Cyanobacterial toxins: Microcystin

Background document for development of WHO Guidelines for Drinking-water Quality and Guidelines for Safe Recreational Water Environments

Version for Public Review
March 2020
Preface

Information on cyanobacterial toxins, including microcystins, is comprehensively reviewed in a recent volume published by the World Health Organization, “Toxic Cyanobacteria in Water” (TCiW; Chorus & Welker, in press). This covers chemical properties of the toxins and information on the cyanobacteria producing them as well as guidance on assessing the risks of their occurrence, monitoring and management. Therefore, the sections 1 – 3 and 8 of this document are largely summaries of respective chapters in TCiW and references to original studies can be found therein. In contrast, this background document focuses on reviewing the toxicological information available for guideline value derivation for microcystins in water.

(to be completed by WHO)
Acknowledgements

(to be completed by WHO)
**Abbreviations used in the text**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>ASP</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>bw</td>
<td>body weight</td>
</tr>
<tr>
<td>C</td>
<td>daily water consumption</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunoassay</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>GGT</td>
<td>gamma-glutamyl transferase</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GST-P</td>
<td>glutathione S-transferase placental form-positive</td>
</tr>
<tr>
<td>GV</td>
<td>guideline value</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LOAEL</td>
<td>lowest-observed-adverse-affect level</td>
</tr>
<tr>
<td>MC</td>
<td>microcystin(s)</td>
</tr>
<tr>
<td>MC-LA</td>
<td>microcystin-leucine-alanine</td>
</tr>
<tr>
<td>MC-LF</td>
<td>microcystin-leucine-phenylalanine</td>
</tr>
<tr>
<td>MC-LR</td>
<td>microcystin-leucine-arginine</td>
</tr>
<tr>
<td>MC-LW</td>
<td>microcystin-leucine-tryptophan</td>
</tr>
<tr>
<td>MC-RR</td>
<td>microcystin-arginine-arginine</td>
</tr>
<tr>
<td>MC-YR</td>
<td>microcystin-tyrosine-arginine</td>
</tr>
<tr>
<td>MMPB</td>
<td>2-methyl-3-methoxy-4-phenylbutyric acid</td>
</tr>
<tr>
<td>NOAEL</td>
<td>no-observed-adverse-affect level</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase-1</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase-2A</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TDI</td>
<td>tolerable daily intake</td>
</tr>
<tr>
<td>UF</td>
<td>uncertainty factor</td>
</tr>
</tbody>
</table>
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1.0 EXECUTIVE SUMMARY

2.0 GENERAL DESCRIPTION

2.1 Identity

Microcystins (MCs) were first characterized in the early 1980s and named after the cyanobacterium *Microcystis aeruginosa* from which they were initially isolated. MCs share a common cyclic heptapeptide structure of cyclo-(D-Ala\(^1\)-X\(^2\)-D-Masp\(^3\)-Z\(^4\)-Adda\(^5\)-D-Glu\(^6\)-Mdha\(^7\)) in which X and Z are variable L-amino acids, D-Masp is D-erythro-β-methyl-isoaspartic acid, Mdha is N-methyldehydroalanine and Adda is \((2S,3S,4E,6E,8S,9S)-3\text{-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid})\) (Figure 2.1). A structurally related group of hepatotoxins, the nodularins primarily produced by *Nodularia spumigena*, are cyclic pentapeptides that lack the two sites for the variable L-amino acids. Adda is the most characteristic moiety of MCs and nodularins, as it occurs exclusively in these cyanobacterial peptides. Further characteristics are the presence of five (5) D-amino acids, usually not found in ribosomally-synthesized peptides, and these gave an early indication of a non-ribosomal synthesis of these cyclic peptides.

To date more than 250 different MCs have been identified, with molecular weights in the range of 882 – 1101 Da. (Spoof & Catherine 2017, Bouaïcha et al. 2019). Structural modifications exist in all seven amino acids; the most frequent variations are substitution of L-amino acids at positions 2 and 4, substitution of Mdha by dehydrobutyryne (Dhb) or serine in position 7, and a lack of methylation of amino acids at positions 3 and/or 7 (Figure 2.1); the structural modifications observed in the Adda, although not frequent, can be of relevance, since they may impact analytical tests using Adda as a marker. The principle nomenclature of MCs is based on the variable L-amino acids in position 2 and 4, e.g. using the standard one-letter codes for amino acids, microcystin-LR contains L-leucine (L) in position 2 and L-arginine (R) in position 4. All other modifications in the molecule are suffixed to the respective variant, e.g., [D-Asp\(^3\)]MC-LR lacks the methyl group in position 3.

![Fig. 2.1: Generic structure of microcystins. X and Z denote variable L-amino acids in positions 2 and 4. R\(^1\), R\(^2\), R\(^3\), R\(^4\) are sites where common substitutions have been observed, respectively. MC-LR: X\(^2\)=L-leucine, Z\(^4\)=L-arginine, R\(^1\)=CH\(_3\), R\(^2\)=CH\(_3\), R\(^3\)=H, R\(^4\)= CH\(_3\).]
### 2.2 Physicochemical Properties

MCs and nodularins are extremely heat stable. They can exhibit a range in solubility at different pH (de Maagd et al. 1999, Liang et al. 2011). Most MCs are hydrophilic, and Santori et al. (2020) determined and compared the LogP obtained by using the OECD test guideline no 117 method: at pH=7 and at pH=5 resulting in the ranking from the most to the least polar as: MC-RR, MC-YR, MC-LR, MC-LW, MC-LF. Dependence of solubility on pH should influence detection methods used. For instance, the use of an acidic mobile phase for final preparative HPLC (e.g. trifluoroacetic acid) results in a change in net charge and thereby reduces solubility in water, especially at high MC concentration. Further, using methanol as a dissolution solvent in conjunction with MCs purified with trifluoroacetic acid has been shown to form methyl esters (Foss et al. 2018, Harada et al. 1996). These aspects are important when preparing MCs for testing and toxicological investigations.

**Table 2.1**: Physical and chemical properties of selected microcystins and nodularin for which data on these characteristics are available, in the order of decreasing polarity. N/A: not applicable. For further MCs, see TCiW, Fastner and Humpage, in press).

<table>
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<tr>
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<tr>
<td>Chemical Formula</td>
<td>Ca₉H₂₅N₆O₁₁</td>
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<td>Ca₉H₂₅N₆O₁₁</td>
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<td>Average MW² (g/mol)</td>
<td>1038.224</td>
<td>824.983</td>
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<td>910.087</td>
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<td>Monoisotopic MW (g/mol)</td>
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<td>824.443</td>
<td>1044.528</td>
<td>994.549</td>
<td>980.533</td>
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<td>Color/Physical State</td>
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<td>LogPow⁶ at pH 5</td>
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<td>2.85</td>
<td>3.84</td>
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<tr>
<td>LogPow⁶ at pH 7</td>
<td>1.7</td>
<td>2.25</td>
<td>2.54</td>
<td>3.84</td>
<td>2.54</td>
<td>3.95</td>
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<td></td>
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<tr>
<td>Solubility in Other Solvents</td>
<td>Dimethyl sulfoxide, methanol, ethanol (note that solvents may alter structures and in vitro processes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

¹ Molecular Abstracts Service Registry Number  
² Molecular Weight  
³ from Santori et al. 2020; note that for the other MCs in the table, no experimental data on LogPow or Kow are available

### 2.3 Organoleptic properties

While none of the known cyanobacterial toxins (MCs, cylindropermopsins, saxitoxins, anatoxins) has been shown to affect the taste or odour of water, some cyanobacterial species produce other compounds such as geosmin and methyl-isoborneol that do affect taste or odour of water, thus indicating the presence of cyanobacteria in raw water. However, as this applies only to some strains of some species, the absence of tastes or odours is not a reliable indicator for the presence/absence of cyanotoxins (for an overview of the relationship between organoleptic properties and toxins, see TCiW, Kaloudis, in press).

### 2.4 Major uses and sources

MCs occur naturally (although high concentrations are typical for waterbodies influenced by human activity, i.e. effluents from wastewater or run-off from agricultural land which introduce nutrients that fertilise the growth of phototrophic organisms, including cyanobacteria). There
are no known commercial applications of MCs. In surface waters, MCs are produced by a number of cyanobacterial species, however primarily by Chroococcales (Microcystis), Nostocales (Anabaena/Dolichospermum, Nostoc), Oscillatoriales (Planktothrix, Limnothrix), and Stigonematales (Fischerella). MC production is not restricted to the latter taxa albeit these taxa are the most prominently represented in water blooms. Toxigenic (containing the required genes for MC synthesis) and non-toxigenic strains of a given species often co-occur. Although many toxigenic strains simultaneously produce several MC variants, usually only one to three of the variants make up the bulk of total MCs. The variants as well as the amount occurring in a given bloom are largely determined by its composition of strains (or genotypes), and the environmental factors driving this are not yet understood (Testai et al, 2016).

Variations in growth conditions affect the MC content of individual strains only moderately, i.e., by a factor of not more than 2-3 in most studies and maximally of five, while differences in MC contents among individual toxigenic strains can vary much more widely, from trace amounts to more than 10 mg/g dry weight. Cell quotas of up to 550 fg MCs/cell and toxin contents up to 14 µg MCs/mm³ biovolume have been reported for individual Microcystis sp. strains, although these values seem to be exceptionally high and a number of other studies suggest maximum cell quota or toxin contents of some 200 fg MCs/cell or 5 µg MCs/mm³ biovolume, respectively (see discussion in TCiW, Ibelings et al., in press). Similar ranges of cell quota and toxin contents have been reported for Planktothrix agardhii and P. rubescens, with MC values for P. rubescens reported as high as 850 fg MCs/cell and 20 µg MCs/mm³ biovolume, respectively.

MCs are synthesized via a hybrid polyketide synthase/non-ribosomal peptide synthetase (PKS/NRPS) enzyme complex, similar to other cyanobacterial secondary metabolites. The genes (mcyA-J) are coded in a gene cluster of approx. 60 kbp. Sequences of the corresponding PKS/NRPS gene clusters are available for multiple strains and taxa, including Microcystis, Planktothrix, Dolichospermum, Nostoc, and Fischerella, allowing for the profiling of cyanobacterial strains or field populations to assess the presence of the MC gene cluster and thus the potential of MC production. The presence of the biosynthesis cluster in the genome of a particular strain is generally an indicator of MC production, although strains with inactive gene clusters are known, in particular in P. rubescens.

For more details on MC producing organisms, biosynthesis, and environmental fate, including the references on which the assessments presented here are based, see TCiW (Fastner & Humpage, in press).

Cyanobacteria producing MCs are known from freshwaters, including lakes and reservoirs used for drinking-water production or for recreation, but also from marine environments. Therefore, exposure to MCs is primarily through contact with contaminated water, through drinking the water, by dermal contact, accidental ingestion during recreational activities, or ingestion of MC-contaminated fish and shellfish. Further possible exposure pathways include haemodialysis and vascular infusions when inappropriate water is used for such purposes (e.g. from contaminated surface waters) as well as the use of cyanobacterial preparations sold as health foods or dietary supplements.

3.0 ENVIRONMENTAL LEVELS AND HUMAN EXPOSURE

3.1 Air
MCs are not volatile and so exposure via inhalation would require their dissolution in aerosols. It could also occur through cyanobacterial cells carried in spray, e.g. during storms or in the wake of a power boat. Estimates of air concentrations of MCs above a lake in a situation with high concentrations, i.e. >120,000 cells/ml of *Microcystis* sp. (up to 2140 µg/L total MCs, 0.7-45% of which was extracellular) were only in the low pg/m$^3$ range (Wood & Dietrich, 2011).

3.2 Food

MCs – as other cyanobacterial toxins – have been found in some foods such as algal dietary supplements and fish or seafood (mussels, shrimp, clams, crabs, etc.) taken from waters contaminated with cyanobacterial blooms. Many publications on MC contents of matrices other than water lack adequate validation and may not be reliable.

Data from publications with robust analytical methods suggest that MC contents in fish muscle are largely below 100 µg/kg fresh weight, whereas higher values were reported from fish liver or whole fish. Similar contents have been detected in molluscs and crustaceans (for more details see Testai et al., 2016). Therefore, exposure to MCs from ingestion of contaminated seafood will vary greatly depending upon local circumstances.

Bioaccumulation (uptake of the toxins from the water or cyanobacteria) occurs in the food web, but there is no strong evidence for biomagnification (uptake and concentration in organisms at higher trophic levels). Hence, highest MC contents are expected in animals that recently fed on cyanobacteria.

In temperate regions, MCs are most likely to be seasonally present and year-round exposure is unlikely. However, greater risks are possible for communities in tropical locations with persistent cyanobacterial blooms, particularly communities who rely on local fish for subsistence and frequently consume the entire fish, particularly as MCs are resistant to boiling. Any guidance for MC contamination of foods is therefore best based on local data, as available, on the frequency and magnitude of contamination as well as local habits of the consumption of local fish and mussels. An example of such an approach can be found in Mulvenna et al. (2012).

Accumulation of MCs in plants and vegetables has been reported following irrigation with surface water with cyanobacterial blooms. Although most of the available data refer to experiments irrigating with extreme concentrations of cells and/or toxins and may be therefore poorly representative of the majority of scenarios likely to be realistic, these studies do indicate that accumulation can occur. Moreover, cyanobacterial cells containing MCs can adhere to surfaces of leafy crops such as lettuce. Hence, where irrigation with bloom-infested surface waters or use of cyanobacterial scum as fertilizer is a common practice in agriculture, agricultural produce is an additional potential source of exposure to consider when estimating summary health risks caused by MCs.

In a number of samples of commercial cyanobacterial dietary supplement products MCs were detected in contents above 1 µg/g. However, the proportion of positive samples as well as the maximum reported MC contents vary widely between studies, most likely reflecting the variable composition of natural cyanobacterial blooms from which the raw material for products is harvested. Consumption of MC-contaminated dietary supplements, due to the amount of ingestion (ranging up to 20 g/person day$^{-1}$), can become the major exposure source to MCs.
MCs were not found in the milk of dairy cattle that were exposed to *Microcystis aeruginosa* via drinking-water, albeit most of the MCs to which the cattle were exposed were quite hydrophilic.

For more details on MC in food and dietary supplements see TCiW (Dietrich, in press; Ibelings & Chorus, in press).

### 3.3 Water

In many settings the primary water-borne route of human exposure to MCs will be the consumption of drinking-water, where it is produced from surface waters with insufficiently effective or non-existent treatment. A further exposure route – important in some settings – is the recreational use of lakes and rivers. Depending on the seasonal patterns of cyanobacterial blooms and water body use, patterns of exposure may be episodic, and recreational exposure more often be associated with acute intoxication risks.

MCs are the most commonly reported cyanotoxin, with reports from surface waters demonstrating a worldwide occurrence. Concentrations of MCs in surface waters largely coincide with the abundance of potentially toxigenic cyanobacterial species. In the environment, MCs are mostly confined to viable cells, i.e., intracellular, but they can be released upon cell lysis that is induced, for example, by adverse growth conditions, phage activity or algicidal treatment. Extracellular MCs (either dissolved in water or bound to other materials) typically make up less than 10% of the total MC concentration in raw water but higher shares may occur occasionally.

