR disease OR diseased OR diseases OR exposed OR exposure OR ill OR illness OR illnesses OR infect OR infected OR infection OR infections OR morbidity OR mortality OR

poison OR poisoned OR poisoning OR poisonings OR sick OR sickness OR toxic OR toxicity OR diagnose OR diagnosis OR diagnosed OR diagnoses)

AND

(feline OR felines OR cat OR cats OR kitten OR kittens OR "*F. Catus*" OR "*Felis Catus*" OR bird OR birds OR avian OR waterfowl)

Filters: English
Date search was conducted: 2/1/2016
Publication dates searched: 1/1/2012–2/1/2016

Web of Science Search:

("*lemmermannii Raphidiopsis mediterranea*" OR *flos-aquae* OR anatoxin OR *Aphanizomenon* OR cylindrospermopsin OR "*C. raciborskii*" OR *Cuspidothrix* OR *Cylindrospermopsis* OR *Cylindrospermum* OR "*Cylindrospermopsis raciborskii*" OR *Dolichospermum* OR "*M. aeruginosa*" OR *Microcystis* OR microcystin OR microcystins OR *Oscillatoria* OR *Planktothrix* OR *Phormidium* OR *Tychonema* OR *Woronichinia* OR Cyanobacteria OR cyanotoxin OR Cyanotoxins OR "harmful algae" OR "harmful algal bloom" OR blue green algae)

AND

("health effect" OR "health endpoint" OR "health outcome" OR dead OR death OR deaths OR died OR disease OR diseased OR diseases OR exposed OR exposure OR ill OR illness OR illnesses OR infect OR infected OR infection OR infections OR morbidity OR mortality OR poison OR poisoned OR poisoning OR poisonings OR sick OR sickness OR toxic OR toxicity OR diagnose OR diagnosis OR diagnosed OR diagnoses)

AND

(feline OR felines OR cat OR cats OR kitten OR kittens OR "*F. Catus*" OR "*Felis Catus*" OR bird OR birds OR avian OR waterfowl)

Filters: English
Date search was conducted: 2/1/2016
Publication dates searched: 1/1/2012–2/1/2016

APPENDIX D. REVIEW OF THE STATE OF THE SCIENCE ON CYANOBACTERIAL CELLS HEALTH EFFECTS

D.1 Introduction

This appendix provides information gathered and reviewed to determine the state of the science on health effects from cyanobacterial cells. EPA conducted literature searches to identify studies relevant to the health effects from cyanobacterial cells. Detailed information on the design and implementation of these searchers is provided in Appendix C. Results from these literature searches were reviewed for relevance to cyanobacterial cell exposures and health effects. This appendix builds on the cyanobacterial bloom information included in the main document by discussing additional detail on the nature of cyanobacterial cells as stressors and, in particular, the health effects associated with exposures to cyanobacterial cells.

D.1.1 Animal Studies

Cyanobacterial cells cause allergenicity and irritation in animals, independent of whether the cyanobacterial cells produce toxin. Three animal studies (Shirai et al. 1986; Stewart et al. 2006c; Torokne et al. 2001) demonstrated hypersensitivity reactions and dermal and eye irritation in several species. Results from Torokne et al. (2001) indicated that hypersensitization reactions do not correlate with microcystin content. Although the number of studies is limited and different species were evaluated in each study, these studies provide evidence to support hypersensitivity reactions in animals from exposure to cyanobacteria when cyanotoxins are not present (Shirai et al. 1986; Torokne et al. 2001) and when they are (Stewart et al. 2006c).

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 not detected to 2.21 mg/g. To determine sensitization, guinea pigs were initiated with an intradermal injection of freeze-dried cyanobacteria followed 7 days later by topical application at the injection site. Sensitization was moderate to strong in 30–67 percent of guinea pigs and did not correlate with microcystin content. The *Aphanizomenon ovalisporum* sample (a nontoxin-producing strain) sensitized 91percent of the animals and was the strongest allergen. Skin irritation tests in albino rabbits showed slight or negligible irritation, except for *Aphanizomenon ovalisporum*, which showed moderate irritation. The eye irritation evaluation in rabbits was positive for four of the five samples containing *Microcystis*.

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 sonicated *Microcystis* cells. A positive reaction, as assessed by footpad swelling, was seen in mice immunized with either live cells or sonicated cells. Both toxic and nontoxic *Microcystis* cells induced delayed-type hypersensitivity in this mouse study. Because this strain of mouse is unresponsive to lipopolysaccharide (LPS), the footpad delayed-type hypersensitivity was not related to LPS, thus, the antigenic component of the sonicated cyanobacterial cells is not known.

Stewart et al. (2006c) conducted a mouse ear swelling test in which cylindrospermopsin and *C. raciborskii* solutions generated irritation of the abdominal skin exposed during induction

(2 percent w/v lysed cell solution containing 73 μg/mL cylindrospermopsin). Subsequent dermal exposures to the *C. raciborskii* solution produced hypersensitivity reactions ($p = 0.001$). The cyanobacteria *Microcystis aeruginosa* and *Anabaena circinalis* elicited no responses in this test.

Two of the cyanobacterial cell studies in animals found that rodents became sensitized after exposure and subsequent challenge to nontoxin strains (Shirai et al. 1986; Torokne et al. 2001). Torokne et al. (2001) found that a nontoxic strain was more sensitizing and irritating than the toxic strains evaluated. These experiments support the conclusion that there is no relationship between the cyanotoxin content and the allergenic effect of cyanobacteria.

D.1.2 Clinical and Laboratory Human Studies

Several types of studies and reports provide information on associations between cyanobacteria exposure and health effects. Clinical and *in vitro* studies (Bernstein et al. 2011; Geh et al. 2015; Pilotto et al. 2004; Stewart et al. 2006a) have been able to assess associations between cyanobacteria exposure and human health effects including dermal and allergenic reactions. Three clinical studies assessed dermal exposure to cyanobacterial cells using skin-patch or skin-prick testing in humans (Bernstein et al. 2011; Pilotto et al. 2004; Stewart et al. 2006a). Some of the exposed individuals showed mild irritation or allergenicity. No statistically significant dose-response relationships were found between skin irritation and increasing cyanobacterial cell concentrations. The allergenicity study suggests that cyanobacteria are allergenic, particularly among people with chronic rhinitis (Bernstein et al. 2011).

Skin-patch testing in humans was performed by Pilotto et al. (2004) with laboratory-grown cylindrospermopsin-producing *C. raciborskii* cells, both whole and lysed, which were applied using adhesive patches at concentrations ranging from < 5,000 to 200,000 cells/mL to the skin of 50 adult volunteers. After 24 hours, patches were removed and evaluation of the erythematous reactions were graded. Analysis of participants' reactions to patches treated with whole cells showed an OR of 2.13 and a 95% Confidence Interval (CI) of 1.79–4.21 ($p < 0.001$). Lysed cells patch analysis showed an OR of 3.41 and a 95% CI of 2.00–5.84 ($p < 0.001$). No statistically significant increase or dose-response between skin reactions and increasing cell concentrations for either patches (whole or lysed) was observed. Subjects had skin reactions to the cylindrospermopsin, and positive control patches more frequently than to the negative control patches. The mean percentage of subjects with a reaction was 20% (95% CI: 15–31%). When subjects reacting to negative controls (39) were excluded, the mean percentage was 11percent (95% CI: 6–18%). Evaluation of erythematous reactions showed that mild irritations (grade 2) were resolved in all cases within 24 to 72 hours.

Stewart et al. (2006a) conducted a skin-patch test with 39 volunteers (20 dermatology outpatients; 19 controls) who were exposed to 6 cyanobacterial suspensions, including toxigenic species, nontoxigenic species, mixed suspensions, and two cyanobacterial LPS extracts. All cyanobacterial suspensions of lyophilized cells were tested at three concentrations, 0.25 percent w/v, 0.05 percent w/v, 0.005 percent w/v, and the estimated doses of cyanotoxins were 2.4 ng/kg cylindrospermopsin and 2.6 ng/kg microcystins. Only one subject showed significant responses to cyanobacterial suspensions, specifically to two suspensions of cyanobacterial cells: *C. raciborskii* and mixed *M. aeruginosa* and *C. raciborskii*, both of which contained one or more cyanotoxins. This subject showed no evidence of any dose-response effect in the dermal reactions. None of the participants reacted to the cyanobacterial LPS extracts, which ranged from

260 ppb to 31 ppm. This small clinical study demonstrated that dermal hypersensitivity reactions to cyanobacteria exposure occur infrequently, and further research into risk factors for predisposition to this type reaction could be beneficial.

