APPENDIX I

DSREH Document: ***Recommended NJ Action Level and Health Advisory Guidelines for Recreational Exposure to Microcystin-LR, Cylindrospermopsin, and Anatoxin –A***

RECOMMENDED NJ ACTION LEVEL AND HEALTH ADVISORY GUIDELINES FOR RECREATIONAL EXPOSURE TO MICROCYSTIN-LR, CYLINDROSPERMOPSIN, AND ANATOXIN-A

Thomas Atherholt, Alan Stern, and Gloria Post
NJDEP Division of Science, Research and Environmental Health
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Introduction

Harmful algal bloom events (HABs) result from excessive growth of cyanobacteria (i.e. photosynthetic bacteria also known as "blue-green algae") in waterbodies. HABs are ephemeral in nature. Although certain environmental conditions are known to favor the development of a HAB (sunlight, high concentrations of nutrients, stagnant water, warm temperatures), scientists have not been able to determine a method that can accurately predict when a HAB event will occur. In addition, the location within a waterbody where a HAB occurs often depends on the prevailing wind direction and/or currents. Some species of cyanobacteria can produce chemicals (cyanotoxins) that are toxic to humans and animals if sufficient exposure occurs. However, cyanotoxins are not always produced during a cyanobacteria bloom event, and when they are produced, the comparative amount produced can be low or high. Over a hundred different cyanotoxins (including "variants" within a toxin "family") have been identified. The specific toxins involved and their respective amounts can differ from one HAB event to another.

Therefore, it is difficult at best to accurately quantify human health risk during recreational exposure to a HAB event. Despite this difficulty, USEPA (2016) developed draft recreational criteria/swimming advisories for two cyanotoxins, while a number of states, as well as the World Health Organization (WHO), have derived their own "action levels" or health advisory guidelines based on cyanobacteria cell counts and/or concentrations of a few of the more toxic, commonly-occurring cyanotoxins.

The Bureau of Freshwater and Biological Monitoring (BFBM) of the NJ Department of Environmental Protection has developed the laboratory capability to measure levels of three of the most toxic, commonly observed cyanobacterial toxins in freshwater lakes, namely microcystins, cylindrospermopsin, and anatoxin-a. The first two are hepatic (liver) toxins while anatoxin-a is a neurotoxin. The methods are provided in Appendix 1.

There are two general types of health effects concerns for recreational exposures to toxins from blue green algae/cyanobacteria:

[1] In May 2015, the USEPA provided guidance values for two cyanotoxins, microcystin-LR and cylindrospermopsin, for drinking water exposure. See USEPA (2015a) and USEPA (2015b).

1. Allergic and irritation reactions which occur from dermal contact with cyanobacterial substances in general (not specifically cyanotoxins) in some individuals at cyanobacteria levels below those which are of concern for liver toxicity.
2. Higher exposure levels with potential for liver or neurological toxicity.

The Division of Science, Research and Environmental Health (DSREH) was asked by BFBM to recommend action levels for recreational exposure to HABs, including health advisory guidance values for the three cyanotoxins mentioned above, based on review of the cyanotoxin guidance values developed by the WHO and by various states, and other relevant information. WHO recommendations are discussed in Appendix 2, and recommendations of other states are presented in Appendix 3. DSREH recommendations provided in this document will be reviewed in the context of the USEPA recreational criteria/swimming advisories for these cyanotoxins when they are finalized.

Recommended action level and health advisory guidance levels

Action level based on cyanobacterial cell count

Low concentrations of cyanobacteria may cause **allergenic and/or irritative** effects to a portion of an exposed population. These effects are caused by endotoxins (mainly the lipopolysaccharide component of the cyanobacterial cell wall) rather than cyanotoxins. Therefore, county or local authorities may wish to post advisories for any freshwater lake or pond in which cyanobacterial cell counts reach a level of concern.

DSREH recommends that if the cyanobacterial cell count equals or exceeds **20,000 cells/ml in an area where primary recreational contact is likely to occur**, county or local authorities have the option to post advisory signs. When cell counts exceed this level, monitoring for cyanotoxins should be initiated. This recommendation is based on WHO(2003a) guidance described in detail in Appendix 2.

Health advisory guidance levels for individual cyanotoxins

DSREH recommends the following guidance values for recreational exposure to individual cyanotoxins. Their basis, including derivation of Reference Doses and explanation of exposure assumptions, is provided in Appendix 4.

- **Microcystins: 3 µg/L**
- **Cylindrospermopsin: 8 µg/L**
- **Anatoxin-a: 27 µg/L**

Discussion

The recommended water concentrations are intended to be protective of a range of exposures and are probably highly conservative (i.e., protective) for the exposures most likely to occur. The uncertainties in the risk estimates (discussed in Appendix 4), as well as the inherent uncertainty in the temporal variability of the toxins in any given waterbody, should be considered when providing advice to the public regarding recreation in affected waterbodies. It should be noted that these recommendations do not address the risk to pets, livestock and wild fauna, nor do they address the risk associated with consuming fish from affected waters or the combined risk from swimming and fish consumption.

It should be noted that multiple congeners of microcystin are produced by cyanobacteria, and that the analytical assay used by BFBM measures multiple microcystin congeners as one value, i.e., it does not measure individual congeners. The recommended guidance value for microcystins in general is based on toxicity of microcystin-LR. Microcystin-LR is one of the more prevalent and toxic microcystin isomers and the toxicological database for other microcystin isomers are insufficient for Reference Dose development. Therefore, it is recommended that the guidance value based on microcystin-LR apply to total microcystins.

Citations for this document including the Appendices are found at the end of the document.

APPENDIX 1: METHODS USED BY BFBM FOR CYANOTOXIN ANALYSIS

Cyanotoxins

Test Method: Microcystins/Nodularins (ADDA), ELISA kit. #520011 (Abraxis, Inc.)
Detection Limit: 0.1 µg/L Reacts with all cyclic peptide toxin congeners.
Interferences: ≥ 5% methanol, ≥ 2.5% seawater.
"Positive results requiring regulatory action should be confirmed by an alternative method."

Cylindrospermopsin

Test Method: Cylindrospermopsin ELISA kit #522011 (Abraxis, Inc.)
Detection Limit: 0.04 µg/L. Reacts with cylindrospermopsin, deoxycylindrospermopsin.
Interferences: ≥ 20% methanol, ≥20% seawater.
"Positive results requiring regulatory action should be confirmed by an alternative method."

Anatoxin-a

Test Method: Anatoxin-a ELISA kit #520060 (Abraxis, Inc.)
Detection Limit: 0.15 µg/L. Reacts with (+)anatoxin-a, homoanatoxin-a.
Immediate sample preservation required (anatoxin-a will degrade in presence of light, high pH.
Interferences: ≥2.5 % methanol.
"Positive results requiring regulatory action should be confirmed by an alternative method."

APPENDIX 2: WORLD HEALTH ORGANIZATION (WHO) GUIDANCE FOR RECREATIONAL EXPOSURE TO ALGAL BLOOMS AND CYANOTOXINS

WHO (2003a) has developed guidance based on the concentration of cyanobacteria cells, chlorophyll-a, and one type of microcystin, microcystin-LR. These guidance values form the basis for recreational water advisories adopted by many states. Microcystins are liver toxins. In addition to causing acute liver toxicity, microcystins are promoters for liver carcinogenicity. Additionally, a recent study published subsequent to the WHO (2003a) guidance suggests that microcystins may also cause nephrotoxicity, impairing renal function (Lin. et al., 2016).

WHO (2003a) states that approaches to recreational water safety **should address the occurrence of cyanobacteria as such** (i.e., **cell counts**) for the following reasons: (1) It is unclear whether all important cyanotoxins have been identified. (2) The health outcomes observed after recreational exposure, particularly irritation of the skin and mucous membranes, are probably related to cyanobacterial substances other than the well-known toxins.

Depending upon dose, there are comparatively mild (e.g., irritation) effects in addition to adverse health effects related to toxicity of the cyanotoxins. To address these various effects, WHO (2003) developed three guidance levels.

1. ***Low probability of adverse health effects***

Guidance: A level of **20,000 cyanobacterial cells/ml** (equivalent to **10 µg/L of chlorophyll-a**) for protection for a relatively low probability of **irritative and allergenic effects**. This level would be associated with **2-4 µg/L of microcystin-LR if microcystin-producing cyanobacteria are dominant, with 10 µg/L possible in highly toxic blooms**. At this level, less than 30% of people experienced symptoms, although a small number of people had mild irritation at lower levels (5,000 cells/ml).

Recommended action: Inform authorities to initiate further surveillance of the site. The WHO notes that it is difficult to define "safe" concentrations of cyanobacteria in recreational water for allergenic effects or skin reactions, as individual sensitivities vary greatly.

2. ***Moderate probability of adverse health effects***

Guidance: A level of **100,000 cyanobacterial cells/ml** (equivalent to **50 µg/L of chlorophyll-a**) for potential skin irritations and gastrointestinal effects. This level would be associated with **20 µg/L of microcystin-LR,** assuming an "average" microcystin content per cell, **with 50-100 µg/L possible in highly toxic blooms**.

The 20 µg/L human health value for microcystin-LR is based on the Tolerable Daily Intake (TDI, similar to Reference Dose) of 0.04 µg/kg/day and recreational exposure assumptions for adults (60 kg body weight, ingestion of 100 ml per swimming event). The TDI was developed by WHO, 2003b for its drinking water guidance of 1 µg/L) and is based on liver toxicity in a subchronic (13 week) mouse study (Fawell et al., 1994).