Reported *in situ* concentrations of total MCs (extracellular plus intracellular) in raw water vary widely, from non-detectable or less than 0.1 µg MCs/L to more than 100 mg MCs/L. However, on average – e.g. in integrated water samples the MC concentration rarely exceeds 10-20 µg MCs/L and often ranges in the low µg/L. Some toxigenic cyanobacteria can form surface blooms through buoyancy regulation, and such blooms can further accumulate by wind-driven dislocation to form massive scums. Depending on actual weather conditions and water body morphology, concentration gradients over depths and area may be very steep and often change rapidly, i.e. within less than one hour. Elevated MC concentrations are most likely at downwind, near-shore sites with massive scums of *Microcystis* sp. Therefore, for health risk assessment, the actual site of water use (raw water abstraction, bathing site, etc.) needs to be considered while averaged MC concentrations may underestimate the risk at respective sites. Blooms of *Planktothrix agardhii* occur generally in well-mixed, shallow water bodies and only rarely form light surface blooms generally resulting in more spatially homogeneous MC concentrations. Blooms of other planktonic MC-producing cyanobacteria (e.g., *Dolichospermum*) show an intermediate tendency to from surface blooms.

A particular pattern of spatial gradients of MC concentrations is given by *Planktothrix rubescens*, a species that can accumulate in the metalimnion (i.e. between the warm upper and cold deeper layer) of deep, thermally stratified mesotrophic lakes. Therefore, this species does not cause maximum MCs at the surface (although occasionally *P. rubescens* forms surface blooms) but rather in a narrow layer at some metres depth, sometimes coinciding with depths of drinking-water offtakes in reservoirs.
High, lake-wide concentrations of extracellular MCs are most likely following algicide treatment of dense cyanobacterial blooms, with up to 1,800 μg/L reported. Massive release of MCs following natural lysis, for example through phages or heat-stress, has not frequently been reported and tends to be locally confined to sites of scum formation. MCs are moderately resistant to chemical and microbial breakdown in situ, the latter often setting in only after a lag phase followed by a rapid degradation. Therefore, it is important to avoid cell lysis during drinking-water production (see below). A number of taxonomically diverse heterotrophic bacteria has been isolated that are capable of degrading MCs.

While surveys investigating MC occurrence in finished drinking-water have mostly found levels below 1.0 μg/L, a number of samples contained higher concentrations of up to 12.5 μg MCs/L. Such findings are likely due to bloom situations in conjunction with inadequate treatment processes. For example, in August 2014, the city of Toledo, Ohio, USA, was required by the State of Ohio to issue a “do not drink or boil advisory” to nearly 500,000 customers in response to the presence of total MCs in the finished drinking-water at levels briefly exceeding 1 μg/L and up to 2.5 μg MCs/L in some samples due to a bloom near the drinking-water intake located within Lake Erie. The advisory was lifted two days later after water treatment adjustments reduced cyanotoxin concentrations to non-detect in most samples and below 0.5 μg/L in the remaining few samples.

Recreational activity in surface waters with cyanobacterial blooms can cause exposure to MCs (and other toxins in blooms and scums) primarily through unintentional swallowing of water. Recreational activity typically takes place at near-shore sites where surface blooms or scums accumulate, sometimes leading to extremely high concentrations of MCs, so that even small volumes of ingested water can contain MCs in the low mg range. Inhalational exposure may be a relevant pathway for specific recreational activities, such as water skiing or jet skiing, and for specific occupational situations involving spray with water containing bloom material, such as spray irrigation or dust suppression. Therefore, recreational activity and in some cases occupational activity may be a potentially substantial exposure route, albeit in most cases over a limited time span.

For more details on MC occurrence in the environment and drinking-water see TCiW (Ibelings et al., in press; Fastner & Humpage, in press).

3.4 Estimated total exposure and relative contribution of drinking-water

Drinking-water is the most likely source of exposure to MCs where surface water sources are used. However, this assumption serves as a starting point only: country- or region-specific circumstances should consider the contribution of potentially contaminated foods, which in certain settings may be a significant source of exposure. Likewise, recreational activities in lakes with cyanobacterial blooms may expose individuals intermittently to high concentrations of MCs. Significant dermal or inhalational uptake of MCs during recreational exposure at present appears unlikely for most situations, possibly with the exception of substantial inhalation of aerosol during activities such as jet skiing or water skiing. Spray irrigation water containing bloom material may also be a source of dermal or inhalation exposure. For most situations for the general population this is likely to leave the oral route as the main route of concern.

For specific population groups, the parenteral route of exposure may also occur, with risks potentially being high if water from contaminated surface waters is used for haemodialysis.
and risks also being possible where it is used for infusions; haemodialysis was the major source of exposure (including lethal) documented in the cases discussed in TCiW (Azevedo, in press). Further as noted in section 3.2, contaminated dietary supplements can also become a major exposure source to MCs. However, due to the varying degrees of MC contamination and lack of information on extent of use of supplements, it is difficult to estimate the actual contribution of these dietary supplements in an overall health risk context.

Exposure patterns and durations are strongly influenced by region and lifestyle. Estimating total exposure or the relative contribution of particular exposure sources (e.g., food, drinking-water) requires specific analyses of MC concentrations in samples from the respective media. Chapter 5 of TCiW gives further guidance and background information on assessing routes of exposure.

4.0 KINETICS AND METABOLISM IN LABORATORY ANIMALS AND HUMANS

4.1 Absorption

The dose and route of exposure play crucial roles in MC absorption. Due to the lipid-water partition coefficient of many MC variants, oral cavity and sublingual absorption would not be expected. However, this has not been investigated. Once in the gastrointestinal tract, several factors impact MC absorption, such as disruption of intestinal epithelium and transport mechanisms.

Oral gavage administration of MC-LR in mice at a high dose (500 μg/kg bw) suggested that the stomach absorbed the toxin to some degree, with primary absorption through the villi of the small intestine (Ito et al. 2000). MC-LR absorption into the systemic circulation may have been aided by villi erosion at this high dose. Parenteral administration (e.g. intraperitoneal) bypasses the digestive tract. However, intraperitoneal administration of MC-LR to mice also resulted in damage to intestinal villi and cells (Botha et al. 2004).

Specific mechanisms of MC transport across intestinal membranes to the circulatory system have been described in various cell types, although not fully elucidated. Available data indicate that the Organic Acid Transporter Polypeptide (OATP) family of receptors facilitates the absorption of toxins, including MCs, from the intestinal tract into liver, brain, and other tissues (Falconer et al., 1992; Cheng et al., 2005; Fischer et al., 2005; Fischer et al., 2010; Svoboda et al., 2011). However, the specific OATP(s) responsible for intestinal absorption have yet to be identified. Variability in OATP expression due to genetic polymorphism, regulation and age-related expression may be important factors in absorption and distribution of MCs, but are poorly understood at present (Alam et al., 2018). Scant data are available about efflux proteins involved in MC transport. Intestinal absorption/permeability studies involving Caco-2 cells support facilitated uptake of MC-LR (Zeller et al., 2011, Henri et al. (2014) reported that in Caco-2 cell monolayers, MC-LR (1-10-75 μM) was rapidly taken into cells from the apical (intestinal lumen) side, but after 30-45 min, the majority of toxin was re-excreted back into the apical compartment, likely by efflux proteins, with only 0.3 to 1.35% of the toxin reaching the basolateral compartment over 24h.

MCs are not volatile and are not likely to be present in gaseous form in air at ambient temperatures. However, they can be present in very low concentrations in aerosols generated by wind and during showering or swimming, thereby contacting the respiratory epithelium. Pulmonary absorption of MC-LR (purified from a cyanobacterial bloom sample) was
demonstrated by intratracheal instillation of a sublethal dose of 50 μg/kg bw or a lethal dose of 100 μg/kg bw in mice (Ito et al., 2001). Immunostaining of the lung for MCs was evident within 5 minutes. A lag period of 60 minutes occurred after the lethal dose and 7 hours after the sublethal dose before staining was observed in the liver. These data suggest that uptake from the lungs into systemic circulation can occur.

No dermal absorption studies for MCs were identified.

4.2 Distribution

Once absorbed and in the circulatory system, MC distribution to tissues and other body fluids is dependent on many factors, including, but not limited to, OATP expression, degree of blood perfusion and congener chemistry.

The expression of OATPs, especially OATP1B1 and OATP1B3, on the basolateral (sinusoidal) side of hepatocytes, allows for the considerable uptake of MCs by this organ (Fischer et al., 2005, 2010). OATP1A2 has been identified as the responsible MC transporter at the blood-brain barrier (Fischer et al., 2005), but is also heavily expressed in human kidney (Lee et al. 2005). Competition with other substrates, such as bile acids, has been shown to limit transport (Thompson & Pace, 1992; Fischer et al 2010). Studies (in vivo and in vitro) inhibiting specific OATPs have shown reduction or elimination of MC-induced liver toxicity, further supporting their role in MC active transport (Komatsu et al., 2007; Lu et al., 2008; Feurstein et al., 2010; Fischer et al., 2010; Jasionek et al., 2010; Teneva et al., 2016). Transport is congener-specific, as indicated by studies with human hepatocytes, with much greater uptake of MC-LW and MC-LF than of MC-LR and even lower uptake of MC-RR (Fischer et al., 2010). These authors also demonstrated a 5-10-fold difference in sensitivity of primary human hepatocytes from two different donors for MC-LR and MC-RR, but not for MC-LW and MC-LF. Genetic polymorphism, regulation and variability in OATP gene expression may further affect distribution (Alam et al., 2018, Lee et al. 2005).

Understanding of MC distribution in vivo has been derived primarily from studies utilizing radiolabeled MCs. Therefore, it should be noted that the site of MC radiolabeling may impact distribution profiles. Indeed, [3H]-labeling at the Mdha7 residue alters the ability of MCs to bind covalently with protein phosphatases and other thiols (e.g. glutathione) (Hilborn et al., 2007; Miles et al. 2016).

Organ distribution studies after a single gavage administration of [3H]dihydroMC-LR in female ICR mice showed most of the dose was detected (6 h) in the contents of the gastrointestinal tract (38%) and liver (0.7%), followed by small intestine, large intestine, cecum, kidney, stomach and brain (Nishiwaki et al., 1994).

Studies addressing distribution of unlabeled MC after oral administration were conducted with mice and pigs. Following oral gavage administration of MC-LR to BALB/C and ICR mice at a high dose (500 μg/kg bw up to 6 times over 13 weeks), immunostaining showed that MC-LR was located primarily in the villi of the small intestine in both the surface epithelial cells and the lamina propria. Immunostaining was also present in the blood plasma, and the tissues of the liver > lungs > kidneys, with greater staining in those animals that died. Staining in the heart, pancreas and spleen was only seen in the blood plasma within those tissues (Ito et al. 2000).
Male Duroc pigs (n=6) were dosed daily by gavage with 2 µg MC-LR/kg bw for 35 d, and serum samples were collected weekly. MC-LR and MC-LR-GSH in methanolic extracts as well as total Adda by the 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) method were measured in blood and tissues (Greer et al. 2018). MC-LR, GSH conjugate and Adda fragment ion were not detected in the serum of any animal at any time. The livers of 3/6 pigs contained MMPB-detectable MC-LR (26.4 µg MC-LR per liver, about 1.1% of the total administered dose) (Greer et al. 2018). However, some limitations in the analytical detection methods in tissues, and the presence of lasalocid A (Welten et al., 2019), an OATP inhibitor potentially interfering with MC-LR uptake and limits the weight of the paper, especially regarding quantitative aspects.

In one study investigating canine intoxication following oral exposure to a bloom of Microcystis, total MCs was determined 2 days after exposure to be highest in urine, followed by bile, kidney, liver and lastly blood in a deceased canine (Foss et al. 2019).

Most studies of MC distribution have used non-oral routes of exposure. Hepatic accumulation of [3H]dihydroMC-LR in female ICR mice was observed following a single i.p. administration, with 72% of the total dose observed in the liver after 1 h (Nishiwaki et al., 1994). However, hepatic content of [3H]-dihydroMC-LR, as percent of administered dose, was about 80-fold lower after oral administration, with most of the radioactivity remaining in the gastrointestinal tract (Nishiwaki et al., 1994). After i.v. injection of 35 µg MC-LR/kg bw in male VAF/plus CD-1 mice 67% of the dose of [3H]-dihydroMC-LR was detected in the liver, with a biphasic plasma clearance (t½ 1/2 = 0.8 and 6.9 min, respectively) (Robinson et al., 1991).

The biphasic plasma clearance rates (t½ = 0.8 and 6.9 min) coupled with rapid uptake in the liver (t½ = 6.8 min) suggest the hepatic tissue accumulation was a major factor in plasma clearance (Robinson et al. 1991). Partition coefficients (30 min) of 206, 4 and 11 for liver, kidney and intestine, respectively, calculated for mice support MC accumulation in other organs beyond the liver (Robinson et al., 1991).

[3H]dihydroMC-LR i.v. administration in swine also resulted in rapid biphasic blood clearance at two doses (25 and 75 µg/kg bw), with slower clearance observed for the higher dose (t½ = 3 and 269 min) than the lower dose (t½ = 3.7 and 134 min) (Stotts et al. 1997a). Hepatic damage at the higher dose rate may have resulted in slower clearance, with interspecies differences likely accounting for the longer swine β-phase t½. When swine were dosed with [3H]dihydroMC-LR via ileal loop (75 µg/kg bw), toxin concentrations were higher in the portal circulation than in peripheral blood, indicating first-pass effect. In all swine dosing groups, liver uptake (4 – 5 h) predominated, at 47 – 65% of the total dose. Distribution to swine kidney, lungs, heart, ileum and spleen was also observed (Stotts et al. 1997b).

One study investigated distribution after i.v. administration in Wistar rats of crude extract of MCs (mixture of MC-LR and MC-RR) at approximately 80 µg/kg using unlabeled MCs (Wang et al. 2008, confirming that unmetabolized MC-LR and MC-RR were detected in kidney>lung>stomach>liver (Wang et al. 2008). However, this study, as many using unlabeled MCs, only analyzed for non-metabolized MC-LR and MC-RR, limiting interpretations.

Available results indicate that the route of administration (parenteral vs oral) greatly influences MC kinetics and therefore the internal dose concentration at the target organ (Buratti et al., 2017), warranting further investigation. The available data indicate a much lower uptake, as a
percent of dose, into the liver after oral exposure when compared to that observed after i.p. and i.v. administrations, indicating that intestinal uptake is slow and limits systemic exposure.

Overall, it can be summarised that, following uptake from the gut and entry into the portal vein, MCs are taken up by the liver, which rapidly removes the toxin from the blood due to the first pass clearance. However, MCs are also partially distributed to organs beyond the liver. Clearance from the blood is slower at higher dose levels, presumably due to the liver toxicity and circulatory shock observed at high doses (Health Canada, 2018). Additional work is required to further expand the knowledge on MC kinetics to real world exposure scenarios to elucidate mechanisms responsible for the transport both pre- and post- metabolism using appropriate analytical techniques.