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. The authors found that 86 percent of the 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 indicated that cyanobacterial allergenicity is associated with the non-toxic portion of the cyanobacteria.

Geh et al. (2015) studied the immunogenicity of extracts of toxic and non-toxic strains of *M. aeruginosa* in patient sera (18 patients with chronic rhinitis and 3 non-atopic healthy subjects as documented in Bernstein et al. [2011]). Enzyme Linked Immunosorbent Assay (ELISA) test was used to test IgE-specific reactivity, and gel electrophoresis, followed by immunoblot and mass spectrometry, was done to identify the relevant sensitizing peptides. The authors found an increase in specific IgE in those patients tested with the non-toxic *Microcystis* extract than the extract from the toxic strain. 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-toxin-producing strains of cyanobacteria are more allergenic than toxin-producing strains in allergic patients, and that the toxin may have an inhibitory effect on the allergenicity of the cyanobacterial cells.

D.1.3 Epidemiological Studies and Case Reports

Among the epidemiological studies discussed here, some identified significant associations between cyanobacteria exposure and a range of health outcomes including dermal, eye/ear, GI, and respiratory effects. Several of these studies also measured one or more cyanotoxins and found no association between cyanotoxin occurrence or exposure and health effects. Additional evidence from outbreak and case reports provides support for health effects associated with cyanobacteria exposure. Overall, these studies provide evidence of significant associations between cyanobacterial cell exposure and human health effects even in the absence of cyanotoxins. However, the reported associations between cyanobacterial cell densities and health outcomes are not consistent. The studies vary in study design, methods used, size of study population, cyanobacterial species evaluated, health effects identified, and cyanobacterial cell densities associated with human health effects. Therefore, substantial uncertainty remains regarding the associations between cyanobacterial cell exposure and human health effects.

Eight epidemiological studies evaluated short-term health effects associated with recreational exposure to cyanobacterial blooms (El Saadi et al. 1995; Lévesque et al. 2014; Lin et al. 2015; Philipp 1992; Philipp & Bates 1992; Philipp et al. 1992; Pilotto et al. 1997; Stewart et al. 2006d). See Table D-1 for a summary list of these studies. The health outcomes evaluated included dermal, GI, respiratory, and other acute effects, such as eye or ear symptoms. Seven studies evaluated recreational exposure to freshwater cyanobacteria, and one evaluated exposure to marine water cyanobacteria (Lin et al. 2015). Two studies included field sites in the continental United States or Canada (Lévesque et al. 2014; Stewart et al. 2006d), three occurred

in the United Kingdom (Philipp 1992; Philipp & Bates 1992; Philipp et al. 1992), and three were conducted in sub-tropical and tropical regions in Australia (El Saadi et al. 1995; Pilotto et al. 1997) and Puerto Rico (Lin et al. 2015). These epidemiological studies are discussed below in chronological order.

Table D-1. Cyanobacteria Epidemiological Studies Summary

Reference	Study Design, n, and Location	Cyanobacteria Identified	Cyanotoxins Measured	Health Association[a]	Lowest Significant Cyanobacterial Cell Density (cells/mL)
Philipp (1992)	Cross-sectional n = 246 UK (Hampshire)	*Microcystis* sp., *Gleotrichia* sp.	-	No statistically significant health associations	No quantitative cyanobacterial cell densities provided
Philipp and Bates (1992)	Cross-sectional n = 382 UK (Somerset)	*Microcystis* sp., *Gleotrichia* sp.	-	No statistically significant health associations	No quantitative cyanobacterial cell densities provided
Philipp et al. (1992)	Cross-sectional n = 246 UK (Lincolnshire, South Yorkshire)	*Oscillatoria* sp., *Aphanizomenon* sp., *Aphanothece* sp., *Merismopedia* sp.	-	No statistically significant health associations	No quantitative cyanobacterial cell densities provided
El Saadi et al. (1995)	Case-control n cases = 102 GI, 86 dermatological n controls = 132 Australia (South Australia)	*Anabaena* sp., *Aphanizomenon* sp., *Planktothrix* sp., *Anabaena circinalis*, *Microcystis aeruginosa*	-	No statistically significant health associations	No quantitative cyanobacterial cell densities provided
Pilotto et al. (1997)	Cross-sectional n = 295 exposed n = 43 unexposed Australia (South Australia, New South Wales, Victoria)	*Microcystis aeruginosa*, *Microcystis* sp., *Anabaena* sp., *Aphanizormenon* sp., *Nodularia spumigena*	Hepatotoxins detected by mouse bioassay	Significant positive association between combined symptoms (GI, dermal, respiratory, fever, eye or ear irritation) and cyanobacteria	> 5,000
Stewart et al. (2006d)	Cohort (prospective) n = 1,331 Australia (Queensland, New South Wales) and Florida	Cyanobacteria identified, species not specified	Microcystins detected by high-performance liquid chromatography (HPLC) with photodiode array detection or ELISA; cylindrospermopsin and anatoxin-a detected by HPLC-MS/MS; saxitoxins not detected by HPLC with fluorescence detection	Significant positive association between respiratory symptoms and cyanobacteria Significant positive association between combined symptoms (GI, dermal, respiratory, fever, eye or ear irritation) and cyanobacteria	> 100,000[b]
Lévesque et al. (2014)	Cohort (prospective) n = 466 Canada (Quebec)	Cyanobacteria identified, species not specified	Microcystins detected by ELISA	Significant positive association between GI symptoms with fever and cyanobacteria	20,000–100,000

Reference	Study Design, n, and Location	Cyanobacteria Identified	Cyanotoxins Measured	Health Association[a]	Lowest Significant Cyanobacterial Cell Density (cells/mL)
Lin et al. (2015)[c]	Cohort (prospective) n = 15,726 Puerto Rico (Boquerón)	Cyanophyte filament, *Pseudanabaena* sp., Picocyanophyte, *Synechococcus* sp., *Synechocystis* sp., Cyanophyte cell pair, *Phormidium* sp., *Lyngbya* sp., *Trichodesmium* sp., *Aphanothece* sp., *Johannesbaptistia* sp., *Komvophoron* sp., Cyanophyte colony, Cyanophyte unicell sphere	Lyngbyatoxin-a and debromo-aplysiatoxin measured but not detected by HPLC-MC	Significant positive association between respiratory illness and cyanobacteria other than picocyanobacteria	36.7–237.4
				significant positive association between rash and cyanobacteria other than picocyanobacteria	> 237.4

[a] Includes only significant associations between recreational cyanobacteria exposure and health effects.
[b] Values were converted from cyanobacterial cell surface area (>12.0 mm2/mL) to cyanobacterial cell density (>100,000 cells/mL) using conversions in NHMRC (2008). Relationship between biomass and cyanobacterial cell density can vary by species and cell size (Lawton et al. 1999; Stewart et al. 2006d).
[c] Lin et al. (2015) evaluated picocyanobacteria and cyanobacteria other than picocyanobacteria separately.

Three cross-sectional studies were conducted by Philipp et al. (Philipp 1992; Philipp & Bates 1992; Philipp et al. 1992) to evaluate health effects related to exposure to cyanobacteria from recreational activities including sailing, windsurfing, and fishing in water bodies in the United Kingdom. Questionnaires were administered to participants who visited one of six inland lakes to evaluate exposure and morbidity (including dermal, eye/ear, GI, and respiratory symptoms). Several species of cyanobacteria were identified and, in some cases, cyanobacterial levels exceeded the National Rivers Authority threshold for "potential to cause harm." Only minor morbidity was identified among recreators, and no statistically significant associations between cyanobacteria exposure and morbidity were identified.

El Saadi et al. (1995) conducted a case-control study in Australia to evaluate exposure to river water with detectable levels of cyanobacteria and GI and dermatological symptoms evaluated by a medical practitioner. This river was used as a source for drinking water, domestic water, and recreational water. The authors found no significant association between recreational exposure to river water with cyanobacteria and GI or dermatological symptoms. Cyanotoxins were not measured, but species of cyanobacteria were present that were capable of producing cyanotoxins.

These four earlier studies (El Saadi et al. 1995; Philipp 1992; Philipp & Bates 1992; Philipp et al. 1992) provided no quantitative data on cyanobacterial cell densities. Therefore, they could not help inform determination of a quantitative level associated (or not associated) with health effects.