The risk of liver toxicity is primarily from incidental ingestion during swimming, playing in the water, water skiing, boating, etc. Dermal exposure is very unlikely to cause liver toxicity. WHO (2003a) notes that the 20 µg/L is based on adult exposure assumptions and that a 15 kg child could consume 250 ml of water in a swimming event and thus receive 10-times the exposure of an adult from recreational activities. It is also noted that there is increased risk to people with liver disease such as chronic hepatitis B.

Recommended actions: Post warning signs, discourage swimming, daily inspection to watch for scum formation, inform authorities.

3. ***High probability of adverse health effects***

Guidance: Cyanobacteria **scum-containing waters**. Potential for acute poisoning in addition to any of the symptoms noted at the lower levels. Surface scums can represent thousand-fold to million-fold concentrations of cyanobacterial cell populations. The **ingestion of 5–50 mg of microcystin could be expected to cause acute liver injury in a 10-kg child**. Reports of up to 24 mg microcystin/litre from scum material have been published.

Recommended actions: prohibit all water-contact activities, inform authorities.

APPENDIX 3: STATE GUIDANCE/ACTION LEVELS AND RECOMMENDED ACTIONS FOR RECREATIONAL EXPOSURE TO HABs AND CYANOTOXINS

USEPA has summarized recreational water guidance/action levels and recommended actions developed by 21 states for cyanobacteria and cyanotoxins at http://www2.epa.gov/nutrient-policy-data/policies-and-guidelines#what3. It should be noted that this state list may not be current in some cases, and that additional states may have developed guidance values since USEPA posted its list. For example, Pennsylvania adopted Ohio's guidance values for Lake Erie.

State guidance/action levels are based on various parameters including visual assessment of the presence of scum, mats, and/or discolored water, cyanobacterial cell counts, and/or concentrations of microcystin-LR and other cyanotoxins. For advisories based on microcystin-LR concentrations, most but not all states use the WHO toxicity factor (tolerable daily intake, equivalent to Reference Dose) of 0.04 µg/kg/day as their basis. However, many states use different exposure assumptions than WHO and have developed values lower than the 20 µg/L WHO value. Specifically, a number of states have guidance values lower than 20 µg/L based on assumptions for recreational exposures for children rather than adults, in order to be public health protective.

Recommended actions may include various levels of recommended/mandatory restrictions based on the level of cyanobacteria or toxins that are present (e.g. posted advisories, recommendations or reducing or avoiding contact with the water, or closure of swimming areas). As with the WHO, some states have tiered guidance levels. For example, Ohio's Public Health **Advisor**y Warning recommends no swimming and wading at 6 µg/L for microcystin (with guidance levels also for 3 other toxins) and gives precautions for the very young, the very old, and those with compromised immune systems at these levels. At higher levels there is a more stringent Recreational **No Contact** Advisory.

Listed below are some websites with information on recreational exposure to HABs and cyanotoxins:

California: http://www.swrcb.ca.gov/water_issues/programs/peer_review/docs/calif_cyanotoxins/cyanotoxins053112.pdf
Indiana: http://www.in.gov/idem/algae/
Kansas: http://www.kdheks.gov/algae-illness/download/HAB_response_plan.pdf
Massachusetts: http://www.mass.gov/eohhs/docs/dph/environmental/exposure/protocol-cyanobacteria.rtf
Ohio: http://epa.ohio.gov/Portals/35/hab/beachmanagersguide.pdf

Oregon:
http://public.health.oregon.gov/HealthyEnvironments/Recreation/HarmfulAlgaeBlooms/Documents/HABPublicHealthAdvisoryGuidelines%2020150619.pdf
Rhode Island:
http://www.dem.ri.gov/programs/benviron/water/quality/surfwq/pdfs/cyan1013.pdf
Vermont: http://healthvermont.gov/enviro/bg_algae/documents/BGA_guide.pdf
Virginia:
http://www.vdh.virginia.gov/epidemiology/DEE/HABS/documents/VDHMicrocystisGuidance.pdf
Washington: http://search.usa.gov/search?affiliate=dohwagov&query=cyanotoxins

The basis for guidance values developed by states, USEPA, and WHO for the three cyanotoxins evaluated herein is presented in the tables below:

Microcystins

Agency	Year	Form	RfD (µg/kg/day)	Exposure Assumptions			Guidance value (µg/L)
				Body weight (kg)	Water ingestion rate (L/hr)	Duration of swimming event (hrs)	
California EPA	2012	-LR, LA, -RR, -YR	0.006	30.25	0.05	5	0.8
New Jersey DEP	**2017**	**Total**	**0.01**	**31.8**	**0.12**	**1**	**3**
Ohio DOH	2015	Total	0.04	15	0.05	2	6
Oregon Health Authority	2015	Not stated	0.05	20	0.05	2	10
USEPA*	2016	Total	0.05	31.8	0.33	2.7	4
WHO**	2003	Total	0.04	60	0.1 (per swimming event)		20

*From USEPA Draft Human Health Recreational Ambient Water Quality Criteria (USEPA, 2016).
**Adult exposure assumptions; noted that 15 kg child could receive 10-fold higher exposure

Cylindrospermopsin

Agency	Year	RfD (µg/kg/day)	Exposure Assumptions			Guidance value (µg/L)
			Body weight (kg)	Water ingestion rate (L/hr)	Duration of swimming event (hrs)	
California EPA	2012	0.033	30.25	0.05	5	4
New Jersey DEP	**2017**	**0.03**	**31.8**	**0.12**	**1**	**8**
Ohio DOH	2015	0.03	15	0.05	2	5
Oregon Health Authority	2015	0.1	20	0.05	2	20
USEPA*	2016	0.1	31.8	0.33	2.7	8

*From USEPA Draft Human Health Recreational Ambient Water Quality Criteria (USEPA, 2016)

Anatoxin-a

Agency	Year	RfD (µg/kg/day)	Exposure Assumptions			Guidance value (µg/L)
			Body weight (kg)	Water ingestion rate (L/hr)	Duration of swimming event (hrs)	
California EPA*	2012	2.5	30.25	0.05	5	90
New Jersey DEP	**2017**	**0.1**	**31.8**	**0.12**	**1**	**27**
Ohio DOH	2015	0.5	15	0.05	2	80
Oregon Health Authority	2015	0.1	20	0.05	2	20

*Considered dermal and inhalation exposure in addition to ingestion exposure. Dermal exposure was about twice ingestion exposure. Inhalation exposure was not significant.

APPENDIX 4

DERIVATION OF HEALTH ADVISORY GUIDANCE LEVELS FOR CYANOTOXINS
March 10, 2017

Derivation of Reference Doses (RfDs)

The studies summarized here appear to be the only relevant mammalian *in vivo* oral dosing studies available for each of the hazardous algal toxins addressed in this assessment. They include all of the oral exposure studies addressed by WHO and the other states referenced in appendix 3 and other relevant studies cited in the USEPA Health Advisories and Support documents for microcystins, cylindrospermopsin and anatoxin-a (USEPA, 2015a-e).

The assessment of these cyanotoxins is limited to those animal studies that administered the toxin as the specifically isolated chemical. It is also generally limited to those studies in which the toxin was administered orally, either by gavage or through drinking water. Data from studies that included injection sub-studies are included where those data are useful in informing the results of the oral administration. As explained in the Exposure Scenario section, only studies with less than sub-chronic to sub-chronic duration were considered for quantitative derivation of an RfD. Finally, these assessments are limited to studies that evaluated at least one endpoint suitable for RfD development. Studies that only presented data on sub-clinical or mechanistic endpoints are not considered here.

I. Microcystin-LR (MC-LR)

Review of toxicologic data:

Fawell et al., (1999)[2] - Mice (CD-1) were exposed to MC-LR obtained from a commercial laboratory. (This study also initially investigated rats as well as mice, but mice were found to be a more sensitive species). It was stated that MC-LR was obtained from a commercial laboratory as a "pure" substance, but no further details were provided. Fawell et al. (1994) do not indicate that the material was tested for purity. As MC-LR is a (hepta)peptide, this is somewhat less of a concern than for more structurally complex chemicals. However, this does introduce some uncertainty into the quantitative assessment of the reported results.

Single dose study: Groups of 5 male and female mice were given a single dose of MC-LR in aqueous solution by gavage (500, 1580, 5000 µg/kg) or by intraperitoneal injection (50, 158, 500 µg/kg). This study was intended as a range-finding study and no control groups were used. By

[2] The Fawell et al. (1999) paper provides a journal-based version of the report-based Fawell et al. (1994) report-based version of this research. There does not appear to be significant disagreement between these two publications, but the report-based version is more complete.

oral exposure, there was no mortality among males at the two lower doses and 60% mortality at the high dose. Among the females, there was no mortality at the low dose, and 20% and 40% at the two highest doses. By intraperitoneal (i.p.) injection there was no mortality at the lowest dose and 100% mortality for males and females at the two highest doses. The oral LD_{50}, therefore, is approximately 5,000 µg/kg.

Liver effects from the single dose study were also examined. For the oral dose, "minimal" diffuse hemorrhage was observed at the low (500 µg/kg) dose in 2/5 males, and in 1/5 at each of the two highest doses. There were also 2/5 and 5/5 observations of "moderate" centrilobular hemorrhage in 2/5 males at 1580 µg/kg and 5/5 males at 5000 µg/kg, and in 2/5 females at 5000 µg/kg. In addition, centrilobular necrosis was observed in 1/5 males and females at the highest dose. Diffuse hemorrhage was also evident at all i.p. doses.