4.3 Metabolism

Regarding MC biotransformation, the major reaction is conjugation with thiolic compounds, glutathione (γ-glutamyl-cysteinyl-glycine, GSH) and cysteine (Cys), which has been described both in vitro and in vivo. However, other MC metabolites have been observed in vivo, which were attributed to possible epoxidation of the Adda, but this has not been confirmed (Kondo et al. 1996).

Conjugation with Cys and GSH is considered a detoxication reaction, since MC-LR and MC-YR conjugates exhibit less toxicity than the parent, with 2- to 17-fold higher mouse LD50 values (Kondo et al., 1992). MC-LR conjugates (GSH, Cys, γ-GluCys) have also been shown to be weaker inhibitors of PPI and PP2A in vitro (PPase inhibition is considered the molecular initiating event), suggesting that at least some of the reduced potency is due to reduced affinity for the PPase active site (Metcalf et al. 2000). In addition, in vivo treatment of rats with an inhibitor of GSH synthesis together with i.p. injection of MC-LR resulted in toxin accumulation and higher hepatotoxicity (Li et al., 2015a), likely due to decreased conjugation.

The α,β-unsaturated carbonyl group (Mdha7/Dha7) is considered to be the main site for thiol conjugation (Kondo et al. 1992). MCs have been shown to be conjugated with physiological thiols in vitro (Kondo et al. 1992, Foss et al. 2018), as well as with mercaptoethanol used for derivatization prior to analytical detection (Miles et al. 2012, 2013). Metabolic GSH and Cys conjugation was confirmed in vivo (Kondo et al. 1996, He et al. 2012, Li et al. 2018). MC-Cys and γ-GluCys can be considered as breakdown metabolites of MC-GSH conjugate formation (Li et al. 2018), but MC-Cys may also be a proteolysis product of PP bound MCs (Smith et al. 2010).

GSH conjugation largely occurs via a spontaneous reaction in vivo and in physiological conditions in vitro, but is also catalyzed by a range of glutathione S-transferase (GST) isoforms (Buratti et al., 2011). Human recombinant GSTs, with GST-T1 showing the highest clearance, and liver cytosol have been shown to catalyze conjugation of GSH with MC-LR, MC-RR, MC-LW, MC-YR (Buratti et al., 2011, 2013, Santori et al., 2020), while MC-LF is very poorly conjugated. For all the tested variants, the non-enzymatic reaction predominated in normal cell conditions in vitro with a rate that is approximately 10 times higher than the enzymatic reaction (Santori et al, 2020). The GSH conjugation efficiency is congener-dependent: the total in vitro detoxication reaction (spontaneous plus enzymatic) favours variants exhibiting higher hydrophilicity, with MC-RR being conjugated at the highest rate. A differential rate of
conjugation in response to MC Mda\textsuperscript{7} moiety chemistry was also observed using mercaptoethanol as a model thiol: the kinetics of conjugation with Mda\textsuperscript{7}/Dha\textsuperscript{7} has been shown to be much faster ($t_\frac{1}{2} = 0.21 – 0.84$ h) than conjugation with Dhb\textsuperscript{7} ($t_\frac{1}{2} \geq 50$ h), which has been applied analytically to distinguish these variants (Miles et al. 2012, Miles et al. 2013).

When GSH depletion occurred, the enzymatic reaction became predominant with MC-LR and MC-RR, but not with MC-YR and MC-LW (Buratti & Testai, 2015, Santori et al, 2020). Species differences were also noted, with higher enzymatic conjugation occurring with rats/mice than with human cytosol with MC-LR and MC-RR (Buratti & Testai, 2015).

Conjugation with thiols has been shown to be reversible under a variety of \textit{in vitro} conditions (Miles et al. 2016) and \textit{in vivo} with bighead carp and rats (Li W et al. 2014, Li et al. 2018). Injection of MC-RR-GSH in rats resulted in the rapid formation of MC-RR-Cys, but also deconjugation to MC-RR (Li et al. 2018). Higher deconjugation (to MC-RR) from MC-RR-GSH has been observed when compared to MC-RR-Cys conjugates (Li W et al. 2014).

Although indicative of reversibility, the quantitative data on MC detection in matrices other than water have to be considered with caution: methods can be prone to matrix effects, resulting in inaccuracies due to ion enhancement or suppression (Testai et al. 2016), especially in electrospray ionization (ESI) mode, as used by the authors of the two \textit{in vivo} publications.

The site of MC metabolism is largely dictated by route of exposure, distribution and dose. Since conjugation with GSH does not necessarily require enzymatic catalysis, cells that take up MCs can form MC-GSH until depletion. GSH concentration is highest within hepatocytes, and following oral exposure, there is a high first pass clearance, hence the liver is a major site of metabolism. The liver is also the primary site of metabolism after i.p. injection, although this route of exposure is not representative of drinking-water exposure and shows relevant kinetic differences when compared to the oral route.

### 4.4 Elimination

Primary elimination of both parent and metabolized MCs has been described to occur by the urinary and faecal routes. Secondary elimination through hair follicles (Foss et al. 2019), and bird feathers (Metcalf et al. 2006) has been proposed, but other pathways (e.g. perspiration, salivation, lactation) have not been studied.

The oral bioavailability of the MCs is limited due to poor passive membrane permeability. Excretion of unabsorbed parent MCs into the faeces would be expected after oral exposure. In support of this, a faecal sample collected 8 days post exposure of a canine exposed to a \textit{Microcystis} bloom contained intact, non-metabolized MC-LA (Rankin et al. 2013). However, the unmetabolized MCs may have been excreted by the liver to bile. Multidrug resistance-associated protein 2 (MRP2) has been shown to transport MC-LF > MC-LR > MC-RR, but not MC-LR-GSH (Kaur et al 2019), providing one mechanism for hepatic excretion of unmetabolized MCs. Once excreted from the liver, MCs may be available for reabsorption and elimination via urine or with faeces.

Results indicated not only a MC congener and conjugate specific export of MCs from cells but more importantly, major differences in affinity and capacity of the various exporters for MCs and their conjugates. Similar to the case with OATP1B1 and 1B3, MC-LF had higher affinity for MRP2 than either MC-LR or MC-RR. Hence, although MC-LF may be taken up more
rapidly than the other congeners by hepatocytes, it may also be re-exported to the bile canaliculus faster as well (Kaur et al. 2019).

Faecal elimination has been observed in radiolabelled MC studies. Intravascular administration of $[^3]$HdihydroMC-LR in mice showed that 24% of the administered dose was excreted over a 6-day period, 9% in urine and 15% in faeces (Robinson et al., 1991), supporting the importance of the biliary route of excretion. The majority of the excreted compound (> 60%) was the parent compound, indicating that some excretion was of metabolized $[^3]$HdihydroMC-LR despite the site of thiol conjugation being reduced. Similarly, in isolated perfused rat liver, 1.7% of $[^3]$HdihydroMC-LR was recovered in the bile by the end of the 60-minute perfusion (Pace et al., 1991). In the bile collected during the perfusion, 78% of the radiolabel was associated with the parent toxin, whereas the remaining radiolabel was associated with more polar metabolites. Specific metabolite identities were not elucidated in these radiolabelled MC studies.

Following oral gavage of MC-LR, immunostaining techniques showed excretion of MC-LR in the mucous from goblet cells in both the small intestine and large intestine. The kidneys were also stained, indicating a renal route of elimination (Ito et al. 2000). Following the i.v. administration of a crude extract to Wistar rats, MC-RR and MC-LR concentrations were consistently higher in the kidney than liver throughout the experiment, indicating excretion of unmetabolized MCs through the kidney (Wang et al. 2008). Unmetabolized MC-LR was also observed (11 – 33 ng/mL) in mouse urine 5 h post i.p. administration of MC-LR (40 µg/kg bw). However, evidence suggests that the bulk of MCs excreted in the urine is metabolized. Urinalysis of dogs 1-2 days post accidental exposure to a Microcystis bloom, 0.34 – 1.63% of total Adda MCs (via the MMPB technique) was unmetabolized MC-LR, the dominant congener detected in the source water (Foss et al. 2019). Continued elimination following the event resulted in MCs still detectable (via MMPB) in urine 10 – 68 days post exposure in 3 surviving dogs. Bile contained high MCs upon mortality of one canine (5,400 ng/mL), but levels were an order of magnitude higher in urine (41,000 ng/mL), supporting the renal route playing a significant role in MC elimination for canines.

GSH and Cys conjugates of MC-LR were primarily distributed to the kidney and intestine following intratracheal instillation in mice (Ito et al., 2001), demonstrating that MC conjugates are excreted from the hepatocytes via the sinusoidal as well as the biliary side; this suggests that the lower toxicity of GSH and Cys conjugates may be also related to their distribution to excretory organs and elimination of metabolites in vivo.

Li et al. (2018) investigated the excretion patterns of i.p. injected MC-RR-GSH (0.55 µmol/kg bw) in male Sprague-Dawley rats. LC-MS/MS analysis of urine and faeces indicated that excretion was evident by the first time point (0.25 h) in both excreta and reached a peak 2 h after dosing, sharply dropping and levelling off at 6 h for constant excretion thereafter. In urine, MC-RR-Cys was the major form seen, followed by MC-RR-GSH and MC-RR, respectively. In faeces, MC-RR was the predominant form. Overall, excretion of all forms was significantly higher via urine than in the faeces.

Limited information on the elimination of MCs from the human body is available from follow-up of dialysis patients exposed to MCs in dialysis water. The total clearance seems to be a long-lasting process since more than 50 days after exposure MCs were still detected in patients’ serum by ELISA (Soares et al., 2006; Hilborn et al., 2007).

5.0 EFFECTS ON HUMANS
Cyanobacteria have been reported to cause animal and human poisoning when present in lakes, ponds, and dugouts in various parts of the world for over 100 years. It is unclear in most instances which cyanotoxin (if any) was potentially involved because they had not been characterised at the time of the incident, were not analysed for, or multiple cyanotoxins were present. Nevertheless, given our current understanding of the widespread occurrence of MC-producing cyanobacteria, it seems likely that MCs would have been present in many of the reported cases. Cyanobacterial blooms tend to occur repeatedly in the same water supply. Therefore, depending on the level of water treatment available, some human populations are at risk of repeated ingestion of cyanobacterial toxins, particularly MCs. However, many of the reported symptoms in historical reports are quite general and cannot be considered in isolation as diagnostic of MC poisoning. Effects reported in humans include gastrointestinal illness, hepatic toxicity and pneumonia. However, gastrointestinal and respiratory symptoms may well be due to other less well described cyanobacterial metabolites or to bloom-associated microorganisms or their metabolites. Furthermore, where blooms were treated with copper sulphate, high copper concentrations may be an explanation for symptoms such as diarrhoea, vomiting, stomach cramps and nausea; however, this would require concentrations above the range of 1-2 mg/L at which it is used as an algicide (see WHO, 2011 for a discussion of copper toxicity and derivation of respective guideline values).

5.1 Case Studies

Drinking-water

Consumption of drinking-water originating from surface raw water infested by cyanobacterial blooms producing MCs and not properly treated gave rise to some outbreaks in the late past century (Funari & Testai 2008; USEPA, 2015). Symptoms range from gastroenteritis, abdominal pain, flu-like symptoms, irritation and rashes, to kidney and liver damage. In Harare, Zimbabwe (published as Salisbury, Rhodesia at the time), seasonal acute childhood gastroenteritis during the years 1960–1965 was linked to annual blooms of Microcystis in the lake serving as the water supply. An adjacent water supply was not similarly affected and was not associated with this disease (Zilberg, 1966).

An outbreak with a high death rate attributed to cyanobacterial toxins in drinking-water occurred in the Paulo Alfonso region of Bahia State in Brazil followed the flooding of the newly constructed Itaparica Dam reservoir in 1988. Some 2,000 gastro-enteritis cases were reported over a 42-day period, and 88 deaths, mostly children, occurred (Teixera et al., 1993). Blood and faecal specimens from gastroenteritis patients were subjected to bacteriological, virological and toxicological testing, and drinking-water samples were examined for micro-organisms and heavy metals. No infectious agent was identified, and cases occurred in patients who had been drinking only boiled water. The cases were restricted to areas supplied with drinking-water from the dam. Clinical data and water sample tests were reviewed, and it was concluded that the source of the outbreak was water from the dam and that a toxin produced by cyanobacteria present in the water (Anabaena and Microcystis in high densities) was the most likely responsible agent, although it could not be identified.

Possible liver damage, as evidenced by significant increases in GGT, was seen in persons drinking water supplied from sources containing blooms of Microcystis after treatment with copper sulfate (Malpas Dam, Armidale, Australia) compared with persons drinking water from another supply (Falconer et al., 1983). MC-YM had been identified in these blooms.
Recreational Exposure

Exposure during recreational activity can be associated with three different routes: oral, due to accidental ingestion of water contaminated with algal cells, dermal, due to the direct contact, and inhalation, providing MCs are aerosolized or cyanobacterial cells contained in spray. Inhalation of MC-containing aerosols (range 0.052-2.89 ng MCs/m³) in 81 individuals resulted in MC detection in the nasal swabs but not in plasma (LOD:1 µg/L) (Backer et al., 2008, 2010). A special case may result from intranasal or intratracheal exposure during full immersion water activities. Animal studies employing these routes of exposure resulted in both localised tissue damage and liver toxicity similar to i.p. dosing (Ito et al., 2001; Fitzgeorge et al., 1994, see section 6.1 for more details). Pneumonia or severe respiratory distress was described in several case studies in which full immersion occurred (Pearson et al. 1990; Giannuzzi et al. 2011, see below). However, it is not possible to conclude from current knowledge whether these symptoms were MC-related or due to other compounds or cells in the water.

In Saskatchewan, Canada, 10 children became sick with diarrhoea after swimming in a lake covered with cyanobacteria. Anabaena cells were found in the stools of one child (Dillenbergh & Dehnel, 1960). Anabaena (Dolichospernum) has since been shown to be a major MC producer in Canada (Winter et al., 2011). In the United Kingdom, 10 of 18 army recruits on a military exercise in a reservoir with a bloom of M. aeruginosa suffered abdominal pain, nausea, vomiting, diarrhoea, sore throat, dry cough, blistering at the mouth, and headache. Two were hospitalized and developed an atypical pneumonia. Serum enzymes, indicative of liver damage, were elevated. Microcystin-LR was identified in the bloom material (Pearson et al., 1990). However, high levels of Escherichia coli were also found in reservoir water two weeks later. The authors suggested that exposure to MCs may have had a role in some of the clinical symptoms.