Four more recent epidemiological studies assessed the association between exposure to recreational waters containing cyanobacteria and human health and provide quantitative density data for cyanobacterial cells (Lévesque et al. 2014; Lin et al. 2015; Pilotto et al. 1997; Stewart et al. 2006d). These studies reported at least one statistically significant association between

exposure to cyanobacteria and human health outcomes., including GI illness (Lévesque et al. 2014), respiratory symptoms (Lin et al. 2015; Stewart et al. 2006d), dermal symptoms (Lin et al. 2015), or combined symptomology (GI, dermal, respiratory, and other symptoms) (Pilotto et al. 1997; Stewart et al. 2006d). These associations were linked to a range of densities of cyanobacterial cells from as low as > 5,000 cells/mL (Pilotto et al. 1997) to as high as 100,000 cells/mL (analogous to ≥ 12 mm^2/mL (NHMRC 2008; Stewart et al. 2006d). In contrast to the studies that examined all cyanobacteria, Lin et al. (2015) evaluated picocyanobacteria, larger cyanobacterial cells, and total phytoplankton, and reported health effects associated with 37–1,461 cells/mL for cyanobacteria other than picocyanobacteria.

Pilotto et al. (1997) investigated the health effects from recreational exposures (including jet-skiing, water-skiing, swimming, and windsurfing) to cyanobacteria in Australia. The study included 852 participants, 777 who had water contact and were considered exposed, and 75 not exposed. There were 338 recreators (295 exposed, 43 not exposed) after exclusion of those who experienced symptoms or had recreational exposure in the 5 days prior to the initial interview at the water recreation site (the *after exclusion* study group). Health outcomes evaluated included diarrhea, vomiting, flu-like symptoms (e.g., cough), skin rashes, mouth ulcers, fevers, or eye or ear infections. Water samples were collected for evaluation of cyanobacterial cell counts, hepatotoxins, and neurotoxins.

In the *after exclusion* study group, when all symptoms were combined, the authors found a significant trend of increasing symptom occurrence with duration of exposure at 7 days post-exposure (*p-value* for trend =0.03). Similarly, in the *after exclusion* study group there was a significant trend of increasing symptom occurrence with increasing cyanobacterial cell count (*p-value* for trend = 0.04). To account for the combined effect of duration of exposure and cyanobacterial cell density, unexposed participants were compared with those exposed for up to 60 minutes and for more than 60 minutes to water with up to 5,000 cells/mL and to water with more than 5,000 cells/mL. For the *after exclusion* study group, a significant trend of increasing symptom occurrence with increasing levels of exposure was identified (*p-value* for trend =0.004). In addition, participants with recreational exposure for more than 60 minutes to cyanobacterial densities above 5,000 cells/mL had a significantly higher symptom occurrence rate at 7 days post-exposure than unexposed participants (OR = 3.44, CI: 1.09–10.82). In this study, the significant trends observed in the *after exclusion* study group were not observed when all participants were included.

Pilotto et al. (1997) reported toxicity data collected by the Australia Water Quality Center. Presence or absence of particulate (intracellular) hepatotoxins in concentrated surface water phytoplankton samples was measured by mouse bioassay. The authors reported that hepatotoxins were identified at one site on two separate interview days and at three sites for one day each. No evidence of neurotoxins was detected. They reported that no significant association was found between the presence of hepatotoxins and symptom occurrence at two and seven days after exposure. Data and analysis methods were not provided. The authors point out that trends were observed at seven days and not at two days after exposure and this might suggest a delayed rather than an immediate allergic response. The authors also stated they could not rule out other causative factors, such as other microorganisms, that could co-occur with cyanobacteria. The results from this study informed the recommendations made by WHO in *Guidelines for Safe Recreational Water Environments* (WHO 2003).

Stewart et al. (2006d) conducted a prospective cohort study to investigate the incidence of acute symptoms in individuals exposed to cyanobacteria via recreational activities in lakes and rivers in Australia and Florida. This study included 1311 recreators with any water contact-related activity (e.g., swimming, boat entry/egress). Cyanobacterial cell densities were characterized in terms of cell surface area rather than cell counts (to normalize for cell size differences among different species). Authors evaluated incidence of acute symptoms in recreators exposed to low, medium and high levels of cyanobacteria.

Study subjects were asked to complete a self-administered questionnaire before leaving for the day after enrollment and to submit to a telephone follow-up interview. The questionnaire and follow-up interview forms gathered information on various acute illnesses, their onset and severity. Respiratory symptoms among study participants in the high recreational exposure group (total cyanobacterial cell surface area > 12 mm^2/mL on day of recreation) were significantly greater compared to participants in the low recreational exposure group (< 2.4 mm^2/mL) (adjusted OR = 2.1, 95% CI: 1.1–4.0). Respiratory symptoms were defined as difficulty breathing, dry cough, productive cough, runny nose, unusual sneezing, sore throat, or wheezy breathing. Reports of any symptom among study participants in the high exposure group were significantly greater compared to reports among study participants in the low recreational exposure group (adjusted OR = 1.7, 95% CI: 1.0–2.9). However, when subjects with recent prior recreational water exposure were excluded the result remained positive but not significant (adjusted OR = 1.6, 95% CI: 0.8–3.2). A dose-response relationship between increased cyanobacterial biomass and increased symptom reporting was not identified. The authors speculated that the pattern in their data could be due to a threshold effect. No other significant associations with health effects were identified.

For water samples that contained potentially toxic cyanobacteria, Stewart et al. (2006d) measured cyanotoxins including microcystins, saxitoxins, cylindrospermopsin and anatoxin-a by HPLC or HPLC-MS/MS methods. Cyanotoxins were infrequently identified and only at low levels. Microcystins were detected on two occasions (1 and 12 µg /L). Cylindrospermopsin was found on seven occasions (ranging from 1-2 µg /L). Anatoxin-a was identified on a single recruitment day at a concentration of 1 µg/L. A statistically significant increase in symptom reporting was found to be associated with anatoxin-a exposure, but the number of exposed subjects was very low (n =18). No relationship between fecal indicator bacteria (fecal coliforms) and symptoms was identified.

Lévesque et al. (2014) conducted a prospective study of health effects including GI, respiratory, dermal, eye/ear, and other symptoms associated with cyanobacteria and microcystin exposure at three lakes in Canada (Quebec), one of which was a local supply of drinking water. The study evaluated acute symptoms in humans (466 subjects included in analysis) living in proximity to lakes affected by blooms and analyzed recreational exposure (full and limited contact) and drinking-water exposure scenarios for both cyanobacterial cells and microcystins.

More severe GI symptoms, defined as diarrhea, vomiting, nausea and fever, or abdominal cramps and fever, were associated with recreational contact (full and limited) and cyanobacteria. For the more severe GI symptoms, the adjusted relative risk RR increased with cyanobacterial cell counts providing evidence of a dose-response relationship (*p-value* for trend= 0.001, < 20,000 cells/mL: RR = 1.52, 95% CI: 0.65–3.51; 20,000–100,000 cells/mL: RR = 2.71, 95% CI: 1.02–7.16; > 100,000 cells/mL: RR = 3.28, 95% CI: 1.69–6.37). No evidence of a dose-response relationship for cyanobacterial cell counts and the less severe GI symptoms was found.

No relationship was observed between duration of contact or head immersion and risk of GI symptoms. A significant increase for both the less and the more severe GI symptoms was found with contact in the more highly impacted lakes (median cell densities 20,001–21,485 cells/mL), but not in the less impacted lake (median 1,032 cells/mL). No relationship was observed between microcystin concentrations and risk of GI symptoms. No significant associations between recreational exposures to cyanobacteria and health effects other than GI effects were identified.

To evaluate possible co-exposures, authors measured microcystin concentrations and *E. coli* as a fecal indicator. Lévesque et al. (2014) measured particulate (intracellular) and dissolved microcystins by ELISA and found that microcystins concentrations varied by lake and by sample location (littoral vs. limnetic). Microcystin was detected in all three lakes. At Lake William the median values were below the limit of detection at littoral and limnetic stations, with maximum values of 0.63 μg/L and 0.02 μg/L respectively. At Lake Roxton littoral stations, the median concentration was 0.23 μg/L (range: 0.008 μg/L–108.8 μg/L) and at limnetic stations the median was 0.12 μg/L (range: 0.04 μg/L–1.12 μg/L). The Mallets Bay littoral stations had a median of 0.70 μg/L (range: under limit of detection – 773 μg/L) and the limnetic stations had a median of 0.35 μg/L (range: 0.001 μg/L–125 μg/L).