It is clear that the liver is a target for MC-LR. The apparent LOAEL for acute liver effects is 500 µg/kg from oral exposure for minimal diffuse hemorrhage in males, with no NOAEL identified; although the lack of this effect at higher doses and with repeated dosing makes this conclusion somewhat unclear. It should be noted that diffuse hemorrhage occurred at lower doses (50 and 158 µg/kg) from i.p. injection.

Developmental study**:** Pregnant female mice (n = 14-26/dose group) were exposed by gavage from GD 6-15 to 0, 200, 600, or 2,000 µg/kg/d in aqueous solution with necropsy on GD 18. In the high dose group, 9 females died or were sacrificed *in extremis*. Body weight gain among surviving dams was not affected. No effects are reported for the dams exposed at the two lower doses. The number of implantations, live fetuses, and post-implantation loss were not clearly affected by MC-LR. There was a small (6%), but significant reduction, in fetal weight in males and females compared to controls at the highest maternal dose, but no clear effect on fetal weight at the other doses. There was no clear effect of MC-LR on fetal visceral or skeletal structure at any dose.

MC-LR does not appear to be strongly fetotoxic. The high maternal dose (2,000 µg/kg/d) appears to have a mild effect on fetal weight gain and should be considered a LOAEL for fetal effects, although this dose also resulted in maternal lethality. The NOAEL is, therefore, 600 µg/kg/d.

Less than sub-chronic duration study (14 days)**:** Male and female mice (5/sex/dose group) were exposed by gavage daily to 0, 40, 200, or 1,000 µg/kg/d (presumably in aqueous solution, as this was the method in the longer-term follow-up 13 week study) for 14 days. Histopathology was conducted on lung and liver. Organ weights were not determined. There was no dose-related mortality, and there were no effects on body weight, body weight gain, or food consumption. Macroscopic findings were not remarkable with the possible exception of red discoloration of an accentuated lobular pattern on the liver for 2/5 males at 1,000 µg/kg/d, and a red discoloration of

the lungs for 1/5 males at 40 µg/kg/d and 2/5 males at 1,000 µg/kg/d. Histopathology found hepatic centrilobular cellular rarefaction (decreased density of cell count) at 1,000 µg/kg/d.

1,000 µg/kg/day appears to be a minimal LOAEL and 200 µg/kg/d the NOAEL from this study.

Sub-chronic study (13 weeks): Males and female mice (15/sex/dose group) were exposed by daily gavage to 0, 40, 200, or 1,000 µg/kg/d in aqueous solution for 13 weeks (91 d). Animals were assessed for body weight; food consumption; hematology; blood chemistry; organ weights (adrenals, kidneys, liver and testes); organ/tissue pathology; and histopathology. For males at 1,000 µg/kg/d, there was one death on day 7 with no obvious pathology and one case of frank neurologic morbidity at day 91. There was a significant decrease in body weight gain (15%) at the 40 and 200 µg/kg/d doses, and body weight was also decreased at the 1,000 µg/kg/d dose but was not statistically significant. For females, there was a significant (29%) *increase* in body weight at the 200 µg/kg/d dose only. For both males and females, there was a significant increase in food consumption (14% and 20%) at 1,000 µg/kg/d.

In females, there was a significant increase (10-12%) in hemoglobin concentration, RBC count, and packed RBC volume at 1,000 µg/kg/d. In males, liver enzyme concentrations in blood were significantly increased at 200 µg/kg/d (ALT, AST) and 1,000 µg/kg/d (ALP, ALT, AST). There was also an overall decrease in gamma-glutamyl transferase (GGT), but this was significant only for males at 200 µg/kg/d. Total blood protein and albumin were also significantly decreased at 200 and 1,000 µg/kg/d in males. Although not significant, there was also a decrease in these two parameters at 40 µg/kg/d. No dose-related effects on organ weight were observed. Significant histopathological effects were seen only in the liver. For both males and females, there was a monotonic increase in the incidence of generalized chronic liver inflammation. The authors did not provide an analysis of statistical significance for this observation. However, a chi-squared analysis indicates that for males and females, there was a statistically significant difference only between the controls and the 1,000 µg/kg/d dose.

Although 200 µg/kg/d is a clear effect level in this study based on decreased weight gain in males, increased serum liver enzymes, and decreased total blood protein, and albumin in males, it is not entirely clear from these data that 40 µg/kg/d is a NOAEL. This conclusion is based on the significant decrease in body weight gain in males, and is also supported by changes in other parameters (total blood protein, albumin, and chronic liver inflammation) that were not statistically significant at this dose. Therefore, it appears appropriate to characterize 40 µg/kg/d as a minimal LOAEL.

Heinze (1999) - Adult male hybrid rats (WELS x BDIX) received MC-LR [Calbiochem] through drinking water at doses of 50 or 150 µg/kg/d. There were 10 animals/dose group including controls. Animals were sacrificed after 28 days of exposure. Measurements consisted of: body weights; organ weights (liver, kidneys, adrenals, thymus, and spleen); erythrocyte and leukocyte

counts; hemoglobin concentration; hematocrit; and serum enzymes. Histopathological examinations were conducted on liver and kidney.

There was no dose-related effect on body weight. Relative liver weight was significantly increased relative to controls at both doses. There were no other significant changes in organ weight. There was a significant increase in leukocytes and lymphocytes at the 150 μg/kg/d dose only. There was a significant increase in the serum levels of LDH and ALP (but not ALA or AST) at both doses, suggesting liver toxicity. Liver pathology, characterized by the author as "toxic hepatosis" was observed diffusely throughout the parenchyma at both doses. This included degenerative and necrotic hepatocytes (with and without hemorrhages).

This study yields clear evidence of liver damage at both doses and evidence of hematologic abnormalities at the high dose. Based on these observations, 50 μg/kg/d is a clear LOAEL. No NOAEL was identified from this study. It should be noted that this study was chosen by the USEPA (2015a) as the basis for its Health Advisory for MC-LR.

Li et al. (2014) – Adult male Sprague-Dawley rats were exposed by gavage to commercial MC-LR (reported as > 95% purity) mixed with methanol and diluted to administered concentrations with distilled water. The reported administered amounts were 0, 0.2, 1.0, or 5.0 μg/kg every two days for a total of 8 weeks (56 d), corresponding to effective doses of 0, 0.1, 0.5, and 2.5 μg/kg/d (n = 8/dose group). The primary analysis in this study was the potential effect of MC-LR on spatial learning as measured in the Morris water maze test administered 24 hrs after the final dosing. In addition, 24 hrs after the behavioral studies, blood was drawn and analyzed for serum liver enzymes, cholinesterase, total protein and albumin. The rats were sacrificed 24 hrs after the completion of the behavioral studies. Histopathology was conducted on the neurons of the hippocampal region of the brain, and on the liver.

Rats showed decreasing performance on most of the Morris water maze parameters as a function of dose, with significant decrements in the 2.5 μg/kg/d group and a significant decrement in the 0.5 μg/kg/d group in one parameter (platform zone frequency). Serum cholinesterase was significantly increased at 2.5 μg/kg/d. No significant treatment related histopathology was noted in either the hippocampus or the liver. However, the authors did report an increase in the density of N-20+ cells in the hippocampus as a function of dose. This effect reached statistical significance relative to the control animals only at the highest dose, however. N-20+ is an immunologic marker for the expression of the NOS2 gene that codes for nitric oxide synthetase. This gene is inducible by cytokines and the increase of N-20+ cells was interpreted by the authors as in indication of an inflammatory response in the hippocampus. The interpretation of the toxicological significance of this observation, however, is not entirely clear.

Based on the performance decrements in the Morris water maze test, 0.5 μg/kg/d appears to be a LOAEL for neurologic effects from this study, and 0.1 μg/kg/d is the NOAEL. However, as the USEPA (2015c) points out, the administered doses of MC-LR also delivered doses of methanol

that increased in proportion to the administered MC-LR dose, but given the lack of data on the volume of the gavage solution, the actual methanol dose cannot be determined. Methanol is a known neurotoxicant and the potential for a synergistic effect of methanol and MC-LR cannot be ruled out.

Li et al. (2015) – Adult female Sprague-Dawley rats (n = 7/dose group) were dosed by gavage with MC-LR (mixed with methanol and diluted with distilled water) at 0, 1.0, 5.0 or 20.0 μg/kg every two days for 8 wks. This resulted in effective doses of 0, 0.5, 2.5, or 10.0 μg/kg/d. One day following the final dosing, the rats were mated with unexposed males. Maternal body weight was recorded during gestation, and reproductive parameters were recorded. From each litter, 4 M and 4 F pups at PND 7 were evaluated in a series of behavioral tests: motor development (surface righting, negative geotaxis, cliff avoidance); and at PND 28 and 60 were evaluated for open field and Morris water maze. Histopathology was also conducted on one male and one female pup brain from each dam 24 hrs after each behavioral test.

There was no treatment related maternal toxicity and no effect on maternal body weight. The number of pregnant females appears to have declined and the number of dead fetuses appears to have increased with MC-LR dose. However, these parameters did not differ significantly from controls. Performance of the pups in the cliff avoidance test declined significantly at all doses. Pup performance in the Morris water maze was negatively affected by treatment. This was most evident with respect to the frequency-in-platform-zone parameter which showed statistically significant reduced performance at all doses. No treatment related effect was seen in brain histopathology. There was, however, an increase in markers of oxidative stress in the hippocampus that were significant at the highest dose and to a lesser extent at 2.5 μg/kg/d.