Acute intoxication with a MC-producing cyanobacterial bloom in recreational water was reported in Argentina in 2007 (Giannuzzi et al., 2011). A 19 year old male was immersed in a Microcystis bloom for at least 2 hours. A concentration of 48.6 µg/L (total MCs, i.e. cell-bound and dissolved) was detected in the water within 4 hours of exposure. A few hours after exposure, the patient exhibited fever, nausea, and abdominal pain, and 4 days later, he was admitted to a medical centre. He was found initially to have hypoxaemia and renal failure, along with reduced platelet count and increased leukocytes, but within 3 days of admission developed signs of liver injury (increased AST, ALT and GGT but normal bilirubin and ALP). Tests for HIV, Epstein-Barr virus, Clamidia pneumoniae and Mycoplasma were negative. The patient recovered completely within 20 days.

In Montevideo, Uruguay, in 20-month old child was hospitalised following repeated recreational exposure to Microcystis blooms containing up to 8,200 µg/L MCs (Vidal et al. 2017). Faecal coliforms were below the local health alert limit of 1,000 cfu/dL. Three adult family members reported diarrhoea that was self-limiting but the child continued to have diarrhoea and vomiting, and over 5 days developed fatigue and jaundice before being admitted to intensive care. Serum ALT, AST and bilirubin were elevated. The patient tested negative for hepatitis A, B, C viruses, Epstein-Barr virus and cytomegalovirus. The initial diagnosis was autoimmune hepatitis type II but the patient failed to respond to immunosuppressants (metilprednisolone and cyclosporin). A liver transplant was performed 20 days after admission following which the child recovered. Histopathological examination of the excised liver showed extensive hepatocellular damage, haemorrhage and nodular regeneration without
inflammation. A methanolic extract of a 20 g sample of the liver was found to contain 2.4 ng/g MC-LR and 75.4 ng/g of [D-Leu]$^4$MC-LR.

Numerous reports of skin irritations and local effects associated with recreational contact with MC-producing cyanobacteria in lakes or freshwater basins are available, but no correlation was found between the MC levels and the local or allergic reaction. On the basis of results obtained in animals with pure toxins and extracts, it seems that dermal effects in humans (including skin sensitization and allergies) are associated with exposure to cyanobacterial cell components other than the known cyanotoxins. This was confirmed in a human study with 259 chronic rhinitis patients tested with nontoxic cyanobacterial extract, showing that cyanobacterial allergenicity (IgE response in a skin-prick test) resides in non-toxin-containing components of this organism (Bernstein et al., 2011). Further testing, with sera from patients and cyanobacterial extracts from toxic and non-toxic strains, identified phycobiliprotein complexes in *M. aeruginosa* as the sensitizing agent (Geh et al., 2015). These findings suggest that the relevant metric for the dermal effects is not the MC concentration (dissolved or total), but rather biovolume or cell surface area, as proposed in some epidemiological studies (Stewart et al., 2006), but not in others (Pilotto et al., 1997).

A Canadian prospective case–control study found a correlation of several gastrointestinal symptoms (e.g., diarrhea or abdominal pain, nausea or vomiting) with the presence of cyanobacterial cells in a lake affected by MC-producing blooms (Lévesque et al., 2014). Similarly, health surveillance data from the US have also linked outbreaks of illness (such as rash, skin and eye irritation, gastrointestinal and respiratory symptoms) in water users with the presence of toxic cyanobacteria and their toxins, including MCs, in recreational freshwaters (Hilborn et al. 2014; Backer et al. 2015; Trevino-Garrison et al. 2015). However, association does not equate to cause and many other etiological agents could be posited to be responsible for some or all of these effects. For example, a role of the bacterial community associated with cyanobacteria, particularly *Aeromonas* strains, in causing the gastrointestinal symptoms reported has been suggested (Berg et al., 2009).

**Dialysis**

In 1996 in Caruaru, Brazil, an outbreak of acute liver failure occurred in a dialysis clinic where dialysis water was contaminated with MCs and possibly cylindrospermopsin. Of the 130 patients who were treated at that time, 116 reported symptoms of headache, eye pain, blurred vision, nausea and vomiting. One hundred of the affected patients developed acute liver failure and, of these, 76 died (Carmichael et al., 2001; Jochimsen et al., 1998). The serum methanol extractable or “free”-MC concentrations ranged from <0.16 to 28.8 ng/mL (median 1.53 ng/mL, Hilborn et al., 2007). The authors estimated total (free plus protein bound) MCs in a subset of samples, finding a median of 52.8 ng/mL and a maximum of 112.9 ng/mL. From these data, the authors estimated that free MCs amounted to between 8% and 51% of the total MC burden (Hilborn et al., 2007). Liver samples from 39 patients who died contained an average 223 ng/g methanol-extractable MCs (Carmichael et al., 2001). It is uncertain whether conjugated MCs would have been detected by the ELISA methods employed in these studies.

A second poisoning event occurred at a dialysis centre in Rio de Janeiro, Brazil in 2001, when 44 dialysis patients were potentially exposed to MCs. During a survey of dialysis clinics an MC concentration of 0.32 μg/L was detected in the water filtered by activated carbon in an intermediate step for treating drinking-water to prepare dialysate, and so patients were recruited for follow-up (Soares et al., 2006). A concentration of 0.4 μg/L MCs was detected in the
drinking-water supply prior to onsite treatment. Serum samples were collected from 12 dialysis patients for eight weeks. MCs in the serum ranged from 0.46 to 0.96 ng/mL (ELISA), with the highest concentrations occurring 31 to 38 days after MCs was last detected in water samples. Results were consistent with a mild to moderate liver injury (Hilborn et al., 2013). MCs were not detected in water during weekly monitoring after the first detection and so the authors concluded that the patients were not continuously exposed to the toxin, suggesting instead that the toxin detected in the serum after eight weeks may have been present in the form of bound toxin in the liver (Soares et al., 2006).

5.2 Epidemiological Studies

Although there are several epidemiological studies regarding the association of microcystin exposure via contaminated drinking-water and cancer risk, they generally provide only for weak associations. In view of the contradictory results no conclusions can yet be drawn from these epidemiologic studies (Health Canada, 2018, IARC 2010). Indeed, these studies are limited by their study design, poor measures of exposure, potential co-exposure to other contaminants, and the lack of control for confounding factors, e.g. hepatitis B infection and aflatoxin exposure (USEPA, 2015). Numerous epidemiological studies (some published in Chinese) conducted in an area of southeast China that is endemic for hepatocellular carcinoma (HCC) have been reviewed extensively elsewhere (IARC, 2010, Chen and Kensler, 2014; USEPA, 2015; Health Canada, 2018).

Having in mind the above-mentioned limitations, a positive association was found between the risk for HCC and water source from surface waters, with estimates of relative risk ranging from 1.5 to 4. Consumption of pond or ditch untreated water was associated with an 8-fold increase in liver cancer incidence when compared with well water consumption (Yu, 1995). Microcystin-producing cyanobacteria are abundant in surface waters in this area, with significant amounts of MCs detected in pond and ditch waters, whereas no detectable levels were found in deep well water; this suggests that MCs in drinking-water may have been partially responsible for the higher incidence of HCC, although many other toxic chemicals, such as but not limited to pesticides as well biological vectors, would also be expected to be similarly distributed in these water types. Based on the average MC contents of river and pond/ditch samples, Ueno et al. (1996) estimated an average daily exposure of about 0.2 µg per person during the summer months (this exposure is miscalculated in the paper as 1.9 pg/d). Later studies from China have associated slightly higher exposure rates from food and water combined (0.36 to 2.03 µg/person/day) with detectable serum MCs and increased levels of serum liver enzymes (Chen et al., 2009; Li Y et al., 2011), see below.

A later case control study in Haimen city, China, confirmed that HBV infection was the greatest risk factor for HCC, but did not find an association with the drinking-water source (Yu et al., 2002). However, this study did not analyse or control for the confounding prevalence of aflatoxin-B1 antigens. Animal studies suggest that MCs act as promoters of the aflatoxin-mediated DNA adducts to increase mutations and thus elevate rates of liver cancer (Sekijima et al., 1999; Liu et al., 2018) whereas this may not be the case for HBV-related HCC (Lian et al., 2006).

An increase in serum markers for hepatotoxicity (AST, ALP, ALT and lactate dehydrogenase, LDH) was observed in a cohort study of Chinese fishermen exposed to MC-RR, MC-YR and
MC-LR in Lake Chaohu through consumption of contaminated water and food (Chen et al., 2009). The fishermen had a median serum MC concentration of about 0.2 ng/ml and an estimated daily intake of MCs of 2.2–3.9 µg MC-LReq (Chen et al., 2009). The relative proportions of the three variants were similar in the fishermen’s blood as in the carp and duck tissues used as typical food.

Li Y et al. (2011) conducted a cross-sectional study assessing the relationship between liver damage in children (n > 1000) and MC levels in drinking-water and aquatic food (carp and duck) in China. MC levels measured in three local sources of drinking-water were classified as null controls, low and high with children in the low-exposure group consuming an estimated 0.36 µg MCs/day and high-exposure children consuming 2.03 µg MCs/day. Mean serum levels of MCs in the groups were <0.1 (LOD), 0.4 and 1.3 µg MC-LReq/L in the no, low- and high-exposure groups, with detection rates of 1.9% (1 of 54 samples), 84.2% and 91.9%, respectively. MC was associated with increases in AST and ALP, but not ALT or GGT. The odds ratio (OR) for liver damage associated with MCs was 1.72 (95% CI: 1.05–2.76), after adjustment for HBV infection and use of hepatotoxic medicines as confounding factors, suggesting a possible association of MCs with liver pathology in children.

None of these epidemiologic studies allow for causal relationships to be established between MC exposure and liver tumors.

6.0 EFFECTS ON EXPERIMENTAL ANIMALS AND IN VITRO SYSTEMS

6.1 Acute exposure

Fatalities in animals have been reported following the consumption of water containing large numbers (>10⁶/ml) of cyanobacterial cells (Stewart et al., 2008; Mancini et al., 2010; Wood et al., 2010).

Fawell et al. (1999) estimated oral LD₅ₒ values of about 5000 µg/kg bw for both mice and rats for MC-LR (commercial product; MC-LR purity and age of animals not specified). Yoshida et al. (1997) estimated an oral LD₅ₒ of 10.9 mg/kg bw for purified MC-LR (> 95% pure by HPLC) in 6-week old female BALB/c mice and hepatic effects ranged from fibrosis to focal or diffuse hepatocellular necrosis in the centrilobular and midzonal regions. Both of these studies reported toxin-related effects in some animals at the lowest dose tested.

Two studies have suggested age-related differences in sensitivity of mice to single doses of MCs (Ito et al., 1997b, Rao et al., 2005), with young animals (5-6 weeks old) showing less severe effects on histopathology, serum enzymes and time to death than aged mice (32-36 weeks old) when given the same lethal dose of toxin (i.p. and oral).

The LD₅ₒ value of MC-LR by the intraperitoneal (i.p.) route is approximately 25–150 µg/kg bw in mice (Fawell et al., 1994) with a very steep dose response and a <2-fold ratio between the no-effect concentration and one inducing severe effects, including mortality (Funari & Testai, 2008). The i.p. LD₅ₒ of several other MCs that have been determined are generally less than a factor of 2 higher than that of MC-LR, although for some (e.g., MC-RR and MC-M(O)R), the i.p. LD₅ₒ values are about 5-10-fold higher (Zurawell et al., 2005).

LD₅ₒ values for mice treated with MC-LR intratracheally (Ito et al., 2001) or intranasally (Fitzgeorge et al., 1994) were similar to those for the i.p. route (75-250 µg/kg bw). These treatments caused toxic effects at the site of exposure as well as on the liver and other organs.
similar to i.p. treatment. Following i.v. administration to mice the LD$_{50}$ value was 28 μg/kg bw (Kondo et al., 1992).

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

6.2 Short-term and subchronic exposure

Among the various short-term/subchronic toxicity studies available, only a few can be considered as key studies for the derivation of reference values, as recently reviewed by Buratti et al. (2017), since most of them have been carried out with poorly characterized extracts, administered via i.p. route (which is an exposure route of limited relevance to risk assessment of human oral exposure via drinking water), and/or by using a single dose level which precludes dose-response assessment. Some of the i.p. studies, although characterized by a much higher internal dose with respect to the oral studies, can provide information on the potential hazard for organs other than the liver, such as kidney, lung, and thyroid (see Buratti et al., 2017).

Ito et al., (2000) treated 7 BALB/C and 39 ICR aged (24 weeks and 32 weeks, respectively) male mice with single gavage doses of 500 μg/kg MC-LR after 0, 1, 6, 7, 12 and 13 weeks, i.e. 6 doses in total. Lethality was 71% in BALB/C after only the second dose whereas it was only 15% in the ICR mice after the full 13 week treatment period.

In the study considered key for risk assessment by many authorities, MC-LR was administered via gavage to 15 CR1:CD-1(ICR)BR(VAF plus) mice per sex and dose at 0, 40, 200, or 1000 μg/kg bw per day for 13 weeks. Thirty-nine organs or tissues from the control and high dose groups as well as lungs, liver and kidneys from the intermediate dose groups were examined microscopically. Blood samples from subsets of 7 animals were analysed for haematology and serum biochemistry. No treatment-related changes were noted at the lowest dose. At 200 μg/kg bw per day, there was slight liver pathology in some male and female mice. At the highest dose, all male and most female mice showed liver changes, which included chronic inflammation, focal degeneration of hepatocytes, and haemosiderin deposits. In male mice at the two highest dose levels, serum transaminases were significantly elevated, serum gamma glutamyl transferase was significantly reduced, and there were small but significant reductions in total serum protein and serum albumin. In female mice, changes in transaminases occurred only at the highest dose. Also at the highest dose, food consumption in both sexes was increased by 14 and 20%, respectively, but body weight was 7% lower in both sexes compared with control mice. The NOAEL for MC-LR was considered to be 40 μg/kg bw per day (Fawell et al., 1994; Fawell et al., 1999).

Heinze (1999) investigated the effects of MC-LR (commercial product; purity not reported) on 11-week-old male hybrid rats (F1 generation of female WELS/Fohm x male BDIX; n=10 per group) given 0, 50 or 150 μg/kg bw per day for 28 days in drinking-water. Dose estimates were not adjusted to account for drinking-water not consumed by the animals (estimated as 3-7%). Haematology and serum enzyme analyses were conducted, as was histopathology of the liver and kidney. Increased leukocyte counts (38%) were observed in the highest dose group while
increased LDH (low dose: 84%, high dose: 100%) and ALP (low dose: 34%, high dose: 33%) were seen in both treatment groups, with no changes in ALT or AST, the known marker of hepatic toxicity. There were dose-dependent increases in relative liver weights (low dose: 17%, high dose: 26%) and absolute liver weights (data not provided). There were no statistically significant changes in other organ weights or in body weights. Slight to moderate liver lesions with or without haemorrhage occurred in both treatment groups with severity increased in the 150 μg/kg bw per day group; other histological effects, including Kupffer cell activation and PAS staining, showed no dose–response. No effects on the kidneys were observed. The LOAEL was 50 μg/kg bw per day, the lowest dose tested.

