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Health Effects Support Document for the Cyanobacterial Toxin Cylindrospermopsin

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

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

This document presents information, including occurrence, toxicology and epidemiology data, for the cyanobacterial 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)

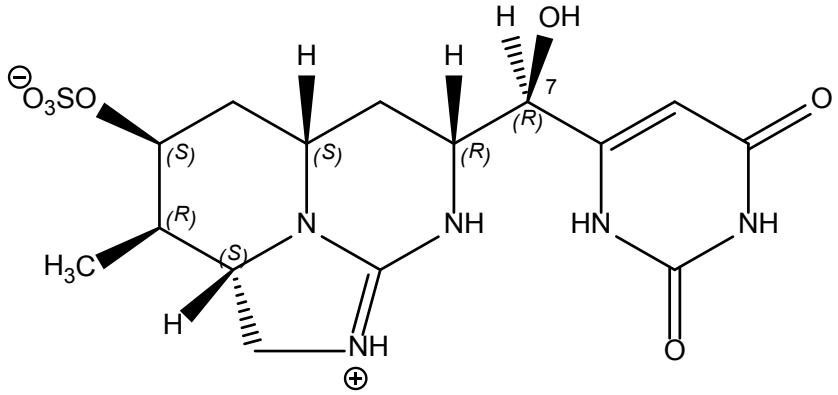


Figure 1-2. Structure of 7-epicylindrospermopsin (de la Cruz et al., 2013)

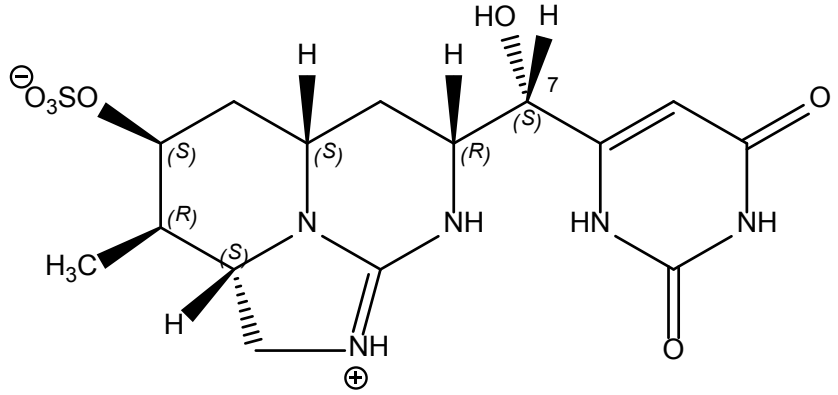
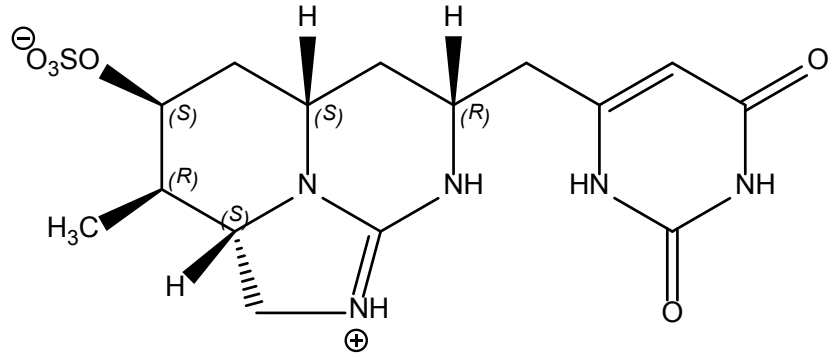


Figure 1-3. Structure of 7-deoxycylindrospermopsin (de la Cruz et al., 2013)



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).