The apparent LOAEL for neurological developmental effects in this study was 0.5 μg/kg/d. There was no NOAEL. However, as with the related Lin et al. (2014) study, MC-LR exposure was associated with methanol exposure, although the actual methanol dose cannot be determined. It is, therefore, difficult to determine the specific effect of MC-LR alone. It should also be noted that the exposure of the females in this study was only pre-conception. While this does not, by itself, limit the applicability of these data for the purpose of identifying an adverse effect, it does make interpretation of the nature of the effects of MC-LR difficult. Because the pharmacokinetics of MC-LR in these animals is unknown, the fetal doses cannot be estimated.

Zhang et al. (2010) – Adult male C57bl/6 mice received commercial MC-LR in drinking water. The purity of the MC-LR was stated to be > 95%. The mice were exposed for 180 days, making this a chronic exposure study. The drinking water concentrations were 0, 1, 40 and 80 μg/L which, according to the authors, corresponded to doses of 0, 0.2, 8.0 and 16.0 μg/kg/d with n = 10 for each group. The primary purpose of this study was to investigate the effect of MC-LR exposure on the expression of matrix metalloproteinases. These are a family of enzymes that function in degrading the extracellular protein matrix. As such, they are linked to tumor progression by providing space for tumor expansion. However, because this assessment focuses

on short-term exposure, tumor progression is not considered a relevant endpoint and changes in matrix metalloproteinase expression are, therefore, considered to be a mechanistic or sub-clinical endpoint that is not appropriate for short-term RfD development, Nonetheless, this study also addresses outcome determinations relevant to RfD derivation: body weight, liver weight, and liver histopathology.

The authors report a statistically significant decrease in body weight and an increase in relative liver weight compared to the controls in the 8.0 and 16.0 μg/kg/d dose groups. However, the specific data are not presented. Histopathological examination of the livers revealed infiltration of lymphocytes and fatty degeneration in the 8.0 and 16.0 μg/kg/d dose groups.

While this study confirms the hepatotoxic potential of MC-LR, as a chronic duration study, it is not quantitatively applicable for derivation of a shorter duration RfD.

Ueno et al. (1999) - Adult female BALB/c mice were exposed to MC-LR isolated from "algal bloom materials and stated to have > 95% purity. Exposure occurred through drinking water for 3 (n = 20), 6 (n = 20), 12 (n = 20) or 18 months (n = 40) at a single concentration of 20 μg/L plus controls. The mean cumulative MC-LR intake over 18 months (548 days) was reported as 35.5 μg/animal. The mean body weight for the animals is not reported. However, the graphical presentation of body weight over the duration of exposure yields an estimated time-weighted body weight of 25.3 g (0.025 kg). Thus the dose of MC-LR can be estimated as 2.6 μg/kg/d. This study is considered a chronic duration study for all exposures except for the 3 month exposure. Analyses included body weight; organ weights; hematology; serum chemistry - liver enzymes, serum glucose, lipids, bilirubin, Ca, and inorganic P. Histopathology was performed on a large number of tissues.

There was no significant difference from controls in survival, body weight, or food or water consumption at intermediate time points or at the termination of the exposure. No significant differences were noted in hematology throughout the study. The only statistically significant treatment related effects seen in serum chemistry were a transient decrease in ALP at 12 months and an increase in total cholesterol at 18 months. A significant decrease in relative thymus weight was observed at months 3-12, but not at 18 months. A significant decrease in absolute (but not relative) heart weight was observed 18 months, but not at earlier time periods. No remarkable outcomes were observed in histopathological analysis (including histopathology of the liver)

Overall this study did not show significant adverse outcomes from MC-LR, and a dose of approximately 2.6 μg/kg/d can be considered to be a chronic NOAEL. However, given the chronic nature of this exposure, this cannot reliably be back-extrapolated to a shorter term RfD. It can, however, be useful in informing an RfD derived from a less than chronic study, especially for liver histopathology, as a NOAEL from a chronic duration study can be expected to be a lower bound estimate for a NOAEL from a less-than-chronic duration study.

Chen et al. (2011) – Adult male SPF mice (age not specified) were orally administered commercial MC-LR daily for either 3 or 6 months. The purity of the material was not provided by the authors. The route of exposure appears to be through drinking water, but this is not explicitly stated. The daily administered concentrations were 0, 1, 3.2, or 10 μg/L (n = 20/dose group). Although a range of body weights is given, the body weights over time are not specified. Thus, the dose (μg/kg/d) cannot be directly calculated from the published data. The USEPA (2015c), in its review of this study, estimates the corresponding daily doses on the basis of species/strain-specific default assumption about water intake and body weight as 0, 0.25, 0.79, and 2.5 μg/kg/d. The 6-month duration exposure is considered to be a chronic duration study. Body weight and testis weight were measured. Sperm count and sperm morphology were assessed, and testis histopathology was evaluated. Serum reproductive hormones were also measured.

No treatment-related effects were observed on body weight or testis weight. At 3 months, a significant decrease in sperm motility was observed at 0.79 and 2.5 μg/kg/d. At 6 months, there was a decrease in sperm count and sperm motility as well as an increase in the frequency of abnormal sperm for 0.79 and 2.5 μg/kg/d. Although serum testosterone appears to have markedly declined for 2.5 μg/kg/d at three months, it apparently did not reach a level of statistical significance. At 6 months, serum testosterone was statistically significantly decreased at 0.79 and 2.5 μg/kg/d. Also, LH was significantly increased at the same doses, while serum FSH was significantly increased at 2.5 μg/kg/d. Histopathology revealed a slight effect on the arrangement of spermatogenic epithelium in the seminiferous tubules at 2.5 μg/kg/d at 3 months. At 6 months, there was slight testicular atrophy at 0.79 μg/kg/d with increasing severity and various morphological abnormalities at 2.5 μg/kg/d.

This study suggests the potential for male reproductive toxicity from chronic MC-LR exposure with an apparent NOAEL of 0.25 μg/kg/d. However, a number of factors render this study problematic for RfD development. As noted above, the dose (μg/kg/d) could not be determined directly from the published data. The age of the mice was not provided and sperm quality can vary as a function of age. There are several potentially significant methodological issues with sperm and tissue analysis, as pointed out by the USEPA (2015c).

Lin et al. (2016) - This was a cross sectional epidemiology study in a population in Southwest China exposed to both MC-LR and aflatoxin. Renal function indicators (blood urea nitrogen, BUN; serum creatine, SCr; estimated glomerular filtration rate, eGFR) were evaluated as a function of estimated MC-LR (and aflatoxin) intake from water and food. The population in this study was apparently exposed long-term (possibly over a lifetime) to microcystin-LR that appears to have been chronically (or at least repeatedly) present in its environment. The mean, median, 75th and 95th percentiles of estimated daily MC-LR intake were 4.05, 3.23, 4.66, and 9.55 ng/kg/d, respectively.

For both the full study population (5,493 people) and the subset of those with abnormal renal function (129-383 people depending on the specific renal function parameter), there was a significant association of renal function indicators with estimated MC-LR (but not aflatoxin), with an apparent dose-response relationship across quartiles (significant for trend) of MCL-LR exposure. This association was seen for each renal function indicator in adjusted models. The odds ratio (OR) for having abnormal renal function indicators was significantly > 1.0 for the third and fourth quartiles of estimated MC-LR intake. The OR for having abnormal renal function indicators relative to the median estimated MC-LR intake in the fully adjusted model was significantly > 1.0 for all three renal function indicators. Although there is documented exposure to aflatoxin in this population, this did not appear to confound the observed associations with MC-LR.

Although this study does not readily lend itself to the calculation of a NOAEL, based on the above summary, the lowest quartile of exposure (i.e., 0.003 μg/kg/d) appears to be the most reasonable estimate of the estimated exposure that is not clearly associated with adverse effects. Nonetheless, the reliability of the exposure estimates in this study is not clear and there was no independent estimate of intake that could be used to ground-truth these estimates. Thus, the quantitative determination of dose-response from this study can be viewed as only suggestive. Importantly, this study suggests that MC-LR is associated with adverse renal function in humans.

RfD Derivation:

Selection of critical study for derivation of an RfD – General considerations

The two Li et al. studies (2014, 2015) on rats both suggest the potential for MC-LR to cause both adult and developmental neurotoxicity with sub-chronic exposure. The apparent LOAEL for both endpoints is 0.5 μg/kg/d. However, interpretation of the results of both studies is complicated by co-exposure to methanol and the possibility that the co-exposure could result in a synergistic response.

The Chen et al. (2011) study suggests the potential for male reproductive effects with an apparent NOAEL 0.25 μg/kg/d. However, as discussed above, there are numerous reporting and methodological issues with this study.

The Lin et al. (2016) study has the advantage of investigating a human population. However, the accuracy of the microcystin-LR exposure estimates in that study is unknown. Furthermore, the statistical analysis does not readily lend itself to the estimate of a NOAEL. In addition, although not entirely clear from the published paper, it appears that this population was chronically exposed to microcystin-LR. Thus, the estimated dose-response relationship from this study may not be appropriate for the purposes of deriving a short-term RfD. Both the Fawell et al. (1994/1994) and Heinze (1999) studies provide appropriate toxicological data for the derivation of an RfD. These studies yield a LOAEL at very similar doses, with no NOAEL identified.