In an “omics” study of MC-LR effects on lipid metabolism, 10 male BALB/C inbred mice were dosed via gavage every other day for 90 days with MC-LR at 0, 40 or 200 μg/kg bw per day (He et al., 2017). Although perturbations of serum and hepatic metabolites suggested non-alcoholic fatty liver disease, only 3 of 10 liver samples from each dose group were examined histopathologically. Such small sample sizes do not allow for statistical analyses, especially when inflammatory infiltrates and apoptosis were also seen in 1 of the 3 control mice. Further, serum chemistry markers were either not affected (e.g. ALT, AST) or not dose-related (e.g. albumin, bilirubin). The methodology also lacked scoring for lipid accumulation and cellularity or staining for inflammation (e.g. F/480), additional preferable parameters when evaluating steatosis.

Shaeffer et al. (1999) reported results from an earlier (1984) unpublished dosing trial in which five Cox-Swiss mice per sex and dose were administered extracts containing MC-LR via the diet for either 21 or 43 days. The doses were estimated to be 43, 83, 123, 167 and 333 μg MC-LR/kg bw per day based on the MC-LR content of the lyophilised Aph. flos-aquae as determined 13 years after the original trial. No effects were seen on body weight, liver, kidney or spleen weight, nor on histopathology at the highest estimated dose of 333 μg MC-LR/kg bw per day (Schaeffer et al., 1999). Although this dosing regimen is more similar to human oral exposure, as with others using cyanobacterial extracts the presence of other cyanobacterial components complicates interpretation of the results.

Sedan et al. (2015) administered by gavage 50 or 100 μg/kg bw MC-LR to male N:NIH-mice every other day for a month, and extrapolated the daily dose to 25 μg/kg bw/day, claiming they obtained effect below the NOAEL identified in the Fawell et al study. This extrapolation is not correct, based on present knowledge of MC kinetics. Alterations in superoxide dismutase (SOD) activity and GSH content, slight hepatic steatosis with no necrosis and a decrease in intraepithelial lymphocytes, without any alterations of hepatic toxicity biomarkers (ALT, AST, and ALP) were reported at the lower dose. SOD and lymphocyte infiltration showed a dose response (higher at 100 μg/kg bw), whereas oxidised GSH and lipid peroxidation did not.

Huang et al. (2011) evaluated the effects of orally administered MC-RR on apoptosis in the liver of adult male ICR mice. Groups of five mice were administered a dose of 0, 4.6, 23, 46, 93 or 186 μg/kg bw per day of MC-RR (commercial product; purity not reported) via gavage for 7 days, after which animals were terminated and livers were analysed. A statistically significant dose-dependent increase in the percentage of apoptotic cells in the liver at doses of 46 μg/kg bw per day and higher was reported. Expression of apoptosis-related proteins BAX and Bcl-2 were also significantly altered at doses of 46 and 23 μg/kg bw per day, respectively, with their ratio (Bax/Bcl-2) significantly increased at all doses greater than 4.6 μg/kg bw per day.
Extract from *M. aeruginosa* was given to groups of five pigs (strain not reported) in their drinking-water for 44 days at MC doses estimated to be 0, 280, 800, or 1310 μg/kg bw per day based on the extract LD₉₀ value in mice (Falconer et al., 1994). The extract contained at least seven MC variants, with MC-YR tentatively identified as the major constituent. A NOAEL for MCs of 280 μg/kg bw per day was reported, with liver injury (evident from histopathology and changes in serum enzymes) observed at the two highest dose levels. As one pig was also affected at the lowest dose level, the LOAEL was 280 μg/kg bw per day.

Greer et al (2018) treated pigs (different breeds in the two trials) by oral gavage with MC-LR (0.04 μg/kg/d for 98 d, n=8, or 2.0 μg/kg/d for 35 d, n=6). They analysed serum and various organs (including liver, kidney, spleen, small and large intestine and brain) for both free and bound MC-LR. MC-LR-GSH was qualitatively analysed for in the livers and kidneys from the high dose group only. As already indicated, the weight of the paper is limited, especially regarding quantitative aspects, due to some inaccuracies in the detection methods. No toxicity data is reported in the paper, but Welten et al (2019) analysed samples from the high dose group of Greer et al (2018) plus those from another 8 pigs dosed by gavage at 8.0 μg/kg/d for 35 d for changes in metabolomic (blood and liver) and lipidomic (liver only) profiles. In addition, AST, ALP, ALT, GGT, LDH, albumin and total protein were measured in plasma samples from day 35 of the 8.0μg/kg/d group. Multivariate analyses of the various profiles showed no treatment-related effects and nor did any of the plasma indicators of liver damage.

Benson et al. (2005) exposed groups of six male BALB/c mice to monodispersed submicrometer aerosols of MC-LR via nose-only inhalation for 30, 60 or 120 minutes each day for 7 consecutive days. The concentration of MC-LR was 260–265 μg/m³, and doses deposited in the respiratory tract were estimated to be 3, 6 and 12.5 μg/kg bw per day. Control mice were exposed to the aerosolized vehicle (20% ethanol). Clinical chemistry analysis, organ weights, and histopathology of the liver, respiratory tract tissues, adrenals, kidney, spleen, thymus, gastrointestinal tract and testes was conducted. No clinical signs or effects on body weight or organ weights were observed. Treatment-related lesions were observed in the nasal cavity in the mid and high dose groups, with the incidence and severity increasing with length of exposure. The nasal cavity lesions observed included necrosis or inflammation of respiratory epithelial cells and degeneration, and necrosis and atrophy of olfactory epithelial cells.

### 6.3 Long-term exposure

#### 6.3.1 Systemic Effects

A growing number of long-term rodent studies have used dosing via drinking water to better mimic the human exposure scenario and avoid potential issues associated with gavage dosing. Many of these studies have based their exposure concentrations directly on the pGV for MC-LR of 1 μg/L (and/or multiples of it). For comparison, if murine water consumption of 1.5mL/10g bw per day is assumed (Chen et al. 2016a), then a mouse GV-equivalent of 0.27 μg MC-LR/L can be calculated from a TDI of 0.04 μg/kg/day.

Ueno et al. (1999) conducted a study in which 6-week-old female BALB/c mice received drinking-water containing 0 or 20 μg MC-LR/L daily for up to 18 months (20 mice per dose). Average cumulative MC-LR intake for the 18 months was estimated at 35.5 μg per mouse. This is equivalent to an exposure of 2.3 μg/kg/day based on the reported average adult body weight of 26.68 g/mouse and the reported 567-day exposure. No clinical signs of toxicity or impacts on survival, body weight, food or water consumption, haematology or liver...
histopathology were observed. A significant increase in serum cholesterol (22%) occurred although immunohistochemical analysis did not provide evidence of hepatic MC-LR accumulation.

Zhang et al. (2010) administered MC-LR (commercial product; ≥ 95% purity) to 8-week-old male C57BL/6 mice (10 per group) via drinking-water at a 0, 1, 40 or 80 μg/L (estimated as average intakes of 0, 0.2, 8.0 or 16.0 μg/kg bw per day) for 180 days. A significant decrease in body weight occurred at 40 and 80 μg/L, accompanied by an increase in relative liver weight (data not given) and hepatic lymphocyte infiltration and fatty degeneration.

Zhou et al., (2020) exposed groups of 10 male mice (strain not specified) to MC-LR at 0, 1, 10 and 100 μg/L in drinking water for 180 days. A significant reduction in weight gain was observed in the 10 and 100 μg/L groups from 154 or 126 days, respectively, of exposure onward.

Labine et al., (2017) exposed CD-1 mice (20 per group) to 1 μg/L MC-LR, MC-LR plus thiacetamide (TAA, 300 mg/L) or TAA only in their drinking water for 28 weeks after which serum was analysed for ALT and total bilirubin while livers were examined by histopathology. Consumption rates of the dose materials were not reported and so exposure in terms of µg/kg/d cannot be calculated. There were no treatment related effects observed in the MC-LR alone group and MC-LR did not potentiate the toxic effects induced by TAA.

Female BALB/C mice were given 1, 10, 40 μg MC-LR/L for 6 months in drinking water (estimated by the authors to correspond to 0.1, 1 and 4 µg/kg bw per day based on “the general amount of water intake”). Alveolar collapse and lung cell apoptosis with altered cell junction integrity occurred at all doses, apparently dose-dependently although no quantitative analysis was provided (Wang et al., 2016).

In male C57BL/6 mice exposed to 1, 5, 10, 20 or 40 μg MC-LR/L in drinking water for 12 months (no estimate of daily dose per kg bw was provided), dose-dependent thickening of alveolar septa or alveolar collapse were reported to have occurred in all but the 1 μg/L group, but no quantitative analysis of these changes was presented. (Li et al., 2016).

ICR mice (13 weeks old) were given either 80 (n=10) or 100 (n=5) gavage doses (5 days per week and therefore for a total of 16 or 20 weeks, respectively) of 80 μg MC-LR/kg bw. A further 7 mice were given 100 gavage doses and then put on a 2-month withdrawal period. “Light” injuries to hepatocytes occurred near the central vein in 8/15 mice terminated immediately after the end of treatment and also in 5/7 mice that had the withdrawal period. There were no changes in liver weight, fibrous or neoplastic lesions, or immunohistochemical evidence of hepatic MCs (Ito et al., 1997a).

6.3.2 Neurological Effects

Li X-B et al., (2014) administered MC-LR (purity >95%) to groups of 8 male SD rats at doses of 0, 0.2, 1.0 and 5.0 μg/kg by gavage every second day for 8 weeks. Twenty-four hours after the last dose, the rats were then subjected to a Morris water maze test for learning and memory consisting of 5 days of training followed by a probe trial on the 6th day. There was a dose-dependent trend for reduced learning (with escape latency significantly higher in the 5 μg/kg group at day 3 of training only) and memory (with a significant reduction in the 1 and 5 μg/kg
groups for one of three parameters measured in the probe trial). No histological lesions were found in the hippocampal regions of treated rats stained with H&E, however immunostaining for markers of astrocyte activation and inflammation was increased in the 5 µg/kg group.

Feurstein et al., (2010) demonstrated the uptake and protein phosphatase inhibitory actions of MC-LR, MC-LW and MC-LF in primary murine neurons expressing at least two mOATPs (mOATP1A5 and the known MC-LR transporter mOATP1B2). The transport of all three MC variants into the neurons was confirmed. At low MC concentrations (0.31–1.25 µmol/L), MC-LR, MC-LW and MC-LF showed a comparable 20% reduction in protein phosphatase activity compared with controls. At 2.5 µmol MC-LR, MC-LW or MC-LF per L, total protein phosphatase activity was reduced by 25%, 30% and 60%, respectively, and 5 µmol MC-LF per L reduced total protein phosphatase activity by 65%. In a follow up study, Feurstein et al. (2011) examined the effects of MC-LR, MC-LF and MC-LW on cytotoxicity, apoptosis, caspase activity, chromatin condensation, and hyperphosphorylation of microtubule-associated Tau protein in isolated murine cerebellar granule neurons (CGN). All 3 congeners reduced cell viability (MTT reduction) and neurite length, with MC-LF >> MC-LW being significantly more potent than MC-LR. However, MC-LF and MC-LW also significantly increased various measures of apoptosis whereas MC-LR did not. Significant differences in Tau protein phosphorylation patterns were also induced. The authors note that these analogues do not significantly differ in inhibitory potency towards protein phosphatases 1 and 2A, thus hypothesised that the observed differences related to affinity for OATPs, i.e. to uptake kinetics.

6.3.3 Reproductive and developmental toxicity

A number of reproductive studies have been reported in which the toxin was administered intraperitoneally. This route of exposure bathes the reproductive organs in essentially pure test solution, rendering results difficult to interpret in terms of human health risk. Hence such studies were not generally reviewed.

The effects of MC-LR on embryonic and fetal development were studied in groups of 26 Cr1:CD-1 (ICR) BR mice dosed by oral gavage with aqueous solutions of MC-LR from gestational day 6 to 15 at 0, 200, 600, or 2000 µg/kg bw per day. Nine of the 26 females dosed with 2000 µg/kg bw per day died or were terminated prematurely. High dose females had abnormal livers but bodyweight gains were comparable to those seen in the other groups including controls. Reduced weight and retardation of skeletal ossification were observed in their fetuses without effects on litter size, post-implantation loss, or fetal sex distribution. The NOAEL for developmental toxicity was 600 µg/kg bw per day (Fawell et al., 1994) equal to the NOAEL for maternal toxicity, both well above the NOAEL for liver toxicity.

Similarly, Falconer et al., (1988) found no evidence of teratogenicity, embryonic mortality, or reduction in fertility in mice exposed to MC-containing M. aeruginosa extract in drinking-water at an estimated dose of 2700 µg MCs/kg bw per day from weaning (17 weeks prior to mating) through mating. Seven of 73 pups from MC-exposed dams were found to have reduced brain size (compared to 0/67 controls). The single small brain examined histologically exhibited extensive damage to the outer region of the hippocampus.

Chernoff et al. (2002) administered MC-LR either i.p. or s.c. to groups of 8 CD-1 female mice daily at 1 of 3 periods during gestation (GD7-8, GD9-10 or GD11-12). I.p. doses were 0, 32, 64 and 128 µg/kg bw while the s.c. dose was 128 µg/kg bw. There was no treatment related effect
on maternal weight or survival, litter size, fetal weight or incidence of supernumerary ribs. In a second trial using a batch of MC-LR that proved to be more toxic, 19 of 35 dams administered 64 μg/kg bw died. However, there was no difference from control in terms of survival or postnatal growth over 4 days of newborns from dams that survived this dose. No effects were seen in foetuses of dams given lower doses.

MC-LR (commercial product with purity not reported) was administered to groups of 20 mice (age and strain not reported) for 3 or 6 months in drinking-water at a concentration of 0, 1, 3.2 or 10 μg/L (Chen et al., 2011). Body weight and amount of water consumed were reported to have been measured, but were not presented and daily doses were not calculated (they can be estimated from default values as approximately 0, 0.25, 0.79 and 2.5 μg/kg bw per day; USEPA, 2015). Clinical signs of toxicity were not observed, and water consumption, body and testes weight were not affected by treatment. Sperm or hormone parameters were not affected at 1 μg/L. At 3.2 and 10 μg/L, sperm counts were reduced by 34% and 50%, respectively at 3 months and 37% and 69%, respectively, at 6 months. Motility was reduced by 25% and 50%, respectively, at 3 months and by 59% and 70% at 6 months, while sperm abnormalities were not increased at 3 months but were more than double control values in the 3.2 and 10 μg/L dose groups by 6 months. Mid- and high-dose mice had significantly lower serum testosterone and higher luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels after 6 months. Histopathology of the testes of the mid- and high-dose mice showed dose- and time-related effects. In particular, Leydig cell apoptosis was significantly increased at 3.2 and 10 μg/L after 6 months exposure. This finding is consistent with the hormonal changes observed (reduced testosterone and increased FSH and LH). The study had a number of short-comings (e.g., use of methanol as a vehicle for MC-LR not administered to control animals, effects present also in the control group, poorly described analysis on sperm, no purity of the test item reported, nor check for MC-LR concentration in the administered drinking-water, as well as questionable statistical evaluation) that limit its usefulness for human health risk assessment (USEPA, 2015; Health Canada, 2018; Buratti et al., 2017).