Lévesque et al. (2014) reported that as a whole the microcystin concentrations during contact were relatively low (1st tertile: < 0.0012 μg/L; 2nd tertile: 0.0012–0.2456 μg/L; 3rd tertile: > 0.2456 μg/L). Symptoms were examined in relation to recreational and drinking water exposure to cyanobacteria and microcystin. Only GI symptoms were associated with recreational contact. The highest concentration of microcystin at which an episode of GI symptoms was reported was 7.65 μg/L. There was no significant increase in adjusted relative risk of GI symptoms with recreational exposure to more than 1 μg/L microcystin. Adjusted relative risks (adjusted for gender, gastrointestinal symptoms reported in the two weeks prior to data collection, residence's source of drinking water) for GI illness without fever and GI illness with fever were 1.06 (95% CI=0.32–3.52) and 1.48 (95% CI = 0.41–5.23), respectively. There were significant increases in adjusted relative risk of several symptoms in participants who received their drinking water from a source contaminated by cyanobacteria (muscle pain, GI illness, skin, and ear symptoms).

Lévesque et al. (2014) found that the geometric mean of *E. coli* at the three lakes ranged from 0 to 145 CFU per 100 mL, and there was no association between GI illness and *E.coli* levels. The authors noted that GI symptoms could have other causes, such as *Aeromonas* infections; however, the symptoms were not related to fecal contamination as measured by culturable *E. coli*. They also noted that people avoided full recreational contact during blooms and more people engaged in limited contact recreation at higher cell counts. This observation explains the counterintuitive finding that participants with limited contact exposure (fishing, watercraft without direct water contact) had higher likelihood of symptom reporting compared to participants with full contact.

A follow-up analysis (Lévesque et al. 2016) characterized the same health data as Lévesque et al. (2014) to evaluate the relationship of bacterial endotoxin (lipopolysaccharides or LPS) concentration to GI symptoms. Endotoxin concentrations were slightly correlated with cyanobacterial counts (polychoric correlation coefficient = 0.57). The highest tertile of endotoxin concentration (> 48 endotoxin units/mL) was significantly associated with GI illness both with and without fever (GI illness without fever relative risk (RR) = 2.87, CI: 1.62–5.08; GI illness with fever RR = 3.11, CI: 1.56–6.22). Adjustment to the level of cyanobacteria did not

alter the relationship between endotoxin and GI illness and authors hypothesize that other gram negative bacteria might play a role in the relationship between endotoxin levels and GI illness as has been suggested in a previous study (Berg et al. 2011). Authors note that they stored filtered water samples at -80 ºC for several months prior to conducting endotoxin testing and that another study (O'Toole et al. 2009) showed a 44 percent mean decline in the concentration of endotoxins in samples stored at -80 ºC for several weeks compared to samples stored at 4 ºC for 24 hours. Lévesque et al. (2016) caution that concentrations reported could be underestimated and should be interpreted on an ordinal basis. Two other studies conducting endotoxin testing on frozen samples found concentrations of a similar magnitude as this study (Berg et al. 2011; Rapala et al. (2002).

Lin et al. (2015) conducted a prospective study based on data collected in 2009 at Boquerón, Puerto Rico for 26 study days involving 15,726 enrollees to examine the association between phytoplankton cell counts and illness among beachgoers. Three categories of phytoplankton were evaluated: picocyanobacteria, cyanobacteria other than picocyanobacteria, and total phytoplankton. The analysis compared people exposed at phytoplankton cell count levels > 25th percentile (e.g., 25th to 75th percentile, > 75th percentile) to people exposed at levels < 25th percentile (range of cyanobacteria other than picocyanobacteria: < 37–1461 cells/mL).

The study reported significant associations between recreational exposure to cyanobacteria other than picocyanobacteria and respiratory symptoms, rash, and earache. For the other symptoms measured, including eye irritation, no significant associations were observed. More specifically, cyanobacterial (other than picocyanobacterial) densities of 37 to 237 cells/mL (> 25th to < 75th percentile) and densities ≥ 237 cells/mL (≥ 75th percentile) were associated with increased respiratory symptoms (> 25th to < 75th percentile, odds ratio (OR) = 1.30, 95% CI = 1.08–1.56; ≥ 75th percentile, OR = 1.37, 95% CI = 1.12–1.67) in study participants who reported body immersion. Respiratory symptom occurrence was defined as any two of the following: sore throat, cough, runny nose, cold, or fever. Cyanobacterial (other than picocyanobacterial) densities >237 cells/mL were associated with rash (OR = 1.32, 95% CI = 1.05–1.66) and earache (OR = 1.75, 95% CI = 1.09–2.82). Study participants who reported head submersion or swallowing of water showed no relationship between recreational exposures to cyanobacteria (other than picocyanobacteria) and respiratory symptoms. There was no association between recreational exposures to cyanobacteria (other than picocyanobacteria) and respiratory symptoms in study participants who reported head submersion or swallowing of water. A statistically significant association between cyanobacterial cell exposure (other than picocyanobacterial cell exposure) and all health effects combined was also observed.

Lin et al. (2015) measured the dermatotoxins, debromoaplysiatoxin and lyngbyatoxin, using high performance liquid chromatography-mass spectrometry and did not detect levels above the limit of detection of 1.0 ppb. Authors reported that debromoaplysiatoxin and lyngbyatoxin-a are photolabile and are unlikely to persist in the water column (Moikeha & Chu 1971). They noted that the health effects identified in this study were consistent with previous blooms of *Lyngbya majuscula*, which can produce these toxins, though *Lyngbya* only comprised 3 percent of total planktonic cyanobacteria (other than picocyanobacteria). It is also possible that the cyanobacterial cells could be having direct health effects as cyanotoxins levels were below the limit of detection.

To evaluate possible co-exposures, some studies measured cyanotoxins and fecal indicators. Lin et al. (2015), Lévesque et al. (2014), Pilotto et al. (1997), and Stewart et al. (2006d) measured one or more cyanotoxins or total hepatotoxins. In some cases, cyanotoxin levels were below the limit of detection. To determine if study participants possibly were exposed to fecal contamination, three of the studies (Lévesque et al. 2014; Lin et al. 2015; Stewart et al. 2006d) measured bacterial fecal indicators at some study locations and times. Of the studies that measured bacterial fecal indicators, none found an association between bacterial fecal indicators and health effects. Of these studies, the only one with data available for viral fecal indicators or concentrations of waterborne pathogens was Lin et al. (2015) provided in Wade et al. (2010) and Soller et al. (2016).

In summary, although four studies identified significant associations between cyanobacteria exposure and health effects, the type of health effect identified varied. One study reported a significant association between GI illness and exposure to cyanobacteria (Lévesque et al. 2014). Stewart et al. (2006d) and Lin et al. (2015) identified statistically significant associations between cyanobacterial cell exposure and respiratory effects. Lin et al. (2015) also found a statistically significant association between earache and cyanobacterial densities (other than picocyanobacteria). Both Pilotto et al. (1997) and Stewart et al. (2006d) found statistically significant associations between cyanobacterial cell exposure and all symptoms combined. The three cross-sectional studies conducted in the United Kingdom in 1990 found no statistically significant associations, although some minor elevated morbidity was observed in exposed individuals (Philipp 1992; Philipp & Bates 1992; Philipp et al. 1992). Another 1992 case-control epidemiological study in Australia found no statistically significant symptoms for exposed recreators (El Saadi et al. 1995).

The Centers for Disease Control and Prevention (CDC) has collected information on illness outbreaks associated with HABs, which commonly involve cyanobacteria. This information includes human health effects and water-sampling results voluntarily reported to the Waterborne Disease Outbreak Surveillance System via the National Outbreak Reporting System and the Harmful Algal Bloom Related Illness Surveillance System. CDC published summary information on HAB-associated outbreaks from recreational exposures focusing on 2009–2010 with limited additional information available for outbreaks that occurred in 2001, 2004, and 2011–2012 (Dziuban et al. 2006; Hilborn et al. 2014; Hlavsa et al. 2014; Yoder et al. 2004). CDC defines a recreational water-associated outbreak as the occurrence of similar illnesses in two or more persons, epidemiologically linked by location and time of exposure to recreational water or recreational water-associated chemicals volatilized into the air surrounding the water.