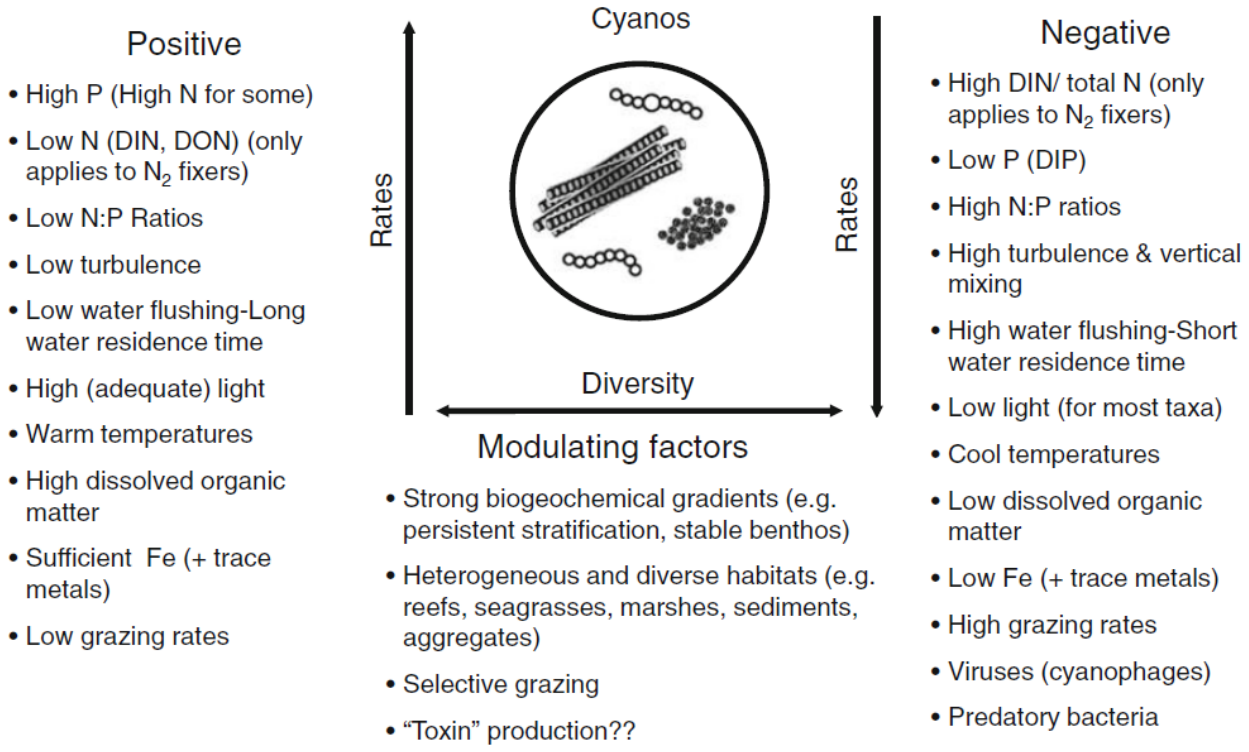
Little is known about how nitrogen affects cylindrospermopsin production. Saker and Neilan (2001) observed the highest concentration (on a dry-weight basis) of cylindrospermopsin in cultures of *C. raciborskii* in the absence of a fixed nitrogen source (Saker and Neilan, 2001). Some studies have suggested 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

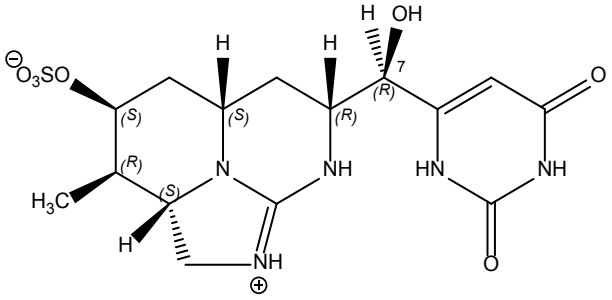
There is some evidence that the most toxic form of cylindrospermopsin is an unidentified metabolite produced by hepatic CYP450. Pretreatment of hepatocytes with known inhibitors of CYP450 diminished the *in vitro* cytotoxicity of cylindrospermopsin (Froscio et al., 2003). Similarly, Norris et al. (2002) 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.

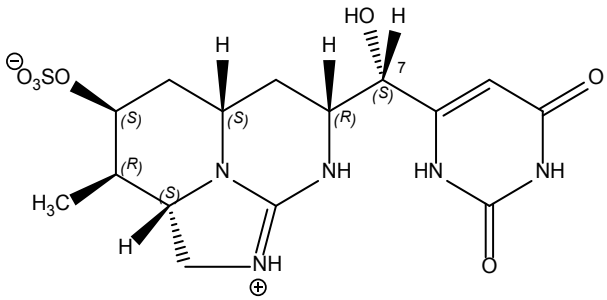
Norris et al. (1999) treated male white Quackenbush mice with a 0.8 mg/kg i.p. dose of 7-deoxycylindrospermopsin, an analogue of cylindrospermopsin isolated and purified from *C. raciborskii.* After 5 days of administration, 7-deoxycylindrospermopsin did not appear to be toxic.

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)

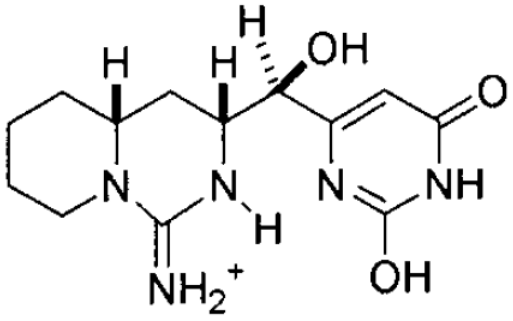


Cylindrospermopsin



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.