Eighty male mice (strain not specified) were divided into groups of ten and provided with MC-LR in drinking water at concentrations of 0, 1, 10, or 100 μg/L for 90 or 180 days to examine effects on the testis (Zhou et al. 2020). Histopathology of the testes showed significant increases in abnormal seminiferous tubules in the 100 μg/L group at 90 days and the 10 and 100 μg/L groups at 180 days. Proteomic analysis demonstrated changes in expression of a range of testicular proteins. The method used to fix the testes (4% paraformaldehyde) has been questioned due to potential generation of artefacts (USEPA 2015).

Groups of 10 male BALB/C mice were provided with 0, 1, 10, 20 or 30 μg MC-LR/L in drinking water for 90 or 180 days to investigate effects on the prostate (Pan et al 2018). Prostate weight as percent body weight was significantly increased in the 20 μg/L group at 90 days and in both higher dose groups at 180 days. Body weight was significantly reduced in those given 30 μg/L at 180 days, but not in any group at 90 days, or in lower dose groups at 180 days. Prostate specific antigen and prostate acid phosphatase, markers of prostatic cancer, were dose-dependently increased in the 3 highest dose groups after 180 days of exposure.

In a study examining MC-LR effects on the ovaries, 30 female BALB/C mice per dose group were treated for 3–6 months with drinking-water containing 0, 1, 10 or 40 μg MC-LR/L (isolated from Microcystis aeruginosa, purity ≥95%, with body weight and amount of water consumed not reported). Ovary weight as % bodyweight and serum estradiol were significantly reduced at 3 months (40 μg/L) and 6 months (10 and 40 μg/L), with atretic follicles (showing
degeneration of oocytes) increasing in parallel (significant with 40 µg/L at 3 months and all treatment groups at 6 months). Progesterone was significantly increased in the 10 and 40 µg/L groups at 3 months but only the 40 µg/L group at 6 months. Some perturbations of the estrous cycle were observed that were essentially the same at both time points, as was a significant increase in stillbirths in the 10 and 40 µg/L dose groups (Wu et al., 2015). However, as there are questions regarding the statistical evaluation (a two-way ANOVA with post-test should have been used rather than a one-way ANOVA, the number of replicates are too low for the number of parameters evaluated, not all parameters have a normal distribution), the determination of significant values is questionable.

Li et al. (2015b) exposed female Sprague-Dawley rats (28 days old, 7 per dose group) to 0, 1.0, 5.0 or 20.0 µg MC-LR/kg in 2 x 10^{-3} (v/v%) methanol by gavage once every 48 h for 8 weeks and then, 24 hr after the last dose, each female was mated with an unexposed male. Pups from all MC-LR-treated groups had significantly lower scores in the cliff avoidance test. The Morris water maze test of cognitive impairment revealed significantly lower frequencies of entering the platform zone for male offspring from all treatment groups, significantly lower frequencies of entering the enlarged platform zone for the females (5.0 and 20.0 µg MC-LR/kg), and significantly decreased swimming speed in the female offspring from the group treated with 20.0 µg MC-LR/kg. Malondialdehyde contents in the hippocampus were significantly increased in the male offspring at 5.0 µg MC-LR/kg and in both male and female offspring at 20.0 µg MC-LR/kg. Total SOD activities were also significantly increased in the hippocampus of the male and female offspring at 20.0 µg MC-LR/kg. However, exposure of the dams to MC-LR ceased before mating and conception, making it difficult to quantify the relationship between the dosing of the dams and the exposures of the pups in the absence of data on half-life for MC-LR.

Male and female Sprague-Dawley rats were randomly paired and mated, then on GD8 (chosen because organogenesis occurs between GD6 and GD16) the dams were subcutaneously implanted with osmotic pumps designed to deliver either 0.9% saline or 10 µg MC-LR/kg/d (presumed to be 5 dams each though not explicitly stated; Zhao et al 2015). Dosing continued for 28 days, until postnatal day 15. Dams delivered naturally and pups were euthanised on postnatal day 15. Free MC-LR content of pooled brain samples was determined to be 3.75 ± 0.94 ng/g dw by LC/MS/MS but maternal and newborn serum MC-LR contents were not reported. Transmission electron microscopy examination of the cerebrum of offspring showed astrocyte and neuronal swelling with ultrastructural changes to mitochondria and endoplasmic reticulum. Malondialdehyde was increased while GSH content and acetylcholine esterase activity were reduced compared to controls. However, due to the unusual route of exposure, and hence different MC kinetics, it is not possible to make any comparison with the oral route.

The ability of MCs to decrease cell viability of spermatogonia, Leydig cells and Sertoli cells in vitro (Li et al., 2008; Zhang et al., 2011; Li & Han, 2012; Zhou et al., 2012) suggests that MC transport may occur in the testes. OATPs are active in the testes (Svoboda et al., 2011) and spermatogonia (Zhou et al., 2012) but their potential role in the toxicity of MCs to the testes has yet to be elucidated. In contrast, human placental trophoblasts in vitro were relatively resistant to MC-LR despite expressing OATP1B3 (Douglas et al., 2016).

6.3.4 Genotoxicity and carcinogenicity

Genotoxicity studies of pure MCs have generally been negative (IARC 2010; Sieroslawska, 2013). Both in vitro and in vivo genotoxicity studies of cyanobacterial extracts have shown positive
results with DNA damage induced by formation of reactive oxygen species as well as inhibition of repair pathways. The available data on the genotoxicity of cyanobacterial toxins, including MCs, has been reviewed (USEPA, 2015; Buratti et al., 2017). Current evidence indicates that the MCs are not bacterial mutagens and that discrepancies in results from cyanobacterial extracts are likely due to differences in source of the cyanobacteria and composition of the complex extract mixtures. Cellular DNA damage observed after in vitro treatments with pure MCs may be due to induction of apoptosis and cytotoxicity rather than direct effects on the DNA (USEPA, 2015).

MCs have been classified as Group 2B, possibly carcinogenic to humans (IARC, 2010), based on their tumour promotional activity mediated via protein phosphatase inhibition (a threshold effect) rather than direct carcinogenicity.

MCs appear to cause adverse effects on DNA replication. Observations of polyploidy in MC-LR-treated cells (Humpage & Falconer, 1999; Lankoff et al., 2003) may be related to its effects on cytokinesis. Lankoff et al. (2003) showed that MC-LR, through its effect on microtubules, damages the mitotic spindle, leading to the formation of polyploid cells. Microcystin-LR disrupted chromatin condensation in Chinese hamster ovary cells at the end of interphase and the beginning of metaphase (Gácsi et al., 2009). However, neither MC-LR nor cyanobacterial extracts resulted in an increase in micronucleus formation in cultured human lymphocytes (Abramsson-Zetterberg et al., 2010).

Two long term oral dosing studies of purified MC-LR as a potential tumour initiator are available. Ito et al. (1997a) administered 80 µg MC-LR/kg/day by gavage to mice for 80 or 100 days over 28 weeks (7 months). This single dose level did not induce fibrous changes or neoplastic nodules in the liver. A concurrent trial in which 20 µg MC-LR/kg/day was injected i.p. to mice 100 times over 28 weeks did induce detectable nodules (Ito et al., 1997a). Microcystin-LR and its cysteine conjugate were detected in the livers of mice i.p.-dosed but not in those of the gavage-dosed ones.

In the second study four groups of adult male CD-1 mice (n = 20/group) were dosed for 28 weeks with water, water containing 1.0 µg MC-LR/L, water containing thioacetamide, and water containing both MC-LR and thioacetamide. No tumours were present in the control or MC-LR-alone groups, while in the other two groups 4 and 5 mice developed liver tumours, whose mean size, Ki-67 staining, number of atypical mitoses and liver cancer gene expression profiles were similar (Labine and Minuk, 2014).

Three studies that used i.p. dosing suggest that MC-LR is a tumour promoter in the liver. In these studies, animals were first exposed to substances known to be tumour initiators (e.g. N-methyl-N-nitroso urea or NDEA) alone, or in combination with MC-LR at i.p. doses known to have no significant impact on liver weight. The combination of the initiator and the MC-LR significantly increased the number and area of glutathione S-transferase placental form-positive (GST-P) foci when compared to treatment with the initiator alone. The same was true for situations where the initiator treatment was combined with a partial hepatectomy (to stimulate tissue repair) and then exposed to MC-LR i.p. (Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994). In a study that examined possible synergism between aflatoxin B1 (AFB1) and MC-LR, male Fischer 344 rats were given i. p. injections of diethylaminoethylamine (DEN) and/or AFB1 as initiators then partial hepatectomy and MC-LR (1 or 10 µg/ kg bw twice weekly for 6 weeks) as promoters. Animals were killed 8 weeks after injections of initiator and their livers examined for GST-P positive foci. (Sekijima et al., 1999). MC-LR alone did not cause an increase in the number or size of foci. However, MC-LR did significantly increase both number and size of foci in mice treated with either DEN or AFB1, but not in those treated with a combination of the two. GST-P positive foci
are regarded as tumour precursors and thus indicators for potential tumour formation. The results from these studies support the conclusion that of MC-LR is a tumour promoter in the liver.

Some studies that used oral exposures to *Microcystis* extracts have provided evidence of tumour promotion in non-hepatic tissues, but a direct link with MCs cannot be established. Falconer and Buckley (1989) reported evidence of skin tumour promotion by extracts of *Microcystis* in drinking-water in DMBA-initiated mice. Total skin tumour weight was increased although the number of tumours per mouse was only slightly increased (Falconer and Buckley, 1989). Humpage et al. (2000) administered *M. aeruginosa* extract in drinking-water to mice pre-treated with azoxymethane. A concentration-dependent increase in the mean area of aberrant crypt foci of the colon was observed, although the number of foci per colon and the number of crypts per focus were not different among the groups. The authors proposed that an increase in cell proliferation caused the increase in the size of foci. In contrast, when *Microcystis* extract was provided in the drinking-water of mice pre-treated with two oral doses of *N*-methyl-*N*-nitrosourea, no evidence of promotion of lymphoid or duodenal adenomas and adenocarcinomas was observed, and no primary liver tumours were observed (Falconer & Humpage, 1996).

**Immunotoxicity**

A few studies have suggested that exposure to MCs (from cyanobacterial extracts) at doses ranging from 4.97 to 50 μg MC-LReq/kg bw by single or multiple i.p. injections may alter immune function (Shi et al., 2004; Yuan et al., 2012). Effects reported include reduced phagocytic capacity, B-lymphocyte proliferation, humoral immune response, altered plasma leukocyte, and cytokine and interferon levels. Chen et al., (2004, 2005) reported a dose-dependent inhibition of nitric oxide production in activated macrophages, as well as reduction of cytokine formation at the mRNA level, following exposure to 1–1000 nmol MC-LR/L.

MC toxicity is characterized by the influx of neutrophils to affected organs, with release of proteolytic enzymes and reactive oxygen and nitrogen metabolites (Babior, 2000), stimulating possible inflammatory responses (Kujbida et al., 2009). Furthermore, in Caco-2 cells, 100 μM MC-LR induced a five-fold greater IL-8 secretion compared to MC-RR, although no differences in intracellular ROS production were observed (Huguet et al., 2013), suggesting that other mechanisms can occur with different patterns depending on the toxin.

Further research is required to elucidate the immunotoxicity of MCs, particularly following oral exposure.

**Hematological effects**

As reviewed by others (USEPA, 2015; Health Canada, 2018), thrombocytopenia seen in laboratory animals treated with MCs or bloom extracts containing MCs may be secondary effects of liver haemorrhage. *In vitro* studies have shown that MC-LR does not affect the aggregation of platelets. MC-LR exposure in human erythrocytes *in vitro* at 0.1 μM and above resulted in the formation of echinocytes, haemolysis, conversion of oxyhaemoglobin to methaemoglobin, and a decrease in membrane fluidity. In addition, measures of oxidative stress were affected in treated erythrocytes.

**6.4 Mode of Action**
Mechanistic studies of MC cellular effects have helped elucidate the mode of toxic action. The need for membrane transporters for systemic uptake and tissue distribution of MCs by all exposure routes determines the specificity of organ effects (Fischer et al., 2005; Feurstein et al., 2010). Members of the OATP transporter family regulate uptake from the intestines and into tissues such as the liver, kidney, testes, brain, lung, heart, and placenta (Falconer et al., 1992; Augustine et al., 2005; Fischer et al., 2005; Fischer et al., 2010). The importance of the transporters to tissue access is demonstrated by the data that indicate a reduction in, or lack of, liver damage when OATP is inhibited (Hermansky et al., 1990 a,b; Thompson & Pace, 1992) or nearly absent in OATP1B2-null mice (Lu et al., 2008). In contrast, the transporters responsible for MC efflux are less characterized despite biliary and urinary metabolites of MCs having been reported. Recent findings by Kaur et al. (2019) demonstrated that MC-LR is transported by MRP2 but not by MDR, BSEP and BCRP. Nor was there any MRP2 transport of the MC-LR-GSH conjugate.

The MC-LR mechanism of action has been extensively reviewed by Liu and Sun (2015) and Buratti et al. (2017). MCs cause protein phosphatase (PP1, 2A, and 5) inhibition, with PP5 being 2-10-fold less sensitive than PP1 and 2A for a range of MC congeners (Altaner et al., 2019), which is considered to be the molecular initiating event, and subsequent loss of coordination between kinase phosphorylation and phosphatase dephosphorylation of cytokeratins, resulting in destabilization of the cytoskeleton and microtubuli (Falconer and Yeung, 1992; Feurstein et al. 2011); this event initiates altered cell function followed by cellular apoptosis and necrosis. In the liver, loss of cell morphology and cell to cell adhesion allows haemorrhage into that organ due to damage of sinusoidal capillaries as the results of acute relatively high doses. At low doses (below 20 μg/kg bw; Gehringer 2004) typical of repeated long-term exposure, phosphatase inhibition induces cellular proliferation and hepatic hypertrophy and tumour promotion activity.

The increased protein phosphorylation related to PP2A inhibition and to protein kinase (MAPK) pathway activation induces abnormal signalling in multiple pathways, triggering a cascade of events leading to a series of cellular responses. Most of the information on these intracellular perturbations comes from in vitro studies, with many of the cell lines used lacking metabolic competency, thus their toxicological significance to humans in vivo at relevant exposure concentrations is unclear. Toxicity induced by MCs is a complex process that may result from ‘cross-talk’ and cooperative effects between different candidate pathways, responsible for:

- Modification of cytoskeleton and disruption of actin filaments. The alteration of microtubules seems to be the early event associated with MC-LR-induced PP2A inhibition, including in cells of human origin (Sun et al., 2014), due to hyperphosphorylation of different types of microfilament-associated proteins, such as Tau (Feurstein et al. 2011), vasodilator-stimulated phosphoprotein (VASP) and HSP27 (Zeng et al., 2015; Sun et al., 2015). Dysfunction of the cytoskeleton, crucial in maintaining cellular architecture, is also related to disruption of cell division, migration and signal transduction (Zhou et al., 2015).