The 2009–2010 reporting cycle was notable, as almost half (46 percent) the recreational water outbreaks reported to CDC were associated with HABs (Hilborn et al. 2014). Three of the outbreaks confirmed the presence of cyanobacteria, and four confirmed the presence of cyanotoxins. Gastrointestinal and dermatologic symptoms were the most commonly reported symptom categories associated with HAB-related outbreaks in freshwater (Dziuban et al. 2006; Hilborn et al. 2014; Hlavsa et al. 2014; Yoder et al. 2004). For the cyanobacteria-associated outbreaks with reported symptom counts, the most common symptoms reported were GI related, including vomiting, diarrhea, and nausea (estimated to be > 40 percent). The second most frequent outbreak symptom reported was skin rash (> 27 percent cases reported). Fever, earache, skin irritation, and headache were the next most frequently reported symptoms (11 percent, 9 percent, and 9 percent of cases reported, respectively).

Hilborn et al. (2014) analyzed the HAB outbreak data from 2009–2010 and found 66 percent of case patients were individuals aged 1–19 years (n = 38 of 58 total) and 35 percent were aged 9 years or younger (n = 20). In addition, in a cyanobacteria-associated outbreak in 2001, 42 children were affected. These data are limited and might be underreported, but they suggest that children could be at increased risk for cyanobacteria-associated illness via recreational exposure.

In addition to reports related to freshwater exposure, health effects including dermal, eye/ear, and respiratory effects have been reported following exposure to marine cyanobacteria and/or cyanotoxins including *Lyngbya majuscula* which can produce the cyanotoxins lyngbyatoxin A and debromoaplysiatoxin (Osborne & Shaw 2008).

D.2 Mode of Action

Few mechanistic investigations have been completed on how exposure to cyanobacterial cells might lead to inflammatory response. Torokne et al. (2001) evaluated the sensitization and irritation potential of *Microcystis*, *Anabaena*, *Cylindrospermopsis*, and *Aphanizomenon* bloom and strain samples and found no correlation between the cyanotoxin content and allergenicity. For example, the nontoxic *Aphanizomenon* was the most allergenic sample, more allergenic than the most toxic cyanobacterial cells they studied, *Microcystis aeruginosa*. Stewart et al. (2006e) concluded that cutaneous effects strongly suggest allergic reactions, and symptoms such as rhinitis, conjunctivitis, asthma, and urticaria (or hives) also indicate immediate hypersensitivity responses, which are probably explained by a cascade action of pro-inflammatory cytokines.

Bernstein et al. (2011) suggested that the allergenic structure of cyanobacteria might be associated with a nontoxin-producing part of the organism. Building on this conclusion, Geh et al. (2015) conducted a series of experiments to identify the cyanobacteria allergen(s) responsible for sensitization. Study participants were given skin-prick tests with extracts from nontoxic *M. aeruginosa* strains. Serum from these individuals was collected from a subset of 15 patients who elicited strong skin test responses to *M. aeruginosa* and from 3 healthy control subjects. The lysate from nontoxic *M. aeruginosa* strains was significantly ($p < 0.01$) more immunoreactive than the lysate from the toxin-producing strains, which suggests that the nontoxic strain was more allergenic than the toxic strain. They found, however, that IgE binds to *M. aeruginosa* peptides present in lysates of both the toxic and nontoxic strains. Geh et al. (2015) also performed a β-hexosaminidase release assay, as a surrogate assay for measuring histamine release, to identify functional activity of the *M. aeruginosa* extracts using rat basophil leukemia cells. The authors concluded that the same allergen is present in toxic and nontoxic *M. aeruginosa* lysates, but suggest the toxic *M. aeruginosa* lysate might contain an endogenous inhibitor that prevents IgE from effectively binding to the specific allergen. The further analysis by Geh et al. (2015) of the sera of individuals exposed to nontoxic *M. aeruginosa* lysate indicated that either linker core-membrane peptide or phycocyanin, or both, are potentially responsible for *M. aeruginosa* allergenicity.

Epidemiological studies and case reports suggest respiratory effects that could be consistent with an allergic or hay fever type reaction (Giannuzzi et al. 2011; Stewart et al. 2006e). Inhalation exposure to bacterial endotoxins (i.e., a toxin that is part of the cyanobacterial cell as opposed to exotoxins such as microcystins and cylindrospermopsin) has been found to be associated with pulmonary disease, including asthma, chronic obstructive airway disease, and

emphysema (Stewart et al. 2006b). A recent review of the structure and effects of cyanobacterial lipopolysaccharide suggested that it could act as an antagonist of the TLR4 receptor and inhibit the inflammatory response pathway (Durai et al. 2015).

Stewart et al. (2006e) also noted that, although symptoms and time to onset can be disparate, several reports described:

> "a collective group of symptoms resembling immediate or Type-I hypersensitivity reactions. Immediate hypersensitivity reactions are commonly associated with atopy, which is the familial tendency to react to naturally occurring antigens, mostly proteins, through an IgE-mediated process. Atopy frequently manifests as a spectrum of diseases, e.g., seasonal rhinitis, conjunctivitis, asthma, and urticaria."

Documentation of this type of respiratory response is consistent with results from Geh et al. (2015) and further supports that immune system response follows exposure to cyanobacteria.

In older literature, cyanobacterial lipopolysaccharide was suspected as being a cause of inflammatory response because this cell structure, also found in many gram-negative bacterial species, has been observed to initiate acute inflammatory responses in mammals that are typical of a host reaction to tissue injury or infection (Stewart et al. 2006b). The Stewart et al. (2006e) review, however, found evidence to support this mechanism lacking. Although all cyanobacteria contain the pigment phycocyanin, not all species of cyanobacteria have shown dermal reactions. Also, some species of cyanobacteria produce toxins that are known dermal irritants (e.g., lyngbyatoxin-a). Pilotto et al. (2004), however, found that 20–24 percent of the study participants exposed to cyanobacterial cells via skin patches for 24 hours showed dermal reactions to cyanobacteria species, both whole and lysed cells.

Stewart et al. (2006b) noted that the effects of microcystin- and cylindrospermopsin-producing bacteria on the GI tract could suggest that cyanotoxins and lipopolysaccharide from the cyanobacteria or other bacteria residing in the gut might cross a gut mucosal barrier that has been disrupted and enhance the adverse effects of cyanotoxins.

An aquatic invertebrate study using brine shrimp (*Artemia salina, Daphnia magna* and *Daphnia galeata*) to determine the toxicity of microcystin and cylindrospermopsin in combination with cyanobacterial lipopolysaccharide found that pre-exposure to LPS increased the lethal concentration (LC_{50}) of cylindrospermopsin 8-fold (Lindsay et al. 2006). The authors concluded that the decrease in susceptibility to cylindrospermopsin was due to the effects of lipopolysaccharide on detoxification enzyme pathways; lipopolysaccharide decreased toxic metabolites of cylindrospermopsin by suppressing the invertebrate cytochrome P450 system, thus decreasing toxicity.

D.3 References

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APPENDIX E. INCIDENTAL INGESTION EXPOSURE FACTOR COMBINED DISTRIBUTION ANALYSIS

EPA combined the distributions of incidental ingestion rate per hour and recreational exposure duration to generate a hybrid distributions using R version 3.3.1. Table E-1 presents the parameters used to fit the distributions, and Table E-2 provides summary statistics for the combined distributions. The R code follows these tables.