In the Fawell et al. (1994/1999) study, the 40 µg/kg/d dose is identified as a minimal LOAEL based on the significant decrease in body weight gain in males, and is also supported by changes in other parameters (total blood protein, albumin, chronic liver inflammation) that were not statistically significant at this dose, but are predictive of significant effects at higher doses. This study also provides information on effects from developmental exposures. In the Heinze (1999) study, the 50 µg/kg/d dose is a clear LOAEL that reflects liver toxicity based on increased liver weight and elevated serum liver enzymes. Liver toxicity was also observed in the Fawell et al. (1994/1999) study, but only appears to have reached statistical significance at 1,000 µg/kg/d. Although the LOAEL from the Fawell et al. (1994/1999) study (40 µg/kg/d) is slightly lower than the LOAEL from the Heinze (1999) study (50 µg/kg/d), the LOAEL from Fawell et al. (1994) is judged to be a minimal LOAEL, whereas the LOAEL from Heinze (1999) is a LOAEL for more significant adverse effects. In addition, the length of exposure in the Heinze (1999) study (28 days) was less than that in the Fawell et al. (1994/1999) study (91 days). However, the USEPA (2015a) cites a study (Guzman and Solter, 1999) in which rats were intraperitoneally infused with MC-LR. In that study, the route of exposure resulted in direct exposure of the liver. Adverse liver effects were observed in that study, and the NOAEL and LOAEL doses were separated by a factor of two. The USEPA thus argues that a full factor of 10 is not necessary to estimate the NOAEL from the observed LOAEL for adverse effects in the Heinze (1999) study, and the alternative UF of 3 is appropriate. Given these considerations it appears more appropriate to identify the minimal, but lower LOAEL of 40 µg/kg/d for small, but significant decreased body weight in male mice in Fawell et al. (1994/1999) as the point of departure for RfD derivation, noting that applying a UF of 3 to estimate the NOAEL from the minimal LOAEL in Fawell et al. (1994/1999) also adequately addresses the NOAEL for liver effects in the Heinze (1999) study based on the argument presented by the USEPA (2015a).

Uncertainty factor analysis - A total UF of 3,000 was applied to the LOAEL based on the following individual UFs:

UF – study duration = 1
Although this was a sub-chronic duration study, it appears appropriate to the relevant exposure scenarios.

UF – LOAEL-NOAEL = 3
The moderate decrease in male body weight gain at the 40 µg/kg/d dose is identified as a minimal LOAEL.

UF – animal-human = 10
Standard assumption - this includes factors of 3 each for interspecies toxicokinetic and toxicodynamic variability.

UF – sensitive human populations = 10
Standard assumption – includes children as a sensitive group.

UF – database = 10

The only studies that address neurotoxicity/developmental neurotoxicity are the two studies of Li et al. (2014, 2015). Both of these studies yield a LOAEL of 0.5 µg/kg/day. The interpretation of both of these studies is potentially confounded by co-exposure to methanol, a known neurotoxicant. However, the extent of confounding by methanol in either Li et al. study is unknown, and the neurotoxicity and developmental neurotoxicity effects in these studies could, in fact, be independent of the methanol exposure. If it is assumed that the application of a UF of 3 to account for the use of a minimal LOAEL in the absence of a NOAEL from the Fawell et al. (1994/1999) study appropriately estimates a NOAEL from that study (i.e., 13 µg/kg/d), then application of an additional UF of 3 for database uncertainty would yield a value of 4 µg/kg/day (leaving aside the other UFs that are independent of the treatment of the Li et al. studies). This would still be an order of magnitude larger than the LOAEL from the Li et al. (2014, 2015) studies. Alternatively, application of a full uncertainty factor of 10 (rather than 3) to the estimated NOAEL (13 µg/kg/d) from Fawell et al. (1994/1999) to account for database uncertainty regarding the potential for neurotoxicity/developmental toxicity would result in a value of 1.3 µg/kg/d. This value is still approximately twice the LOAEL from the Li et al. studies. The application of a full UF of 10 for database uncertainty also appears to address all other database issues.

UF-total = 3,000

RfD calculation

RfD = LOAEL ÷ UF-total
= 40 µg/kg/d ÷ 3,000
= 0.013 rounded to **0.01 µg/kg/d**

Comparison to USEPA RfD and WHO Tolerable Daily Intake (TDI) - The RfD of 0.01 µg/kg/d is smaller than the Tolerable Daily Intake (TDI) (0.04 µg/kd/d) developed by WHO (2003b) as well as the RfD value developed by the USEPA (2015a) for MC-LR of 0.05 µg/kg/d. The difference is almost entirely due to the consideration of the database uncertainty regarding the potential for neurotoxicity/developmental neurotoxicity introduced by the Li et al (2014, 2015) studies. This uncertainty was not addressed directly by either WHO or USEPA.

II. Cylindrospermopsin (CYN)

Review of toxicological data:

Humpage and Falconer (2003) - Male mice (Swiss albino) were dosed with cylindrospermopsin derived from *C. raciborskii* cells (strain AWT 205) in overlapping studies, the first with

exposure by drinking water for 10 weeks with a CYN dose range of 216-657 μg/kg/d, and the second by gavage for 11 weeks with a dose range of 30-240 μg/kg/d. The drinking water study was conducted using a crude cell extract, while the gavage study utilized a purified extract, assayed as 47% CYN, 53% phenylalanine. The n for controls was 12 in the drinking water study was 12 and 10 for the gavage study. The n was 10 for all dose groups except the highest doses, which had an n of 5-6. The study protocols appear valid although the body weights of the controls at sacrifice in the two studies differed despite being in the same range at the start of dosing. There was a 23% decrease in body weight of gavage controls compared to the drinking water controls. The authors speculate that this was due to discomfort resulting from the gavage treatment *per se*.

In the drinking water study, body weight was significantly decreased at the two highest doses, and liver and kidney weight were significantly increased at all doses. Spleen weight was not significantly affected. At the two lowest doses, 30 and 60 μg/kg/d, in the gavage study, body weight was significantly increased compared to controls, but it was significantly decreased at the two highest doses in the drinking water study. Relative liver weight increased monotonically with an apparent LOAEL of 216 μg/kg/d in the drinking water study, and a NOAEL and LOAEL of 120 μg/kg/d and 240 μg/kg/d, respectively, in the gavage study. In the drinking water study, with kidney weight increased monotonically and significantly across all dose groups compared to controls. In the gavage study, kidney weight increased monotonically, with a LOAEL of 60 μg/kg/d and a NOAEL of 30 μg/kg/d (the lowest dose). There were no other significant changes in organ weight. Urine protein/creatinine ratio decreased monotonically with a LOAEL of 120 μg/kg/d in the gavage study. There was minimal liver histopathology (unspecified) at 120 and 240 μg/kg/d, but no changes in serum liver enzymes in the gavage study. Serum bilirubin was significantly increased and serum total bile acids were significantly decreased at 216 and 432 μg/kg/d in the drinking water study, but were not significantly altered in the gavage study.

The authors hypothesize that CYN inhibits protein synthesis. Decreased urinary protein (presumably small proteins, not reflective of glomerular or tubular damage) is consistent with this hypothesis, although the decreased protein/creatinine ratio in conjunction with decreased creatinine concentration at the high dose in the gavage study could reflect increased creatinine due to protein catabolism as well as, or instead of, decreased overall protein synthesis at the high dose.

The study NOAEL is 30 μg/kg/d based on increased relative kidney weight. While increased relative kidney weight is considered an adverse effect, the mode of action leading to the effect in this study is unclear. The authors hypothesize that increased kidney weight could reflect an increase in cellular volume or cellularity in response to inhibition of protein and general metabolic synthesis. While this explanation appears to be speculative, the linkage of this effect to more frank adverse effects at higher doses including decreased body weight argues for the

validity of this NOAEL. However, there would be more confidence in this value if there had been a lower dose that also showed no adverse effect.

Chernoff et al. (2011) – Pregnant CD-1 mice were exposed to commercial CYN (>98% pure) by intraperitoneal injection daily for 5 days at doses of 50 μg/kg/d during GD 8-12 or GD 13-17. Animals were sacrificed (n = 2-5) 24 hrs after the final injection, or on post-treatment days 7 and 14. Measurements included maternal weight, serum chemistry, and histopathology of liver and kidney.

Decreased weight gain in the GD 8-12 group occurred starting with the first dose, followed by vaginal bleeding, reduced activity, blood accumulation in the tail, and hemorrhaging around the eyes. There was mortality and morbidity during dosing with some mortality through GD 18. Gestational length was not affected. These effects were milder in the GD 13-17 group. Treated animals in this group gave birth earlier in the day compared to controls. Serum liver enzymes (AAT, AST, and ALT) were significantly elevated in the GD 8-12 group and in the GD 13-17 group. LDH and SDH were elevated in the GD 13-17 group. Serum albumin was significantly reduced in both groups. BUN and creatinine were significantly increased in the GD 8-12 group, and serum glucose was significantly decreased in both groups. Blood chemistry parameters returned to normal values 7 days post-dosing in both groups. Relative liver weight was not affected in the GD 8-12 group, but was elevated in the GD 13-17 group and did not recover. Minimal-moderate centrilobular hepatocyte necrosis and apoptosis was elevated in both treatment groups compared to controls. Elevated minimal-moderate chronic interstitial nephritis was seen in the GD 8-12 group.