- Oxidative stress. The increased formation of reactive oxygen species (ROS) and/or GSH depletion, leading to oxidative stress, drives cells toward apoptotic cell death, rather than to necrosis, due to release of apoptotic factors in vitro (Zegura et al., 2004; Meng et al., 2015) and in vivo (Qin et al., 2015), with time-dependent alterations of GSH levels in rat liver, significant changes of antioxidant enzymes, including GSH peroxidase (GPX) and GSH reductase (GR), activation of NF-kB and the expression of p53, Bax and Bcl-2 (Chen et al., 2016b). The latter finding is supported by the oxidative
stress suppression and decreased apoptosis observed when antioxidants, such as N-acetylcysteine and vitamin C were used as pre-treatment (Meng et al., 2015; Xue et al., 2015). However the concentrations of MCs and antioxidants employed were extremely high and it remains questionable whether ROS is a major contributor in the development of acute and, more importantly, chronic toxicity.

- Induction of apoptosis, due also to mechanisms other than oxidative stress, including the phosphorylation of p53, Bcl-2 and Bax, cytochrome c and caspases (Liu et al., 2016; Wang et al., 2013). However, rats appear to be more prone to induction of apoptosis than mice or humans, in which necrosis has been shown to dominate (Woolbright et al., 2017).
- Reduced DNA repair (Douglas et al., 2001; Kleppe et al., 2015).
- Cell proliferation leading to tumour promotion, associated with the known tumour suppressor role of PP2A. The alteration of cell proliferation, division, signal transduction and gene expression, due to the hyperphosphorylation of transcriptio

### Toxic equivalency

MC-LR has been shown to be one of the most potent MC variants, with the lowest reported i.p. LD$_{50}$ values, ranging between 50 and 60 μg/kg bw. However, MC-LA has a similar LD$_{50}$ to MC-LR, while LD$_{50}$ values for other more lipophilic MC congeners (MC-LF, MC-WR) have yet to be determined (Zurawell et al., 2005). Intraperitoneal LD$_{50}$s have been reported for a range of other variants (Zurawell et al., 2005) and toxicity equivalency factors (TEFs) limited to acute toxicity have been proposed by Wolf and Frank (2002) for MC-LR (1.0), MC-LA (1.0), MC-YR (1.0) and MC-RR (0.1). However, comparative testing of in vitro protein phosphatase inhibition (IC$_{50}$) for MC-LR, MC-YR and MC-RR resulted in IC$_{50}$ values of 0.016, 0.014 and 0.034 μmol/L, respectively (Yoshizawa et al., 1990). Comparable results from other studies (Hoeger et al., 2007; Monks et al., 2007; Fischer et al., 2010; Vesterkvist et al., 2012; Altaner et al., 2019) indicate that most MC variants may be relatively similar with respect to protein phosphatase inhibition potency, despite differences in their variable amino acids. Therefore, pharmacokinetic differences among the variants may be at least partially responsible for observed variations in lethal potency (Ito et al., 2002; Fischer et al., 2010; Buratti & Testai, 2015, Santori et al., 2020). Differences in uptake, first pass clearance and distribution from the gut may be important determinants of potency via the oral route, limiting the utility of currently available i.p. or in vitro comparative data for human health risk assessment. Therefore, extrapolation of toxicological information from MC-LR, the most studied congener, to the whole MC group might be difficult as far as repeated dose toxicity is concerned.

### 7.0 OVERALL DATABASE AND QUALITY OF EVIDENCE

#### 7.1 Summary of Health Effects

Acute and sub-chronic oral exposure studies in animals and human data from dialysis-related poisoning events confirm the liver as a major target organ for MCs, although other organs may be affected as well. Data from animal studies have provided a reasonably detailed understanding of the mechanisms underlying these hepatic effects (see section 6.4). However,
the human data are limited by lack of quantitative exposure information and by potential co-exposure to other microorganisms and contaminants. There are no long-term studies of MC carcinogenicity. However, evidence from i.p. and in vitro studies using pure MCs (referring almost exclusively to MC-LR) indicate that it is not a DNA-reactive genotoxin. DNA damage observed after in vitro treatments with pure MCs may be due to induction of apoptosis and cytotoxicity rather than direct effects on the DNA (Žegura et al., 2011; USEPA, 2015; Buratti et al., 2017). The weight of evidence suggests that tumour promotion in a range of tissues may be a potential outcome of long-term exposure (IARC, 2010).

There are limited data on developmental toxicity, but an oral study in mice using dosing during organogenesis did not show any adverse effects on embryofetal survival or development. There is some evidence, mainly from in vitro or i.p. studies in vivo, that both male and female reproductive tissues in rodents may be adversely affected by MCs. However, in addition to other limitations in many of those studies, i.p. dosing causes the reproductive tissues be bathed in test material, leading to much higher exposure concentrations than would be achievable via oral exposure. Currently available oral dosing studies that employed drinking water exposure to very low doses to MC-LR are unfortunately affected by methodological and reporting deficiencies that limit their use for human health risk assessment. Therefore, further high-quality oral dosing studies are required to corroborate these findings and quantify the dose-response relationship. A mechanistic understanding of the observed reproductive effects is not yet available. However, this area will be an important focus for research in coming years because the early evidence suggests that these tissues may be highly sensitive to MC effects.

There is currently only limited data to assess adverse effects in other organs or tissues (neurological, immunological, haematological).

In addition, data related to variants other than MC-LR are very scant. Although in vitro kinetic data clearly indicate that inhibitory capacity of single MC congeners on protein phosphatases is comparable (Altaner et al., 2019), toxicokinetics may play an important role in vitro in determining the variability of toxic effects exerted by different variants (Santori et al. 2020), but further research is warranted on these aspects.

### 7.2 Quality of Evidence

There are a number of deficiencies in the available database including a paucity of studies that used highly purified and well characterized toxins, and that used the oral route of exposure. There is only limited information on a number of key endpoints including neurotoxicity, reproductive effects and effects of chronic exposure. There are significant methodological limitations in many of the reproductive and developmental studies as described in section 6.3.3. A lack of comparative in vivo oral studies of MC congeners severely limits the risk assessment of variants other than MC-LR. Many in vitro mechanistic studies used uptake- and biotransformation-incompetent immortalized cell-lines that have unclear relevance to in vivo exposures. Available epidemiological studies have lacked adequate exposure assessments and control of potential confounders, as described in section 5.2. Nevertheless, general agreement on key processes in MC toxicity has emerged, for example, the consistency of evidence that the liver is the primary target for acute toxicity due to the high uptake rate in this organ facilitated by active transport into the hepatocyte and protein phosphatase inhibition as the primary biochemical target, which triggers a cascade of dose related events leading to the observed toxic outcomes.
8.0 PRACTICAL CONSIDERATIONS

Microcystins are the most commonly reported and best researched of the cyanotoxins. Where blooms occur MC concentrations can fluctuate widely due to uneven distribution of blooms in a water body, heterogeneity of clones within blooms and to some extent also variation in the amount of toxin produced by individual clones.

Chapters 7 - 10 of TCiW give guidance on multiple barriers against cyanotoxins in water including controlling nutrient loads from the catchment, managing water bodies, optimizing sites for drinking-water offtakes or recreation, applying drinking-water treatment to remove cyanobacteria and cyanotoxins and providing information or warnings for recreational use of water bodies with blooms. This includes guidance on planning, managing and documenting the barriers used to mitigate cyanotoxin risks through developing a water safety plan (TCiW; Chorus & McKeown, in press).

8.1 Source control

For planktonic toxic cyanobacteria the prevention of blooms in source waters is the key to long-term control of the risks they represent. The most sustainable approach to achieve this is to keep concentrations of plant nutrients low, as many cyanobacteria typically proliferate under eutrophic conditions i.e., at elevated concentrations of nutrients, in particular of phosphorus, and total phosphorus concentrations below 20-50 µg/L will limit the development of cyanobacterial blooms in many situations. Exceptions include large, deep and quite clear lakes and reservoirs in which significant scums can develop even from low concentrations of cells or in which Planktothrix rubescens develop at the interface between the upper, warm and lower cold water layers: bloom control in such situations may require less than 10 µg/L of total phosphorus (TCiW; Chorus & McKeown, in press; Zessner & Chorus, in press). A number of measures within water bodies can mitigate cyanotoxin occurrence, including e.g. artificial water column mixing, nutrient reduction through sediment removal or treatment, or biomanipulation. Their success is highly dependent on the specific conditions in the water body, as discussed in TCiW (Burch et al., in press).

Many reservoir off-take structures (towers) can take water from multiple depths to account for vertical heterogeneity. Variable offtakes enable avoiding water layers containing the highest concentrations of cyanobacteria. If multiple off-takes are not available (e.g. in small systems) it may be possible, as a temporary measure, to siphon water from a specific depth. Where conditions allow, the use of bank filtration between source waters and treatment plant inlets can be effective both for removing cyanobacteria and for biodegradation of dissolved MCs (TCiW; Brookes et al., in press). Where possible, sites for recreational activities are best located upwind of bays where scums tend to accumulate.

8.2 Monitoring

Depending on a range of conditions, including climate, cyanobacteria can be present in surface waters throughout the year or as short-lived seasonal blooms, in both cases potentially producing significant concentrations of toxins. Monitoring of source waters should include surveillance for factors that can promote the growth of cyanobacteria including total phosphorus, temperature, water residence time, pH and Secchi disc transparency (for detail see TCiW; Padičák et al., in press). On site visual assessment of turbidity with greenish discoloration or scums and microscopy are effective low cost direct methods that can trigger
increased vigilance if MC-producing cyanobacteria are observed. In many cases monitoring over several seasons can establish the likely occurrence and timing of favourable conditions for cyanobacterial growth as well as the taxonomic composition and magnitude of blooms. For example, a lake with regular seasonal blooms of *Microcystis* in late summer is unlikely to shift to perennial blooms of *Planktothrix* from one year to the next (TCiW; Ibelings *et al.*, in press).

Monitoring programs should be adaptive with sampling being increased when there is evidence of increasing cell numbers. An Alert Level Framework (ALF) has been described for drinking-water which includes various criteria to trigger particular analyses and risk mitigation measures (TCiW; Chorus & Testai, in press; Humpage, *et al.*, in press). As described in the ALF monitoring of source waters can start with simple site inspections for appearance of visible blooms, assessing transparency using a Secchi disc. However, not all MC producers form surface scums or strong discoloration, and these may be overlooked. Therefore, if the presence of cyanobacteria is suspected, microscopic examination for the presence of potentially MC producing cyanobacteria is important. As blooms develop monitoring can be expanded to include quantitative measures of cyanobacterial biomass indicating potential toxin concentrations such as cyanobacterial biovolumes or chlorophyll-α, or direct analyses of MC concentrations. Wherever possible, particularly when estimates of MCs derived from biomass indicators approach guideline values, toxin testing should be performed as concentrations associated with blooms can vary substantially and toxin data may well allow avoiding or lifting restrictions of site use where these were based on biovolume or chlorophyll-α concentrations.

### 8.3 Analytical methods and achievability

Analytical techniques are available for the range of parameters associated with cyanobacterial blooms and MCs. The complexity, expertise requirements and costs of monitoring increase from relatively simple visual inspections to testing for phosphorus, pH, Secchi disc transparency, cell numbers, species identification, biovolumes, chlorophyll-α determination, to toxin analysis. There are multiple techniques for testing MCs, with caveats to each.

For cell-bound and total MCs (cell-bound and extracellular), extraction (e.g. freeze-thaw) is performed prior to analysis. Filtration can be used to separate cells for the purpose of testing intra- and extra-cellular fractions separately. To efficiently extract more hydrophobic MC-variants from cells collected on filters, extraction with aqueous methanol has been successful. Samples or crude extracts can be concentrated through evaporation or with solid-phase extraction (SPE), with the latter also providing sample clean-up. For absorbance detection (UV, PDA) pre-concentration and clean-up may be required, and many methods for the analysis of dissolved MCs require prior concentration of samples from larger volumes of water by SPE for achieving a limit of quantification below 1 µg/L.

The method exhibiting the highest specificity and sensitivity is liquid chromatography tandem mass spectrometry (LC-MS/MS), with quantification limits well below 1 µg/L possible. However, accurate quantification requires reference standards (certified or self tested for purity and quantity) for each individual MC congener because responses vary substantially between congeners. Reference standards are commercially available for some MC-variants, but not for all of the MCs that may be relevant in a given sample.

In contrast LC separation coupled with absorbance detection (UV, PDA) allows for a reasonably accurate quantification of MC congeners for which no reference standards are available, based on calibration curves established using only a few MC standards whose signals are representative.
for all. This is due to the fact that the chromophore of MCs is the conjugated double bond of the Adda moiety that is conserved among all known structural variants and hence, molar extinction coefficients of individual MC variants are similar (ISO 2005). A caveat of absorbance detection may be an overestimation of peaks not fully separated from other substances in the matrix, in particular when peak intensity is low.

In order to account for total MCs, a few non-specific methods can be used. One approach allows for the quantification of total MCs (and nodularin) through the oxidative cleavage of the Adda and subsequent analysis of the product, 2-methyl-3-methoxy-4-phenylbutanoic acid (MMPB). More commonly used commercially available tests include immunological (e.g. enzyme-linked immunosorbent assay) ELISA and biochemical (e.g. protein-phosphatase inhibition assay) techniques. While such tests do not allow for the identification of specific MC congeners, they are useful for screening.

These methods were developed for the analysis of water samples, and applying them to more complex matrices such as food or stomach/tissue contents requires prior clean-up and determination of recovery rates through spiking of samples with known amounts of MC.

Molecular methods have been developed to identify the presence of genes involved in the production of MCs. These methods do not provide information about actual toxin production or concentrations but can provide early warning of potential occurrence.

The choice of methods depends on local or regional accessibility, costs and in particular on the purpose of the analyses which may range, for example, from screening for risk assessment over assessing compliance to Guideline values to research (for more details see TCiW; Lawton et al., in press).

8.4 Treatment methods and performance

Treatment processes to reduce MCs in drinking-water are based on two approaches; reducing cell-bound MCs by physical removal of cells and reducing dissolved MCs (TCiW; Newcombe et al, in press). Unless blooms are decaying, a high proportion of MCs are cell-bound and therefore effectively removable by physical processes, i.e. coagulation followed by flocculation, clarification and rapid media filtration as well as by slow sand filtration or membrane filtration. Filtration processes require care to avoid shear-stress that may cause rupture of cells. Care also needs to be taken to avoid or minimise pre-filtration treatments such as chlorination as this causes cell lysis and release of MCs. Further, as cells may lyse in more acidic water the pH should be kept above 6. Care also needs to be taken to ensure that cyanobacterial and MC concentrates (e.g. filter backwash, sludges and sludge supernatants) are not returned to the head of the filtration plant during a bloom.