Table E-1. Parameters Used to Fit Distributions

Parameter	Ingestion rate (L/hr)	Exposure duration (hr/d)
Mean	0.0501	2.737
Standard deviation	0.0401	1.733
Minimum		0.417
Maximum		7.5
μ (ln transformed)	-3.241	
σ^2 (ln transformed)	0.704	
Minimum (ln transformed)	-10^{10}	
Maximum (ln transformed)	-1.59	

Table E-2. Summary Statistics of Hybrid Distribution

Combined Distribution #	Ingestion rate (L/hr)*	Exposure Duration (hr/d)*	Summary Statistics for Ingestion (L/d)							
			Min	Q1	Median	Mean	Q3	Max	Percentile at 0.33 L/d	Percentile at 0.60 L/d
1	Normal	Normal	0.0000	0.0702	0.1424	0.1768	0.2465	1.2850	0.86	0.99
2	LN	Normal	0.0023	0.0564	0.1051	0.1448	0.1872	1.5340	0.91	0.99
3	LN	LN	0.0023	0.0479	0.0873	0.1241	0.1580	1.4730	0.94	0.99
4	LN	Gamma	0.0018	0.0467	0.0888	0.1281	0.1646	1.4580	0.93	0.99
5	Gamma	Gamma	0.0000	0.0396	0.0871	0.1307	0.1736	1.4020	0.92	0.99

* All input distributions were truncated to reflect observed minimum and maximum values

R Code

```
#Cyanotoxin recAWQC WA

#This script is to combine two distributions and generate histogram using five different distribution combinations

rm(list=ls()); # Remove all current R objects from memory

library(truncnorm) #import library for truncated normal distribution
```

```
# Convert exposure data from min/day to hr/day
mean_dur_min<-164.2 #mean exposure duration min/day
sd_dur_min<-103.97 #sd exposure duration min/day
med_dur_min<-140 #median exposure duration min/day
min_dur_min<-25 #minimum exposure duratrion min/day
max_dur_min<-450 #maximum exposure duration min/day

mean_dur<-mean_dur_min/60 #mean exposure duration hr/day
sd_dur<-sd_dur_min/60 #sd exposure duration hr/day
med_dur<-med_dur_min/60 #median exposure duration hr/day
min_dur<-min_dur_min/60 #minimum exposure duration hr/day
max_dur<-max_dur_min/60 #maximum exposure duration hr/day

#(1) Truncated normal ingestion and normal exposure duration distribution
mean_ing <- 0.05 #mean ingestion rate L/hr
sd_ing <- 0.04 #sd ingestion rate L/hr
min_ing<- 0 #minimum ingestion rate L/hr
max_ing<-0.205 #maximum ingestion rate L/hr

n = 100000 #number of samples

ingperhr_trunc<-rtruncnorm(n, a=min_ing, b=max_ing, mean_ing, sd_ing)

duration_hr_trunc<-rtruncnorm(n, a=min_dur, b=max_dur, mean_dur, sd_dur)

ingperday_trunc<-ingperhr_trunc*duration_hr_trunc

summary(ingperday_trunc)

hist(ingperday_trunc,xlab="Ingestion rate (L/day)",ylab="Frequency", main ="Normal distribution fit, truncated", xlim=c(0, 1.0), ylim=c(0, 400))
h=hist(ingperday_trunc)
h$density=h$counts/sum(h$counts)
plot(h,xlab="Ingestion rate (L/day)",ylab="Probability", main ="Truncated Normal distribution fit", xlim=c(0, 1), ylim=c(0, 0.6), xaxp=c(0,1.5,15), freq=FALSE)

#Determine percentiles in combined normal distribution
```

```
#(a)  97th ingestion rate =0.12 L/hr and mean exposure duration of 2.74 hr/day = 0.33 L/day

ecdf(ingperday_trunc)(0.33)

#(b)  97th ingestion rate =0.12 L/hr and 90th exposure duration of 5.0 hr/day = 0.60 L/day

ecdf(ingperday_trunc)(0.60)

#(2) Truncated Log-normal ingestion and normal exposure duration distribution

#transform mean and std of ingestion rate

sd_ing_ln<-sqrt(log((sd_ing/mean_ing)^2+1))

mean_ing_ln<-log(mean_ing)-((sd_ing_ln^2)/2)

min_ing_ln<- -10^10

max_ing_ln<-log(max_ing)

ingperhr_ln_trunc<-exp(rtruncnorm(n, a=min_ing_ln, b=max_ing_ln, mean=mean_ing_ln, sd=sd_ing_ln)) #truncated log normal distribution

duration_hr_trunc<-rtruncnorm(n, a=min_dur, b=max_dur, mean=mean_dur, sd=sd_dur) #truncated normal distribution

ingperday_ln_trunc<-ingperhr_ln_trunc*duration_hr_trunc #combine distributions

summary(ingperday_ln_trunc) #summary statistics about the combined distribution

#Generate histogram

hist(ingperday_ln_trunc,xlab="Ingestion rate (L/day)",ylab="Probability", main ="Truncated hybrid distribution fit", xlim=c(0, 2.0), ylim=c(0, 1))

h=hist(ingperday_ln_trunc)

h$density=h$counts/sum(h$counts)

plot(h,xlab="Ingestion rate (L/day)",ylab="Probability", main ="Truncated LN-Normal hybrid distribution fit", xlim=c(0, 1), ylim=c(0, 0.6), xaxp=c(0,1.5,15), freq=FALSE)

#Generate empirical cumulative distribution function

plot(ecdf(ingperday_ln_trunc), main="")

#Determine percentiles in combined distribution

#(a)  97th ingestion rate =0.12 L/hr and mean exposure duration of 2.74 hr/day = 0.33 L/day

ecdf(ingperday_ln_trunc)(0.33)

#(b)  97th ingestion rate =0.12 L/hr and 90th exposure duration of 5.0 hr/day = 0.60 L/day
```

```
ecdf(ingperday_ln_trunc)(0.60)

#(3) Truncated Log-normal ingestion and log-normal duration

sd_dur_ln<-sqrt(log((sd_dur/mean_dur)^2+1))

mean_dur_ln<-log(mean_dur)-((sd_dur_ln^2)/2)

min_dur_ln<-log(min_dur)

max_dur_ln<-log(max_dur)

ingperhr_ln_trunc<-exp(rtruncnorm(n=n, a=min_ing_ln, b=max_ing_ln,
mean=mean_ing_ln, sd=sd_ing_ln)) #truncated log normal distribution

duration_hr_ln_trunc<-exp(rtruncnorm(n=n, a=min_dur_ln, b=max_dur_ln,
mean=mean_dur_ln, sd=sd_dur_ln))

ingperday_ln2_trunc<-ingperhr_ln_trunc*duration_hr_ln_trunc #combine distributions

summary(ingperday_ln2_trunc) #summary statistics about the combined distribution

#Generate histogram

hist(ingperday_ln2_trunc,xlab="Ingestion rate (L/day)",ylab="Probability", main
="Truncated hybrid distribution fit", xlim=c(0, 2.0), ylim=c(0, 1))

h=hist(ingperday_ln2_trunc)

h$density=h$counts/sum(h$counts)

plot(h,xlab="Ingestion rate (L/day)",ylab="Probability", main ="Truncated log-normal
distribution fit", xlim=c(0, 1), ylim=c(0, 0.6), xaxp=c(0,1.5,15), freq=FALSE)

#Generate emperical cumulative distribution function

plot(ecdf(ingperday_ln2_trunc), main="")

#Determine percentiles in combined distribution

#(a)    97th ingestion rate =0.12 L/hr and mean exposure duration of 2.74 hr/day = 0.33
L/day

ecdf(ingperday_ln2_trunc)(0.33)

#(b)    97th ingestion rate =0.12 L/hr and 90th exposure duration of 5.0 hr/day = 0.60
L/day

ecdf(ingperday_ln2_trunc)(0.60)


# (4) Truncated log-normal ingestion distribution and gamma duration distribution (beta
distribution for duration added by arun)

vr_dur<- sd_dur^2 # variance of the duration distribution

theta_dur<-vr_dur/mean_dur # scale parameter of the gamma distribution

k_dur<-mean_dur/theta_dur # shape parameter of the gamma distribution

rgamma_trunc<-function(n,k,theta,min,max){
```

```
 i<-1
 gv<-matrix(,n,1)
 while(i<=n) {
 a<-rgamma(1,shape=k,scale=theta)
 if (a>min & a<max){
  gv[i]<-a
  i<-i+1 } # end of if operation
 } # end of while loop
 return(as.vector(gv))
 } # end of function
duration_hr_gm<-rgamma_trunc(n,k_dur,theta_dur,min_dur,max_dur)  #truncated gamma distribution
ingperday_ln_gm<-ingperhr_ln_trunc*duration_hr_gm #combine ln and gm distributions
summary(ingperday_ln_gm) #summary statistics about the combined distribution
#Generate histogram
hist(ingperday_ln_gm,xlab="Ingestion rate (L/day)",ylab="Probability", main ="Truncated hybrid distribution fit", xlim=c(0, 2.0), ylim=c(0, 1))
h=hist(ingperday_ln_gm)
h$density=h$counts/sum(h$counts)
plot(h,xlab="Ingestion rate (L/day)",ylab="Probability", main ="Truncated LN-Gamma hybrid distribution fit", xlim=c(0, 1), ylim=c(0, 0.6), xaxp=c(0,1.5,15), freq=FALSE)
#Generate emperical cumulative distribution function
plot(ecdf(ingperday_ln_gm), main="")
#Determine percentiles in combined distribution
#(a)     97th ingestion rate =0.12 L/hr and mean exposure duration of 2.74 hr/day = 0.33 L/day
ecdf(ingperday_ln_gm)(0.33)

#(b)     97th ingestion rate =0.12 L/hr and 90th exposure duration of 5.0 hr/day = 0.60 L/day
ecdf(ingperday_ln_gm)(0.60)
# (5) Truncated gamma ingestion distribution and gamma duration distribution
vr_ing<- sd_ing^2 # variance of the duration distribution
theta_ing<-vr_ing/mean_ing # scale parameter of the gamma distribution
k_ing<-mean_ing/theta_ing # shape parameter of the gamma distribution
```

```
duration_hr_gm<-rgamma_trunc(n,k_dur,theta_dur,min_dur,max_dur)  #truncated gamma distribution

ingperhr_gm<-rgamma_trunc(n,k_ing,theta_ing,min_ing,max_ing)  #truncated gamma distribution

ingperday_gm<-ingperhr_gm*duration_hr_gm #combine ln and gm distributions

summary(ingperday_gm) #summary statistics about the combined distribution

#Generate histogram

hist(ingperday_gm,xlab="Ingestion rate (L/day)",ylab="Probability", main ="Truncated hybrid distribution fit", xlim=c(0, 2.0), ylim=c(0, 1))

h=hist(ingperday_gm)

h$density=h$counts/sum(h$counts)

plot(h,xlab="Ingestion rate (L/day)",ylab="Probability", main ="Truncated Gamma distribution fit", xlim=c(0, 1), ylim=c(0, 0.6), xaxp=c(0,1.5,15), freq=FALSE)

#Generate emperical cumulative distribution function

plot(ecdf(ingperday_gm), main="")

#Determine percentiles in combined distribution

#(a)     97th ingestion rate =0.12 L/hr and mean exposure duration of 2.74 hr/day = 0.33 L/day

ecdf(ingperday_gm)(0.33)

#(b)     97th ingestion rate =0.12 L/hr and 90th exposure duration of 5.0 hr/day = 0.60 L/day

ecdf(ingperday_gm)(0.60)
```