The injection route of exposure and the single dose nature of this study preclude its use in RfD derivation. However, this study provides evidence of the potential of CYN to result in adverse metabolic, liver and kidney effects.

Chernoff et al. (2014) - Pregnant CD-1 mice were exposed to CYN by i.p. injection during either gestation day (GD) 8-12, or GD 13-17. Animals were dosed on five successive days with 50 μg/kg/d of CYN. Groups of dams were sacrificed after each day of dosing and at various times up to 13 weeks post-dosing. Maternal body weight, visual signs of toxicity, serum chemistry, and liver and kidney histopathology were analyzed. The number of animals examined (generally 4-21) varied by endpoint and number of doses.

Maternal weight decreased significantly during the dosing period in both gestational period groups. Vaginal hemorrhaging and visual signs of morbidity were also observed in both groups, although the late gestational period group showed effects after the first dose compared to the third dose in the early gestational group. ALT, SDH, and total bile acids were significantly increased in both groups both during, and to varying extents, post-dosing, indicating liver toxicity. Hemoglobin and hematocrit were reduced in both groups with a greater sensitivity in the later gestational group. Significant increases in liver weight were seen in the late gestational

group. Liver histopathology including hepatocellular necrosis; hepatocellular cytoplasmic alterations; and chronic centrilobular inflammation was observed in both groups. In the kidney, acute tubular necrosis was also observed in both groups. In addition, a significant decreased platelet count was observed during the last two days of dosing.

The injection route of exposure and the single dose nature of this study preclude its use in RfD derivation. However, this study provides further evidence of the potential of CYN to result in metabolic, liver, kidney and hematologic adverse effects.

de Almeida et al. (2013) – Pregnant Wistar rats were exposed to CYN by gavage at 0, 0.03, 0.3 and 3.0 μg/kg/d for GD 1-20 (n = 10/dose group). Body weight was measured during and at the end of treatment. Organ weights were measured for ovaries, uterus, kidney, pancreas, adrenal gland, heart and spleen. Histopathology was conducted on liver and kidney. Reproductive parameters were recorded. Half of the fetuses from each litter were examined for visceral malformation, and the other half were examined for skeletal malformation.

No significant differences from controls were observed for body weight or histopathology. No differences were observed in organ weights, or in the incidence of visceral or skeletal malformations.

This study yields a free-standing NOAEL of 3.0 μg/kg/d for maternal, reproductive and teratological effects from gestational exposure.

Sukenik et al. (2006) – Four-week old ICR mice (M and F) were exposed to a cell-free, but unpurified solution of CYN in water. The exposure protocol in this study is somewhat unusual. In order to minimize the number of animals, the authors gradually increased the dose to all of the animals over the course of the study. Blood was drawn from the tails every four days for determination of hematocrit and cholesterol and half of the animals were sacrificed at 20 weeks and the remainder at 42 weeks. The authors state that the dose increased from 10 μg/kg/d at the start of the study to > 50 μg/kg/d in the last 22 weeks of exposure. Although not explicitly stated in the paper, the dose at the 20-week sacrifice was approximately 30 μg/kg/d based on the graphic presentation in the paper. This study is, therefore, a chronic duration study. The authors state that analysis of the drinking water revealed only CYN and the related compound 7-epi/CYN. However, the purity of the CYN is not known. Blood samples were obtained every four weeks. There were initially 20 males and 20 females in the control and exposure groups. Liver, spleen, kidney and testes were weighed and examined by histopathology. Cholesterol was determined in RBC membranes, plasma, and liver homogenate.

No effect on body weight gain was observed. Relative liver weight was significantly increased at 42 weeks (but not at 20 weeks) in M and F. Relative kidney weight was significantly increased in M and F at both 20 and 40 weeks, and relative testes weight was significantly increased at 42 weeks. Relative spleen weight was not affected. Hematocrit was significantly increased compared to controls in M at all time periods of measurement except for the final (36

week) period when it was significantly decreased compared to controls. Female hematocrit was also significantly increased for all time periods except for the final measurement period (36 weeks). This was accompanied by deformed RBCs. RBC membrane cholesterol was significantly increased in M and F at 42 weeks. Plasma cholesterol was slightly (but significantly) increased in F at 42 weeks. In liver homogenate, however, cholesterol was significantly decreased in M at 20 weeks and in M and F at 42 weeks.

This study suggests that chronic exposure to moderate levels of CYN can result in adverse effects in liver, kidney and testes weight, hematologic parameters and cholesterol levels. However, the chronic nature of this study renders it not appropriate for derivation of a shorter term RfD. Additionally, the inability to link effects to specific CYN doses and the potential contribution of the 7-epi/CYN compound precludes the use of this study for quantitative assessment for CYN.

Rogers et al. (2007) - Pregnant CD-1 mice were injected (i.p.) with 8-128 μg/kg/d CYN, stated to be free from organic impurities and with >98% purity, during GD 8-12. Term fetuses were examined for viability and structural abnormalities. Significant lethality in the dams was observed for doses > 32 μg/kg/d, but there were no adverse effects on litter size, fetal weight, or incidence of anomalies. However, as the number of surviving dams at doses > 32 μg/kg/d was small, the number of fetuses available for evaluation at the larger doses was also small and conclusions about lack of fetal effects at doses > 32 μg/kg/d are weak. Subsequently, dams were injected with 50 μg/kg/d during GD 8-12 or 13-17. Maternal toxicity, including lethality and hemorrhaging, was noted in dams exposed during both stages of gestation although the incidence and severity was less for exposure during the later period. In the dams exposed during the later period, birth occurred earlier in the day compared to controls. A reduction in litter size compared to controls was noted for exposure during both gestational periods. Pups of dams exposed during GD 13-17 had significantly reduced body weight. There was decreased fetal survival among the pups in the GD 13-17 dosing group and indication of gastrointestinal hemorrhage. Following cross-fostering to control dams, pups of dams exposed during GD 13-17 (but not GD 8-12) had decreased viability and weight gain.

The injection route of exposure is not appropriate for deriving an oral RfD. In addition, the design of this study is not amenable to deriving a useful LOAEL or NOAEL. However, this study does provide qualitative evidence of reproductive/developmental effects of CYN.

Reference Dose (RfD) derivation:

Selection of critical study – Only two repeat dose studies with specific estimates of daily dose were identified, Humpage and Falconer (2003) and de Almeida et al. (2013). The de Almeida et al. (2013) study, however, yields only a freestanding NOAEL that is an order of magnitude lower than the NOAEL from Humpage and Falconer (2003). The Humpage and Falconer (2003) study with a critical effect of increased relative kidney weight is, therefore, the more appropriate study

for the derivation of a short term RfD. The Humpage and Falconer (2003) study is also the study selected by the USEPA for its Drinking Water Health Advisory for cylindrospermopsin (USEPA, 2015b). This study yields a NOAEL of 30 µg/kg/d based on increased relative kidney weight.

Uncertainty factor (UF) analysis - A **total UF of 1,000** was applied to the NOAEL based on the following individual UFs:

UF – study duration = 1
Although this was a less-than sub-chronic duration (11 week) study, it is consistent with the range of study durations applicable to the derivation of a short-term RfD (see Exposure Scenario section).

UF – LOAEL-NOAEL = 1
The study yields a reasonable estimate of the NOAEL.

UF – animal-human = 10
Standard assumption - this includes factors of 3 each for interspecies toxicokinetic and toxicodynamic variability.

UF – sensitive human populations = 10
Standard assumption – includes children as a sensitive group.

UF – database = 10
It is noted that the USEPA (2015b) applied an uncertainty factor of 3 for database insufficiency for CYN citing the same study. The study of Rogers et al. (2007) provides evidence that CYN can produce reproductive/developmental effects. However, the nature of this study does not permit the derivation of a meaningful LOAEL or NOAEL. Furthermore, there are no data that permit an assessment of potential neurological or immunologic effects. Thus, although there is evidence indicating that CYN is capable of causing reproductive/developmental effects, there is no basis for deriving a reproductive/developmental-specific RfD, and there is no basis for determining the NOAEL based on increased relative kidney weight is protective against reproductive/developmental effects. Based on this consideration as well as the lack of data on possible neurological and immunological effects, a full UF of 10 for database insufficiency appears justified.

UF - total = 1,000

Calculation of RfD

RfD = NOAEL ÷ UF-total
= 30 µg/kg/d ÷ 1,000
= **0.03 µg/kg/d**

Comparison to USEPA RfD - USEPA (2015d) derived an RfD of 0.1 µg/kg/d for CYN. The USEPA RfD uses the same NOAEL of 30 µg/kg from Humpage and Falconer (2003), but differs in applying a total UF of 300 rather than the value of 1,000 derived here. The basis for this difference is described above.

III. Anatoxin-a

Review of toxicological data:

Astrachan and Archer (1981) - Anatoxin-a was isolated from NRC-44-1 strain of *A. flos-aquae*. Anatoxin-a is known from previous work (Carmichael et al., 1975) to be a neurotoxin acting as a nicotinic agonist acting by stimulation followed by a depolarizing blockade to produce death with ataxia and convulsion. Based on Carmichael et al. (1975), the single dose injection (intraperitoneal) LD_{50} is approximately 250 µg/kg in rats and mice.