Dissolved MCs can be removed by adsorption onto powdered or granular activated carbon (PAC or GAC). Efficacy of removal can be influenced by the type of activated carbon, carbon doses and points of application (PAC), contact times (PAC), flow rates (GAC filters) and water quality. Biological degradation of MCs during slow sand filtration or on GAC filters can be very effective, although it may require a lag phase for the degrading bacteria to establish and verification of performance is important, as the type of activated carbon as well as the age of a GAC filter and the biologically active microbial film it has established may considerably affect performance.
Oxidation by chlorine or ozone can be effective for degradation of dissolved MCs. However, as emphasised by Newcombe et al. (in press), both the type and concentration of organic substance as well as pH strongly affect the amount of disinfectant needed, and elevated DOC in bloom situations will substantially increase the disinfectant demand. It is therefore important to validate the disinfectant dose and contact time under the specific conditions of the treatment train and at the point of disinfection. Other oxidants such as chloramine and chlorine dioxide have been shown to be ineffective against MCs. Ozone or chlorine can be used to reduce concentrations of MCs prior to filtration, and pre-oxidation is sometimes practiced to enhance flocculation. Depending on the amounts applied, oxidants can both lyse the cells, causing toxin release, while at sufficiently high oxidant concentration they can also degrade the released toxins. However, elevated cyanotoxin concentrations typically occur during blooms which cause a high organic load to the treatment plant. As oxidising this without prior filtration is likely to cause high concentrations of disinfection by-products, filtration prior to oxidation is recommended.

The treatment methods discussed above are able to reduce MC concentrations below 1 µg/L. However, validation of efficacy under local conditions is important as described above for oxidation, but also for any other methods applied, including physical removal of cells. Validation may include field trials and laboratory investigations such as jar testing. Verification of toxin removal during blooms should be undertaken by monitoring of MCs in the finished drinking-water.

After effective treatment it is important to ensure drinking-water remains free of cyanobacterial regrowth. This can be accomplished by ensuring that any channels and storages are covered and dark, so that cyanobacteria lack light necessary for growth. Maintaining chlorine residuals throughout distribution systems will also suppress cyanobacterial regrowth.

9.0 CONCLUSIONS

9.1 Derivation of the provisional guideline-value

There are insufficient data to derive a guideline value for microcystin variants except for MC-LR.

The two key oral toxicity studies of the effects of MC-LR on liver toxicity on which human health–based guideline values can be calculated are:

- Fawell et al. (1999). Mice of both sexes given MC-LR by gavage at 40 µg/kg bw per day for 13 weeks did not show treatment related effects in the parameters measured. Only slight hepatic damage was observed at the lowest observed effect level (LOAEL) of 200 µg/kg bw per day in a limited number of treated animals, whereas at the highest dose tested (1 mg/kg bw per day) all the animals showed hepatic lesions, consistent with the known action of MC-LR.

- Heinze (1999). Exposure of male rats (females were not included) to MC-LR in drinking water for 28 days at doses as low as 50 µg/kg bw per day (identified as the LOAEL) resulted in increased liver weight, liver lesions (with haemorrhages) and increased ALP and LDH, but no changes were measured in the mean levels of AST (aspartate aminotransferase) and ALT (alanine aminotransferase) as early markers for hepatotoxicity. Some of the histological effects, including Kupffer cell activation and PAS staining, showed no dose–response since all ten animals at the low and high doses displayed similar degree of damage.
A number of studies have investigated MC effects on reproductive organs and on embryofetal development. Some of them did not observe any relevant reproductive or developmental effects. In a developmental study in mice, Fawell et al. (1999) showed a NOAEL for maternal and developmental toxicity of 600 µg MC-LR/kg bw per day by gavage (GD6-15), well above the NOAEL for liver toxicity. Falconer et al. (1988) did not find evidence of teratogenicity, embryonic mortality, or reduction in fertility in mice exposed to MCs at 2700 µg/kg bw per day in drinking water from weaning (17 weeks prior to mating) through mating. Chernoff et al. (2002) did not observe any effects on fetal survival or postnatal growth in mice born to dams administered a maternally toxic i.p. dose of MC-LR.

In a series of studies of MC-LR effects on reproductive organs, (Chen et al., 2011; Wu et al., 2015; Pan et al., 2018; Zhou et al., 2020), the exposure of mice via drinking water to relatively low doses of MC-LR were reported to cause adverse effects in the testis (reduced sperm count and motility, altered expression of some reproductive hormones and altered testis proteome patterns), prostate (hyperplasia) and ovaries (follicular histology, hormonal changes). Other studies (Li X-B, et al 2014; Li et al 2015a; Zhao et al 2015) in which MC-LR was administered by gavage or subcutaneously described effects on learning in pups. However, lack of reporting of key data and some limitations in the study design preclude use of these endpoints to determine a point of departure (PoD). Inhalation exposure to aerosolized MC-LR (doses equivalent to 6 and 12.5 µg/kg) has been reported to induce lesions in the nasal cavity of mice. However, systemic effects following inhalation exposure in humans seem unlikely due to the reported very low concentrations of aerosolised MCs detected in air above a dense bloom (Wood and Dietrich, 2011), although limited evidence suggests that intratracheal or intranasal exposure to MC-containing water may lead to systemic effects. No specific studies addressed systemic effects following dermal exposure. The oral route (through involuntary intake of contaminated water) is considered more likely to lead to systemic effects during most recreational activities.

The findings of Ito et al. (1997a), describing only mild “injuries to hepatocytes” around the central vein and no other effects (including liver weight change) in mice dosed by gavage with 80 µg/kg bw per day over 28 weeks supports the NOAEL of 40 µg/kg bw identified by Fawell et al. (1999). The Sedan et al. (2015) study, in which some injury was observed (slight hepatic steatosis with no necrosis and a decrease in intraepithelial lymphocytes, without any alterations of hepatic toxicity biomarkers) at 50 µg/kg bw per day given every 48h to mice by gavage for 1 month, can support both the NOAEL coming from the Fawell study and the LOAEL from the Heinze study.

A disadvantage of the Fawell et al (1999) study is that gavage dosing may affect uptake. The bolus dose to the intestine once daily may limit absorption to the period of small intestinal transit. While the Heinze study has the advantage of having administered MC-LR via drinking-water, the Fawell study has some other advantages:

- it produced a NOAEL instead of a LOAEL (affected by a higher degree of uncertainty)
- there were more dose groups, covering an appreciably wider dose range
- the duration of the study was longer (13 weeks vs 28 days in the Heinze study), making it the more suitable for derivation of a lifetime guideline value. There are longer studies than Fawell et al. (1999) but none provide the comprehensive analysis of effects required in a key study.
A further consideration is evidence there may be fundamental differences in the mechanisms leading to hepatocellular death between rats on one hand and mice and humans on the other (Woodbridge et al. 2018).

Although the duration of the Heinze (1999) study is shorter and more similar to the exposure duration envisaged for application of the short-term guideline value, the advantages of the Fawell study described above include no need to include an additional uncertainty factor for extrapolation from a LOAEL to a NOAEL that would increase the total uncertainty and reduce the confidence in the derivation of the short-term guideline value. For this reason, the NOAEL derived by Fawell et al. (1999) was selected as the basis for the short-term and recreational guideline values as well as the lifetime guideline value.

The Tolerable Daily Intake (TDI) for lifetime exposure includes an Uncertainty Factor (UF) of 1000, consisting of a factor of 10 each for the interspecies differences and intraspecies variability in humans and a factor of 10 due to use of a subchronic study and other database deficiencies as described in section 7 and further described below. For deriving the provisional lifetime guideline value, the allocation factor of 80% was chosen because drinking-water is usually the most likely long-term source of exposure.

No uncertainty factor for database deficiencies was included to calculate the provisional short-term and recreational guideline values, since the PoD is based on a sufficiently relevant period of exposure, which in the envisaged scenarios is short. Regarding developmental toxicity, there is an adequate developmental mouse oral study by Fawell et al (1999), with a very high NOAEL of 600 µg/kg bw/d, well above the NOAEL identified the hepatic effects, supported by another two studies (Falconer et al 1988; Chernoff et al, 2002). Studies on other possible end-points, such as male reproductive organs are conflicting. Some studies, reporting adverse effects at doses estimated to range from 0.79-25 µg/kg bw/d via the drinking water (Chen et al 2011; Wu et al., 2015; Pan et al 2018; Zhou et al 2020) used longer exposures, but are considered inadequate; all of these publications came from the same University, suffer from a number of methodological and reporting deficiencies, and require replication in another laboratory to provide confidence in the findings. These studies do not provide evidence for adverse effects from short-term exposures. Moreover, Falconer et al (1988) found no effect on male mouse fertility at doses up to 2700 µg/kg bw/d for 17 weeks via the drinking water, and the reported histopathological findings in the testis from Chen et al (2011) are inconsistent with the results from the 13-week study of Fawell et al (1999), which did not report any histopathological effects in organs and tissues other than liver, at gavage doses up to 1000 µg/kg bw/d. MCs have been classified as Group 2B, possibly carcinogenic to humans (IARC, 2010), based on their tumour promotional activity mediated via protein phosphatase inhibition (a threshold effect) rather than direct carcinogenicity, so there is no need to consider this endpoint in relation to short-term exposures.

However, the evidence does suggest that MCs are likely to accumulate in the liver over longer-term exposure, potentially reaching harmful concentrations. There was considered to be much greater data uncertainty regarding this long-term process and so was a further consideration in the application of a database uncertainty factor of 10 for the derivation of the lifetime guideline value.

The provisional recreational guideline value, aimed to protect from systemic effects, is based on a conservative scenario of a 15 kg child swallowing 250 ml of water (WHO, 2003).
Calculation of provisional Tolerable Daily Intake for microcystin-LR:

\[
TDI_{MC,\text{chronic}} = \frac{\text{NOAEL}}{\text{UF}} = \frac{40}{1000} \, \text{µg/kg/d} = 0.04 \, \text{µg/kg/d}
\]

For comparison, if the LOAEL from Heinze (1999) is used as the PoD, and incorporating uncertainty factors of 10 for inter- and intraspecies variability and 10 for database uncertainties including use of a LOAEL (as per WHO policy), then the TDI would be 0.05 µg/kg/d.

Calculation of provisional lifetime drinking-water guideline value for microcystin-LR:

\[
GV_{MC,\text{chronic}} = \frac{\text{NOAEL} \times \text{bw} \times P}{\text{UF} \times \text{C}} = \frac{40 \times 60 \times 0.8}{1000 \times 2} \, \text{µg/L} = 0.96 \, \text{µg/L} \approx 1 \, \text{µg/L}
\]

\(P:\) proportion of exposure considered to be due to drinking-water;
\(bw:\) body weight (WHO standard = 60 kg);
\(C:\) daily water consumption (assumed to be 2 litres per day);
\(GV_{\text{chronic}}:\) chronic (lifetime) guideline value;
\(NOAEL:\) 40 µg/kg bw based on Fawell et al. (1999);
\(UF:\) uncertainty factor (100 for interspecies and intraspecies extrapolation × 10 for use of a subchronic study and database deficiencies).

Calculation of provisional short-term drinking-water guideline value for microcystin-LR:

\[
GV_{MC,\text{shortterm}} = \frac{\text{NOAEL} \times \text{bw} \times P}{\text{UF} \times \text{C}} = \frac{40 \times 60 \times 1}{1000 \times 2} \, \text{µg/L} = 12 \, \text{µg/L} \approx 10 \, \text{µg/L}
\]

\(P:\) proportion of exposure considered to be due to drinking-water (1 or 100%);
\(bw:\) body weight (WHO standard = 60 kg);
\(C:\) daily water consumption (assumed to be 2 L for an adult);
\(GV_{\text{shortterm}}:\) Short-term guideline value;
\(NOAEL:\) 40 µg/kg bw based on Fawell et al. (1999);
\(UF:\) uncertainty factor (100 for interspecies and intraspecies extrapolation).

Calculation of provisional recreational water guideline value for microcystin-LR:

\[
GV_{MC,\text{recreation}} = \frac{\text{NOAEL} \times \text{bw} \times P}{\text{UF} \times \text{C}} = \frac{40 \times 15}{100 \times 0.25} \, \text{µg/L} = 24 \, \text{µg/L} \approx 20 \, \text{µg/L}
\]

\(bw:\) body weight (assumed to be 15 kg for a child);
\(C:\) incidental water (L) ingested during primary contact (energetic play, swimming, falling out of a boat, etc.);
\(GV_{\text{recreation}}:\) recreational guideline value;
\(NOAEL:\) no-observed-adverse-effect level (40 µg/kg per day);
UF: uncertainty factor (100 for interspecies and intraspecies extrapolation).

9.2 Considerations in applying the provisional guideline values

Informing the public about cyanobacterial blooms in source waters is important for recreational use of the source water and when the source water is used for producing drinking-water. This is particularly important if toxin concentrations in finished drinking-water temporarily exceed the guideline values. Furthermore, cyanobacterial blooms tend to impair the taste and odour of drinking-water even when cyanotoxins are absent and informing the public about the safety of its use is important in order to avoid people turning to other, less safe sources of water.

For recreational sites with blooms, monitoring often cannot occur at sufficiently short time intervals (i.e. daily rather than weekly) to ensure that it captures situations with heavy scums. Site users therefore need information about avoiding scum contact and ingestion as well as situations with pronounced greenish turbidity, i.e. to the extent that one cannot see one’s feet when knee-deep into the water. Temporary closure of sites is an option if blooms contain high toxin concentrations, exceeding the recreational guideline values (for further detail see TCiW, d’Anglada et al.).

The provisional guideline values are based on toxicological data for MC-LR. However, MCs usually occur as mixtures. In the absence of oral toxicity data for other congeners, it is recommended that these values be applied to total MCs as gravimetric or molar equivalents based on the assumption that all MCs have similar toxicity to MC-LR. The highlighted kinetic differences among variants warrant further investigation of MC variants other than MC-LR in order to reduce this relevant source of uncertainty.

The provisional lifetime guideline value for drinking-water applied an allocation factor of 80% because drinking-water is usually the most likely long-term source of exposure. However, in some regions, food could be a significant source of exposure, particularly in tropical locations where the duration of blooms is long and there is high consumption of local seafood. In such situations, consideration should be given to reducing the allocation factor based on relative consumption data from the exposed population.

The short-term drinking-water guideline value is intended to provide guidance on how much the lifetime guideline value can be exceeded for short periods of about 2 weeks until enhanced water treatment or other measures can be implemented. It is not intended to allow for repeated seasonal exceedances of the lifetime guideline value.

The short-term drinking-water guideline value is based on exposure of adults. Since infants and children can ingest a significantly larger volume of water per body weight (e.g. up to 5 times more drinking-water/kg bw for bottle-fed infants compared to an adult), it is recommended that alternative water sources such as bottled water are provided for bottle-fed infants and small children when MC concentrations are greater than 3 µg/L for short periods, as a precautionary measure.

10.0 REFERENCES


Heinze R (1999). Toxicity of the cyanobacterial toxin microcystin-LR to rats after 28 days intake with the drinking water. Environ Toxicol. 14:57-60.