APPENDIX F. INFORMATION ON CELLULAR CYANOTOXIN AMOUNTS AND CONVERSION FACTORS

The information in the tables below was generated from a brief survey of the peer-reviewed and published scientific literature. This survey was not a formal systematic literature search and was conducted to evaluate the availability of data needed to calculate a cyanobacterial cell density potentially associated with a specific cyanotoxin concentration.

Table F-1. Cell Quotas for Cyanotoxins Available from a Spot Check of the Literature

Toxin	Species	Site/Clone	Toxin Quota	Reference	Notes
Cylindrospermopsin	*Cylindrospermopsis raciborskii*	16 sites in 3 reservoirs in Queensland, Australia	4.5 – 55.8 fg[a] $cell^{-1}$; 10.0 – 49.4 fg $cell^{-1}$	Orr et al. (2010)	has values for cylindrospermopsin and d-cylindrospermopsin as cell versus cylindrospermopsin concentration in table
	Cylindrospermopsis raciborskii	New South Wales, Australia	31 (12 – 52) fg $cell^{-1}$	Hawkins et al. (2001)	also has biomass conversions (Table 2)
	Cylindrospermopsis raciborskii	Saudi Arabia lake	0.6 – 14.6 pg[b] $cell^{-1}$	Mohamed and Al-Shehri (2013)	
	Cylindrospermopsis raciborskii	Queensland, Australia	13.4 (± 2.6) – 14.9 (± 3.4) fg $cell^{-1}$	Davis et al. (2014)	range is for two strains
	Cylindrospermopsis raciborskii		12.1 (5.6) – 24.7 (9.5) ng[c] 10^{-6} cells; 3.2 (0.67) – 5.7 (1.4) ng 10^{-6} cells; 0.049 (0.002) – 0.094 (0.001) cylindrospermopsin chlorophyll a^{-1}; 0.016 (0.005) – 0.11 (0.003) cylindrospermopsin chlorophyll a^{-1}	Carneiro et al. (2013)	
	Cylindrospermopsis raciborskii	Queensland, Australia	0.28 x 10^{-2} (0.2 x 10^{-2}) – 1.8 x 10^{-2} (0.4 x 10^{-2}) pg $cell^{-1}$	Willis et al. (2015)	
	Cylindrospermopsis raciborskii	Queensland, Australia	19 (3) – 26 (4) fg $cell^{-1}$; 416 (67) – 447 (69) 10^3 fg μm^{-3}	Pierangelini et al. (2015)	breaks out cylindrospermopsin and d-cylindrospermopsin
Microcystin (MC)	*Planktothrix agardhii*	Paris, France lake	1.5 – 19 fg MC-LR $cell^{-1}$	Briand et al. (2008)	
	Planktothrix agardhii	England; Turkey	0.7 – 1.9 fg μm^{-3}; 75.6 – 91.2 fg $cell^{-1}$	Akcaalan et al. (2006)	
	Planktothrix rubescens	England; Turkey	1.4 – 2.9 fg μm^{-3}; 103.9 – 235.6 fg $cell^{-1}$	Akcaalan et al. (2006)	
	Planktothrix rubescens	Italy lake	1.0 – 3.9 μg mm^{-3};	Salmaso et al. (2014)	
	Planktothrix rubescens	France lake	0.13 (0.16) – 0.16 (0.27) pg $cell^{-1}$	Briand et al. (2008)	

Toxin	Species	Site/Clone	Toxin Quota	Reference	Notes
Microcystin (continued)	Model Cyanobacteria		91.5 fg cell^{-1}	Jähnichen et al. (2001)	Cites Long et al. (2001), Orr and Jones (1998), Jähnichen et al. (2001), and Watanabe et al. (1989) for quotas
	Microcystis aeruginosa	MASH01-A19	50 – 170 fg cell^{-1}	Orr and Jones (1998)	estimated from Figure 5
	Microcystis aeruginosa	Lake Huron, United States	140 fg cell^{-1}	Fahnenstiel et al. (2008)	
	Microcystis aeruginosa	Portugal lake	0.06 – 0.22 pg cell^{-1}	Vasconcelos et al. (2011)	
	Microcystis aeruginosa		18 (0.95) – 23.7 (0.96) fg cell^{-1}	Jähnichen et al. (2007)	
	Microcystis aeruginosa	PCC 7806	34.5 – 81.4 fg cell^{-1}	Wiedner et al. (2003)	
	Microcystis aeruginosa	France	0.05 – 3.8 pg cell^{-1}	Sabart et al. (2010)	estimated from Figure 3
	Microcystis aeruginosa	New Zealand	0.1 – 1.55 pg cell^{-1}	Wood et al. (2012)	estimated from Figure 1

[a] fg = femtogram
[b] pg = picogram
[c] ng = nanogram

Table F-2. A Brief Summary of Cell Concentration – Cyanotoxin Conversions Available from a Spot Check of the Literature

Toxin	Species	Site/Clone	Conversion	Reference	Notes
Cylindrospermopsin	*Cylindrospermopsis raciborskii*	16 sites in 3 reservoirs in Queensland, Australia	has cell versus cylindrospermopsin concentration in table	Orr et al. (2010)	additional data available but need to be digitized
	Cylindrospermopsis raciborskii	New South Wales, Australia	0.13% (0.06 – 0.35%) dry weight; 0.57 (0.18 – 1.52 %) fg μm^3 biovolume	Hawkins et al. (2001)	
	Cylindrospermopsis raciborskii	Queensland, Australia	1% w/w (10 mg cylindrospermopsin per g of dry weight) to 1 µg/g of dry weight	Eaglesham et al. (1999)	has cylindrospermopsin versus Trichomes mg^{-1} (x 10^{-3}) conversion in Figure 4; data need to be digitized
Microcystin (MC)	*Planktothrix agardhii*	England; Turkey	29.4 (2.3) – 34.9 (3.7) pg $filament^{-1}$; 0.2 (0.06) – 1.1 (0.6) fg μm^{-3} biovolume	Akcaalan et al. (2006)	
	Planktothrix agardhii	55 German lakes	1,500 – 2,200 µg g^{-1} dry weight; 0.25 – 0.5 µg MC µg chlorophyll a^{-1}	Fastner et al. (1999)	derived from Figure 2
	Planktothrix rubescens	England; Turkey	28.2 (7.1) – 53.6 (20.6) pg $filament^{-1}$; 0.9 – 3.4 (1) fg μm^{-3}	Akcaalan et al. (2006)	
	Planktothrix rubescens	55 German lakes	1,600 – 4,000 µg g^{-1} dry weight; 0.22 – 0.5 µg MC µg chlorophyll a^{-1}	Fastner et al. (1999)	derived from Figure 2
	Planktothrix rubescens	Italy lake		Salmaso et al. (2014)	has regression formulas for both MC versus chlorophyll and MC versus biovolume
	Planktothrix spp.		0.38 – 6.01 µg mg dry $weight^{-1}$ 0.17 – 4.5 µg mm^{-3} biovolume	Kurmayer et al. (2016)	low and high MC producing strains of *P. rubescens* and *P. agardhii*