Female adult Sprague-Dawley rats were exposed to anatoxin-a through either drinking water (0, 51 or 510 µg/kg/d, 20/dose group) for up to 7 weeks, or by i.p. injection (0 or 89 µg/kg/d for 21 days, 18/dose group). The oral doses are estimated to be 0.8% and 8% of the oral LD_{50} respectively, and the intraperitoneal dose was estimated by the authors to be 25% of the i.p. LD_{50}. Based on the body weight provided in study, however, this dose appears to be 36% of the i.p. LD_{50}. Animals exposed by both routes were assessed for body weight, food consumption, behavior; gross lesions; liver, spleen, kidney weight, and histopathology of these organs; RBC and WBC count; and serum liver enzymes (cholinesterase, AP, SGTP, GGTP).

No significant differences were observed between the control and dosed groups in any of the parameters assessed. Although there was a transient increase in white blood cell count in the high dose animals at 5 weeks, this parameter was not different from the control or low dose value at 7 weeks, and the significance, if any, of this observation is unclear. Thus, 510 µg/kg/d is identified as a free-standing NOAEL from this study. The USEPA (2015e) identifies the high dose in this study as a LOAEL (rather than a NOAEL) on the basis of the transient white blood cell count. Although most of the data are presented only as summary narrative statements, the study design and results appear to be valid. However, there would be more confidence in the NOAEL if this study had identified a LOAEL.

Astrachan et al. (1980) - Teratology studies - Astrachan et al. (1980) isolated anatoxin-a from a laboratory culture of NRC-44-1 and reported that it was "essentially pure" by TLC and HPLC. Pregnant golden hamsters were dosed by i.p. injection on either GD 8-11, or 12-14. The only single daily dose was 200 µg/kg on GD 8-11. Other doses consisted of multiple daily injections (3 x 125 µg/kg = 375/µg/kg/d; and 3 x 200 µg/kg = 600 µg/kg/d) with an identical schedule for GD 8-11 and GD 12-14. Dams were sacrificed on GD 15 and the fetuses examined.

The authors do not report statistical significance for fetal resorption. However, for all dose groups and each gestation period, the percent resorption was greater than for controls – including greater than four-fold the control rate (GD 8-11, 375 μg/kg/d). Fetal weight was significantly decreased compared to controls for all dose groups for dosing on GD 8-11, and for the 375 μg/kg/d group on GD 12-14. The authors refer to "stunting" of the fetuses. This refers to reduced body weight. In addition, in one litter with maternal dosing at 375 μg/kg/d, all 10 of the fetuses had hydrocephaly. No other soft tissue or skeletal malformations were noted.

The injection route of exposure as well as the absence of a NOAEL preclude the direct use of this study in the derivation of an RfD. In addition, precise interpretation of these findings is hampered by incomplete and inexact reporting. Nonetheless, this study provides evidence that anatoxin-a exposure during gestation can have developmental effects including, at a minimum, decreased fetal weight.

Fawell and James (1994)/Fawell et al. (1999)[3]:
In each of the studies in this publication, Fawell and James (1994) used commercial anatoxin-a hydrochloride. The doses given below reflect adjustment to the dose as anatoxin-a (i.e., the parent molecule without the hydrochloride salt).

Single dose studies - Male CD-1 mice received anatoxin-a hydrochloride by a single intravenous injection (6 per dose group) at doses of 0, 8.2, 24.5 and 81.7 μg/kg. Mice were observed through 4 hrs post-injection and evaluated according to the Irwin protocol (a checklist of *in vivo* clinical observations). No observed effect occurred for the 8.2 or 24.5 μg/kg doses. Two animals in the 25 μg /kg dose group died and those that survived showed increased salivation, respiration and hyperactivity. No effects were observed in the low dose group (8 μg/kg anatoxin-a/kg). At 81.7 μg/kg, all animals died within 1 min of dosing with neurological symptoms consistent with a cholinergic effect. It should be noted that only a factor of approximately 3 separates the no-effect dose (relative to these parameters) from the lethal dose.

Male CD-1 mice received intravenous anatoxin-a as a single injection of 0, 24.5, 40.8, or 49 μg/kg (6/dose group). Animals were tested on the rotarod 15 min post injection. One animal died at 24.5 μg/kg, and two animals died at 40.8 μg/kg. Animals at 49 μg/kg experienced convulsions, hypersalivation, micturition, an elevated tail, and hyperactivity, and all of them died within one minute of dosing. The "majority" of animals at 24.5 μg/kg and all animals at 40.8 μg/kg had increased respiration with a duration of approximately 1 minute. Recovery of all surviving animals occurred within a few minutes. All surviving animals remained on the rotarod, indicating retention of significant neuromuscular function/coordination. Contrary to the

[3] Fawell et al. (1999) is a reiteratioin of a portion of the studies in Fawell and James (1994). The former is a journal article and the latter is a study report.

earlier intravenous injection study in this paper, 24.5 µg/kg is a LOAEL not a NOAEL here due to the apparent dose-related death at this dose.

Repeated dose studies - Two male and two female mice were dosed five times by oral gavage at each of the dose levels below. Although not stated explicitly, it appears that surviving animals were sacrificed 24 hrs after the last dose. For animals receiving 1,225 or (apparently) 2,450 µg/kg/d, dosing was on successive days. For the 6,125 and 12,250 µg/kg/d doses, dosing was every other day. All animals receiving 12,250 µg/kg/d and one female receiving 6,125 µg/kg/d died within 3 min. of dosing. At 6,125 µg/kg/d, males were hyperactive after the third dose. Body weight and food consumption were unaffected and no unusual results were observed on necropsy. No toxicity was observed at 1,225 or 2,450 µg/kg/d.

In a longer duration component of this study, mice (10 males and 10 females per dose group) received 0, 98, 490, or 2,450 µg/kg/d by gavage for 4 wks. Animals were bled without sacrifice during the final week of dosing and assessed for hematology and blood chemistry, and weight and histopathology of multiple organs was assessed at sacrifice.

One animal receiving 98 µg/kg/d was reported to have died with evidence of infection. One male receiving 490 µg/kg/d and one female receiving 2,450 µg/kg/d died within 2.5 hrs of dosing with no obvious pathology. Although there is no clear evidence linking these two deaths to the dosing, the timing relative to receiving the doses in both cases is suspicious and such a connection cannot be dismissed. There was no dose-related effect on body weight, body weight gain, or ophthalmoscope examination.

Small, but statistically significant increases in mean cell hemoglobin (Hb) (males) or mean cell Hb concentration (females) were observed. However, the increase relative to controls was small (5-6%) and the significance of this observation is unclear. A relatively large (maximum = 30%), but not statistically significant, increase in AST was observed in males at the two highest doses. However, none of the other serum liver enzymes concentrations (ALP, ALT) were remarkable, and there was no abnormal liver histopathology. There was also a relatively small (2%), but statistically significant, increase in serum Na in females. There was no significant effect on body weight or organ weight and no remarkable histopathology.

In a developmental toxicity component of this study, time-mated female CD-1 were gavage dosed once per day at 2,450 µg/kg/d (n = 10), or with sterile water (controls) during GD 6-15 (n = 12). Dams were observed for clinical signs. On GD 18, animals were sacrificed and maternal necropsy was performed. Fetal implants, live and dead fetuses, fetal weight, sex and external abnormalities were recorded. Results were only provided in narrative form with few quantitative specifics.

No maternal toxicity was observed, including no effect on body weight or weight gain. No abnormalities were noted on necropsy. Implantations, live fetuses, post-implantation losses and

sex ratio were unaffected. Mean fetal weight was "marginally" lower in the exposed group. The authors attribute this to small differences in litter size. No differences in physical abnormalities were observed between control and treated fetuses. This dose can be considered a NOAEL for developmental effects.

Although there were no clear adverse toxicological results at any of the doses, the two unexplained deaths at 490 and 2,450 µg/kg/d dictate that the clearest NOAEL is 98 µg/kg/d. A NOAEL of 98 µg/kg/d is reasonably consistent with a single dose LOAEL of 24.5 µg/kg from the i.v. injection study given the likely difference in toxicokinetics between the i.v. and gavage routes of exposure. However, it should be noted that in both studies, the LOAEL is based on lethality. This NOAEL is smaller than the freestanding NOAEL of 510 µg/kg/d in rats via drinking water from the study of Astrachan and Archer (1981).

Yavasoglu et al. (2008) - Male mice (strain not specified) were exposed by intraperitoneal injection for 7 consecutive days to 0, 50, 100, or 150 µg/kg/d of anatoxin-a. Animals were evaluated for sperm count and light microscope histopathology of the testes. There were no significant effects on body weight. However, epididymis weight decreased monotonically with significant decreases compared to controls at the two higher doses. Sperm count also decreased monotonically with all doses resulting in a significant decrease relative to controls. Histopathological changes in the testes were reported. Although counts for individual effects were not reported, the effects (including degeneration of seminiferous tubules, dissociation of spermatogenic cells with resulting sloughing of germ cells into the lumen, vacuolization of Sertoli cells, and loss of germ cells) were stated to be greater at 150 µg/kg/d than at 100 or 50 µg/kg/d. The epithelial thickness of the seminiferous tubules decreased monotonically with dose and was significantly less than controls at all doses. The injection nature of the dosing in this study precludes the quantitative use of these data in RfD derivation. However, all of the doses produced adverse effects and these results raise concerns for potential effects on male fertility with oral exposure.

RfD Derivation:

Selection of critical study – The following table summarizes the NOAELs and LOAELs for lethality from the available studies.