Toxin	Species	Site/Clone	Conversion	Reference	Notes
Microcystin (continued)	*Planktothrix* spp.	UTEX 2388	87.9 (5.2) – 339 μg MC-LR g^{-1} dry weight; 0.56 (0.03) – 2.47 (0.03) mg MC-LR g^{-1} protein; 467 (8.1) – 773.5 g MC-RR g^{-1} dry weight; 3.00 (0.05) – 5.63 (0.03) mg MC-LR g^{-1} protein	Oh et al. (2000)	has values for MC-LR and MC-RR
	Microcystis aeruginosa	Portugal lake		Vasconcelos et al. (2011)	has cell versus MC concentration in Figure 4
	Microcystis aeruginosa	UTEX 2388	$y = 0.661x - 38.9$ ($r^2 = 0.569$); y: (MC μg g^{-1}), x: (chlorophyll *a* μg L^{-1})	Lee et al. (2000)	
	Microcystis aeruginosa	France lake		Sabart et al. (2010)	has MC versus cell concentrations in Figures 2 and 3
	Microcystis aeruginosa	Lake Biwam, Japan		Ozawa et al. (2005)	has MC versus cell concentration, chlorophyll *a* in Figure 2
	Microcystis aeruginosa	MASH01-A19	1.2 – 9.3 mg g^{-1} dry weight	Orr and Jones (1998)	estimated from Figure 4
	Microcystis spp.	San Francisco estuary	0 – 1 μg g^{-1} dry weight	Lehman et al. (2008)	derived from Figure 6
	Microcystis spp.	55 German lakes	0 – 1000 μg g^{-1} dry weight 0.08 – 0.31 μg MC μg chlorophyll a^{-1}	Fastner et al. (1999)	derived from Figure 2; derived from Figure 7
	Microcystis spp.	Lake Suwa, Japan	1.20 – 136 μg MC-RR 100 mg^{-1} dry weight; 4 – 89.8 μg MC-RR 100 mg^{-1} dry weight;	Park et al. (1998)	estimated from Figure 7
	Unknown	Quebec, Canada		Giani et al. (2005)	has biomass (g C L^{-1}) versus MC (mg g^{-1}) in Figure 4

Appendix F References

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APPENDIX G. TABLES OF STATE-ISSUED GUIDELINE SPECIFIC TO ANIMAL CYANOTOXIN POISONING

G.1 California

Table G-1. California Environmental Protection Agency (2012) Action levels for Selected Pet and Livestock Scenarios

	Microcystins[a]	Cylindrospermopsin	Media (units)
Subchronic water intake, dog[b]	2	10	water (µg/L)
Subchronic crust and mat intake, dog	0.01	0.04	crusts and mats (mg/kg dw)[c]
Acute water intake, dog[d]	100	200	water (µg/L)
Acute crust and mat intake, dog	0.5	0.5	crusts and mats (mg/kg dw)[c]
Subchronic water intake, cattle[e]	0.9	5	water (µg/L)
Subchronic crust and mat intake, cattle[e]	0.1	0.4	crusts and mats (mg/kg dw)[c]
Acute water intake, cattle[e]	50	60	water (µg/L)
Acute crust and mat intake, cattle[e]	5	5	crusts and mats (mg/kg dw)[c]

[a] Microcystins LA, LR, RR, and YR all had the same RfD so the action levels are the same.
[b] Subchronic refers to exposures over multiple days.
[c] Based on sample dry weight (dw).
[d] Acute refers to exposures in a single day.
[e] Based on small breed dairy cows because their potential exposure to cyanotoxins is greatest.

Table G-2. California Environmental Protection Agency (2012) Reference Doses and Acute and Subchronic Action Levels for Canine Exposure to Cyanotoxins in Drinking Water

	Microcystin	Cylindrospermopsin
Water consumption L/kg-d	0.085	0.085
Uncertainty factor (unitless)	3	3
Acute RfD[a] mg/kg/d	0.037	0.04
Acute action level µg/L	100	200
Subchronic RfD mg/kg/d	0.00064	0.0033
Subchronic action level µg/L	2	10

Reference:

Butler N, Carlisle J, Kaley KB, & Linville R (2012). Toxicological Summary and Suggested Action Levels to Reduce Potential Adverse Health Effects of Six Cyanotoxins. http://www.waterboards.ca.gov/water_issues/programs/peer_review/docs/calif_cyanotoxins/cyanotoxins053112.pdf. Last Accessed: 08/03/2016.

G.2 Oregon

Table G-3. Oregon Dog-specific Guideline Values for Cyanotoxins in Recreational Waters (µg/L)

	Microcystin	Cylindrospermopsin
Dog Guidance Value	0.2	0.4

Note: All dog-specific guideline values have been changed in this revision because California EPA's estimate of the amount of water an exercising dog consumes per kilogram body weight was updated in 2012 (from 0.168 to 0.255 L/kg-day). Current dog-specific guideline values are now consistent with the California EPA update. The dog-specific value for saxitoxins was further modified by application of an uncertainty factor to the dog-specific TDI for interspecies differences in sensitivity between humans (the species in the critical study) and dogs.

Reference:

Oregon Health Authority (2016). Oregon Harmful Algae Bloom Surveillance (HABS) Program Public Health Advisory Guidelines: Harmful Algae Blooms in Freshwater Bodies. https://public.health.oregon.gov/HealthyEnvironments/Recreation/HarmfulAlgaeBlooms/Documents/HABPublicHealthAdvisoryGuidelines.pdf.

G.3 Grayson County, Texas

Table G-4. Grayson County Texas Microcystin Guidelines for Dogs

Quantity of Lake Water Ingested to Receive a Potentially Lethal Dose of Microcystin, Assuming that Mouse and Dog Toxic Responses are Equivalent

	Gallons of Water	*Pounds of Water*
10 pound dog	2.70	22.50
80 pound dog	21.57	180.00

Quantity of Lake Water Ingested to Receive a Potentially Lethal Dose of Microcystin, Assuming that Mouse and Dog Toxic Responses are Equivalent (at actual concentrations found in Grand Lake, Oklahoma, in June 2011)
Highest measured concentration of Microcystin was 358 ppb.

	Gallons of Water	*Pounds of Water*
10 pound dog	0.15 (19.3 ounces)	1.26
80 pound dog	1.21	10.06

*This is not including additional dose amounts that could be ingested from a dog self grooming algae scum off its fur.
**LD50 for Microcystin- mouse used in Calculations = 45 mcg/kg
***20 ppb Microcystin is algal toxin threshold for BGA Warning (condition red)

Quantity of Lake Water Ingested to Receive a Potentially Lethal Dose of Cylindrospermopsin, Assuming that Mouse and Dog Toxic Responses are Equivalent
20 ppb Cylindrospermopsin in Lake Water

	Gallons of Water	*Pounds of Water*
10 pound dog	263	2200
80 pound dog	2109	17601

*This is not including additional dose amounts that could be ingested from a dog self grooming algae scum off its fur.
**LD50 for Cylindrospermopsin- mouse used in Calculations = 4400 mcg/kg
***20 ppb Cylindrospermopsin is algal toxin threshold for BGA Warning (condition red)

Reference:
Lillis J, Ortez A, & Teel JH (2012). *Blue-Green Algae Response Strategy*. Sherman, Texas. http://www.co.grayson.tx.us/users/Health_Dept/Docs/Blue-Green_Algae_Response_Strategy.pdf