Study	NOAEL (µg/kg/d)	LOAEL (lethality) (µg/kg/d)	Route of Exposure
Astrachan and Archer (1981)	510 (freestanding)		Drinking water
Fawell and James (1994)/ Fawell et al. 1999)	98	490	gavage
Fawell and James (1994)/ Fawell et al. (1999)		24.5	intravenous

Anatoxin-a appears to cause lethality through a rapid onset neurologic toxicity that produces hyperactivity and convulsions. Following intravenous injection (Fawell and James, 1994), lethality occurred within 1 min. Following gavage exposure, lethality occurred in 3 min. (Fawell and James, 1994). Thus, the much larger NOAEL from Astrachan and Archer (1981) is likely due to the slower absorption and distribution following drinking water exposure (which is directly analogous to incidental ingestion while swimming). This argues that LOAELs for lethality based on intravenous or gavage routes of exposure are likely to be conservative estimates of the LOAELs for lethality by the oral (drinking water) route of exposure. However, there is insufficient information on which to estimate the ratio between the oral NOAEL and the (unobserved) LOAEL for lethality. Furthermore, although the recreational (i.e., swimming) exposure scenario utilized in this assessment (see below) is based on empirical data, it cannot account for unusual (but possible) short-term high volume ingestions while swimming. Therefore, given the potential for lethality, conservative (i.e., protective) assessment of the relationship between the oral NOAEL and the LOAELs for lethality from the other exposure routes, particularly gavage, is appropriate.

The free standing NOAEL of 2,450 μg/kg/d (maternal exposure) from the developmental toxicity study of Fawell and James (1994)/Fawell et al. (1999) is the largest of the NOAELS. However, that NOAEL is based on a study with limited data reported, including inadequate reporting of the maternal pathology analyses that were carried out. The next largest NOAEL of 510 μg/kg/d from the 7 week drinking water study in rats of Astrachan and Archer (1981) reflects more complete analyses and reporting. However, this dose is close to the LOAEL of 490 μg/kg/d from the 4 week repeated dosing gavage study in mice of Fawell and James (1994)/Fawell et al. (1999). In addition, although the 510 μg/kg/d dose in the Astrachan and Archer (1981) study is assessed here as a NOAEL, it could be argued (per USEPA, 2015e) that this is a minimal LOAEL (for increased white blood cells) rather than a NOAEL. The endpoint yielding the LOAEL from the Fawell and James (1994)/Fawell et al. (1999) study is lethality that is, presumably, dose-related. The possibility that the observed lethality is dose-related is supported by the LOAEL from the i.v. single dosing portion of this study (i.e., 24.5 μg/kg) that is also based on lethality. Thus, the free standing NOAEL (drinking water) from Astrachan and Archer (1981) differs from the LOAEL (gavage) of 490 μg/kg/d for lethality from Fawell and James (1994)/Fawell et al. (1999) by only 16% and does not provide sufficient protection against lethality. The NOAEL of 98 μg/kg/d from Fawell and James (1994)/Fawell et al. (1999) is a factor of five below the LOAEL (based on lethality) from that study and is, therefore identified as the preferable point of departure for RfD derivation.

Uncertainty factor (UF) analysis - A **total UF of 1,000** was applied to the NOAEL based on the following individual UFs:

UF – study duration = 1
Although this was a less-than sub-chronic duration study, it appears appropriate to the relevant exposure scenarios.

UF – LOAEL-NOAEL = 1
The study yields a NOAEL.

UF – animal-human = 10
Standard assumption - this includes factors of 3 each for interspecies toxicokinetic and toxicodynamic variability.

UF – sensitive human populations = 10
Standard assumption– includes children as a sensitive group.

UF – database = 3
There is evidence from Astrachan et al. (1980) that anatoxin-a can cause developmental effects. However, that study does not yield a NOAEL. The Fawell and James (1994)/Fawell et al. (1999) study yields a NOAEL for developmental effects of 2,450 µg/kg/d (maternal dose). However, the method and data reporting for that portion of the study is inadequate. Thus, it is not entirely clear whether the NOAEL in the Fawell and James (1994)/Fawell et al. (1999) study is protective of developmental effects. In addition, there are no data that would allow an assessment of whether this NOAEL is protective of reproductive or immunotoxic effects.

UF – modifying factor = 3
The NOAEL from the critical study is less than an order of magnitude smaller than the LOAEL from the same study that reflects lethality.

UF-total = 1,000

RfD Calculation

RfD = NOAEL ÷ UF-total
= 98 µg/kg/d ÷ 1,000
= 0.098 µg/kg/d
which rounds to 0.1 µg/kg/d

Exposure Scenario - Water ingestion while swimming

Swimming is the most direct and pervasive activity that is likely to lead to exposure to hazardous algal toxins in surface water. Although there appears to be a potential for exposure to these algal toxins through inhalation and dermal exposure, direct ingestion appears to be the predominant

route of exposure while swimming (USEPA, 2016). Therefore, the exposure scenario is based on ingestion while swimming.

Water ingestion rate:

The USEPA Exposure Factors Handbook (EFH) (USEPA, 2011) provides guidance on incidental water ingestion while swimming. The rate of incidental water ingestion is greater (on both an absolute and body-weight adjusted basis) for children than for adults. For children (defined in the guidance as less than 18 years old), the guidance for the mean ingestion rate is 37 ml/event (45 min in the study used to derive this guidance) and 49 ml/hour. The upper percentile (reported as the 97th percentile of the distribution in the source study (Dufour et al., 2006) guidance for children is 90 ml/event (45 min) and 120 ml/hr. For episodic (as opposed to chronic) exposures, consistent with swimming events, the upper percentile values appear to be more appropriate. The EFH provides the value as generated by the recommended study based on the measurement time of 45 min. However, the EFH also provides a linear extrapolation of this value to a 1 hour swimming exposure in its recommendation. The length of a swimming event can be longer than the specific swimming event in the study used in the EFH, and a duration of one hour is assumed. It is recognized that the total recreational time spent near surface water used for swimming can be considerably longer than 1 hour. However, this duration of exposure refers specifically to time spent in the water.

Ingestion rate (upper percentile) for a 1 hour swimming event is 120 ml (0.12 L).

Frequency of exposure:

This is a difficult parameter to anticipate or model since it depends both on the frequency of swimming over the course of swimming season and the persistence of harmful algal blooms during that period. These appear to be highly variable. Rather than attempt to estimate this parameter quantitatively, the less-than sub-chronic to sub-chronic RfDs derived above will be assumed to be applicable as derived under the broad assumption that swimming events in water contaminated with toxins from harmful algal blooms can occur during multiple events over the course of the swimming season.

Body weight:

Because the source study used in the EFH to derive the recommended value for children's water ingestion (Dufour et al., 2006) does not specify the ages or age range of the children, the corresponding body weight cannot be derived directly from the recommended ingestion rate. The EFH provides recommendations for body weights for the entire range of childhood. Since hazardous algal blooms occur in natural waters (as opposed to, e.g., pools), it is assumed that an exposure scenario envisioning multiple swimming events would be most applicable to children who can swim and/or participate in water activities by themselves. The youngest age range addressed by the EFH that corresponds to this criterion is 6 to <11 years old. The mean body weight for this age group is given as 31.8 kg.

Body weight (ages 6 to <11 years) is 31.8 kg.

Calculation of recommended target concentrations of hazardous algal toxins

Equation

The recommended target water concentration of a toxin is given as:

$$C = \frac{RfD \times BW}{I}$$

Where:

C = the concentration of the toxin in the swimming water (µg/L, ppb)
RfD = the Reference Dose for the specific toxin (µg/kg-body wt/day)
BW = the assumed body weight of the child (31.8 kg)
I = the ingestion rate of swimming water (0.12 L/day)

Recommended target concentrations

Microcystin-LR
C = (0.01 µg/kg/d x 31.8 kg)/0.12 L/day = 2.65 µg/L
This is rounded to 3 µg/L.

Cylindrospermopsin
C = (0.03 µg/kg/d x 31.8 kg)/0.12 L/day = 7.95 µg/L
This is rounded to 8 µg/L.

Anatoxin-a
C = (0.1 µg/kg/d x 31.8 kg)/0.12 L/day = 26.5 µg/L
This is rounded to 27 µg/L.

Discussion

There are numerous uncertainties related to the recommended values for these hazardous algal toxins. The literature appropriate for consideration in RfD derivation was quite limited and in most cases only a single applicable study was available. The application of uncertainty factors of 1,000 (cylindrospermopsin and microcystin) and 1,000 (anatoxin-a) reflects the incompleteness of the databases for these toxins as well as the lack of clarity about the significance of reported outcomes. The specification of the likely exposure scenarios is highly uncertain due to lack of information about the nature, duration, and frequency of the actual exposures, and the likely duration of the toxins in any given waterbody. In particular, the density of blue-green algae at the surface of a body of water is subject to rapid change resulting from wind conditions. The uneven pattern of algal growth and the rapid shift in bloom density make obtaining a representative sample difficult.

Given these multiple uncertainties, the recommended water concentrations given here are intended to be protective of a range of exposures and are probably highly conservative (i.e., protective) for the most likely exposures. Nonetheless, the extent of this conservatism is not known. The uncertainty in the risk estimates as well as the inherent uncertainty in the temporal variability of the toxins in any given waterbody should be taken into account when considering advice to the public regarding recreation in affected waterbodies.

These recommendations do not address the risk to pets, livestock and wild fauna, nor do they address the risk associated with consuming fish from affected waters or the combined risk from swimming and fish consumption.

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